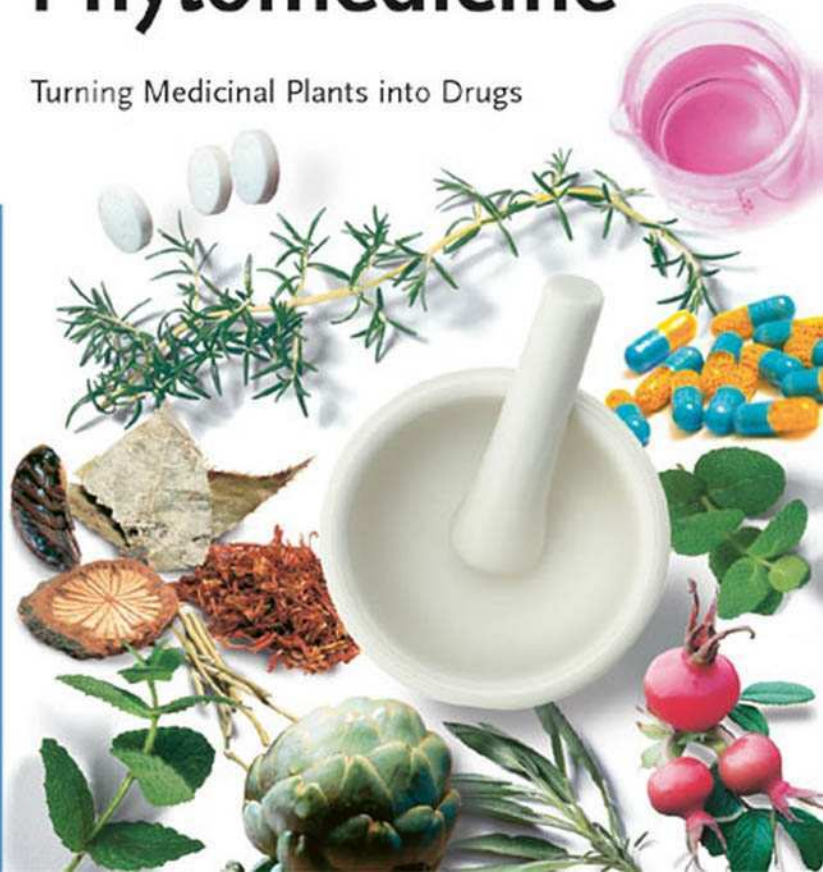
Iqbal Ahmad, Farrukh Aqil and
Mohammad Owais (Ed.)

WILEY-VCH

Modern Phytomedicine

Turning Medicinal Plants into Drugs



Modern Phytomedicine

Edited by
Iqbal Ahmad, Farrukh Aqil,
and Mohammad Owais

Related Titles

Kayser, O., Quax, W. (eds.)

Medicinal Plant Biotechnology

From Basic Research to Industrial Applications

2006

ISBN 3-527-31443-1

Liang, X.-t., Fang, W.-s. (eds.)

Medicinal Chemistry of Bioactive Natural Products

2005

ISBN 0-471-66007-8

Fischer, R., Schillberg, S. (eds.)

Molecular Farming

Plant-made Pharmaceuticals and Technical Proteins

2004

ISBN 3-527-30786-9

Lewis, W. H., Elvin-Lewis, M. P. F.

Medical Botany

Plants Affecting Human Health

2003

ISBN 0-471-62882-4

Modern Phytomedicine

Turning Medicinal Plants into Drugs

Edited by
Iqbal Ahmad, Farrukh Aqil, and Mohammad Owais

WILEY-VCH Verlag GmbH & Co. KGaA

The Editors

Dr. Iqbal Ahmad
Department of Agricultural Microbiology
Aligarh Muslim University
Aligarh 202002
India

Farrukh Aqil
Department of Agricultural Microbiology
Aligarh Muslim University
Aligarh 202002
India

Dr. Mohammad Owais
Interdisc. Biotechnology Unit
Aligarh Muslim University
Aligarh 202002
India

Library of Congress Card No.
applied for

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

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available in the Internet at http://dnb.d-nb.de.

Printed in the Federal Republic of Germany
Printed on acid-free paper

Composition Fotosatz Detzner, Speyer
Printing Strauss GmbH, Mörlenbach
Bookbinding Litges & Dopf Buchbinderei GmbH, Heppenheim

ISBN-13: 978-3-527-31530-7
ISBN-10: 3-527-31530-6

Contents

Preface

Medicinal preparations derived from natural sources, especially from plants, have been in widespread use since time immemorial. Ancient texts of India and China contain exhaustive depictions of the use of a variety of plant-derived medications. In fact, plants remain the main source of medicines for a large proportion of the world's population, particularly in the developing world, despite the advent of the pharmaceutical chemistry during the early twentieth century, which brought with it the ability to synthesize an enormous variety of medicinal drug molecules and allowed the treatment of previously incurable and/or life-threatening diseases.

Not surprisingly, chemically synthesized drug gained popularity and became the basis of pharmaceutical industry. Over the years, however, synthetic drugs have been plagued by unwanted side-effects, toxicity, and inefficiency, among other problems. In addition, the search for new drugs against a variety of illnesses through chemical synthesis and other modern approaches has not been encouraging. These factors, as well as the emergence of new infectious diseases, the proliferation of disorders such as cancer, and growing multidrug resistance in pathogenic microorganisms, have prompted renewed interest in the discovery of potential drug molecules from medicinal plants.

Herbal medicine is now globally accepted as a valid alternative system of therapy in the form of pharmaceuticals, functional foods, etc., a trend recognized and advocated by the World Health Organization (WHO). Various studies around the world, especially in Europe, have been initiated to develop scientific evidence-based rational herbal therapies. Though ancient medical treatises have documented a large number of medicinal plants, most have remained undocumented and uncharacterized, the knowledge of their use being passed down from generation to generation by word of mouth. New plant sources of medicine are also being discovered.

Here we have made an attempt to bring together recent work and current trends in the field of modern phytomedicine from different parts of the world. Although there are a number of books available on medicinal plants and phytocompounds, this book has unique contributions in the form of chapters from experts in the field starting from the concept of phytoscience, screening biological activities against problematic infectious agents such as multidrug-resistant bacteria, fungi, and viruses. Discussion of types of herbal remedies, problems associated with herbal

medicines, such as efficacy, adulteration, safety, toxicity, regulations, and drug delivery etc. are included as contributions by different learned experts.

This book is intended to cover recent trends in phytomedicine and future perspectives in human health care. It is intended that this book will be useful to students, teachers, and researchers in universities, R & D institutions, pharmaceutical and herbal industries as well as to health organizations.

With great pleasure and respect, we extend our sincere thanks to all the contributors for their timely responses, excellent and updated contributions, and consistent cooperation. We express deep gratitude to Prof. M. Shamim Jairajpuri, FNA, Prof. M. Saleemuddin, Prof. Javed Musarrat, and Prof. Akhtar Haseeb who have been a great source of inspiration. We also thank our colleagues Dr. S. Hayat, Dr. M. Saghir Khan, Dr. Abdul Malik, and our research students, Miss Farah Ahmad and Mohd Imran, for their cooperation and critical suggestions.

The technical support and continued encouragement received from the book publishing team at Wiley-VCH (Germany) is also acknowledged.

The financial assistance rendered by University Grant Commission, New Delhi in the form of Major Research Project, India is greatly acknowledged. Finally, we acknowledge the Almighty God, who provided all the channels to work in cohesion and coordination right from the conception of the idea to the development of the final version of this book *Modern Phytomedicine: Turning Medicinal Plants into Drugs*.

Iqbal Ahmad
Farrukh Aqil
Mohammad Owais

List of Contributors

Farah Ahmad
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh 202002
India

Iqbal Ahmad
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh 202002
India

Nadeem Ahmad
Department of Pharmaceutics
Jamia Hamadard University
New Delhi-110062
India

Maroof Alam
Interdisciplinary Biotechnology Unit
Aligarh Muslim University
Aligarh 202002
India

Farrukh Aqil
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh 202002
India

Buket Cicioğlu Arıdoğan
University of Süleyman Demirel
Faculty of Medicine
Microbiology and Clinical Microbiology Department
Isparta
Turkey

Wickramasinghe M. Bandaranayake
Australian Institute of Marine Science
Cape Ferguson, Townsville, MSC 4810
Queensland
Australia

Deepa Bisht
Department of Biochemistry
Central JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR)
Agra; UP 282001
India

Debprasad Chattopadhyay
ICMR Virus Unit
I.D. & B.G. Hospital
GB 4, First Floor, 57
Dr. Suresh C. Banerjee Road
Beliaghata, Kolkata 700 010
India

Jacobus N. Eloff
Phytomedicine Programme
Department of Paraclinical Sciences
University of Pretoria
Private Bag X04
Onderstepoort 0110
South Africa

Shazia Khan
Department of Pharmaceutics
Jamia Hamdard University
New Delhi-110062
India

Umber Khan
Department of Pharmaceutics
Jamia Hamdard
New Delhi-11062
India

Mustapha Larhsini
Laboratory of Medicinal Plants and Phytochemistry
Department of Biology
Faculty of Sciences – Semlalia
POB 2390
40000 Marrakesh
Morocco

Lyndy J. McGaw
Phytomedicine Programme
Department of Paraclinical Sciences
University of Pretoria
Private Bag X04
Onderstepoort 0110
South Africa

Ricardo Ramos Mendonça-Filho
Instituto de Microbiologia Professor Paulo de Góes
Departamento de Microbiologia Geral
Universidade Federal do Rio de Janeiro
UFRJ, Cidade Universitária, CCS, bloco I – Ilha do Fundão
Rio de Janeiro, RJ 21941-590
Brazil

Javed Musarrat
Department of Agricultural Microbiology
Faculty of Agricultural Sciences
Aligarh Muslim University
Aligarh-202002
India

Antonia Nostro
Pharmaco-Biological Department
Microbiology Section, Faculty of Pharmacy
University of Messina
Contrada Annunziata
98168 Messina
Italy

Mohammad Owais
Interdisciplinary Biotechnology Unit
Aligarh Muslim University
Aligarh-202002
India

Enzo A. Palombo
Environment and Biotechnology Centre
Faculty of Life and Social Sciences
Swinburne University of Technology
PO Box 218
Hawthorn Victoria 3122
Australia

Yoshikazu Sakagami
Faculty of Agriculture
Kinki University
3327-204 Nakamachi
Nara City, Nara, 631-8505
Japan

Jin-ichi Sasaki
Ex-Professor of Clinical Immunology
Hirosaki University
Hirosaki 036-8568
Aomori
Japan

7-1 Nishi-Aoyama 2 Chome
Morioka IWATE JAPAN
Area Cord No.020-0132
Japan

Krishnamurthy Venkatesan
Department of Biochemistry
Central JALMA Institute for Leprosy
and Other Mycobacterial Diseases
(ICMR)
Agra UP. 282001
India

Jenny M. Wilkinson
Faculty of Health Studies
School of Biomedical Sciences
Charles Sturt University
Wagga Wagga, NSW 2678
Australia

1
Bioactive Phytocompounds: New Approaches in the Phytosciences

Ricardo Ramos Mendonça-Filho

Summary

Today's use of medicinal plants and bioactive phytocompounds worldwide and our scientific knowledge of them comprises the modern field of the "phytosciences." The phytosciences have been created from the integration of disciplines that have never been linked before, combining diverse areas of economic, social, and political fields, chemistry, biochemistry, physiology, microbiology, medicine, and agriculture.

The field is unique among the biomedical sciences in that instead of testing a hypothesis, in the phytosciences researchers try to determine whether plants commonly used in traditional medicine brings benefits for health and, if so, what their mechanisms of action are.

Despite the common belief that phytocompounds are safe, they all have inherent risks just like synthetic compounds. Thus it is within the scope of the phytosciences to elucidate side-effects, appropriate doses, identify bioactive phytocompounds and ways of extraction and conservation. Besides these, legal aspects regarding regulation of the prescription and commercial sale of medicinal plants are a matter of debate all around the world. The varied regulations in different jurisdictions regarding the prescription and sale of these products add confusion to the formal use of phytocompounds.

As a multidisciplinary science, research in the phytosciences is almost unlimited, which makes it impossible to discuss all aspects of this emerging science in just one chapter. Therefore, we have focussed here mainly on the antimicrobial activity of bioactive phytocompounds, discussing their use against multidrug-resistant (MDR) bacteria and fungi, their mechanisms of action, and their interactions with macromolecules and potential for toxicity in mammalian cells. Technical aspects regarding the development of fast and reliable methods of extraction, high-output screening systems, and bioautography of essential oils and crude extracts and fractions have also been discussed. Problems related to the efficacy, stability, drug delivery systems and quality control are also commented on.

Overall this chapter aims to provide a better understanding of the modern field of the phytosciences and its application in the world today.

1.1 Introduction

To trace the history of phytotherapy is to trace the history of humanity itself. The discovery of the curative properties of certain plants must have sprung from instinct. Primitive peoples first used plants as food and, as result of this ingestion, the link with some plant properties would have been learnt. Medicinal plants were the main source of products used to sustain health until the nineteenth century, when the German chemist Friedrich Wöhler in 1828, attempting to prepare ammonium cyanate from silver cyanide and ammonium chloride, accidentally synthesized urea. This was the first organic synthesis in history and heralded the era of the synthetic compound.

a)

b)

Fig. 1.1 Pedanius Dioscorides, *De Materia Medica* (AD 65). Greek physician Pedanius Dioscorides (c. 40–c. 90) was from Anazarbus, a small town near Tarsus in what is now south-central Turkey. As a surgeon with the Roman army of Emperor Nero, Dioscorides traveled through Italy, Gaul, Spain, and North Africa, recording the existence and medicinal value of hundreds of plants. He compiled an extensive listing of medicinal herbs and their virtues in about AD 70. Originally written in Greek, Dioscorides's herbal was later translated into Latin as *De Materia Medica*. It remained the authority in medicinal plants for over 1500 years.

During the 100 years following Wöhler's discovery phytomedicine was largely forgotten by Western science. In the early 1980s, however, there was a resurgence of interest in the use of natural substances generally known today as bioactive phytocompounds. This interest can be easily understood in the light of questions concerning the safety, cytotoxicity, and side-effects of synthetic compounds, and the need to find new medicines, including new antibiotics to manage infectious diseases caused by multiresistant pathogens and substances to treat chronic diseases.

Today, the use of medicinal plants and their bioactive phytocompounds and our scientific knowledge about them comprises the modern field of the phytosciences. This is a science created from the integration of a range of disciplines that have never been linked before, combining several different areas of economic, social, and political fields, chemistry, biochemistry, physiology, microbiology, medicine, and agriculture.

The phytosciences are different from the other biomedical sciences in that instead of testing a hypothesis, researchers try to determine whether plants commonly used in traditional medicine bring benefits for health and, if so, what are their mechanisms of action. Despite the common belief that bioactive phytocompounds are safe, they have inherent risks just like all active chemical compounds. Researchers within the phytosciences are working to elucidate the side-effects, calculate appropriate dosages, identify the bioactive components, and define the best methods of extraction and conservation. Besides these, legal aspects regarding the prescription and trade in medicinal plants are a matter of debate all around the world. The varying regulations in different jurisdictions allowing the prescription and sale of these products add confusion to the formal use of bioactive phytocompounds.

As a multidisciplinary science the research in this field is almost unlimited, which makes it impractical to discuss all the aspects of this emerging science in just one chapter. Therefore, this review discusses the antimicrobial activity of bioactive phytocompounds, particularly their use against multidrug-resistant bacteria and fungi, their mechanisms of action, and their interactions with macromolecules and potential toxicity for mammalian cells. It also discusses technical aspects regarding the development of fast and reliable methods of extraction, high-output screening systems and bioauthography of essential oils and crude extracts and fractions. Problems related to the efficacy, stability, drug delivery systems and quality control will also be discussed.

1.2
Development of Fast Reliable Methods of Extraction and High-Throughoutput Screening (HTS) of Crude Plant Extracts: New Challenges

Medicinal plants have formed the basis of health care throughout the world since the earliest days of humanity and are still widely used and have considerable importance in international trade. Recognition of their clinical, pharmaceutical, and economic value is still growing, although this varies widely between countries. Plants are important for pharmacological research and drug development, not on-

ly when bioactive phytocompounds are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds. Regulation of their exploitation and exportation is therefore essential to ensure their availability for the future [1].

Plant preparations have a very special characteristic that distinguishs them from chemical drugs: a single plant may contain a great number of bioactive phytocompounds and a combination of plants even more. This complexity is one of the most important challenges to phytoscientists attempting to identify a single bioactive phytocompound or chemical group in the enormous universe that comprises a single crude extract.

Biotechnology in the 1970s and 1980s made tremendous strides and ushered in a new era for the pharmaceutical industry. Many enzymes and receptor proteins of therapeutic interest were made available in large quantities by recombinant expression, while signal transduction pathways could be interrogated by reporter gene carrying cellular constructs. Such mechanism-based *in vitro* assays are amenable to large scales of operation, and the concept of high-throughput screening rapidly became the paradigm for lead discovery [2].

High-throughput screening, often abbreviated as HTS, is a method of scientific experimentation especially relevant to the fields of biology and chemistry. Through a combination of modern robotics and other specialized laboratory hardware, it allows a researcher to effectively conduct hundreds of scientific experiments at once. In essence, HTS uses a brute-force approach to collect a large amount of experimental data, usually observations about how some biological entity reacts to exposure to various chemical compounds in a relatively short time. A screen, in this context, is the larger experiment, with a single goal to which all this data may subsequently be applied [3].

A necessary precondition for the success of the HTS approach is a large and diverse compound collection. In the early days, this largely comprised in-house archives and natural product extracts. The former represented the efforts of chemists internally over the years, supplemented by purchase from external sources. Neither the total number of compounds, nor their chemical diversity, was appropriate to feed HTS. These deficiencies created the science of combinatorial chemistry in the late 1980s and early 1990s and an unanticipated repercussion of high-throughput chemical synthesis was a steady waning of interest in natural product screening, leading to its complete abandonment by many companies [4].

Just like drugs of synthetic origin, bioactive phytocompounds range from simple to complex structures. Either way, the evaluation of a bioactive phytocompound or a natural product leads to benefits from modern HTS for the generation of analogs [5]. Thus, paradoxically, the same combinatorial chemistry that initially caused the decline in natural product screening now promises to be an essential tool in rejuvenating it. Academic groups in particular are used to allocating significant resources of time and staff towards the total synthesis of bioactive phytocompounds. The ability to adapt such routes for the preparation of analogs is an obvious strategy for leveraging the initial expenditure, and is now increasingly evident in the literature. Because of the stricter timelines, large-scale combinatorial programs

based on natural products are less common in industry, but are still practiced in the absence of more tractable synthetic leads [6].

Combinatorial chemistry has come a long way in the past two decades. Industrially, it competed with natural product extracts and purified bioactive phytocompounds for HTS resources and emerged as the preferred option. Unfortunately this technique has not produced a wealth of high-quality drug candidates. Instead, the integration of combinatorial chemistry with other mechanisms for lead generation is now rightly considered the correct strategy. A natural product lead is a legitimate starting point for combinatorial chemistry, and this process can often discover novel analogs [7]. In some cases, such compounds are more potent than the natural product or can possess superior drug-like properties. In others, the synthetic analogs display new biological activities not seen with the original molecule [4].

The ability to rapidly identify undesirable or desirable compounds in natural product extract libraries is a critical step in an efficiently run natural products discovery program. This process, commonly called dereplication [8], is important to prevent the unnecessary use of resources for the isolation of compounds of little or no value for development from extracts used in the screening process. Resources can then be focussed on samples containing the most promising leads. The recent application of HTS technologies to assay natural products extracts for biological activity has intensified the need for efficient dereplication strategies [9].

Dereplication of the bioactive phytocompounds in crude natural product extracts requires some form of feedback from the bioassay, which was initially used to detect the biological activity. This is necessary regardless of the separation technique and analytical method used. A common strategy has been to collect fractions from the high-performance liquid chromatography (HPLC) separation in deep-dish microtiter plates or tubes and then resubmit the individual fractions to the original assay. This approach requires desiccation of fractions to remove the HPLC solvents, which are usually incompatible with the bioassay, resuspending the fractions in a compatible solvent (water, DMSO, or Tween), and then individual assaying of each fraction. This process is not cost effective, being both time and labor intensive. Consequently, as a result of the increasing emphasis on the generation of new lead compounds, faster cycle times, and high efficiency, many pharmaceutical companies have moved away from the natural products area.

Currently, almost every large pharmaceutical company has established HTS infrastructures and possesses large combinatorial compound libraries, which cover a wide range of chemical diversity. However, the ability to detect the desired biological activity directly in the HPLC effluent stream and to chemically characterize the bioactive phytocompound on-line, would eliminate much of the time and labor taken in the fraction collection strategy. This way, cycle times, expenses, and the isolation of known or undesirable compounds would be reduced dramatically, allowing natural products to be screened in an efficient and cost effective manner [10].

Recently, such an on-line HPLC biochemical detection (BCD) system, in the following referred to as high-resolution screening (HRS) system, has been described for a range of pharmacologically relevant targets, such as the human estrogen receptor, cytokines, leukotrienes, and the urokinase receptor [11]. In contrast to con-

ventional microtiter-type bioassays, the interactions of the extracts and the biochemical reagents proceed at high speed in a closed continuous flow reaction detection system. When sufficient chromatographic separation is achieved, the individual contribution of the bioactive phytocompounds to the total bioactivity is obtained within a single run. Moreover, by combining on-line biochemical detection with complementary chemical analysis techniques, such as mass spectrometry (HRS-MS), chemical information that is crucial for the characterization and identification of bioactive phytocompounds is obtained in real time. Biochemical responses are rapidly correlated to the recorded MS and MS/MS data, thus providing chemical information such as molecular weight and MS/MS fingerprints [12]. Compared with traditional screening approaches of complex mixtures, which are often characterized by a repeating cycle of HPLC fractionation and biological screening, HRS-MS analysis speeds up the dereplication process dramatically. Moreover, the technology enables drug discovery programs to access the enormous chemical diversity offered by complex mixtures as a source of novel drug-like molecules [13]. The use of chromatographical assays is discussed in the next section of this chapter.

1.3 Antimicrobial Bioactive Phytocompounds from Extraction to Identification: Process Standardization

Different approaches to drug discovery using higher plants can be distinguished: random selection followed by chemical screening; random selection followed by one or more biological assays; biological activity reports and ethnomedical use of plants [14]. The latter approach includes plants used in traditional medical systems; herbalism, folklore, and shamanism; and the use of databases. The objective is the targeted isolation of new bioactive phytocompounds. When an active extract has been identified, the first task to be taken is the identification of the bioactive phytocompounds, and this can mean either a full identification of a bioactive phytocompound after purification or partial identification to the level of a family of known compounds [15].

In Fig. 1.2 an extraction-to-identification flowchart is proposed in order to optimize bioactive phytocompound identification. For screening selection, plants are collected either randomly or by following leads supplied by local healers in geographical areas where the plants are found. Initial screening of plants for possible antimicrobial activities typically begins by using crude aqueous or alcohol extractions followed by various organic extraction methods [16]. Plant material can be used fresh or dried. The aspects of plant collection and identification will be discussed further in this chapter. Other relevant plant materials related to antimicrobial activity are the essential oils. Essential oils are complex natural mixtures of volatile secondary metabolites, isolated from plants by hydro or steam distillation and by expression (citrus peel oils). The main constituents of essential oils (mono and sesquiterpenes), along with carbohydrates, alcohols, ethers, aldehydes, and ke-

tones, are responsible for the fragrant and biological properties of aromatic and medicinal plants. Due to these properties, since ancient times spices and herbs have been added to food, not only as flavoring agents but also as preservatives. For centuries essential oils have been isolated from different parts of plants and are also used for similar purposes.

The activities of essential oils cover a broad spectrum. Various essential oils produce pharmacological effects, demonstrating anti-inflammatory, antioxidant, and anticancerogenic properties [17–19]. Others are biocides against a broad range of organisms such as bacteria, fungi, protozoa, insects, plants, and viruses [20–22].

The dispersion of the hydrophobic components of essential oils in the growth medium is the main problem in testing the activity of essential oils. Different organic solvents must be used as solubilizing agents, which may interfere with the results of antimicrobial assays. The solution to this problem is the use of nonionic emulsifiers, such as Tween 20 and Tween 80. These molecules are relatively inactive and are widely applied as emulsifying agents. Control tests must guarantee that these emulsifying agents do not interfere in the experiments.

Plants can be dried in a number of ways: in the open air (shaded from direct sunlight); placed in thin layers on drying frames, wire-screened rooms, or in buildings; by direct sunlight, if appropriate; in drying ovens/rooms and solar dryers; by indirect fire; baking; lyophilization; microwave; or infrared devices. Where possible, temperature and humidity should be controlled to avoid damage to the active chemical constituents. The method and temperature used for drying may have a considerable impact on the quality of the resulting medicinal plant materials. For example, shade drying is preferred to maintain or minimize loss of color of leaves and flowers; and lower temperatures should be employed in the case of medicinal plant materials containing volatile substances [23]. The drying conditions should be recorded. In the case of natural drying in the open air, medicinal plant materials should be spread out in thin layers on drying frames and stirred or turned frequently. In order to secure adequate air circulation, the drying frames should be located at a sufficient height above the ground. Efforts should be made to achieve uniform drying of medicinal plant materials to avoid mold formation [24].

Drying medicinal plant material directly on bare ground should be avoided. If a concrete or cement surface is used, the plant materials should be laid on a tarpaulin or other appropriate cloth or sheeting. Insects, rodents, birds and other pests, and livestock and domestic animals should be kept away from drying sites. For indoor drying, the duration of drying, drying temperature, humidity and other conditions should be determined on the basis of the plant part concerned (root, leaf, stem, bark, flower, etc.) and any volatile natural constituents, such as essential oils. If possible, the source of heat for directs drying (fire) should be limited to butane, propane or natural gas, and temperatures should be kept below 60 °C [25]. If other sources of fire are used, contact between those materials, smoke, and the medicinal plant material should be avoided.

Since researches are trying to identify bioactive phytocompounds in medicinal plant extracts generally used by local population to treat diseases and based on empiric knowledge that they have the searched bioactivity, the solvent chosen must be

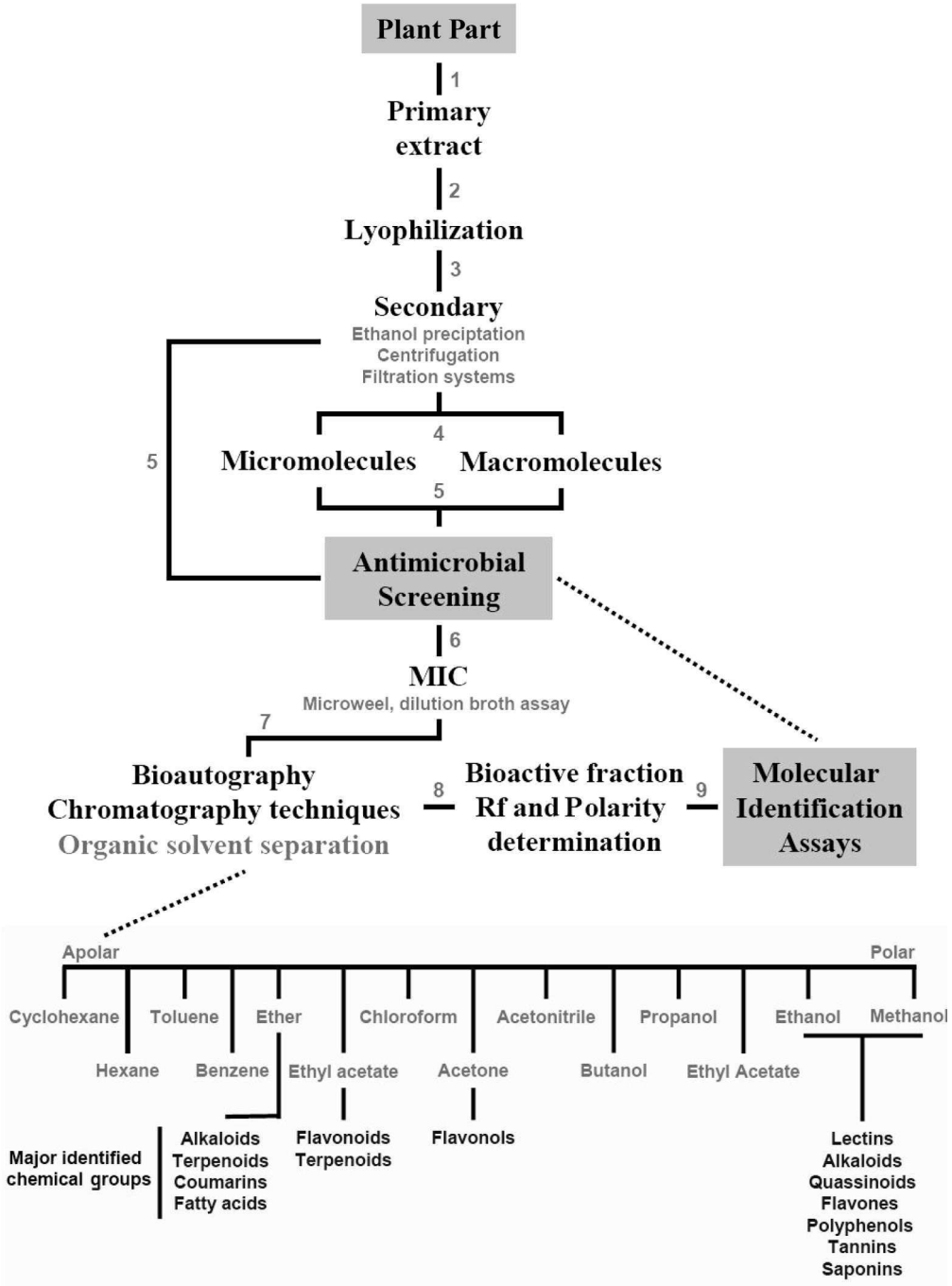

Plant Part
1
Primary extract
2
Lyophilization
3
Secondary
Ethanol preciptation
Centrifugation
Filtration systems
4
5
Micromolecules
Macromolecules
5
Antimicrobial Screening
6
MIC
Microweel, dilution broth assay
7
Bioautography
Chromatography techniques
Organic solvent separation
8
Bioactive fraction
Rf and Polarity
determination
9
Molecular Identification Assays
Apolar
Polar
Cyclohexane
Toluene
Ether
Chloroform
Acetonitrile
Propanol
Ethanol
Methanol
Hexane
Benzene
Ethyl acetate
Acetone
Butanol
Ethyl Acetate
Major identified chemical groups
Alkaloids
Terpenoids
Coumarins
Fatty acids
Flavonoids
Terpenoids
Flavonols
Lectins
Alkaloids
Quassinoids
Flavones
Polyphenols
Tannins
Saponins

Fig. 1.2 Standardization flowchart: from extraction to identification of bioactive phytocompounds. (1) Plants can be chosen either randomly, based on the literature or following consulation with local healers. After choosing the right material, plant collection must be followed by botanical identification and a voucher specimen must be placed in the local herbarium. All data about the collection must be observed and documented, such as climate conditions, season, geographical localization, environmental conditions, etc. in order to elucidate future differences in bioactivity compared with other results found. Any plant part can be used but consultation of the literature or with local healers is very useful to reduce research time. (2) Collected plant material can be used fresh or dried. Several studies have started extractions with both fresh and dried material in order to compare the chemical composition of the extracts. They must be ground to optimize the solvent contact during the extraction process. Weight standardization must be used (i.e. 300 g of plant material to 1000 mL of solvent). More than 90% of the studies for antimicrobial activity in the literature start extraction with methanol, ethanol or water because it is proved that ethanol extraction is more effective in isolating the bioactive phytocompound. The primary extractions methods are very variable but the idea is to research activity cited in popular use, and to choose the same extraction method. This is especially useful to corroborate the *in vivo* activity found in popular use. (3) After extraction the volume must be concentrated by lyophilization or another concentration technique before screening. Usually, after the lyophilization process ground powder is obtained. This must be resuspended in water at a higher concentration (i.e. 1 g mL^{-1}) for initial drop test screening. The high concentration of the extract guarantees the identification of the bioactivity, if present. Using low concentrations in drop tests may lead to false negative results. (4) Due to the complex composition of the extract primary separation may be used to facilitate the identification process. Micromolecules can be separated from macromolecules (proteins and carbohydrates) by very simple techniques such as ethanol precipitation (30% v/v), centrifugation (10 000g for 10 min) and filtration systems such as Centricon and Amicon (Millipore). Supernatant and precipitate phases are obtained and can be separated in drop tests. As discussed previously, antimicrobial activity is commonly present in micromolecules (supernatant) phase. (5) The antimicrobial screening by drop test (formerly disk diffusion agar assay) is the most efficient and inexpensive assay to identify antimicrobial activity. The extract is dropped (i.e. 15 µL) onto an agar surface previously inoculated with the desired microorganism. Note that is very important to count by McFarland scale or Newbauer chamber (i.e. 10^5 UFC mL^{-1} for bacteria; 10^6 cells mL^{-1} for fungi) the microorganism inoculums; this permits the antimicrobial activity to be compared within antibiotic controls and between different microorganism groups. (6) When antimicrobial activity is detected the minimum inhibitory concentration (MIC) must be determined to continue other antimicrobial assays of interest. The MIC is usually established by the broth dilution method. The use of 96-microwell plates to minimize costs is very effective, reducing the culture media quantities drastically and enhancing the test capacity (in one plate up to eight different extracts can be tested in 10 different concentrations plus 1 negative and 1 positive controls, also see Fig. 1.3). (7) Bioguided chromatography techniques such as bioautography preceded by solvent separation is essential to initiate the bioactive phytocompound identification process; fraction collection with HPLC or FPLC assays, preparative TLC are also valid techniques. Bio-guided fraction and purification confirms previous results leading to isolation of a bioactive phytocompound. (8) By TLC assays, Rf values can be determined and polarity or even chemical groups (use of specific dyes) elucidated (Fig. 1.3). (9) NMR, HPLC/MS, and GC/MS are used to identify a bioactive phytocompound as discussed in this chapter.

the same as that used in popular treatment. As we know, water and ethanol are by far the most commonly used, and for this reason most studies begin with water or ethanol as solvents.

Water is almost universally the solvent used to extract activity. At home, dried plants can be ingested as teas (plants steeped in hot water) or, rarely, tinctures (plants in alcoholic solutions) or inhaled via steam from boiling suspensions of the parts. Dried plant parts can be added to oils or petroleum jelly and applied externally. Poultices can also be made from concentrated teas or tinctures.

Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained initially through ethanol and water extraction [26]. Some water-soluble compounds, such as polysaccharides like starch and polypeptides, including fabatin [27] and various lectins, are commonly more effective as inhibitors of virus adsorption and would not be identified in the screening techniques commonly used [28]. Occasionally tannins and terpenoids may be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents (Fig. 1.2).

Another concern during the extraction phase is that any part of the plant may contain active components. For instance, the roots of ginseng plants contain the active saponins and essential oils, while eucalyptus leaves are harvested for their essential oils and tannins. Some trees, such as the balsam poplar, yield useful substances in their bark, leaves, and shoots [29]. The choice of which part to use must be based on ethnopharmacological studies and review of the literature.

For alcoholic extractions, plant parts are dried, ground to a fine texture, and then soaked in methanol or ethanol for extended periods. The slurry is then filtered and washed, after which it may be dried under reduced pressure and redissolved in the alcohol to a determined concentration. When water is used for extractions, plants are generally soaked in distilled water, blotted dry, made into slurry through blending, and then strained or filtered. The filtrate can be centrifuged (approximately 10 000 g for 10 min) multiple times for clarification [30]. Crude products can then be directly used in the drop test and broth dilution microwell assays (Fig. 1.2) to test for antifungal and antibacterial properties and in a variety of assays to screen bioactivity (Fig. 1.3).

In order to reduce or minimize the use of organic solvents and improve the extraction process, newer sample preparation methods, such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) or pressurized liquid extraction (PLE) have been introduced for the extraction of analytes present in plant materials. Using MAE, the microwave energy is used for solution heating and results in significant reduction of extraction time (usually in less than 30 min). Other than having the advantage of high extraction speed, MAE also enables a significant reduction in the consumption of organic solvents. Other methods, such as the use of SFE that used carbon dioxide and some form of modifiers, have been used in the extraction of compounds present in medicinal plants [31].

To identify the bioactive phytocompounds, liquid chromatography with an isocratic/gradient elution remains the method of choice in the pharmacopeia, and re-

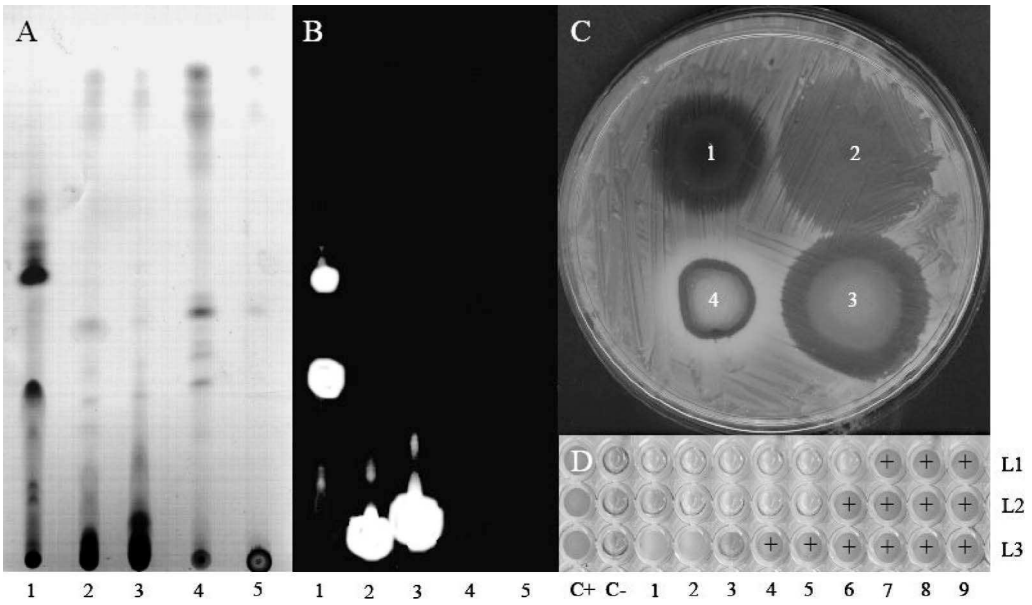


Fig. 1.3 Current assays to identify bioactivity and start molecule identification. (A/B) Bioauthography technique: (A) Thin-layer chromatography (TLC) of aqueous extracts of (1) *Ocimun gratissimum*, (2) *Anadenanthera macrocarpa*, (3) *Croton cajucara* Benth. (4) *Cymbopogon citrates*, and (5) *Juglans regia* performed in silica gel G60 F_{254} aluminum plates (5 _ 8). Plates were developed with *n*-butanol:acetic acid:water (8:1:1, v/v) and were visualized under ultraviolet light or after staining with cerric sulfate plate. (B) Alternatively, plates were placed inside Petri dishes and covered with over solid media (10 mL BHI with 1% phenol red). After overnight incubation for diffusion of the separated components, the plate was inoculated with *Candida albicans* (ATCC 51501) 10^6 cells per plate and incubated for 48 h at 37 °C. Growth inhibition can be seen in (1, 2, and 3) after spraying with methylthiazollyltetrazolium chloride (MTT) at 5 mg mL^{-1}. (C) Drop test at same concentrations (200 µg mL^{-1}) of (1) aqueous extract from *Punica granatum* and commercially available antifungal agents, (2) fluconazole, (3) flucytosine, and (4) anphotericin. (D) MIC microwell dilution test of (L1) *Punica granatum*, (L2) fluconazole, and (L3) flucytosine against *Candida albicans* (ATCC 51501). (C+) positive control, (C–) negative control, (1) 200 µg mL^{-1}, (2) 100 µg mL^{-1}, (3) 50 µg mL^{-1}, (4) 25 µg mL^{-1}, (5) 12.5 µg mL^{-1}, (6) 6.75 µg mL^{-1}, (7) 3.4 µg mL^{-1}, (8) 1.7 µg mL^{-1}, and (9) 0.8 µg mL^{-1}. (+) means fungi growth.

versed octadecyl silica (C-18) and ultraviolet detection mode is the most commonly used method. Gradient elution HPLC with reversed phase columns has also been applied for the analysis of bioactive phytocompounds present in medicinal plants extracts [32].

The advantages of liquid chromatography include its high reproducibility, good linear range, ease of automation, and its ability to analyze the number of constituents in botanicals and herbal preparation. However, for the analysis of multiple bioactive phytocompounds in herbal preparations with two or more medicinal plants, coeluting peaks were often observed in the chromatograms obtained due to

the complexity of the matrix. The complexity of matrix may be reduced with additional sample preparation steps, such as liquid–liquid partitioning, solid-phase extraction, preparative LC and thin-layer chromatography (TLC) fractionation.

Capillary electrophoresis (CE) proved to be a powerful alternative to HPLC in the analysis of polar and thermally labile compounds. Reviews on the analysis of natural medicines or natural products in complex matrix by CE are well reported. Many publications showed that all variants of CE, such as capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC), and capillary isoelectric focusing (cIEF), have been used for the separation of natural products. The separation in CZE is based on the differences in the electrophoretic mobilities resulting in different velocities of migration of ionic species in the electrophoretic buffer in the capillary. For MEKC, the main separation mechanism is based on solute partitioning between the micellar phase and the solution phase. Factors that are known to affect separation in CZE and MEKC include the pH of the running buffer, ionic strength, applied voltage, and concentration and type of micelle added. From the review articles, CE has been used to determine the amount of catechin and others in tea composition, phenolic acids in coffee samples and flavonoids and alkaloids in plant materials.

Chromatographic separation with mass spectrometry for the chemical characterization and composition analysis of botanicals has been growing rapidly in popularity in recent years. Reviews on the use of mass spectrometry and high-performance liquid chromatography mass spectrometry (HPLC/MS) on botanicals have been reported. The use of hyphenated techniques, such as high-resolution gas chromatography mass spectrometry (HRGC/MS), high performance liquid chromatography/mass spectrometry (HPLC/MS), liquid chromatography tandem mass spectrometry (HPLC/MS/MS) and tandem mass spectrometry (MS/MS) to perform on-line composition and structural analyses provide rich information that is unsurpassed by other techniques.

HRGC/MS remains the method of choice for the analysis of volatile and semi-volatile components, such as essential oils and others in botanicals and herbal preparations, along with high-resolution separation with capillary column coupling with mass spectrometry using electron impact ionization (EI).

In analyzing bioactive phytocompounds, HPLC/MS has played an increasingly significant role as the technique is capable of characterizing compounds that are thermally labile, ranging from small polar molecules to macromolecules, such as peptides/proteins, carbohydrates, and nucleic acids. The most common mode of ionization in HPLC/MS includes electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Mass analyzers, such as single quadruple, triple quadruple, ion-trap, time-of-flight, quadruple time-of-flight (Q-TOF) and others, are also used. With tandem mass spectrometry, additional structural information can be obtained about the target compounds. However, methods using HPLC/MS are still limited to conditions that are suitable for MS operations. There are restrictions on pH, solvent choice, solvent additives and flow rate for LC in order to achieve optimal sensitivity.

For the identification of bioactive phytocompounds by HRGC/MS or HPLC/MS, the following conditions are useful when standards are available: a suspect peak has to show a retention time similar to the average retention time of the pure standard or control sample and mass spectra for the suspect peaks have to show relative abundance ±10% (arithmetic difference) of the relative abundance of the standard analyzed that day. With HPLC/MS, applying the right separation, with the right ionization interface and mass analyzer, significant information can be obtained with regards to the target compounds. However, for the quantification of bioactive phytocompounds in plant materials, the system precision will be higher compared to that obtained using HPLC with ultraviolet detection. For on-line HPLC/MS, the internal diameter of the column selected will be an important consideration.

Another important chromatography technique is bioautography (Fig. 1.3). Bioautography is often used as an option to identify chemical groups of bioactive phytocompounds or even a single bioactive phytocompound when padrons are available. The complex chemical composition of plant extracts is generally a limiting obstacle to the isolation of antimicrobial compounds. Nevertheless, the use of bioautography agar overlay bioassays allows the detection of active components in a crude plant extract. This method permits the localization of antimicrobial active components that have been separated by TLC [33]. Precipitation with ethanol of plant aqueous extracts allows the separation of polymers, such as polysaccharides and proteins, from micrometabolites [34, 27]. By this technique, the solvation between molecules is changed, and in the same way, the interaction between molecules. Polymers (macromolecules) will be found in the water-soluble precipitate and micrometabolites in the supernatant. The precipitation of macromolecules can also be achieved by ammonium sulfate and acetone. The association of bioautography and ethanol precipitation techniques allows the detection of otherwise nondetectable bioactive phytocompounds [35].

An extremely important aspect of chromatography techniques is to identify nonnatural molecules, such as paracetamol, that may be present in or added to health supplements and commercially available herbal preparations.

1.4 Problems Associated with the Efficacy, Stability and Quality Control of Herbal Drugs Preparations

The number of reports of patients experiencing negative health consequences caused by the use of herbal medicines has increased in recent years [36]. Analysis and studies have revealed a variety of reasons for such problems. One of the major causes of reported adverse events is directly linked to the poor quality of herbal medicines, including raw medicinal plant materials. It has therefore been recognized that insufficient attention has been paid to the quality assurance and control of herbal medicines [37].

Quality control directly impacts the safety and efficacy of herbal medicinal products [38]. The implementation of good agricultural and collection practises for medicinal plants is only the first step in quality assurance, on which the safety and efficacy of herbal medicinal products directly depend, and also plays an important role in the protection of natural resources of medicinal plants for sustainable use.

Some reported adverse events following the use of certain herbal medicines have been associated with a variety of possible explanations, including the inadvertent use of the wrong plant species, adulteration with undeclared other medicines and/or potent substances, contamination with undeclared toxic and/or hazardous substances, overdosage, inappropriate use by health care providers or consumers, and interactions with other medicines, resulting in adverse drug effects [39].

The safety and quality of raw medicinal plant materials and finished products depend on factors that may be classified as intrinsic (genetic) or extrinsic (environment, collection methods, cultivation, harvest, post-harvest processing, transport, and storage practises). Inadvertent contamination by microbial or chemical agents during any of the production stages can also lead to deterioration in safety and quality. Medicinal plants collected from the wild population may be contaminated by other species or plant parts through misidentification, accidental contamination, or intentional adulteration, all of which may have unsafe consequences.

The collection of medicinal plants from wild populations can give rise to additional concerns related to global, regional, and/or local over-harvesting, and protection of endangered species. The impact of cultivation and collection on the environment and ecological processes, and the welfare of local communities should be considered [40].

It is well established that intrinsic and extrinsic factors, including species differences, organ specificity, diurnal and seasonal variation, environment, field collection and cultivation methods, contamination, substitution, adulteration, and processing and manufacturing practises greatly affect botanical quality. Intrinsically, botanicals are derived from dynamic living organisms, each of which is capable of being slightly different in its physical and chemical characters due to genetic influence.

Diurnal and seasonal variations are other intrinsic factors affecting chemical accumulation in both wild and cultivated plants. Depending on the plant, the accumulation of chemical constituents can occur at any time during the various stages of growth. In the majority of cases, maximum chemical accumulation occurs at the time of flowering, followed by a decline beginning at the fruiting stage. The time of harvest or field collection can thus influence the quality of the final herbal product. There are many extrinsic factors affecting the qualities of medicinal plants. It has been well established that factors such as soil, light, water, temperature, and nutrients can, and do, affect phytochemical accumulation in plants,

The methods employed in field collection from the wild, as well as in commercial cultivation, harvest, post-harvest processing, shipping, and storage can also influence the physical appearance and chemical quality of botanical source materials. Contamination by microbial and chemical agents (pesticides, herbicides, heavy metals), as well as by insect, animal, animal parts, and animal excreta during any

of the stages of source plant material production can lead to lower quality and/or unsafe materials. Adulteration of herbal medicines with synthetic drugs represents another problem in product quality.

In the following paragraphs technical aspects of medicinal plant production will be discussed. According to the World health Organization [37] the botanical identity, scientific name (genus, species, subspecies/variety, author, and family) of each medicinal plant under cultivation should be verified and recorded. If available, the local and English common names should also be recorded. Other relevant information, such as the cultivar name, ecotype, chemotype, or phenotype, may also be provided, as appropriate. For commercially available cultivars, the name of the cultivar and of the supplier should be provided. It's essential that a voucher botanical specimen used in the experiments be placed in a regional or national herbarium for identification and further consultation by other researchers; it is almost impossible and not advised to publish without the registration numbers.

Cultivation of medicinal plants requires intensive care and management. The conditions and duration of cultivation required vary depending on the quality of the medicinal plant materials required. If no scientific published or documented cultivation data are available, traditional methods of cultivation should be followed, where feasible. Otherwise a method should be developed through research. The principles of good plant husbandry, including appropriate rotation of plants selected according to environmental suitability, should be followed, and tillage should be adapted to plant growth and other requirements. Risks of contamination as a result of pollution of the soil, air, or water by hazardous chemicals should be avoided. The impact of past land uses on the cultivation site, including the planting of previous crops and any applications of plant protection products should be evaluated.

The quality and growth of medicinal plants can also be affected by other plants, other living organisms, and by human activities. The introduction of nonindigenous medicinal plant species into cultivation may have a detrimental impact on the biological and ecological balance of the region. The ecological impact of cultivation activities should be monitored over time, where practical.

The social impact of cultivation on local communities should also be examined to ensure that negative impacts on local livelihood are avoided. In terms of local income-earning opportunities, small-scale cultivation is often preferable to large-scale production, especially if small-scale farmers are organized to market their products jointly. If large-scale medicinal plant cultivation is or has been established, care should be taken that local communities benefit directly from, for example, fair wages, equal employment opportunities, and capital reinvestment.

Climatic conditions, for example, length of day, rainfall (water supply), and field temperature, significantly influence the physical, chemical, and biological qualities of medicinal plants. The duration of sunlight, average rainfall, average temperature, including daytime and night-time temperature differences, also influence the physiological and biochemical activities of plants, and prior knowledge should be considered.

The soil should contain appropriate amounts of nutrients, organic matter, and other elements to ensure optimal medicinal plant growth and quality. Optimal soil

conditions, including soil type, drainage, moisture retention, fertility, and pH, will be dictated by the selected medicinal plant species and/or target medicinal plant part. The use of fertilizers is often necessary in order to obtain large yields of medicinal plants. It is, however, necessary to ensure that correct types and quantities of fertilizers are used through agricultural research. In practise, organic and chemical fertilizers are used.

Human excreta must not be used as a fertilizer owing to the potential presence of infectious microorganisms or parasites. Animal manure should be thoroughly composted to meet safe sanitary standards of acceptable microbial limits and to destroy the germination capacity of weeds. Any applications of animal manure should be documented. Chemical fertilizers that have been approved by the countries of cultivation and consumption should be used. All fertilizing agents should be applied sparingly and in accordance with the needs of the particular medicinal plant species and supporting capacity of the soil. Fertilizers should be applied in such a manner as to minimize leaching.

Any agrochemical used to promote the growth of or to protect medicinal plants should be kept to a minimum, and applied only when no alternative measures are available. Integrated pest management should be followed where appropriate. When necessary, only approved pesticides and herbicides should be applied at the minimum effective level, in accordance with the labeling and/or package insert instructions of the individual product and the regulatory requirements that apply for the grower and the end-user countries. Only qualified staff using approved equipment should carry out pesticide and herbicide applications. Growers and producers should comply with maximum pesticide and herbicide residue limits, as stipulated by local, regional and/or national regulatory authorities.

Medicinal plants should be harvested during the optimal season or time period to ensure the production of medicinal plant materials and finished herbal products of the best possible quality. The time of harvest depends on the plant part to be used. It is well known that the concentration of biologically active constituents varies with the stage of plant growth and development. This also applies to nontargeted toxic or poisonous indigenous plant ingredients. The best time for harvest (quality peak season/time of day) should be determined according to the quality and quantity of bioactive phytocompounds rather than the total vegetative yield of the targeted medicinal plant parts. During harvest, care should be taken to ensure that no foreign matter, weeds, or toxic plants are mixed with the harvested medicinal plant materials. Medicinal plants should be harvested under the best possible conditions, avoiding dew, rain, or exceptionally high humidity. If harvesting occurs in wet conditions, the harvested material should be transported immediately to an indoor drying facility to expedite drying so as to prevent any possible deleterious effects due to increased moisture levels, which promote microbial fermentation and mold. Cutting devices, harvesters, and other machines should be kept clean and adjusted to reduce damage and contamination from soil and other materials. They should be stored in an uncontaminated, dry place or facility free from insects, rodents, birds and other pests, and inaccessible to livestock and domestic animals.

Contact with soil should be avoided as far as possible so as to minimize the microbial load of harvested medicinal plant materials. The harvested raw materials should be transported promptly in clean, dry conditions. They may be placed in clean baskets, dry sacks, trailers, hoppers, or other well-aerated containers and carried to a central point for transport to the processing facility.

1.5 Novel Bioactive Phytocompounds Against Multidrug-Resistant Bacteria/Fungi: The Management of Infectious and Chronic Diseases

Long before the discovery of the existence of microbes, the idea that certain plants had healing potential, indeed, that they contained what we would currently characterize as antimicrobial principles, was well accepted. Since antiquity, humans have used plants to treat common infectious diseases, and some of these traditional medicines are still included as part of the habitual treatment of various maladies. For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*), and tee tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents. That being said, it has generally been the essential oils of these plants rather than their extracts that have had the greatest use in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal, and biliary systems, as well as on the skin. In the case of *Melaleuca alternifolia*, for example, the use of the essential oil (tee tree oil) is a common therapeutic tool to treat acne and other infectious troubles of the skin.

Antimicrobial resistance is one of the biggest challenges facing global public health. Although antimicrobial drugs have saved many lives and eased the suffering of many millions, poverty, ignorance, poor sanitation, hunger and malnutrition, inadequate access to drugs, poor and inadequate health care systems, civil conflicts and bad governance in developing countries have tremendously limited the benefits of these drugs in controlling infectious diseases. The development of resistance in the responsible pathogens has worsened the situation, often with very limited resources to investigate and provide reliable susceptibility data on which rational treatments can be based as well as the means to optimize the use of antimicrobial agents. The emergence of multidrug-resistant isolates in tuberculosis, acute respiratory infections, and diarrhea, often referred to as the diseases of poverty, has had its greatest toll in developing countries. The epidemic of HIV/AIDS, with over 30 million cases in developing countries, has greatly enlarged the population of immunocompromised patients. The disease has left these patients at great risk of numerous infections and even greater risk of acquiring highly resistant organisms during long periods of hospitalization.

Antibiotic resistance can occur via three general mechanisms: prevention of interaction of the drug with target, efflux of the antibiotic from the cell, and direct

destruction or modification of the compound. The emergence of multidrug resistance in human and animal pathogenic bacteria as well as undesirable side-effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin.

Ahmad and Beg [41] tested alcoholic extracts of 45 traditionally used Indian medicinal plants against drug-resistant bacteria and fungi (*C. albicans*) both related to the critical prognosis and treatment of infectious diseases in immunocompromised, AIDS and cancer patients. Of these, 40 plant extracts showed varied levels of antimicrobial activity against one or more test bacteria. Anticandidal activity was detected in 24 plant extracts. Overall, broad-spectrum antimicrobial activity was observed in 12 plants (*L. inermis, Eucalyptus* sp., *H. antidysentrica, H. indicus, C. equistifolia. T. belerica, T. chebula, E. officinalis, C. sinensis, S. aromaticum* and *P. granatum*). Several other studies have also demonstrated the importance of new bioactive phytocompounds against multidrug-resistant bacteria/fungi.

Useful antimicrobial phytochemicals can be divided into several categories summarized in Table 1.1. Scientists from divergent fields are investigating plants anew with an eye to their antimicrobial usefulness. A sense of urgency accompanies the search as the pace of species extinction continues. Laboratories of the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro*. More of these compounds should be subjected to animal and human studies to determine their effectiveness in whole-organism systems, including in particular toxicity studies as well as an examination of their effects on beneficial normal microbiota. It would be advantageous to standardize methods of extraction and *in vitro* testing so that the search could be more systematic and interpretation of results facilitated. Also, alternative mechanisms of infection prevention and treatment should be included in initial activity screenings. Disruption of adhesion is one example of an anti-infection activity not commonly screened currently. Attention to these issues could usher in a badly needed new era of chemotherapeutic treatment of infection by using plant-derived principles.

1.6 Mode of Action of Bioactive Phytocompounds and their Interactions with Macromolecules and Toxicity

The mode of action of antimicrobial agents depends on the type of microorganism under consideration and is mainly related to their cell wall structure and the outer membrane arrangement. Gram-negative bacteria (e.g. *Pseudomonas aeruginosa*) display an intrinsic resistance to a wide variety of essential oils, which is associated with the hydrophilic surface of their outer membrane, rich in lipopolysaccharide molecules. A permeability barrier against toxic agents is formed. Small hydrophilic molecules are not prevented from passing through the outer membrane because of the action of abundant porin proteins. However, hydrophobic macromolecules, such as essential oils constituents, are unable to penetrate the barrier.

Table 1.1 Plants and identified antimicrobial bioactive phytocompounds.

Scientific name	*Compound class*	*Compound*	*Activity (most relevant)*	*Ref.*
Allium sativum	Sulfoxide	Allicin	Broad spectrum[a]	42
Anacardium pulsatilla	Polyphenols	Salicylic acids	*P. acnes*	–
Anemone pulsatilla	Lactone	Anemonins	Bacteria	–
Berberis vulgaris	Alkaloid	Berberine	Protozoa and bacteria	43
Camellia sinensis	Flavonoid	Catechin	Broad spectrum[a], viruses	44
Carum carvi	–	Coumarins	Viruses, broad spectrum[a]	45
Centella asiatica	Terpenoid	Asiatocoside	*Mycobacterium leprae*	–
Cinchora sp.	Alkaloid	Quinine	*Plasmodium* spp.	–
Citrus sinensis	Terpenoid	–	Fungi	46
Croton cajucara	Essential oil	Linalool	*Leishmania amazonenis*, fungi and bacteria	20
Erythroxylum coca	Alkaloid	Cocaine	Bacteria	–
Eucalyptusglobulus sp.	Polyphenol	Tannin	Bacteria and viruses	–
Gloriosa superba	Alkaloid	Colchicina	Broad spectrum[a]	–
Hydrastis canadensis	Alkaloid	Berberine	Bacteria, *Giargia duodenale*	47
Malus sylvestris	Flavonoid derivate	Phloretin	Broad spectrum[a]	–
Matricaria chamomilla	Phenolic acid	Anthemic acid	*M. tuberculosis* and *S. typhimurium*	–
Melissa officinalis	Polyphenols	Tannins	Viruses	48
Millettia thonningii	Flavone	Alpinum-isoflavone	*Schistosoma* sp.	49
Ocimum basilicum	Essential oil	Terpenoids	Bacteria, *Salmonella* sp.	50
Olea europaea	Aldehyde	Hexanal	Broad spectrum[a]	51
Onobrychis viciifolia	Polyphenols	Tannins	Bacteria	52
Panax notoginseng	Saponins	–	Bacteria	–
Pimenta dioica	Essential oil	Eugenol	Broad spectrum[a]	53
Piper betel	Essential oil	Cathecol	Broad spectrum[a]	50
Piper nigrum	Alkaloid	Piperine	Fungi, *Lactobacillus* sp.	54
Podocarpus nagi	Flavonol	Totarol	*P. acnes* and Gram-positive bacteria	55
Rabdosia trichocarpa	Terpene	Trichorabdal	*Helicobacter pylori*	56
Rhamnus purshiana	Polyphenols	Tannins	Viruses, broad spectrum[a]	–
Satureja montana	Terpenoid	Carvacrol	Broad spectrum[a]	
Vaccinium spp.	Monosaccharide	Fructose	*Escherichia coli*	57
Vicia faba	Thionin	Fabatin	Bacteria	–
Vinca minor	Alkaloid	Reserpine	Broad spectrum[a]	–
Curcuma longa	Terpenoids	Curcumin	Protozoa and bacteria	58
Aloysia tripphylla	Essential oil	Terpenoid	*Ascaris* sp.	–
Mentha piperita	Terpenoids	Menthol	Broad spectrum[a]	–
Artemisia dracunlus	Polyphenols	Tannins	Helminthes and viruses	48

[a] Active against Bacteria (Gram + and Gram –) and Fungi

It has been proved that the effectiveness of the antibacterial agent generally increases with its lipophilic properties as a result of the action on cytomembranes. On the other hand, essential oils usually express low aqueous solubility, which prevents them from reaching a toxic level in cytomembranes, even if the oils have quite good affinity with the membranes. Some oil components of phenolic nature (e.g. carvacrol and thymol) cause a disruption of the lipopolysaccharide outer layer followed by partial disintegration of the outer membrane.

The mechanism of action of essential oils and other bioactive phytocompounds towards microorganisms is complex and has not yet been fully explained. It is generally recognized that the antimicrobial action of essential oils depends on their hydrophilic or lipophilic character. Terpenoids may serve as an example of lipid-soluble agents that affect the activities of membrane-catalyzed enzymes, for example their action on respiratory pathways. Certain components of essential oils can act as uncouplers, which interfere with proton translocation over a membrane vesicle and subsequently interrupt ADP phosphorylation (primary energy metabolism). Specific terpenoids with functional groups, such as phenolic alcohols or aldehydes, also interfere with membrane-integrated or associated enzyme proteins, stopping their production or activity.

Recent scientific research has shown that many plants used as food or in traditional medicine are potentially toxic, causing allergic processes, intoxication, mutagenic, and carcinogenic. The following plants are highly toxic because they cause both DNA damage and chromosomal aberrations: *Antidesma venosum* E. Mey. ex Tul. (Euphorbiaceae), *Balanities maughamii* Sprague (Balanitaceae), *Catharanthus roseus*, *Catunaregam spinosa* (Thunb.) Tirveng. (Rubiaceae), *Chaetacme aristata*, *Croton sylvaticus* Hochst. (Euphorbiaceae), *Diospyros whyteana* (Hiern) F. White (Ebenaceae), *Euclea divinorum* Hiern (Ebenaceae), *Gardênia volkensii* K. Schum. (Rubiaceae), *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. var. *abyssnica* (A. Rich.) H. Wolff (syn. *Heteromorpha trifoliata* (H.L. Wend.) Eckl., Zeyh.) (Apiaceae), *Hypoxis colchicifolia* Baker (Hypoxidaceae), *Ornithogalum longibractaetum* Jacq. (Hyacinthaceae), *Plumbago auriculata*, *Prunus africana* (Hook. f.) Kalkm. (Rosaceae), *Rhamnus prinoides* L'Hér. (Rhamnaceae), *Ricinus communis*, *Spirostachys africana* Sond. (Euphorbiaceae), *Trichelia emetica* Vahl subsp. *Emetica* (Meliaceae), *Turraea floribunda* Hochst. (Meliaceae), *Vernonia colorata* and *Ziziphus mucronata*.

In an extensive screening program of plants used in traditional medicine, researchers provided scientific evidence for their rational use in treating infections and diseases, inflammation, and disorders of the central nervous system. Using the ethnobotanical approach and bioassay-guided fractionation, several compounds with biological activity were isolated and identified. Genotoxicity studies also showed that several plants used for medicinal purposes cause damage to the genetic material and, therefore, should be used with caution.

In vitro screening programm, using the ethnobotanical approach, are important in validating the traditional use of herbal remedies and for providing leads in the search for new active principles. Whereas activity identified by an *in vitro* test does not necessarily confirm that a plant extract is an effective medicine, nor a suitable

candidate for drug development, it does provide basic understanding of a plant's efficacy and, in some cases toxicity.

The nonprescription use of medicinal plants is cited today as an important health problem, in particular their toxicity to the kidneys. Several factors, such as active uptake by tubular cells and high concentration in the medullary interstitium, make the kidneys particularly vulnerable to toxic substances that may be present in plant preparations; the risk of kidney injury is even higher in renal patients. For instance, they may contain underestimated amounts of potassium, interact with drugs used for the treatment of renal diseases, or have vasoconstrictive properties.

The use of traditional plant remedies has been implicated in 35% of all cases of acute renal failure in Africa [59–63]. Precise identities of the culprit substances are mainly unknown, as well as the toxicological characteristics and pathogenetic mechanisms involved. Most data published are case reports, with no clear identification of the herbal product involved in the renal toxic effect. Various renal syndromes have been reported after the use of medicinal plants. They include acute tubular necrosis, acute interstitial nephritis, Fanconi's syndrome, hypokalemia, hypertension, papillary necrosis, chronic interstitial nephritis, nephrolithiasis, urinary retention, and cancer of the urinary tract. Conversely, herbal medicine also may be hazardous for renal patients because it may interact with such drugs as cyclosporine or carry significant amounts of potassium.

1.7 Bioactive Phytocompounds and Future Perspectives

The integration of herbal medicine into modern medical practises, including treatments for infections and cancer, must take into account the interrelated issues of quality, safety, and efficacy [64]. Quality is the paramount issue because it can affect the efficacy and/or safety of the herbal products being used. Current product quality ranges from very high to very low due to intrinsic, extrinsic, and regulatory factors. Intrinsically, species differences, organ specificity, diurnal and seasonal variations can affect the qualitative and quantitative accumulation of active chemical constituents in the source medicinal plants. Extrinsically, environmental factors, field collection methods such as cultivation, harvest, post-harvest transport, and storage, manufacturing practises, inadvertent contamination and substitution, and intentional adulteration are contributing factors to the quality of herbal medicinal products. Source plant materials that are contaminated with microbes, microbial toxins, environmental pollutants, or heavy metals; or finished products that are adulterated with foreign toxic plants or synthetic pharmaceutical agents can lead to adverse events. Substandard source materials or finished products will yield therapeutically less effective agents. Herbal medicine quality can also be attributed to regulatory practises. In a number of countries, herbal medicines are unregulated, which has led to product quality differences.

Product quality improvement may be achieved by implementing control measures from the point of medicinal plant procurement under Good Agricultural Practises (GAPs) and the manufacture of the finished botanical products under Good Manufacturing Practises (GMPs), plus postmarketing quality assurance surveillance. The lack of pharmacological and clinical data on the majority of herbal medicinal products is a major impediment to the integration of herbal medicines into conventional medical practise. For valid integration, pharmacological and especially, clinical studies, must be conducted on those plants lacking such data. Adverse events, including drug–herb interactions, must also be monitored to promote a safe integration of efficacious herbal medicine into conventional medical practises.

For the developing countries, the approval as drugs of standardized and formulated plant extracts might be the starting point of an innovative and successful local pharmaceutical industry, which can compete with the large pharmaceutical companies, not only for the treatment of minor diseases, but also for the treatment of severe and life-threatening diseases. It can be stated that the major activities of natural products research of the past decades have clearly demonstrated that natural products represent an unparalleled reservoir of molecular diversity to drug discovery and development, and are complementary to combinatorial libraries.

The major disadvantage is the time taken to isolate and to characterize the active components from the extracts. By improving the diversity and quality of sample source and screen suitability, by accelerating dereplication and by automating and standardizing early isolation steps, the effectiveness of natural products research can be enhanced. The efforts to establish collaboration between universities and local pharmaceutical companies to produce new medicines with scientific proof of safety, quality and efficacy are relevant to progress in this area. This interaction between the pharmaceutical industry and the universities has in turn stimulated the appearance of preclinical pharmacological studies and of well-controlled and randomized clinical trials to prove their worth. Furthermore, emphasis on domestication, production, and biotechnological studies, followed by genetic improvements to medicinal plants, are other fields of science that emerge from such progress in the use of medicinal plants in the world.

Scientists have dedicated significant efforts to the publishing of both basic and clinical studies on herbal medicines, and thus certainly will create the scientific basis for the physician's prescription of herbal drugs. In spite of this, so far insufficient data exist to provide an accurate assessment of the quality, efficacy, and safety of most of the herbal medicines currently available on the market. For all these reasons, a great effort in training more scientists in the relevant areas is still necessary in order to establish rational and sustainable exploitation of the world's biodiversity.

References

1 Newman D.J., Cragg, G.M., Snader, K.M. *J. Nat. Prod.* **2003**, *6*, 1022–1037.
2 Ganesan, A. *Drug Discov. Today* **2002**, *7*, 47–55.
3 Mehta, G., Singh, V. *Chem. Soc. Rev.* **2002**, *31*, 324–334.
4 Breinbauer R., Vetter, I.R., Waldmann, H. *Angew. Chem. Int. Ed.* **2002**, *41*, 2879–2890.
5 Lee M.L., Schneider, G. *J. Comb. Chem.* **2001**, *3*, 284–289.
6 Bajorath, J. *J. Comput. Aided Mol. Des.* **2002**, *16*, 431–439.
7 Hall, D.G., Manku, S., Wang, F. *J. Comb. Chem.* **2001**, *3*, 125–150.
8 Hook, D.J., Pack, E.J., Yacobucci, J.J., Guss, J. *J. Biomol. Screen.* **1997**, *2*, 145–152.
9 Wolfender, J-L., Rodriguez, S., Hostettmann, K. *J. Chromatogr.* **1998**, *A794*, 299–316.
10 Silva, C.J., Brian, P., Peterson, T. *Drugs Pharmac. Sci.* **2001**, *114*, 357–382.
11 Kuiper, G.G.J.M., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J-Ä. *Endocrinology* **1998**, *139*, 4252–4263.
12 Corley, D.G., and Durley, R.C. *J. Nat. Prod.* **1994**, *57*, 1484–1490.
13 Pannell, L.K., Shigematsu, N. *Am. Lab.* **1998**, *30*, 28–30.
14 Eloff, J.N. *J. Ethnopharmacol.* **1998**, *60*, 1–8.
15 Miles, D.H., Nguyen, C.L., Miles, D.H. *Curr. Med. Chem.* **1998**, *5*, 421–440.
16 Rojas, A., Hernandez, L., Pereda–Miranda, R., Mata, R. *J. Ethnopharmacol.* **1992**, *35*, 275–283.
17 Mazzanti, G., Battinelli, L., Salvatore, G. *Flav. Fragr. J.* **1998**, *13*, 289–294.
18 Lopes, D., Bizzo, H.R., Sobrinho, A.F.S., Pereira, M.V.G. *Oil Res.* **2000**, *12*, 705–708.
19 Grynberg, N. F., Echevarria, A., Lima, J.E., Pamplona, S.S., Pinto, A.C., Maciel, M.A. *Planta Med.* **1999**, *65*, 687–689.
20 Rosa, M.S.S., Mendonça Filho, R.R., Bizzo, H.R. Rodrigues, I.A., Soares, R.M.A., Souto-Padrón, T., Alviano, C.S., Lopes, A.H.C.S. *Antimicrob. Agents Chemother.* **2003**, *47*, 1895–1901.
21 Tassou, C.C., Drosinos, E.H., Nychas, G.J.E. *J. Appl. Bacteriol.* **1995**, *78*, 593–600.
22 Esquenazi, D., Wigg, M.D., Miranda, M.M.F.S., Rodrigues, H.M., Tostes, J.B.F., Rozental, S., Da Silva, A.J.R., Alviano, C.S. *Res. Microbiol.* **2002**, *153*, 647–652.
23 Ibanez, E., Kubatova, A., Senorans, F.J., Cavero, S., Reglero, G., Hawthorne, S.B. *J. Agric. Food Chem.* **2003**, *51*, 375–382.
24 Ju, Z., Howard, L. *J. Agric. Food Chem.* **2003**, *51*, 5207–5213.
25 Awad, R., Arnason, J.T., Trudeau, V.L., Bergeron, C., Budzinski, J.W., Foster, B.C., Merali, *Z. Phytomed.* **2003**, *10*, 640–649.
26 Lewis, W.H., Elvin-Lewis, M.P. *Ann. Mo. Bot. Gard.* **1995**, *82*,16–24.
27 Zhang, Y., Lewis, K. *FEMS Microbiol. Lett.* **1997**, *149*, 59–64.
28 Balzarini, J., Schols, D.J., Neyts, E. Van Damme, W. Peumans., E. De Clercq. *Antimicrob. Agents Chemother.* **1991**, *35*, 410–416.
29 Hui, K.M., Wang, X.H., Xue, H. *Planta Med.* **2002**, *66*, 91–93.
30 Kubo, I., Muroi, H., Himejima, M. *J. Nat. Prod.* **1993**, *56*, 220–226.
31 Moerman, D.E. *J. Ethnopharmacol.* **1996**, *52*, 1–22.
32 Heimhuber, B., Galensa, R., Herrmann, K. *J. Chromatogr.* **1988**, *739*, 481–483.
33 Saxena, G., Towers, G.H.N., Farmer, S., Hancock, R.E.W. *Phytochem. Anal.* **1995**, *6*, 125–129.
34 Wu, A.M., Jiang, Y.J., Hwang, P.Y., Shen, F.S. *Biochim. Biophys. Acta* **1995**, *1243*, 157–160.
35 Schmourlo, G., Mendonça–Filho, R.R., Alviano, C.S., Costa, S.S.C. *J. Ethnopharmacol.* **2005**, *96*, 563–568.
36 Farnsworth N.R. *Herbal Gram* **1993**, *29*, 36A–367H.
37 World Health Organization, *WHO Guidelines for Methodologies on Research and Evaluation of Traditional Medicine*. World Health Organization, Geneva, **2001**.
38 Bannerman, R., Burton, J., Chen, W.C. eds. *Traditional Medicine and Health Care Coverage*. World Health Organization, Geneva, **1983**.

39 Awang, D.V.C. *Food Drug Law J*. **1997**, *52*, 341–344.

40 World Health Organization, Good Manufacturing Practices for pharmaceutical products: main principles. In *WHO Expert Committee on Specifications for Pharmaceutical Preparations*. Thirty-seventh report. WHO Technical Report Series, No. 908. World Health Organization, Geneva, **2003**, Annex 4.

41 Ahmad, I., Beg, A.Z. *J. Ethnopharmacol*. **2001**, *74*, 113–123.

42 Naganawa, R., Iwata, N., Ishikawa, K., Fukuda, H., Fujino, T., Suzuki, A. *Appl. Environ. Microbiol*. **1996**, *62*, 4238–4242.

43 Omulokoli, E., Khan, B., Chhabra, C.S. *J. Ethnopharmacol*. **1997**, *56*, 133–137.

44 Ooshima, T., Minami, T., Aono, W., Izumitani, A., Sobue, S., Fujiwara, T., Kawabata, S., Hamada, S. *Caries Res*. **1993**, *27*, 124–129.

45 Bose, P.K. *J. Indian Chem. Soc*. **1958**, *58*, 367–375.

46 Stange, R.R., Midland, S.L., Eckert, J.W., Sims, J.J. *J. Nat. Prod*. **1993**, *56*, 1627–1629.

47 Freiburghaus, F., Kaminsky, R., Nkunya, M.H.H., Brun, R. *J. Ethnopharmacol*. **1996**, *55*, 1–11.

48 Wild, R. (ed.) *The Complete Book of Natural and Medicinal Cures*. Rodale Press, Emmaus, PA, **1994**.

49 Perrett, S., Whitfield, P.J., Sanderson, L., Bartlett, A. *J. Ethnopharmacol*. **1995**, *47*, 49–54.

50 Wan, J., Wilcock, A., Coventry, M.J. *J. Appl. Microbiol*. **1998**, *84*, 152–158.

51 Kubo, A., Lunde, C.S., Kubo., I. *J. Agric. Food Chem*. **1995**, *43*, 1629–1633.

52 Jones, G.A., McAllister, M.A., Muir, A.D., Cheng, K.J. *Appl. Environ. Microbiol*. **1994**, *60*, 1374–1378.

53 Martinez, M.J., Betancourt, J., Alonso-Gonzalez, N., Jauregui, A. *J. Ethnopharmacol*. **1996**, *52*, 171–174.

54 Ghoshal, S., Krishna Prasad, B.N., Lakshmi, V. *J. Ethnopharmacol*. **1996**, *50*, 167–170.

55 Kubo, I., Muroi, H., Kubo, A. *J. Nat. Prod*. **1994**, *57*, 9–17.

56 Kadota, S., Basnet, P., Ishii, E., Tamura, T., Namba, T. *Zentbl. Bakteriol*. **1997**, *286*, 63–67.

57 Ofek, I., Goldhar, J., Sharon, N. *Adv. Exp. Med. Biol*. **1996**, *408*, 179–183.

58 Apisariyakul A., Vanittanakom, N., Buddhasukh, D. *J. Ethnopharmacol*. **1995**, *49*, 163–169.

59 Seedat, Y.K. *South Afr. Med. J*. **1978**, *54*, 427–431.

60 Ojogwu L.I., Anah C. *East Afr. Med. J*. **1983**, *60*, 478–484.

61 Kadiri S., Ogunlesi A., Osinfade K., Akinkugbe O. *Afr. J. Med. Med. Sci*. **1992**, *21*, 91–96.

62 Randeree, I.G., Czarnocki, A, Moodley, J., Seedat, Y. *Ren. Fail*. **1995**, *17*, 147–153.

63 Adelekun, T.A., Ekwere, T.R., Akinsola, A. *West Afr. J. Med*. **1999**, *18*, 60–63.

64 Schwartsmann, G., Ratain, M.J., Cragg, G.M., Wong, J.E., Saijo, N., Parkinson, D.R., Fujiwara, Y., Pazdur, R., Newman, D.J., Dagher, R., DiLeone, L. *J. Clin. Oncol*. **2002**, *20* (Suppl. 18), 47S–59S.

2
Quality Control, Screening, Toxicity, and Regulation of Herbal Drugs

Wickramasinghe M. Bandaranayake

Summary

Medicinal plants constitute a source of raw materials for both traditional systems of medicine (e.g. Ayurvedic, Chinese, Unani, Homeopathy, and Siddha) and modern medicine. Nowadays, plant materials are employed throughout the industrialized and developing world as home remedies, over-the-counter drugs, and ingredients for the pharmaceutical industry. As such, they represent a substantial proportion of the global drug market. Most rural populations, especially in the developing world, depend on medicinal herbs as their main source of primary health care. Although most medicinal herbs are not, in their natural state, fit for administration, preparations suitable for administration are made according to pharmacopeia directions. The therapeutic potential of a herbal drugs depends on its form: whether parts of a plant, or simple extracts, or isolated active constituents. Herbal remedies consist of portions of plants or unpurified plant extracts containing several constituents, which often work together synergistically.

The herbal drug preparation in its entirety is regarded as the active substance and the constituents are either of known therapeutic activity or are chemically defined substances or group of substances generally accepted to contribute substantially to the therapeutic activity of the drug. Phytochemical screening involves botanical identification, extraction with suitable solvents, purification, and characterization of the active constituents of pharmaceutical importance. Qualitative chemical examination employing different analytical techniques is conducted to detect and isolate the active constituent(s). In general, all medicines, whether they are synthetic or of plant origin, should fulfill the basic requirements of being efficacious and safe. Ultimate proof of these can only be achieved by some form of clinical research. A defined and constant composition of the drug is therefore one of the most important prerequisites for any kind of clinical experiment.

Quality control for the efficacy and safety of herbal products is essential. The quality control of phytopharmaceuticals may be defined as the status of a drug, which is determined either by identity, purity, content, and other chemical, physical or biological properties, or by the manufacturing process. Compared with syn-

thetic drugs, the criteria and the approach for herbal drugs are much more complex.

Phytopharmaceuticals are always mixtures of many constituents and are therefore very variable and difficult to characterize. The active principle(s) in phytopharmaceuticals are not always known. The quality criteria for herbal drugs are based on a clear scientific definition of the raw material. Depending on the type of preparation, sensory properties, physical constants, moisture, ash content, solvent residues, and adulterations have to be checked to prove identity and purity. Microbiological contamination and foreign materials, such as heavy metals, pesticide residues, aflatoxins, and radioactivity, also need to be tested for. To prove the constant composition of herbal preparations, appropriate analytical methods have to be applied and different concepts have to be used in order to establish relevant criteria for uniformity.

Are there rigorous trials to show that herbal treatments work? With many of these herbal medicines we do not fully understand how they work. Nor do we always know which component is pharmacologically active. Even though herbal remedies may be effective, do their benefits outweigh their risks? In some countries herbal remedies are sold as food supplements, thus evading safety regulations. Can herbal medicines save money? Not all plant-based medicines are cheap.

Even though global herbal resources have a great potential as natural drugs and are of great commercial importance, they are very often procured and processed without any scientific evaluation, and launched onto the market without any mandatory safety and toxicology studies because there is no effective machinery to regulate manufacturing practices and quality standards. Although some herbal medicines are efficacious, there is unquestionably a need for more reliable information, a demand that must be met adequately by doctors, pharmacists, and other health care professionals.

Policy and regulation in their use, are two of the most sensitive aspects of developing and using plant-based medicines and health products. At present there is almost no policy worth its name to regulate the procurement and sale of medicinal plants in developing countries. Neither are the products derived from medicinal plants subject to control.

Stringent quality control should be enforced. Growing evidence of effectiveness is counterbalanced by inadequate regulation. The present review will address some of these issues.

2.1 Introduction

Since ancient times humanity has depended on the diversity of plant resources for food, clothing, shelter, and traditional medicine to cure myriads of ailments. Early humans recognized their dependence on nature in both health and illness. Physical evidence of the use of herbal remedies has been found from some 60 000 years ago in a burial site of a Neanderthal man uncovered in 1960 in a cave in northern

Iraq [1]. Here, scientists found great quantities of plant pollen, some of which came from medicinal plants still used today. The first written records detailing the use of herbs in the treatment of illness are in the form of Mesopotamian clay tablet writings and Egyptian papyrus [2]. Led by instinct, taste, and experience, primitive men and women treated illness by using plants, animal parts, and minerals that were not part of their usual diet. Herbal medicine is the oldest form of health care known to humanity and has been used in all cultures throughout history. Primitive people learned by trial and error to distinguish useful plants with beneficial effects from those that were toxic or nonactive, and also which combinations or processing methods had to be used to gain consistent and optimal results. Even in ancient cultures, tribal people methodically collected information on herbs and developed well-defined herbal pharmacopeias. Traditional medicine evolved over centuries, depending on local flora, culture, and religion [3–5]. Indeed, well into the twentieth century, much of the pharmacopeia of scientific medicine was derived from the herbal lore of native people. This knowledge of plant-based drugs developed gradually and was passed on, thus laying the foundation for many systems of traditional medicine all over the world.

Herbal medicine can broadly be classified into a few basic systems:

- Ayurvedic herbalism (derived from the Sanskrit word *ayurveda*, meaning "the science of life"), which originated in India more than 5000 years ago and was also practiced in neighboring countries such as Sri Lanka.
- Chinese herbalism, which is a part of traditional oriental medicine.
- African herbalism.
- Western herbalism, which originated from Greece and Rome and then spread to Europe and North and South America.

Chinese and Ayurvedic herbalism have developed into highly sophisticated systems of diagnosis and treatment over the centuries. Both have a long and impressive history of effectiveness. Western herbalism today is primarily a system of folk medicine. A European healing tradition, sometimes called the "wise woman" also focuses primarily on herbal healing.

Medicinal plants have played a key role in world health. They are distributed worldwide, but they are most abundant in tropical countries. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants [6–23].

By definition, a herb is a plant or a part of a plant valued for its medicinal, aromatic, or savoury qualities. Herbs can be viewed as biosynthetic chemical laboratories, producing a number of chemical compounds. Herbal medicine or herbalism is the use of herbs or herbal products for their therapeutic or medicinal value. They are also referred to as botanicals, biomedicines, or herbal supplements. Herbal drugs range from parts of plants to isolated, purified active constituents. They may come from any part of the plant but are most commonly made from leaves, roots, bark seeds, and flowers. They are eaten, swallowed, drunk, inhaled, or applied to the skin [24].

Typically, there is no one single herb that is recommended for a given health disorder; and there is no one single health disorder linked with just one single herb. Herbal products often contain a variety of biochemicals found naturally in the plants and many different biochemicals contribute to a plant's medicinal benefit. Chemicals known to have medicinal benefits are referred to as "active ingredients," and their presence depends on the plant species, the way the herb is prepared, the time and season of harvest, the type of soil, etc. Most herbal products contain plant parts or plant materials in the crude or processed state as active ingredients and certain excipients, such as solvents, diluents, or preservatives. In most cases, the active principles responsible for their pharmacological action are unknown.

A herb might be considered a "diluted" drug. To achieve the desired benefit, an individual must take an adequate amount over a certain length of time. Each herb is different. While some are safe and effective for specific uses, others are not. The general perception that herbal drugs are very safe and free from side effects is not true. Herbs can produce undesirable side effects and can be toxic. A particular plant part will have many constituents and some of them may well be toxic. However, it may take more to cause toxicity, because herbs usually are not as potent as manufactured drugs, and compared with synthetic drugs the adverse effects of most herbal drugs are relatively infrequent [25–27].

Herbal medicines are very different from well-defined synthetic drugs. For example, the availability and quality of the raw materials are frequently problematic; the active principles are frequently unknown; and standardization, stability, and quality control are feasible but not easy. In comparison with modern medicine, herbal medicines cost less, are more often used to treat chronic diseases, and the occurrence of undesirable side effects seems to be less frequent.

A vast number of plants have medicinal properties; in fact, many pharmaceutical drugs were originally derived from plants. Ethnopharmacology – the scientific study of indigenous medicines – is an interdisciplinary science practiced all over the world. Phytotherapeutic agents or phytomedicines are standardized herbal preparations that contain, as active ingredients, complex mixtures of plant materials in the crude or processed state. One basic characteristic of phytotherapeutic agents is the fact that they normally do not possess an immediate or strong pharmacological action. For this reason, these agents are not suitable for emergency treatment.

During the past decade, there has been increasing acceptance and public interest in natural therapies in both developing and developed countries. Due to poverty and limited access to modern medicine, about four billion people, 80% of the world's population, living in developing countries use herbal medicine as their source of primary health care [25, 28–30]. In these communities, traditional medical practice is often viewed as an integral part of their culture.

In the West, people are attracted to herbal therapies for many reasons, the most important reason being that, like our ancestors, we believe they will help us live healthier lives. Herbal medicines are often viewed as a balanced and moderate approach to healing. Individuals who use them as home remedies and over-the-counter drugs spend billions of dollars on herbal products. As such, they represent a substantial proportion of the global drug market [16, 19–21, 23, 24, 27, 28, 31–36].

This recent resurgence of interest in plant remedies has been spurred on by several factors [21, 23, 26, 31]:

- The effectiveness of plant medicines.
- The preference of consumers for natural therapies, a greater interest in alternative medicines and a commonly held erroneous belief that herbal products are superior to manufactured products.
- A dissatisfaction with the results from synthetic drugs and the belief that herbal medicines might be effective in the treatment of certain diseases where conventional therapies and medicines have proven to be inadequate.
- The high cost and side effects of most modern drugs.
- Improvements in the quality, efficacy, and safety of herbal medicines with the development of science and technology.
- Patients' belief that their physicians have not properly identified the problem; hence they feel that herbal remedies are another option.
- A movement towards self-medication.

Medicinal plants provide the raw materials for the pharmaceutical industry. Indeed, about 25% of the prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material. Many pharmacological classes of drugs include a natural product prototype. Aspirin, atropine, morphine, quinine are just a few of the drugs that were originally discovered through the study of traditional cures and folk knowledge of indigenous people [37]. Herbal therapies, on the other hand, consist of the chemical components of a plant as they occur naturally [8]. Some are made from plant extracts, others are synthesized to mimic a natural plant compound. Pharmaceutical drugs derived from plants are made by isolating the active chemicals and concentrating them to the medication. Pharmacognosy is the scientific study of drugs from natural products.

In most countries herbal products are launched into the market without proper scientific evaluation, and without any mandatory safety and toxicological studies. There is no effective machinery to regulate manufacturing practices and quality standards. Consumers can buy herbal products without a prescription and one might not recognize the potential hazards in an inferior product. A well-defined and constant composition of the drug is therefore one of the most important prerequisites for the production of a quality drug. Given the nature of products of plant origin, which by definition are never constant and are dependent on and influenced by many factors, quality control plays a significant role for the industry to thrive and be successful [38, 39].

2.2 Preparation of Herbal Drugs

Herbal therapies are usually prepared by grinding or steeping the parts of a plant that are believed to contain medicinal properties. The ground plant matter is called the "macerate." The macerate is soaked in a liquid referred to as the "menstruum" in order to extract the active ingredients. Herbal infusions are prepared by treating

the herb with water or alcohol (ethanol) or mixtures of the two; coarsely bruised drug boiled in water for a definite period is known as a decoction and tinctures are solutions of the active principles of the drug in alcohol and water. This extraction process leads to the production of the herbal preparations in the form of fresh juice, hot and cold infusions, decoctions, tinctures, pastes, and powders referred to as "pulverata." The resulting therapies come in several forms, including oral tablets, capsules, gel caps, extracts, and infusions. Solid or powdered extracts are prepared by evaporation of the solvents used in the process of extraction of the raw material. Some phytotherapeutic agents are greatly concentrated in order to improve their therapeutic efficacy. In this process, it is possible to remove some secondary metabolites present in the plants, which may produce undesirable side effects [40]. The extracts also contain marker compounds which are, by definition, chemically defined constituents that are of interest for control purposes, independent of whether they have any therapeutic activity or not.

2.3 Quality Control of Herbal Drugs

Quality control for efficacy and safety of herbal products is of paramount importance [14–16, 19, 20, 41–45]. Quality can be defined as the status of a drug that is determined by identity, purity, content, and other chemical, physical, or biological properties, or by the manufacturing processes. Quality control is a term that refers to processes involved in maintaining the quality and validity of a manufactured product. For the quality control of a traditional medicine, the traditional methods are procured and studied, and documents and the traditional information about the identity and quality assessment are interpreted in terms of modern assessment. In general, all medicines, whether they are of synthetic or of plant origin, should fulfill the basic requirements of being efficacious and safe, and this can be achieved by suitable clinical trials. This applies both to the multinational pharmaceutical company conducting a multi-center, double-blind placebo-controlled study with a herbal extract, and to the health practitioner in a rural village who applies a locally produced herbal mixture.

Natural products in medicine constitute a vast array of "raw materials," making clear definitions important. Quality criteria are based on clear scientific definitions of the raw material. The term "herbal drugs" denotes plants or plant parts that have been converted into phytopharmaceuticals by means of simple processes involving harvesting, drying, and storage [46]. Hence they are capable of variation. This variability is also caused by differences in growth, geographical location, and time of harvesting. A practical addition to the definition is also to include other crude products derived from plants, which no longer show any organic structure, such as essential oils, fatty oils, resins, and gums. Derived or isolated compounds in the processed state such as extracts or even isolated purified compounds (e.g. strychnine from *Strychnos nux-vomica*) or mixtures of compounds (e.g. abrin from *Abrus precatorius*) are, as a rule, not included in the definition. Combinations with chemical-

ly defined active substances or isolated constituents, and homeopathic preparations which frequently contain plants, are not regarded as herbal medicines. Their production is already based on adequate quality control of the respective starting materials. The following paragraphs will focus on quality control of herbal drugs in compliance with the above definition.

In general, quality control is based on three important pharmacopeial definitions:

- Identity: Is the herb the one it should be?
- Purity: Are there contaminants, e.g., in the form of other herbs which should not be there?
- Content or assay: Is the content of active constituents within the defined limits?

It is obvious that the content is the most difficult one to assess, since in most herbal drugs the active constituents are unknown. Sometimes markers can be used which are, by definition, chemically defined constituents that are of interest for control purposes, independent of whether they have any therapeutic activity or not [46, 47]. To prove identity and purity, criteria such as type of preparation sensory properties, physical constants, adulteration, contaminants, moisture, ash content and solvent residues have to be checked. The correct identity of the crude herbal material, or the botanical quality, is of prime importance in establishing the quality control of herbal drugs.

Identity can be achieved by macro- and microscopical examinations. Voucher specimens are reliable reference sources. Outbreaks of diseases among plants may result in changes to the physical appearance of the plant and lead to incorrect identification [40, 48]. At times an incorrect botanical quality with respect to the labeling can be a problem. For example, in the 1990s, a South American product labeled as "Paraguay Tea" was associated with an outbreak of anticholinergic poisoning in New York. Subsequent chemical analysis revealed the presence of a class of constituents that was different from the metabolites normally found in the plant from which Paraguay tea is made [49].

Purity is closely linked with the safe use of drugs and deals with factors such ash values, contaminants (e.g. foreign matter in the form of other herbs), and heavy metals. However, due to the application of improved analytical methods, modern purity evaluation also includes microbial contamination, aflatoxins, radioactivity, and pesticide residues. Analytical methods such as photometric analysis, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) can be employed in order to establish the constant composition of herbal preparations. Depending upon whether the active principles of the preparation are known or unknown, different concepts such as "normalization versus standardization" have to be applied in order to establish relevant criteria for uniformity.

Content or assay is the most difficult area of quality control to perform, since in most herbal drugs the active constituents are not known. Sometimes markers can be used. In all other cases, where no active constituent or marker can be defined for the herbal drug, the percentage extractable matter with a solvent may be used

as a form of assay, an approach often seen in pharmacopeias. The choice of the extracting solvent depends on the nature of the compounds involved, and might be deduced from the traditional uses. For example, when a herbal drug is used to make a tea, the hot water extractable matter, expressed as milligrams per gram of air-dried material, may serve this purpose [18, 50].

A special form of assay is the determination of essential oils by steam distillation. When the active constituents (e.g. sennosides in *Senna*) or markers (e.g. alkydamides in *Echinacea*) are known, a vast array of modern chemical analytical methods such as ultraviolet/visible spectroscopy (UV/VIS), TLC, HPLC, GC, mass spectrometry (MS), or a combination of GC and MS (GC/MS), can be employed [51].

Several problems not applicable to synthetic drugs influence the quality of herbal drugs:

- Herbal drugs are usually mixtures of many constituents.
- The active principle(s) is (are), in most cases unknown.
- Selective analytical methods or reference compounds may not be available commercially.
- Plant materials are chemically and naturally variable.
- Chemo-varieties and chemo cultivars exist.
- The source and quality of the raw material are variable.
- The methods of harvesting, drying, storage, transportation, and processing (for example, mode of extraction and polarity of the extracting solvent, instability of constituents, etc.) have an effect.

Strict guidelines have to be followed for the successful production of a quality herbal drug. Among them are proper botanical identification, phytochemical screening, and standardization. Quality control and the standardization of herbal medicines involves several steps. The source and quality of raw materials, good agricultural practices and manufacturing processes are certainly essential steps for the quality control of herbal medicines and play a pivotal role in guaranteeing the quality and stability of herbal preparations [32, 35, 36, 47, 52–56].

The quality of a plant product is determined by the prevailing conditions during growth, and accepted Good Agricultural Practices (GAP) can control this. These include seed selection, growth conditions, use of fertilizers, harvesting, drying and storage. In fact, GAP procedures are, and will be, an integral part of quality control. Factors such as the use of fresh plants, age and part of plant collected, period, time and method of collection, temperature of processing, exposure to light, availability of water, nutrients, drying, packing, transportation of raw material and storage, can greatly affect the quality, and hence the therapeutic value of herbal medicines. Apart from these criteria, factors such as the method of extraction, contamination with microorganisms, heavy metals, and pesticides can alter the quality, safety, and efficacy of herbal drugs. Using cultivated plants under controlled conditions instead of those collected from the wild can minimize most of these factors [36, 38, 57–59].

Sometimes the active principles are destroyed by enzymic processes that continue for long periods from collection to marketing, resulting in a variation of compo-

sition. Thus proper standardization and quality control of both the raw material and the herbal preparations should be conducted.

Standardization involves adjusting the herbal drug preparation to a defined content of a constituent or a group of substances with known therapeutic activity by adding excipients or by mixing herbal drugs or herbal drug preparations. Botanical extracts made directly from crude plant material show substantial variation in composition, quality, and therapeutic effects. Standardized extracts are high-quality extracts containing consistent levels of specified compounds, and they are subjected to rigorous quality controls during all phases of the growing, harvesting, and manufacturing processes. No regulatory definition exists for standardization of dietary supplements. As a result, the term "standardization" may mean many different things. Some manufacturers use the term standardization incorrectly to refer to uniform manufacturing practices; following a recipe is not sufficient for a product to be called standardized. Therefore, the presence of the word "standardized" on a supplement label does not necessarily indicate product quality. When the active principles are unknown, marker substance(s) should be established for analytical purposes and standardization. Marker substances are chemically defined constituents of a herbal drug that are important for the quality of the finished product. Ideally, the chemical markers chosen would also be the compounds that are responsible for the botanical's effects in the body.

There are two types of standardization. In the first category, "true" standardization, a definite phytochemical or group of constituents is known to have activity. Ginkgo with its 26% ginkgo flavones and 6% terpenes is a classic example. These products are highly concentrated and no longer represent the whole herb, and are now considered as phytopharmaceuticals. In many cases they are vastly more effective than the whole herb. However the process may result in the loss of efficacy and the potential for adverse effects and herb–drug interactions may increase. The other type of standardization is based on manufacturers guaranteeing the presence of a certain percentage of marker compounds; these are not indicators of therapeutic activity or quality of the herb.

In the case of herbal drug preparations, the production and primary processing of the medicinal plant or herbal drug has a direct influence on the quality of the active pharmaceutical ingredients (APIs). Due to the inherent complexity of naturally growing medicinal plants and the limited availability of simple analytical techniques to identify and characterize the active constituents solely by chemical or biological means, there is a need for an adequate quality assurance system. This assurance is also required during cultivation, harvesting, primary processing, handling, storage, packaging, and distribution. Deterioration and contamination through adulteration, especially microbial contamination, can occur at any one of these stages. It is extremely important to establish good agricultural, harvesting, and manufacturing practices for herbal starting materials in order to minimize these undesirable factors.

In this regard producers, processors, and traders of medicinal plants or herbal drugs have an obligation and a role to play. The manufacturers and suppliers of herbal products should adhere to quality control standards and good manufactur-

ing practices. Currently, only a few manufacturers adhere to complete quality control and good manufacturing procedures including microscopic, physical, chemical, and biological analysis. Organizations such as Health Canada help safeguard Canadians' health by carrying out premarket reviews of all drugs before they are authorized for sale. The products available in the market are analyzed regularly to ensure that they are free of unsafe ingredients and that the products actually contain the ingredients indicated on the labels.

The potency and quality of an individual herbal product may be unclear because of lack of regulation. It is obvious that for a given plant product its quality will also be determined by the prevailing conditions during the growth cycle of the plant. Therefore, for cultivated plants the GAP system has been introduced, under which each step, including seed selection, growing conditions, use of fertilizers, and optimization of harvest time, harvesting, and drying, has to adhere to a set of criteria. It is likely that GAP procedures will become an integral part of quality control in the near future.

2.3.1 Parameters for Quality Control of Herbal Drugs

2.3.1.1 Microscopic Evaluation

Quality control of herbal drugs has traditionally been based on appearance and today microscopic evaluation is indispensable in the initial identification of herbs, as well as in identifying small fragments of crude or powdered herbs, and detection of foreign matter and adulterants. A primary visual evaluation, which seldom needs more than a simple magnifying lens, can be used to ensure that the plant is of the required species, and that the right part of the plant is being used. At other times, microscopic analysis is needed to determine the correct species and/or that the correct part of the species is present. For instance, pollen morphology may be used in the case of flowers to identify the species, and the presence of certain microscopic structures such as leaf stomata can be used to identify the plant part used. Although this may seem obvious, it is of prime importance, especially when different parts of the same plant are to be used for different treatments. Stinging nettle (*Urtica urens*) is a classic example where the aerial parts are used to treat rheumatism, while the roots are applied for benign prostate hyperplasia [60].

2.3.1.2 Determination of Foreign Matter

Herbal drugs should be made from the stated part of the plant and be devoid of other parts of the same plant or other plants. They should be entirely free from moulds or insects, including excreta and visible contaminant such as sand and stones, poisonous and harmful foreign matter and chemical residues. Animal matter such as insects and "invisible" microbial contaminants, which can produce toxins, are also among the potential contaminants of herbal medicines [54–56]. Macroscopic examination can easily be employed to determine the presence of foreign matter, although microscopy is indispensable in certain special cases (for example,

starch deliberately added to "dilute" the plant material). Furthermore, when foreign matter consists, for example, of a chemical residue, TLC is often needed to detect the contaminants [17, 19, 60].

2.3.1.3 Determination of Ash

To determine ash content the plant material is burnt and the residual ash is measured as total and acid-insoluble ash. Total ash is the measure of the total amount of material left after burning and includes ash derived from the part of the plant itself and acid-insoluble ash. The latter is the residue obtained after boiling the total ash with dilute hydrochloric acid, and burning the remaining insoluble matter. The second procedure measures the amount of silica present, especially in the form of sand and siliceous earth [60].

2.3.1.4 Determination of Heavy Metals

Contamination by toxic metals can either be accidental or intentional. Contamination by heavy metals such as mercury, lead, copper, cadmium, and arsenic in herbal remedies can be attributed to many causes, including environmental pollution, and can pose clinically relevant dangers for the health of the user and should therefore be limited [42, 60–62]. The potential intake of the toxic metal can be estimated on the basis of the level of its presence in the product and the recommended or estimated dosage of the product. This potential exposure can then be put into a toxicological perspective by comparison with the so-called Provisional Tolerable Weekly Intake values (PTWI) for toxic metals, which have been established by the Food and Agriculture Organization of the World Health Organization (FAO-WHO) [14, 15, 48].

A simple, straightforward determination of heavy metals can be found in many pharmacopeias and is based on color reactions with special reagents such as thioacetamide or diethyldithiocarbamate, and the amount present is estimated by comparison with a standard [41]. Instrumental analyses have to be employed when the metals are present in trace quantities, in admixture, or when the analyses have to be quantitative. The main methods commonly used are atomic absorption spectrophotometry (AAS), inductively coupled plasma (ICP) and neutron activation analysis (NAA) [63, 51, 64].

2.3.1.5 Determination of Microbial Contaminants and Aflatoxins

Medicinal plants may be associated with a broad variety of microbial contaminants, represented by bacteria, fungi, and viruses. Inevitably, this microbiological background depends on several environmental factors and exerts an important impact on the overall quality of herbal products and preparations. Risk assessment of the microbial load of medicinal plants has therefore become an important subject in the establishment of modern Hazard Analysis and Critical Control Point (HACCP) schemes.

Herbal drugs normally carry a number of bacteria and molds, often originating in the soil. Poor methods of harvesting, cleaning, drying, handling, and storage may also cause additional contamination, as may be the case with *Escherichia coli* or *Salmonella* spp. While a large range of bacteria and fungi are from naturally occurring microflora, aerobic spore-forming bacteria frequently predominate.

Laboratory procedures investigating microbial contaminations are laid down in the well-known pharmacopeias, as well as in the WHO guidelines [17, 65]. Limit values can also be found in the sources mentioned. In general, a complete procedure consists of determining the total aerobic microbial count, the total fungal count, and the total Enterobacteriaceae count, together with tests for the presence of *Escherichia coli*, *Staphylococcus aureus*, *Shigella*, and *Pseudomonas aeruginosa* and *Salmonella* spp. The European Pharmacopoeia also specifies that *E. coli* and *Salmonella* spp. should be absent from herbal preparations [66]. However it is not always these two pathogenic bacteria that cause clinical problems. For example, a fatal case of listeriosis was caused by contamination of alfalfa tablets with the Gram-positive bacillus *Listeria monocytogenes* [67].

Materials of vegetable origin tend to show much higher levels of microbial contamination than synthetic products and the requirements for microbial contamination in the European Pharmacopoeia allow higher levels of microbial contamination in herbal remedies than in synthetic pharmaceuticals. The allowed contamination level may also depend on the method of processing of the drug. For example, higher contamination levels are permitted if the final herbal preparation involves boiling with water [66].

The presence of fungi should be carefully investigated and/or monitored, since some common species produce toxins, especially aflotoxins. Aflatoxins in herbal drugs can be dangerous to health even if they are absorbed in minute amounts [65, 68]. Aflatoxin-producing fungi sometimes build up during storage [61]. Procedures for the determination of aflatoxin contamination in herbal drugs are published by the WHO [65]. After a thorough clean-up procedure, TLC is used for confirmation.

In addition to the risk of bacterial and viral contamination, herbal remedies may also be contaminated with microbial toxins, and as such, bacterial endotoxins and mycotoxins, at times may also be an issue [61, 69–72]. There is evidence that medicinal plants from some countries may be contaminated with toxigenic fungi (*Aspergillus*, *Fusarium*). Certain plant constituents are susceptible to chemical transformation by contaminating microorganisms.

Withering leads to enhanced enzymic activity, transforming some the constituents to other metabolites not initially found in the herb. These newly formed constituent(s) along with the molds such as *Penicillium nigricans* and *P. jensi* may then have adverse effects [61].

2.3.1.6 **Determination of Pesticide Residues**

Even though there are no serious reports of toxicity due to the presence of pesticides and fumigants, it is important that herbs and herbal products are free of these chemicals or at least are controlled for the absence of unsafe levels [61]. Her-

bal drugs are liable to contain pesticide residues, which accumulate from agricultural practices, such as spraying, treatment of soils during cultivation, and administering of fumigants during storage. However, it may be desirable to test herbal drugs for broad groups in general, rather than for individual pesticides. Many pesticides contain chlorine in the molecule, which, for example, can be measured by analysis of total organic chlorine. In an analogous way, insecticides containing phosphate can be detected by measuring total organic phosphorus.

Samples of herbal material are extracted by a standard procedure, impurities are removed by partition and/or adsorption, and individual pesticides are measured by GC, MS, or GC/MS. Some simple procedures have been published by the WHO [17, 43, 65] and the European Pharamacopoeia has laid down general limits for pesticide residues in medicine [48, 60, 66, 73, 74].

2.3.1.7 **Determination of Radioactive Contamination**

There are many sources of ionization radiation, including radionuclides, occurring in the environment. Hence a certain degree of exposure is inevitable. Dangerous contamination, however, may be the consequence of a nuclear accident. The WHO, in close cooperation with several other international organizations, has developed guidelines in the event of a widespread contamination by radionuclides resulting from major nuclear accidents. These publications emphasize that the health risk, in general, due to radioactive contamination from naturally occurring radio nuclides is not a real concern, but those arising from major nuclear accidents such as the nuclear accident in Chernobyl, may be serious and depend on the specific radionuclide, the level of contamination, and the quantity of the contaminant consumed. Taking into account the quantity of herbal medicine normally consumed by an individual, they are unlikely to be a health risk. Therefore, at present, no limits are proposed for radioactive contamination [60, 61, 65].

2.3.1.8 **Analytical Methods**

Published monographs in a pharmacopeia are the most practical approach for quality control of herbal drugs and there are many available [15, 17, 18, 41, 43, 45, 55, 75]. When pharmacopeial monographs are unavailable, development and validation of analytical procedures have to be carried out by the manufacturer. The best strategy is to follow closely the pharmacopeial definitions of identity, purity, and content or assay. Valuable sources for general analytical procedures are included in the pharmacopeias, in guidelines published by the WHO [60, 65, 76]. Additional information, especially on chromatographic and/or spectroscopic methods can be found in the general scientific literature. The plant or plant extract can be evaluated by various biological methods to determine pharmacological activity, potency, and toxicity. A simple chromatographic technique such as TLC may provide valuable additional information to establish the identity of the plant material. This is especially important for those species that contain different active constituents.

Qualitative and quantitative information can be gathered concerning the presence or absence of metabolites or breakdown products [60].

TLC fingerprinting is of key importance for herbal drugs made up of essential oils, resins, and gums, which are complex mixtures of constituents that no longer have any organic structure. It is a powerful and relatively rapid solution to distinguish between chemical classes, where macroscopy and microscopy will fail. Chromatograms of essential oils, for example, are widely published in the scientific literature, and can be of invaluable help in identification.

The instruments for UV-VIS determinations are easy to operate, and validation procedures are straightforward but at the same time precise. Although measurements are made rapidly, sample preparation can be time consuming and works well only for less complex samples, and those compounds with absorbance in the UV-VIS region.

HPLC is the preferred method for quantitative analysis of more complex mixtures. Though the separation of volatile components such as essential and fatty oils can be achieved with HPLC, it is best performed by GC or GC/MS.

The quantitative determination of constituents has been made easy by recent developments in analytical instrumentation. Recent advances in the isolation, purification, and structure elucidation of naturally occurring metabolites have made it possible to establish appropriate strategies for the determination and analysis of quality and the process of standardization of herbal preparations. Classification of plants and organisms by their chemical constituents is referred to as chemotaxonomy. TLC, HPLC, GC, quantitative TLC (QTLC), and high-performance TLC (HPTLC) can determine the homogeneity of a plant extract. Over-pressured layer chromatography (OPLC), infrared and UV-VIS spectrometry, MS, GC, liquid chromatography (LC) used alone, or in combinations such as GC/MS, LC/MS, and MS/MS, and nuclear magnetic resonance (NMR), electrophoretic techniques, especially by hyphenated chromatographies, are powerful tools, often used for standardization and to control the quality of both the raw material and the finished product. The results from these sophisticated techniques provide a chemical fingerprint as to the nature of chemicals or impurities present in the plant or extract [44, 77–79].

Based on the concept of photoequivalence, the chromatographic fingerprints of herbal medicines can be used to address the issue of quality control. Methods based on information theory, similarity estimation, chemical pattern recognition, spectral correlative chromatograms (SCC), multivariate resolution, the combination of chromatographic fingerprints and chemometric evaluation for evaluating fingerprints are all powerful tools for quality control of herbal products.

2.3.1.9 **Validation**

The validation of herbal products is a major public health concern both in developed and resource-poor countries, where a fake businesses selling adulterated herbal medicines are common. In this regard, there is no control by the government agencies, despite the existence of certain guidelines in some individual countries

and those outlined by the WHO. If the herbal products are marketed as therapeutic agents, and irrespective of whether the products really have any positive effects to cure and reduce the severity of the disease, it is necessary to ensure scientific validation and periodic monitoring of the quality and efficacy by drug control administrators.

It is feasible that the introduction of scientific validation would control the production of impure or adulterated herbal products and would eventually ensure their rational use. This could also lead to the regulation of the industry so that only qualified physicians and health providers are allowed to prescribe the medication.

Several of the principal pharmacopeias contain monographs outlining standards for herbal drugs. The major advantage of an official monograph published in a pharmacopeia is that standards are defined and available, and that the analytical procedures used are fully validated. This is of major importance, since validation can be a rather time-consuming process.

By definition, validation is the process of proving that an analytical method is acceptable for its intended purpose for pharmaceutical methods. Guidelines from the United States Pharmacopeia (USPC, 1994–2001), the International Conference on Harmonization (ICH), and the US Food and Drug Administration (FDA) provide a framework for performing such validations. In general, validation investigations must include studies on specificity, linearity, accuracy, precision, range, detection, and quantitative limits, depending on whether the analytical method used is qualitative or quantitative [80]. Also of utmost importance is the availability of standards. For macroscopic and microscopic procedures in general this means that reliable reference samples of the plant must be available. A defined botanical source (e.g. voucher specimens) will normally solve this problem. Standards for chromatographic procedures are less easy to obtain. Characteristic plant constituents, either active or markers, are seldom available commercially. Sometimes an LC/MS approach can be referred to as a mode of characterization. Going one step further, after isolation of such a compound, elucidations to prove its definite structure will not be easy. The method often employed is to use readily available compounds that behave similarly in the chosen chromatographic systems, and to calculate retention values and/or times towards these compounds as a standard.

Qualitative chemical examination is designed to detect and isolate the active ingredient(s). TLC and HPLC are the main analytical techniques commonly used. In cases when active ingredients are not known or too complex, the quality of plant extracts can be assessed by a "fingerprint" chromatogram [81–87].

2.4 Herbal Supplements

A botanical is a plant or part of a plant valued for its medicinal or therapeutic properties, flavor, and/or scent. Herbs are subsets of botanicals. To be classified as a dietary supplement, a botanical must meet the following criteria:

1. It is intended to supplement the diet.
2. It contains one or more dietary ingredients (including amino acids, vitamins, minerals, herbs, or other botanicals, etc.).
3. It is intended to be taken orally as a pill, capsule, tablet, or liquid.
4. It is labeled as being a dietary supplement.

A herbal supplement labeled "Natural" does not mean it is safe or without any harmful effects. Herbal products can act the same way as drugs. Their safety depends on factors such as their chemical make-up, how they work in the body, method of preparation, and dosage. In the US, the FDA regulates herbal and other dietary supplements. This means that they do not have to meet the same standards as drugs and over-the-counter medications, they are not required to be standardized, and no legal or regulatory definitions exist for standardization. As a result, manufacturers are not required to demonstrate the safety and effectiveness of their products before they reach the market. In addition, they do not have to adhere to any of the quality control measures applicable to drugs; hence the composition may vary greatly from one batch to another.

The use of some herbal supplements has been reported to be associated with ailments such as oral manifestations, including swelling, irritation, and bleeding of the tongue. These potential effects of herbal supplements, in conjunction with factors related to regulation restrictions, suggest that the use of these products may be associated with various adverse reactions that can affect health. The active ingredient(s) in many herbal supplements are not known, and some have been found to be contaminated with metals, unlabeled prescription drugs, and microorganisms. Under its current regulatory authority, the FDA can remove a herbal supplement from the market only after it has been shown to be unsafe. There has been an increase in the number of Internet websites that sell and promote herbal supplements. Unfortunately, some of them make inaccurate claims and statements regarding their products and claim unsubstantiated effects in curing disease and disease conditions. In the US, distributors of herbal products are under the jurisdiction of the Federal Trade Commission (FTC), which monitors advertising for truthful statements that do not mislead.

2.5 Adulteration of Herbal Drugs

Direct or intentional adulteration of drugs usually includes practices in which a herbal drug is substituted partially or fully with other inferior products. Due to morphological resemblance to the authentic herb, many different inferior commercial varieties are used as adulterants. These may or may not have any chemical or therapeutic potential. Substitution by "exhausted" drugs entails adulteration of the plant material with the same plant material devoid of the active constituents. This practice is most common in the case of volatile oil-containing materials, where the dried exhausted material resembles the original drug but is free of the

essential oils. Foreign matter such as other parts of the same plant with no active ingredients, sand and stones, manufactured artifacts, and synthetic inferior principles are used as substitutes [29].

The practice of intentional adulteration is mainly encouraged by traders who are reluctant to pay premium prices for herbs of superior quality, and hence are inclined to purchase only the cheaper products. This encourages producers and traders to sell herbs of inferior quality. Rarity of a herbal product is another factor that influences adulteration. Sometimes sale of inferior products may be unintentional. In the absence of proper means of evaluation, an authentic drug partially or fully devoid of the active ingredients may enter the market. Factors such as geographical sources, growing conditions, processing, and storage are all factors that influence the quality of the drug. Deterioration may contribute to indirect adulteration, and crude drugs are often prone to deterioration, especially during storage, leading to the loss of the active ingredients, production of metabolites with no activity and, in extreme cases, the production of toxic metabolites. Physical factors such as air (oxygen), humidity, light, and temperature can bring about deterioration directly or indirectly [88]. These factors, alone or in combination, can lead to the development of organisms such as molds, mites, and bacteria. Oxidation of the constituents of a drug can be brought about by oxygen in the air, causing some products, such as essential oils, to resinify or to become rancid. Moisture or humidity and elevated temperatures can accelerate enzymatic activities, leading to changes in the physical appearance and decomposition of the herb.

Dried herbs are particularly prone to contamination with spores of bacteria and fungi present in the air. Bacterial growth is usually accompanied by the growth of molds, whose presence is evidenced by changes in appearance, break down of the plant material, and smell. Mites, nematode worms, insects/moths, and beetles can also destroy herbal drugs during storage.

Control measures to protect against deterioration include the use of airtight containers made of materials that will not interact physically or chemically with the material being stored. Storage in ventilated, cool, dry areas and periodic spraying of the stored area with insecticides will help to prevent the spread of infestation. Sterilization of crude drugs is achieved by treatment of bulk consignments with ethylene oxide, and methyl bromide under controlled conditions and complying with acceptable limits for toxic residues [29, 47, 88]. World markets from time to time experience wild fluctuations in the price of herbals. One reason for this is indiscriminate harvesting which leads to the extinction of natural populations – still the only source of bioresources. This in turn encourages producers to replace the required herb with other supplements.

2.6
Contamination of Herbal Drugs and Herb–Drug Interactions

Conventional synthetic pharmaceuticals such as synthetic corticosteroids, nonsteroidal anti-inflammatory drugs and other prescription drugs, potent drugs such as

phenylbutazone, in fact examples of almost every therapeutic drug class have been found in certain herbal remedies as contaminants. A recent study by Ramsay et al. found that potent corticosteroids had been deliberately added to herbal creams in order increase their efficacy [89]. This problem is widespread, and occurs in both Oriental and European countries [90–94]. These "adulterated" herbal medicines sometimes result in serious ailments such as acute renal failure [10, 95–99].

Many people, especially those living with HIV/AIDS, use both herbal medicines and prescription drugs. A number of clinically significant interactions between prescribed and herbal medicines have been identified. When these medications are used together, they can interact in the body, causing changes in the way the herbs and/or the drug works. Such changes are called herb–drug interactions. Concurrent use of herbal or homeopathic remedies alongside prescribed or over-the-counter medicines are frequent, and may mimic, magnify, or oppose the effect of the drug [100].

Herb–drug interactions are not chemical interactions between a drug and a herbal component to produce something toxic. Instead, the interactions generally cause either an increase or decrease in the amount of drug in the bloodstream. As with conventional medicines, herbal medicines interact with drugs in two general ways: pharmacokinetically and pharmacodynamically. *Pharmacokinetic* interactions result in alterations in the absorption, distribution, metabolism, or elimination of the drug or natural medicine. These interactions affect drug action by quantitative alterations, either increasing or decreasing the amount of drug available to have an effect. *Pharmacodynamic* interactions cause alterations in the way a drug or natural medicine affects a tissue or organ system. These actions affect drug action in a qualitative way, either through enhancing or antagonizing effects.

Herb–drug interactions change the effectiveness of the treatment, sometimes resulting in potentially dangerous side effects, possibly leading to toxicity, and/or reduced benefits. They can modify the mode of action of the drug, leading to unexpected complications or enhancement of the therapeutic effect, possibly leading to overmedication and an impact on health. Drug interactions are a significant problem in association with the use of St John's wort [101, 102].

The risk of herb–drug interactions is not limited to synthetic drugs. Herbal supplements and certain foods can interact with medications. Unfortunately very little is known about these interactions and there is little available scientific research on herb–drug interactions. When combining herbal therapies with other medications, it is important to watch for potential symptoms and to inform health care providers. It is essential to train doctors to appreciate that drug interactions exist and to emphasize the importance of the need for physicians and naturopathic doctors to work together.

Currently, there is very little information published on herb–drug interactions [103–109]. Controlled clinical studies are needed to clarify and determine their clinical importance and more research is required to define them.

2.7 Toxicity of Herbal Drugs

For several reasons it is not possible to establish absolute safety standards for herbal preparations based solely on epidemiological studies. First, these types of studies would be costly. Second, there is little published data in countries where the major use of medicinal plants occurs and thus general standards based on a limited number of reports would have little meaning. Third, the exact identification of the products implicated in side effects claimed for medicinal plants is usually lacking. In spite of these inadequacies, there are a number of general comments that can be made with regard to avoiding potential serious side effects from herbal medicines.

The definition of "toxic" is ultimately a matter of viewpoint. Traditionally, herbs and herbal products have been considered to be nontoxic and have been used by the general public and traditional medicinal doctors worldwide to treat a range of ailments. The fact that something is natural does not necessarily make it safe or effective. The active ingredients of plant extracts are chemicals that are similar to those in purified medications, and they have the same potential to cause serious adverse effects. Whilst the literature documents severe toxicity resulting from the use of herbs, on many occasions the potential toxicity of herbs and herbal products has not been recognized [108]. In certain countries, such as Taiwan, herbs can be obtained from temples, night markets, street vendors, herbal stores, neighborhoods, or relatives, and from traditional medicine practitioners. Ordinary people recommend the medicines to others without safety considerations. The general public and many practitioners also believe that the herbs are nontoxic. Apparently, this cultural style/concept needs more attention in terms of drug safety education. Herbs and herbal preparations can cause toxic adverse effects, serious allergic reactions, adverse drug interactions, and can interfere with laboratory tests [110–117]. High-risk patients such as the elderly, expectant mothers, children, those taking several medications for chronic conditions, those with hypertension, depression, high cholesterol or congestive heart failure, should be more cautious in taking herbal medicine.

It is axiomatic that pregnancy should be a time of minimal medical intervention, and herbalists in particular regard pregnancy as a "contraindication" to taking herbal medicines [106, 110, 118, 119].

Two kinds of side effects have been reported for herbal medicines. The first, considered to be intrinsic to herbal drugs themselves, is mainly related to predictable toxicity due to toxic constituents of the herbal ingredients and overdosage, and the second is allergy. Many cases of allergic reactions have been reported for herbal drugs. It is impossible to completely eliminate the possibility of any substance, including prescription drugs, herbal remedies, or cosmetics, producing an allergic response in people exposed to them. Herbal medicines do not present any more of a problem in this respect than any other class of widely used foods or drugs.

Based on published reports, the side effects or toxic reactions associated with herbal medicines in any form are rare. This could be due to the fact that herbal

medicines are generally safe, that adverse reactions following their use are underreported, or because the nature of the side effects or minor allergic reactions are such that they are not reported.

Perhaps the major problem with regard to the safety of herbal medicines is related to the manufacturing practice, including contamination, substitution, incorrect preparation and dosage, intentional addition of unnatural toxic substances, interactions involving synthetic prescriptions, drugs, and herbal medicines, either intentional or unintentional mislabeling, and the presence of natural toxic contaminants. Many ordinary foods contain constituents that could be regarded as poisonous. Alpha gliadin produced by gluten in wheat, oats, and rye, the cyanogenic glycosides in many fruit skins and seeds, thiocyanates of the brassica vegetables, and lectins of many pulses including soya and red kidney bean are such examples. Cyanogenetic glycodides present in the kernel of many fruits can undergo gastric hydrolysis, resulting in the release of hydrogen cyanide. Viscotoxins, which are constituents of mistletoe, are both cytoxic and cardiotoxic [101, 120]. Nonetheless, these foods are generally regarded as safe. Similarly, both water and oxygen can kill in excessive amounts! So quantity is often an important consideration.

A number of cases have been reported in the literature in which herbal medicines, used for a number of years with safety, suddenly appear to be unsafe, and to date there has been no satisfactory explanation for these adverse effects.

In this context herbs can be broadly classified into three major categories:

- The *food herbs* – medicines such as peppermint, ginger, garlic, hawthorn, nettles, lemon, and balm are gentle in action, have low toxicity, and are unlikely to cause any adverse response. They can be consumed in substantial quantities over long periods of time without any acute or chronic toxicity. However they may bring about allergic reactions in certain individuals.
- The *medicinal herbs* – these are not daily "tonics" and need to be used with greater knowledge (dosage and rationale for use) for specific conditions (with a medical diagnosis) and usually only for a limited period. They have a greater potential for adverse reactions and in some cases drug interactions. They include aloe vera, black cohosh, comfrey, echinacea, ephedra, ginkgo biloba, ginseng, kava kava, milk thistle, and senna.
- The *poisonous herbs* have a strong potential for either acute or chronic toxicity and should only be prescribed by trained clinicians who understand their toxicology and appropriate use. Fortunately, the vast majority of these herbs are not available to the public and are not sold in health food or herbal stores. Aconite, *Arnica* spp., *Atropa belladonna*, digitalis, datura, male fern, gelsemium, and veratrum are some examples [116].

There are herbs such as *Lobelia* and *Euonymus* spp. that have powerful actions, often causing nausea or vomiting, although they are safe under appropriate conditions. There is also an idiosyncratic grouping of herbs that have been alleged, with some scientific support, to exhibit specific kinds of toxicity. The best known example is the hepatotoxicity of pyrrolizidine alkaloid-containing plants such as *Symphy-*

tum (comfrey), *Dryopteris* (male fern), *Viscum* (mistletoe), and *Corynanthe* (Yohimbe) [9, 121].

2.8 Screening of Herbal Drugs

Once the botanical identity of a herb is established, the next step is phytochemical screening, which involves bioassays, extraction, purification, and characterization of the active constituents of pharmaceutical importance [17, 44, 50, 76]. The herb or herbal drug preparation in its entirety is regarded as the active substance. These constituents are either of known therapeutic activity or are chemically defined substances or a group of substances generally accepted to contribute substantially to the therapeutic activity of a herbal drug. In any program in which the end product is to be a drug, some type of pharmacological screening, or evaluation, must obviously be done.

Pharmacological screening programs are not without problems. Ideally the active principles should be isolated, preferably using bioassay guided isolation processes, which can be problematic. The ideal pharmacological screen would be to identify those extracts or pure compounds that are highly active and nontoxic. Such a screen is rare to find. Failure to duplicate pharmacological results is another problem.

There are many pharmacological screening tests available [87]. In the random selection program of the National Cancer Institute (NCI) in the US, plants are randomly selected, extracted, and the extracts are evaluated against one or more *in vitro* tumor systems and *in vitro* cytotoxicity tests. An extension of this procedure is to isolate metabolites or "active compounds" from the plant that had shown most promising activity and subject them to pharmacological tests. In another approach, plants containing specific types or classes of chemical compounds, for example alkaloids, are tested. Simple tests such as color reactions are carried out on various parts of the plant in the field, and assays are carried out in the laboratories [87]. In terms of cost–benefit ratio, these "shotgun" approaches are considered to be very unsatisfactory.

Another method involves random collection of plants and subjection of their extracts to several broad screening methods and pharmacological tests. The success of this method depends on the number of samples assayed, adequate funding, and appropriate predictable bioassay protocols. Broad-based empirical screening, which is time consuming and expensive, can detect novel activities but is not suited for screening large numbers of samples [29, 81, 82, 122, 123].

Diagnosis by observation, a method introduced by the "father" of medicine, Hippocrates, is still one of the most powerful tools of today's physicians. *In vitro* screening methods, though restricted to the detection of defined activities, are simpler and more useful [124]. Recently, biochemical and receptor–ligand binding assays have gathered momentum. This has been made possible by the increasing availability of human receptors from molecular cloning, and extracts and compounds can be tested for binding directly to the presumed therapeutic target pro-

tein. Clone receptors can be expressed in a functional state linked to receptor proteins in cells such as yeast, and this has been made possible by applications of molecular biology. Combined with automated instrumentation and computer databases, hundreds of such assays can be completed in relatively short periods of time [83, 88, 125–129]. These screening processes are successfully used by international agencies such as the National Cancer Institute (NCI) in the United States and the Central Drug Research Institute in India [29, 124, 130].

The technology of plant medicinal screening processes has even advanced to enzyme isolation. The enzymes that cause the disease are first isolated and the plant extracts are tested to determine if they block enzyme action [131]. An enzyme immunoassay for the quantification of femtomole quantities of therapeutically important alkaloids has been established [132]. Ethanolic extracts, tinctures, and pure plant compounds from commercially available herbs have been analyzed for their *in vitro* cytochrome P450 3A4 (CYP3A4) inhibitory capability via a fluorometric microtiter plate assay. These studies indicate that high-throughput screening methods for assessing CYP3A4 inhibition by natural products have important implications for predicting the likelihood of potential herb–drug interactions [133].

Higher plants contain both mutagens and antimutagens and are susceptible to mutagenesis, but screening programs for the detection of antimutagenesis rarely employ higher plant systems. However, using modified screening tests to detect antimutagenic agents, higher plants have been shown to contain a variety of structurally novel antimutagenic agents [134–136]. Short-term bacterial and mammalian tissue culture systems are the standard methods employed.

2.9 Labeling of Herbal Products

The quality of consumer information about the product is as important as the finished herbal product. Warnings on the packet or label will help to reduce the risk of inappropriate uses and adverse reactions [70]. The primary source of information on herbal products is the product label. Currently, there is no organization or government body that certifies an herb or a supplement as being labeled correctly. It has been found that herbal remedy labels often cannot be trusted to reveal what is in the container. Studies of herbal products have shown that consumers have less than a 50% chance of actually getting what is listed on the label, and published analyses of herbal supplements have found significant differences between what is listed on the label and what is in the bottle. The word "standardized" on a product label is no guarantee of higher product quality, since there is no legal definition of the word "standardized." Consumers are often left on their own to decide what is safe and effective for them and the lack of consistent labeling on herbal products can be a source of consumer frustration.

Certain information such as "the product has been manufactured according to Pharmacopoeia standards," listing of active ingredients and amounts, directions such as serving quantity (dosage) and frequency of intake of the drug, must be in-

cluded on the labels of all herbal products and packages. The label should also indicate the method of extraction and relative amount of macerate and menstruum used, and possible side effects. It should indicate that the product's content has been standardized to contain a particular amount of a specified biochemical constituent. Standardization gives the buyers a measure of potency by which to judge the quality of the product and to compare dosage with those indicated by clinical trials. This will also ensure that the correct herb has been used. In addition to the above information, the label should include the name and origin of the product, its intended use, net quantity of contents, other ingredients such as herbs and amino acids, and additives, for which no daily values have been established, storage conditions, shelf life or expiry date, warnings, disclaimer, and name and address of manufacturer, packer or distributor.

A herb categorized as a nutritional supplement cannot claim any health benefits or "disease claims" on the label, leaving the consumer with little information [137]. Marketing plays a big role in the use of herbal products and the media help significantly to provide information about natural health products. One of the problems with mass media "propaganda" is scientific inconsistency. Unless the packaging contains a medical claim, herbal products are not reviewed by any government agency. Food and drug administrations that regulate prescription drugs only review a herbal product if the item is suspected of being harmful or if the label contains medical claims. Scientists use several approaches to evaluate botanical dietary supplements for their potential health benefits and safety risks, including their history of use and laboratory studies using cell or animal models. Studies involving people can provide information that is relevant as to how botanical dietary supplements are used.

2.10
Policies and Regulations

It is a widely held myth that modern drugs are dangerous foreign chemicals with side effects, while herbals are natural, gentle and safe. The truth is that some herbs can be dangerous and can bring about serious diseases and even lead to death. Unlike conventional drugs, herbal products are not regulated for purity and potency and this could cause adverse effects and can even lead to drug interactions [138, 139]. There are fewer studies on herbal medicines than on conventional drugs, mainly because, unlike synthetic chemicals, herbs cannot be patented, so there is little money to be made by funding such research.

It is important that consumers are made aware of interactions herbs might have with other drugs they are taking. Unfortunately this information is not available with herbals. Herbals are also frequently adulterated with prescription drugs. In certain countries, herbal products used for diagnosis, cure, mitigation, treatment, or prevention of disease are normally treated as drugs, and hence regulated by legislation. However, in most countries, including the United States, such legislation does not exist and in fact, most botanical products are marketed as dietary supple-

ments. Herbal products categorized as nutritional or dietary supplements are not regulated [139–142]. In many countries these medicines are not required to pass any regulatory analysis to be sold as health food supplements.

It is clear that the herbal industry needs to follow strict guidelines and that regulations are needed. The food and drug administrations that regulate prescription drugs only review a herbal product if the item is suspected of being harmful or if the label contains a medical claim. Although research is being done, it is very limited and only a few herbal drugs have been studied adequately by well-controlled clinical trials. Even though evidence should always be presented to support claims of products, most herbs are still marketed with little or no research [24, 36, 54, 137, 143, 144]. To be registered as drugs, these products need to be tested to prove their safety and clinical efficacy. However, so far, few programs have been established to study the safety and efficacy of herbal medicines as originally proposed in the WHO guidelines for the assessment of herbal medicines [27, 44, 53, 146, 147].

The future of herbal drugs is overshadowed by the pervading lack of regulatory control [145, 148–151]. In 1993, the WHO sponsored a symposium on the use of medicinal plants. The result was a standard guideline for the assessment of herbal medicines and a recommendation that governments of the world should protect medicinal plants, improve regulation of herbal medicines, and respect traditional medicine approaches [50, 91–93, 146, 151–153].

More recently the Health Directorate of Canada developed a new regulatory framework for natural health products, which came into effect in January 2004. Among other things, the new regulations call for improved labeling, good manufacturing practices, product and site licensing, and provision of a full range of health claims that will be supported by evidence. However, even in Canada, the only regulatory requirements enforced are that all products intended for medicinal use, including natural health products, are issued a Drug Identification Number (DIN). These numbers are not required for raw materials such as bulk herbs.

In the US, access to herbal medicines is restricted by FDA regulations. Before any new chemical or herbal drug is approved, research must prove that it is both safe and effective. As a result of these restrictions, packages of herbal medicines are labeled as food supplements, which do not require pre-approved testing. Food supplements cannot make any healing claims or issue warnings about potential risks. In the US, plant-based derivatives already appear in a quarter of the prescription medicines produced. However, many other plants with healing properties are shunned by the medical community despite scientific data from other countries showing their effectiveness. The misconception that herbs are old fashioned and unscientific has helped to promote a general distrust of phytotherapy. The American Botanical Council contends that, in many cases, herbal medicines are safer than prescription drugs. According to the Council, herbal medicines react more slowly and often include their own antidotes to counteract any toxic effects [135].

With proper enforcement of regulations, more products that are legitimate will enter the market and the consumers will see justifiable claims on labels. In fact, it is predicted that appropriate regulations will rejuvenate the market in response to growing concerns about the regulatory environment for herbal remedies.

2.11 Trends and Developments

The rationalization of the new multidrug and multitarget concept of therapy in classical medicine is likely to have great implications on the future basic research in phytomedicine and evidence-based phytotherapy. It requires concerted cooperation between phytochemists, molecular biologists, pharmacologists, and clinicians, with the aim of using modern high-tech methods for standardization of phytopreparations, of integrating new molecular biological assays into the screening of plant extracts and plant constituents, and of increasing studies on the efficacy proof of phytopreparations using controlled clinical trials. This should be paralleled or followed by pharmacokinetic and bioavailability studies.

One major concern will be the investigation of the multivalent and multitarget actions of plant constituents and standardized extracts, with the aim of rationalizing the therapeutic superiority of many plant extracts over single isolated constituents.

Increased effort in three major research areas will be crucial: (1) efforts to develop suitable standardization methods for phytopreparations; (2) the integration of molecular biological assays into the screening of plant extracts, single isolated compounds thereof and phytopreparations; and (3) the performance of further placebo-controlled, mono- or double-blind, clinical trials, paralleled or followed by pharmacokinetic and bioavailability studies [154].

Herbs are still marketed without sufficient research but evidence must always be shown to consumers to support claims of products [24, 36, 54, 137, 143, 144]. More clinical studies are needed and doctors, along with other professionals, should work towards on untangling this herbal maze. Standards should be developed for each natural health product and the same regulatory standards that apply to manufactured pharmaceuticals should apply equally to herbal products as well. Unlike conventional drugs, herbal products are not regulated for purity and potency and this could cause adverse effects and drug interactions [108]. Herbal manufacturing processes should be refined in order to improve the purity, safety and quality of products and the herbal industry needs to follow strict guidelines, for herbal products are now classified as medicines. Manufacturers and producers tend to resist these laws because such laws will increase cost, which will have to be passed on to consumers, and thus the appeal or herbal drugs might then be lost. The media help significantly to provide information about natural health products to consumers. One of the biggest problems with many mass media stories today is scientific inconsistency. With proper enforcement of regulations, more products that are legitimate will come to the market and the consumer will see justifiable claims on labels and these regulations will rejuvenate the market. Herbal medicines still have value because they have a long history.

Finally, it is sometimes asked whether natural health food stores require legislation. The answer should be yes. Promoting herbal products for medical conditions should be regulated in a similar fashion to shops that dispense pharmaceutical products.

2.12 Conclusions

Plant materials are used throughout the developed and developing world as home remedies, in over-the-counter drug products, and as raw material for the pharmaceutical industry, and they represent a substantial proportion of the global drug market. Therefore, it is essential to establish internationally recognized guidelines for assessing their quality. Certain herbs have become popular over the years, but the public, medical practitioners, and the media still have a poor understanding of herbal medicine. Evidence is emerging on the dangers of herbs. As in most situations, the truth lies hidden under the media hype, poorly understood science, and exaggerated claims. Seeing herbal medicines as either panaceas or poisons blinds us to the reality that in most cases they are neither! Lack of experience, information, and education about herbs make consumers, physicians, and other orthodox health care providers easy victims of market exploitation and herbal myths.

There is no rational reason behind the tendency to equate "natural" with "harmlessness." The fact that something is natural does not necessarily make it safe or effective. In addition, a lack of knowledge of phytochemistry leads to misinterpretation and misunderstanding. It is very likely that some herbs will have side effects, interact with other medications, and be toxic. Information on isolated constituents should not be applied directly to the whole herb and studies on *in vitro* forms should not be confused with oral administration. The gold standard for proof of efficacy for a medication is the controlled double-blind trial, which can offer proof of activity and effectiveness. In addition to this, well-designed unblended and clinical trials, epidemiological, animal, and phytochemical studies can provide useful information on the herbal drug. It is not uncommon for studies to be carried out on animals and the results extrapolated to humans even though they have different metabolic processes. Many herbs have not been subjected to this type of study. We do not fully understand how many of these herbal medicines work, nor do we know which component is pharmaceutically active. Even though herbal remedies may be effective, do their benefits outweigh the risks?

With rationing looming in virtually all health care systems, the question whether herbal medicines can save money is important. Not all plant medicines are cheap. Botanicals are not patentable (they can be patented for use); hence herbal remedies are not viable candidates for the existing drug approval processes. Pharmaceutical companies will not risk a loss, and herbal producers, especially in developing countries, lack the financial resources even to consider conducting research or seeking approval. In contrast to the United States, many European and Asian countries have taken a more holistic approach to researching the efficacy of herbal remedies.

Companies supplying standardized extracts with the greatest degree of quality control typically offer the highest quality products. Most standardized extracts are currently made under strict guidelines set forth by individual members of the European Community (EC) as well as those proposed by the EC. The EC production of standardized extracts serves as a model for quality control processes for all forms

of herbal preparations. Herbal products and nutritional supplements are not the same. Most herbal remedies in the United Kingdom and the United States are sold as food supplements [138]. Thus, they evade regulation of their safety.

The possibility of herb–drug interactions is important but "under-research" is an issue. The World Health Assembly in resolutions WHA31.33 (1978), WHA40.33 (1987), and WHA42.43 (1989) has emphasized the need to ensure the quality of medicinal plant products by using modern control techniques and applying suitable standards [42, 148, 149]. These resolutions describe a series of tests for assessing the quality of medicinal plant materials. The tests are designed primarily for use in national drug quality control laboratories in developing countries, and complement those described in the international pharmacopeia, which provide quality specifications only for the few plant materials that are included in the WHO Model List of Essential Drugs. This manual does not constitute a herbal pharmacopeia, but a collection of test procedures to support the development of national standards based on local market conditions, with due regard to existing national legislation and national and regional norms [14, 15].

The test procedures cannot take account of all possible impurities. Common sense and good pharmaceutical practice should be applied in deciding whether an unusual substance not detectable by the prescribed tests can be tolerated. The international pharmacopeia provides quality specifications only for the few plant materials that are included in the WHO Model List of Essential Drugs [14, 15, 52, 146].

There is a lack of open interpretation in the area of safety and efficacy, especially for bibliographic studies. Such interpretations are particularly relevant for herbal medicinal products because they have been used for long periods of time, sometimes over centuries, and a wealth of literature is available. It is desirable that this documented knowledge is exploited in order to avoid unnecessary tests with animals and clinical trials. Scientific evaluation of the traditional knowledge is needed. In many societies much of the knowledge resides in the hand of the healers, where oral transmission of information is the unwritten rule. In most cases, the information is not documented. As a result, in many regions, this knowledge is endangered because the younger generation is unwilling to carry on the profession of the elders. Knowledge that has been refined over thousands of years of experimentation with herbal medicine is being lost. A major research opportunity in this field would be to catalogue information on herbal medicines by traditional healers in cultures where these skills are normally transmitted through an apprentice system [141].

Opinion about the safety, efficacy, and the appropriateness of medicinal herbs varies widely among medical and health professionals in countries where herbal remedies are used. In most cases the safety and efficacy of drugs of herbal origin cannot be attributed to one single chemical constituent. Various pharmaceutical particulars, including the production and collection of the starting material and the extraction procedures, need to be assessed. Some professionals, however, accept historical, empirical evidence as the only necessary criterion for the efficacy of herbal medicines. Others would ban all herbal remedies as dangerous or of question-

able value. Herbal medicines have the potential for improving public health at low cost. Phytomedicines, if combined with preventive medical practice, could be a cost-effective, practical way to shift modern health care from treatment to prevention.

Manufacturers and distributors should attempt to certify that the herbal medicines available to the public meet certain standards by answering questions such as: Does the product meet recognized standards of quality? Does the label accurately reflect what is in the product? Is the product reasonably free of contaminants such as heavy metals or pesticides? Was the product produced and packaged under clean and safe conditions? Good housekeeping is required to prove that a product is safe and effective. To obtain this certification, a manufacturer must submit research-based evidence that the product does what it claims to do and that it does so without harming the consumer. Clinical trials should be conducted to establish facts such as average effective dose for any drug, as well as potential side effects a compound may cause. Recommendations on product information such as dosage limits and any warnings should also be supplied to the consumer [69–71].

Two paradigm shifts in medicine characterize the beginning of the twenty-first century: the gradual renunciation of the long-standing reliance on monosubstance therapy in favor of a multidrug therapy and the transition to a new kind of multitarget therapy, through which the interference of drugs with protective, repair, and immunostimulatory mechanisms of the human body, rather than with single disease-causing agents, gains more and more importance. Phytomedicine research has a good chance of contributing to these new strategies through the development of new and better drugs for an evidence-based and rational phytotherapy. One major concern will be to investigate the multivalent and multitarget actions of plant constituents and standardized extracts, with the aim of rationalizing the therapeutic superiority of many plant extracts over single isolated constituents. Phytomedicine and chemosynthetic pharmaceutical research find themselves in a race to develop new medicines, with fewer or no side effects, for therapeutic and preventive application in illness for which causality-based treatments are nonexistent or imperfect [154].

It has now become evident that there is need for a holistic approach to health care, and the untapped potential of traditional medicines should be utilized. However, this will not be easy, as it requires a thorough search for medicinal plants, proper guidelines for their identification, validation of the scientific methods of isolation of active ingredients, preclinical evaluation of their pharmacological and toxicological profiles, and clinical evidence of their usefulness. Clinical trials should be conducted to establish facts such as the average effective dose for any drug, as well as potential side effects a compound may cause. In short, these herbal drugs need to be analyzed in the same way as any modern drug, that is with randomized controlled clinical trials.

As doctors and researchers continue to explore the safety and effectiveness of herbal medicines, more is learned about both their promises and their pitfalls. At the same time, legislators at the national level should continue to press for effective laws to protect consumers from potentially harmful herbal drugs. In the mean

time, your own scrutiny and curiosity are your best protection. Quality control for efficacy and safety of herbal products is of utmost importance. The assurance of the safety of a herbal drug requires monitoring of the quality of the finished product as well as the quality of the consumer information on the herbal remedy.

References

1 Solecki, R.S., Shanidar IV, *Science* **1975**, *190*, 880.
2 Bensky, D., Gamble, A. *Chinese Herbal Medicine: Materia Medica*. Eastland Press, Seattle, **1993**.
3 Cassileth, B.R. *The Alternative Medicine Handbook*. W.W. Norton and Co., New York, **1998**.
4 Lans, C. Harper, T. Georges K., Bridgewater, E. *BMC Complement. Altern. Med*. **2001**, *10*, 1
5 Cragg, G.M., Newman. D.J. *Ann. NY Acad. Sci*. **2001**, *953*, 3.
6 Ackerknecht, E.H. *Therapeutics: from the Primitives to the Twentieth Century*. Hafner Press, New York, **1973**.
7 Majno, G.M. *Healing Hand: Man and Wound in the Ancient World*. Harvard University Press, Cambridge, MA, **1975**.
8 Farnsworth, N.R., Morris, R.W. *Am. J. Pharm. Educ*. **1976**, *148*, 46.
9 Duke, J.A., Martinez, R.V. *Handbook of Ethnobotanicals (Peru)*. CRC Press, Boca Raton, FL, **1994**.
10 De Smet, P.A.G.M. *Drug Safety* **1995**, *13*, 81.
11 Cragg, G.M. Newman, D.J. Snader, K.M. *J. Nat. Prod*. **1997**, *60*, 52.
12 Shu. Y.Z. *J. Nat. Prod*. **1998**, *61*, 1053.
13 Liu, Z., Liu, Y. *J. Herbal Phytother*. **2001**, *1*, 25.
14 WHO. *The International Pharmacopeia*, Vol. 1: *General Methods of Analysis*, 3rd edn. World Health Organization, Geneva, **1979**.
15 WHO. *The International Pharmacopeia*, Vol. 2: *Quality Specifications*, 3rd edn. World Health Organization, Geneva, **1981**.
16 WHO. *The Use of Essential Drugs*. Eighth report of the WHO Expert committee. World Health Organization, Geneva, **1990**.
17 WHO. *Quality Control Methods for Medicinal Plant Materials*, World Health Organization, Geneva, **1998**.
18 WHO. *Guidelines for the Appropriate Use of Herbal Medicines*. WHO Regional Publications, Western Pacific Series No 3, WHO Regional office for the Western Pacific, Manila, **1998**.
19 WHO. *Quality Control Methods for Medicinal Plant Materials*. World Health Organization, Geneva, **1999**.
20 WHO. *WHO Monographs on Selected Medicinal Plants*, Vol. 1, World Health Organization, Geneva, **1999**.
21 WHO. *WHO Monographs on Selected Medicinal Plants*, Vol. 2, World Health Organization, Geneva, **2002**.
22 WHO. *Ancient Remedies, New Disease*. World Health Organization, Geneva, **2002**.
23 WHO. *WHO Global Atlas of Traditional, Complementary and Alternative Medicine*. 2 vol. set. Vol. 1 contains text and Vol. 2, maps. World Health Organization, Geneva, **2005**.
24 Akerele, O. *HerbalGram*. **1993**, *28*, 13.
25 Bisset, N.G. (ed.). *Herbal Drugs and Phytopharmaceuticals*. CRC Press, Boca Raton, FL, **1994**.
26 Calixto, J. B. *Braz. J. Med. Biol. Res*. **2000**, *33*, 179.
27 Pal, S.K., Shukla, Y. *Asian J. Cancer Prev*. **2003**, *4*, 281.
28 Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D., Guo, Z. *World Health Organ*. **1985**, *63*, 965.
29 Mukherjee, P.W. *Quality Control of Herbal Drugs: An Approach to Evaluation of Botanicals*. Business Horizons Publishers, New Delhi, India, **2002**.
30 Bodeker, C., Bodeker, G., Ong, C.K., Grundy, C.K., Burford, G., Shein, K. *WHO Global Atlas of Traditional, Complementary and Alternative Medicine*. World Health Organization, Geneva, **2005**.
31 Kong, J.M., Goh, N.K., Chia, L.S., Chia, T.F. *Acta Pharm. Sinic*. **2003**, *24*, 7.

32 De Smet, P. A. G. M. *Drugs*, **1997**, *54*, 801.

33 Blumenthal, M. *HerbalGram* **1999**, *45*, 68.

34 Grunwald, J. *HerbalGram* **1995**, *34*, 60.

35 Roberts, J.E., Tyler, V.E. *Tyler's Herbs of Choice. The Therapeutic Use of Phytomedicinals*. The Haworth Press, New York, **1997**.

36 Blumenthal, M., Brusse, W.R., Goldberg, A., Gruenwald, J., Hall, T., Riggins, C. W., Rister, R.S. *The Complete German Commission E Monographs. Therapeutic Guide to Herbal Medicines*. The American Botanical Council, Austin, TX, **1998**.

37 Midley, J.M. *Pharm. J*. **1988**, *241*, 358.

38 Bauer, R., Tittel, G. *Phytomedicine*. **1996**, *2*, 193.

39 Baur, R. *Drug Inf. J*. **1998**, *32*, 101.

40 Schier, W., Sachsa, B., Schultze, W. *Dtsch Apoth. Ztg*. **1994**, *134*, 4569.

41 WHO. *The International Pharmacopeia*, Vol. 3: *Quality Specifications for Pharmaceutical Substances, Excipients, and Dosage Forms*, 3rd edn. World Health Organization, Geneva, **1988**.

42 WHO. *Basic Tests for Drugs, Pharmaceutical Substances, Medicinal Plant Materials and Dosage Forms*. World Health Organization, Geneva, **1998**.

43 WHO. *Quality Assurance of Pharmaceuticals: A Compendum of Guidelines and Related Materials*, Vol. 2: *Good Manufacturing Practices and Inspection*. World Health Organization, Geneva, **1996**.

44 WHO. *General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine*. World Health Organization, Geneva, **2002**.

45 EMEA. *Guidelines on Quality of Herbal Medicinal Products/Traditional Medicinal Products*, EMEA/CVMP/814OO Review. European Agency for the Evaluation of Medicinal Products (EMEA), London, **2005**.

46 EMEA. *Quality of Herbal Medicinal Products. Guidelines*. European Agency for the Evaluation of Medicinal Products (EMEA), London, **1998**.

47 WHO. *Quality Control Methods for Medicinal Plant Materials*. World Health Organization, Geneva, **1992**.

48 De Smet. P.A.G.M. *Drug Inf. J*. **1999**, *33*, 717.

49 Hsu, C.K., Leo, P., Shastry, D., Meggs, W., Weisman, R., Hoffman, R.S. *Arch. Intern. Med*. **1995**, *155*, 2245.

50 WHO. *Guidelines for the Assessment of Herbal Medicines*. WHO Technical Report Series, No 863. World Health Organization, Geneva, **1996**.

51 Watson, D.G. *Pharmaceutical Analysis*. Churchill Livingstone, Edinburgh, **1999**.

52 WHO. *Quality Control Methods for Medicinal Plant Materials*. World Health Organization, Geneva, **1988**.

53 WHO. *General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine*. World Health Organization, Geneva, **2000**.

54 WHO. *WHO Guidelines on Good Agricultural and Collection Practices* (GACP). World Health Organization, Geneva, **2003**.

55 WHO. *Guidelines on Good Agricultural and Collection Practices (GACP) for Medicinal Plants*. World Health Organization, Geneva, **2004**.

56 EMEA. *Points to Consider on Good Agricultural and Collection Practice for Starting Materials of Herbal Origin*. EMEA/HMPWP/31/99 Review. European Agency for the Evaluation of Medicinal Products (EMEA), London, **2002**.

57 Eskinazi, D., Blumenthal, M., Farnsworth, N., Riggins, C.W. *Botanical Medicine: Efficacy, Quality, Assurance, and Regulation*. Mary Ann Liebert, New York, **1999**.

58 Keller, K. *Drug Inf. J*. **1996**, *30*, 933.

59 Li, F., Sun, S., Wang, J., Wang, D. *Biomed. Chromatogr*. **1998**, *12*, 78.

60 AOAC. *Official Methods of Analysis of AOAC International*, 18th edn. AOAC International, Gaithersburg, MD, **2005**.

61 De Smet, P.A.G.M., Keller, K. Hansel, R., Chandler, R.F. *Adverse Effects of Herbal Drugs*, Vol. 1. Springer-Verlag, Heidelberg, **1992**.

62 Lazarowych, N., Pekos. P. *Drug Inf. J*. **1998**, *32*, 497.

63 Sarmani, S. B., Abugassa, I., Hamzah, A., Yahya, M.D. *Biol. Trace Elem. Res*. **1999**, *71–72*, 365.

64 Boumans, P.W.J.M. *Inductively Coupled Plasma-emission Spectroscopy Part 1*. John Wiley & Sons, New York, **1999**.

65 WHO. *The WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification 2000–2202* (WHO/PCS/01.5). International Programme on Chemical Safety, World Health Organization, Geneva, **2000**.

66 Anonymous. *European Pharmacopoeia*, 3rd edn. Strasbourg Council of Europe, **1996**.

67 Farber, J.M., Carter, A.O., Varughese, P.V., Ashton, F.E., Ewan, E.P. *New Engl. J. Med.* **1990**, *322*, 338.

68 Kneifel, W., Czech, E., Kopp, B *Planta Med.* **2002**, *68*, 5.

69 De Smet, P.A.G.M., in *Adverse Effects of Herbal Drugs*, De Smet, P.A.G.M., Keller, K., Hansel, R., Chandler, R.F. (eds), Vol. 3. Springer-Verlag, Heidelberg, **1997**, p. 137.

70 De Smet, P.A.G.M., in *Adverse Effects of Herbal Drugs*, , De Smet, P.A.G.M., Keller, K., Hansel, R., Chandler, R.F. (eds), Vol. 3. Springer-Verlag, Heidelberg, **1997**, p. 145.

71 De Smet, P.A.G.M., in *Adverse Effects of Herbal Drugs*, , De Smet, P.A.G.M., Keller, K., Hansel, R., Chandler, R.F. (eds), Vol. 3. Springer-Verlag, Heidelberg, **1997**, p. 129.

72 Sinha, K.K. (ed.). *Mycotoxins in Agriculture and Food Safety*. Marcel Dekker, New York, **1998**.

73 Anonymous. *Deutsches Arzneibuch 2000*. Deutscher Apotheker Verlag, Stuttgart, **2000**.

74 Anonymous. *Momoopatiscen Arzneibuch 2000*. Deutscher Apotheker Verlag, Stuttgart, **2000**.

75 van Valkenburg, J.L.C.H., Bunyapraphatsara, N. (eds). *Plant Resources of South-East Asia 12 (2): Medicinal and Poisonous Plants 2*. Backhuys Publ., Leiden, **2001**.

76 WHO. *The International Pharmacopeia*, Vol. 4: *Tests Methods, and General Requirements*, 3rd edn. World Health Organization, Geneva, **1988**.

77 Rózylo, J.K., Zabinska, A., Matysiak, J., Niewiadomy, A. *J. Chromatogr. Sci.* **2002**, *40*, 581.

78 Bilia, A.R., Bergonzi, M.C., Lazari, D., Vincieri, F.F. *J. Agric. Food Chem.* **2002**, *50*, 5016.

79 Ebel, S., Gigalke, H.J., Voelkl, S. *AMD-HPTLC Analysis of Medicinal Plants*. Proceedings of 4th International Symposium of Instrumental HPTLC, Selvino/Bargamo at Italy, **1987**, p. 113.

80 Ko, R.J., Greenwald, M.S., Loscutoff, S.M., Au, A.M., Appel, B.R., Kreutzer, R.A., Haddon, W.F., Jackson, T.Y., Boo, F.O., Presicek, G. *West. J. Med.* **1996**, *164*, 71.

81 Farnsworth, N.R., Henry, L.K., Svoboda, G.H., Blomster, R.N., Yates, M.J., Euler, K.L. *Lloydia* **1966**, *29*, 101.

82 Farnsworth, N.R., Henry, L.K., Svoboda, G.H., Blomster, R.N., Fong, H.H.S., Quimby, M.W., Yates, M.J. *Lloydia* **1968**, *35*, 35.

83 WHO. *The International Pharmacopeia*, Vol. 4: *Test Methods and General Requirements. Quality Specifications for Pharmaceutical Substances, Excipients, and Dosage Forms*, 3rd edn. World Health Organization, Geneva, **1994**.

84 WHO. *The International Pharmacopeia*, Vol. 2: *Quality Specifications*, 3rd edn. World Health Organization, Geneva, **1983**.

85 *The International Pharmacopeia*, Vol. 4: *Test Methods and General Requirements. Quality Specifications for Pharmaceutical Substances, Excipients, and Dosage Forms*, 3rd edn. World Health Organization, Geneva, **1994**.

86 IPCS. *Pyrrolizidine alkaloids, Health and Safety Guide*. Standards on Chemical Safety Health and Safety Guide No. 26. International Programme on Chemical Safety, Geneva, **1989**.

87 Farnsworth, N.R., Bingel, A.S., in *New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity*. A.H. Wagner, P. Wolff (eds), Proceedings of the First International Congress on Medicinal Plant Research. Springer-Verlag, Berlin, Heidelberg, New York, **1977**.

88 Evans, W.C. *Br. J. Phytother.* **1990**, *1*, 16.

89 Ramsay, H.M., Goddard, W., Gill, S., Moss, C. *Arch. Dis. Child.* **2003**, *88*, 1056.

90 Segasothy, M., Samad, S. *Nephron* **1991**, *59*, 199.

91 Chan, T.Y., Chan, J.C., Tomlinson, B., Critchley, J.A. *Lancet* **1993**, *342*, 1532.

92 Anonymous. *Evaluation of Certain Food Additives and Contaminants*. 22nd Report of the Joint FAO/WHO Expert committee on Food Additives. Technical Report Series 631. World Health Organization, Geneva, **1978**.

93 Anonymous. *Evaluation of Certain Food Additives and Contaminants*. 33rd Report of the Joint FAO/WHO Expert committee on Food Additives. Technical Report Series 776. World Health Organization, Geneva, **1989**.

94 Anonymous. *Evaluation of Certain Food Additives and Contaminants*. 41st Report of the Joint FAO/WHO Expert committee on Food Additives. Technical Report Series 837. World Health Organization, Geneva, **1993**.

95 Ross, J.J.W., Pelders, M.G., De Smet, P.A.G.M. *Pharm. World Sci*. **1999**, *21*, 44.

96 Abt, A.B., Oh, J.Y., Huntington, R.A., Burkhart, K.K. *Arch. Intern. Med*. **1995**, *155*, 211.

97 Nelson, L., Shih, R., Hoffman, R. *J. Toxicol-Clin. Toxicol*. **1995**, *33*, 467.

98 Gertner, E., Marshall, P.S., Filandrinos, D., Potek, A.S., Smith, T.M. *Arthritis Rheum*. **1995**, *38*, 614.

99 Van der Stricht, B.I., Parvais, O.E., Vanhaelen-Fastre, R.J., Vanhaelen, M.H. *BMJ* **1994**, *308*, 1162.

100 Pitetti, R., Singh, S., Hornyak, D. *Pediatr. Emerg. Care*. **2001**, *17*, 165.

101 Herr, S.M. *Herb-Drug Interaction Handbook*, 3rd edn. ISBN 0967877326, **2005**.

102 Barnes, J., Anderson, L.A., Phillipson, J.D. *J. Pharm. Pharmacol*. **2001**, *53*, 583.

103 Smolinske, S.C. *J. Am. Med. Womens Assoc*. **1999**, *54*, 191.

104 Fugh-Berman, A. *Lancet* **2000**, *355*, 144.

105 Meneilly, G. Top ten tips for avoiding drug-herb interactions. *Living* + **2000**, 6. http://www.bcpwa.org/issue6/tentips.htm. Accessed January 2004.

106 Ernst, E. *Eur. J. Pediatr*. **2003**, *162*, 72.

107 Janetzky, K., Morreale, A.P. *Am. J. Health Syst. Pharm*. **1997**, *54*, 692.

108 Jowell. T. *Herbal Medicines*. House of Commons official report (Hansard). **1999**, *26*, 426.

109 Mathews, J. Holisticonline.com 1998–2005. http://www.holistic-online.com/alt_medicine/altmed_definitions.htm. Accessed August **2005**.

110 Moore, B., Adler, R. *Pediatrics*. **2000**, *106*, 600.

111 Florido-Lopez, F., Gonzalez-delgado, P., Desanpedro, B.S., Perez-Miranda, C., De Saavedra, J.M.A., Marinpozo, J.F. *Int. Arch. Allergy Immun*. **1995**, *108*, 170.

112 D'Arcy, P.F. *Adverse Drug React. Toxicol. Rev*. **1991**, *10*, 189.

113 D'Arcy, P.F. *Adverse Drug React. Toxicol. Rev*. **1993**, *12*, 147.

114 Rosenblatt, M., Mindei, J. *N. Engl. J. Med*. **1997**, *336*, 1108.

115 Pillans, P.I. *N. Z. Med. J*. **1995**, *108*, 469.

116 Winston, D., Nvwoti. in *American Herbalism*, Tierra, M. (ed.). The Crossing Press, Freedom, CA, **1992**.

117 McRae. S. *Can. Med. Assoc. J*. **1996**, *155*, 293.

118 Marcus, D.M., Snodgrass., W.R. *Obstet. Gynecol*. **2005**, *105*, 1119.

119 Marcus, D.M., Snodgrass, W.R. *Obstet. Gynecol*. **2005**, *106*, 640.

120 Tomassoni, J., Simone, K. *Curr. Opin. Pediatr*. **2001**, *13*, 162.

121 Brinker, F. *An Introduction to the Toxicology of Common Botanical Medicines*. NCNM (National College of Naturopathic Medicine), Oregon, **1983**. AMA. *AMA Handbook of Poisonous and Injurious Plants*. American Medical Association, Chicago, **1985**.

122 Sharma, M.L., Chandokhe, N., Ray Ghatak, B.J., Jamwal, K.S., Gupta, O.P., Singh, G.B., Ali, M.M., Thakur, R.S., Handa, K.L., Rao, P.R., Jamwal, P.S., Sareen, Y.K. *Indian J. Exp. Biol*. **1978**, *16*, 228.

123 Aswal, B.S., Bhakuni, D.S., Goel, A.K., Kar, K., Mehrotra, B.N. *Indian J. Exp. Biol*. **1984**, *22*, 487.

124 Boyd, N.G. *Princ. Pract. Oncol*. **1989**, *3*, 1.

125 Farnsworth, N.R. *HerbalGram* (Special Suppl.), **1993**, *29*, 36A–36H.

126 Farnsworth, N.R., Loub, W.D., in *Plants: the Potential for Extracting Proteins, Medicines, and Other Useful Chemicals*. Workshop proceedings. OTA-BP-F-23. US Congress, Office of Technology Assessment, Washington, DC, **1983**.

127 Loub, W.D., Farnsworth, N.R., Soejarto, D.D., Quinn, M.L. *J. Chem. Inf. Comput. Sci*. **1985**, *25*, 99.

128 Strosberg, A.D., Marullo, S. *Trends Pharmacol. Sci*. **1992**, *13*, 95.

129 Hobden, A.N., Harris, T.J.R. *Proc. R. Soc. Edin. B*. **1992**, *99*, 37.

130 Cragg, G.M., Boyd, M.R. Drug development at the national cancer institute, in Balick, M.J., Elisabetsky, E., Laird, S.A. (eds) *Medicinal Resources of the Tropical Forest*. Columbia University Press, New York, **1999**.

131 Hallowell, C. *Time* **1997**, *150*, 16.

132 Poulev, A., Deus-Neumann, B., Zenk, M.H. *Planta Med*. **1993**, *59*, 442.

133 Budzinski, J.W., Foster, B.C., Vandenhoek, S., Arnason J.T. *Phytomedicine* **2000**, *7*, 273.

134 Mitscher, L.A., Telikepalli, H., Wang, PB-B, Kuo, S., Shankel, D.M.,. Stewart. S. *Mutat. Res*. **1992**, *267*, 229.

135 Williamson, N. *Am. Hortic*. **1995**, *74*.

136 Tona, L., Kambu, K., Mesia, K., Cimanga, K., Apers, S., De Bruyne, T., Pieters, L., Totte, L., Vlietinck, A.J. *Phytomedicine*. **1999**, *6*, 59.

137 Gaedcke, F., Steinhoff, B. *Herbal Medicinal Products. Scientific and Regulatory Basis for Development, Quality Assurance and Marketing Authorisation*. CRC Press, Boca Raton, **2003**,

138 Snider, S. *FDA Consum*. **1991**, 31.

139 Drew, A., Myers, S.P., *Med. J. Aust*. **1997**, *166*, 538.

140 Wichtl, N.G., Wichtl, M. (eds). *Asian J. Can. Prev*. **2000**.

141 Arvigo, R., Balick, M. *Rainforest Remedies: 100 Healing Herbs of Belize*. Lotus Press, Twin Lakes, WI, **1993**.

142 Rousseaux, C.G., Schachter, H. *Birth Defects Res. B: Dev. Reprod. Toxicol*. **2003**, *68*, 505.

143 Zeisel, S.H. *Science* **1999**, *285*, 1853.

144 Petrovick, P.R., Marques, L.C., Paula, I.C., *J. Ethnopharmacol*. **1999**, *66*, 51.

145 Haden, B.L., Haden, C.R. *Alternative Health Care: The Canadian Directory*. Nobel Agnes Publishing, Toronto, **1997**.

146 Mukherjee, P.K., Verpoorte R. (eds) *GMP for Botanicals: Regulatory and Quality Issues on Phytomedicines*. Business Horizons, New Delhi, **2003**.

147 WHO. *Legal Status of Traditional Medicine and Complementary/Alternative Medicine*. World Health Organization, Geneva, **2002**.

148 WHO. *Bulletin of the World Health Organization. Regulatory Situation of Herbal Medicines. A Worldwide Review*. World Health Organization, Geneva, **1998**.

149 WHO. *Regulatory Situation of Herbal Medicines: a Worldwide Review*. World Health Organization, Geneva, **1998**.

150 WHO. *Guidelines for the Regulatory Assessment of Medicinal Products for Use in Self Medication*. World Health Organization, Geneva, **2002**.

151 WHO. *Research Guidelines for the Safety and Efficacy of Herbal Medicines*. World Health Organization, Geneva, **1993**,

152 Chaudhury R.R. *Herbal Medicine for Human Health*. SEARO, No. 20. World Health Organization, New Delhi, **1992**.

153 Lueng, A. *HerbalGram* **2000**, *48*, 63.

154 Wagner, H. Natural products chemistry and phytomedicine research in the new millennium: new developments and challenges. http://www.arkat-usa.org/ark/journal/2004/I07_Antus/SA-1008R/1008R.asp. Accessed 26 September 2005, **2004**.

3
Herbal Medicines: Prospects and Constraints

Iqbal Ahmad, Farrukh Aqil, Farah Ahmad, and Mohammad Owais

Summary

Herbs and herbal preparations have been used to treat ailments throughout the history of humanity. A World Health Organization (WHO) survey has reported that about 70–80% of the world's population rely chiefly on traditional medicines, mainly of herbal sources, in their primary health care. Towards the end of the twentieth century herbal medicine became more mainstream throughout the world, partly as a result of the recognition of the value of traditional medicinal systems, particularly of Asian origin. We have also seen an increase in the popularity and use of natural remedies in developed countries, including herbs, herbal medicines, over-the-counter health foods, neutraceuticals, harbal medicinal products. The use of herbal medicines is especially prevalent in primary health care and for many chronic diseases. Overall, the world market for herbal medicine and products is increasing rapidly, especially for Chinese, German, and Indian herbal medicines.

Major problems associated with herbal medicine are the lack of standardization, consistency, toxicity, safety, quality, and, in some countries, regulations. The correct identification of herbal materials and pharmacologically active constituents, standardization, pharmacological basis of efficacy, toxicity, clinical and nonclinical trials, adopting Good Agricultural Practices (GAP), Good Sourcing Practices (GSP), Good Manufacturing Practices (GMP), and strict implementation of regulation are needed to improve the acceptability, quality, and possible integration of herbal medicines with modern medicine for the effective management of health problems. These issues are discussed in this chapter.

3.1
Introduction

Herbs and herbal preparations have been used to treat ailments since prehistoric times, and the treatment of various diseases with plant-based medicines has re-

mained an integral part of many cultures across the globe. The World Health Organization (WHO) estimates that 80% of the people living in developing countries almost exclusively use traditional medicine. Such medicines, derived directly or indirectly from plants, constitute 25% of the pharmaceutical arsenal. Herbal medicine has now become mainstream worldwide since the latter part of twentieth century. This is primarily due to the recognition of the value of traditional and indigenous pharmacopeias, the need to make health care affordable for all, and the perception that natural remedies are somehow safer and more efficacious than remedies that are pharmaceutically derived [1].

Over the past two decades we have witnessed two apparently unrelated trends in the biomedical and biotechnological development of medicinal products. There has been rapid development of recombinant DNA technology and related procedures to provide biomedical proteins and related therapeutic drugs, prophylactic vaccines, and diagnostic agents [2]. At the same time the growth in popularity of over-the-counter (OTC) health foods (nutraceuticals) and herbal products has taken a very large share of the health care market [3].

The WHO defines complementary and alternative medicine (CAM) as all forms of health care provision that usually lie outside the official health sector. There are over 100 different therapies available as CAM treatments, but the five discrete clinical disciplines (acupuncture, chiropractic, herbal medicine, homeopathy, and osteopathy) are distinguished by having established foundations of training and professional standards [4]. CAM treatments are recommended for chronic pain affecting the spine, joints, and muscles, for the control of nausea, eczema, and other skin complaints, asthma, cancer, and migraine, etc. [5].

Herbal medicine occupies an important position, with the lowest level (7.6%) of reported adverse effects compared with other CAMs [6]. All over the world, there are numerous therapeutic approaches based on medicines of plant origin. The Chinese and Indian systems of traditional medicine and German phytomedicine are of international importance. Other common traditional therapeutic approaches of regional significance include Indusyunic medicine (Pakistan), Islamic medicine (Middle East), kampo (Japan), Korean medicine (Korea), aromatherapy, herbalism, and homeopathy (European), and botanicals (USA).

WHO guidelines define herbal medicines as finished labeled medicinal products containing an active ingredient that is obtained from the aerial or underground parts of botanicals or other plant materials or their combination [7]. Plant materials include juices, gums, fatty oils, essential oils, and any other substances of this nature. Medicines containing plant material combined with chemically defined isolated constituents of plants are not considered to be herbal medicines. Exceptionally, in some countries herbal medicines may also contain, by tradition, natural organic or inorganic active ingredients that are not of plant origin.

3.1.1 Traditional Systems of Medicine

3.1.1.1 Asian Medicinal System

The most established herbal therapeutic systems are Ayurveda, Unani and Siddha of Indian origin, WU-Hsing (China) and kampo (Japan). Most of the herbal remedies are mixtures of plants, sometimes also containing animal parts and minerals. The basis of preparation is synergistic or additive therapeutic value of the preparation. Under ideal conditions, care is taken by traditionally trained practitioners to identify the ingredients carefully, to harvest the plants at very specific times to ensure appropriate levels of bioactivity, to prepare the remedies under strict rules, and to prescribe them to achieve an appropriate clinical response [8].

3.1.1.2 European Herbalism

European traditional medicine has its roots mostly in ancient Mediterranean civilizations and in plants from abroad. By the nineteenth century some of the medicinal plants had become part of the pharmacopeias of allopathy, naturopathy, and homeopathy. Usually when compounds are isolated and sometimes synthesized their pharmaceutical uses are more carefully regulated [9].

3.1.1.3 Neo-Western Herbalism

In its totality European traditional medicine has matured along with American herbal medicine into Neo-Western herbalism. In this system single plant preparations that have been either selected from formulations found in ancient pharmacopeias or derived from medicinal plants valued in other countries, including those of indigenous origin, are sold alone or as mixtures in an assortment of combinations [8, 10–12].

3.1.2 Modern Phytomedicine

In Europe, most notably in German-speaking countries, one special feature has been the emergence of phytotherapy as a separate therapeutic system based on the traditional usage of plants in medicine and the extraction of active substances from plants. Phytotherapy may be further differentiated as rational phytotherapy (herbal medicinal products) and traditional phytotherapy. In rational phytotherapy appropriate pharmacological investigations and clinical studies in patients have documented the efficacy of the products employed. In traditional phytotherapy, on the other hand, the efficacy of phytopharmaceuticals or herbal teas has not yet been established in that way.

The European Medicine Licensing Agency (EMLA) established the term Herbal Medicinal Products (HMPs) in guidelines related to quality and specifications of products used in rational therapy. HMPs also include products referred to as bo-

Table 3.1 Herbal medicinal products and supplements available in the USA [3].

Therapeutic	*Description*	*Examples*
Drugs New Chemical Entity (NCE)	Mostly single active ingredients, pharmaceuticals originating from plants	Vinblastin, taxol
Botanical drugs	Clinically validated and standardized phytochemical mixtures	None in USA, Several in clinical trails
Dietary supplements/ nutraceuticals	A plant component with health benefit	Garlic, echinacea extract
Functional/medicinal foods	A food engineered or supplemented to provide health benefits	Healthy canola oil, golden rice, edible vaccine

tanicals or botanical drugs in the US or as phytopharmaceuticals in scientific literature [13]. In the USA various forms of herbal medicinal products and herbal supplements are available (Table 3.1) [3].

3.2 Prospects for Herbal Medicine

Herbal medicine and other plant-derived therapeutic or prophylactic products in various forms have been available for many hundreds of years for the treatment of diseases in both Eastern and Western cultures. About one-quarter of marketed orthodox pharmaceutical medicines are either derived from plant sources or from derivatives of secondary plant metabolites. Some of the most economically important pharmaceuticals or their precursors derived from plants as listed by several workers are shown in Table 3.2 [3].

The US Food and Drug Administration (FDA) has published guidelines for standardized multicomponent plant extracts referred as botanical drugs, thus making it possible to market these products under the New Drug Administration (NDA) approved process [16]. Common botanical dietary supplements sold in the USA are *Echinacea purpurea*, *Panax ginseng*, *Serono repens*, *Ginkgo biloba*, *Hypericum perforatum* (St. Johns wort), *Valeriana officinalis*, *Allium sativum*, *Hydrastis canadensis*, *Matricaria chamomilla*, *Silybum marianum*, *Trigonella foenum-graecum*, *Tanacetum parthenium*, *Ephedra sinica*, and *Cimicifuga racemosa* [3]. At present the basis for marketing of these products in the US is the Dietary Supplements Health and Education Act (DSHEA) of 1994, which allows manufacturers to market products as dietary supplements without the rigorous testing required for other drug products [17].

The approach of the Canadian Health Protection Branch with respect to herbal products is very similar to the FDA's, whereas several European countries have more advanced legislative regulations of herbal products. Rapid growth has been

Table 3.2 Some of the most economically important pharmaceuticals or their precursors derived from plants [3, 14, 15].

Plant names	*Compounds*	*Class*	*Therapeutic use*
Apocyanaceae, Rubiaceae spp.	Yohimbine	Indole alkaloid	Aphrodisiac
Artemisia annua L.	Artemisinin	Sesquiterpene lactone	Antimalarial
Camptotheca acuminata Dence	Camptothecin	Indol alkaloid	Antineoplastic
Capsicum spp.	Capsaicin	Phenylalkyl-amine alkaloid	Topical analgesic
Cassia angustifolia Vahl.	Sennosides A and B	Hydroxy anthracene glycosides	Laxatine
Catharanthus roseus L.	Vinblastin, vincristine	Bis-indole alkaloid	Antineoplastic
Cephaelis ipecacuanha (Brot.) A. Rich.	Ipecac	Mixture of ipecac alkaloids and other components	Emetic
Cephaelis ipecacuanha (Brot.) A. Rich.	Emetine	Isoquinoline alkaloid	Antiamoebic
Chondodendron tomentosum Ruiz, *Strychnos toxifera* Bentham	Tubocurarine	Bisbenzyl isoquinolone alkaloid	Skeletal muscle relaxant
Cinchona spp.	Quinine	Quinoline alkaloid	Antimalarial
Cinchona spp.	Quinidine	Quinoline alkaloid	Cardiac depressant
Colchium autumnale L.	Colchicine	Isoquinoline alkaloid	Antigout
Digitalis spp.	Digoxin, digitoxin	Steroidal glycosides	Cardiotonic
Dioscorea spp.	Diosgenin, hecogenin, stigmasteroll	Steroids	Oral contraceptives and harmona
Erythroxylum coca Lamarck	Cocaine	Cocaine alkaloid	Local anesthetic
Leucojum aestivum L	Galanthemine	Isoquinoline alkaloid	Cholinesterase inhibitors
Nicotiana spp.	Nicotine	Pyrrolidine alkaloid	Smoking cessation therapy
Papaver somniferum L	Codeine, morphine	Opium alkaloid	Analgesic, antitussive
Physostigma venenosum Balfor	Physostigmine	Indole alkaloid	Cholinergic
Pilocarpus jaborandi Holmes	Pilocarpin	Imidazole alkaloid	Cholinergic
Podophyllum peltatum L.	Podophyllotoxin	Lignan	Antineoplastic
Rauwolfia serpentina L.	Reserpine	Indole alkaloid	Antihypertensive, psychotropic
Solanaceous spp.	Atropine, hyoscyamine, scopolamine	Tropane alkaloid	Anticholinergic
Taxus brevifolia Nutt.	Taxol and other taxoids	Diterpenes	Antineoplastic

seen in the herbal medicine market in recent years, as increasing numbers of consumers are persuaded by the benefits of plant extracts as an alternative to medicinal products with chemically derived APIs (Active Pharmaceutical Ingredients) [18].

In 1999 the global market for herbal supplements exceeded US$15 billion, with a US$7 billion market in Europe, US$2.4 billion in Japan, and US$2.7 billion in the rest of Asia, and US$3 billion in North America [19]. It has been estimated that the market for branded nonprescription herbal medicine has grown from $1.5 billion in 1994 to $4.0 billion in 2000 in the US alone. A similar trend is also being followed in European countries [20].

3.2.1 Indian System-Based Herbal Medicine

India has been identified as one of the top 12 megadiversity centers of the world with an immensely rich medicinal and aromatic plant population occurring in diverse ecosystems. These medicinal plants are used both for primary health care and for treating chronic diseases such as AIDS, cancer, hepatitic disorders, heart disease, and age-related diseases such as memory loss, osteoporosis, and diabetic wounds etc. (Table 3.3). In the Indian coded system (Ayurveda, Unani, Siddha, Amchi), Ayurveda currently utilizes as many as 1000 single drugs and over 8000 compound formulations of recognized merit [21]. Similarly 600–700 plants are utilized by other systems such as Unani, Siddha, and Amchi.

About 70% of Indian medicinal plants are found in tropical and subtropical forest and less than 30% are found in the temperate and high altitude forest [22]. These medicinal plants species belong to a wide range of plant types, including trees, herbs, lianas, woody climbers, and twiners [24] (Fig. 3.1). In India more than 90% of plant species used by industry are collected from the wild and over 70% of the collection involves destructive harvesting, using different parts of the plants

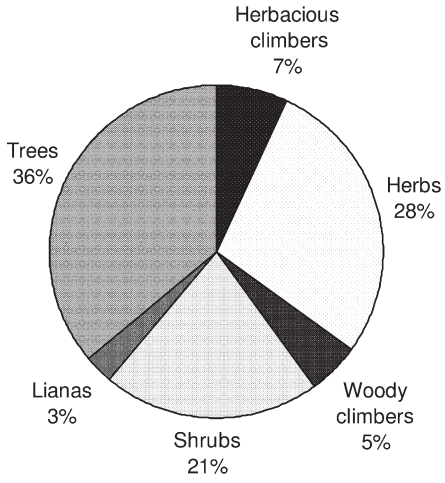


Fig. 3.1 Types of medicinal plants [24].

(roots, stem, bark, wood, whole plants) [25] (Fig. 3.2). However, about 20 species of medicinal plants in India are under large-scale cultivation and a number of superior varieties of medicinal plants have been developed. These cultivated materials are mostly used to derive modern medicines.

Table 3.3 Health care areas in which there is an emerging need for medicinal plant preparations that can be used for common ailments [26].

Emerging health care areas	***Status***	***Medicinal plants suited for herbal medicine/formulations***
Protozoan diseases	Widespread	*Artemisia annua, Cinchona* sp.
Amebic diseases	More than 60 million sufferers	*Cephalis ipecacuantha, Terminalia bellerica, Tylophora indica*
Ulcer diseases	General occurrence	*Glycyrrhiza glabra, Terminalia* sp., *Aloe barbadensis*
Cardiovascular diseases	Number one killer in the world,	*Ammi visnaga, Cloeus forskohlii, Digitalis* spp. *Nardostachys jatamansi, Rauvolfia serpentina, Swertia chirta*
Cancer	Insidious	*Catharanthus roseus, Podophyllum emodi, Taxus baccata*
Age-related diseases, rheumatism, etc.	Occur widely in old age,	*Commiphora wightii, Withania somnifera Pluchea lanceolata, Berberis vulgaris*
Lifestyle disorders: diabetes, stress, piles, and hypertension	17 million suffering in India	*Catharanthus roseus, Mimordica charantia, Salancia prinoides, Syzygium cumini, Gymnema silvestre, Curcuma longa, Zingiber officinale, Ocimum sanctum*
Constipation disorders	Common occurrence	*Plantago ovata, Cassia senna*
Autoimmune disorders	General occurrence	*Withania somnifera, Asparagus racemosus, Tinospora cordifolia, Picrorhiza kurroa, Acorus calamus, Sida cordifolia, Azadiracta indica, Crocus sativus, Glycyrrhiza glabra, Panax gineng*

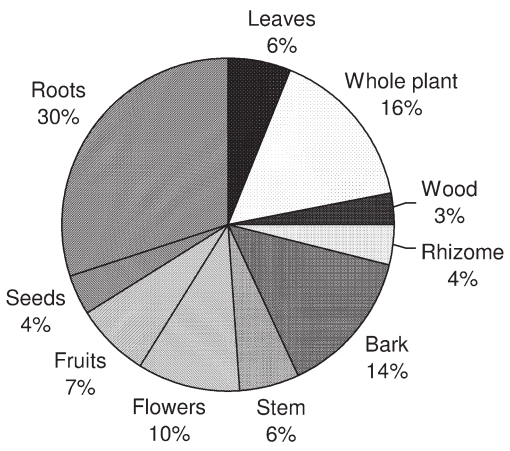


Fig. 3.2 Parts of medicinal plants used [25].

The major contribution in this area has been by the Central Institute of Medicinal and Aromatic Plants (CIMAP, Lucknow), and several Agricultural Institutes of Indian Council of Agricultural Research (India) [23].

The domestic Indian System of Medicine (ISM) market, comprising Ayurveda, Unani, Siddha, and homeopathy, has been estimated to exceed Rs42 billion (US$950 million) and India at present exports herbal medicines and materials to the tune of Rs5.5 billion (US$124 million) (Fig. 3.3). The world trade in medicinal plants is estimated to be about US$62 billion, with the major players being the European Union at 45%, Asia 17%, and Japan 16% [24] (Fig. 3.4). In India the classical patent or proprietary medicines of these systems are manufactured by over 9000 licensed pharmacies/manufacturing units. Some of these medicines are also exported to the Middle East. Major destination countries are the USA, Japan, Nepal, Sri Lanka, Germany, Italy, Nigeria, and the UAE.

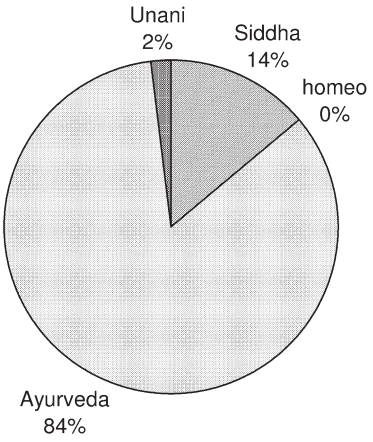


Fig. 3.3 The Indian System of Medicine and the divisions of the herbal market in India [25].

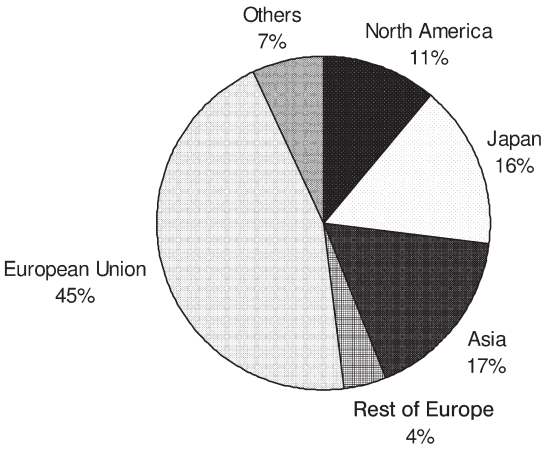


Fig. 3.4 Global markets for herbal medicines [25].

Recent market trends indicate that the export market in India is growing faster than the domestic market. The Indian medicinal plant industry is facing many problems and is affected by a number of factors, including lack of proper defined policies and strategies for the promotion of cultivation and post-harvest technologies, including research, patenting and marketing.

3.2.2
Progress in the Pharmacokinetics and Bioavailability of Herbal Medicine

In general, herbal medicine has relied on tradition that may or may not be supported by empirical data. The popularity and use of herbal medicine in recent years, especially in developed countries, has increased tremendously. Market-driven information about natural products is widespread and has further fostered their use in daily life. In most countries the evidence-based verification of the efficacy of herbal medicine is still lacking. However in recent years, data on the evaluation of the therapeutic and toxic activity of herbal medicinal products has become available. Establishing the pharmacological basis of the efficacy of herbal medicine is a constant challenge. Of particular interest is the question of bioavailability to assess to what degree and how fast compounds are absorbed after administration of a herbal medicine [13]. Research in this area is difficult due to the complex composition of herbal medicines and the ever-increasing list of their putative active constituents. Indeed the task becomes even more difficult where the active constituents and synergistic interactions are not known. With increasing knowledge of putative active compounds and highly sensitive and modern analytical methods (gas chromatography–mass spectroscopy (GC/MS), high-performance liquid chromatography–mass spectrometry (HPLC/MS)/MS and HPLC/CoulArray, HPLC/UV, etc.), data on certain herbal medicines are now increasingly reported in literature.

The herbal medicinal products most widely studied for their active constituents and pharmacology bioavailability in clinical and nonclinical trials, as well as drug interactions, are of Asian and European origin and are as follows [27]:

- *Ginkgo biloba* L. (Ginkgoaceae) [28–30]
- St John's wort (*Hypericum perforatum* L.) [31–36]
- *Spiraea ulmaria*, *Gaultheria procumbens*, and *Salix* sp. [37]
- Horse chestnut [38–40]
- Milk thistle (*Carduus marianus*) [41, 42]
- Quercetin [13, 43]
- Essential oils (e.g. peppermint oil, eucalyptus oil, pine oil, thyme oil) [44].

An extensive literature survey indicated that a considerable amount of scientific data are now available on the above and other standardized herbal medicines. Similar efforts should be made for all other herbal medicines to assess their real therapeutic potential and safety [8, 13, 43, 45].

3.3
Constraints in Herbal Medicine

3.3.1
Reproducibility of Biological Activity of Herbal Extracts

One of the major constraints in using plants in pharmaceutical discovery is the lack of reproducibility of activity for over 40% of plant extracts [46]. Reproducibility is the major problem, as the activities detected in screens often do not repeat when plants are re-sampled and re-extracted. This problem is largely due to differences in the biochemical profiles of plants harvested at different times and locations, differences in variety, and variation in the methods used for extraction and biological activity determination. Furthermore, the activity and efficacy of plant extracts/medicines often results from additive or synergistic interaction effects of the components. Therefore, a strategy should be used to evaluate the qualitative and quantitative variations in the content of bioactive phytochemicals of plant material. It is important to identify the different agroclimatic or stress locations, climate, microenvironment, physical and chemical stimuli often called elicitors, which quantitatively and qualitatively alter the content of bioactive secondary metabolites. Thus, elicitation-induced reproducible increases which might be otherwise undetected in screen, should significantly improve reliability and efficiency of plant extracts in drug discovery. Standardization, optimization, and full control of growing conditions could result in the cost-effective and quality-controlled production of many herbal medicines.

3.3.2
Toxicity and Adverse Effects

The general belief is that herbs are safer than pharmaceuticals because they are natural. But the fact is, healing herbs are neither completely safe nor poisonous. They are like other medicines. In low amounts they may be in effective while in the right amounts they may prove beneficial. Their use in high quantities and over prolonged periods may prove to be injurious.

Toxicity in herbal medicine may be due to (1) accidents due to a mistake in botanical identification, (2) accidental ingestion of cardiotonic plants, (3) inappropriate combinations, including the use of potentially toxic plants, (4) or plants that interfere with conventional pharmacological therapy, such as plants containing coumarinic derivatives, a high content of tyramine, estrogenic compounds, plants causing irritation and allergic problems, plant containing photosensitive compounds etc. [47–51]. Recent scientific research has demonstrated that many traditionally used herbal medicines are potentially toxic and some are even mutagenic and carcinogenic [52–54]. The toxicity benchmarks for herbal drugs therefore depend on purity, herbs containing toxic substances, bioavailability, and reported adverse effects.

3.3.3
Adulteration and Contamination

Adulteration and contamination of herbal medicines appears to be common in countries that are lenient with regard to controls regulating their purity. Adulterations in herbal medicine are particularly disconcerting because they are unpredictable. Often they remain undetected unless they can be linked to an outbreak or epidemic. An example is veno-occlusive disease due to ingestion of plants containing pyrrolidizine alkaloids, which can be life threatening or fatal [55, 56].

In many cases contaminated or adulterated herbal medicines can cause significant medical problems, especially in children [57, 58]. In a recent review on heavy metal poisoning in children consuming herbal medicines, 13 reports were identified from Singapore, Hong Kong, the USA, the UK, and the UAE from 1975 to 2002.

Ayurvedic medicines are sometimes prepared using inorganic active constituents. Combined with environmental contamination this may increase the heavy metal content above permissible limits in developed countries.

The Indian Government has initiated a major program under which the pharmacopeial standards for the drugs used in the Ayurveda, Unani, and Siddha systems of medicine are being developed. The resultant pharmacopeia will help in knowing more about the herbal drugs in use. Simultaneous use of more than one herbal products or the use of herbal products in combination with pharmaceuticals needs to be checked. There are chances of adverse interactions. Some of the contradictions associated with poisonous drugs of the ISM are listed in Table 3.4.

Adulteration in Asian medicines mostly results from the misidentification of plants. This has resulted in a number of serious events, primarily due to poisoning with digitalis, belladonna, skullcap, etc. [8]. In 1998, the California Department of Health reported that 32% of Asian patent medicines sold in the US contained undeclared pharmaceuticals or heavy metals [60, 61]. The FDA and other investigators have also reported the presence of prescription drugs, including glyburide, sildenafil, colchicines, adrenal steroids, alprazolam, etc. in products claiming to contain only natural ingredients [62].

3.3.4
Herb–Drug Interactions

Herbal medicines can act through a variety of mechanism to alter the pharmacokinetic profile of concomitantly administered drugs [63]. St John's wort, for example, induces the cytochrome P450 isozyme CYP 3A4 and intestinal P-glycoproteins, accelerating the metabolic degradation of many drugs including cyclosporin, antiretroviral agents, digoxin, and warfarin [64].

Numerous examples exist of drug and herbal interactions. These effects may potentiate or antagonize drug absorption or metabolism, the patient's metabolism, or cause unwanted side reactions such as hypersensitivity [65–67]. Care should be

Table 3.4 Some commonly used poisonous drugs in the Indian System of Medicine [59, 79].

Plant name	*Vernacular name*	*Part used*	*Common use*	*Adverse effect (in large doses)*
Aborus precatorius L.	Indian liquorice	Seed	Diarrhea, dysentery, paralysis and skin diseases, antiseptic, uterine stimulant and anticancerouss,	Abrin causes edema and ecchymosi inflammation antifertility activity, antiestrogenic activity, abortifacient and oxytocic activity
Aconitum casmanthum Stappex Holm	Aconite	Rhizome	Neuralgia, rheumatism, cardiac tonic and nerve poisons	Narcotic, powerful sedative, arrhythmia and hypertension
Gloriosa superba L.	Malanbar glory lily	Root	Anthelmintic, purgative, emetic, antipyretic, expectorant and toxic	Antifertility, vomiting, purging, gastrodynia and burning sensation
Croton tiglium L.	Croton	Seed	Abdominal disorders, constipation, helminthiasis, inflammation, leukoderma and dropsy	Depressor responses and neuromuscular blockade
Calotropis gigantea L.	Gigantic swallow wort	Latex and leaf	Paralysis, purgative and intermittent fevers	Violent purgative and gastrointestinal irritant
Cannabis sativa L.	Hemp	Leaf	Antidiarrhetic, intoxicating, stomachic and abdominal disorders,	Neurotoxic, respiratory arrest, nausea tremors, insomnia, sexual impotence and gastrointestinal disturbance
Datura metel L.	Thorn apple	Seed and leaf	Antihelminthic and anticancerous	Insanity
Euphorbia neriifolia	Milk hedge	Latex	Insecticidal and cardiovascular	Emetic, irritant, apnea and pathological changes in liver, heart and kidney
Papaver somniferum L.	Poppy	Exudate	Diarrhoea, dysentery, sedative, narcotic and internal hemorrhages	Highly narcotic
Semecarpus anacardium	Marking nut	Fruit	Antiseptic, cardiotoxic, anticarcinomic liver tonic and uterine stimulants	Abortive
Nerium indicum Mill	Oleander	Fruit and leaf	Antibacterial, ophthalmic and cardiotonices	Cardiac poison, paralysis and depress respiration, gastrointestinal, neurological and skin rashes
Strychnos nux vomica L.	Snake wood	Seed	Appetizer, anthelmintic, purgative and stomachic	Paralysis

taken to understand the effects of foods or herbal medicines during anticoagulant therapy, in the treatment of diabetes, depression, pain, asthma, heart conditions, or blood pressure disorders, and during slimming [8]. The scientific data about the interactions of various herbal medicines with a drug and its pharmacokinetics and bioavailability should be evaluated to assess the potential toxicity as well as the pharmacological basis of efficacy [13].

3.3.5 Standardization

Standardization is an important step where the active constituents are known. However, for many herbs the active constituents are not known. In such cases, products may be standardized on the content of certain marker compounds. However herbal medicines rarely meet this standard for several reasons, including the lack of scientific information about the acting pharmacological principles. The variability in the content and concentration of constituents of plant material, together with the range of extraction techniques and processing steps used by different manufacturers results in marked variability in content and quality of commercially available herbal products [68]. The use of chromatographic techniques and marker compounds to standardize herbal preparations promotes batch-to-batch consistency but does not ensure consistent pharmacological activity or stability.

Consistency in composition and biological activity are prerequisites for the safe and effective use of therapeutic agents. But standardization of correct dosage forms is not always easy, especially in polyherbal preparations or single plants that are not cultivated under controlled condition. And there is no guarantee that a product contains the amount of the compound stated on the label [51].

3.3.6 Regulatory Challenges of Asian Herbal Medicine

Overall the incidence of serious adverse reactions is significantly lower with most herbal medicines when compared with pharmaceutically derived drugs [8]. However, the need still exists to more closely monitor practitioners and formulators of any traditional medicine, including those of Indian origin, so that unethical practices are reduced.

For most herbal products, verification is difficult if not impossible after processing has occurred. In traditional medicines that are prepared in Asian countries and exported, the task of ensuring safety is even more difficult since the incorporation of certain levels of potentially toxic herbs or heavy metals may not be considered harmful in the country of origin [69]. Some Chinese and Indian Ayurvedic medicines have been rejected by US, Canada and other countries on the grounds that they contain high levels of potentially toxic elements, including heavy metals.

In the view of above problem, the authorized body for traditional medicine "Ayush" has adopted strict guidelines for all herbal medicines (Unani, Ayurveda, and Siddha) to be exported from India. Ayush has made it mandatory for all ISM

medicines to be exported to meet the international standards for contamination including heavy metals in 2005. These guidelines can be accessed on the Ayush website (http://www.indianmedicine.org).

3.4
Good Manufacturing Practice (GMP) for Herbal Medicine

In India there are about 10 000 licenced pharmacies of ISM and herbal medicines producing medicines [70]. With the increase in commercialization, some unscrupulous manufacturing practices have crept in to this profession, resulting in the use of shortcuts to replace certain tedious and necessary processes, poor and inaccurate labeling, and several other poor manufacturing practices. These have all necessitated the introduction of statutory Good Manufacturing Practices (GMPs) for all ISM drug-manufacturing industries. The Government of India came up with guidelines for the adoption of GMP standards by June 2002, and the details of the provision of GMP for Ayurveda, Siddha, and Unani drugs are provided in the Drugs and Cosmetics Amendment Rules, 2000. GMPs are prescribed to ensure that: (1) raw materials used in the manufacturer of drugs are authentic, of prescribed quality, and free from contamination; (2) manufacturing processes are as has been prescribed to maintain the standards; (3) adequate quality control measures are adopted; and (4) manufactured drugs that are released for sale are of acceptable quality.

In addition to these guidelines, it is also required that at the factory in which the medicines are prepared there must be adequate space for (a) receiving and storing raw materials, (b) processing/manufacturing activities, (c) a quality control section, (d) storage of finished goods, and (e) a proper office for record maintenance including storage of rejected drugs/goods.

3.5
Improving the Quality, Safety and Efficacy of Herbal Medicine

Herbal medicine products have been used for thousands of years for the prevention and treatment of various diseases in India, China, and other countries. Herbal medicine occupies an important position with regard to adverse reactions, having a lower percentage (7.6%) of reported adverse effects than other CAM therapies, such as manipulation (15.8%), acupuncture (12.5%), and homeopathy (9.8%) [6, 71, 72].

Problem and difficulties arise, however, in the quality assurance of herbal medicinal products because of the complex nature of unidentified chemical entities in the finished products and our lack of knowledge about the actual bioactive compounds. Recent advances in analytical chemistry and related disciplines have a promising role in elucidating complex chemical compositions. Chemical and analytical techniques can be applied at different stages of good practice in quality assu-

rance of herbal medicine. Major stages at which techniques such as GC, HPLC, high-performance thin-layer chromatography (HPTLC), ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), MS, X-ray diffraction, GC/MS, and LC/MS, etc. may be applied include Good Agricultural Practise (GAP), Good Sourcing Practise (GSP), Good Manufacturing Practise (GMP), and Good Clinical Trial Practise (GCTP) [6].

The problem still is not solved in cases where synergistic action provided by some chemically unknown or isolated ingredients in composite herbal medicine have proven effectiveness from double clinical trials. On the other hand, production of active secondary metabolites may be influenced by physiological conditions and closely related species may contain similar chemical components, causing problems in botanical identification.

3.5.1 Quality Management

The raw material passes through different stages of processing, evaluation, and development before the final product is released. In the farm sector, many abiotic and biotic environmental factors will affect a crop's composition and yield, resulting in variation of desired quality and yield, leading to a variation in the quality of the product. Appropriate production and quality management measures, including quality assurance, are required both at farms and in the herbal industrial sector. Cultivation not only ensures a consistent, generally predictable supply of plant material without destroying our natural heritage in wild flora but also ensures the selection of genetically superior plants with a high level of sustainable biomass and an enhanced quality of the finished product [26, 73, 74].

The main source of raw materials for herbal medicines at present, however, is wild plants. There is huge demand for raw plant material due to the widespread and increasing use of herbal medicine. Continued harvesting is causing loss of genetic diversity and habitat destruction. Therefore, domestic cultivation should be encouraged. Domestic cultivation also offers the opportunity to overcome some of the problems inherent in herbal medicine/extracts: misidentification, genetic and phenotypic variability, extract variability and instability, toxic components, and contaminants. Conventional plant breeding methods can improve both agronomic and medicinal traits and molecular markers coupled with assisted selection will be used increasingly in the future [75].

3.5.2 Encouraging Mediculture

The concept of growing crops for health rather than food or fiber is slowly changing plant biotechnology and medicine. The rediscovery of the connection between plants and health is responsible for launching a new generation of botanical therapeutics that include plant-derived pharmaceutical, multicomponent botanical drugs, dietary supplement, functional foods and plant products, and recombinant

proteins. Mediculture is defined as the cultivation of medicinal plants on a scientific basis. The emphasis on genetic stability and uniformity of plant population is important in order to ensure reproducible results.

3.5.3
Correct Identification of Plant Material

Classical methods of plant taxonomy for the identification of plant material provide an authentic and viable methodology. However, in many situations, for example when whole plants are not available to the taxonomist, a genetic approach will be more reliable. DNA molecules are more reliable markers than chemicals based on proteins or caryotyping because the genetic composition is unique for each individual and it is less affected by age, physiological and environmental conditions. The DNA can be extracted from leaves, stems, and roots of herbal material. Thus DNA fingerprinting can be very useful tool to assess and confirm the species contained within a plant material of interest.

3.5.4
Minimizing Contamination in Herbal Medicine

Herbal medicines in Asia and other countries consist of a mixture of crude or raw herbs collected from the wild, some from cultivated fields, as well as prepared herbal extracts provided by other agencies. Toxic chemicals and other contaminants, including microbes, may come from (1) environmental and agricultural conditions where the plants have been grown or collected. (2) transport and storage conditions, and (3) during manufacturing, processing, and packaging.

In order to ensure safety, it is desirable to ensure quality by removing such contaminants through the application of radiation processing technology [76]. In order to increase the quality of production for domestic use and export, the quality control and assurance of raw materials from the farm as well as from forest sources should be defined in terms of the genetic variation in the natural product content and crop quality [77].

3.6
Conclusions

Herbal medicines make an enormous contribution to primary health care and have shown great potential in modern phytomedicine against numerous ailments and the complex diseases and ailments of the modern world. There will always be risks when appropriate regulations do not mandate the appropriate formulation of the remedies or when self-medication fosters abuse.

Quality is the paramount issue because it can affect both the efficacy and the safety of the herbal medicines being used. Current product quality ranges from very high to very low as a result of intrinsic, extrinsic, and regulatory factors. Intrin-

sically, species differences, diurnal and seasonal variations can affect the qualitative and quantitative accumulation of chemical constituents in the source medicinal plants. Extrinsically, environmental factors, field conditions, cultivation, harvest and post-harvest transport and storage, manufacturing practises, inadvertent contamination and substitution, and intentional adulteration are contributing factors to the quality of herbal medicines. Plant materials that are contaminated with microbes, microbial toxins, or environmental pollutants, or finished products that are adulterated with toxic plants or synthetic pharmaceuticals can lead to adverse events.

To overcome environmental, toxic, and contamination problems like pesticides, heavy metals, microbial, toxins, control measures need to be introduced to implement necessary standard operating procedures, as are applied for foods and the pharmaceutical industry, as well as GAPs and GSPs at source. GLPs and GMPs are also needed to produce quality medicinal products. The quality of herbal medicines can also be related to regulatory practises [6]. The WHO guidelines for herbal medicine should be strictly implemented and monitored by the concerned regulatory agency. Most traditional medicinal herbs are used in the form of an aqueous decoction. Therefore scientific data should be generated on the development of analytical and biological procedures for use to give quality assurance and control and clinical assessment of efficacy and safety of these products. There is still a need for more scientific evaluation of Asian herbal medicines including their active constituents, synergistic interactions, formulation strategies, herb–drug interactions, standardization, pharmacological and clinical evaluation, toxicity, safety and efficacy evaluation and quality assurance. Furthermore, in order to ensure the use of genuine raw materials, more priority should be given to encouraging the organic cultivation of medicinal plants. Countries interested in promoting herbal medicine should generously provide funds for fundamental research on above aspects.

Clearly, strategic planning for research in herbal medicine is needed. The lack of a pharmacological basis for the efficacy and toxicity and clinical data on the majority of herbal medicines is the major constraint to the integration of herbal medicine into conventional medicinal practises. Adverse events, including drug–herb interactions, must also be monitored to promote the safe integration of efficacious medicines into conventional medical practises [78].

Acknowledgments

We are grateful to University Grant Commission, New Delhi for financial assistance in the form of UGC-Major Research Project No. F.3-58/ 2002 (SR-II) on medicinal plants.

References

1 Murphy, J.M. *Am. Org. Reg. Nurses J.* **1999**, *69*, 173–183.
2 Chan, K. *Biologist* **1996**, *43*, 50–51.
3 Raskin, I., Ribnicky, D.M., Komarnystky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D.A., Ripoll, C., Yakoby, N., O'Niel, J.M., Cornwell, T., Pastor, I., Fridlender, B. *Trends Biotechnol.* **2002**, *20*, 1–10.
4 British Medical Association. *Complementary Medicine: New Approaches to Good Practice*. Oxford University Press, Oxford, **1993**.
5 Lewith, G., Kenyon, J., Lewis, P. *Complementary Medicine: An Integrated Approach*. Oxford University Press, Oxford, **1996**.
6 Chan, K. *Chemosphere* **2003**, *52*, 1361–1371.
7 World Health Organization. *Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines*. Regional Office for the Western Pacific, Manila, **1993**
8 Elvin-Lewis, M.J. *Ethnopharmacology* **2001**, *75*, 141–164.
9 De Smet, P.A.G.M. *Drugs* **1997**, *54*, 801–840.
10 Lewis, W.H., Elvin-Lewis, M. *Medical Botany, Plants Affecting Man's Health*. John Wiley Interscience, New York, **1977**.
11 De Smet, P.A.G.M. *Drug Safety* **1995**, *13*, 81–93.
12 Soller, W. *HerbalGram* **2000**, *49*, 64–67.
13 Bhattaram, V.A., Graefe, U., Kohlert, C., veit, M., Derendorf, H. *Phytomedicine* **2002**, *9*, 1–33.
14 Business Communication Company, Inc. *Plant Derived Drugs: Products Technologies and Applications*. Study RB.121, **1998**.
15 Balandrin, M. F. et al. *ACS Symp. Ser.* **1993**, *534*, 2–12.
16 U.S. Department of Health and Human Services, Food and Drug Administration for Drug Evaluation and Research. *Guidance for Industry Botanical Drug Products*. http://www.Fda.gov/cder/guidance/1221dft.htm# P131_3293.
17 Senate, U.S. Dietary Supplement Health and Education Act. 103rd Congress, 2nd session. **1994**, pp. 103–104.
18 Greger, J.L. *J. Nutr.* **2001**, *131*, 1339S–1343S.
19 Glasser, V. *Nat. Biotechnol.* **1999**, *17*, 17–18.
20 De Smet, P.A., Bonsel, G., Van Der kyu, A., Hakster, Y.A., Pronk, M.H., Brorens, M.J., Lockefeer, J.H., Nuijten, M.J. *Pharmacoeconomics* **2000**, *18*, 1–7.
21 Anonymous. *The Ayurvedic Formulary of India* (Pt. 1). Ministry of Health and Family Welfare, Government of India, **1978**.
22 Kumar, S., Singh, J., Saha, N.C., Ranjan, V. *Indian Medicinal and Aromatic Plants Facing Genetic Erosion*. Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India, **1997**.
23 Singh, J., Srivastava, R.K., Singh, A.K., Kumar, S. *J. Med. Aromatic Plant Sci.* **2000**, *22*, 564–571.
24 Singh, J., Singh, A.K., Pravesh, R. *Proc. First NIM on Medical Aromatic Plants* **2003**, 50–58.
25 Ved, D.K., Anjana, M., Shankar, D. *Curr. Sci.* **1998**, *75*, 341–344.
26 Krishna, A. *Proc. First NIM on Medical Aromatic Plants* **2003**, 209–214.
27 Wagner, H. *Environ. Health Perspect.* **1999**, *107*, 779–781.
28 Mauri, P., Simonetti, P., Gardana, C., Minoggio, M., Morazzoni, P., Bombardelli, E., Pietta, P. *Rapid Commun. Mass. Spectrom.* **2001**, *15*, 929–934.
29 Biber, A. Koch, B. *Planta Med.* **1999**, *65*, 192–193.
30 Duche, J.C., Barre, J., Guinot, P., Duchier, J., Cournot, A., Tillement, J.P. *Int. J. Clin. Pharmacol. Res.* **1989**, *9*, 165–168.
31 Draves, A.H., Walher, S.E. *J. Chromatogr B Biomed. Sci. Appl.* **2000**, *749*, 57–66.
32 Kasper, S. *Pharmacopsychiatry* **2001**, *34*, 51–55.
33 Muller, W.E., Singer, A., Wonnemann, M. *Schwiz. Rundsch. Med. Prax.* **2000**, *89*, 2111–2121.
34 Nathan, P.J. J. *Psychopharmacology* **2001**, *15*, 47–54.
35 Jacobson, J.M., Feinman, L., Liebes, L., Ostrow, N., Koslowski, V. *Antimicrob. Agents Chemother.* **2001**, *45*, 517–524.

36 Biber, A., Fiscer, H., Romer, A., Chatterjee, S.S. *Pharmacopsychiatery* **1998**, *31*, 36–43.

37 Chrubaic, S. Eisenberg, E., Balan, E., Weinbereger, T., Luzzati, R., Condradt, C. *Am. J. Med.* **2000**, *109*, 9–14.

38 Yoshikawa, M., Murakami, T., Yamahara, J., Matsuda, H. *Chem. Pharm. Bull. (Tokyo)* **1998**, *46*, 1764–1769.

39 Low, D. Schroedter, A., Schwankl, W., Marz, R.W. *Methods Find. Clin. Pharmacol.* **2000**, *22*, 537–542.

40 Meyer-Bernarth, J., Kaffarnik, H. *Arzneimittelforschung* **1970**, *20*, 147–148.

41 Venkataramanan, R., Ramachandran, V., Komoroski, B.J., Zhang, S., Sciff, P.L., Strom, S.C. *Drug Metab. Dispos.* **2000**, *28*, 1270–1273.

42 Gatti, G., Perucca, E. *Int. J. Clin. Pharmacol. Ther.* **1994**, *32*, 614–617.

43 De Smet, P.A.G.M., Keller, K., Hansel, R., Chandler, R.F. *Adverse Effects of Herbal Drugs, I and II*. Springer-Verlag, Berlin, **1992**.

44 Bruneton, J. *Pharmacognosy, Phytochemistry, Medicinal Plants*. Lavosier, Paris, **1995**.

45 Chan, K. Understanding the toxicity of Chinese Herbal Medicinal products. In *The Way Forward for Chinese Medicines*, Chan, K., Lee, H (eds). Taylor & Francis, London, **2002**.

46 Cordell, G.A. *Phytochemistry* **2000**, *55*, 463–480.

47 Rates, S.M.K. *Toxicon* **2001**, *39*, 603–613.

48 Thomson, S.A. *South African Government Genoside and Ethnopiracy*. The Gaia Research Institute, South Africa, **2000**.

49 Stewart, M. J., Steenkamp, V. *South Afr. Ethnobot.* **2000**, *1*, 32–33.

50 Wojcikowski, K., Johnson, D.W., Gobe, G. *Nephrology* **2004**, *9*, 313–318.

51 Goldman, P. *Ann. Intern. Med.* **2001**, *135*, 594–600.

52 Shimmer, O., Haefele, F., Kruger, A. *Mutat. Res.* **1988**, *206*, 201–208.

53 Shimmer, O., Kruger, A., Paulini, H., Haefele, F. *Pharmazie* **1994**, *49*, 448–451.

54 De SaFerrira, I.C.F., Ferrago Vargas, V.M. *Phytother. Res.* **1999**, *13*, 397–400.

55 Chauvin, P., Dillon, J.C., Moren, A. *Sante* **1994**, *4*, 263–268.

56 Drew, A.K., Myres, S.P. *Med. J. Aust.* **1997**, *166*, 538–541.

57 Saper, R.B., Kales, S.N., Paquin, J., Burns, M.J., Eisenberg, D.M., Davis, R.B., Phillips, R.S. *JAMA* **2004**, *292*, 2868–2873.

58 Ernst, E., Coon, J.T. *Clin. Pharmacol. Ther.* **2001**, *70*, 497–504.

59 Seth, P.K. Kakkar, P. *Proc. First NIM on Medicinal Aromatic Plants* **2003**, 29–41.

60 Ko, R.J. *N. Engl. J. Med.* **1998**, *339*, 847.

61 Marcus, D.M., Grollman, A.P. *N. Engl. J. Med.* **2002**, *347*, 2073–2076.

62 Ernst, E. *J. Intern. Med.* **2002**, *252*, 107–113.

63 Gugh-Berman A. *Lancet* **2000**, *355*, 134–138.

64 Moore, L.B., Goodwin, B., Jones, S.A., Wisely, G.B., Serabjit-Singh, C.J., Willson, T.M., Collins, J.L., Kliewer, S.A. *Proc. Natl Acad. Sci. USA* **2000**, *97*, 7500–7502.

65 Brinker, F. *Herb Contraindications and Herb Interactions*. Electic Institute, **1997**.

66 Cupp, M.J. *Am. Fam. Physician* **1999**, *59*, 1239–1245.

67 Blumenthal, M. *HerbalGram* **2000**, *49*, 52–63.

68 Schulz, V., Hansel, R., Tyler, V.E. *Rational Phytotherapy. A Physicians Guide to Herbal Medicine*, 4th edn. Springer, Berlin, **2000**.

69 Shaw, D., Leon, C., Kolev, S., Murray, V. *Drug Safety* **1997**, *17*, 342–356.

70 Akerele, O. *HerbalGram* **1993**, *26*, 13–20.

71 Abbot, N.C., White, A.R., Ernst, E. *Nature* **1996**, *381*, 361.

72 Barnes, J. *Pharm. J.* **1997**, *258*, 76–77.

73 Akhtar, H. *Essential Oils and their Cultivation*. CIMAP Publication, Lucknow, India, **1993**.

74 Akhtar, H. *Medicinal Plants and their Cultivation*. CIMAP Publication, Lucknow, India, **1993**.

75 Canter, P.H., Thomas, H., Ernst, E. *Trends Biotechnol.* **2003**, *23*, 180–185.

76 Sharma, R. *J. Food. Sci.* **1989**, *54*, 589–590.

77 Forester, S. *Forest Pharmacy – Medicinal plants in American Forest*. Forest History Society, Durham, NC, **1995**.

78 Fong, H.H. *Integr. Cancer Ther.* **2002**, *1*, 287–293.

79 Chopra, R.N., Nayer, S.L., Chopra, I.C. *Glossary of Indian Medicinal Plants*, 3rd Edn. Council of Scientific and Industrial Research, New Delhi, 7–246, **1992**.

4
Bioactive Phytocompounds and Products Traditionally Used in Japan

Jin-ichi Sasaki

Summary

For centuries, East Asian people have used traditional herbs or functional foods as folk medicine to treat or prevent diseases, long before the introduction of Western medicine. Although Western medicine is often effective in curing acute diseases, it is not necessarily applicable to the prevention of diseases.

In the twenty-first century, there has been a great upsurge of interest in the biological functions of food ingredients in relation to their physiological activities in vivo. A mass of scientific data accumulated has stressed the presence of an interrelationship between lifestyle-related diseases, such as cancer, heart disorder, and diabetes, and daily food intake, and these kinds of diseases are considered to be preventable through diet modification.

In order to use foods to improve health, we first need to clarify scientifically the functions of the foods, and to give reasonable scientific answers to questions such as How well does this work? How safe is it? What type of conditions is it best used for? Research aimed at answering these kinds of questions is still considered a less scientifically valid field than conventional medicine.

In addition, foods or herbs with various biological functions are gradually being recognized as having a use as second medicines, able to intervene in the prevention or therapy of diseases. Post-operative cancer patients are actually employing their own devised foods menu to inhibit the recurrence of disease due to remaining malignant cells.

In this chapter I describe the functions of general vegetables and plants available in Japan, such as garlic powder (antibacterial activity, prolongation of blood coagulation, antioxidant activity), garlic odor (antibacterial activity), Japanese cypress oil and oil flavor (antibacterial activity), edible mushrooms (*Grifola frondosa*, maitake) or nonedible mushrooms (*Lampteromyces japonicus* Singer, tsukiyotake), and sweetcorn powder (cancer-curing activity).

4.1 Introduction

Throughout the world, there has been an upsurge of interest in the topic of functional foods in relation to lifestyle-related diseases, such as arterial sclerosis, hypertension, malignancy, diabetes, and others. The Ministry of Health and Welfare in Japan has stated that the modification of the daily diet can reduce the incidence of a wide variety of cancers by 30%.

In the US the National Cancer Institute launched a major project 15 years ago called the "Designer Foods Project," which aimed to provide citizens with beneficial scientific information to restrain an outbreak of cancer [1]. Since then, nationwide studies have been carried out with the same objective to improve the health of the Japanese population.

In 1996 there was a serious outbreak of food poisoning in Japan caused by enterohemorrhagic *Escherichia coli* O157:H7. It was estimated to have affected over 12 000 people and resulted in at least 12 deaths. However, despite intensive investigations, the source and carrier of the infection remained unclear. The whole nation went into panic about the unknown source of the infection and many studies were immediately initiated to seek for functional foods with bacteria-killing potency.

To date, although numerous food-related books have been published in this area in Japan, many of them lack their own data, and are just collections from other books. Information is passed around from book to book just by changing the titles and they are repeatedly using the same old data.

In this chapter, I will advance the debate on the food functions of garlic, vegetable odors (flavors), sweetcorn, mushrooms, and Japanese cypress oil (hinokitiol) by introducing our own new data.

4.2 Garlic

4.2.1 Introduction

Garlic is found almost everywhere in the world (from Polynesia to Siberia) and has been used in traditional medicine for over 4000 years to treat disorders of arthritis, common cold, diabetes, malaria, and tuberculosis [1]. The microbiologist Louis Pasteur studied the bactericidal properties of garlic, and during the Second World War garlic was called "Russian penicillin" because the Russian government turned to this ancient treatment for its soldiers when supplies of antibiotics had been exhausted.

It has been shown experimentally that garlic possesses therapeutic and preventive activities against bacterial infection, atherosclerosis, high total cholesterol, and hypertension; it also protects against free radicals and also aids in prolongation of blood coagulation time [1, 2]. It is ranked at the top as the strongest cancer preventive vegetable on the "Designer Foods Project" [1].

In Japan, garlic has traditionally been used as a folk medicine from ancient times. Aomori prefecture is a region well known for the production of high-quality garlic and its crop represents 60% of Japan's output. We describe here the new functions of garlic that were recently researched in our laboratory.

4.2.2 Biological Effect of Garlic

4.2.2.1 Antibacterial Effects

Against Enterohemorrhagic Escherichia coli O157:H7

Food poisoning caused by enterohemorrhagic *Escherichia coli* O157:H7 was first reported in 1983 by Riley et al. [3]. The symptoms of this unusual gastrointestinal illness were characterized by the sudden onset of severe abdominal cramps and bloody diarrhea with no fever or low-grade fever. The illness sometimes develops a hemolytic uremic syndrome (HUS), which differentiates it from other types of food poisoning and can often be fatal to the patient, especially in infants.

After the huge outbreak of O157-caused food poisoning in Japan in 1996, sporadic outbreaks continued to occur nationwide, and are still reported even now. This suggests that O157 has already become indigenous to Japan and our surroundings are polluted by this bacterium. O157 is a remarkably resistant organism that can survive for over three years just in water without any nutrients. It can also change certain biochemical characters, often leading to microbiological misdiagnosis.

We began our study by looking for Japanese foods that have anti-O157 activity, and soon found that garlic powder was effective [4]. The garlic powder used was prepared from garlic harvested one year previously. Briefly, garlic bulbs were dried in the shade for one year, cut into small pieces followed by drying at 60 °C for 6 h, and grinding into a powder with a mill. Fresh garlic powder was similarly prepared without air-drying from fresh garlic after harvesting, and used for antibacterial tests against O157. The antibacterial activity of garlic powder was mainly tested by the test-tube method, combined with the nutrient agar plate method. Garlic powder easily killed O157 as shown in Table 4.1.

Table 4.1 Anti-O157 activity of garlic powder prepared from old or fresh garlic bulbs.

Sample	*Number of O157 (cfu mL^{-1})*		
	0 h	*6 h*	*24 h (treatment)*
1% Old garlic powder	4.0×10^{7}	8.0×10^{6}	0
1% Fresh garlic powder	4.0×10^{7}	0	0
Control (water alone)	4.0×10^{7}	8.0×10^{7}	8.0×10^{8}

cfu, colony forming unit.

Using the nutrient agar plate test, it was additionally found that garlic powder killed other species of pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus subtilis* (Fig. 4.1). For this test half of the nutrient agar in the Petri dish was replaced by the garlic powder-supplemented nutrient agar. Then the bacteria were streaked on both types of agar to examine the growth inhibition activity of the samples.

It is known that the processing of garlic and onion into extracts, essence, and dehydrated foods leads to the formation of products with significantly different physicochemical and biological characteristics [2]. The garlic oil extracted by distillation in boiling water consists of dimethylsulfides, diallylsulfides, methyl allyl sulfides, and others, which have all been shown to process biological properties such as antioxidant effects. However, it lacks bactericidal and antithrombotic activity. When garlic is extracted with ethanol and water at room temperature, it yields the oxide of diallyl disulfide, allicin, which is the source of the garlic odor. Under the influence of allinase the precursor alliin decomposes to 2-propenesulfenic acid. Allicin possesses hypolipidemic, antimicrobial, and hypoglycemic activities [2], and heat-unstable allicin is considered to be a principal antibacterial constituent [5]. However, as shown in Table 4.2, heat treatment at 100 °C for 20 min could not eliminate the bactericidal potency and its activity remained in the garlic powder [4]. Thus it seems that garlic contains two types of antibacterial ingredients: the heat-labile allicin and heat-stable sulfur compounds [6], both of which work together against bacteria.

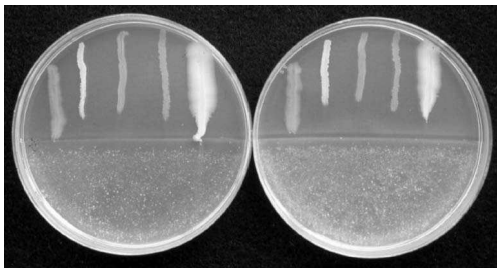


Fig. 4.1 Bactericidal activity of garlic powder prepared from old garlic bulbs. Pathogenic bacteria were streaked on both sides of a Petri dish (upper: control, bottom: 1% (left) and 2% (right) garlic-supplemented). Bacteria failed to grow on the lower side. From left to right: *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, enterohemorrhagic *E. coli* O157, and *Bacillus subtilis*.

Table 4.2 Thermostability of anti-O157 activity in powder prepared from old garlic bulbs.

Sample	***Number of O157 (cfu mL^{-1}) after 24 h incubation***
1% Garlic powder	0
1% Garlic powder (100 °C, 10 min)	0
1% Garlic powder (100 °C, 20 min)	0
Control (water)	6.2×10^{7}

cfu, colony forming unit. Garlic powder solution was heat-treated above described conditions.

Against *Bacillus anthracis*

The outbreak of anthrax in the USA in 2002, thought to have been linked with terrorism, killed four people and generated widespread panic in the US. Anxious citizens were reported to be asking doctors for antibiotics to prevent infection.

Anthrax is primarily a disease of cattle and sheep; horses and pigs are also susceptible, but are less commonly affected. The bacillus is almost always transmitted to humans from lower animals rather than from other humans. The pulmonary form of anthrax, transmitted by inhalation of the microorganisms (spores) floating in the air, is the most dangerous [7].

In serial experiments on the functional activities of garlic, we found that 1% of garlic powder in water killed Bacillus anthracis at 10^7 mL^{-1} after 3 h of treatment [8] (Table 4.3). Most experiments reported were carried out *in vitro* [4, 9, 10], and there have been very few *in vivo* studies.

Next we designed an experiment with mice to find out how garlic powder worked against living bacteria in the intestine. Briefly, 1% of garlic powder in water was administered orally to mice by catheter once daily for three days, and the number of living bacteria in feces were counted. It was found that oral administration of garlic powder worked effectively *in vivo* to reduce the number of living bacteria in the intestine (Table 4.4). This result suggests that garlic (powder) probably works *in vivo* against an invading pathogen. However, it is not recommended to take raw garlic in large doses, because it can cause numerous symptoms, such as stomach disorders, heartburn, nausea, vomiting, diarrhea, and others.

Table 4.3 Bacillus anthracis-killing potency of garlic powder prepared from old garlic bulbs.

Incubation time (h)	*Number of living bacteria (cfu mL^{-1})*	
	In 1% garlic powder	*In distilled water*
0	2.0×10^7	2.0×10^7
1	4.1×10^4	ND
3	0	1.0×10^7
6	0	4.0×10^7

cfu, colony forming unit.
B. anthracis was added to 1% garlic powder in water and kept at room temperature for analysis [8].

Table 4.4 Effect of feeding garlic powder to mice on the number of living bacteria in the feces.

Group	*Number of living bacteria (cfu/excrement)*
1% Garlic powder fed	2.3×10^5
Water fed	5.4×10^6

cfu, colony forming unit one excrement.
One per cent solution of garlic powder was fed by catheter once daily for three days, then animals were sacrificed on day 4 for analysis.

Antibacterial Activity of Garlic Odor

A variety of foodstuffs and plants are known to produce odor (flavor) either in the raw state or in the process of cooking. Some studies suggest that odor modulates mental activity to reduce stress and aids recovery from distress [11, 12]. However, there has been little research to date on the odor (flavor) of vegetables or plants and little scientific information has accumulated.

Our recent data showed that garlic's odor (flavor) had a bactericidal potency due to the volatiles released from grated garlic or its juice. For this experiment, grated garlic or other samples were placed in the lid of the Petri dish, which was then covered with the bacteria-streaked agar dish. After sealing with Scotch tape, the dish was cultivated. The result obtained is shown in Fig. 4.2. Other types of foodstuffs, such as onion, horseradish, and *Houttuynia cordata*, produced similar results and their odor also killed bacteria.

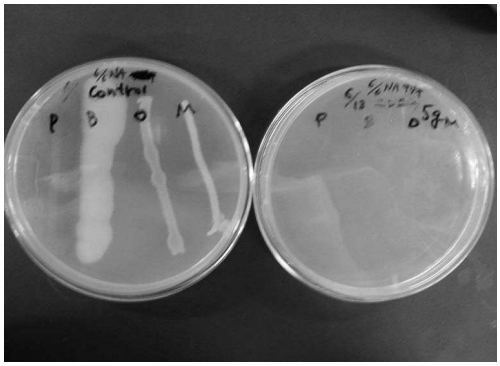


Fig. 4.2 Inhibition of bacterial growth by garlic odor released from chopped fresh garlic bulb. Pathogenic bacteria failed to grow when exposed to garlic odor released from grated bulbs placed on the lid of Petri dish (right). Control dish without garlic odor (left). From left to right: *Pseudomonas aeruginosa*, *Bacillus subtilis*, enterrohemorrhagic *E. coli* O157, and methicillin-resistant *Staphylococcus aureus* (MRSA).

The volatile allicin in garlic is primarily responsible for garlic's odor and sulfur compounds are produced when cells are ruptured, resulting in the formation of different thiosulfinates and related sulfonic acid-derived compounds by reaction taking place between the enzyme allinase and the volatile precursor alliin [13]. It can also blister the skin and kill bacteria, viruses, and fungi. The evidence suggests that garlic uses allicin for protection against bacteria and parasitic threats. This is a kind of defense system acquired over evolution to guard against attack [1].

4.2.2.2 **Anticoagulation Effects**

It has long been known that garlic and onion have an anti-aggregation effect on platelets, and several mechanisms appear to be associated with this process, such as modification of the platelet membrane properties, inhibition of calcium mobil-

ization, and inhibition steps of the arachidonic acid cascade in blood platelets [14, 15].

Our animal experiments also suggested a prolongation of blood coagulation time in garlic powder-fed mice. In this experiment, 1 mL of 5% garlic powder in water was administered orally once daily for three days by catheter and coagulation time was measured. Garlic-fed mice clearly demonstrated a prolonged blood coagulation time (Table 4.5, Fig. 4.3).

Administration of 800 mg of garlic powder to a human over a period of four weeks made spontaneous platelet aggregation disappear [13]. Blood thinning as a positive action of garlic sometimes leads to negative effect such as induction of bleeding. Because of this care should be taken in ingesting garlic (pills) prior to surgery or labor and delivery, due to the risk of excessive bleeding. Similarly, gar-

Table 4.5 Prolongation of blood coagulation time in mice fed with garlic powder.

Mouse number (n = 3)	*Coagulation time (s)*	*Average*
Before feedingof garlic powder		
Mouse 1	150	170 ± 28.3
Mouse 2	150	
Mouse 3	210	
After feeding of 5% garlic powder		
Mouse 1	240	390 ± 106.8
Mouse 2	480	
Mouse 3	450	

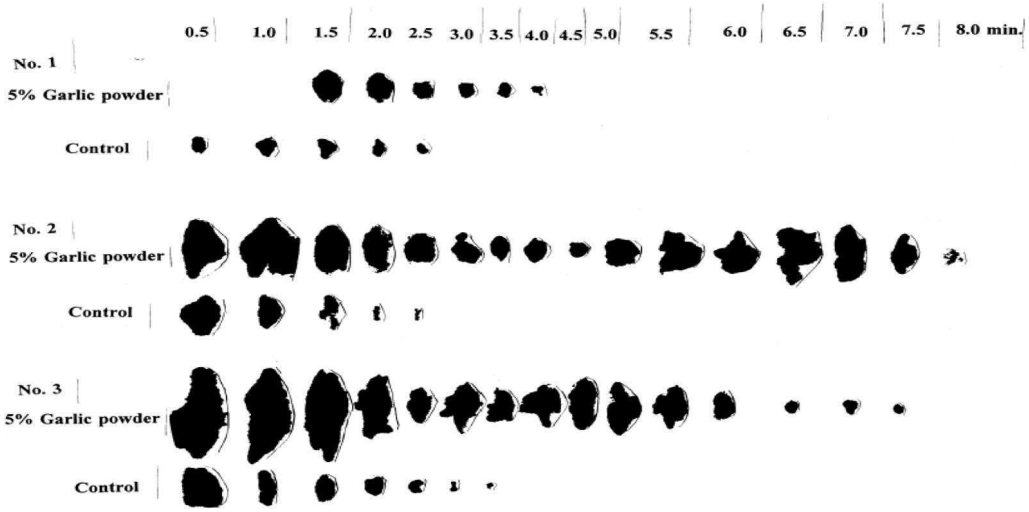


Fig. 4.3 Prolongation of blood coagulation time in garlic powder-fed mice. These pictures correspond to the results given in Table 4.5. The blood coagulation time in three mice was prolonged after they were fed with garlic powder.

lic should not be combined with blood-thinning drugs, such as warfarin, heparin, aspirin, or pentoxifylline [1].

Concerning safety issues of garlic, no negative effects were observed in rats fed with high doses of garlic (200 mg kg^{-1} body weight) for six months [2, 16]. However, care should be observed in taking excessive raw garlic as it produces numerous symptoms as described above.

4.2.2.3 **Antioxidant Activity**

Antioxidation is one of the most important mechanisms for preventing or delaying the onset of major degenerative diseases [2]. Active oxygen (hydroxyl, peroxy radicals, and single oxygen) is highly toxic and one of the strongest causative agents of many diseases, including cancer, heart disease, cataracts, and cognitive disorder. Antioxidants block the oxidation processes that contribute towards these chronic diseases and delay the onset of degenerative diseases of aging [17, 18].

The antioxidative activity of garlic powder has been evaluated to compare it with that of horseradish and shellfish extracts. Garlic powder showed the strongest antioxidant activity, and its activity was dose dependent (Table 4.6).

Antioxidant properties in garlic extracts are mostly attributed to the presence of allicin, as antioxidant activity of allicin-free garlic extracts was much lower than that of the garlic extracts [19]. Antioxidant mechanisms are believed to exert their effects by blocking oxidative processes and free radicals that contribute to the causation of these chronic diseases [2, 17, 18]. Like the constituents of grapes, such as catechins, flavonols, anthocyanins, and tannins [20], garlic is thought to possess similar antioxidant activity.

Table 4.6 Antioxidant activity of garlic, Japanese horseradish, Western horseradish, and scallop extracts.

Specimen	*Concentration (mg mL^{-1})*	*Comparative activity to BHA (1 mg per 100 mL) (%)*
Garlic	5	66.5
	2.5	60.1
	1.25	53.2
Japanese horseradish	5	56.0
	2.5	23.0
	1.25	22.2
Western horseradish	5	–28.6
	2.5	–39.1
	1.25	8.5
Scallop extracts	5	36.7
	2.5	19.0
	1.25	5.6

BHA, butylated hydroxyanisole.

4.2.2.4 Therapeutic Effects of Garlic Powder in the Organophosphate Compound Poisoning Mouse as a Model of SARS

In 2002, an outbreak of severe acute respiratory syndrome (SARS) occurred in Guangdong Province, China, and 800 of 8000 infected people became the victims of the SARS coronavirus infection. There are very few effective antibiotics or chemicals for the treatment of this virus infection, and patients have to wait over 10 days for the production of virus-specific antibody to recover from virus-caused infections.

My co-worker, Dr Lu Changlong of the China Medical University, found a novel biological function in garlic powder, which was effective in detoxifying organophosphate compound poisoning in mice used as a SARS model. This alternative SARS model shows close similarity on pathohistological findings in lung to those of the SARS-infected human.

After a week's administration of 1% garlic powder solution, the organophosphate solution was given orally to mice to develop the SARS mimicking disease. The curative effect induced by the garlic powder was more than that expected and 75% of the garlic powder-fed mice (9/12) recovered from the disease, whereas in the control group only 8% survived (1/12) (Fig. 4.4).

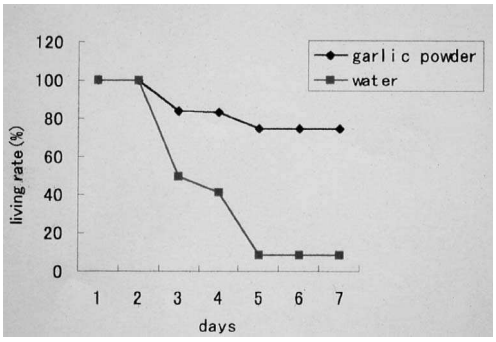


Fig. 4.4 Therapeutic effects of garlic powder in response to organophosphate poisoning used as a SARS model in mice. An improved survival rate was clearly observed in the garlic-treated group.

The detoxification potency of the organophosphate by garlic powder was probably due to the chelating activity of garlic powder. This newly found property in garlic is a very promising complementary therapeutic approach for the treatment of cases of organophosphate poisoning.

4.3 Mushroom

4.3.1 Introduction

There are over 1500 mushroom species growing in Japan, of which around 300 species are edible. In the autumn, Japanese enjoy harvesting mushrooms, especially in the mountains, and a variety of mushroom dishes are appreciated at home

and in restaurants. Some of the mushrooms are pickled or dried to use as preserved foods for the special occasions, such as at the year end and new year. Recently, biotechnological devices have allowed the cultivation of a variety of mushrooms in greenhouses, which means that they are constantly supplied all year round.

Mushrooms are represented commonly in Japanese folk medicine and have been used for cancer therapy since ancient times in Japan. However, in Europe and America, mushrooms are not included as herbal plants, and there are few descriptions of the therapeutic properties of mushrooms in the literature published in other countries.

Recently, the healing powers of mushrooms, ranging from curing cancer to preventing heart disease, have been reviewed scientifically to lend support to these ancient beliefs in the form of reliable evidence. Some Japanese pharmaceutical companies have already developed anticancer medicines, such as Krestin, Lentinan, and Sizofiranm, which are now being administered clinically to cancer patients in hospital.

The nonedible toadstool tsukiyotake, which causes most of the mushroom intoxication in Japan, also contains antitumor substances. In this section, we look at the anticancer properties of the edible mushroom maitake (*Grifola frondosa*) and the poisonous mushroom tsukiyotake (*Lampteromyces japonicus* Singer) (Fig. 4.5).

Fig. 4.5 Maitake mushroom (*Grifola frondosa*) (left) and poisonous Tsukiyotake mushroom (*Lamterumyces japonica* Singer) (right).

4.3.2 Biological Effects

4.3.2.1 Antitumor Activity

Edible Mushroom Maitake (*Grifola frondosa*)

Maitake is one of the most popular mushrooms used as a medicine, and is now easily available in numerous stores throughout the year because of the artificial cultivation. An American book recently outlined maitake's medicinal effects, but it

stated that there has been no reliable research to determine whether any of these ancient beliefs are really true or not and that formal safety studies have not been performed [1]. We maintain that the safety of maitake does not need to be questioned because it has been taken daily by many people for generations and no cases of medical problems have been reported to date.

In our laboratory, boiled water extracts of maitake showed anticancer activity with a cure rate at 60% against Meth A tumor of BALB/c mice, using three intratumor injections of 5 mg (Table 4.7). The ethanol precipitate (ET-pre) from the boiled water extracts was stronger in therapeutic potency than that of the boiled water extracts, and its cure rate was 80%, using three intratumor injections of 1 mg [21] (Table 4.8).

The ET-pre was further fractionated into the low (s-R) and high (r-R) molecular RNA, and the water-soluble b-glucan (ASAS). The antitumor potencies of these components were verified and are summarized in Table 4.9 [22].

Since the boiled water extracts did not show cytotoxicity against Meth A tumor cells, maitake extracts probably strengthen the immune system *in vivo* to inhibit the growth of tumors.

Table 4.7 Antitumor potency of boiled water extracts of maitake against Meth A tumor of BALB/c mice.

Treatment	***Cure rate***	***Tumor size in noncured mice (mm^2)***
Experiment 1		
500 mg, 3 shots	0/5	534
5 mg, 3 shots	1/5	220
Control (no treatment)	0/5	527
Experiment 2		
5 mg, 3 shots	1/5	116
Control	0/5	375
Experiment 3		
5 mg, 3 shots	3/5	47
Control	0/5	457

Mice were treated with sample on days 2, 4 and 6 after tumor transplantation. Antitumor potency was evaluated three weeks after tumor transplantation.

Table 4.8 Antitumor potency of ethanol precipitates of maitake against Meth A tumor.

Dosage	***Cure rate***
ET-pre (1 mg, 3 shots)	4/5
Ether-washed ET-pre. (1 mg, 3 shots)	1/5
Control	0/5

ET-pre, ethanol precipitate.
Mice were treated with sample on days 2, 4 and 6 after tumor transplantation. Antitumor potency was evaluated three weeks after tumor transplantation.

Table 4.9 Tumor curative potency of RNA and β-glucan separated from maitake extracts against Meth A tumor.

Fraction	*Cure rate*
Low molecular RNA (1 mg, 3 shots)	4/5
High molecular RNA (1 mg, 3 shots)	2/5
β-Glucan (1 mg, 3 shots)	1/5
Control	0/5

Tumor-transplanted mouse was treated with sample on days 2, 4, and 6 after tumor transplantation. Antitumor activity was evaluated three weeks after tumor transplantation.

A principal constituent in maitake extracts with antitumor activity is considered to be β-D-glucan, which might affect the human immune system in complex ways [2]. However, our data showed that the RNA fraction in maitake extracts was more effective in antitumor activity than that of β-glucan (Table 4.9), suggesting that the RNA also contributes substantially to the antitumor activity of maitake, working together with β-glucan.

Effectiveness of Maitake's extracts is suggested against the liver cancer, breast cancer and leukemia, and stomach and brain cancer were less responsive to Maitake's treatment. Other proposed uses of Maitake are for diabetes, hypertension, high level of cholesterol, however, clinically definitive scientific evidences should deserve further serious investigations that Maitake really functions in this way [2].

Other proposed uses for maitake are for diabetes, hypertension, high levels of cholesterol, but scientific evidence of these effects is lacking.

Tsukiyotake (*Lampteromyces japonicus* Singer) (Fig. 4.5)

There are about 40 species of poisonous mushrooms growing in Japan, and some of them are very like the edible mushrooms in appearance, which often results in mushroom intoxication. Inedible tsukiyotake (*Lampteromyces japonicus*) closely resembles the edible mushroom hiratake (*Pleurotus ostreatus Que*l) and is a leading cause of mushroom poisoning, with symptoms of nausea and diarrhea. The main toxic substance in tsukiyotake is "Illudin S," which induces vomiting and diarrhea 30 min after ingestion, but few lethal cases have been reported. Amazingly, people in mountainous village have knowledge of how to detoxify poisonous mushroom and make them edible.

Boiled water extracts and two fractionates (Fr. I and II) were first used in mouse toxin tests, then in antitumor tests. Fr. I induced diarrhea in mouse and Fr. II showed lethal toxicity after 5 mg intraperitoneal injection (Table 4.10). Intraperitoneal injection of 1 mg Fr. II showed no lethal toxicity, and this dosage did not affect blood cell constituents in mouse (Table 4.11). Oral administration of Fr. II prior to tumor transplantation effectively inhibited growth of the tumor by 80% (Table 4.12), but Fr. I did not show antitumor activity.

Table 4.10 Toxicity test of tsukiyotake (*Lampteromyces japonicus*) fractionates in a mouse model.

Time after administration	*Fr. I (5 mg, i.p.)*	*Fr. II (5 mg, i.p.)*
4 h	1/5 (diarrhea)	No change
7 h	5/5 (diarrhea)	No change
24 h	5/5 (diarrhea)	No change
2 days	All recovered	No change
11 days	Normal	4/5 (died)
12 days	Normal	5/5 (died)

i.p., intraperitoneal injection.

Table 4.11 Blood cell constituents in mice injected with tsukiyotake Fr. II.

Group (n = 4)	*RBC ($\times 10^4$)*	*WBC ($\times 10^3$)*	*Ht (%)*	*Hb (%)*
Fr. II	992 ± 17.1	56 ± 4.0	57.2 ± 1.4	16.7 ± 0.2
Control	924 ± 5.2	57 ± 6.8	54.3 ± 0.3	16.7 ± 0.2

RBC, red blood cell; WBC, white blood cell; Ht, hematocrit; Hb, hemoglobin.
No toxicity was observed for Fr. II by 1 mg intraperitoneal injection.

Table 4.12 Antitumor activity of tsukiyotake toadstool Fr. II by oral administration in a Meth A tumor model.

Dosage of Fr. II	*Cured mice*	*Tumor size in noncured mice (mm^2)*
1 mg	4/5	35
5 mg	2/5	54 ± 30
Control	0/5	592 ± 112

Fr. II was administered orally for a week before tumor transplantation, then antitumor activity was evaluated three weeks after tumor transplantation.

Oral administration of Fr. II resulted in a higher cure rate against mouse tumor than intratumor injection. The mechanisms of action remain unclear because there is no cytotoxicity against Meth A tumor cells and no effect on the number of immune cells (CD8+ T cells, natural killer (NK) cells) in peripheral blood. Ubiquitous constituent(s) with anti-tumor potency such as glucan presumably exists among both edible (Maitake) and non-edible (Tsukiyotake) mushrooms, which is a principal reason for two types of mushrooms could demonstrate anti-tumor activity.

4.4 Sweetcorn

4.4.1 Introduction

The diet of the younger generation in Japan is rapidly becoming Westernized, including items such as bread, soup, coffee, or tea taken at meals. Consumption of corn soup is increasing and it is available in every supermarket throughout the country. Hokkaido at the northern end of Japan is a principal corn-producing area, and provides a wide range of species to the nation.

One of aims of my laboratory is to search out more effective antitumor substances from natural resources. Our previous findings were that glycogen or glycogen-like substances extracted from scallops possessed strong antitumor activity against mouse tumors [23]; however, other researchers could not agree with our results. Also, a highly purified glycogen as a chemical reagent did not show any antitumor activity.

This discrepancy led us to carry out further experiments to confirm our results (hypothesis) in collaboration with the Kewpie Institute in Tokyo by using the phytoglycogen prepared from sweetcorn.

4.4.2 Biological Effects

4.4.2.1 Antitumor Activity of Sweetcorn

Native sweetcorn powder and three types of phytoglycogen extracted from sweetcorn powder (PG, PG-S, PG-LS) were tested for antitumor activity in a mouse model. The phytoglycogen PG-S revealed antitumor activity and gave a cure rate of 40% (2/5) when administered by intratumor injection (Table 4.13) [24]. Oral administration, before or after tumor transplantation, was adopted for the evaluation of its activity.

Table 4.13 Antitumor activity of sweetcorn's phytoglycogen by intratumor injection.

Sample	*Cured mice*	*Tumor size in noncured mice (mm^2)*
PG, 1 mg	0/5	521 (59.9%)
Control	0/5	869
PG-S, 1 mg	2/5	358 (56.4%)
PG-S, 5 mg	0/5	438 (69.0%)
Control	0/4	634
PG-LS, 5 mg	0/5	570 (108.4%)
Control	0/5	692

Tumor-bearing mice were treated by test sample on days 2, 4, and 6 after tumor transplantation.

Oral administration of sweet corn powder before tumor transplantation (pre-oral administration) was very effective to prevent growth of tumor, and mice at eighty percent (4/5) completely inhibited the growth of tumor (Table 4.14). The antitumor effectiveness by oral administration of sweetcorn powder was better than that by intratumor treatment, as shown in Table 4.13. It is possible that tumor at a very early stage can be preventable and eradicable by regularly intake of sweetcorn powder.

Table 4.14 Tumor growth inhibition by pre-oral administration of sweetcorn powder.

Dosage	*Cured mice*	*Tumor size in noncured mice (mm^2)*
200 μg	1/	66 ± 32 (19.4%)
1 mg	4/5	96 ± 0 (27.8%)
5 mg	0/5	214 ± 42 (62.3%)
Control	0/4	345 ± 248

Sweetcorn powder in water was orally given by catheter once daily for a week, then tumor cells were transplanted intradermally.

The phytoglycogen PG-S weakly increased the number of CD8+ T cells and NK cells in the peripheral blood of mice, and weakly enhanced phagocytic activity of macrophages, but these data are not enough to explain the antitumor mechanism of sweetcorn (Table 4.15).

Table 4.15 Profile of lymphocyte subsets in blood of mice injected with intraperitoneal phytoglycogen (PG-S).

Group	*CD4+ T cells (%)*	*CD8+ T cells (%)*	*Natural killer cells (%)*
PG-S injected	69.5 ± 1.3	13.0 ± 0.4	6.7 ± 0.04
Control (saline)	66.9 ± 1.3	10.6 ± 0.03	5.5 ± 0.3

The phytoglycogen contained 45% corn powder as a major constituent, which probably played an important role in healing the tumors. In a structural analysis of scallop glycogen in relation to antitumor activity, it became clear that the glycogen with antitumor potency was highly branched with a shorter chain than the glycogen with no antitumor activity. These results suggest that short and highly branched saccharide chains with nonreducing terminals are essential to maintain antitumor activity [16].

The biological functions of glycogen or glycogen-related compounds still remain obscure and research in this field should be carried out to answer these questions in the near future.

4.5
Oil and Flavor of Tree Hiba (Japanese Cypress) (Hinokitiol)

4.5.1
Introduction

The hiba (Japanese cypress) is a tree that grows in Japan and produces high-quality timber for housing materials with a range of characteristics, such as durability, antihumidity, antiseptic, and a fresh flavor (Fig. 4.6). It is known by woodmen through experience that fewer insects and weeds are found around this tree than near other species, which suggests that there is a continuous release of certain volatiles (flavor) from the tree.

Fig. 4.6 Tree hiba (Japanese cypress) and leaves enlarged.

The bactericidal potential of hiba oil has already been reported and overviewed by others [25, 26]. Interestingly, recent work has revealed that pathogenic bacteria are easily killed by exposure to hiba flavor. This novel finding in flavor function could expand its availability of oil or crystal into daily necessities such as cleaning air in the house or hospital by using bacteria killing potency of flavor together with induction of mental relaxation. Actually the Hiba oil is widely utilizing as ingredient for soap, toothpaste, clothing, et al. and the Hiba-wooden bed is recently commercialized using our experimental data of flavor. Number of researches on flavor is increasing in our country with expectation to cultivate novel medicinal function.

4.5.2
Biological Effects

4.5.2.1 Antibacterial Activity of Flavor Released from Hiba Oil and Hinokitiol Crystal

Steam distillation of sawdust from the hiba tree yields 1% oil that consists of phenolic acid and a terpenoid type oil (neutral volatile oil). Crystal hinokitiol was a

principal constituent in the phenolic acid oil with a fresh tree flavor. Hiba oil and crystal are now widely used as ingredients for daily necessities, such as soap, toothpaste, clothing, and other products, because of their nontoxicity and aromatic activity. Our works confirmed the bactericidal potential of the flavor from hiba oil and crystal.

The experiment was carried out using the hinokitiol crystal. Hinokitiol crystal was placed on the lid of a Petri dish, and was covered by a bacteria-streaked nutrient agar dish for cultivation at 37 °C. Flavor from the crystal was effective in inhibiting the growth of bacteria (Table 4.16).

Table 4.16 Growth inhibition of pathogenic bacteria by hinokitiol-released flavor.

Dosage (mg per Petri dish)	*MRSA*	*O157*	*Ps. aeruginosa*
100	+	+	+
10	+	+	+
5	+	+	PG
2.5	PG	PG	PG

+, Complete growth inhibition; PG, partial growth inhibition.
Test was carried out using the crystal hinokitiol.

In addition to the bactericidal effect, the psychological functions of hiba flavor as an aromatherapy were recently reported in tests of chronic hemodialysis patients [27]. The presence of the oil's flavor significantly decreased scores on the Hamilton Rating Scale for Depression (HAMD) and the Hamilton Rating Scale for Anxiety (HAMA). It was concluded that the odor of the oil was very effective for the treatment of depression and anxiety in chronic hemodialysis patients [28]. The use of hiba oil and hinokitiol crystals is now being expanded from the medical field into the production of daily necessities to create amenities and improve mental health conditions.

4.6 Conclusions

The biological functions of plants traditionally used in Japan have been introduced and discussed. Surprisingly, the odor of garlic and the flavor of a tree oil (Japanese cypress) showed antibacterial activity against pathogenic bacteria. These findings suggest that there are still undeveloped research fields that could contribute more to the medical area.

One of the important tasks that should be conducted urgently is a broad review of the analyses of functional foods, including fruit, seaweed, fish, shellfish, and other natural sources. The results of these experiments are essential if we are to create effective therapeutic strategies for disease treatments combining functional foods and herbs with Western medicines.

Acknowledgments

The author gratefully acknowledges my laboratory's students, Y. Itoh, N. Kumaki, C. Sutoh, T. Satoh, A. Norigami, K. Chiba, C. Lu, for assistance in the experiments, and Drs H. Uchisawa and N. Yamaguchi for a their contribution to the purification and chemical analyses of materials. These studies were financially supported by Grant Aid from Kieikai (Kewpie Ltd., Tokyo).

References

1 Bratman, S. (ed.), *Herbs*. Prima Publishing, Roseville, CA, **2000**.
2 Mazza, G. (ed.), *Functional Foods*, Technomic Publishing, Lancaster, PA, **1998**.
3 Riley, L.R., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A., Cohen, M.L. *N. Engl. J. Med*. **1983**, *308*, 681–685.
4 Sasaki, J., Kita, T., Ishita, K., Uchisawa, H., Matsue, H. *J. Nutr. Sci. Vitaminol*. **1999**, *45*, 785–790.
5 Block, E., *Sci. Am*. **1985**, *252*, 114–119.
6 Steiner, M., Khan, A.H., Holbert, D., Lin, RI-S. *Am. J. Clin. Nutri*. **1996**, *64*, 866–870.
7 Burrows, W. (ed.), *Textbook of Microbiology*, W.B. Saunders Company, Philadelphia, **1968**.
8 Sasaki, J., Kita, J. *J. Nutr. Sci. Vitaminol*. **2003**, *49*, 297–299.
9 Satoh, A., Terao, M., Ishibashi, M. *Sokihin Eiseigakushi* **1993**, *34*, 63–67 (in Japanese).
10 Satoh, A., Terao, M., Konn, M., Kobatake, M. *Nihon Shokuhin Biseibutsugakushi* **1996**, *13*, 121–125 (in Japanese).
11 Okabe, T., Hiraoka, K., Saito, K., Inuma, K. *Fragrance J*. **1992**, *20*, 106–111 (in Japanese).
12 Itai, T., Amayasu, H., Kuribayashi, M., Kawamura, N., Okada, M., Momose, A., Takayama, T., Narumi, K., Uematsu, W., Kaneko, S. *Psychiatry Clin Neurosci*. **2000**, *54*, 393–397.
13 Nakamura, Y.K., Matsuo, T., Shimoi, K., Nakamura, Y., Tomita, I. *Biosci. Biotechnol. Biochem*. **1996**, *60*, 1439–1443.
14 Ariga, T., Oshiba, S., Tamada, T. *Lancet* **1981**, *2*, 150–154.
15 Apitz-Castro, R., Cabrera, S., Cruz, M.R., Ledezma, E., Jain, M.K. *Thromb. Res*. **1986**, *42*, 303–311.
16 Kiesewetter, H., Jung, F., Pindur, G., Jung, E. M., Mrowietz, C., Wenzel, E. *Int. J. Clin. Pharmac. Toxicol*. **1991**, *29*, 151–155.
17 Ames, B.N. *Sciences* **1983**, *221*, 1256–1264.
18 Block, G. *Nutr. Rev*. **1992**, *50*, 207–213.
19 Okada, Y., Tanaka, K., Fujita, I., Sato, E., Okajima, H. *Redox Rep*. **2005**, *10*, 96–102.
20 Decker, E.A. *Nutr. Rev*. **1995**, *53*, 49–58.
21 Sasaki, J., Kita, T., Ishita, K., Uchisawa, H., Matsue, H., Sugawara, C., Lu, C., Yan, J., Liu, J. *BCG BRM Ther*. **1999**, *23*, 17–21 (in Japanese).
22 Sasaki, J., Uchisawa, H., Matsue, H., Kita, T., Ishita, K., Sugawara, C., Takahashi, K., Nohtomi, T., Yoshida, K. *BCG BRM Ther*. **2000**, *24*, 25–28 (in Japanese).
23 Takaya, Y., Uchisawa, H., Ichinohe, H., Sasaki, J., Ishita, K., Matsue, H. *J. Mar. Biotechnol*. **1998**, *6*, 208–213.
24 Sasaki, J., Soga, H., Shinohara, C., Itoh, Y., Sasahara, R., Yoshida, K. *BCG BRM Ther*. **2003**, *27*, 27–31 (in Japanese).
25 Okabe, T., Saitoh, K. *Wood Preservation* **1993**, *19*, 18–28 (in Japanese).
26 Okabe, T., Saitoh, K., Ootomo, Y., Kudoh, Y. (eds.), *Mysterious Tree Aomori Hiba*, Hirosaki Sogo, Co., Hirosaki, Japan, **1990**.
27 Itai, T., Amayasu, H., Kuribayashi, M., Kawamura, N., Okada, M., Momose, A., Takayama, T., Narumi, K., Uematsu, W., Kaneko, S. *Psychiatry and Clinical Neurosciences* **2000**, *54*, 393–397.
28 Hiruma, T., Yabe, H., Sato, Y., Sutoh, T., Kaneko, S. *Biol. Psychol*. **2002**, *61*, 321–331.

5
Plant Extracts Used to Manage Bacterial, Fungal, and Parasitic Infections in Southern Africa

Jacobus Nicolas Eloff and Lyndy Joy McGaw

Summary

Infectious diseases are prevalent in many areas of the world, particularly in developing countries. With the high incidence of acquired immune deficiency syndrome (AIDS) in many sub-Saharan African countries, opportunistic pathogens such as *Candida albicans* and *Cryptococcus neoformans*, as well as other fungal, bacterial, and parasitic infections, are becoming major health problems. Added to this problem is the deficiency of health care clinics adequately equipped to cope with these challenges. Southern Africa has a long history of medicinal plant use by traditional healers and other community members to combat infections in humans and animals. As a consequence of the widespread use of a diverse array of plants to treat infectious diseases, coupled with the renowned plant diversity in South Africa, we have spectacular potential to discover anti-infective activity in extracts of these plants.

Many methods may be used to select plants for bioactivity testing, including ethnobotanical and ethnoveterinary leads, random selection and chemotaxonomic approaches. In the Phytomedicine Programme we have concentrated on investigating species belonging to the family Combretaceae, yielding much useful information about biological activity relationships in the family. Traditional uses of plants and random screening have also proved their value as methods of plant selection for phytomedicinal investigations. Our aim is two-pronged: first to bring to light highly active plant extracts, and second to concentrate on using bioassay-guided fractionation to isolate and identify the chemical constituents responsible for activity.

The bioassays forming the focus of our investigations comprise antibacterial, antifungal, and antiparasitic activity tests, among others. The antibacterial and antifungal test organisms include Gram-negative and Gram-positive bacteria recommended by the United States National Committee for Clinical Laboratory Standards (NCCLS), various fungal species (plant, animal, and human pathogens), as well as *Mycobacterium* species and methicillin-resistant *Staphylococcus aureus*. Plant extracts are tested for antiparasitic activity against the animal helminth parasites *Haemonchus contortus* and *Trichostrongylus colubriformis*, as well as against the

free-living nematode *Caenorhabditis elegans*. To ensure that biological activity is not due to a general toxic effect, we also carry out cell line cytotoxicity studies and the brine shrimp larval mortality assay. Toxicity screening in laboratory systems is a necessary aspect of the preliminary safety evaluation of plant-derived extracts and compounds prior to further development and commercialization.

Bioassay-guided fractionation on crude plant extracts with excellent activity in our laboratories has successfully yielded isolated active compounds with some potential for commercialization. Extracts from plants which have been developed and potentized without isolation of constituents have many applications, particularly where it appears that a number of compounds in the extract may have synergistic effects.

Herbal extracts and isolated compounds with biological activity discovered in our group are being applied to the development of treatments for human and animal diseases. Tests *in vivo* performed thus far have identified extracts and compounds with exceptional antibacterial and antifungal activity. In addition, we are investigating antifungal compounds in plant extracts that assist in protecting cultivated plants from fungal attack. These successes emphasize the potential value of newly developed plant products, particularly from a region as rich in plant biodiversity as South Africa.

We conclude that there is a much better opportunity to develop commercially useful products by focusing on plant extracts rather than the isolated compounds.

5.1 Introduction

Southern Africa is fortunate to possess a remarkable diversity of indigenous plants, coupled with rich cultural traditions on the use of plants for medicine. The lack of easy access to Western primary health care and veterinary services in many rural parts of the country has supported the use of traditional medicine to treat both humans and animals. Even where clinics and orthodox medicines are readily available, a large proportion of the population uses traditional medicines together with, or in preference to, Western medicine. Exploration of the uses of plants affords scientists a rich source of research opportunities in disease control, particularly infectious diseases which are so prevalent in the developing world. Research targeted at investigating the biological activity and potential toxicity of medicinal plants is a priority, not only in South Africa but worldwide.

As an example of South African research on the use of plants for treating infections in animals and humans we focus on the Phytomedicine Programme (Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, website http://www.up.ac.za/academic/veterinary/depts_paracl_phyto.htm). Reference will be made where possible to related research projects occurring in other groups in the country. There are several recent reviews available on the status of South African ethnobotanical, phytochemical, and pharmacological investigations. These reviews provide detailed information about research projects in other groups working on various aspects of medicinal plant use [1–4].

In this chapter a number of general aspects will be discussed followed by procedures that we have used to deliver products that are patented and licensed to be used in industry. We will share our experience and shortcuts that we have developed.

5.2 Biodiversity in Southern Africa

The concept of biodiversity encompasses the number and variety of organisms inhabiting a specified geographic region. Owing to its diverse range of climatic and topographic conditions, southern Africa possesses a wealth of plant and animal species. It is considered to have the richest temperate flora in the world, with a floristic diversity of about 24 000 species and intraspecific taxa in 368 families. With only 2.5% of the world's land surface, it contains more than 10% of the world's vascular plant flora [5]. Southern Africa also contains a major proportion of the 50 500 taxa present in sub-Saharan Africa [6].

South Africa has a flourishing diversity of cultures, with 11 official languages, giving an indication of the many different communities present in this area of the African continent. Studies of the varying cultural practices, together with methods of traditional healing using the extensive array of available plants, are yielding valuable information to researchers.

5.3 Use of Plants in Southern African Traditional Medicine

Globally, natural products and their derivatives represent about 50% of all drugs in clinical use, and higher plants contribute 25% to this figure [7–8]. It is well-known that plants were originally a source of medicines, and there is currently a strong interest in natural medicines as a source of new remedies and bioactive compounds. This phenomenon is reflected in South Africa, which has a long history of medicinal plant use. South Africa has contributed to worldwide medicines with natural teas and remedies such as Cape aloes (*Aloe ferox*), rooibos (*Aspalathus linearis*), buchu (*Agathosma betulina*), honeybush (*Cyclopia intermedia*), and devil's claw (*Harpagophytum procumbens*).

There are an estimated 200 000 indigenous traditional healers in South Africa [9]. They are known by different names according to the different cultures, for example "inyanga" and "isangoma" (Zulu), "ixwele" and "amaquira" (Xhosa), "nqaka" (Sotho), "bossiedokter" and "kruiedokter" (Afrikaans). There is often a basic general knowledge of medicinal plant use among the elderly members of the community.

A survey in Durban (KwaZulu-Natal) indicated that over 80% of the black population relies on both Western and traditional health care systems [10], and this figure is likely to be reflected country-wide. The market for medicinal plants is vast, and it has been estimated that 20 000 tonnes of plant material are traded in South

Africa every year [10]. Based on information on herbarium sheets, 3689 taxa in 215 families and 1240 genera are used ethnomedicinally in the southern African region [11]. This represents about 15% of the flora and includes 159 threatened Red Data Listed taxa [11]. The sustainable use and conservation of these plants is of immense importance to researchers and traditional medicine consumers alike.

The use of plant remedies to treat animals developed concomitantly with human ethnomedicine, and ethnoveterinary healing remains an integral part of animal health care in developing countries [12]. The scope of ethnoveterinary medicine incorporates traditional veterinary theory, diagnostic procedures, medicines, surgical methods, and animal husbandry practices [13]. Ethnobotany constitutes an essential element of ethnoveterinary medicine, as plants form the basis of many treatments.

5.4 The Need for Anti-Infective Agents

The emergence of antibiotic resistance in both Gram-negative and Gram-positive bacteria is on the increase, and in spite of attempts to control the use of antibiotics, the incidence of resistance threatens to overwhelm modern health care systems [14]. Several risk factors have been implicated as causal factors of antibiotic resistance, for example the irresponsible use of antibiotics, and antibiotics used as prophylactics in food production. There is an increasing need for new antibiotic agents to treat the multidrug-resistant pathogens that are frequently encountered both in hospitals and in the community. This worrying situation is reflected in the drug resistance encountered against disease-causing helminth and protozoan parasites of humans and animals worldwide.

In southern African countries rural populations, particularly children, commonly suffer from diarrhea, gastrointestinal parasites, and bilharzia [14]. The expense of orthodox medicines and the frequent lack of easy access to Western health care facilities encourages the use of traditional healers in rural regions. African traditional healers have a holistic approach and treat the putative cause of the ailment as well as the symptoms of the disease. Treatment often has a major psychological component involving ancestral spirits. Research on the efficacy and potential toxicity of medicinal plants used to combat infectious diseases may lead to interesting leads for new plant extracts or isolated compounds with antibacterial activity.

5.5 Selection of Plant Species to Investigate

The choice of plant selection method for phytochemical and biological activity screening is often difficult. The abundance of plants available to researchers in South Africa lends support to a rational, methodical approach that will supply the greatest potential for discovery of interesting, biologically active chemicals. There

are three main methods of plant selection with the aim of isolating and identifying active substances, namely the ethnobotanical, chemotaxonomic, and random selection approaches.

5.5.1 Ethnobotanical Approach

This approach entails selecting plants used in traditional medicine on the reasonable premise that remedies used to treat a certain ailment may have an associated biological activity. To enhance chances of success in detecting significant biological activity, we have concentrated on plants used to treat easily diagnosed illnesses such as sores, wounds, and intestinal parasites. Diagnosis by traditional healers of internal problems, such as cardiovascular disease and cancer, is more difficult to verify. The value of the ethnobotanical approach in our research is highlighted later in the chapter.

In South Africa, a number of ethnobotanical works have documented the use of indigenous plants by traditional healers and local communities for various medical conditions [15–17]. Although some studies on the ethnobotanical uses of plants for treating livestock and domestic animals in different parts of the country are available [18–20], this remains a neglected area of ethnobotanical investigation. The comprehensive, systematic documentation of indigenous knowledge on traditional plant use in South Africa, as indeed in many other countries, is essential before this knowledge disappears.

5.5.2 Chemotaxonomy

If the taxonomic classification used (based mainly on morphological parameters) approximates a natural classification there should be a good correlation between the taxonomy and occurrence of plant secondary compounds. It may be possible to discover related compounds in related species, genera, or families.

Several members of the family Combretaceae have been used to treat bacterial diseases in southern Africa [15, 16]. Different degrees of antibacterial activity in the different species may have some taxonomic predictive value [21]. We have successfully used the chemotaxonomic approach to reveal significant biological activity of many members of this family [21–27], and this is described in more detail later in the chapter.

5.5.3 Random Selection

The value of the random method of selecting plants should not be underestimated, taking into consideration the diversity of plant life in South Africa. Balick [28] has found that after dereplication there is no difference between the anti-HIV activity of species randomly screened and that of species with ethnomedicinal use.

5.6 Collecting, Drying, and Storage of Plant Material

Any fungal contamination on leaves leads to large differences in the chemical composition and biological activity of leaf extracts. Any plant material with visible fungal growth or insect attack is therefore discarded. We do not collect material that is wet or before dew has dried off completely, and collected material is dried as quickly as possible.

Collecting plant material in nature poses difficulties with regard to drying under controlled conditions but we follow guidelines to facilitate the process. We never store material even for short periods in plastic containers. We use paper bags to collect the leaves and within a short period spread leaves out on paper in a dust-free environment in the shade. When large quantities of material are collected this is more difficult and we then use open-mesh (c. 5 mm) bags that are used to sell oranges and other fruit. We fill bags about half full with plant material and then suspend them from strings in a room at room temperature in the shade for approx. 10 days until there is no mass change.

In most cases scientists have used dried material for extraction of biologically active compounds. This makes sense because during drying, membranes of plant organelles containing different secondary compounds are destroyed, making extraction more efficient. Labile compounds may, however, be destroyed during the drying process or if hydrolase enzymes are released when vacuolar membranes are broken during the drying process. Artifacts may be formed during drying, and this is a major disadvantage in plant metabolic studies. This process is probably unimportant in the investigation of biologically active compounds because the artifact may be the active compound useful in ethnomedicine. For practical reasons, traditional healers and especially traders in traditional medicine use predominantly dried material. The difficulty with using fresh leaves is that it is tedious to remove water from an extract. When bulbs or corms are used, it takes a long time to dry the bulbs and undesirable changes may occur if bulbs are cut into slices and then dried.

The drying process has a major effect on the antibacterial activity of *Combretum erythrophyllum* leaves. Freeze drying led to lower activity than other drying procedures, probably because volatile antibacterial compounds were lost. Slow drying at very low temperatures yielded the highest activity (IE Angeh, personal communication).

One would expect that there would be differences in biological activity and chemical composition between dried and fresh material after extraction. When fresh *Acacia* leaves were extracted, it led to a higher yield but lower antibacterial activity than dried leaf extracts [29]. When bulbous material is extracted the water content of the material should be brought into consideration in determining the composition of the extractant. If, say, 70% acetone is used, the water content of the plant material should be taken into consideration to ensure that reproducible results are obtained [30].

Storage conditions may also affect the activity and chemical constituents of plant material. It appears that plant material stored in a dry condition in the dark does

not lose any biological activity over a long period. Leaves of *C. erythrophyllum* collected in the same area and stored in herbaria for up to 92 years did not lose any antibacterial activity and the chemical composition was very similar to that of recently collected material [31].

5.7
Extraction of Plant Material

5.7.1
Which is the Best Extractant?

Scientists have used many different solvents to extract plant material. To examine which extractants would be the most useful, freeze dried and finely ground leaves of two plants with known antimicrobial activity, *Anthocleista grandiflora* [32] and *Combretum erythrophyllum* [22], were extracted with a series of extractants of varying polarity (methylene dichloride, acetone, ethanol, methanol, methanol/chloroform/water and water) at a 1 to 10 ratio of dry material to solvent in each case [33].

The following parameters were investigated with the different extractants: the quantity extracted, the rate of extraction, the diversity of different compounds extracted, the diversity of inhibitory compounds extracted, the ease of subsequent handling of the extracts, the toxicity of the solvent in the bioassay process, and the potential health hazard of the extractants. The different solvents were compared by grading on an arbitrary five-point weighted scale. As shown in Table 5.1, acetone gave the best results with these plants with an arbitrary value of 102 followed by methanol/chloroform/water (MCW, 81), methylene dichloride (MDC, 79), methanol (MeOH, 71), ethanol (EtOH, 58) and water with 47 [33].

Table 5.1 Comparison of extractants on different parameters based on a five-point scale (0–4) and with different weights allocated to the different parameters (A = results for *A. grandiflora* and C = results for *C. erythrophyllum*) [33].

	Weight	*Acetone*		*EtOH*		*MeOH*		*MCW*		*MDC*		*Water*	
Parameter		***A***	***C***	***A***	***C***	***A***	***C***	***A***	***C***	***A***	***C***	***A***	***C***
Quantity extracted	3	6	3	9	6	12	12	12	12	3	3	9	9
Rate of extraction	3	12	15	12	12	12	12	12	12	15	15	9	9
Number of compounds extracted	5	20	20	10	15	15	20	10	15	10	15	5	5
Number of inhibitors extracted	5	20	20	0	10	20	15	20	20	20	15	0	0
Toxicity in bioassay	4	16	16	8	8	0	0	8	8	8	8	16	16
Ease of removal	5	20	20	5	5	10	10	10	10	20	20	0	0
Hazardous to use	2	8	8	8	8	2	2	6	6	6	6	8	8
Total		102	102	52	64	71	71	78	83	79	79	47	47

5.7.2 Extraction Period and Efficiency

Very finely ground plant material suspended in an inert dosing vehicle mobilizes from the rat peritoneal cavity into the blood almost as fast as if it had been injected in a soluble form [34]. It may therefore be possible to shorten the extraction period by grinding the leaves finer and by shaking at a very rapid rate for a short period. The average diameter of the particles of the plants that we ground using a mill was about 10 μm. After three 5-min extractions, 49% of the *A. grandiflora* and 38% of the *C. erythrophyllum* dry mass was extracted [33]. These values were even higher than values obtained after 24 h in a shaking machine with less finely ground material. Four 5-min sequential extractions of very finely ground *A. grandiflora* shaken with solvent at a high rate extracted 97% of the total antimicrobial activity [33].

5.7.3 Selective Extraction

We set out to investigate whether it is possible to simplify extracts to facilitate the isolation of antibacterial compounds from the complex mixture of chemicals in the plant by using different extractants [25]. Intact dried ground leaves of *Combretum microphyllum* were extracted with a series of extractants of varying polarity (i.e. hexane, carbon tetrachloride, di-isopropylether, ethyl ether, methylene dichloride, tetrahydrofuran, acetone, ethanol, ethyl acetate, methanol and water). Thin-layer chromatography (TLC) was used to determine chemical composition, and antibacterial activity of extracts was determined by a microplate serial dilution method. The different solvents extracted from 2.6 to 17.4% of the dry weight. Methanol, methylene dichloride, and tetrahydrofuran extracted the most components. The chemical composition of the nonpolar components of the different extracts was remarkably similar. The minimum inhibitory concentration for the different extractants varied from 0.01 to 1.25 mg mL^{-1} with the four test organisms used (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterococcus faecalis*). The extracts had similar activity towards Gram-negative and Gram-positive bacteria. Di-isopropyl ether, ethanol, ethyl ether, acetone, and ethyl acetate extracted high antibacterial activity with a lower quantity of other nonactive compounds and appear to be useful for isolating bioactive compounds.

In another application of simplifying plant extracts using selective extraction, there is rationale for using extracts to treat infectious diseases in preference to single compounds. It is likely that interactions between various compounds present in an extract result in synergistic effects which lead to heightened activity [35]. There is a distinct possibility that active principles with differing mechanisms of action may be present in a crude extract, thus slowing the onset of antibiotic resistance. Therefore, it may be worthwhile to seek to potentize plant extracts for antiinfectivity in preference to solely aiming for isolation of active compounds. Enhancing the activity of plant extracts by selectively removing bulky nonactive components is a relatively simple process. These potentized preparations may find ap-

plication chiefly in the arena of primary health care for humans and animals in developing countries. An example is the selective extraction of plant material from *Combretum woodii* which resulted in an extract with high antibacterial and antioxidant activity [36].

5.7.4
Redissolving Extracts for Quantitative Data

To enable valid comparisons, all data have to be expressed on a quantitative base (e.g. activity per mg extract). Extracts therefore have to be dried and subsequently redissolved to make up a known concentration of the extract. Frequently, dried extracts are not freely soluble even if the same solvent is used and this causes complications. To avoid this problem we do not dry extracts. To determine the concentration of the extract for quantification purposes we take a small aliquot, dry it, and use the values obtained to calculate the original concentration [37].

5.7.5
Storage of Extracts

Extracts are kept at 3–7 °C, not in a deep freeze where precipitation may take place. We had difficulties in storing aqueous extracts because in our experience fungal growth invariably occurs after some time, even at low temperatures. This is probably because good carbon and nitrogen resources for fungal growth such as sugars and amino acids may be present in the aqueous extracts.

Selection of containers used to store acetone extracts is important. Acetone plant extracts lose up to 87% of the acetone if stored in glass containers with polyethylene stoppers at 40 °C for a month. Overall, Teflon film is the best, followed by rubber, aluminum film, and polyethylene stoppers [38].

One would expect that dried extracts would be very stable, but we were surprised that acetone extracts of members of the Combretaceae retained antibacterial and anti-inflammatory activity over prolonged periods even when stored in a dissolved state at room temperature [23]. This may be due to the antibacterial and antifungal activity of these compounds [21, 27].

5.8
Evaluating Quantitative Antimicrobial Activity

Agar diffusion techniques are used widely to assay plant extracts for antimicrobial activity, but there are problems associated with this technique. A microdilution technique was developed using 96-well microplates and tetrazolium salts to indicate bacterial growth [39]. *p*-Iodonitrotetrazolium violet (0.2 mg mL^{-1}) gave better results than tetrazolium red or thiazolyl blue. The method is quick, worked well with *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Escherichia coli* and with nonaqueous extracts from many different plants. These bac-

terial species were selected because they are responsible for most nosocomial diseases in hospitals [40]. The method gives reproducible results, requires only 10–25 µL of extract to determine minimal inhibitory concentrations (MIC), distinguishes between microcidal and microstatic effects and provides a permanent record of the results. Using *S. aureus*, and a *Combretum molle* extract, the technique was 32 times more sensitive than agar diffusion techniques and was not sensitive to culture age of the test organism up to 24 h [39]. The *S. aureus* culture could be stored up to 10 days in a cold room with little effect on the assay results. This method is useful in screening plants for antimicrobial activity and for the bioassay-guided isolation of antimicrobial compounds from plants. MIC values determined for sulfisoxazole, norfloxacin, gentamicin, and nitrofurantoin were similar to values indicated in the literature but values obtained with trimethroprim and ampicillin were higher with some bacteria [39]. This method also works well with fungi [27].

5.9 Evaluating Qualitative Biological Activity

Bioautography can determine how many biologically active compounds are present in an extract or can be used to ensure that a compound isolated earlier is not isolated again.

The components of an extract are separated, usually by TLC. If microorganisms can grow on the plate it is usually easy to determine the R_f value of the compound that inhibits growth. Some authors have tried blotting the wet TLC plate with filter paper and then spraying the filter paper with a culture of the test organism. Others have applied an agar layer over the chromatogram by spraying with semi-liquid agar containing the test organism.

Neither of these techniques gave good results in our hands. We tried spraying the culture directly on the chromatogram, but it is difficult to visualize microbial growth. If a fungus that has visible spores is used this does work. We then used a technique developed by Begue and Kline [41]. In this technique the eluent is removed from the chromatogram, which is sprayed with a concentrated microbial suspension and after overnight incubation, the chromatogram is sprayed with tetrazolium violet. Microbial growth is indicated by red-purple areas and inhibition by clear areas. This method worked very well with aerobic bacteria (Fig. 5.1). By adding carbon dioxide and by including anaerocult the method also worked well with micro-aerophilic/anaerobic bacteria [42].

The bioautography technique is more difficult with fungi because they grow slower and contamination can be a problem. We have succeeded in developing a technique that works well with several fungi including *C. albicans* [43].

Bioautographic techniques work extremely well if antioxidant activity is determined using the free radical DPPH (1,1-diphenyl-2-picryl-hydrazyl) as a spray reagent (Fig. 5.2).

By applying these qualitative techniques, one can obtain a good idea of which species would be the most interesting to investigate further. By comparing bioau-

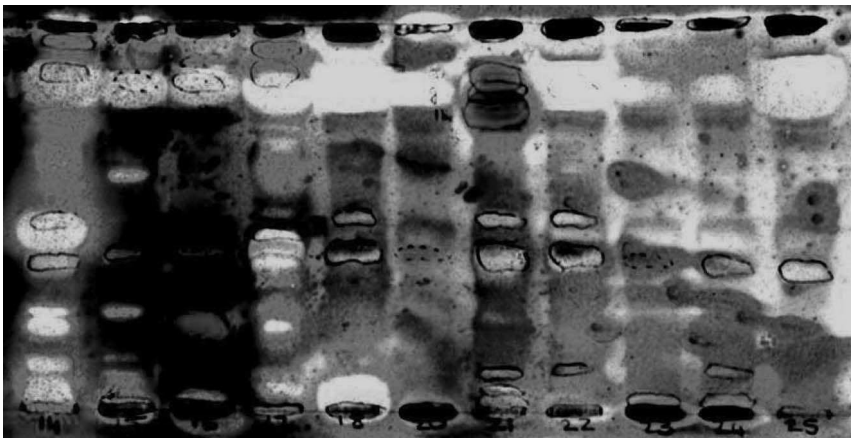


Fig. 5.1 Bioautography of acetone leaf extracts of eight *Combretum* species sprayed with *Enterococcus faecalis*, incubated, and then sprayed with tetrazolium violet. White areas indicate the presence of antibacterial compounds.

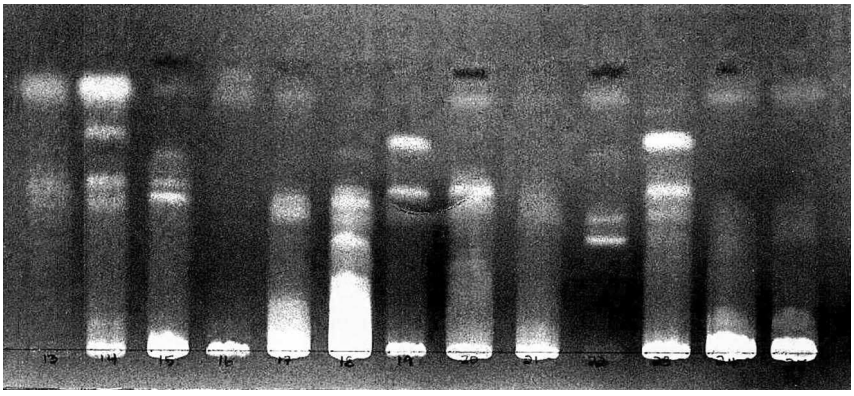


Fig. 5.2 Antioxidant activity of acetone leaf extracts of several Combretum species determined by spraying with methanolic DPPH solution. White areas indicate the presence of antioxidant compounds in extracts.

tograms measuring different activities one can identify compounds with antibacterial, antifungal, and antioxidant activities.

5.10 Expression of Results

Authors have used different ways of expressing the biological activity of plant extracts based on the technique used. Initially the agar diffusion method led to results being expressed in the width of the inhibition zone. Later the MIC values of

extracts were determined. Both of these techniques gave information on the activity of the extracts and were used to isolate biologically active components or evaluate whether the ethnobotanical use of plants could be justified. These techniques gave little quantitative information about the plants. We proposed that the quantity of material extracted from 1 g of dried plant material be divided by the MIC value to give the total activity of the plant. The unit is mL g^{-1}, and indicates the largest volume to which the biologically active compounds in 1 g can be diluted and still inhibit the growth of bacteria. If the results of other bioassays are also expressed in relative quantity of activity present in plants investigated, the most promising plants to use in rural areas for traditional health care can be identified [44].

This technique can also be of great value in bioassay-guided fractionation if the total activity of a fraction is expressed in milliliters per fraction by dividing the mass in milligrams by the MIC in mg mL^{-1}. This volume indicates to what level that fraction can be diluted and still inhibit growth of the test organism. By following this approach, any loss of activity is soon detected and it ensures that minor biologically active compounds are not isolated in the mistaken belief that they are major active components [37].

5.11 Antibacterial Activity

Most of the research conducted at the Phytomedicine Programme comprises studies on the antibacterial activity of plants. This is primarily a consequence of the development of the rapid and reproducible serial dilution method [39] used for obtaining MIC values of plant extracts against bacterial species. As stated earlier, acetone is routinely selected as a solvent to prepare extracts for the initial screening process as this solvent among several tested was found to yield the best results with reference to quantity and diversity of compounds extracted, number of inhibitors extracted, toxicity in a bioassay, and ease of removal of solvent among other factors [33]. Bioautographic techniques [41, 45] as described earlier are also employed to estimate the number and R_f values of antibacterial constituents in a plant extract of interest.

The test organisms we routinely employ in the preliminary screening of plant extracts for antibacterial activity are the Gram-positive *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213) and the Gram-negative *Escherichia coli* (ATCC 27853) and *Pseudomonas aeruginosa* (ATCC 25922). These ATCC reference strains are recommended by the National Committee for Clinical Laboratory Standards (NCCLS), Villanova, Pennsylvania, USA, for antibacterial testing [46]. We have recently included a strain of methicillin-resistant *Staphylococcus aureus* and *Mycobacterium smegmatis* in the range of bacteria against which activity is tested.

A plethora of publications have reported the antibacterial activity of South African plant extracts and compounds isolated from them using bioassay-directed fractionation (see, for example, refs [47–56]). There is a great deal of ongoing research in this field and much useful and interesting information is being generated.

5.12
Results on Antibacterial Activity Obtained with Members of the Combretaceae

5.12.1
Introduction

When we began this work we decided to select a plant family for focused investigation. Some of the parameters considered were: ethnobotanical leads and conditions when ethnobotanical leads are valuable, the quantity of material used in the natural medicine trade, the use of the plant, pharmacological activity in related taxa, availability of plant material and co-operators. Analysis of available data [57] indicated that the Combretaceae was one of the main plant families used in KwaZulu-Natal with an average of 20.2 tonnes consumed per year. We decided to start investigating this family especially since Noristan found indications of antibacterial activity with *Combretum erythrophyllum* extracts (B Fourie, personal communication). In addition, preliminary studies had reported on the antimicrobial testing of selected *Combretum* plant extracts [58].

5.12.2
Combretum erythrophyllum

In a preliminary investigation on the antibacterial activity of *Combretum erythrophyllum* acetone leaf extracts, liquid/liquid extraction gave better group separation of compounds than solid phase extraction on normal or reversed phase silica gel. The six fractions obtained inhibited the four test organisms to different degrees. *Staphylococcus aureus* was the most sensitive (100%) followed by *Enterococcus faecalis* (36%), *Escherichia coli* (11%), and *Pseudomonas aeruginosa* (3%). With *S. aureus* as test organism, the chloroform-soluble fraction contained by far the largest quantity of inhibiting components (100%), followed by the fractions soluble in water (23%), 35% methanol in water (18%), butanol (5%), carbon tetrachloride (2%), and hexane (traces). The lowest MIC value for *S. aureus* was 0.05 mg mL^{-1} at this stage of purification, compared with MIC values of 0.08 and 0.16 mg mL^{-1} for ampicillin and chloramphenicol respectively. There were at least 14 different inhibitors with a wide range of polarities present in the different fractions. The polar components apparently did not contain polysaccharides and were probably basic according to their chromatographic behavior [22].

5.12.3
Antibacterial Activity of Southern African Members of the Combretaceae

Based on the results obtained with *Combretum erythrophyllum*, other members of the Combretaceae were examined to find the best source for isolating antibacterial compounds. Leaves of 27 species of *Combretum*, *Terminalia*, *Pteleopsis*, and *Quisqualis* were collected, dried, milled, and extracted with acetone. The MIC of extracts was determined by our microplate serial dilution technique using *Staphylococcus*

aureus, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Escherichia coli* as test organisms. All extracts inhibited the growth of the four test isolates with MIC values generally between 0.1 and 6 mg mL^{-1} and an average of 2.01 mg mL^{-1}. After storing extracts for six weeks at 7 °C there was a slight loss of activity with MIC values increasing from 1.75 mg mL^{-1} to 2.24 mg mL^{-1}. The Gram-positive strains were slightly more sensitive with an average MIC of 1.8 mg mL^{-1} than the Gram-negative strains with an MIC of 2.22 mg mL^{-1}. Based on the MIC values and the total content in each plant, the seven plants with the highest antibacterial activity were *C. molle*, *C. petrophilum*, *C. moggii*, *C. erythrophyllum*, *C. padoides*, *C. paniculatum*, and *C. mossambicense* [21].

5.12.4 Stability of Extracts

Because extracts retained activity for several months we investigated the stability of the antibacterial activity of dried leaves of herbarium samples of *Combretum erythrophyllum* growing in the Pretoria area and collected between 92 and 12 years ago on the one hand, and freshly collected dried leaves on the other. *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* were used as test organisms. There were no differences in the MIC values of the different samples. There were only minor differences in chromatograms separating steroids and flavonoids. Light fungal infection indicated by small spots on herbarium leaves did not influence the MIC value or the chromatographic profile, but heavy fungal attack decreased the biological activity of the extracts. If biologically active components in other plants are stable, the examination of herbarium material may be a useful first step in establishing a scientific base for the use of plants in traditional medicine [31].

5.12.5 Anti-Inflammatory Activity

Combretum species are used throughout Africa for the relief of pain of different origins, which implies that extracts may have an anti-inflammatory effect. Initial studies indicated that some *Combretum* species have cyclooxygenase-inhibiting activity and that the activity was stable. A similar stability in antibacterial activity was observed earlier. We compared the anti-inflammatory activity and stability of 20 *Combretum* species growing under the same environmental conditions. All the extracts had anti-inflammatory activity with an average 65% inhibition of cyclooxygenase activity. The inhibition was remarkably stable with no loss of activity after storage for three months at room temperature (78% inhibition). There was a fair to moderate correlation between total anti-inflammatory activity and total antibacterial activity of the same taxa studied earlier. This suggests that similar compounds may be responsible for the biological activity, especially since in both cases the bioactivity is stable [23].

5.12.6
Other Activities of Extracts of *Combretum* Species

Leaf extracts of 20 *Combretum* species, many of which are used in southern African traditional medicine, were screened for anti-inflammatory, anthelmintic, antibilharzia (antischistosomal), and DNA-damaging activity. Significant activity in more than one bioassay was exhibited by *C. apiculatum*, *C. hereroense*, *C. molle*, and *C. mossambicense*. Ethyl acetate extracts were generally most active, followed by acetone and then water extracts [24].

5.12.7
Isolation and Biological Activity of Antibacterial Compounds from *C. erythrophyllum*

C. erythrophyllum leaf extracts were examined in more detail and seven antibacterial compounds were isolated. Four of these compounds were identified as flavonols, namely 5,6,4′-trihydroxyflavonol (kaempferol), 5,4′-dihydroxy-7-methoxyflavonol (rhamnocitrin), 5,4′-dihydroxy-7,5′-dimethoxyflavonol (rhamnazin), and 7,4′-dihydroxy-5,5′-dimethoxyflavonol (quercetin-5,3′-dimethylether) and three were identified as flavones, namely 5,7,4′-trihydroxyflavone (apigenin), 5,4′-dihydroxy-7-methoxyflavone (genkwanin), and 5-hydroxy-7,4′-dimethoxyflavone. Six of these flavonoids were reported for the first time in Combretaceae. Isolated compounds were identified using nuclear magnetic resonance (NMR) and mass spectroscopy (MS). R_f values of the flavonoids in three TLC solvent systems were provided to facilitate dereplication [26].

The biological activity of five of these compounds was examined in more detail. All had good activity against *Vibrio cholerae* and *Enterococcus faecalis*, with MIC values in the range of 25–50 μg mL^{-1}. Rhamnocitrin and quercetin-5,3′-dimethylether also inhibited *Micrococcus luteus* and *Shigella sonei* at 25 μg mL^{-1}. With the exception of 5-hydroxy-7,4′-dimethoxy-flavone, the flavonoids were not toxic towards human lymphocytes. This compound is potentially toxic to human cells and exhibited the poorest antioxidant activity whereas rhamnocitrin and rhamnazin exhibited strong antioxidant activity. Genkwanin, rhamnocitrin, quercetin-5,3′-dimethylether, and rhamnazin had higher anti-inflammatory activity than the positive control mefenamic acid. Although these flavonoids are known, this is the first report of biological activity with several of these compounds.

5.12.8
Combretum woodii

Another member of the Combretaceae to receive closer attention was *Combretum woodii*. Dried ground leaves of *C. woodii* were extracted with 10 different solvents (hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, ethanol, methanol, and water) to determine the best extractant for subsequent isolation and characterization of antibacterial compounds. With the exception of the water extract, which had no antibacterial activity, the oth-

er extracts were bioactive with at least one of them exhibiting minimum inhibitory concentration values of 0.04 mg mL^{-1} against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, or *Enterococcus faecalis*. Intermediate polarity solvents extracted about 10% of the dry mass compared with about 3% with the more polar or nonpolar solvents. These solvents also had higher antibacterial activity than more polar or nonpolar extractants [59].

Ethyl acetate was the best extractant with an average MIC value of 0.08 mg mL^{-1} for the four pathogens, followed by acetone and methylene dichloride with values of 0.14 mg mL^{-1}. The average MIC values for the positive controls were 0.13 (ampicillin) and 0.12 mg mL^{-1} (chloramphenicol). By taking the quantity extracted from the leaf powder into consideration, the total activity was highest for methylene dichloride (1309 mL g^{-1}) followed by acetone (1279 mL g^{-1}) extracts. The antibacterial activity was high enough to consider the use of extracts for clinical application and to isolate and characterize antibacterial compounds from the extracts. Based on the R_f values of the antibacterial compounds determined by bioautography, the antibacterial compound was not a polyphenol or a tannin [59].

Acetone extracts of *C. woodii* leaf powder were separated by solvent–solvent partitioning into six fractions. The highest total activity was in the chloroform fraction. This fraction contained mainly one compound active against *S. aureus*. This compound was isolated by bioassay-guided fractionation using silica gel open column chromatography and identified by NMR and MS as the stilbene 2′,3′,4-trihydroxyl, 3,5,4′-trimethoxybibenzyl (combretastatin B5) previously isolated from the seeds of *C. kraussii*. It showed significant activity against *S. aureus* with an MIC of 16 µg mL^{-1} but with lower activity towards *P. aeruginosa* (125 µg mL^{-1}), *E. faecalis* (125 µg mL^{-1}), and slight activity against *E. coli*. This is the first report of the antimicrobial activity of combretastatin B5. Its concentration in the leaves was in the order of 5–10 mg g^{-1} which makes the use of nonpolar leaf extracts a viable proposition in treating some infections, particularly in resource-poor settings [60].

5.12.9
Unpublished Work on Other Members of the Combretaceae

Two antibacterial flavonoids were isolated from *Combretum apiculatum* subsp. *apiculatum* [61]. In his PhD study, Angeh isolated three antibacterial compounds, a new oleanene-type triterpenoid glycoside and two known triterpenoids from *Combretum padoides* [62]. He also isolated a new antibacterial pentacyclic triterpenoid and four antibacterial triterpenoids with known structures from the leaves of *Combretum imberbe* [62].

5.13
Antifungal Activity

We have adapted our procedures to facilitate the investigation of antifungal activities of plant extracts. These methods were applied in the investigation of the anti-

fungal activity of *Combretum* and *Terminalia* (another genus of the Combretaceae) species [27]. The rising incidence of opportunistic mycotic infections associated with AIDS, as well as those developing after treatment with immunosuppressive drugs, has supplied impetus to the search for new antifungal drugs. There have been several publications produced by diverse research groups on antifungal effects of South African plants [27, 53, 63, 64].

We concentrate on researching the antifungal properties of plants against fungi implicated in causing opportunistic diseases in immunocompromised patients, such as *Candida albicans* and *Cryptococcus neoformans*. The microplate method for detecting antibacterial activity has been modified to result in a method appropriate for the antifungal testing of extracts [27]. Using this method, significant antifungal activity was found in several *Terminalia* species (Combretaceae) against a range of fungal organisms [27]. The antifungal activity of acetone, hexane, dichloromethane, and methanol leaf extracts of six *Terminalia* species (*T. prunioides*, *T. brachystemma*, *T. sericea*, *T. gazensis*, *T. mollis*, and *T. sambesiaca*) was tested against five fungal animal pathogens (*Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Microsporum canis*, and *Sporothrix schenckii*). Fungi cultured from clinical cases of disease in animals were used in the screening procedure. These fungi represent the different morphological forms of fungi, namely yeasts (*Candida albicans* and *Cryptococcus neoformans*), thermally dimorphic fungi (*Sporothrix schenckii*) and moulds (*Aspergillus fumigatus*) and are the most common and important disease-causing fungi of animals. Methanol extracted the highest quantity, but the acetone extracts had the highest antifungal activity. Some of the extracts had antioxidant activity. Most of the antifungal extracts had MIC values of about 0.08 mg mL^{-1}, some with MIC values as low as 0.02 mg mL^{-1}. *Microsporum canis* was the most susceptible microorganism and *T. sericea* extracts were the most active against nearly all microorganisms tested.

Recent research being prepared for publication indicates that more than 30 species from the family Combretaceae display antifungal MIC values of less than 20 µg mL^{-1} against fungal species *in vitro*. This work has been extended to include an *in vivo* rat model, where plant extracts with good antifungal activity are applied to fungally inoculated lesions on rat skin. The healing of the wound is compared with nontreated infected controls, and this method is producing good results.

Currently, efforts are being directed at discovering extracts with activity against a spectrum of plant pathogenic fungi. The aim is to develop plant extracts with the ability to protect other plants (crops and ornamentals) from fungal attack, both before and after harvesting.

5.14
Antiparasitic Activity

The extensive use of anthelmintics in livestock has led to the development of resistance to one or more of the widely available anthelmintics in many countries [65].

Parasite infestations caused by helminth species are prevalent in poor rural parts of southern Africa. Internal parasites in humans and animals, particularly worm infestations, are commonly treated with traditional remedies, and a wide variety of plant species is employed for this purpose. As it is time-consuming and expensive to verify the performance of extracts of these plants *in vivo*, we use *in vitro* models as a preliminary screening technique.

Assaying plant extracts against the free-living nematode *Caenorhabditis elegans* [66, 67] is much simpler, quicker, and cheaper than using parasitic nematodes, which are often difficult to maintain in culture. Most of the commercially available broad-spectrum anthelmintic drugs in popular use demonstrate activity against *C. elegans* [66]. There are clearly limitations to extrapolating activity against a free-living nematode to activity against parasitic nematodes [68]. In some broad screening studies, it was found that a large proportion of plant extracts tested showed activity against the free-living *Caenorhabditis* nematode [51, 69, 70], providing some rationale for the use of these plants in treating helminth infestations in both humans and their livestock. Members of the Combretaceae featured in other anthelmintic activity investigations, and leaf extracts of some members of the family revealed noteworthy anthelmintic activity against *C. elegans* [24].

We also apply plant extracts to *in vitro* assays against parasitic nematode species. Larval development and egg hatch assays against *Haemonchus contortus* and *Trichostrongylus colubriformis* form the basis of these investigations. These two species are among the most important nematodes causing disease in livestock in southern Africa. The parasitic nematodes are cultured in monospecifically infected lambs, and eggs of each species are collected from the feces when needed for assay. The larval development assay [71] analyzes the ability of the test substance to retard the development of eggs to infective larvae .The egg hatch assay [72] determines inhibition of hatching of freshly collected nematode eggs and, when combined with the larval development assay, supplies a reasonable indication of the anthelmintic activity of plant extracts.

In a study in which the *in vitro* anthelmintic activity of 20 plants was tested against the parasitic nematodes *H. contortus* and *T. colubriformis*, interesting results were obtained (JB Githiori, personal communication). The criteria of plant selection included firstly the prior discovery of anthelmintic activity against the free-living nematode *Caenorhabditis elegans* [24, 51]. A second criterion was the documentation of plant usage in ethnoveterinary medicine for the treatment of nematode parasites of ruminant livestock [73]. Thirdly, the ethnoveterinary use of plants by rural farmers (D Luseba, personal communication) was taken into account, and lastly, information from available literature sources was incorporated. Aqueous and acetone plant extracts were prepared and submitted to the egg hatch and larval development assays described above. Most of the plants showed minimal effects on egg hatching and larval development with the exception of *Aloe* species, and these results are being prepared for publication. Another study tested the activity of acetone extracts of the leaf, bark, and root of *Peltophorum africanum*, a plant traditionally used to treat helminthosis, against *H. contortus*. At a concentration of 0.2 mg mL^{-1}, the extracts inhibited egg hatching and larval development, providing

a degree of support for the traditional use of the plant, but this needs to be confirmed with further studies.

Research into correlations between lethal effects of plant extracts against parasitic nematodes on the one hand and free-living nematodes on the other hand remains an ongoing area of interest in our group.

5.15
Other Anti-Infective Research in South Africa

Malaria remains a significant problem in southern African countries and there is active research on new antimalarial compound discovery from indigenous plants in South Africa [74–78]. Presently, many of the African medicinal plants tested for antiplasmodial activity have shown potential to be developed as new antimalarial drugs [3].

Tuberculosis (TB) is a major concern in South Africa, and efforts are being directed towards the search for new effective anti-TB medications present in plants [56, 79, 80]. The Novel Drug Development Platform (NDDP, www.sahealthinfo.org/noveldrug) is a collaborative South African project aiming to develop new medicines from indigenous medicinal plants effective against tuberculosis and malaria, among other diseases.

Bilharzia, or schistosomiasis, is a public health concern in many developing countries, and it is estimated that 95% of rural communities in and around South Africa make use of traditional remedies to treat the disease [81]. Most research has, however, focused on the development of plant molluscicides rather than treating the human stage of the disease. Recently, an *in vitro* method of testing activity against infective schistosomula worms by plant extracts was optimized and used to screen numerous plant extracts [82].

Treatments for animal diseases apart from those caused by bacteria and fungi are also receiving attention. Antibabesial activity (*in vitro*) has been reported in *Elephantorrhiza elephantina*, a commonly used plant in ethnoveterinary medicine [83]. Studies are in progress on the antirickettsial effect of ethnoveterinary plants and promising results are anticipated. Plants showing mechanisms of action different to those of commercially available drugs are particularly of interest. Work has also been undertaken on detecting the effects of plant extracts against ticks, the vectors of many diseases [84].

5.16
Cytotoxicity

As a routine part of our anti-infective agent investigations, we have begun including cytotoxicity studies to rule out false positive bioactivity results ensuing from a general toxic effect of a plant extract [70]. The brine shrimp assay [85] involves incubating test substances with freshly hatched brine shrimp larvae and examining

the larvae for mortality after incubation. This assay has been frequently used to detect *in vitro* cytotoxic or pharmacological effects [85], but does not detect activity in those compounds requiring metabolic activation. We have found that few extracts of plants known to be toxic to livestock animals displayed activity in the brine shrimp assay [69]. Therefore it would appear that this cytotoxicity assay has limited applicability in detecting toxic effects of plant extracts.

At present we are employing a cell-line cytotoxicity assay, where viable cell growth after incubation with test compound is determined spectrophotometrically using a tetrazolium-based colorimetric assay [86]. The LC_{50} values are calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells. A number of different cell lines are suitable for use in the assay.

Several other approaches have been taken in the assessment of the toxicity of medicinal plants, including testing for genotoxic effects using *in vitro* bacterial and mammalian cell assays such as the Ames test, micronucleus test, and comet assay [3]. The genotoxicity of various South African medicinal plants has been reported [87, 88].

5.17 Ethnoveterinary Research

Many plants are used for ethnoveterinary purposes in South Africa, particularly in rural areas. Little work appears to have been carried out concerning the evaluation of the *in vitro* or *in vivo* efficacy of these plant preparations. In one such study [70] extracts of 17 plant species employed to treat infectious diseases in cattle were prepared using solvents of differing polarities. Antibacterial activity of the extracts was determined against a range of bacteria and anthelmintic activity was evaluated against the free-living nematode *Caenorhabditis elegans*. Cytotoxic effects were assessed using the brine shrimp larval mortality test. Most of the plant extracts exhibited antibacterial activity, with the best MIC being 0.1 mg mL^{-1}. More than a third of the extracts displayed a level of anthelmintic activity. Slightly toxic effects against brine shrimp larvae were shown by 30% of extracts, with the lowest LC_{50} recorded as 0.6 mg mL^{-1}. The promising biological activity displayed by a number of plant extracts may provide support for the ethnoveterinary use of these plants, but it is clear that *in vivo* tests are required to substantiate their medicinal properties and possible toxicity.

Following results obtained from this preliminary screening of ethnoveterinary plants, an MSc study was undertaken to determine the antibacterial constituents of *Ziziphus mucronata* (Rhamnaceae), a common tree in southern Africa. From the leaves, 2,3-dihydroxylup-20-en-28-oic acid and betulinic acid (zizyberanalic acid) were isolated [89]. The first compound displayed excellent activity against *Staphylococcus aureus*, adding support to the use of *Z. mucronata* leaf paste in treating bacterial infections in animals as well as humans. The antibacterial activity of the isolated compounds was not previously known.

Peltophorum africanum (Fabaceae) is a deciduous tree widespread in southern Africa. The plant has many ethnomedical and ethnoveterinary uses. The root and bark decoctions are used to treat diarrhea, dysentery, sore throat, wounds, back and joint pains, HIV-AIDS, venereal diseases, and infertility. Pastoralists and rural farmers use the root and bark extracts to treat diarrhea, dysentery, infertility, and to promote well-being and resistance to diseases in cattle. To evaluate these ethnobotanical leads, dried leaves, stem bark, and root bark were extracted with ethanol, acetone, dichloromethane, and hexane.

Polyphenols in the extract were determined by the Folin–Ciocalteu method with gallic acid as standard. Qualitative antioxidant activity was screened by spraying TLCs of the extracts with 0.2% 1,1-diphenyl-2-picryl hydrazyl (DPPH), and quantified with the Trolox equivalent antioxidant capacity (TEAC) assay. MIC and total antibacterial activity (TAA) values were determined by serial microplate dilution for *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*, with gentamicin as standard and tetrazolium violet as growth indicator. Acetone and ethanol extracted the largest quantity of material. The polyphenol concentration was 49.2% in the acetone extract of the root and 3.8% in the dichloromethane extract of the leaf. Antioxidant activity of at least five antioxidant compounds as measured by TEAC ranged from 1.34 (ethanol extract of the root) to 0.01 (hexane extract of the leaf). The total antibacterial activity (volume to which active compounds present in 1 g plant material can be diluted and still inhibit bacterial growth) was 1263 mL g^{-1} for the ethanol extract of the root against *S. aureus* and 800 mL g^{-1} for the acetone extract of the root against *P. aeruginosa*. There was substantial activity against both Gram-positive and Gram-negative bacteria, with MIC values of 0.08 mg mL^{-1} for *S. aureus* and 0.16 mg/ml for *P. aeruginosa*. There is therefore a rationale for the traditional use of root and bark of *P. africanum* in treating bacterial infection-related diseases [90].

Rhizome extracts of *Gunnera perpensa* (Gunneraceae) are used in South Africa to treat endometritis in animals as well as in humans. A study was conducted to investigate whether antibacterial activity could be responsible for the efficacy of the *G. perpensa* extract. It was concluded that although some degree or activity was present, the relatively weak antibacterial activity was unlikely to justify the use of *G. perpensa* rhizomes in the traditional treatment of endometritis [91]. It seems likely that the slightly antibacterial nature of the rhizomes may contribute an additive effect, along with their known uterotonic activity, to the overall efficacy of the preparation.

5.18
Determining the *in vivo* Efficacy of Extracts and Isolated Compounds

An indication of the *in vivo* efficacy of plant extracts and isolated compounds showing *in vitro* activity is necessary to take into account factors that are not present in the assays used to test activity *in vitro*, such as metabolic activation of the plant constituents. We have developed procedures to test the effectiveness of plant extracts

on healing skin infections caused by bacteria [92] and fungi (P Masoko, personal communication).

To date one of these extracts has found some application in the herbal medicine market. The issue of *in vivo* toxicity is also important, as cell-based assays detecting cytotoxic effects are not sufficient to indicate toxic effects of ingested or topically applied medications. The avenue of investigating *in vivo* efficacy of plant extract preparations and isolated active compounds is a major area of future exploration for the Phytomedicine Programme.

5.19 Conclusion

Plant-based remedies used in human and animal medicine are an essential part of the primary health care system in South Africa, especially with regard to commonly encountered infectious diseases. Antibiotic resistance and the incidence of side effects in currently used drugs are additional factors leading scientists to the plant world in the search for new anti-infective agents. Ethnobotanical and ethnoveterinary leads can provide valuable information on potentially highly active plant extracts. We have recently had success also with a random selection tree screening project, which has highlighted several antibacterial and antifungal plant extracts that have no history of human medicinal use. The chemotaxonomic approach using members of the family Combretaceae as a starting point for antibacterial, antifungal, and other bioactivity investigations, has also proved successful, resulting in the detection and isolation of many active compounds. It is important to bear in mind that while activity *in vitro* does not necessarily verify the efficacy of a plant extract, it does provide a preliminary indication of the usefulness and potential toxicity of the plant [3].

Natural products research has clearly shown that natural products represent an incomparable source of molecular diversity. It is time-consuming to isolate and identify active components from extracts, but biologically active extracts can be extremely useful in their entirety, taking into account synergistic and other effects. The approval as drugs of standardized and formulated plant extracts might be the starting point in developing countries of a successful pharmaceutical industry which can compete with the Western pharmaceutical companies for the treatment of a range of diseases [93].

In this chapter we have highlighted some of the research being carried out in the field of antibacterial, antifungal, and antiparasitic investigations of plant extracts. Unavoidably, we have concentrated in this brief review on the work performed in our own Phytomedicine research group at the University of Pretoria, but would like to acknowledge the world-class research emanating from the many botanical and other centers and institutes around the country. It is clear that South Africa, with its bountiful biological diversity and active research groups, has much to offer regarding the management of infectious diseases and phytomedicine in general.

References

1 George, J., Laing, M.D., Drewes, S.E. *S. Afr. J. Sci.* **2001**, *97*, 93–105.
2 Van Wyk, B-E. *S. Afr. J. Bot.* **2002**, *68*, 1–13.
3 Fennell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M., van Staden, J. *J. Ethnopharmacol.* **2004**, *94*, 205–217.
4 Light, M.E., Sparg, S.G., Stafford, G.I., van Staden, J. *J. Ethnopharmacol.* **2005**, *100*, 127–130.
5 Germishuizen, G., Meyer, N.L. *Plants of Southern Africa, An Annotated Check List*. Strelitzia 14, iv. National Botanical Institute, Pretoria, **2003**.
6 Klopper, R.R., Smith, G.F., Gautier, L., Chatelain, C., Spichiger, R. *The African Plant Checklist and Database Project*. Programme and Abstracts, pp. 93–94, Conference South African Association for Botanists, Port Elizabeth, January, **2006**.
7 Farnsworth, N.F. The role of medicinal plants in drug development. In *Natural Products and Drug Development*, Krogsgaard-Larsen, P., Brogger Christensen, L., Kofold, H. (eds). Munksgaard, Copenhagen, **1984**, pp. 17–28.
8 O'Neill, M.J., Lewis, J.A. The renaissance of plant research in the pharmaceutical industry. In *Human Medicinal Agents from Plants*, Kinghorn, A.D., Balandrin, M.F. (eds). ACS Symposium Series 534. American Chemical Society, Washington, DC, **1993**, pp. 48–55.
9 Van Wyk, B-E., van Oudtshoorn, B., Gericke. N. *Medicinal Plants of South Africa*. Briza Publications, Pretoria, **1997**.
10 Mander, M. *The Marketing of Indigenous Medicinal Plants in South Africa, A Case Study in KwaZulu–Natal*. Forest Products Marketing Programme, FAO, Rome, **1998**.
11 Arnold, T.H., Prentice, C.A., Hawker, L.C., Snyman, E.E., Tomalin, M., Crouch, N.R., Pottas-Bircher, C. *Medicinal and Magical Plants of Southern Africa, an Annotated Checklist*. Strelitzia 13, 89. National Botanical Institute, Pretoria, **2002**.
12 Cunningham, A.B., Zondi, A.S. *Cattle Owners and Traditional Medicines Used for Livestock*. Investigational Report No. 69, Institute of Natural Resources, Pietermaritzburg, **1991**.
13 Mathias, E., McCorkle, C.M., Schillhorn van Veen, T.W. Introduction, ethnoveterinary research and development. In *Ethnoveterinary Research and Development*, McCorkle, C.M., Mathias, E., Schillhorn van Veen, T.W. (eds). Intermediate Technology Publications, London. **1996**.
14 WHO Scientific Working Group. *Bull. World Health Org.* **1996**, *71*, 335–336.
15 Watt, J.M., Breyer-Brandwijk, M.G. *The Medicinal and Poisonous Plants of Southern and Eastern Africa*, 2nd edn. Livingstone, London, **1962**.
16 Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A.B. *Zulu Medicinal Plants, an Inventory*. University of Natal Press, Pietermaritzburg., **1996**.
17 Van Wyk, B-E., Gericke, N. *People's Plants, a Guide to Useful Plants of Southern Africa*. Briza Publications, Pretoria, **2000**.
18 Masika, P.J., Van Averbeke, W., Sonandi, A. *J. S. Afr. Vet. Assoc.* **2000**, *71*, 87–91.
19 Masika, P.J., Afolayan, A.J. *Pharm. Biol.* **2002**, *41*, 16–21.
20 Van der Merwe, D., Swan, G.E., Botha, C.J. *J. S. Afr. Vet. Assoc.* **2001**, *72*, 189–196.
21 Eloff, J.N. *S. Afr. J. Sci.* **1999**, *95*, 148–152.
22 Martini, N.D., Eloff, J.N. *J. Ethnopharmacol.* **1998**, *62*, 255–263.
23 Eloff, J.N, Jäger, A.K., van Staden, J. *S. Afr. J. Sci.* **2001**, *97*, 291–293.
24 McGaw, L.J., Rabe, T., Sparg, S.G., Jäger, A.K., Eloff, J.N., van Staden, J. *J. Ethnopharmacol.* **2001**, *75*, 45–50.
25 Kotze, M., Eloff, J.N. *S. Afr. J. Bot.* **2002**, *68*, 62–67.
26 Martini, N.D., Katerere, D.R.P., Eloff, J.N. *J. Ethnopharmacol.* **2004**, *93*, 207–212.
27 Masoko, P., Picard, J., Eloff, J.N. *J. Ethnopharmacol.* **2005**, *99*, 301–308.
28 Balick, M.J. Ethnobotany, drug development and biodiversity conservation-exploring the linkages. In *Ethnobotany and the Search for New Drugs*, Prance, G.T. (ed.). Ciba Foundation Symposium 185, Wiley, Chichester, **1994**, pp. 4–24.

29 Katerere, D.R., Eloff, J.N. *S. Afr. J. Bot.* **2004**, *70*, 303–305.
30 Naidoo, V., Katerere, D.R., Swan, G.E., Eloff, J.N. *Pharm. Biol.* **2004**, *42*, 529–533.
31 Eloff, J.N. *J. Ethnopharmacol.* **1999**, *67*, 355–360.
32 Eloff, J.N. *S. Afr. J. Bot.* **1998**, *64*, 209–212.
33 Eloff, J.N. *J. Ethnopharmacol.* **1998**, *60*, 1–8.
34 Malone, M.H. *J. Ethnopharmacol.* **1983**, *8*, 127–147.
35 Williamson, E.M. *Phytomedicine* **2001**, *8*, 401–409.
36 Zishiri, V.K. Potentising and application of a *Combretum woodii* leaf extract with high antibacterial and antioxidant activity. MSc thesis, University of Pretoria, **2005**.
37 Eloff, J.N. *Phytomedicine* **2004**, *11*, 370–371.
38 Eloff, J.N. *S. Afr. J. Bot.* **2003**, 69, 603–604.
39 Eloff, J.N. *Planta Med.* **1998**, *64*, 711–713.
40 Sacho, H., Schoub, D.B. *Current Perspectives on Nosocomial Infections*. Pietermaritzburg, Natal Witness Printing and Publishing, **1993**.
41 Begue, W.J., Kline, R.M. *J. Chromatogr.* **1972**, *64*, 182–184.
42 Beukes, S. *In vitro* antimicrobial activity of phytomedicine against acidogenic oral bacteria. MSc thesis, University of Pretoria, **2004**.
43 Masoko, P., Eloff, J.N. *Afr. J. Biotechnol.* **2005**, *4*, 1425–1431.
44 Eloff, J.N. *S. Afr. J. Sci.* **2000**, *96*, 116–118.
45 Hamburger, M.O., Cordell, G.A. *J. Nat. Prod.* **1987**, *50*, 19–22.
46 NCCLS. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 4th edn. Approved Standard. NCCLS Document M2–A4. The National Committee for Clinical Laboratory Standards, Villanova, PA, **1990**.
47 Afolayan, A.J., Meyer, J.J.M. *J. Ethnopharmacol.* **1997**, *57*, 177–181.
48 Rabe, T., van Staden, J. *J. Ethnopharmacol.* **1997**, 56, 81–87.
49 Grierson, D.S., Afolayan, A.J. *J. Ethnopharmacol.* **1999**, *66*, 103–106.
50 Kelmanson, J.E., Jäger, A.K., van Staden, J. *J. Ethnopharmacol.* **2000**, *69*, 241–246.
51 McGaw, L.J., Jäger, A.K., van Staden, J. *J. Ethnopharmacol.* **2000**, *72*, 247–263.
52 Rabe, T., van Staden, J. *J. Ethnopharmacol.* **2000**, 73, 171–174.
53 Masika, P.J., Afolayan, A.J. *J. Ethnopharmacol.* **2002**, *83*, 129–134.
54 Rabe, T., Mulholland, D., van Staden, J. *J. Ethnopharmacol.* **2002**, *80*, 91–94.
55 Steenkamp, V., Mativha, E., Gouws, M.C., van Rensburg, C.E.J. *J. Ethnopharmacol.* **2004**, *95*, 353–357.
56 Weigenand, O., Hussein, A.A., Lall, N., Meyer, J.J.M. *J. Nat. Prod.* **2004**, *67*, 1936–1938.
57 Cunningham, A.B. *Mitt. Inst. Allg. Bot. Hamburg* **1990**, *23b*, 979–990.
58 Alexander, D.M., Bhana, N., Bhika, K.H., Rogers, C.B. *S. Afr. J. Sci.* **1992**, *88*, 342–344.
59 Eloff, J.N., Famakin, J.O., Katerere, D.R. *Afr. J. Biotechnol.* **2005**, *4*, 1161–1166.
60 Eloff, J.N., Famakin, J.O., Katerere, D.R. *Afr. J. Biotechnol.* **2005**, *4*, 1166–1171.
61 Serage, A. Isolation and characterization of antibacterial compounds in *Combretum apiculatum* Sond subsp *apiculatum* Excell. MSc thesis, University of Pretoria, **2003**.
62 Angeh, J. Isolation and characterization of antibacterial compounds present in members of *Combretum* Section Hypocrateroptis. PhD thesis, University of Pretoria, **2005**.
63 Afolayan, A.J., Grierson, D.S., Kambizi, L., Madamombe, I., Masika, P.J. *S. Afr. J. Bot.* **2002**, *68*, 72–76.
64 Motsei, M.L., Lindsey, K.L., van Staden, J., Jäger, A.K. *J. Ethnopharmacol.* **2003**, *86*, 235–241.
65 Maingi, N., Bjørn, H., Dangolla, A. *Vet. Parasitol.* **1998**, *77*, 133–145.
66 Simpkin, K.G., Coles, G.C. *J. Chem. Technol. Biotechnol.* **1981**, *31*, 66–69.
67 Rasoanaivo, P., Ratsimamanga-Urverg, S. *Biological Evaluation of Plants with Reference to the Malagasy Flora*. Napreca, Madagascar, **1993**, pp. 9–43, 72–83.
68 Geary, T.G., Thompson, D.P. *Vet. Parasitol.* **2001**, *101*, 371–386.
69 McGaw, L.J., Eloff, J.N. *S. Afr. J. Bot.* **2005**, *71*, 302–306.
70 McGaw, L.J., Van der Merwe, D., Eloff, J.N. *Vet. J.* (in press, doi,10.1016/j.tvjl. **2005**.09.004). **2005**.
71 Coles, G.C., Tritschler, J.P., Giordano, D.J., Laste, N.J., Schmidt, A.L. *Res. Vet. Sci.* **1988**, *45*, 50–53.
72 Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor,

M.A., Waller, P.J. *Vet. Parasitol.* **1992**, *44*, 35–44.
73 Van der Merwe, D. Use of ethnoveterinary medicinal plants in cattle by Setswana–speaking people in the Madikwe area of the North West Province. MSc thesis, University of Pretoria, **2000**.
74 Prozesky, E.A., Meyer, J.J.M., Louw, A.I. *J. Ethnopharmacol.* **2001**, *76*, 239–245.
75 Van Zyl, R.L., Viljoen, A.M. *S. Afr. J. Bot.* **2002**, *68*, 106–110.
76 Van Zyl, R.L., Viljoen, A.M. *S. Afr. J. Bot.* **2003**, *69*, 265.
77 Clarkson, C., Maharaj, V.J., Crouch, N.R., Grace, O.M., Pillay, P., Matsabisa, M.G., Bhagwandin, N., Smith, P.J., Folb, P.I. *J. Ethnopharmacol.* **2004**, *92*, 177–191.
78 Kamatou, G.P.P., Viljoen, A.M., Gono–Bwalya, A.B., van Zyl, R.L., van Vuuren, S.F., Lourens, A.C.U., Başer, K.H.C., Demirci, B., Lindsey, K.L., van Staden, J., Steenkamp, P. *J. Ethnopharmacol.* **2005**, *102*, 382–390.
79 Lall, N., Meyer, J.J.M. *J. Ethnopharmacol.* **1999**, *66*, 347–354.
80 Lall, N., Das Sarma, M., Hazra, B., Meyer, J.J.M. *J. Antimicrob. Agents Chemother.* **2003**, *51*, 435–438.
81 Clark, T.E., Appleton, C.C., Kvalsvig, J.D. *Acta Tropica* **1997**, *66*, 93–107.
82 Sparg, S.G., van Staden, J., Jäger, A.K. *J. Ethnopharmacol.* **2000**, *73*, 209–214.
83 Naidoo, V., Zweygarth, E., Eloff, J.N., Swan, G.E. *Vet. Parasitol.* **2005**, *130*, 9–13.
84 Nchu, F., Magano, S.R., Eloff, J.N. *J. S. Afr. Vet. Assoc.* **2005**, *76*, 99–103.
85 Solís, P.N., Wright, C.W., Anderson, M.M., Gupta, M.P., Phillipson, J.D. *Planta Med.* **1993**, *59*, 250–252.
86 Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.
87 Elgorashi, E.E., Taylor, J.L.S., Maes, A., de Kimpe, N., van Staden, J., Verschaeve, L. *S. Afr. J. Bot.* **2002**, *68*, 408–410.
88 Elgorashi, E.E., Taylor, J.L.S., Verschaeve, L., Maes, A., van Staden, J., de Kimpe, N. *Toxicol. Lett.* **2003**, *143*, 195–207.
89 Moloto, M.P. Isolation and characterization of antibacterial, anthelmintic, antioxidant and cytotoxic compounds present in *Ziziphus mucronata*. MSc thesis, Medical University of Southern Africa, **2004**.
90 Bizimenyera, E.S., Swan, G.E., Chikoto, H., Eloff, J.N. *J. S. Afr. Vet. Assoc.* **2005**, *76*, 45–48.
91 McGaw, L.J., Gehring, R., Katsoulis, L., Eloff, J.N *J. Vet. Res.* **2005**, *72*, 129–134.
92 Kruger, J. Isolation and characterization of antibacterial compounds from three *Terminalia* spp. PhD thesis, University of Pretoria, **2004**.
93 Pieters, L., Vlietinck, A.J. *J. Ethnopharmacol.* **2005**, *100*, 57–60.

6
Biological and Toxicological Properties of Moroccan Plant Extracts: Advances in Research

Mustapha Larhsini

Summary

In Morocco, the use of traditional remedies is common practise and a large number of plants are used. Some reports of ethnobotanical surveys of Moroccan herbal remedies have been published in different areas of the country. In addition, several studies on the pharmacological properties have also been undertaken in recent years and have tested various biological activities, including antimicrobial, antidiabetic, and molluscicidal.

This chapter reviews the primary results obtained from Moroccan medicinal plants in the field of ethnopharmacology as well as the different secondary metabolites isolated and identified by the different working groups. Finally, some toxicological aspects are discussed.

6.1
Introduction

For centuries people have used plants for healing. The earliest records of the use of medicinal plants date back at least 5000 years to the Sumerians. During the twentieth century, although we have seen the spectacular development of synthetic compounds, the benefits of modern drugs have been felt primarily in developed countries. In developed countries, people continue to rely largely on traditional remedies. The World Health Organization (WHO) estimates that 80% of the world's population continue to use herbal remedies to cure many diseases and for prevention. Ethnomedical plant data has been used in many forms and has been heavily utilized in the development of formularies and pharmacopoeias, as well as contributing substantially to the drug development process.

Plant products are an excellent and exceptional source of complex chemicals, possessing a wide variety of biological activities and therefore having great potential for new drugs and biological entities (e.g. digitoxin from *Digitalis purpurea* or vinblastin and vincristin from *Catharantus roseus*). Today's plant-based prescrip-

tion medicines come from plants belonging only to 95 of the estimated 250 000 known species worldwide. From the small number of species of flowering plant that have been investigated, about 120 therapeutic agents of known structure have been isolated for commercial purpose from about 90 species of plants [1]. In addition, 74% of these 120 plant therapeutic compounds were discovered based on ethnomedical records.

In 1968, the Organization of African Unity's scientific and technical commission (OAU/STRC, 1968, Publication No. 104, Lagos) organized an inter-African symposium in Dakar, Senegal, in which a large number of researchers on the medicinal plants of Africa participated. It was decided to gather information and documentation about African ethnomedical practises and to intensify studies to confirm the claims of traditional healers. In response to this Sofowora [2] reported an overview of the pharmacological screening of African medicinal plants. Half of the publications dealing with the biological activities of African plants discuss of antimicrobial, molluscicidal, antimalarial, toxicology, and antitumor-related activities. Since then, a large number of studies have been carried out to identify and characterize the efficacies of traditional treatments in order to provide instruction to local populations and because it is necessary from a scientific point of view to establish a rational relationship between the chemical, biological, and therapeutic properties of traditional remedies.

Morocco has a long history of traditional medicine, and folk medicine continues to play an important role in the treatment of most diseases, especially in rural areas (55% of the whole population), where people have poor access to modern health care systems. Furthermore, the preparations used in traditional remedies are relatively cheap and accessible since they can be prepared from locally grown and produced plant products.

Medicinal plants of Morocco represent a precious resource from which bioactive compounds can be isolated and developed into invaluable therapeutic agents. A wide spectrum of bioassays can be employed for the detection of bioactivity in extracts, fractions as well as purified compounds of herbal origin. In recent years there has been a substantial increase in the number of Moroccan laboratories working on medicinal plants. Although some 90% of total publications still fall within purely ethnopharmacological research, the remainder deal with phytochemical isolation of plant constituents as well as toxicological testing.

Despite this activity, data about the toxicological and safety of Moroccan medicinal herbs are still limited in a number of ways and the clinically important information conducted on patients is still nonexistent.

In this chapter we report and discuss the main published biological and toxicological investigations undertaken to date on Moroccan plant extracts.

6.2
Ethnobotanic and Ethnopharmacology of Traditional Moroccan Plants

6.2.1
Ethnobotanic Surveys

Many studies have been published regarding the Moroccan pharmacopoeia [3–5]. The geographical position of Morocco is in the extreme north-west of Africa (Fig. 6.1) and the great diversity of its climate and ecology, including mountainous, littoral, and desert areas, has favored the development of a rich flora which is estimated at 4200 native plants and about 1500 introduced species [6, 7].

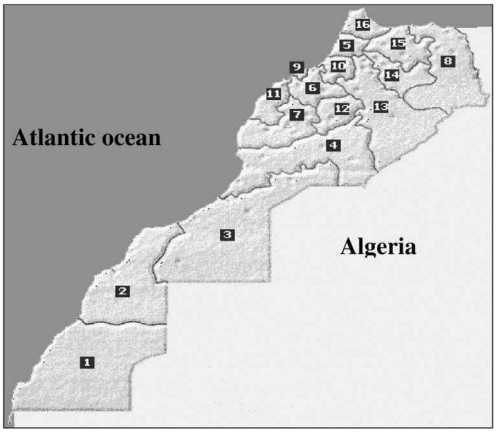


Fig. 6.1 Regions of Morocco. 1 Oued Eddahab-Lagouira, 2 Laayoune-Boujdour-Sakia El Hamra, 3 Guelmim-Es Smara, 4 Souss-Massa-Draa, 5 Gharb-Chrarda-Beni Hssen, 6 Chaouia-Ourdigha, 7 Marrakech-Tensift-El Haouz, 8 Oriental, 9 Casablanca, 10 Rabat-Salè-Zemmour-Zaar, 11 Doukkala-Abda, 12 Tadla-Azilal, 13 14 Fès-Boulman.

Pharmacobotanical studies have been undertaken in different regions of the country and have demonstrated the richness of the plants used and the important place of traditional medicine in Moroccan society for primary health care. Almost all the botanical families, such as Apiaceae, Asteraceae, and Lamiaceae, are represented and the traditional Moroccan pharmacopoeia concerns a large spectrum of diseases.

Several authors have reported the most frequently used plants and the diseases for which they are prescribed [3–5, 8, 9]. From these studies it appears that the major diseases cured by Moroccan traditional medicines are related to digestive pathology (mainly intestinal antiseptic and anthelmintic), skin and health care, bronchopulmonary, urinary system, and liver disorders [3]. The activities of midwives related to reproductive functions (emmenagogue and other gynecologic treatment) are also well represented. In general, people use infusions or decoctions and often use more than one plant either separately or mixed together [9].

6.2.2 Biological Activities

6.2.2.1 Antimicrobial Properties

The development of microbial resistance towards antibiotics has heightened the importance of the search for new potential effective plants and plant constituents against pathogenic microorganisms. Because infectious diseases are usually characterized by clear symptoms, traditional practises have been able to recognize such diseases easily and have developed plant preparations against such infections. Fungal infections play an increasingly important role in many illnesses and are the direct causative agents in serious complications of diseases such as AIDS. In fact, treatment with immunosuppressive drugs and the spread of AIDS has shown that diseases caused by weakness in immunity are becoming more prevalent. The most common opportunistic fungus associated with immunocompromised patients is the genus *Candida*, and it has been reported that 36–85% of HIV-infected patients have *Candida* infections [10–12].

Biologically active compounds from plant sources have always been of great interest to scientists working on infectious diseases. In recent years there has been a growing interest in the evaluation of plants possessing antibacterial activity for various diseases [13]. Numerous broad-based screening programmes have been initiated recently, in which a large number of plant species have been evaluated for their antimicrobial activity in different regions of the world [14–20].

Since infectious diseases are common in Morocco, the search for anti-infective agents has occupied many Moroccan research laboratories, and in recent years several studies have looked at the antimicrobial activities of extracts of Moroccan plants. Table 6.1 gives a summary of the extracts and phytochemicals isolated from Moroccan plants that have proven antimicrobial properties.

The methods used to study Moroccan plant extracts are the agar dilution or diffusion methods. It is well known that many factors such as temperature [21], inoculum size, and medium composition [22] can influence the results and then make it difficult to compare results from different authors. Rios et al. [23] have reported a review of the methods used to screen natural products with antimicrobial activity. They suggested the use of the agar dilution method for essential oils and nonpolar plant extracts and the diffusion method for preliminary screening of pure substances. They also recommend that the diffusion method should never be used as a definitive method or to determine the minimum inhibitory concentration (MIC) value of a sample. In addition, it has also been shown that the extraction procedure has strong effects on the antimicrobial activity of a selected plant, especially the pH of the extracting medium [24].

Regarding the different Moroccan publications on antimicrobial activity we noted the absence of a standard method for investigation and found that the testing methodology varied considerably from laboratory to laboratory in detail. Large numbers of authors used the dilution method for testing but the choice of test microorganisms is often not defined. In general, traditional remedies are used in aqueous form but many workers preferred to assay essential oils or plant organic

Table 6.1 Some Moroccan plant extracts and phytochemicals with antimicrobial activities.

Plant	*Extracts or compounds used*	*Organisms tested*	*References*
Centaurea spp.	Sesquiterpene lactones	*Cunninghamella echinulata*	27
Origanum compactum and *Thymus glandulosus*	Whole plant, essential oil	*Botrytis cinerea*	28
Sium nodiflorum	Aerial part (ethylether, ethylacetate, butanol)	Fungi	29
Pulicaria odora	Root, essential oil	Bacteria, fungi	30, 33
Aristolochia paucinervis	Rhizome, leaf (methanol, hexane, chloroform, ethylacetate, butanol)	Bacteria	26, 34, 35
Cotula cinerea	Whole plant, ethylether, ethylacetate, butanol	Bacteria	36
Cistus incanus and C. monspeliensis	Leaf, water, ethylacetate	Bacteria, fungi	37
Cystoseira tamariscifolia	Diterpenoid	Bacteria, fungi	38
Chrysanthemum viscidehirtum	Aerial part, essential oil	Bacteria	39
Calotopis procera	Ethanol	Fungi	31
Eugenia caryophyllata	Water	Bacteria	40

solvent extracts, using solvents of increasing polarity (Table 6.1). Rios and Recio [25] mentioned that it has generally been the essential oils of the plants rather than their extracts that had the greatest use in the treatment of infectious pathologies. The germs used for the assay are Gram-positive and Gram-negative bacteria, usually Gram-positive bacteria *Staphylococcus aureus*. This bacterium is usually found to be sensitive to Moroccan extracts. Some authors have assayed efficacy against a particular bacterium or fungus, for example *Helicobacter pylori* [26], *Cunninghamella echinulata* [27], and *Botrytis cinerea* [28]. A review of articles published between 1978 and 1988 revealed that Gram-positive bacteria are the most susceptible germs and phenolics are the most active constituents [25].

In the case of fungi, *Candida albicans* is often used for assay and is found to be sensitive [29–31]. When screening 1248 extracts from higher plants, Mitscher et al. [32] found frequent activity against *S. aureus* (15%) and *C. albicans* (7%). It is also important to mention the harvesting stage of the plant since extracts are generally richest in antimicrobial agents after the flowering stage.

Very few studies reported bio-guided isolation of the active principles responsible for the activity observed, or at least a fractionation of the active extracts in order to determine more precisely the nature of the active constituents. From the literature it is clear that the chemical structure of the antimicrobial agents found in higher plants belong to the most commonly encountered classes of higher plant secondary metabolites [32, 41]. An example of a compound obtained in correlation with the verification of antimicrobial ethnomedical treatment is 2-isopropyl-4-methylphenol isolated from *Pulicaria odora* essential oil [27]. There are various

Table 6.2 Secondary metabolites isolated from Moroccan medicinal plants.

Plant	*Constituents*	*Reference*
Chrysanthemum viscidehirtum	Flavonoid	45
Ruta montana	Alkaloids	46
Mentha longifolia	Flavonoid	47
Lavandula multifida	Diterpenes	48
Warionia saharae	Sesquiterpene lactones	49
Tetraclinis articulata	Diterpenoids	50
Anvillea radiata	Sesquiterpene lactones	51
Juniperus thurifera and *J. Phoenicea*	Diterpenic acids	52
Silene cucubalus	Saponins	53
Cedrus atlantica	Diterpenes	54
Herniaria fontanesii	Saponins	55
Bupleurum acutifolium	Lignans and polyacetylenes	56
Zygophyllum gaetulum	Saponins	57
Argania spinosa	Saponins	58
Solanaceous species	Alkaloids	59–61

strategies used to study medicinal plants, including the phytochemical approach, in which a particular compound type is regarded as being of interest and attempts made to isolate it. Thus, some Moroccan researchers have focused their work on the isolation and identification of new compounds without following a bio-guided fractionation approach. In Table 6.2, we give the different secondary metabolites identified from Moroccan plant extracts and we think that it will be of value to determine the antimicrobial potential of these isolated compounds and also to check their pharmacological properties using different biological testing models. This evaluation may lead to interesting spectrum activity.

6.2.2.2 **Antidiabetic Activity**

Noninsulin-dependent diabetes mellitus is one of the most common disorders worldwide [42]. It is a group of metabolic disorders characterized by hyperglycemia. The metabolic disorders include alterations in the carbohydrate, fat, and protein metabolism associated with absolute or relative deficiencies in insulin secretion and/or insulin action. Along with hyperglycemia and abnormalities in serum lipids [43], diabetes is associated with microvascular and macrovascular complications, which constitute the main cause of morbidity and mortality of diabetic patients [44].

The prevention of diabetes is an urgent worldwide public health concern. Obesity and insulin resistance induced by overeating and physical inactivity typically characterizes the period preceding onset of type 2 diabetes. Shigeta et al. [62] have shown that caloric restriction and physical exercise have obvious importance. They stress that actively promoting healthy eating and sleeping habits should be considered for the prevention of obesity and insulin resistance.

Epidemiologic studies of diabetes in Morocco are very rare. But it has been noted that there has been a considerable increase in its prevalence in recent decades. Demographic trends and changes in lifestyle related to intensive urbanization are the main causes of the disease. The last national estimation indicated that the prevalence of diabetes was around 6.6% for people over 20 years old. And if we consider people more than 50 years old, the prevalence exceeds 10%. Thus, today approximately a million and half people suffer from diabetes in Morocco. Along with the big increase in the number of diabetic patients, the cost of treatment, especially that accompanying complications in terms of morbidity and mortality, has risen and this constitutes a challenge for government. Since 1995, the Ministry of Public Health has adopted a national program in which primary health care centers play a crucial role in the management of diabetes mellitus, including diagnosis of people at risk, adoption of a standardized diagnostic procedure, insulin therapy if required in due time, and provision of basic education and information about the control of complications when the diagnosis is confirmed.

Experimental Antidiabetic Plants

Various studies have been undertaken in different regions of Morocco in order to select and classify the main medicinal plants used to treat diabetes. Ziyyat et al. [63] conducted an ethnomedical study in eastern Morocco on plants used for diabetes and hypertension and an inventory of 42 plants used has been established. For diabetes, 38 species have been reported; the most used were *Trigonella foenum-graecum* (Leguminosae), *Globularia alypum* (Globulariacea), *Artemisia herba-alba* (Compositae), *Citrullus colocynthis* (Cucurbitaceae), and *Tetraclinis articulata* (Cupressaceae). Three of these species, namely *Artemisia herba-alba*, *Tetraclinis articulata*, and *Trigonella foenum-graecum* are also used for hypertension, which suggests a relationship between hypertension and diabetes.

Bnouham et al. [64] examined antidiabetic effect of an aqueous extract of the aerial parts of *Urtica dioïca* (nettle), a plant used in eastern Morocco for both diabetes and hypertension, on hyperglycemia induced by oral glucose tolerance test (OGTT) and on alloxan-induced diabetic rats. The authors showed a strong antihyperglycemic effect of the plant (250 mg kg^{-1}) during the first hour after glucose loading in rats under OGTT (33% versus control) but a lack of hypoglycemic effect in alloxan-induced diabetic rats. Furthermore, intestinal glucose absorption was significantly reduced *in situ* in jejunum segment, which suggests that the extract may act on glucose homeostasis via an extrapancreatic mechanism.

In another study, continuous perfusion of *U. dioica* aqueous extract progressively reduced arterial blood pressure and increased both diuresis and natriuresis proportionally at the same time [65]. The observed acute hypotensive effect was provoked by an important bradycardia, which is independent of cholinergic and α_1-adrenergic receptors [66]. Preliminary acute toxicity on rats suggested a low toxicity of the water extract since some people in eastern Morocco use the plant as a supplement with salad without any side effects.

On the other hand, an investigation of 2400 patients with diabetes in the Wilaya of Marrakech (southern Morocco) [67] demonstrated that the most used plants are

Trigonella foenum-graecum (19.11%), *Marrubium vulgare* (13.42%), *Artemisia herba alba* (11.24%), *Ammi visnaga* (10.09%) , *Globularia alypum* (9.86%), and *Zygophyllum gaetulum* (5.21%). Patients (15 men and 15 women) treated with a single dose of the infusion of *Z. gaetulum* leaves (Zygophyllaceae), locally known as "Alaagaya", showed a significant fall in blood glucose levels at 3 h (–13.26%) and at 6 h (–13.84%), and this fall was maintained at 9 h (–35.97%) [68]. The same authors have tested the plant but this time on alloxan-induced hyperglycemic rats [69] and shown that oral treatment with the aqueous extract caused a continuous marked reduction of blood glucose levels particularly at 6 and 9 h after treatment (–52.82% and –69.80% respectively). Toxicological assay of an aqueous extract of the plant on rats (32 g kg^{-1} body weight) showed mild central nervous system stimulation, slow respiration, no motor activity, and distending of the stomach [68]. In all doses of aqueous extract examined (up to 16 g kg^{-1} body weight) no significant change in motor activity was observed, skin was found to be normal, with no noticeable changes in behavior (LD_{50} = 15.5 g kg^{-1} body weight). Chemical studies on *Z. gaetulum* have been conducted by Safir and Fkih-Tetouani [57] and three new bidesmosidic triterpene saponins have been isolated and identified, named zygophyloside I, L, and M.

Another medicinal plant, *Globularia alypum* (Globulariacee), known locally as "ain larnab," which is frequently used to cure diabetes in eastern and southern Morocco [63, 67] and is also known to be laxative [3], stomachic, a good purgative, and sudorific [5], has been evaluated for its antihyperglycemic activity. Skim et al. [70], working on an infusion of *G. alypum* leaves, found that the extract exhibited a remarkable hypoglycemic effect 2 h after oral or intraperitoneal treatment (–69.96% and –53.29% respectively). The authors speculate that *G. alypum* activity could be due to an enhancement of the peripheral metabolism of glucose and an enhancement of insulin glucose levels.

In Tafilalet (south-eastern Morocco), *G. alypum* was also found among the most frequently used plants for diabetes, along with *Ammi visnaga*, *A. herba alba*, *T. foenum-graecum*, *Marrubium vulgare*, *Nigella sativa*, *Allium sativum*, *Olea europea, C. colocynthis*, *Aloe succotrina*, and *Rosmarinus officinalis* [71]. In this study 16 plants used for diabetes were also used to treat hypertension and cardiac diseases and we can note some used plants known for their toxicity such as *Peganum harmala* and *Nerium oleander*. *Globularia alypum* was also evaluated by Jouad et al. [72] together with *Rubus fructicosis* (Rosaceae), traditionally used as a depurative and an astringent. The aqueous extracts of these two plants produced a significant decrease of plasma glucose. In contrast, the mechanism of action stipulated was different from that suggested by Skim and workers [70] and was found to be independent of elevation of insulin secretion. This could be due to the difference in the method of preparation, in the doses used, and in the origins of the plants used. However, the authors are in agreement in considering that *G. alypum* leaves extract are free of toxic compounds at the dose tested [70, 72, 73]. The decoction of *Rubus fructicosis* was more potent that metformin [72]. It was also demonstrated in another study that a 20% dried leaves infusion of *Rubus ulmifolius* caused a significant decrease of plasma glucose in streptozotocin rats [74].

In the central northern region of Morocco (Fez-Boulemane), about 90 plants were recorded as traditional remedies for treating diabetes, cardiac, and renal diseases [75], 54 of which were cited for diabetes (over 100 citations). Two of the cited plants have been studied scientifically to confirm the traditional claims that they cure diabetes. Jouad et al. [76] undertook a survey of an aqueous extract of *Spergularia purpurea* (Caryophyllaceae) and found evidence of a plasma glucose lowering effect but no effect on plasma insulin concentration, suggesting that *S. purpurea* extract acts via an insulin-independent mechanism, possibly by stimulating glucose utilization in peripheral tissues. On the other hand, the extract had a low acute toxicity in rats (LD_{50} = 10.75 g kg^{-1}) and can be considered free of side effects.

6.2.2.3 Other Biological Activities

Beside antimicrobial and antidiabetic investigations in Moroccan plants, other bioassays have been carried out, consisting mainly of studies on molluscicidal, larvicidal, cardiovascular, diuretic, and hypotensive effects. Molluscicidal activity has been studied using *Bulinus truncatus*, the mollusc intermediate host of shistosomiasis in Morocco. This disease affects more than 200 million people in 73 tropical and subtropical countries [77] and constitutes one of the major health problems in rural communities living near slow moving water. In our laboratory, we have initiated a program in which some selected plants have been evaluated for molluscicidal and larvicidal potential, such as the latex of *Calotropis procera* [31, 78], some selected Solanaceous plants such as *Solanum elaeagnifolium*, *S. sodomaeum* [79], and *Quercus lusitania* [80]. Our results showed that *C. procera* latex and extracts from *S. elaeagnifolium* were the most promising as molluscicide and larvicide.

Hmamouchi et al. [81] have also tested the ability of some Moroccan medicinal plants to kill *B. truncatus*. They found that the most active extracts were from *Origanum compactum*, *Chenopodium ambrosioides*, and *Ruta chalepensis*. The ethylacetate extracts of *O. compactum* were also shown to be lethal to the cercariae of *Schistosoma haematobium* [82].

In addition to these molluscicidal and larvicidal effects, other properties of Moroccan medicinal plants have also been reported in the literature, such as platelet antiaggregant [83], diuretic [84, 85], antipyretic [86], antitumoral [87], and anti-inflammatory [88] activities.

6.3 Toxicological Assays

To assess efficacy of a plant as a therapeutic tool to treat a particular disease is not sufficient, it is also essential to study its toxicity and toxicity mechanisms towards animals and humans. It is of paramount importance to identify toxic constituents when evaluating an ethnomedical preparation in a pharmacological model because the plant or extract may be too toxic to be considered useful. When we reviewed a number of articles focussed on the toxicity of Moroccan medicinal plants, we found that this aspect was largely unexplored (Table 6.3).

Table 6.3 Moroccan medicinal plants assessed for toxicological effects.

Plant	*Local name*	*Local traditional use*	*Part tested*	*Reference*
Ferula communis	"Fessoukh"	Anthelminthic, magic, dermatitis	Resinous gum	89, 90
Astragalus lusitanicus	"Fwila"	Cataplasm of roots to treat arthritis	Legume	92–94
Argania spinosa	"Argan"	Tonic, to treat dry skin, wrinkles and burns.	Water extract of saponins	95
Herniaria cinerea	"Harrast lahjar"	Used against urolithiasis	Butanol extract	96
Ajuga iva	"Chendgoura"	Intestinal disorders; diabetes	Decoction of whole plant	97

The "fassoukh" or resinous gum collected from the root of *Ferula communis* (Apiaceae) is well known to Moroccan people for its toxicity. Indeed, intoxication is noticed when cattle graze in an area where *F. communis* predominates. In addition, the population consumes the inflorescence of the plant called "Boubal". The acute toxicity of fassoukh extract (0.1%) studied in albino rats showed 100% mortality within nine days [89]. Fraigui et al. [90] tested the toxicity in mice of a coumarin compound called ferulenol isolated from *F. communis*. They found that animals exhibited hypoprothrombinemia with internal and external hemorrhages. Lamnouer [91] demonstrated the anticoagulant effects of coumarin compounds and the role of vitamin K_1 in the recovery of the physiologic disturbance observed.

Plasma collected from lambs given *Astragalus lusitanicus* showed inhibition of beta-glucosidase and beta-galactosidase in liver and kidney tissues [92]. The toxic principles are suggested to be extremely water-soluble compounds since fresh plants or dry powder rather than methanol extracts were highly toxic, causing cardiac and respiratory disorders [93].

Another plant with particular interest is a tree endemic to south-western Morocco called *Argania spinosa* (Sapotaceae). The fruits of argan trees have great ecological and economical importance since they furnish an edible and marketable oil that provides up to 25% of the daily lipid diet in some regions. Charrouf and Guillaume [95] reviewed the traditional knowledge as well as the most recent results concerning the phytochemistry of *A. spinosa*. The argan tree is rich in saponins and many biological data have been obtained from argan saponins such as antifungal, anti-inflammatory, and analgesic activities. The toxic effects of argan saponins have also been studied and have shown an increase in blood creatinine along with focal renal tube deterioration [94].

6.4 Conclusions

Among Moroccan medicinal plants a small number have been investigated experimentally. Studies have shown that the percentage of use of herbal remedies by Mo-

roccan people oscillates between 55 and 90% according to the different areas of the country [63, 75] and traditional herbal healers are frequently consulted (37%) by the majority of patients, rather than pharmacists (1%). In many regions of Morocco herbal drugs are freely available to the population in nature without any restriction and it is generally believed that all naturally derived drugs are harmless and can be administered without any risk. Consequently, there is a crucial need to investigate toxicity levels and duration of treatment before prescription of traditional remedies to patients, and serious effort must be directed towards education of the population to the dangers of toxic medicinal plants. Further systematic investigations into the chemistry and biological efficacy of plant materiel will be needed to prove their medicinal worth.

It is worth noting the need for a standard method for the pharmacological testing of plant extracts or their constituents. To date, each investigation has been a standalone study and there is a lack of a multidisciplinary approach to the study of a particular plant for a particular purpose. We believe that we need to research a plant comprehensively by carrying out study of all aspects of the selected plant, including chemical isolation procedures, biological properties as well as all toxicological side effects on human and animal models. This can be done if close collaboration and a multidisciplinary approach between the different teams working in this field is initiated and sustained.

Finally, high priority should be given to the mechanisms of action of plant extracts, interaction with available commercial compounds (for example antibiotics in the case of antimicrobial activities) and finally, study of the pharmacokinetic profile of the extract [25].

References

1 Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D., Guo, Z. Medicinal plants in therapy. *Bull. World Health Organ.* **1985**, *63*, 965–981.

2 Sofowora, A. *J. Ethnopharmacol.* **1993**, *38*, 209–214.

3 Bellakhder, J., Claisse, R, Fleurentin, J., Younos, C. *J. Ethnopharmacol.* **1991**, *35*, 123–143.

4 Bellakhder, J. *La pharmacopée Marocaine traditionnelle, médecine arabe ancienne et savoir populaire,* Edition le Fennec et Ibiss Press, Casablanca, **1997**, pp. 764.

5 Sijelmassi, A. *Les plantes médicinales du Maroc,* Le Fennec, Casablanca, **1996**.

6 Nègre, R. *Petite flore des régions arides du Maroc occidental*. CNRS, Paris, **1962**.

7 Jahandiez, E., Maire, R. *Catalogue des plantes du Maroc,* 3 Vol., Alger. **1931–1934**.

8 El-Hilaly, J., Hmammouchi, M., Lyoussi, B. *J. Ethnopharmacol.* **2003**, *86*, 149–158.

9 Merzouki, A., Ed-derfoufi, F., Molero Mesa, J. *Fitoterapia* **2000**, *71*, 278–307.

10 Bruatto, M., Vidotto, V., Marinuzzi, G., Raiteri, R., Sinicco, A. *J. Clin. Microbiol.* **1991**, *29*, 726–730.

11 Fan-Havard, P., Capano, D., Smith, S.M., Mangia, A., Eng, R.H.K. *Agents Chemother.* **1991**, *35*, 2302–2305.

12 Holmberg, K., Meyer, R.D. *Scand. J. Infect. Dis.* **1986**, *18*, 179–192.

13 Clark, A.M., Hufford, C.D. Discovery and development of novel prototype antibiotics for opportunistic infections related to the acquired immunodeficiency syndrome. In *Human Medical Agents from Plants*. ACS Symposium series 534, American Chemical Society, Washington, DC, **1993**, pp, 228–241.

14 Verpoorte, R., Dihal, P.P. *J. Ethnopharmacol*. **1987**, *21*, 315–318.
15 Desta, B. *J. Ethnopharmacol*. **1993**, *39*, 129–139.
16 Meyer, J.J.M., Afolayan, A.J. *J. Ethnopharmacol*. **1995**, *47*, 109–111.
17 Vlietinck, A.J., Van Hoof, L., Totté, J., Lasure, A., Vanden Berghe, D., Rwangabo, P.C., Mvukiyumwami, J. *J. Ethnopharmacol*. **1995**, *46*, 31–47.
18 Sanogo, R., Crisafi, G., Germano, M.P., De Pasquale, R., Bisignano, G. *Phytother. Res*. **1998**, *12*, S154–S156.
19 Abreu, P.M., Martins, E.S., Kayser, O., Bindseik, K.U., Siems, K., Seemann, A., Frevert, J. *Phytomedicine* **1999**, *63*, 187–195.
20 Perumal Samy, R.and Ignacimuthu, S. *J. Ethnopharmacol*. **2000**, *69*, 63–71.
21 Odds, F.C. *Antimicrob. Agents Chemother*. **1993**, *37*, 685–691.
22 Cook, R.A., McIntyre, K.A., Galgiani, J.N. *Antimicrob. Agents Chemother*. **1990**, *34*, 1542–1545.
23 Rios, J.L., Recio, MC., Villar, A. *J. Ethnophamacol*. **1988**, *23*, 127–149.
24 Nadir, D.M.T., Abdul-Baqi, J., AL-Sarraj, S.M., Hussein, W.A. *Fitoterapia* **1986**, *5*, 359–363.
25 Rios, J.L., Recio, M.C. *J. Ethnopharmacol*. **2005**, *100*, 80–84.
26 Gadhi, C.A., Benharref, A., Jana, M., and Lozniewski, A. *J. Ethnopharmacol*. **2001**, *75*, 203–205.
27 Barrero, A.F., Oltra, J.E., Alvarez, M., Raslan, D.S., Saude, D.A., Akssira M. *Fitoterapia* **2000**, *71*, 60–64.
28 Chebli, B., Achouri, M., Idrissi Hassani, L.M., Hmamouchi, M. *J. Ethnopharmacol*. **2003**, *89*, 165–169.
29 Larhsini, M., Lazrek, H.B., Amarouch, H., Jana, M. *J. Ethnopharmacol*. **1996**, *53*, 105–110.
30 Ezoubeiri, A., Gadhi, C.A., Fdil, N., Benharref, A., Jana, M., Vanhaelen, M. *J. Ethnopharmacol*. **2005**, *99*, 287–292.
31 Larhsini, M., Bousaid, M., Lazrek, H.B., Jana, M. *Fitoterapia* **1997**, LXVIII, *4*, 371–373.
32 Mitscher, L.A., Drake, S., Gollapudi, S.R., Okwute S.K. *J. Nat. Prod*. **1987** *50*, 1025–1040.
33 EL Hanbali, F., Akssira, M., Ezoubeiri, A., Gadhi, CA., Mellouki, F., Benharref, A., Blazquez, AM., Boira, H. *J. Ethnopharmacol*. **2005**, *99*, 399–401.
34 Gadhi, C.A., Weber, M., Mory, F., Benharref, A., Lion, C., Jana, M., Lozniewski, A. *J. Ethnopharmacol*. **1999**, *67*, 87–92.
35 Gadhi, C.A., Hatier, R., Mory, F., Marchal, L., Weber, M., Benharref, A., Jana, M., Lozniewski, A. *J. Ethnopharmacol*. **2001**, *75*, 207–212.
36 Markouk, M., Redwane, A., Lazrek, H.B., Jana; M., Benjama; A. *Fitoterapia* **1999**, *70*, 314–316.
37 Bouamama; H., Villard, J., Benharref, A., Jana, M. *Thérapie* **1999**, *54*, 731–733.
38 Bennamara, A., Abourriche, A., Berrada, M., Charrouf, M., Chaib, N., Boudouma, M., Garneau, F.X. *Phytochemistry* **1999**, *52*, 37–40.
39 Khallouki, F., Hmamouch, M., Younos, C., Soulimani, R., Bessiere, J.M., Essassi, E.M. *Fitoterapia* **2000**, *71*, 544–546.
40 Larhsini, M., Oumoulid, L., Lazrek, H.B., Wataleb, S., Bousaid, M., Bekkouche, K., Jana, M. *Phytother. Res*. **2001**, *15*, 250–252.
41 Grayer, J.R., Harborne, J.B. *Phytochemistry* **1994**, *37*, 19–42.
42 Zimmet, P. *Diabetologia* **1982**, *22*, 399–411.
43 Taskinen, M.R., Bogarus, C., Kennedy, A., Howard, B.V. *J. Clin. Invest*., **1985**, *76*, 637–644.
44 Huse, D.M., Oster, G., Killen A.R., Lacey, M.G., Golditz, G.A. *JAMA* **1988**, *262*, 2708–2713.
45 Khallouki, F., Hmamouchi, M., Younos, C., Soulimani, R., Essassi, E.M. *Fitoterapia* **2000**, *71*, 413–416.
46 Touati, D., Atta-ur-Rahman, Ulubelen, A. *Phytochemistry* **2000**, *53*, 277– 279.
47 Ghoulami, S., Il Idrissi, A., Fkih-Tetouani, S. *Fitoterapia* **2001**, *72*, 596–598.
48 Politi, M., De Tommasi, N., Pescitelli, G., Di Bari, L., Morelli, I., Braca, A. *J. Nat. Prod*. **2002**, *65*, 1742–1745.
49 Hilmi, F., Sticher, O., Heilmann, J. *J. Nat. Prod*. **2002**, *65*, 523–526.
50 Barrero, A.F., Quilez del Moral, J.F., Lucas, R., Paya, M., Akssira, M., Akkad, S., Mellouki, F. *J. Nat. Prod*. **2003**, *66*, 844–850.
51 El Hassany, B., El Hanbali, F., Akssira , M., Mellouki, F., Haidour, A., Barrero, A.F. *Fitoterapia* **2004**, *75*, 573–576.

52 Barrero, A.F., Quilez del Moral, J.F., Mar Herrador, M., Akssira, M., Bennamara, A., Akkad, S., Aitigri, M. *Phytochemistry* **2004**, *65*, 2507–2515.

53 Larhsini, M., Marston, A., Hostettmann, K. *Fitoterapia* **2003**, *74*, 237–241.

54 Barrero, A.F., Quilez del Moral, J.F., Mar Herrador, M., Arteaga, J.F., Akssira, M., Benharref, A., Dakir, M. *Phytochemistry* **2005**, *66*, 105–111.

55 M'barek, A.N., Guillaume, D., Kol, O., Charrouf, Z. *Phytochemistry* **1996**, *43*, 1075–1077.

56 Barrero, A.F., Herrador, M.M., Akssira, M., Arteaga, P., Romera, J.L. *J. Nat. Prod.* **1999**, *62*, 7, 946–948.

57 Safir, O., Fkih-Tetouani, S. *J. Nat. Prod.* **1998**, *61*, 130–134.

58 Charrouf, Z., Wieruszeski, J.M., Fkih-Tetouani, S., Leroy, Y., Charrouf, M., Fournet, B. *Phytochemistry* **1992**, *31*, 2079–2086.

59 Daali, Y., Bekkouche, K., Cherkaoui, S., Christen, P., Veuthey J.L. *J. Chromatogr. A* **2000**, *903*, 237–244.

60 Cherkaoui, S., Bekkouche, K., Christen, P., Veuthey J.L. *J. Chromatogr. A* **2001**, *922*, 321–328.

61 Bekkouche, K., Daali, Y., Cherkaoui, S., Veuthey J.L., Christen, P. *Phytochemistry* **2001**, *58*, 455–462.

62 Shigeta, H., Shigeta, M., Nakazawa, A., Nakamura, N., Yoshikawa, T. *Diabetes Care* **2001**, *24*, 602–609.

63 Ziyyat, A., Legssyer, A., Mekhfi, H., Dassouli, A., Serhrouchni, M., Benjelloun, W. *J. Ethnopharmacol.* **1997**, *58*, 45–54.

64 Bnouham, M., Merhfour, F., Ziyyat, A., Mekhfi, H., Aziz, M., Legssyer, A. *Fitoterapia* **2003**, *74*, 677–681.

65 Tahri, A., Yamani, S., Legssyer, A., Aziz, M., Mekhfi, H., Bnouham, M., Ziyyat, A. *J. Ethnopharmacol.* **2002**, *73*, 95–100.

66 Legssyer, A., Ziyyat, A., Mekhfi, H., Bnouham, M., Tahri, A., Serhrouchni, M., Hoerter, J., Fischmeister, R. *Phytother. Res.* **2002**, *16*, 503–507.

67 Jaouhari, J.T. Valorisation des plantes médicinales marocaines, contribution à l'étude pharmacologique de certaines plantes réputées hypoglycémiantes dans la wilaya de Marrakech. Thesis of 3th cycle; No. 294, University of Cadi Ayyad, Faculty of Sciences-Semlalia, Marrakech, **1993**.

68 Jaouhari, J.T., Lazrekk, H.B., Seddik, A., Jana, M. *J. Ethnopharmacol.* **1999**, *64*, 211–217.

69 Jaouhari, J.T., Lazrekk, H.B., Jana, M. *J. Ethnopharmacol.* **2000**, *71*, 17–20.

70 Skim, F., Kaaya, A., Jaouhari, J.T., Lazrek, H.B., Jana, M., EL Amri, H. *Fitoterapia* **1999**, *70*, 382–389.

71 Eddouks, M., Maghrani, M., Lemhadri, A., Ouahidi, M.L., Jouad, H. *J. Ethnopharmacol.* **2002**, *82*, 97–103.

72 Jouad, H., Maghrani, M., Eddouks, M. *J. Ethnopharmacol.* **2002**, *81*, 351–356.

73 Skim, F., Lazrek, H.B., El Amri, H., Kaaya, A., Jana, M. *Phytother. Res.* **1998**, *12*, 592–594.

74 Lemus, I., Garcia, R., Delvillar, E., Knop, G. *Phytother. Res.* **1999**, *13*, 91–94.

75 Jouad, H., Haloui, M., Rhiouani, H., El Hilali, J., Eddouks, M. *J. Ethnopharmacol.* **2001**, *77*, 175–182.

76 Jouad, H., Eddouks, M., Lacaille-Dubois, M.A., Lyoussi, B. *J. Ethnopharmacol.* **2000**, *71*, 169–177.

77 McCullough, F.C., Mott, K.E. The role of molluscicides in shistosomiasis control. WHO document, WHO/SCHISTO/83.72, **1983**.

78 Markouk, M., Bekkouche; K., Larhsini, M., Bousaid, M., Lazrek, H.B., Jana, M. *J. Ethnopharmacol.* **2000**, *73*, 293–297.

79 Bekkouche, K., Markouk, M., Larhsini, M., Jana, M., Lazrek, H.B. *Phytother. Res.* **2000**, *14*, 366–367.

80 Redwane, A., Lazrek, H.B., Bouallam, S., Markouk, M., Amarouch, H., Jana, M. *J. Ethnopharmacol.* **2002**, *79*, 261–263.

81 Hmamouchi, M., Lahlou, M., Agoumi; A. *Fitoterapia* **2000**, *71*, 308–314.

82 Lahlou, M. *Ann. Trop. Med. Parasitol.* **2002**, *96*, 587–593.

83 Mekhfi, H., El Haouari, M., Legssyer, A., Bnouham, M., Aziz, M., Atmani, F., Remmal, A., Ziyyat, A. *J. Ethnopharmacol.* **2004**, *94*, 317–322.

84 Haloui, M., Louedec, L., Michel, J.B., Lyoussi, B. *J. Ethnopharmacol.* **2000**, *71*, 465–472.

85 Jouad, H., Lacaille-Dubois, M.A., Eddouks, M. *J. Ethnopharmacol.* **2001**, *75*, 219–223.

86 Larhsini, M., Markouk, M., Jaouhari, J.T., Bekkouche, K., Lazrek, H.B Jana, M. *Phytother. Res.* **2002**, *16*, S97–S98.
87 Lamchouri, F., Settaf, A., Cherrah, Y., Hassar, M., Zemzami, M., Atif, N., Nadori, E.B., Zaid, A., Lyoussi, B. *Fitoterapia* **2000**, *71*, 50–54.
88 Ismaili, H., Milella, L., Fkih-Tetouani, S., Ilidrissi, A., Camporese, A., Sosa, S., Altinier, G., Della Loggia, R., Aquino, R. *J. Ethnopharmacol.* **2004**, 91, 31–36.
89 Fraigui, O., Lamnaouer, D., Faouzi, M.Y., Cherrah, Y., Tijjane, M. *Vet. Hum. Toxicol.* **2001**, *43*, 6, 327–330.
90 Fraigui, O., Lamnaouer, D., Faouzi, M.Y. *Vet. Hum. Toxicol.* **2002**, *44*, 5–7.
91 Lamnouer, D. *Thérapie* **1999**, *54*, 747–751.
92 Abdennebi, E.H., Ouazzani, N., Jossang, A., Benkouka, F., Lamnouer, D. *Vet. Hum. Toxicol.* **2001**, *43*, 266–269.
93 Abdennebi, E.H., Ouazzani, N., Lamnouer, D. *Vet. Hum. Toxicol.* **1998**, *40*, 327–331.
94 Ouazzani, N., Lamnouer, D., Abdennebi, E.H. *Thérapie* **1999**, *54*, 707–710.
95 Charrouf, Z., Guillaume, D. *J. Ethnopharmacol.* **1999**, *67*, 7–14.

7
Anti-MRSA and Anti-VRE Activities of Phytoalexins and Phytoncides Isolated from Tropical Plants

Yoshikazu Sakagami

Summary

Antibacterial compounds belonging to the phytoalexin and phytoncide groups have been isolated from many plants. Tropical plants in particular possess many antibacterial compounds, such as sophoraflavanone G, calozeyloxanthone, α-mangostin, and the stilbene oligomers of gnemonol B and gnetin E. In this chapter, antibacterial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) are discussed. In addition, interactions between the phytoalexins and phytoncides and the commercially available antibiotics, such as ampicillin, gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride, are also covered. The antibacterial activities of these test compounds were evaluated by measuring minimum inhibitory concentration (MIC) values determined by the agar dilution method of the Japanese Society of Chemotherapy. The synergism between the test compounds and the commercially available antibiotics was evaluated using fraction inhibitory concentration (FIC) indices measured by the MIC values of the test compounds, alone or in combination with the antibiotic.

MIC values of calozeyloxanthone, α-mangostin, gnemonol B, and gnetin E against VRE were 6.25–12.5, 3.12–6.25, 12.5, and 12.5–25 μg mL^{-1}, respectively. MIC values of sophoraflavanone G, α-mangostin, gnemonol B, and gnetin E against MRSA were 3.13–6.25, 6.25–12.5, 6.25, and 12.5–25 μg mL^{-1}, respectively. Strong anti-VRE and anti-MRSA activities of these compounds was found.

Synergism between α-mangostin and gentamicin as well as calozeyloxanthone and vancomycin hydrochloride was observed against VRE. Partial synergism was detected between calozeyloxanthone (or α-mangostin) and ampicillin, GM, minocycline, and fosfomycin. Partial synergism between gnemonol B and the commercially available antibiotics were found, and also observed between gnetin E and some antibiotics tested.

Synergism between sophoraflavanone G and vancomycin hydrochloride was found against MRSA. Synergism between sophoraflavanone G and fosfomycin, and partial synergism between gnemonol B and ampicillin, gentamicin, minocy-

cline, fosfomycin, and vancomycin hydrochloride were found. Partial synergism was also found between gnetin E and gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride.

These results suggest that the above-mentioned compounds possess strong anti-VRE and anti-MRSA activities and some of them show synergistic interactions. These compounds could be used in the medical field to decrease infectious bacteria such as VRE and MRSA.

7.1 Introduction

Phytoalexins are low molecular weight compounds (molecular weights are mainly 100 500) produced defensively following infection of plants by pathogenic microorganisms. They are natural antimicrobial compounds which are produced by plants as a defense against the attack of harmful insects and microorganisms. The production of phytoalexins can be induced by nonbiological stress, such as ultraviolet irradiation and by treatment with heavy metals. The detailed production mechanisms of phytoalexins are not clearly understood. The participation of active oxygen is thought to be one of the main reasons for the killing mechanism of phytoalexins.

The main components of phytoncides are easily volatile terpen compounds that act on autonomic nerves, contributing to the stability of mind and concentration.

No toxicity reports of phytoalexins and phytoncides, including the test compounds in this section against humans have been found.

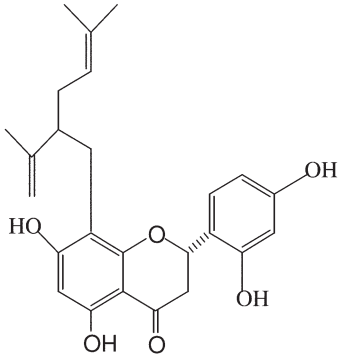
Enterococci and *Staphylococcus aureus* are two of the leading causes of nosocomial infections in long-term health care facilities, and reports on vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) infections in hospitals have increased worldwide [1–4]. In recent years, there have been a number of reports on useful trials carried out to control the infections caused by VRE [5–14] and MRSA [15–20]. However, further trials are needed to find more reliable methods to control VRE and MRSA infections adequately. In this context the use of natural products as anti-VRE and anti-MRSA agents are promising condidates for study towards the prevention and treatment of VRE and MRSA infections. Furthermore, it is very important to investigate the interactions of the active natural products with commercially available antibiotics, with the hope of enhancing their activity.

In this chapter we report on the preparation of some phytoalexins and phytoncides from tropical fruit, and the results of anti-MRSA and anti-VRE activity tests. Furthermore, the synergisms between these test compounds and commercially available antibiotics were also investigated.

7.2
Phytoalexins and Phytoncides

The structures of the phytoalexins and phytoncides sophoraflavanone G, calozeyloxanthone, α-mangostin, and stilbene oligomers, as used in the anti-VRE and anti-MRSA activity tests reported here, are shown in Figs. 7.1–7.4.

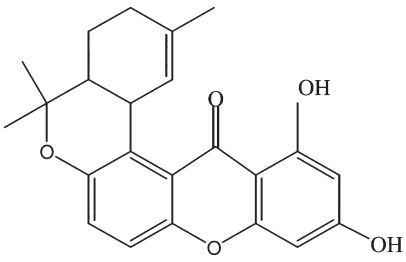
Sophoraflavanone G is a flavanone derivative, calozeyloxanthone and α-mangostin are xanthone derivatives, and stilbene oligomers (gnemonol B and gnetin E) belong to the polyphenol group, respectively.



Sophoraflavanone G

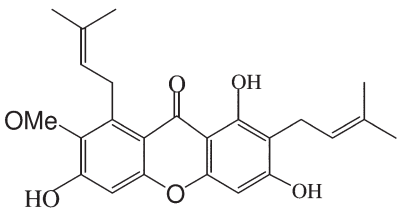
[2 (S)-5,7,2',4'-tetrahydroxy-8-lavandulylflavanone]

Fig. 7.1 The structure of sophoraflavanone G.

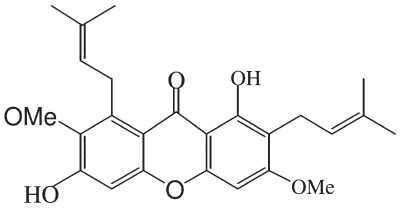


(**1**) Calozeyloxanthone

Fig. 7.2 The structure of calozeyloxanthone.

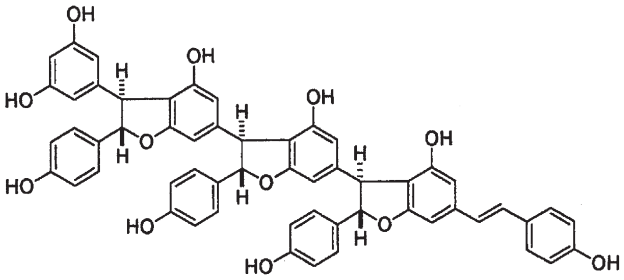


Alpha-mangostin

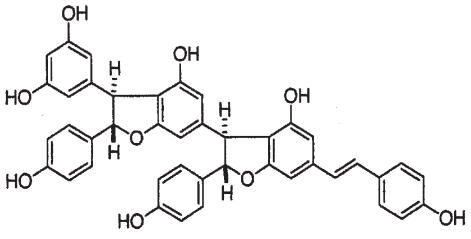


Beta-mangostin

Fig. 7.3 The structure of α-mangostin and β-mangostin.



Gnemonol B (1)



Gnetin E (2)

Fig. 7.4 The structure of stilbene oligomers (gnemonol B and gnetin E).

7.3 Antibiotics

The commercially available antibiotics ampicillin, gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride were used for the test of synergistic studies.

7.4 Bacteria and Broth

7.4.1 VRE

Five strains of VRE (*Enterococcus faecalis* ATCC 51299, *E. faecalis* ATCC 51575, *E. faecium* ATCC 51559, *E. faecium* KIHC-237, and *E. gallinarum* KIHC-241) were used in this experiment. The three ATCC strains were purchased from American Type Culture Collection (ATCC). Two strains of *E. faecium* KIHC-237 and *E. gallinarum* KIHC-241 were supplied by Kobe Institute of Public Health. The genotypes of *E. faecalis* ATCC 51299, *E. faecium* KIHC-237, and *E. gallinarum* KIHC-241 are van B(+), van A(+) and van C1(+), respectively. The genotypes of the other two VRE, *E. faecalis* ATCC 51575 and *E. faecium* ATCC 51559, were unknown.

7.4.2 VSE

Three strains of vancomycin-sensitive enterococci (VSE) (*E. faecalis* IFO 12965, *E. faecium* IFO 3535, and *E. faecalis* ATCC 8459) were used in this experiment. The strains were purchased from Institute for Fermentation of Osaka (IFO), Japan, and ATCC, respectively.

7.4.3 MRSA

Each of three strains (total: nine strains) of methicillin-resistant *Staphylococcus aureus* (MRSA) were kindly donated by Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka National Hospital and Kitano Hospital in 1997.

7.4.4 MSSA

Methicillin-sensitive *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* IFO 13276, *S. aureus* IFO 12732, and *S. aureus* IFO 3060 used in this experiment were purchased from IFO.

7.4.5 Broth

SCD broth (Nihon Pharm. Co., Ltd., Japan) was used for preincubation of VRE, VSE, MRSA, and MSSA. Mueller-Hinton (MH) Agar (Difco Co., Ltd., USA) was used for the measurement of minimum inhibitory concentration (MIC).

7.5 Isolation of Phytoalexins and Phytoncides

Sophoraflavanone G (2(*S*)-5,7,2′,4′-tetrahydroxy-8-lavandulylflavanone) isolated from *Sophora* spp. (Leguminosae) was used in the anti-MRSA activity test [21].

Calozeyloxanthone was isolated from the root bark of *Callophyllum moonii* as a yellow crystalline compound (thin-layer chromatography (TLC) single spot, melting point 236–237 °C). It was identified from the plant material collected from the Kanneliya forest in the southern province of Sri Lanka. The plant specimens were compared with herbarium specimens (specimen no. 24994) at the Royal Botanic Gardens, Peradeniya, Sri Lanka and a voucher specimen was deposited at the natural products laboratories of the Institute of Fundamental Studies [22].

α-Mangostin was isolated as follows. Stem bark of *G. mangostana* L. (1 kg) was dried, powdered, and extracted with hexane, methylene chloride, and methanol, respectively. Silica gel column chromatography (Fluka 6074,1 particle size

0.063 ~ 0.2 mm with hexane, methylene chloride, and methanol as solvents) of the hexane extract (11.9 g) and methylene chloride extract (25 g) gave two major compounds: α-mangostin (11.6 g, 1.16%) and β-mangostin (6.4 g, 0.64%) as yellow needles. These structures were confirmed by direct comparison with authentic samples and spectral data [23].

The stilbene oligomers (gnemonol B and genetin E) isolated from gnetaceous plants were donated by Professor Dr M. Iinuma (Gifu Pharmaceutical University) [24, 25].

7.6
Minimum Inhibitory Concentration

MIC values were determined by the agar dilution method of the Japanese Society of Chemotherapy [26] using a micro-inoculater (Sakuma Seisakusho Co., Ltd., Tokyo). MIC of the five strains of VRE and three strains of VSE described above were measured as 250, 32, 200, 200, and 16 μg mL^{-1}, while MIC values of the nine strains of MRSA and three strains of methicillin were measured as 12.5, 400, 25, 12.5, 400, 1600, 25, 12.5, and 400 μg mL^{-1}, respectively [27].

7.7
Synergism of Antibacterial Compounds with Commercially Available Antibiotics

Antimicrobial compounds were prepared in 50% dimethylsulfoxide solution. A solution of phytoalexin (or phytoncide) in combination with respective antibiotics was prepared by the doubling dilution method with sterilized water, and each solution was poured into sterilized plastic Petri dishes separately. Sterilized MH agar 8 mL (MH agar poured into phytoalexin (or phytoncide) alone or the antibiotic alone) was poured into the above Petri dishes and mixed. After cooling, the MIC values of phytoalexin or phytoncide alone, the antibiotics alone, and their combinations, were examined. The fraction inhibitory concentration (FIC) indices were calculated by the method of Didry et al. [28], and the interactive effects of the phytoalexin or phytoncide and the commercial antibiotics were examined.

FIC index values were judged as follows: FIC index ≤ 0.5: synergetic effect; FIC index 0.5–1.0: partially synergetic effect; FIC index >1.0: no synergetic effect; FIC index ≥ 2.0: antagonistic effect.

7.8
Antibacterial Activities

7.8.1
Sophoraflavanone G

The MIC values of sophoraflavanone G against 27 strains of MRSA are shown in Table 7.1. The values ranged from 3.13 to 6.25 μg mL^{-1}.

Table 7.1 Antibacterial activities of sophoraflavanone G (SFG) against 27 strains of methicillin-resistant *Staphylococcus aureus* (MRSA).

Strain no.	*Coagulase type*	*MIC values to methicillin (μg mL^{-1})*	*MIC values to SFG (μg mL^{-1})*
1[a]	II	1600	6.25
2[a]	II	100	6.25
3	II	1600	6.25
4	II	1600	6.25
5	VII	3200	6.25
6	II	1600	6.25
7	II	1600	6.25
8	II	3200	3.12
9	VII	800	3.12
10[a]	II	400	3.12
11[a]	II	800	3.12
12	III	12.5	6.25
13	II	400	6.25
14	III	12.5	6.25
15	VII	12.5	6.25
16	II	400	6.25
17	II	50	6.25
18	II	400	6.25
19[a]	II	400	3.12
20[a]	II	800	6.25
21	II	400	6.25
22	II	400	6.25
23	II	800	6.25
24	II	1600	3.12
25	II	800	3.12
26	II	800	6.25
27	II	400	6.25

Strain nos 1–9: MRSA isolates from Osaka Medical Center for Cancer and Cardiovascular Diseases.
Strain nos 11–19: MRSA isolates from Osaka National Hospital.
Strain nos 19–27: MRSA isolates from Kitano Hospital.
[a] These strains were used for the test of synergism.

7.8.2 Calozeyloxanthone

The MIC values of calozeyloxanthone against each of the two strains of VRE and VSE are shown in Table 7.2. The antibacterial activity of calozeyloxanthone against VRE and VSE was strong and MIC values observed were 6.25 μg mL^{-1} and 12.5 μg mL^{-1}, respectively.

Table 7.2 Antibacterial activity of calozeyloxanthone against two strains of vancomycin-resistant enterococci (VRE) and vancomycin-sensitive enterococci (VRE).

	MIC (μg mL^{-1})	
	Calozeyloxanthone	***Gentamicin***
Enterococcus faecalis ATCC 51575 (VRE)	12.5	400
Enterococcus faecium ATCC 51559 (VRE)	6.25	25
Enterococcus faecalis ATCC 12953 (VSE)	12.5	12.5
Enterococcus faecalis ATCC 8459 (VSE)	6.25	6.25

MIC, minimum inhibitory concentration.

7.8.3 α-Mangostin

Table 7.3 shows the anti-VRE activity of α-mangostin and β-mangostin, and Table 7.4 shows the anti-MRSA activity of α-mangostin and β-mangostin, respectively.

α-Mangostin was found to be active against five strains of VRE and nine strains of MRSA, with MIC values ranging from 6.25 to 12.5 μg mL^{-1}, respectively.

Table 7.3 MIC values of α-mangostin and β-mangostin mangostin against five strains of vancomycin-resistant enterococci (VRE) and three strains of vancomycin-sensitive enterococci (VSE).

	MIC (μg mL^{-1})		
	α-Mangostin	***β-Mangostin***	***Gentamicin***
Enterococcus faecalis ATCC 51299 (VRE)[a]	3.13	25	>100
Enterococcus faecalis ATCC 51575 (VRE)[a]	3.13	25	>100
Enterococcus faecium ATCC 51559 (VRE)[a]	3.13	25	6.25
Enterococcus faecium KIHC-237 (VRE)[b]	3.13	25	6.25
Enterococcus gallinarum KIHC-241 (VRE)[b]	6.25	25	3.13
Enterococcus faecalis IFO 12965 (VSE)[c]	6.25	25	12.5
Enterococcus faecium IFO 3535 (VSE)[c]	3.13	25	6.25
Enterococcus faecalis ATCC 8459 (VSE)[c]	3.13	25	6.25

MIC, minimum inhibitory concentration.
[a] Purchased from American Type culture Collection (ATCC).
[b] Supplied from Kobe Institute of Public Health.
[c] Purchased from Institute for Fermentation of Osaka (IFO), Japan.

Table 7.4 MIC values of α-mangostin and β-mangostin against nine strains of methicillin-resistant *S. aureus* (MRSA) and three strains of methicillin-sensitive *S. aureus* (MSSA).

	MIC (μg mL^{-1})		
	α-Mangostin	*β-Mangostin*	*Gentamicin*
MRSA-1[a]	6.25	>100	25
MRSA-2[a]	6.25	>100	3.13
MRSA-3[a]	6.25	>100	1.56
MRSA-4[b]	6.25	>100	3.13
MRSA-5[b]	6.25	>100	6.25
MRSA-6[b]	6.25	>100	0.2
MRSA-7[c]	6.25	>100	0.2
MRSA-8[c]	12.5	>100	6.25
MRSA-9[c]	6.25	>100	>100
MSSA 1 (*S. aureus* IFO 13276)[d]	6.25	>100	0.2
MSSA 2 (*S. aureus* IFO 12732)[d]	6.25	>100	0.2
MSSA 3 (*S. aureus* IFO 3080)[d]	6.25	>100	0.2

MIC, minimum inhibitory concentration.
[a] Donated from Osaka Medical Center for Cancer and Cardiovascular Diseases, Japan.
[b] Donated from Osaka National Hospital, Japan.
[c] Donated from Kitano Hospital, Japan.
[d] Purchased from Institute for Fermentation of Osaka (IFO), Japan.

7.8.4
Gnemonol B and Gnetin E

Tables 7.5 and 7.6 show the MIC values of gnemonol B and gnetin E against VRE, MRSA, VSE, and MSSA.

Table 7.5 MIC values of gnemonol B and gnetin E against five strains of vancomycin-resistant enterococci (VRE) and three strains of vancomycin-sensitive enterococci (VSE).

	MIC (μg mL^{-1})		
	Gnemonol B	*Gnetin E*	*Gentamicin*
Enterococcus faecalis ATCC 51299 (VRE)[a]	12.5	12.5	>100
Enterococcus faecalis ATCC 51575 (VRE)[a]	12.5	12.5	>100
Enterococcus faecium ATCC 51559 (VRE)[a]	12.5	12.5	6.25
Enterococcus faecium KIHC-237 (VRE)[b]	12.5	25	6.25
Enterococcus gallinarum KIHC-241 (VRE)[b]	12.5	25	3.13
Enterococcus faecalis IFO 12965 (VSE)[c]	12.5	25	12.5
Enterococcus faecium IFO 3535 (VSE)[c]	12.5	25	6.25
Enterococcus faecalis ATCC 8459 (VSE)[c]	12.5	25	6.25

MIC, minimum inhibitory concentration.
[a] Purchased from American Type culture Collection (ATCC).
[b] Supplied from Kobe Institute of Public Health.
[c] Purchased from Institute for Fermentation of Osaka (IFO), Japan.

Table 7.6 MIC values of gnemonol B and gnetin E against nine strains of methicillin-resistant *S. aureus* (MRSA) and three strains of methicillin-sensitive *S. aureus* (MSSA).

	MIC (μg mL^{-1})		
	Gnemonol B	***Gnetin E***	***Gentamicin***
MRSA-1[a]	6.25	12.5	25
MRSA-2[a]	6.25	12.5	3.13
MRSA-3[a]	6.25	25	1.56
MRSA-4[b]	6.25	12.5	3.13
MRSA-5[b]	6.25	12.5	6.25
MRSA-6[b]	6.25	12.5	0.2
MRSA-7[c]	6.25	12.5	0.2
MRSA-8[c]	6.25	25	6.25
MRSA-9[c]	6.25	25	>100
MSSA 1 (*S. aureus* IFO 13276)[d]	6.25	12.5	0.2
MSSA 2 (*S. aureus* IFO 12732)[d]	6.25	12.5	0.2
MSSA 3 (*S. aureus* IFO 3080)[d]	6.25	12.5	0.2

MIC, minimum inhibitory concentration.
[a] Donated from Osaka Medical Center for Cancer and Cardiovascular Diseases, Japan.
[b] Donated from Osaka National Hospital, Japan.
[c] Donated from Kitano Hospital, Japan.
[d] Purchased from Institute for Fermentation of Osaka (IFO), Japan.

Gnemonol B was found to be active against five strains of VRE and nine strains of MRSA with MIC values of 12.5 and 6.25 μg mL^{-1}, respectively. Gnemonol B was also active against the three strains of VSE and MSSA with MIC values of 12.5 and 6.25 μg mL^{-1}, respectively. Gnetin E also exhibited activities against five strains of VRE, nine strains of MRSA, and three strains of VSE and MSSA with MIC values ranging from 12.5 to 25 μg mL^{-1}.

7.8.5
Summary of MIC Values of Phytoalexin and Phytoncide Against MRSA and VRE

The MIC values of phytoalexin and phytoncide against MRSA and VRE are summarized in Tables 7.7 and 7.8.

Table 7.7 Anti-VRE activities of sophoraflavanone G, calozeyloxanthone, α-mangostin and stilbene oligomers.

	MIC (μg mL^{-1})
Sophoraflavanone G	6.25–12.5
Calozeyloxanthone	6.25–12.5
α-Mangostin	3.13–6.25
Gnemonol B	12.5
Gnetin E	12.5–25
Gentamicin	6.25–400

MIC, minimum inhibitory concentration.

Table 7.8 Anti-MRSA activities of sophoraflavanone G, calozeyloxanthone, α-mangostin and stilbene oligomers.

	MIC (μg mL^{-1})
Sophoraflavanone G	3.13–6.25
α-Mangostin	6.25–12.5
Gnemonol B	6.25
Gnetin E	12.5–25
Gentamicin	1.56–100

MIC, minimum inhibitory concentration.

Sophoraflavanone G, calozeyloxanthone, and α-mangostin possessed strong anti-MRSA and anti-VRE activities compared with gentamicin. Stilbene oligomers (gnemonol B and gnetin E) also possessed anti-MRSA and anti-VRE activities, but they were weaker than those of sophoraflavanone G, calozeyloxanthone, and α-mangostin.

7.9
Synergism Between the Test Compounds and Commercial Antibiotics Against VRE, MRSA, VSE, and MSSA

7.9.1
Sophoraflavanone G

Synergism between sophoraflavanone G and vancomycin hydrochloride or fosfomycin was observed (FIC indices were 0.16 and 0.48), while partial synergism was seen between sophoraflavanone G and other antibacterial agents such as ampicillin, gentamicin, and minocycline (FIC indices were 0.73, 0.69, and 0.65, respectively) (Fig. 7.5).

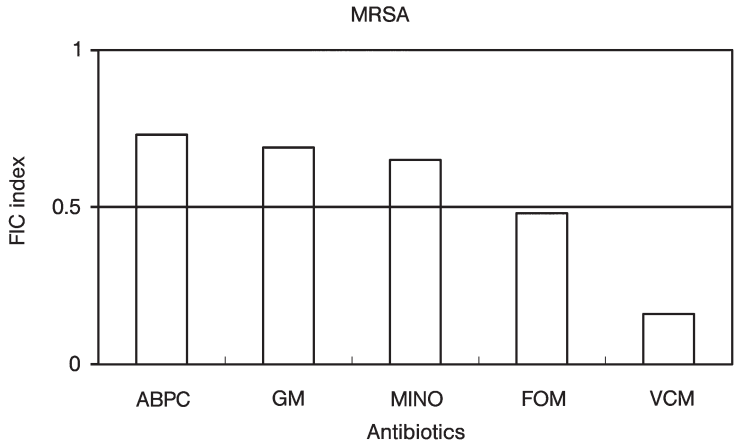


Fig. 7.5 Synergisms between sophoraflavanone G and commercially available antibiotics. FIC, fraction inhibitory concentration; ABPC, ampicillin; GM, gentamicin; MINO, minocycline; FOM, fosfomycin; VCM, vancomycin; MRSA, methicillin-resistant *Staphylococcus aureus*.

7.9.2
Calozeyloxanthone

A marked synergism between calozeyloxanthone and vancomycin hydrochloride against VRE was observed, as shown as Fig. 7.6. The FIC index of calozeyloxanthone and commercially available antibiotics such as ampicillin, gentamicin, minocycline, and vancomycin hydrochloride against VRE were 0.750, 0.625, 0.563, and 0.453, respectively.

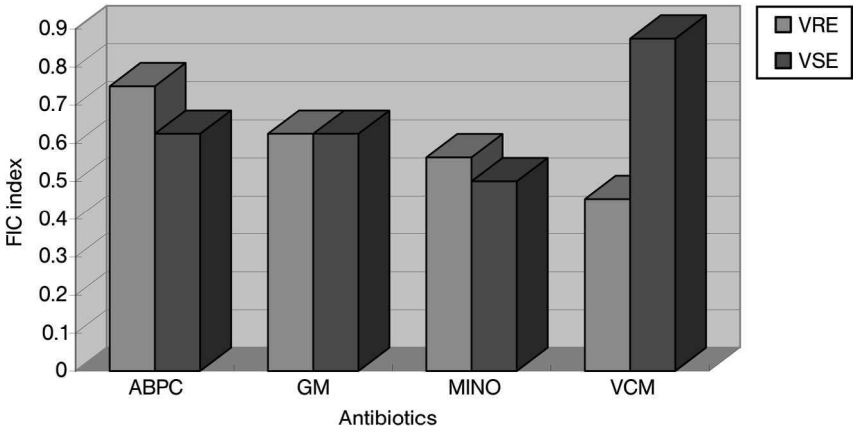


Fig. 7.6 Synergism between calozeyloxanthone and the commercially available antibiotics against each of two strains of vancomycin-resistant enterococci (VRE) and vancomycin-sensitive enterococci (VSE). FIC, fraction inhibitory concentration; ABPC, ampicillin; GM, gentamicin; MINO, minocycline; VCM, vancomycin.

7.9.3
α-Mangostin

The result is given in Fig. 7.7. Synergism between α-mangostin and gentamicin against VRE, and α-mangostin and vancomycin hydrochloride against MRSA was also observed. In the above synergistic studies, the average of FIC index was calculated as 0.451 ± 0.069 and 0.441 ± 0.131, respectively. Partial synergism between α-mangostin and ampicillin, minocycline, fosfomycin, and vancomycin hydrochloride against VRE with FIC indices of 0.606 ± 0.328, 0.969 ± 0.217, 0.826 ± 0.286, and 0.508 ± 0.271 were observed, respectively.

Furthermore, partial synergisms between α-mangostin and ampicillin, gentamicin, minocycline, and vancomycin hydrochloride against MRSA were also observed, and their FIC indices were calculated as 0.779 ± 0.343, 0.667 ± 0.359, 0.586 ± 0.303, and 0.504 ± 0.149, respectively.

On VSE, synergism between α-mangostin and vancomycin hydrochloride was observed, and the FIC index was calculated to be 0.378 ± 0.113. Partial synergisms

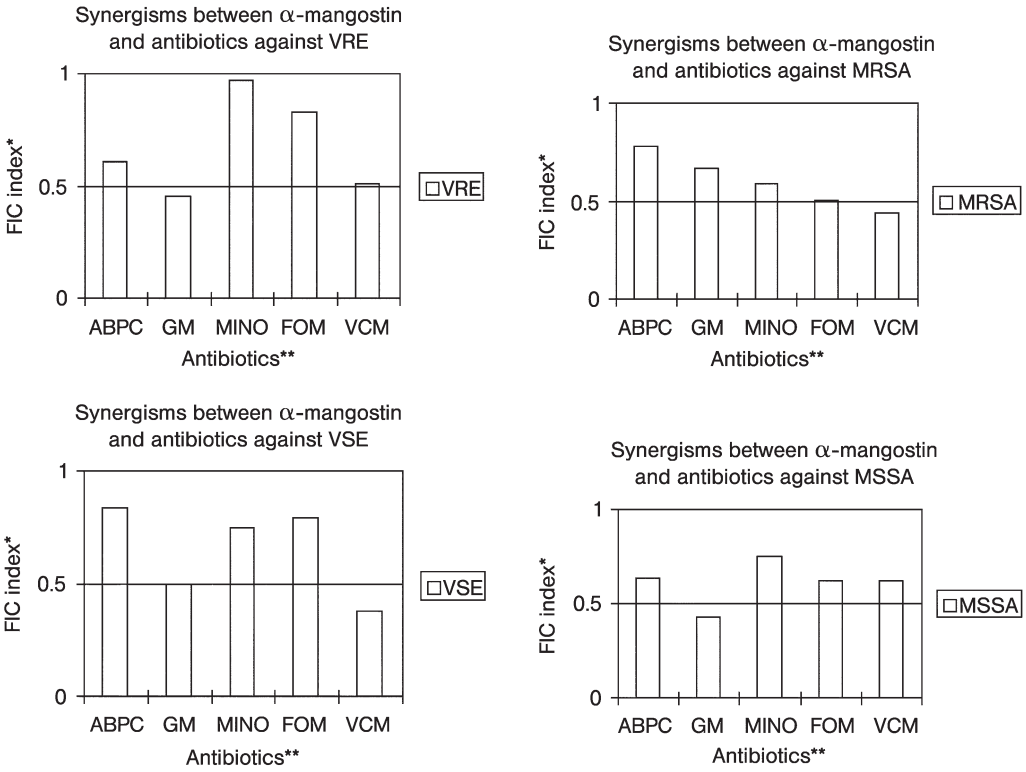


Fig. 7.7 Synergism between α-mangostin and antibiotics against vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-sensitive enterococci (VSE), and methicillin-sensitive *Staphylococcus aureus* (MSSA).

between α-mangostin and the commercially available antibiotics ampicillin, gentamicin, minocycline, and fosfomycin were observed, and their FIC indices were calculated as 0.836 ± 0.284, 0.500 ± 0.108, 0.750 ± 0.000, and 0.792 ± 0.191, respectively.

On MSSA, FIC indices between α-mangostin and the commercially available antibiotics ampicillin, gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride were observed, and their FIC indices were calculated as 0.635 ± 0.325, 0.428 ± 0.209, 0.750 ± 0.000, 0.625 ± 0.217, and 0.625 ± 0.000, respectively.

Synergism between α-mangostin and gentamicin against five strains of VRE, and α-mangostin and vancomycin hydrochloride against nine strains of MRSA were also tested by the evaluation method described by Williamson [29]. The results are shown in Figs 7.8 and 7.9, respectively. Synergism between α-mangostin and gentamicin against VRE, and α-mangostin and vancomycin hydrochloride against MRSA were reconfirmed by this method.

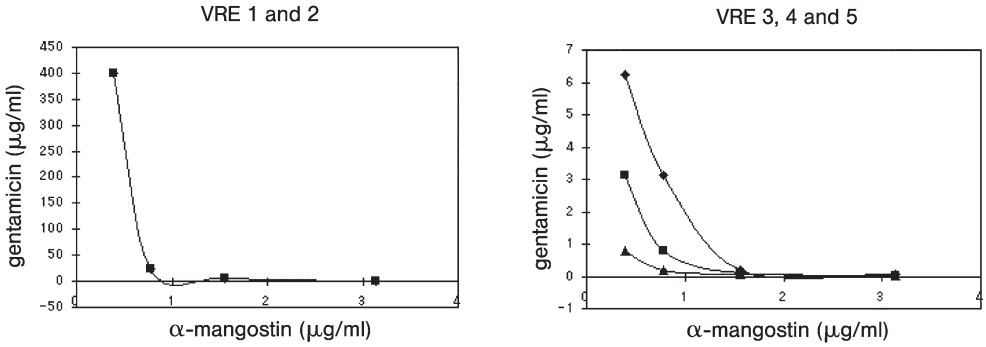


Fig. 7.8 Synergism between α-mangostin and gentamicin against five strains of vancomycin-resistant enterococci (VRE).

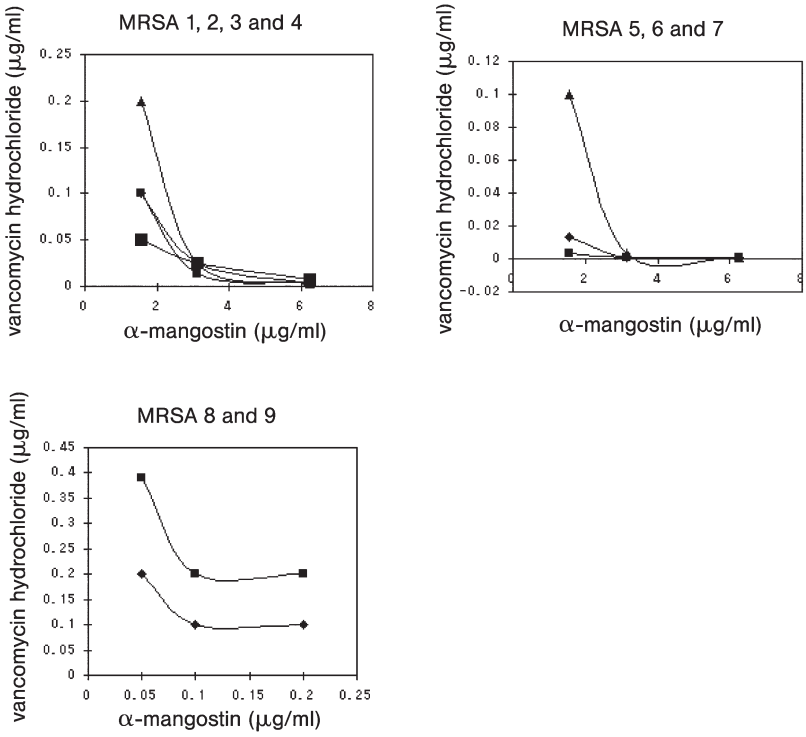


Fig. 7.9 Synergism between α-mangostin and vancomycin hydrochloride against nine strains of methicillin-resistant *Staphylococcus aureus* (MRSA).

7.9.4
Stilbene Oligomer

The results of synergisms of gnemonol B or gnetin E with the commercially available antibiotics against VRE, MRSA, VSE, and MSSA are shown in Figs 7.10 and 7.11, respectively.

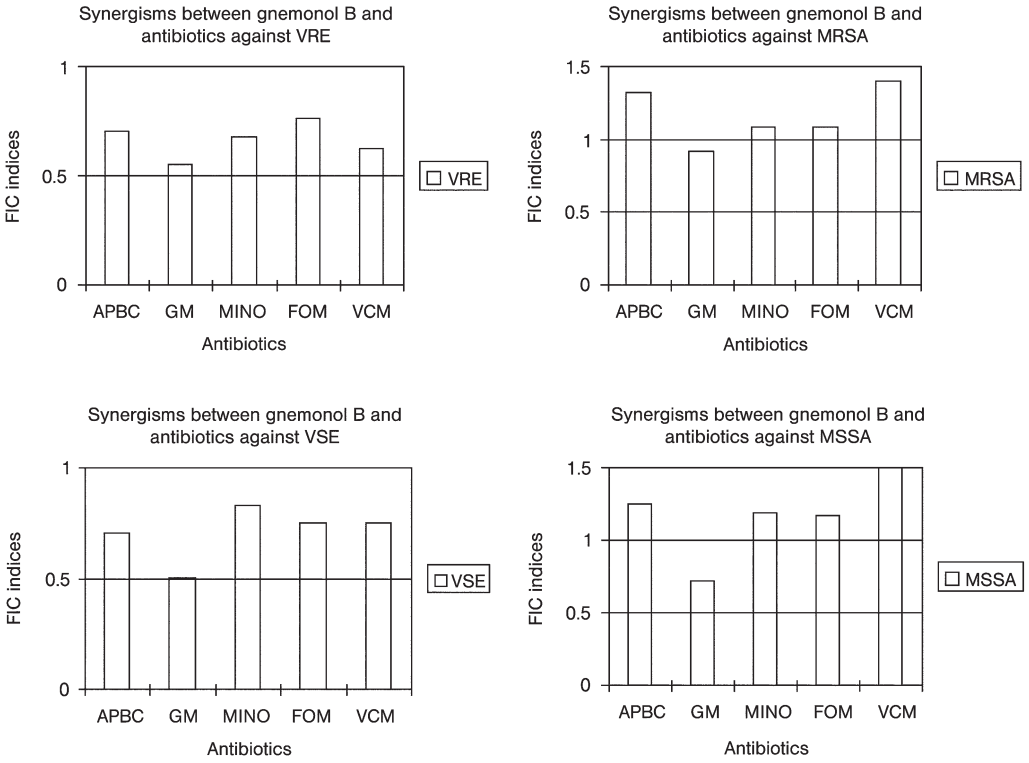


Fig. 7.10 Synergism between gnemonol B and antibiotics against vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-sensitive enterococci (VSE), and methicillin-sensitive *Staphylococcus aureus* (MSSA).

Gnemonol B exhibited a partial synergistic effect in combination with ampicillin, gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride against VRE. It also showed a partial synergism with gentamicin against MRSA. The average of FIC indices against VRE were calculated as 0.703 ± 0.105, 0.550 ± 0.068, 0.678 ± 0.106, 0.763 ± 0.155, and 0.624 ± 0.169. The FIC indices of gnemonol B with ampicillin, gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride against MRSA were also calculated as 0.708 ± 0.072, 0.501 ± 0.249, 0.833 ± 0.144, 0.750 ± 0.000, and 0.750 ± 0.000, respectively (Fig. 7.10).

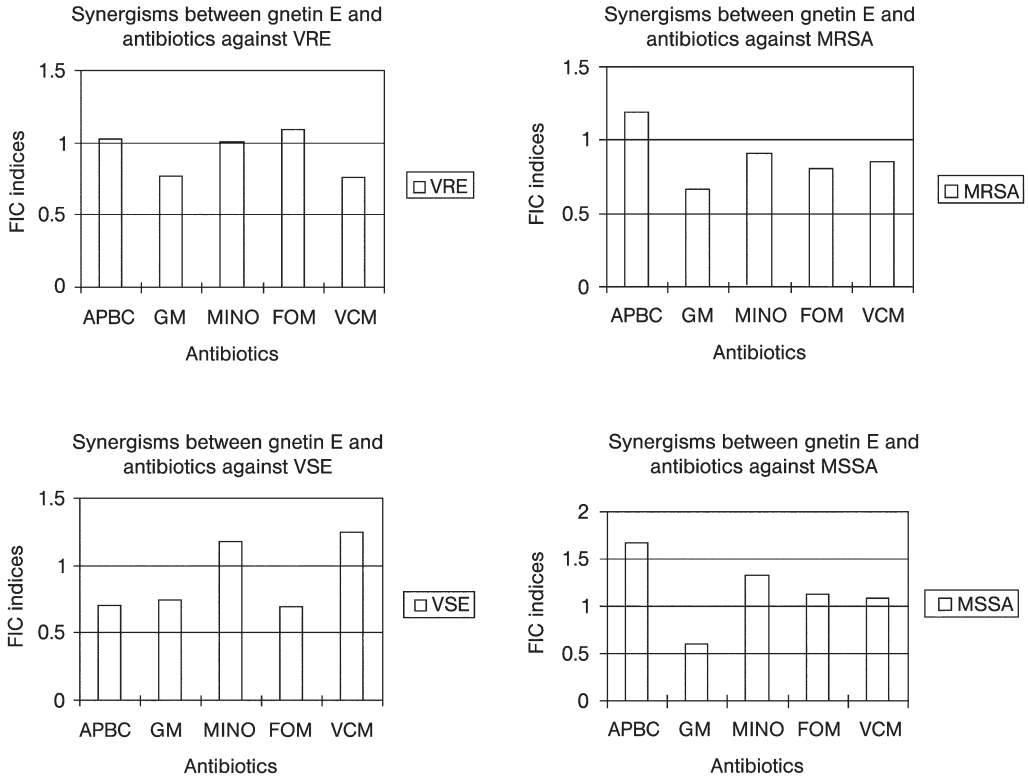


Fig. 7.11 Synergism between gnetin E and antibiotics against vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-sensitive enterococci (VSE), and methicillin-sensitive *Staphylococcus aureus* (MSSA).

In combination of gnemonol B with ampicillin, gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride, partial synergism against VSE was exhibited, and the FIC indices were calculated as 0.836 ± 0.284, 0.500 ± 0.108, 0.750 ± 0.000, 0.500 ± 0.108, and 0.792 ± 0.191, respectively.

Synergism of gnemonol B with gentamicin was observed against MSSA (FIC index 0.719 ± 0.248). FIC indices of the other antibiotics such as ampicillin, minocycline, fosfomycin, and vancomycin hydrochloride were calculated as 1.323 ± 0.332, 0.918 ± 0.306, 1.085 ± 0.124, 1.089 ± 0.121, and 1.403 ± 0.285 (Fig. 7.10).

Partial synergism of gnetin E with gentamicin and vancomycin hydrochloride against VRE, and of gnetin E with gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride against MRSA were observed, respectively. The FIC indices of gnetin E with gentamicin and vancomycin hydrochloride on VRE were 0.770 ± 0.229 and 0.746 ± 0.248, respectively. The FIC indices of gnetin E with ampicillin, gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride on MRSA were 1.195 ± 0.369, 0.667 ± 0.107, 0.913 ± 0.317, 0.809 ± 0.264, and 0.854 ± 0.203, respectively.

Partial synergism of gnetin E in combination with ampicillin, gentamicin, and fosfomycin against VSE were observed, and the FIC indices were 0.705 ± 0.261, 0.750 ± 0.000, and 0.698 ± 0.289.

Against MSSA, the FIC indices of gnetin E in combination with ampicillin, gentamicin, minocycline, fosfomycin, and vancomycin hydrochloride were 1.667 ± 0.577, 0.594 ± 0.054, 1.333 ± 0.144, 1.130 ± 0.117, and 1.083 ± 0.289 (Fig. 7.11).

7.9.5
Summary of Synergistic Effects Between the Test Compounds and the Commercial Antibiotics Against VRE and MRSA

Synergistic effects between the test compounds and the commercial antibiotics against VRE and MRSA are summarized in Table 7.9.

Synergism between sophoraflavanone G and vancomycin hydrochloride or fosfomycin against MRSA was observed. Synergism between calozeyloxanthone and vancomycin hydrochloride against VRE was observed. Synergism between α-mangostin and gentamicin against VRE, and α-mangostin and vancomycin hydrochloride against MRSA was also observed. The other test compounds possessed partial synergism except for the case of gnetin E (ampicillin against MRSA, and ampicillin or fosfomycin against VRE).

Synergism between phytoalexins or phytoncides and commercially available antibiotics could be used to decrease usage of antibiotics, contributing to the decrease of nosocomial infectious bacteria such as MRSA and VRE. The use of phytoalexins or phytoncides isolated from natural products could also be valuable for the prevention of infectious bacteria such as VRE and MRSA etc. No reports of bacteria resistant to antibacterial compounds isolated from the natural products were found. The use of antibiotics could also be decreased because of the partial synergism between the antibacterial compounds and the commercial antibiotics, which means that the detection ratio of the resistant bacteria would become lower.

Table 7.9 Synergisms between the antibacterial compounds and the commercially available antibiotics *in vitro* against VRE or MRSA.

FIC index (average)	***Ampicillin***	***Gentamicin***	***Minocycline***	***Fosfomycin***	***Vancomycin hydrochloride***
SFG (M)	0.73	0.6	nt	0.48	0.16
CZXT (V)	0.750	0.625	0.563	nt	0.453
α-M (VRE)	0.606	0.451	0.969	0.826	0.508
α-M (MRSA)	0.779	0.667	0.586	0.504	0.441
Gnemonol B (M)	0.708	0.501	0.833	0.750	0.750
Gnemonol B (V)	0.703	0.550	0.678	0.763	0.624
Genetin E (M)	1.195	0.667	0.913	0.809	0.854
Genetin E (V)	1.030	0.770	1.008	1.091	0.746

M, methicillin-resistant *S. aureus*; V, vancomycin-resistant enterococci; nt, not tested; SFG, sophoraflavanone G; CZXT, calozeyloxanthone; α-M, α-mangostin.

These findings suggest that phytoalexins or phytoncides alone or in combination with suitable antibiotics against VRE and MRSA might be useful in controlling VRE and MRSA infections.

Recently, many similar reports about the antibacterial activities of the compounds (phytoalexins and phytoncides etc.) isolated from tropical foods and plants have been published [30–44]. These research fields would be more progressive, and would contribute to the prevention of nosocomial infectious bacteria such as VRE and MRSA etc.

References

1 Emori, T.G., Gaynes, R.P. *Clin. Microb. Rev.* **1993**, *6*, 428–442.
2 Leclercq, R., Courvalin, P. *Clin. Microb. Rev.* **1997**, *24*, 545–556.
3 Murry, B. *Am. J. Med.* **1997**, *101*, 284–293.
4 Moellering, R.C. *Clin. Infect. Dis.* **1998**, *26*, 1196–1199.
5 Garner, J.S. *Infect. Control Hosp. Epidemiol.* **1996**, *17*, 53–80.
6 Slaughter, S., Hayden, M.K., Nathan, C., Hu, T.C., Rice, T., Van Voorhis, J., Matushek, M., Franklin, C., Weinstein, R.A. *Ann. Intern. Med.* **1996**, *125*, 448–456.
7 Montecalvo, M.A., Jarvis, W.R., Uman, J., Shay, D.V., Petrullo, C., Rodney, K., Gedris, C., Horowitz, H.W., Wormser, G.P. *Ann. Intern. Med.* **1999**, *131*, 269–272.
8 Nourse, C., Byrne, C., Murphy, H., Kaufman, M.E., Clarke, A., Butler, K. *Epidemiol. Infect.* **2000**, *124*, 53–59.
9 Warren, D.K., Nitin, A., Hill, C., Fraser, V.J., Kollef, M.H. *Infect. Control Hosp. Epidemiol.* **2004**, *25*, 99–104.
10 Calfee, D.P., Giannetta, E.T., Durbin, L.J., Germanson, T.P., Farr, B.M. *Clin. Infect. Dis.* **2003**, *37*, 326–332.
11 Shaikh, Z.H., Osting, C.A., Hanna, H.A., Arbuckle, R.B., Tarr, J.J., Raad, I.I. *J. Hosp. Infect.* **2002**, *51*, 52–58.
12 Montecalvo, M.A., Jarvis, W.R., Uman, J., Shay, D.K., Petrullo, C., Horowitz, H.W., Wormser, G.P. *Control Hosp. Epidemiol.* **2001**, *22*, 437–442.
13 Hanna, H., Umphrey, J., Tarrand, J., Mendoza, M., Raad, I. *Infect. Control Hosp. Epidemiol.* **2001**, *22*, 217–219.
14 Ostrowsky, B.E,, Sinkowitz-Cochran, R.Ll, Quirk, S.B., Jarvis, W.R. *Am. J. Infect. Control.* **2001**, *29*, 53–57.
15 Coolson, B. *Eur. J. Chemother.* **1995**, *78* (suppl. 3), 93–98.
16 Cox, R.A., Conquest, C., Mallaghan, C., Marples, R.R. *J. Hosp. Infect.* **1995**, *29*, 87–106.
17 Voss, A., Milatovic, D., Wallrauch-Schwarz, C., Rosdahl, V.T., Braveny, I. *Eur. J. Clin. Microbiol.* **1994**, *13*, 50–55.
18 Working Party Report. *J. Hosp. Infect.* **1998**, *39*, 253–255.
19 Kotilainen, P., Routamaa, M., Peltonen, R., Evesti, P., Eerola, E., Salmenlinna, S., Vuopio-Varkila, J., Rossi, T. *Arch. Intern. Med.* **2001**, *161*, 859–863.
20 Hori, S. *Nippon Rinsho* **2002**, *60*, 2144–2149 (In Japanese).
21 Sakagami, Y., Mimura, M., Kajimura, K., Yokoyama, H., Iinuma, M., Tanaka, T., Ohyama, M. *Lett. Appl. Microbiol.* **1998**, *27*, 98–100.
22 Sakagami, Y., Kajimura, K., Wijesinghe, W.M.N.M., Dharmaratne, H.R.W. *Planta Med.* **2002**, *68*, 543–545.
23 Sakagami, Y., Iinuma, M., Piyasena, K.G.N.P., Dharmaratne, H.R.W. *Phytomedicine* **2005**, *12*, 203–208.
24 Iliya, I. Tanaka, T, Iinuma, M., Ali, A., Furusawa, M., Nakaya, K., Shirataki, Y., Murata, J, Dornaedi, D. *Chem. Pharm. Bull.* **2002**, *50*, 796–801.
25 Iliya, I., Ali, Z., Tanaka, T., Iinuma, M., Furusawa, M., Nakaya, K., Murata, J., Dornaedi, D. *Helvetica Chim. Acta* **2002**, *85*, 2538–2546.
26 Goto, S, Yo, K., Kawakita, R., Kosakai, N., Mitsuhashi, S., Nishino, T., Ohsawa, N., Tanami, H. *Chemotherapy* **1981**, *29*, 76–79.
27 Sakagami, Y., Kajimura, K. *J. Hosp. Infect.* **2002**, *50*, 140–144.

28 Didry, N., Dubreuil, L., Pinkas, M. *Phytother. Res.* **1993**, *7*, 21–24.
29 Williamson, E.M. *Phytomedicine* **2001**, *8*, 401–409.
30 Iinuma, M., Tosa, H., Tanaka, T., Asai, F., Kobayashi, Y., Shimano, R., Miyauchi, K. *J. Pharm. Pharmacol.* **1996**, *48*, 861–865.
31 Iinuma, M., Tsuchiya, H., Sato, M., Yokoyama, J., Ohyama, M., Ohkawa, Y., Tanaka, T., Fujiwara, S., Fujii, T. *J. Pharm. Pharmacol.* **1994**, *46*, 892–895.
32 Fukai, T., Oku, Y., Hano, Y., Terada, S. *Planta Med.* **2004**, *70*, 685–687.
33 Palombo, E.A., Semple, S.J. *J. Basic Microbiol.* **2002**, *42*, 444–448.
34 Gibbons, S., Leimkugel, J., Oluwatuyi, M., Heinrich, M. *Phytother. Res.* **2003**, *17*, 274–275.
35 Fukai, T., Oku, Y., Hou, A.J., Yonekawa, M., Terada, S. *Phytomedicine* **2005**, *12*, 510–513.
36 Hur, J.M., Yang, C.H., Han, S.H., Lee, S.H., You, Y.O., Park, J.C., Kim, K.J. *Fitoterapia* **2004**, *75*, 603–605.
37 Aqil, F., Khan, M.S., Owais, M., Ahmad, I. *J. Basic Microbiol.* **2005**, *45*, 106–114.
38 Tanaka, H., Sato, M., Oh-Uchi, T., Yamaguchi, R., Etoh, H., Shimizu, H., Sako, M., Takeuchi, H. *Phytomedicine* **2004**, *11*, 331–337.
39 Sato, Y., Oketani, H., Yamada, T., Singyouchi, K., Ohtsubo, T., Kihara, M., Shibata, H., Higuti, T. *J. Pharm. Pharmacol.* **1997**, *49*, 1042–1044.
40 Nostro, A., Cellini, L., Di Bartolomeo, S., Di Campli, E., Grande, R., Cannatelli, M.A., Marzio, L., Alonzo, V. *Phytother. Res.* **2005**, *19*, 198–202.
41 Voravuthikunchai, S.P., Kitpipit, L. *Clin. Microbiol. Infect.* **2005**, *11*, 510–512.
42 Chomnawang, M.T., Surassmo ,S., Nukoolkarn, V.S., Gritsanapan, W. *J. Ethnopharmacol.* **2005**, *101*, 330–333.
43 Rukachaisirikul, V., Tadpetch, K., Watthanaphanit, A., Saengsanae, N., Phongpaichit, S. *J. Nat. Prod.* **2005**, *68*, 1218–1221.
44 Sukpondma, Y., Rukachaisirikul, V., Phongpaichit, S. *Chem. Pharm. Bull.* **2005**, *53*, 850–852.

8
Methods for Testing the Antimicrobial Activity of Extracts

Jenny M. Wilkinson

Summary

There is increasing interest in the use of plant extracts as therapeutic agents, particularly the capacity for these extracts to inhibit the growth of pathogenic microorganisms. In this chapter the main methods for the *in vitro* assessment of antimicrobial activity are discussed and the strengths and limitations of each method highlighted. Methods for the assessment of antibacterial, antifungal, antiviral, and antiparasitic activity are discussed with key issues illustrated by reference to the literature. The aim is to provide an overview of the available methods and to allow the reader to choose the method that best suits their needs.

8.1
Introduction

Over the past decade there has been an explosion of interest in the antimicrobial, particularly antibacterial and antifungal, activity of natural products [1–5]. This is driven by a number of factors including increasing antibiotic resistance and fear of development of even more infectious "superbugs," the impact of infectious diseases on mortality and morbidity, and increasing interest in "natural" therapies and a move to more self-care. Traditional communities also wish to retain their ethnopharmacological heritage and exploration of traditional treatments for a variety of diseases has the potential to empower these communities and improve both their health and economy. This is particularly important in developing nations where the use of conventional antibiotics may be limited due to cost or other factors. In addition these communities often have a rich tradition of use of herbal and other plant products for endemic infections; this serves as a starting point for researchers interested in finding treatments for these diseases.

Recommendations for the use of various natural products (e.g. essential oils, honey, plant extracts) for infectious diseases is widespread and appears in a number of popular and other easily obtainable texts [6–10]. However, despite these nu-

merous claims few products have been comprehensively evaluated for their antimicrobial activity.

One of the difficulties for researchers in this area has been the absence of a single validated and standardized method of testing plant extracts and, in general, methods used for *in vitro* testing of antimicrobial activity have been adopted from the testing of conventional pharmaceuticals. This has several limitations; for example, unlike conventional pharmaceuticals, natural products are complex mixes of tens or hundreds of compounds that may or may not act as expected in the test system. These constituents may also have limited solubility in the aqueous media that is the typical base of many assays. Further, there are no standardized methods for extraction or distillation of products, consequently, the exact composition of the extract being tested may be unknown with some researchers lacking access to funding or equipment to perform gas chromatography–mass spectroscopy (GC-MS) and other chemical analyses of the extracts they are screening.

Essential oils present additional difficulties in that they may interact with disposable laboratory plastics, rendering use of plastics impossible. For example the Australian native essential oil of *Backhousia citriodora* (lemon myrtle) has a high percentage of citral (~95% or higher [11]) and direct contact with the oil, or contact via oil volatiles, can turn standard laboratory plastics into a sticky mess (unpublished observations). As a result, all assays with this oil must be carried out in glass equipment – an additional expense that adds significantly to assay costs.

A survey of the published literature shows that there are a number of different methods used for the assessment of antimicrobial activity; however, there is no one method that is used by all researchers and no comprehensive study to determine which is the best method for *in vitro* assays. This chapter will describe the main methods of testing of antimicrobial activity in plant extracts and highlights the advantages and limitations of each method. Although several plants have been identified as having antibacterial or antifungal activity (e.g. cranberry juice, garlic cloves), the most widely used plant extracts for antibacterial and antifungal activity are the essential oils [12, 13], hence this discussion draws heavily on the essential oil literature.

An important consideration in this discussion is the cost (both financial and time) and need for specialized equipment to complete some assays. Investigations of antimicrobial activity of plant extracts, particular in the early stages of an investigation, may involve the screening of large numbers of extracts and/or large numbers of organisms, screening may also need to be carried out in the field or in locations where laboratory facilities are rudimentary. With these factors in mind there may not be one "best" method, rather a selection of good methods, each best suited to a different circumstance.

8.2 Antibacterial Assays

Perhaps the most common *in vitro* assay used for plant extracts is the assessment of antibacterial activity, with the majority of researchers using one of the three following assays: disk diffusion, agar dilution, or broth dilution/microdilution. These

methods are based on those described for standardized testing of antibiotics [14–17]; however several factors may affect the suitability of these methods for use with plant extracts. These factors include the type of organism being tested, concentration of inoculum, type of media (e.g. IsoSensitest versus nutrient agar) and nature of the extract being tested (pH, solubility) [18–21]. The methods can be used to simply determine whether or not antibacterial activity is present or can be used to calculate a minimum inhibitory concentration (MIC). Table 8.1 summarizes the limitations and advantages of these various methods.

Table 8.1 Comparison of strengths and limitations of various assays for antimicrobial activity.

Method	*Strengths*	*Limitations*
Antibacterial and antifungal assays		
Disk well diffusion	Low cost	Differential diffusion of extract components due to partitioning in the aqueous media
	Results available in 1–2 days	Inoculum size, presence of solubilizing agents, and incubation temperature can affect zone of inhibition
	Does not require specialized laboratory facilities	Volatile compounds can affect bacterial and fungal growth in closed environments
	Uses equipment and reagents readily available in a microbiology laboratory	Data is only collected at one or two time points
	Can be performed by most laboratory staff	
	Large numbers of samples can be screened	
	Results are quantifiable and can be compared statistically	
Agar dilution	Low cost	Hydrophobic extracts may separate out from the agar
	Does not require specialized laboratory facilities	Inoculum size, presence of solubilizing agents and incubation temperature can affect zone of inhibition
	Uses equipment and reagents readily available in a microbiology laboratory	Volatile compounds can affect bacterial and fungal growth in closed environments
	Can be performed by most laboratory staff	Data is only collected at one or two time points
		Use of scoring systems is open to subjectivity of the observer
		Some fungi are very slow growing

Table 8.1 (Continued)

Method	*Strengths*	*Limitations*
Broth dilution	Allows monitoring of activity over the duration	Essential oils may not remain in solution for the duration of the assay; emulsifiers and solvent may interfere with the accuracy of results
	More accurate representation of antibacterial activity	Labor and time-intensive if serial dilutions are used to determine cell counts
	Micro-broth methods can be used to screen large numbers of samples in a cost-effective manner	Highly colored extracts can interfere with colorimetric endpoints in microbroth methods
TLC-bioautography	Simultaneous fractionation and determination of bioactivity	Unsuitable where activity is due to component synergy
		Dependent on extraction method and TLC solvent used
Antiviral assays	Allows simultaneous assessment of cell toxicity with antiviral assay	Labor, time, and cost intensive
	Few methods available therefore comparability across studies is high	Requires access to cell culture and viral containment facilities
		Essential oils may not remain in solution for the duration of the assay
Antiparasitic assays	Methods are well documented	Labor, time, and cost intensive
	Some assays allow simultaneous assessment of cell toxicity	May require access to cell culture facilities
		Essential oils may not remain in solution for the duration of the assay

While the aforementioned methods are those most widely used for *in vitro* testing of plant extracts for antibacterial activity, other methods have also been used. For example, Garedew et al. [22] report on the use of a flow calorimetric method to assess antibacterial activity of honey and demonstrated better sensitivity than other methods and Pitner et al. [23] propose the use of high throughput systems that measure bacterial respiration via a fluorescent signal. However, the practicality of these methods for screening of plant extracts is yet to be determined. An additional method – thin-layer chromatography (TLC)–bioautography – allows for identification of bioactive fractions of extracts within a single assay.

Plant extracts are obtained via aqueous or solvent extraction of flowers, roots, or foliage or can be distilled, as an essential oil, from plant material; hence, there will be a range of solubility and other characteristics that affect assay outcome. These factors will be explored in the following sections. An additional group, the hydrosols (aqueous distillates), of a variety of plants have also gained a reputation as having antimicrobial, among other, activities [24]. However, several studies conducted in our laboratory have failed to show any antimicrobial activity in these plant extracts and hence they have not been discussed further [25, 26].

8.2.1 Semi-Solid Substrate Methods

Both the disk diffusion and agar dilution methods use bacteria grown on a solid agar base to test antibacterial activity. These methods are relatively quick, inexpensive, and do not require sophisticated laboratory equipment; however, they are not without drawbacks.

8.2.1.1 Disk Diffusion Method

The disk diffusion method (also known the zone of inhibition method) is probably the most widely used of all methods used for testing antibacterial activity. It uses only small amounts of the test substance (10–30 μL), can be completed by research staff with minimal training, and as such may be useful in field situations. The method involves the preparation of a Petri dish containing 15–25 mL agar, bacteria at a known concentration are then spread across the agar surface and allowed to establish. A paper disk (6 or 8 mm) containing a known volume of the test substance is then placed in the center of the agar and the dish incubated for 24 h or more. At this time the "cleared" zone (zone of inhibition) surrounding the disk is measured and compared with zones for standard antibiotics or literature values of isolated chemicals or similar extracts. Where the extract is viscous or a semi-solid (e.g. honey) a well can be created in the agar and the substance allowed to diffuse out of the well rather than away from a disk.

Data from these assays are typically presented as mean size of zone of inhibition (with or without standard deviation), although some authors employ a ranking system of +, ++, and +++ to indicate levels of activity. Few authors provide any statistical analysis of their data and levels of activity (slight, moderate, strong) are used without any reference to standardized criteria.

One of the major criticisms of this method is that it relies on the ability of the extract to diffuse through agar and any component of the extract that does diffuse away from the disk will create a concentration gradient, potentially creating a gradient of active antibacterial compounds. All of the antibacterial testing methods use an aqueous base for dispersion of the test substance, either via diffusion in agar or dispersion within nutrient broth, consequently assays using extracts with limited solubility in aqueous media (e.g. essential oils) may not reflect the true antibacterial activity. This has been investigated by Griffin [18] and Southwell et al.

[27] who have demonstrated that many terpenoids, due to their poor water solubility, will not diffuse through aqueous media and hence essential oils high in these terpenoids (e.g. linalool, linalyl acetate, *p*-cymene) will give a "false" result in these assays.

There is also no consensus on the best agar to use for these assays. Oxoid's IsoSensitest agar is the media of choice for conventional antibiotic susceptibility testing [14–16, 27], but several authors have noted that this may not be the case for plant extracts, particularly essential oils. Pauli and Kubeczka [28] found when testing eugenol that zone size varied according to agar used. Moon et al. [21] have also demonstrated differences in zone of inhibition size between IsoSensitest and nutrient agar and have also shown that these differences are not consistent across organisms or essential oil used. Smith et al. [29] found increased sensitivity (i.e. bigger zones of inhibition) when nutrient agar, rather than Difco brain heart infusion agar, was used for a range of methanol plant extracts. These authors also demonstrated that the size of the inoculum and temperature of incubation also affect zone size. Indeed these authors suggest that inoculum density is the single most important factor in the variability of zone size.

A further limitation that has not been directly addressed in the literature, but for which evidence exists, is inference in the assay from vapors liberated from the extract during incubation. This is unlikely to be a major consideration in aqueous or solvent extracts but may be a significant confounder in assays of essential oils. Inouye et al. [30] have shown that the volatile constituents of essentials oils can have a good antibacterial activity; we have also demonstrated this with essential oils from a range of Australian native plants and lavender. It is possible that the results of antibacterial assays completed using a closed Petri dish will reflect the combined actions of oil components diffusing through agar and exposure to gaseous components liberated from the oil. Which is responsible for the majority of the antibacterial activity is yet to be determined.

8.2.1.2 **Agar Dilution Method**

The agar dilution method is another relatively quick method that does not involve the use of sophisticated equipment. Any laboratory with facilities for basic microbiological work can use this method. In this method the test substance is incorporated at known concentrations into the agar and, once set, bacteria are applied to its surface. Replicate dishes can be set up with a range of concentrations of the test substance and by dividing the surface of the agar into wedges or squares, a number of bacterial species may be applied to a single dish. In this way, a large number of bacteria may be screened within a single assay run. The dishes are incubated for 24 h or more and the growth of the bacteria on the extract/agar mix is scored either as present/absent or a proportion of the control (e.g. 0, 25%, 50%, 75%, 100%).

A criticism of this method is that when a scoring system is used it is difficult to guarantee objectivity and to therefore compare one set of results with another.

This method suffers from several other limitations, including many that have been discussed previously: use of larger volumes of test substance than in other

methods, confounding antibacterial actions from volatiles, difficulty of achieving stable emulsions of essential oils in agar and restriction on the maximum concentration that can be used before the agar becomes too dilute to solidify properly. Perhaps the most frustrating of these is the difficulty of stably incorporating essential oils and other hydrophobic extracts into aqueous environments. This problem occurs not just in agar dilution assays but also in broth dilution and other antimicrobial assays. Many a researcher has thought they had incorporated their essential oil into nutrient broth or other media only to find that, on return to the experiment after an hour or so, the oil had separated out and was floating on top of the media. Griffin et al. [18] in their work on tea tree oil found that at concentrations above 2% v/v the oil separated from the agar substrate and was seen as droplets on the agar surface. The most commonly utilized method to overcome this problem is the use of surfactants such as Tween-20, Tween-80, and alkyl dimethyl betaine (ADB). Several authors have described the use of these products and the effect on antibacterial activity. The results of their studies show that surfactants can interfere with calculation of MIC values and the growth of some test organisms [31, 32], however it has also been demonstrated that it is possible to use very small quantities of Tween (<0.5% v/v) to emulsify the essential oil in media and thus avoid the effects on organism growth [18, 20]. Hammer et al. [31] also showed that inclusion of organic matter such as bovine serum albumin in the agar also affected the antibacterial activity of tea tree oil.

8.2.1.3 **Broth Dilution Methods**

Difficulties with partitioning of hydrophobic compounds in agar and a desire to more accurately monitor antibacterial activity over time has resulted in a move to broth dilution methods for testing of plant extracts. In this method, bacteria are grown in test-tubes in a liquid media in the presence of the test substance. At regular time intervals (e.g. every 10 min or every hour) a sample is removed and the bacterial count determined by serial dilution of the sample, subsequent incubation on agar and counting of colony-forming units. In contrast to the single data point (e.g. 24 h incubation) utilized in disk diffusion and agar dilution assays, the broth dilution method allows much finer evaluation of the antibacterial events over time and features such as recovery from the effects of the test substance and proportion of organisms killed at a given time point can be determined. However the method is also time and resource intensive and can be impractical where very large numbers of test substances are to be screened.

As with other testing methods incorporation of hydrophobic compounds and essential oils into the aqueous media is problematic, and as there is no solid phase to trap these compounds they rapidly separate from the media and form a layer across the surface of the media. For organisms sensitive to oxygen tension in the media this can present an additional problem as the oil can inhibit gaseous exchange. Tween or ethanol may be used to enhance incorporation into the aqueous media, however as previously discussed these compounds may interfere with the assay results. Work in our laboratory has shown that essential oils can be stably in-

corporated into broth using 0.02% Tween-80 and that broth dilution assays are more reliable and reproducible than either the disk/well diffusion or agar dilution methods [20].

Micro-broth methods have also been developed, which utilize microtiter plates, thus reducing the volume of extract needed, and have endpoints that can be determined spectrophotmetrically, either a measure of turbidity or use of a cell viability indicator (e.g. resazurin, methylthiazoldiphenyltetrazolium (MTT)) [33]. Mann and Markham [33] propose that the cell viability indicator is the best method of endpoint determination for essential oils as the oil/water interface may interfere with turbidity measures. While these micro-broth methods generally work well for plant extracts, problems arise when the extract is heavily colored as this can interfere with the measurement of the indicator chemical. Further, as these methods use plastic microtiter plates, essential oils that have a solvent action on plastics (e.g. *Letospermum petersonii*, *Backhousia citriodora*) cannot be used. We have also demonstrated that the addition of essential oils to media changes its pH and this may contribute to the observed antibacterial activity [19]; this might be expected to be more significant in small volumes, for example in the micro-broth method. Whether other plant extracts will also have the effect is unknown. Micro-broth methods are also less time and resources intensive than other broth methods as the need for multiple serial dilutions to determine bacterial count is eliminated.

8.2.1.4 **Thin-Layer Chromatography–Bioautography**

While the methods above are used to test whole extracts or extracts fractionated at another time there is an increasing interest in bioassay-guided fractionation, where the separation of extracts into fractions is completed simultaneously with identification of bioactivity. In this method TLC is performed using crude extracts, extract fractions, or whole essential oils. The developed TLC plate is then sprayed with, or dipped into, a bacterial or fungal suspension (direct bioautography) or overlain with agar and the agar seeded with the microorganism (overlay bioautography) [34–37]. The latter method has been particularly used for determining the activity of extract against yeasts such as *Candida albicans*, however Masoko and Eloff [38] suggest that use of fresh cultures of yeasts and shorter incubation times eliminated the previously reported difficulties of using the direct method with yeasts [39]. Following incubation zones of inhibition are observed, either unaided or following development with compounds such as MTT, around those compounds with antifungal or antibacterial activity.

This method has been used to screen a range of crude and solvent-prepared extracts with the activity observed dependent on both the method of extraction and solvents used in the TLC process [40–44]. While this method has the advantage of combining both separation of extract constituents and simultaneous identification of those fractions with bioactivity, it is not a suitable method for detecting activity that is a product of synergy between two or more compounds. Further, the results will be affected by the breakdown or alteration of compounds during the fractionation phase.

8.3 Antifungal Assays

Antifungal assays are regularly used to determine whether plants extracts will have potential to treat human fungal infections (e.g. tinea) or have use in agricultural/horticultural applications. In general these assays are quick, low cost, and do not involve access to specialist equipment. Activity of plant extracts against the yeast candida is typically assessed using the disk or well diffusion methods described above, and many studies report anti-candida activity with antibacterial activity rather than with activity against fungi for this reason (see, for example, refs [45–48]). Activity against filamentous fungi can be evaluated in well diffusion, agar dilution, and broth/micro-broth methods with many of the same limitations and advantages as previously discussed for antibacterial assays [49, 50].

When the well diffusion and disk diffusion techniques are used, fungal plugs are removed from an actively growing colony and placed at a predetermined distance (typically 2 cm) from the center of an agar dish. A well is then bored in the center of the agar and test substance added to the well, or the test substance is added to a paper disk and the disk placed in the center of the agar. (The specific agar to be used, and temperature and time of incubation, will be determined by the fungi to be used.) The growth of the fungi is monitored and any inhibition of mycelial growth noted. This inhibition of growth is then expressed as a percentage of the growth of control colonies. In the agar dilution method (also known as the poison food technique) the test substance is incorporated into the agar substrate and then a sample of actively growing fungus is placed at the center of the plate. The radial growth of the fungus after an appropriate time, depending on the growth characteristics of the fungus, is then measured and compared with control samples. Sridhar et al. [44] used this method to show the activity of essential oils against a range of fungi of agricultural and medical importance.

Alternatively a fungal cell suspension may be inoculated onto the plate and the MIC determined by the lowest concentration of test substance that prevents visible fungal growth [51]. Antisporulation activity can be assessed by using scanning electron microscopy [52], while effects on conidium germination can be evaluated by exposing the conidia to the test substance and subsequently counting the number of conidia with germ tubes equal to 1–1.5 times conidium length [53]. Additional observations of germinated conidia over a set period will also allow evaluation of the effect of the plant extract on germ tube growth.

Inouye and co-workers have investigated the susceptibility of fungi to several essential oils and have shown that MIC values can be calculated using a range of methods [50, 52, 54, 55] . Most significantly, they have shown that when assays are done under closed conditions (i.e. the Petri dish is sealed) the MICs are significantly lower than when performed under open conditions [50]. The action of essential oil and plant extract volatiles on fungal growth has been demonstrated for a range of fungi [55–57] and has important implications for the screening of plant extracts for antifungal activity. Results in these assays will depend not only on the antifungal activity mediated by direct contact with the test substance but also on the vol-

ume of the experimental chamber and whether it is open or closed (and hence the presence and concentration of extract or oil volatiles). The method for evaluating the antifungal activity of extract volatiles is straightforward and involves the placement of a paper disk with test substance on the inverted lid of a Petri dish and subsequent evaluation of fungal growth; however, this is rarely considered in antifungal screening assays. Given the impact that volatiles can have on fungal growth it is recommended that this be included as a standard part of antifungal assessment of plant extracts.

Inouye et al. [50] also showed that the inclusion of Tween-80 resulted in weaker bioactivity in agar dilution assays and the size of the original fungal inoculum had a significant effect with larger inoculums being more resistant to antifungal effects. Shahi et al. [58] in their study of the antifungal activity of essential oils found that the antifungal response was altered by modifying the pH of the fungal growth media. As the media pH become more alkaline the eucalyptus essential oils had a greater inhibitory effect on the fungi (*Trichophyton* spp., *Microsporum* spp, and *Epidermophyton* spp.).

8.4 *In Vivo* Assessment of Antibacterial and Antifungal Activity

The preceding discussion clearly demonstrates the similarity in methods used for *in vitro* antibacterial and antifungal assays of plant extracts and there are many papers in the literature using one of more of the methods. Much of this literature is focussed towards screening of traditional remedies for potential therapeutic agents [4, 5, 47], food preservation [59–61], or investigations of mechanisms of action [33, 45, 62, 63]. A smaller number of research groups have moved beyond the *in vitro* environment and are investigating the *in vivo* efficacy of those extracts that show promise in the laboratory. This is a more complex and costly activity as not only does the activity against the microorganisms need to be evaluated, there must also be consideration of mammalian cell toxicity and allergic reactions [64]. To date most *in vivo* testing of plant extracts has involved the use of essential oils against human skin infections, particularly fungal infections, and testing of extracts follow standard clinical trial protocols. Tea tree oil has been evaluated for use in athletes' foot [65, 66] with equivocal results, PolyToxinol™ (a mix of various essential oils) has shown promise against chronic methicillin-resistant *Staphylococcus aureus* (MRSA) osteomyelitis, and essential oil-containing mouthwashes have demonstrated efficacy against oral bacteria [67, 68]. Perhaps the plant extract best known for its *in vivo* antibacterial activity is honey, with a large number of studies demonstrating *in vivo* activity [69–72].

It is important to note here that demonstrated activity *in vitro* does not always translate to activity *in vivo*. The best example of this is tea tree oil, which has been shown to have excellent activity *in vitro* against the fungi responsible for various tineas (MIC 0.004–0.06%) [49] yet the results from clinical trials have been far from conclusive [65, 66]. This illustrates the caution with which researchers should view

results from *in vitro* assays and reinforces the need for clinical trials of plant extracts that show therapeutic promise.

8.5 Methods for Assessing Antiviral Activity

In addition to antibacterial and antifungal activity, researchers are also investigating the use of plant extracts for antiviral activity; of particular interest is activity against herpes simplex virus (HSV), human immunodeficiency virus (HIV), and hepatitis C virus (HCV). Standard cytopathic assays are used to determine antiviral activity with activity both pre- and post-infection evaluated. As these assays are performed in an aqueous environment the problems of solubility that have been discussed at length previously are also an issue in these assays. These assays also require expertise in cell culture and appropriate laboratory containment facilities for working with viruses; these two features make these assays more expensive and labor intensive than other assays. However as viruses require a cell host this assay has the added benefit of being able to assess cell toxicity of the test substance as part of the antiviral assay protocol. This means that those extracts with significant cell toxicity, and therefore little potential for use, can be eliminated from investigations prior to *in vivo* testing.

Abad et al. [73] tested 10 extracts (both aqueous and ethanol) and demonstrated that aqueous extracts of five plants showed activity against HSV-1 and vesicular stomatitis virus (VSV) with one extract showing activity against poliovirus. These authors suggest that antiviral activity is more likely to be found in aqueous rather than ethanol extracts; this is in contrast to antibacterial and antifungal assays where activity is more commonly seen in solvent extracts and essential oils. However, other studies have identified activity in both aqueous and solvent (ethanol or methanol) extracts of a wide range of plants against the hepatitis C virus [74], HSV-1, VSV [75, 76], and human parainfluenza virus type 2 (HPIV-2) [77]. Few plant extracts/essential oils have been shown to demonstrate antiviral activity *in vivo* [78, 79] with work by Nawawi et al. [76] showing that, as with other *in vitro* assays, activity *in vitro* is not always matched by a similar level of activity *in vivo*.

8.6 Screening of Plant Extracts for Antiparasitic Activity

Parasitic infections are a major public health issue in many parts of the world, causing significant morbidity and mortality, and increasing resistance to the standard treatments for these infections has led to interest in the identification of plant extracts with antiparasitic activity [80, 81]. Upcroft and Upcroft [81] describe the main drug susceptibility methods: essentially the parasite is incubated in the presence of test substance in either a test-tube or microtiter plate and cell counts determined at preset time intervals. Results are then reported as 50% inhibitory concen-

trations (IC_{50}), minimum lethal concentration (MLC), or graphed as a percentage of controls over the length of the incubation period. As with other antimicrobial assays the aqueous environment used in assays for antiparasitic activity can pose difficulties and the need for repeated cell counts makes the assay labor intensive. Microtiter plate methods are less time consuming but have high variability in terms of the gaseous environment in each well, important for anaerobic protozoa, and they cannot be used with essential oils that "eat" plastic. Evaluation of extracts against intracellular parasites (e.g. *Leishmania* and *Plasmodium*) also requires access to an appropriate host cell line, cell culture facilities, and staff with expertise in cell culture.

Despite these difficulties, a large number of plant extracts have been tested against *Leishmania*, *Giardia lamblia*, *Trypanosoma* spp., and *Plasmodium* spp. [82–84], and studies of this nature are regularly published in *Phytotherapy Research* and *Journal of Ethnopharmacology*. A summary of these studies can be found in Saxena et al. [85]. Interestingly, most of the work on antiparasitic activity of plant extracts, and also antiviral activity, has used aqueous and ethanol/methanol extracts of plant parts, with few studies involving essential oils. Why this is the case is unknown, but it may be related to difficulties associated with solubility or to the types of plant products traditionally used for parasitic and viral infections. Perhaps this traditional use reflects the fact that viral and parasitic infections tend to be internal and therefore require an ingestible, easily produced remedy (essential oils are rarely used internally due to toxicity and are produced via steam distillation). We have completed some preliminary studies of the effects of lavender essential oils against *Giardia*, *Hexamita*, and *Trichomonas* and are able to demonstrate excellent antiparasitic activity [25].

8.7 Conclusions

This chapter outlines the main methods used in the evaluation of antimicrobial activity of plant extracts; each method has advantages and limitations and all have been widely cited in the literature. The question of which is the best one to use is essentially unanswerable as preferred methods depend on a variety factors including access to specialized equipment and facilities, the number of samples to be screened and the nature of the plant extract (e.g. volume, extract versus essential oil, chemical composition). For large-scale screening of extracts for antibacterial and antifungal activity disk and agar diffusion methods offer a fast, cost-effective, low-tech, and generally reliable method of sorting those extracts worthy of further investigation from those unlikely to be of value. Broth dilution methods provide more information but are more time and labor intensive and are best used as a follow-up to a large-scale screening of plant extracts. Antiviral and antiparasitic assays are the most time and labor intensive of the *in vitro* antimicrobial testing methods and often require access to cell culture or other specialized laboratory facilities. These are used less frequently than antibacterial and antifungal assays.

Despite the limitations of many of the assay techniques, there is a vast amount of good data demonstrating that some plant extracts possess strong to excellent antimicrobial activity. The next step is to continue this work into the *in vivo* environment and to evaluate the activity of these extracts in the treatment of infectious disease. These extracts and essential oils have enormous potential, a potential we are only just starting to realize.

References

1 Cowan, M.M. *Clin. Microbiol. Rev.* **1999**, *12*, 564–582.
2 Deans, S.G., Waterman, P.G. Biological activity of volatile oils. In *Volatile Oil Crops; their Biology, Biochemistry and Production*, Hay, R.K.M., Waterman, P.G. (eds). Longman Scientific & Technical, Essex, **1993**, pp. 97–111.
3 Hammer, K.A., Carson, C.F., Riley, T.V. *J. Appl. Microbiol.* **1999**, *86*, 985–990.
4 Pieroni, A., Quave, C.L., Villanelli, M.L., Mangino, P., Sabbatini, G., Santini, L., Boccetti, T., Profili, M., Ciccioli, T., Rampa, L.G., Antonini, G., Girolamini, C., Cecchi, M., Tamasi, M. *J. Ethnopharmacol.* **2004**, *91*, 331–334.
5 Shahidi Bonjar, G.H., Aghighi, S., Karimi Nik, A. *J. Biol. Sci.* **2004**, *4*, 405–412.
6 Chevallier, A. *Encyclopedia of Medicinal Plants*. Dorling Kindersley, Sydney, **2001**.
7 Gattefosse, R.-M. *Gattefosse's Aromatherapy*. C W Daniel Co., Essex, **1995**.
8 Lawless, J. *The Encyclopedia of Essential Oils*. Element, Melbourne, **1992**.
9 Rotblatt, M., Ziment, I. *Evidence-Based Herbal Medicine*. Hanley & Belfus, Philadelphia, **2002**.
10 Sullivan, K. *Natural Ways to Natural Health. Natural Home Remedies*. Time Life Books, Virginia, **1997**.
11 Brophy, J., Goldsack, R., Fookes, C., Forester, P. *J. Essential Oil Res.* **1995**, *7*, 237–254.
12 Fetrow, C.W., Avila, J.R. *The Complete Guide to Herbal Medicine*. Springhouse, USA, **2000**.
13 Ulbricht, C.E., Basch, E.M. *Natural Standard Herb & Supplement Reference. Evidence-Based Clinical Reviews*. Elsevier Mosby, New York, **2005**.
14 Andrews, J., Walker, R., King, A. *J. Antimicrob. Chemother.* **2002**, *50*, 479–786.
15 Andrews, J.M. *J. Antimicrob. Chemother.* **2001**, *48*, 29–42.
16 Andrews, J.M. *J. Antimicrob. Chemother.* **2001**, *48*, 43–57.
17 Barry, A.L., Craig, W.A., Nadler, H., Reller, L.B., Sanders, C.C., Swenson, J.M. *Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline*. NCCLS, Pennsylvania, **1999**.
18 Griffin, S. Aspects of antimicrobial activity of terpenoids and the relationship to their molecular structure. PhD thesis, University of Western Sydney, Sydney, Australia, **2000**.
19 Hood, J.R., Cavanagh, H.M.A. Wilkinson, J.M. *Phytother. Res.* **2004**, *18*, 947–949.
20 Hood, J.R., Wilkinson, J.M., Cavanagh, H.M.A. *J. Essential Oils Res.* **2003**, 15, 428–433.
21 Moon, T., Cavanagh, H.M.A., Wilkinson, J.M. *J. Essential Oils Res.* **2006** (in press).
22 Garedew, A., Schnmolz, E., Lamprecht, I. *Thermochim. Acta* **2004**, *415*, 99–106.
23 Pitner, J.B., Timmins, M.R., Kashdan, M., Nagar, M. Stitt, D.T. High-throughput assay system for the discovery of antibacterial drugs. In *AAPS Proceedings*, DB Biosciences. **2000**.
24 Catty, S. Hydrosols. *The Next Aromatherapy*. Healing Arts Press, Vermont. **2001**.
25 Cavanagh, H.M.A. Wilkinson, J.M. *Bioactivity of Lavandula Essential Oils, Hydrosols and Plant Extracts*. RIRDC, Canberra, **2005**.
26 Wilkinson, J.M., Hipwell, M., Ryan, T. Cavanagh, H.M.A. *J. Agric. Food Chem.* **2003**, *51*, 76–81.
27 Southwell, I.A., Hayes, A.J., Markham, J., Leach, D.N. *Acta Horticult.* **1993**, *344*, 256–265.

28 Pauli, A., Kubeczka, K.-H. Evaluation of inhibtory data of essential oil constituents obtained with different microbiological testing methods. In *Essential Oils: Basic and Applied Research, Proceedings of the 27th International Symposium on Essential Oils*, Franz, C., Mathe, A., Buchbauer, G. (eds). Allured Publishing Corporation, Carol Stream, **1996**, pp. 33–36.

29 Smith, R.A., Calviello, C.M., DerManderosian, A., Palmer, M.E. *Pharmaceut. Biol.* **2000**, *38*, 25–29.

30 Inouye, S., Takizawa, T., Yamaguchi, H. *J. Antimicrob. Chemother.* **2001**, *47*, 565–573.

31 Hammer, K.A., Carson, C.F., Riley, T.V. *J. Appl. Microbiol.* **1999**, *86*, 446–452.

32 Remmel, A., Bouchikhi, T., Tantaoui-Elaraki, A. Ettayebi, M. *J. Pharm. Belg.* **1993**, *48*, 352–356.

33 Mann, C.M., Markham, J.L. *J. Appl. Microbiol.* **1998**, *84*, 538–544.

34 Hormas, A.L., Fuchs, A. *J. Chromatogr.* **1970**, *51*, 325–327.

35 Rahalison, L., Hamburger, M., Haostettmann, K., Monod, M., Frenk, E. *Phytochem. Anal.* **1991**, *2*, 199–203.

36 Hamburger, M.O., Cordell, G.A. *J. Nat. Prod.* **1987**, *50*, 19–22.

37 Choma, I. *LC-GC Europe* **2005**, *18*, 482–488.

38 Masoko, P., Eloff, J.N. *Afr. J. Biotechnol.* **2005**, *4*, 1425–1431.

39 Hostettmann, K., Marston, A. *Pure Appl. Chem.* **1994**, *66*, 2231–2234.

40 Nostro, A., Germano, M.P., D'Angelo, V., Marino, A., Cannatelli, M.A. *Lett. Appl. Microbiol.* **2000**, *30*, 379–384.

41 Diallo, D., Marston, A., Terreaux, C., Toure, Y., Smestad Paulsen, B., Hostettmann, K. *Phytother. Res.* **2001**, *15*, 401–406.

42 Nakamura, C.V., Ueda-Nakamura, T., Bando, E., Melo, A.F.N., Cortez, D.A.G., Filho, B.P.D. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro* **1999**, *94*, 675–678.

43 Jassbia, A.R., Zamanizadehnajaria, S., Azarb, P.A., Tahara, S. *Z. Naturforsch.* **2002**, *57c*, 1016–1021.

44 Sridhar, S., R.R, Rajagopal, R.V., Rajavel, R., Masilamani, S., Narasimhan, S. *J. Agric. Food Chem.* **2003**, *51*, 7596–7599.

45 Haraguchi, H., Kataoka, S., Okamoto, S., Hanafi, M., Shibata, K. *Phytother. Res.* **1999**, *13*, 151–156.

46 Iscan, G., Kirimer, N., Kurkcuoglu, M., Husnu Can Baser, K. Demirci, F. *J. Agric. Food Chem.* **2002**, *50*, 3943–3946.

47 Rauha, J.-P., Remes, S., Heinonen, M., Hopia, A., Kahkonen, M., Kujala, T., Pihlaja, K., Vuorela, H., Vuorela, P. *Int. J. Food Microbiol.* **2000**, *56*, 3–12.

48 Wilkinson, J.M., Cavanagh, H.M.A. *Phytother. Res.* **2005**, *19*, 643–646.

49 Hammer, K.A., Carson, C.F., Riley, T.V. *J. Antimicrob. Chemother.* **2002**, *50*, 159–199.

50 Inouye, S., Tsuruoka, T., Uchida, K., Yamaguchi, H. *Microbiol. Immunol.* **2001**, *43*, 201–208.

51 de Aquino Lemos, J., Passos, X.S., de Fatima Lisboa Fernandes, O., de Paula, J.R., Ferri, P.H., e Souza, L.K.H., de Aquino Lemos, A., de Rosario Rodrigues Silva, M. *Mem. Inst. Oswaldo Cruz* **2005**, *100*, 55–58.

52 Inouye, S., Watanabe, M., Nishiyama, Y., Takeo, K., Akao, M., Yamaguchi, H. *Mycoses* **1998**, *41*, 403–410.

53 Antonov, A., Stewart, A., Walter, M. Inhibition of condium germination and mycelial growth of *Botrytis cinerea* by natural products. In *50th Conference Proceeding of the New Zealand Plant Protection Society*, **1997**, pp. 159–164.

54 Inouye, S., Tsuruoka, T., Watanabe, M., Takeo, K., Akao, M., Nishiyama, Y., Yamaguchi, H. *Mycoses* **2000**, *43*, 17–26.

55 Inouye, S., Uchida, K., Yamaguchi, H. *Mycoses* **2001**, *44*, 99–107.

56 Edris, A.E., Farrag, E.S. *Nahrung/Food* **2003**, *2*, 117–121.

57 Inouye, S., Uchida, K., Yamaguchi, H., Miyara, T., Gomi, S., Amano, M. *J. Essential Oils Res.* **2001**, *13*, 69–72.

58 Shahi, S.K., Shukla, A.C., Dikshit, A. *Curr. Sci.* **1999**, *77*, 703–706.

59 Agboola, S.O., Radovanovic-Tesic, M. *Lebensm.-Wiss. u.-Technol.* **2002**, *35*, 575–583.

60 Lean, L.P., Mohamed, S. *J. Sci. Food Agric.* **1999**, *79*, 1817–1822.

61 Lis-Balchin, M., Deans, S.G. *Phytother. Res.* **1998**, *12*, 472–475.

62 Cox, S.D., Mann, C.M., Markham, J.L., Bell, H.C., Gustafsson, J.E., Warmington, J.R. Wyllie, S.G. *J. Appl. Microbiol.* **2000**, *88*, 170–175.

63 Kubo, I., Fujita, K., Hwa Lee, S. *J. Agric. Food Chem.* **2001**, *49*, 1607–1611.

64 Matura, M., Slkold, M., Borje, A., Andersen, K.E., Bruze, M., Frosch, P., Goosssens, A., Johansen, J.D., Svedman, C., White, I.R., Karlberg, A. *Contact Dermatitis* **2005**, *52*, 320–328.

65 Satchell, A.C., Saurajen, A., Bell, C., Barnetson, R.S. *Aust. J. Dermatol.* **2002**, *45*, 175–178.

66 van Heerden, P., Niazov, S., Webb, A., Carson, C., Hammer, K., Riley, T., Parsons, R., Tinley, P. *Aust. J. Pediatr. Med.* **2001**, *35*, 103–106.

67 Claffey, N. *J. Clin. Periodontol.* **2003**, *30*, 22–24.

68 Pan, P., Barnett, M.L., Coelho, J., Brogdon, C., Finnegan, M.B. *J. Clin. Peridontol.* **2000**, *27*, 256–261.

69 Cooper, R.A., Molan, P.C. *J. Wound Care* **1999**, *8*, 161–164.

70 Dunford, C., Cooper, R., Molan, P., White, R. *Nurs. Stand.* **2000**, *15*, 63–68.

71 Moore, O.A., Smith, L.A., Campbell, F., Seers, K., McQuay, H.J., Moore, R.A. *BMC Compl. Altern. Med.* http://www.biomedcentral.com/1472–6882/1/2. **2001**.

72 Natarajan, S., Williamson, D., Grey, J., Harding, K.G., Cooper, R.A. *J. Dermatol. Treatment* **2001**, *12*, 13–36.

73 Abad, M.J., Guerra, J.A., Bermejo, P., Irurzun, A. Carrasco, L. *Phytother. Res.* **2000**, *14*, 604–607.

74 Hussein, G., Miyashiro, H., Nakamura, N., Hattori, M., Kakiuchi, N., Shimotohno, K. *Phytother. Res.* **2000**, *14*, 510–516.

75 Abad, M.J., Bermejo, P., Palomino, S.S., Chiriboga, X., Carrasco, L. *Phytother. Res.* **1999**, *13*, 142–146.

76 Nawawi, A., Nakamura, N., Hattori, M., Kurokawa, M., Shiraki, K. *Phytother. Res.* **1999**, *13*, 37–41.

77 Karagoz, A., Onay, E., Arda, N., Kuru, A. *Phytother. Res.* **2003**, *17*, 560–562.

78 Carson, C.F., Ashton, L., Dry, L., Smith, D.W., Riley, T.V. J. *Antimicrob. Chemother.* **2001**, *48*, 445–458.

79 Hirobe, C., Furukawa, M., Fukuhara, N., Shimotsuura, Y. *Acupuncture Electro-Ther. Res.* **2001**, *26*, 111.

80 Rossignol, J.-F., *Curr. Opin. Infect. Dis.* **1998**, *11*, 597–600.

81 Upcroft, J., Upcroft, P. *Antimicrob. Agents Chemother.* **2001**, *45*, 1810–1814.

82 Asres, A., Bucar, F., Knauder, E., Yardley, V., Kendrick, H., Croft, S.L. *Phytother. Res.* **2001**, *15*, 613–617.

83 Tripathi, D.M., Gupta, N., Lakshmi, V., Saxena, K.C., Agrawal, A.K. *Phytother. Res.* **1999**, *13*, 561–565.

84 Waechter, A.-I., Cave, A., Hocquemiller, R., Bories, C., Munoz, V., Fournet, A. *Phytother. Res.* **1999**, *13*, 175–177.

85 Saxena, S., Pant, N., Jain, D.C., Bhakuni, R.S. *Curr. Sci.* **2003**, *85*, 1314–1329.

9
Targeted Screening of Bioactive Plant Extracts and Phytocompounds Against Problematic Groups of Multidrug-Resistant Bacteria

Farrukh Aqil, Iqbal Ahmad, and Mohammad Owais

Summary

The use of the medicinal plants in the treatment of human diseases is an age-old practise in traditional systems of medicine throughout the world. Medicinal plants are an important source of diverse bioactive and therapeutic compounds, and the recent increase in the numbers of multidrug-resistant (MDR) bacteria has triggered immense interest in new drugs or preparations from natural sources, including plants. Particularly problematic groups of MDR bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), β-lactamase-producing enteric bacteria (*E. coli*, *Salmonella*, *Klebsiella*, *Shigella* spp.) and other MDR *Pseudomonas* spp., *Campylobacter* spp., and *Mycobacterium tuberculosis*. Excessive and indiscriminate use of antibiotics has led to the development of such drug-resistant bacteria both in hospitals and communities all over the world.

Resistance to most commonly used antibiotics, including β-lactam antibiotics and newer synthetic fast-acting fluoroquinolone, is on the rise. Bacteria develop resistance through various mechanisms, encoded by chromosomes, plasmids, and transposons.

Considerable work has been done on the antibacterial activity of plant extracts and phytocompounds. In some cases the mode of action of phytocompounds has been documented. Considering the various mechanisms of drug resistance present in bacteria, the specific activity of plant extracts/compounds may help in combating MDR bacteria. Such novel activity includes (1) MDR pump inhibition activity, (2) inhibition of β-lactamase production or activity, (3) anti-R-plasmid activity (interference with plasmid physiology), (4) synergy of phytocompounds with antibiotics, (5) targeting virulence and pathogenicity of bacteria, and (6) gene transfer mechanisms. Some of these approaches have already been attempted by researchers, while other suitable strategies and methods have to be employed by the scientists and pharmaceutical company involved in screening new antimicrobials from medicinal plants. Careful selection of potential medicinal plants and intelligent design of the test systems is the key to a successful screening outcome.

9.1 Introduction

Infectious diseases are the world's leading cause of premature death, killing almost 50 000 people every day. An increase in antibiotic-resistant bacteria is threatening the human population with a recurrence of infectious diseases (e.g. tuberculosis and cholera) that were once thought to be under control, at least in developed countries [1]. The presence of resistant genes on bacterial plasmids and transposons has played a further important role in the dissemination of drug resistance among bacterial populations [2]. In recent years multiple drug resistance has been commonly reported in the members of the family *Enterobacteriaceae*, especially *E. coli*, *Shigella*, *Salmonella*, and other human pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Campylobacter*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, etc. from all over the world. These multidrug-resistant (MDR) bacteria have also created special problems in treating infections in patients with cancer and AIDS.

Pathogenic bacteria develop resistance to various antibiotics by mutation or acquisition of new genetic markers through various mode of gene transfer [3]. The incidence of several MDR bacteria is on the rise both in hospitals and in communities. There is therefore an urgent need for a holistic targeted approach for screening to search for new antimicrobials from natural sources such as medicinal plants that promise efficacy alone or in combination with antibiotics against problematic MDR bacteria. In this present chapter we review the available information on various possible approaches for screening against MDR bacteria. The concepts and progress made in this area are discussed.

9.1.1 Multiple Antibiotic Resistance in Bacteria

Most of the widely used antibacterial drugs have specific target sites in the physiological processes of microbial cells, which include (1) inhibition of cell wall synthesis, (2) inhibition of protein and nucleic acid synthesis, (3) inhibition of enzyme activity (Fig. 9.1).

Since the introduction of antibiotics into clinical use in the mid 1940s, microorganisms have shown a remarkable ability to protect themselves by developing antibiotic resistance through different mechanisms. The major genetic mechanism for antibiotic resistance is mainly through mutation or acquisition of new gene(s) through genetic exchange mechanisms, like conjugation, transduction, and transformation. Both chromosomal and plasmid-encoded genes are important in the development and dissemination of antibiotic resistance genes. Various antibiotic resistance mechanisms are known in bacteria but the major mechanisms include destruction or modification of antibiotics (e.g. production of β-lactamases and aminoglycosides modifying enzymes), prevention of access to the target (e.g. alteration of permeability), and alteration of the target site. The mechanism of plasmid-encoded resistance is usually quite distinct from the mechanisms observed in

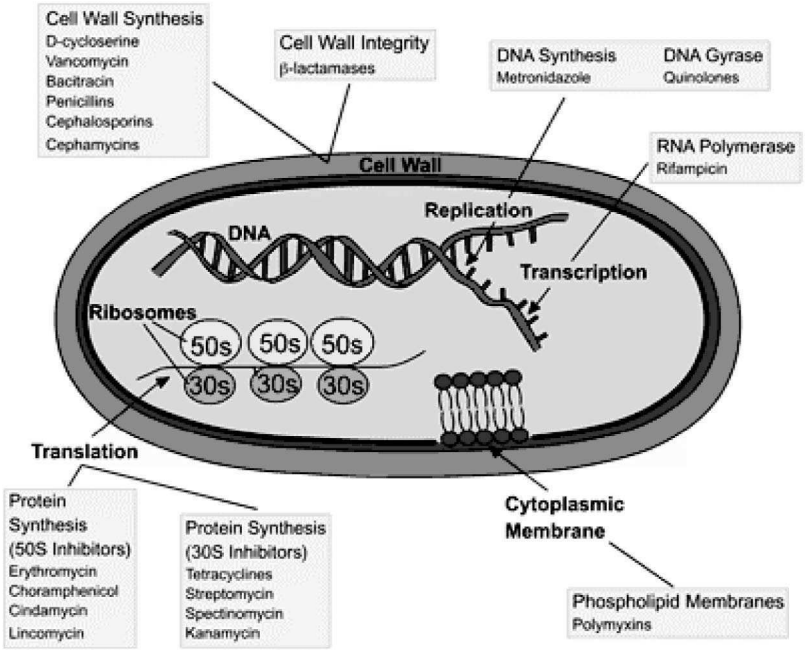


Fig. 9.1 Antibiotics and their sites of action in the bacterial cell.

chromosomal mutants (Table 9.1). In addition to these resistance mechanisms some broad-spectrum efflux pumps that impart low-level resistance to a number of structurally unrelated antimicrobials [4, 5], which results in the mar (multiple antibiotic resistance) phenotype, is a complex bacterial stress response system with which bacteria pump out toxic molecules [6, 7].

The incidence of antibiotic resistance in various pathogenic and opportunistic bacteria indicates that specific groups of bacteria have now become problematic; these include *Staphylococcus aureus* (methicillin and glycopeptides resistant), *Streptococcus pneumoniae* (penicillin and cephalosporins resistant) and *Enterococcus* spp. (vancomycin, ampicillin, and oxazolidonones resistant). Other MDR bacteria like *E. coli*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, *Salmonella* spp., *Shigella* spp. and *Acinetobacter* are widespread both in hospitals and communities [8] (Table 9.2).

MRSA has received much attention in the last decade because it is a major cause of hospital-acquired (nosocomial) infection. β-Lactam antibiotics are the preferred drugs against *S. aureus* infections but *S. aureus* has developed resistance to the β-lactam antibiotics due to the production of chromosomal or plasmid-mediated β-lactamases or penicillin binding proteins (PBPs). All *S. aureus* strains have four PBPs (PBP_1 to PBP_4); only MRSA expresses a special PBP (PBP_2' or PBP_2a) from the *mecA* gene. PBP_2a takes over the biosynthetic function of normal PBPs in the presence of inhibitory concentrations of β-lactams because PBP_2' has a decreased binding affinity to β-lactams [9, 10]. This has resulted in the development of multi-

Table 9.1 Common mechanisms of antibiotic resistance in bacteria [14–16].

Antibiotic	*Chromosomal resistance*	*Plasmid resistance*
β-Lactams	1) Loss of porins 2) β-Lactamase 3) Altered PBP	Cleavage by β-lactamase
Aminoglycosides (Streptomycin, neomycin, gentamycin)	1) Altered ribosomes 2) Defective transport	Modification
Chloramphenicol	Loss of porins	Acetylation
Tetracycline	Various but weak	Impermeability
Erythromycin	Altered ribosomal proteins rRNA	1) Altered 2) Hydrolysis
Sulfonamides	More target enzyme	Resistant enzyme
Trimethoprim	1) More target enzyme 2) Thymine auxotrophy	Resistant enzyme
Nitrofurans	Loss of activating reductase	Not found
Rifamycins	Altered RNA polymerase	Not found
4-Quinolones (norflox, ciproflox)	Mutation in gyrase gene	Found in few cases linked with β-lactamases

PBP, penicillin binding protein.

Table 9.2 Antibiotic-resistant bacteria of major concern [8].

Bacterium	*Antibiotic resistance*
Becoming problematic	
Mycobacterium leprae	Quinolone, depsone
Neisseria meningitides	Penicillin
Pasteurella multocida	Ampicillin, tetracyclines
Vibrio cholerae	Tetracyclines, fluoroquinolones
Yersinia pestis	Streptomycin, tetracyclines
Problematic	
Acinetobacter spp.	Multidrug
Enterococcus spp.	Vancomycin, ampicillin, oxazolidonones
Escherichia coli	Multidrug
Klebsiella pneumoniae	Multidrug
Mycobacterium tuberculosis	Multidrug
Neisseria gonorrhoeae	Multidrug
Non-typhoidal salmonella	Multidrug
Salmonella typhi	Multidrug
Shigella spp.	Multidrug
Staphylococcus aureus	Methicillin and glycopeptides
Streptococcus pneumoniae	Penicillin, cephalosporins

drug resistance against β-lactam and other antibiotics. Increased incidence of vancomycin-resistant MRSA has also been reported [11].

There are various types of β-lactamases produced by Gram-positive (*S. aureus*) and members of the family *Enterobacteriaceae* that act against different β-lactam antibiotics. These are penicillinases, cephalosporinases, and extended spectrum β-lactamases (common in Gram-negative bacteria). β-Lactamases are the commonest cause of bacterial resistance to β-lactam antibiotics. The spread of β-lactamases destroyed the utility of benzyl penicillin against staphylococci and has hugely undermined the use of ampicillin against *Enterobacteria*, *Haemophilus*, and *Neisseria* spp. [12].

New enzymes and new modes of production of old enzymes now threaten the value of extended spectrum cephalosporins against enterobacteria. The incidence of β-lactamase production and resistance to various β-lactam drugs has been reported widely from clinical and environmental origins all over the world [13].

Members of the *Enterobacteriaceae*, including *Salmonella*, *Shigella*, *E. coli*, *Enterococcus* and *Klebsiella*, are the leading cause of mortality and morbidity in children under five years of age, especially in developing countries. Their major mechanisms of resistance are plasmid-encoded multiple drug resistance including the production of extended-spectrum β-lactamases such as TEM-1, TEM-2, and SHV-1 β-lactamases. Extended-spectrum β-lactamases arose by point mutation probably under pressure of excessive use of antibiotics. Various types of β-lactamases produced by these Gram-negative bacteria have been reported [13]. What is even more alarming is the link between extended-spectrum β-lactamase production and fluoroquinolone resistance in *E. coli* and other Gram-negative bacteria (Table 9.1). In a few cases plasmid-encoded fluoroquinolone resistance has also been reported [14, 15].

The emergence of multidrug resistance and fluoroquinolone resistance among enteric bacteria has been disappointing to the clinicians and drug developing agencies. The availability of safe and fast-acting antibiotics against MDR bacteria is decreasing, so we must make great efforts to encourage better usage of these precious antibiotics and continuously update the novel drug development strategies to cope with MDR bacteria.

9.1.2
Plants as a Source of Novel Bioactive Compounds

The use of natural products with therapeutic properties is as ancient as human civilization and for centuries minerals, plants, and animal products were the main source of drugs [17]. About 25% of the drugs prescribed worldwide still come from plants, 121 such active compounds being in current use. It is estimated that 60% of the antitumor and anti-infectious drugs already on the market or under clinical trials are of natural origin [18]. During the twentieth century, the emphasis gradually shifted from extracting medicinal compounds from plants to making these compounds or their analogs synthetically. Despite the current preoccupation with synthetic chemistry as a vehicle to discover and manufacture drugs, the contribution of plants to disease treatment and prevention is still enormous [19]. Even at the start of the twenty-first century, 11% of the 252 drugs considered as basic and

essential by the World Health Organization (WHO) are exclusively of flowering plant origin [20].

In recent years, there has also been growing interest in complementary/alternative therapies and the therapeutic use of natural products, especially those derived from plants [21]. In the context of a modern, social, and economic view of health services, there is a clear need for more research on medicinal plants used in traditional of complementary medicine as they represent a suitable approach for the development of new bioactive compounds/drugs [20, 22]. The potential use of higher plants as a source of new drugs is still poorly explored. Of the estimated 250 000–500 000 plant species, only a small percentage has been investigated phytochemically and an even smaller percentage has been properly studied in term of their pharmacological properties, including the extensive screening conducted by the National Cancer Institute (NCI) of the USA [20, 23].

Screening of plants needs to be multidisciplinary [24]. Careful selection of plants and intelligent design of test system is the key to a successful screening outcome. Screening programs conducted in India and other countries on medicinal plants indicate promising antibacterial potential of several plants against MDR bacteria. Some antibacterials isolated from medicinal plants with known modes of action have been widely documented (Table 9.3) (see also Chapter 10), but their efficacy,

Table 9.3 Phyto-antimicrobial compounds.

Class	*Subclass*	*Example(s)*	*Mechanism*	*References*
Alkaloids	Isoquinoline	Berberine	Intercalate into cell wall and/or DNA	26–28
	Piperidine	Piperine		
Phenolics	Coumarins	Umbelliferone	?	28
	Flavonoids	Chrysin	Bind to adhesins	29–30
	Flavones	Apigenin	Inactivate enzymes, complex with cell wall	28
		Abyssinone	Inactivate enzymes inhibit HIV reverse transcriptase	31–33
	Flavonols	Galangin	Membrane damage	34
	Phenolic acids	Epicatechin	Membrane disruption	35
		Cinnamic acid		36
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzyme	37, 38
	Simple phenols	Catechol	Substrate deprivation	39
	Tannins	Ellagitannin	Bind to proteins	40, 41
			Bind to adhesins	42
			Enzyme inhibition	43–45
			Substrate deprivation	
			Complex with cell wall	
			Membrane disruption	
			Metal ion complexation	
Terpenoids, essential oils	Monoterpenoids	Citral, menthone	Membrane disruption	28
		Capsaicin		46

Partially adapted from Cowan [25]

toxicity, pharmacokinetics, and bioavailability *in vivo* have to be explored in the context of MDR bacteria.

9.2 Approaches to Targeted Screening Against MDR Bacteria

Medical practitioners and researchers have fought back with new antibiotics and drug combination to combat bacterial infections but at the same time bacteria have constantly developed resistance mechanisms either by mutation or acquisition of new genes through genetic exchange mechanisms such as transformation, transduction, and conjugation. Most acquired resistance is contributed by R-plasmids, which encode resistance to one or more antibiotics. Plasmids and transposons have further helped in the development and dissemination of resistance genes in the bacterial community [3]. This has necessitated the development of new antibacterial drugs which can be effectively used against MDR bacteria or which can enhance the efficacy of older antibiotics. In recent years attempts have been made to develop novel approaches to the screening of medicinal plants and other natural and synthetic compounds against MDR bacteria, including screening for bioactive compounds/plant extracts for (1) MDR efflux pump inhibitors, (2) β-lactamase inhibitors, (3) synergistic approaches such as antibiotic–phytocompound synergy, and (4) targeting virulence and pathogenicity of bacteria and use of quorum-sensing inhibitors. However, other viable approaches may include interference of the resistance mechanism, R-plasmid physiology to combat plasmid-encoded drug resistance (use of antiplasmid compounds), and inhibiting gene transfer mechanism. Recent progress made in this direction indicated that the careful screening of potential plants and other natural products might provide novel compounds against MDR bacteria (Tables 9.3 and 9.4).

Table 9.4 Potential bioactive plant extracts/phytocompounds detected as MDR pump inhibitors.

Plants	*Active compound/extract*	*Organism*	*References*
Rheum officinalis	Rhein	G+, G–, Y	62
Plumbago zeylanica	Plumbagin	G+, G–, Y	62
Polyathia memorials	Pyrithione	G+, G–, Y	62
Berberis repens	Berberin, 5′-methoxyhydnocarpin (5′-MHC)	*S. aureus*	59, 62
Zanthoxylum williamsii	Esculatin	G+, G–	62
Berberis aquifolia	Berberine, 5′-MHC	*S. aureus*	59
Berberis fremontii	Berberine, 5′-MHC	*S. aureus*	59
Berberis aetnensis	Crude extract	*S. aureus*	64
Pinus nigra	Hexane extracts	*S. aureus*	65
Rosmarinus officinalis	Carnosic acid, carnosol,12- methoxy-transcarnosic acid, etc.	*S. aureus*	66

Screening of medicinal plants for antimicrobial activity using classical methods has indicated a large number of bioactive compounds against bacteria [25]. But plant antimicrobials are not used as systemic antibiotics at present. The main reason for this is their low level of activity, especially against Gram-negative bacteria. The reported minimum inhibitory concentration (MIC) of plant antibacterials is often in the range of 100–1000 μg mL^{-1}. However, a variety of bioactive phytocompounds are known as antibacterials, and certain plant extracts and phytocompounds can enhance antibiotic activity in one or other way even though they may have weak or no antibacterial activity themselves.

New screening strategies are needed to explore the mode of action of antimicrobials, the synergy with antibiotics, and novel approaches of targeting MDR bacteria, which are still in an early stage of development. We have made an attempt to briefly review literature on the basic concept and progress made in this direction.

9.2.1 MDR Efflux Pump Inhibitors from Plants

Bacteria have evolved numerous defenses against antibacterial agents and drug-resistant pathogens are on the rise. A general and effective defense is conferred by ubiquitous multidrug efflux pumps, membrane translocases that extrude structurally unrelated toxins from the cells [5, 47–50]. Multidrug efflux pumps protect microbial cells from both synthetic and natural antimicrobials. The preferred substrates of most pumps are synthetic hydrophobic cations (amphipathic cations) such as quaternary ammonium antiseptics [51, 52]. Plants produce an enormous array of secondary metabolites and it is commonly accepted that a significant part of this chemical diversity serves to protect plants against microbial pathogens [53]. These phytocompounds are classified as phytoanticipins, which are compounds that are present constitutively, or phytoalexins, whose levels increase strongly in response to microbial invasion.

Plant compounds are routinely classified as antimicrobial on the basis of the susceptibility test that produces MICs in the range of 100–1000 μg mL^{-1}, which is weaker than the microbial produced antibiotics (MIC range from 0.01 to 10 μg mL^{-1}). A compound that is synthesized in response to pathogen invasion and is required to protect the plant from a pathogen but that shows little activity in an *in vitro* sensitivity test is not necessarily an antimicrobial. Such a substance might have a regulatory function, indirectly increasing the level of resistance of the plant. One helpful clue regarding the possible function of plant secondary metabolites is that these compounds often show considerable activity against Gram-positive bacteria but not against Gram-negative bacteria and yeast. Both Gram-negative bacteria and yeast have evolved significant permeability barriers [54]. In Gram-negative bacteria an outer membrane is a fairly effective barrier for amphipathic compounds and a set of MDR pumps exclude amphipathic toxins across the outer membrane [55–58].

In contrast, the single membrane of Gram-positive bacteria is considerably more accessible to permeation by amphipathic toxins and MDR pumps provide limited

protection [54]. Several Gram-positive bacteria invade plants, but the majority of plant pathogens are Gram-negative bacteria, yeast, and fungi.

Stermitz et al. [59] have proposed that plants produce compounds that can be effective antimicrobials, if they find their way into the cell of the pathogens. Thus production of MDR inhibitors by the plant would be one way to ensure delivery of antimicrobial compound. They demonstrated that the *Berberis* plant produces a putative antimicrobial, berberine, and also synthesizes the MDR pump inhibitor 5′-methoxyhydnocarpin D (5′-MHC-D) and pheophorbide A, which facilitate the penetration of berberine into a model Gram-positive *S. aureus*. Whether the *in vitro* ineffectiveness of plant antimicrobials against Gram-negative bacteria is due to poor penetration or efflux by MDR pumps has remained an open question.

Renau et al. [60] screened various synthetic and natural products libraries to search for broad-spectrum efflux inhibitors of the Mex pumps from *Pseudomonas aeruginosa*. They reported the compound MC-207,110 as the lead compound. The compound was active against three multidrug resistance efflux pumps (Mex AB-OprM, MexCD-OprJ,MexEF-OprN) from *P. aeruginosa* and their close *E. coli* efflux pump homolog (AcrAB-TolC). Some workers have shown the inhibition of NorA multidrug transporter of *S. aureus* [56, 61].

Tegos et al. [62] have tested a panel of plant antimicrobials using a set of bacteria representing plant and human pathogens. They demonstrated that the activity of the majority of the plant antimicrobials were considerably greater against Gram-positive bacteria like *S. aureus, B. megaterium* and that disabling of the MDR pumps in Gram-negative species leads to a striking increase in antimicrobial activity. Thus the activity of rhein, the principal antimicrobial from rhubarb was potentiated 100- to 2000-fold (depending upon the bacterial species) by disabling the MDR pumps. Comparable potentiation of activity was observed with plumbagin, resvetrol, gossypol, coumesterol, and berberine. Direct measurement of the uptake of berberine confirms that disabling of the MDR pumps strongly increases the level of penetration of berberine into the cells of Gram-negative bacteria. Tegos et al. have suggested that the plant antimicrobials might be developed into effective broad-spectrum inhibitors of MDR pumps. Thus the findings of considerable potentiation of the activities of plant antimicrobials by MDR pump inhibitors open the possibility for the development of combination therapy. Rhein is now approved for systemic use for the treatment of osteoarthritis, administered as a prodrug [63]. Recent reports are summarized in Table 9.4.

9.2.2 β-Lactamase Inhibitors

The production of β-lactamases/extended-spectrum β-lactamases is the major cause of bacterial resistance to β-lactam antibiotics. The approaches used to solve this resistance problem have evolved via the development of stable penicillins and cephalosporins and the search for β-lactamase inhibitors. Numerous compounds have been included in the list of β-lactamase inhibitors and the sources of these have shown potential clinical usefulness based on their synergistic effects when

they are combined with β-lactamase labile antibiotics. Screening for inhibitors of this type has been carried out extensively in actinomycetes and has resulted in the discovery of clavulanic acid and numerous carbapenem antibiotics. However, only rarely have inhibitors of a non-β-lactam structure been isolated from this group of organisms [67–70].

A combination of β-lactam and β-lactamase inhibitors (sulbactam, clavulanic acid, and tazobactum) is a successful strategy to overcome infection caused by β-lactamase-producing bacteria [71]. But the recent emergence of bacterial strains producing inhibitor-resistant enzymes could be related to the frequent use of clavulanate [72–74]. Furthermore, the appearance of extended-spectrum β-lactamase and IMP-1 (a new β-lactam) is now threatening the value of broad-spectrum cephalosporins and carbapenems against various bacterial infections [75–77]. Now it seems that the introduction of any new β-lactam will be followed by the appearance of a new β-lactamase. Therefore any strategy to prevent inactivation of β-lactam by β-lactamase is of particular significance.

Many attempts have been made to screen plant extracts for β-lactamase-inhibiting activity. In screening programs looking at aqueous and alcoholic extracts from the aerial parts of 179 phanerogamous species belonging to 39 botanical families, only eight plants, representing a wide taxonomic distribution, showed β-lactamase-inhibiting activity. These plants include *Borago officinalis*, *Sinapis alba*, *Spartium junceum*, *Senebiera didyma*, *Ranunculus repens*, and *Allium neapolitanm* [78].

Zhao et al. found that *Camellia sinensis* extracts and metabolites are inhibitors of β-lactamase activity [79]. They demonstrated that the combination of epigallocatechin gallate (EGCg, a main constituent of tea catechins) with penicillin showed synergism against 21 clinical isolates of penicillinase-producing *S. aureus*. Besides binding directly to peptidoglycans, the inhibition of penicillinase activity by EGCg occurs in a dose-dependent fashion, and a 50% inhibitory concentration of 10 μg mL^{-1} was observed [79]. In an other study hexane extracts of the leaf and twigs of *Spondias mombin* exhibited a positive response for β-lactamase inhibition assay. A colorless oil, an anacardic acid derivative (SB-202742), was identified as the active constituent [80].

Screening of natural inhibitors of penicillinases by copolymerization of hydrolyzed starch or glycogen in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was developed as a simple and convenient technique. Using this method anthraquinone-related compounds such as aloe-emodin, emodin, and rhein were detected as penicillinase inhibitors [81].

Similarly, other workers have also demonstrated inhibition of β-lactamase activity by *Papaya carica* (papain) and *Camellia sinensis* (epigalocatechin gallate) [79, 83, 84].

9.2.3
Synergy Between Phytocompounds and Antibiotics

In recent years, increasing attention has been focussed on investigating phytochemicals as possible medicinal agents against MDR bacteria. Plant extracts/phytocompounds exhibiting strong antibacterial activity are expected to interact synergistically with antibiotics. Such interactions may be useful in combination antibio-

tic therapy. Although investigations in this direction are in their infancy, a number of phytocompounds exhibiting synergistic interaction with antibiotics have been isolated and characterized (Table 9.5). The combinational effect of protoanemonum isolated from *Ranunculus bulbosus* with 22 antibiotics was evaluated. In one

Table 9.5 Synergistic interactions of plant extracts/phytocompounds with antibiotics.

Plant name	*Extract/compound*	*Antibiotic*	*Synergy against (organism)*	*Reference*
Calophyllum moonii	Calozeyloxanthone	Vancomycin	VRE	[99]
Camellia sinensis	Ethanol extract water	Tetracycline, ampicillin, gentamycin, methicillin, nalidixic acid	*S. aureus* *Shigella dysenteriae* *Salmonella typhimurium*	[97, 100]
Camellia sinensis	EGCg	Oxacillin, penicillin, ampicillin, methicillin, cephalexin and others	MRSA	[101]
Camellia sinensis	Extracts	Levofloxacin	*E. coli* O157	[102]
Caryophyllus aromaticus	Ethanol extract	Tetracycline, ampicillin, chloramphenicol	*Ps. aeruginosa, K. pneumoniae, Proteus* sp.	[94]
Coptis spp.	Berberine	Ampicillin, oxacillin	MRSA	[103]
Emblica officinalis	Ethanol extracts	Tetracycline	*S. aureus*	[96]
Erythrina variegata	Isoflavonone	Mupirocin	MRSA	[88]
Ferula communis	ferulenol	Isonicotinic acid hydrazide	*Mycobacterium* spp.	[104]
Garcinia mangostana	α-mangostin	Gentamycin, vancomycin, ampicillin, minocycline]	VRE, MRSA	[86,98
Gundelia tournefortii	Methanol extract	Chloramphenicol, gentamycin, cephalosporin	*Ps. aeruginosa*	[105]
Juniperus procera	totarol	Isonicotinic acid hydrazide	*Mycobacterium* spp.	[104]
Lawsonia inermis	Ethanol extract	Tetracycline	*S. aureus*	[97]
Lepidium sativum	Methanol extract	Chloramphenicol, gentamycin, cephalosporin]	*Ps. aeruginosa*	[105
Plumbago zeylanica		Isonicotinic acid hydrazide	*Mycobacterium* spp.	[104]
Propolis	Ethanol extract	Chloramphenicol, gentamycin, tetracycline, netilmicin, vancomycin	*S. aureus* *E. faecalis,* *Salmonella* spp.	[106, 107]
Punica granatum	Ethanol extract	Tetracycline	*S. aureus, Ps. aeruginosa*	[94, 97]
Ranunculus bulbosus	Protoanemonum	22 antibiotics	*S. aureus*	[85]
Sausaria lappa	Ethanol extract	Chloramphenicol	S. aureus	[96]
Scutellaria amoena	Baicalin	Benzyl penicillin	MRSA	[108]
Syzygium joabolanum	Ethanol extract	Tetracycline, ampicillin, chloramphenicol	*Ps. aeruginosa*	[94]
Terminalia chebula	Ethanol extract	Tetracycline	*S. aureus*	[97]
Terminalia belerica	Ethanol extract	Tetracycline	*S. aureus*	[97]
Thymus vulgaris	Ethanol extract	Tetracycline, ampicillin, chloramphenicol	*Ps. aeruginosa*	
Withania somnifera	Methanol, hexane	Tibrin (rifampicin+isoniazid)	*Salmonella typhimurium, Escherichia coli*	[109]

combination, protoanemonum–cefamendole showed strong synergism against *S. aureus* [85].

Iinuma [86] demonstrated the synergistic activity of two xanthones, α-mangostin and rubraxanthon, isolated from *Garcinia mangostana* with antibiotics against MRSA strains. Similarly retin isolated from *Sophora japonica* could be hydrolyzed to quercetin which showed synergistic and additive effects with various antibiotics [87].

An isoflavone from the roots of *Erythrina variegata* (Leguminosae) characterized as 2,4-dihydroxy-8-γ-γ-dimethyl allyl 2′2′-dimethyl pyrano [5′,6′:6,7] isoflavone (bidwillon B) inhibited the growth of 12 MRSA strains with MIC values of 3.13–6.25 mg L^{-1}, while the MIC values of mupirocin were 0.20–3.13 mg L^{-1}. Mupirocin is a naturally occurring agent produced by *Pseudomonas fluorescens* and has successfully been used to reduce substantially the nasal and hand carriage of MRSA [88–91]. Mupirocin consists of a short fatty acid (α-β-unsaturated carboxylic acid), the tail end of which appears to mimic isoleucine. It reversibly binds to isoleucyl tRNA synthetase and prevents the incorporation of isoleucine into growing polypeptide chain [92]. However, a high level of resistance to mupirocin has been reported among MRSA isolates. Sato and co-workers [88] demonstrated a synergistic interaction against 11 MRSA strains with fraction inhibitory concentration (FIC) indices of 0.5–0.75. The minimum bactericial concentration (MBC) of mupirocin in the presence of bidwillon B (3.13 mg L^{-1}) was reduced to 0.05–1.56 mg L^{-1}. They suggested that bidwillon B may prove to be a potent phytotherapeutic and/or combination agent with mupirocin in the elimination of nasal and skin carriage of MRSA. Antibacterial activity of flavones isolated from *Sophora exigua* against MRSA and its interaction with antibiotics have also been reported [93].

Nascimento et al. [94] demonstrated the antibacterial activity of 11 medicinal plants against several Gram-negative and few Gram-positive bacteria. Interestingly the highest activity was observed in the extracts of *Caryophyllus aromaticus* and *Syzygium aromaticum*. The interaction between active plant extracts and ampicillin, chloramphenicol, and/or tetracycline was determined using a synergism assay. Synergistic interactions were observed between antibiotics and extracts from clove, jambolan, pomegranate, and thyme against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Alcoholic extracts of several Indian medicinal plants were tested for the synergistic interactions with tetracycline, streptomycin, and chloramphenicol against an extended-spectrum β-lactamase-producing strain of *E. coli* by the method of Chattopadhyay [95]. Synergistic interactions of the extracts from *Acorus calamus* and *Holarrhena antidysenterica* were demonstrated with tetracycline and ciprofloxacin, while other plant extracts such as *Hemidesmus indicus*, *Plumbago zeylanica*, *Camellia sinensis*, and *Cichorium intybus* showed synergy with tetracycline only. Certain extracts showed synergistic interactions with ampicillin/chloramphenicol against MRSA [96, 97].

α-Mangostin isolated from the stem bark of *Garcinia mangostana* was found to be active against vancomycin-resistant enterococci and MRSA with MIC values of 6.25–12.5 μg mL^{-1}. The compound showed synergistic activity with gentamycin against MRSA. However, partial synergism was found with ampicillin and minocycline [98].

9.2.4 Targeting Virulence and Pathogenicity

Microbial pathogenicity is a multifactorial phenomenon. Bacterial pathogenicity is defined as the ability of bacteria to cause disease and the degree of pathogenicity is called virulence. Various virulence factors related to both structure (flagella, fimbriae, capsule) and products of the bacterial cell are known to influence the pathogenicity of the bacterium. Such factors may be plasmid or chromosomal encoded [110]. The interaction between the pathogen and the host, resulting in the adherence and colonization of the pathogen, is the first step in the process of infection.

Bacterial binding to tissue implicates two main types of interaction, the process of adherence and the rate of adherence, which occur subsequently during colonization. The mechanisms involved in the process of adherence are varied and include lectin-like interactions, electrostatic and hydrophobic forces, and cell surface hydrophobicity. Cell surface hydrophobicity is regarded as an important factor in mediating bacterial adherence to a wide variety of surfaces [111].

The suppression of virulence does not kill the bacteria. It could have a synergistic effect when used with antimicrobial therapies. Balaban et al. [112] investigated a novel way of interfering with virulence factor synthesis by vaccinating mice with an autoinducer called RNAIII-activating peptide (RAP). RNAIII is a regulatory RNA molecule responsible for the synthesis of virulence factors in *S. aureus* and it is induced by RAP. Vaccination of mice with RAP increases their resistance to *S. aureus* challenge from 30% to 70% and decreases the size of lesions. The regulatory mechanism involving autoinducers may be targeted in other bacteria.

Pathogenic microorganisms commonly attach to target tissues by species-specific adhesion receptor mechanisms. However, microbial cell surface hydrophobicity (CSH) is often also associated with binding to the specific cell and tissue receptor of the mucosal surface in the infected host [113]. Therefore, new prophylactic therapies may involve searches for agents that counter the effects of virulence factors, including the hydrophobicity of the pathogenic bacteria. In recent years plant extracts such as wild chamomile and pineapple weed have been shown to be able to decrease the virulence of bacteria by blocking aggregation of *Helicobacter pylori*. The extracts contained small amounts of tannin and did not reveal any antimicrobial activity. Tannic acid, a component of bearberry and cowberry aqueous extracts, showed highest activity in decreasing CSH as well as antibacterial activity against *H. pylori* [114].

The influence of aqueous extracts of bearberry (leaves), St John's wort, wild chamomile, and marigold (flower) on the hydrophobicity of 40 *E. coli* and 20 *Acinetobacter baumanni* strains has been demonstrated [115]. The decoction of bearberry and St. John's wort increased the hydrophobicity remarkably. The infusion of wild chamomile and marigold completely blocked the aggregation properties. These extracts showed poor or no antimicrobial activity (Table 9.6).

Table 9.6 Plant extracts and phytocompounds influencing cell surface hydrophobicity and quorum sensing of bacteria.

Plants	*Active extract*	*Organisms*	*References*
Targeting cell surface hydrophobicity			
Andrographis paniculata	Crude extract	*Streptococcus mutans*	150
Arctostaphylos uva-ursi	Aqueous extracts	*Helicobacter pylori*	114
Arnica montana	Crude extract	*Strep. mutans, Strep. sobrinus*	151
Camellia sinensis	Crude extract	*Strep. mutans*	150
Camomile	Aqueous extracts	*E. coli, A. baumannii*	115
Cassia alata	Crude extract	*Strep. mutans*	150
Harrisonia perforata	Crude extract	*Strep. mutans*	150
Helichrysum italicum	Ethanol extracts	Streptococci	152
Juglandaceae regia	Aqueous and alcoholic extracts	*Strep. mutans*	153
Matricaria matricarioides	Aqueous extracts	*Helicobacter pylori*	114
Matricaria recutita	Aqueous extracts	*Helicobacter pylori*	11
Mikania glomerata	Hexane, ethyl acetate, ethanol fraction	Streptococci	154
Mikania laevigata	Hexane, ethyl acetate, ethanol fraction	Streptococci	154
Propolis extract	Crude extract	*Strep. mutans, Strep. sobrinus*	151
Psidium guajava	Crude extract	*Strep. mutans*	150
St. John's wort	Aqueous extracts	*E. coli, A. baumannii*	115
Streblus asper	Crude extract	*Strep. mutans*	150
Tagetes sp.	Aqueous extracts	*E. coli, A. baumannii*	115
Vaccinium vitis-idaea	Aqueous extracts	*Helicobacter pylori*	114
Targeting quorum sensing			
Allium sativum	Toluene extract		146
Capsicum spp.	Toluene extract		146
Coffea arabica	Toluene extract		146
Daucus carota	Toluene extract		146
Nymphaea odorata	Toluene extract		146
Platanus occidentalis	Plant extract		155
Propolis	Toluene extract		146
Vigna radiata	Toluene extract		146
Yellow pepper	Toluene extract		146

9.2.5
Quorum Sensing Inhibitors

The interaction between the host and a pathogenic bacterium is mainly controlled by bacterial population size. An individual bacterial cell is able to sense other members of the same species and to respond, differentially expressing specific genes.

Such cell-to-cell communication is called quorum sensing (QS) and involves the direct or indirect activation of a response regulator by signal molecules. The major QS signal molecules are *N*-acyl homoserine lactones (AHL) in Gram-negative bacteria and post translationally modified peptides in Gram-positive bacteria. The QS system is used by a wide variety of bacteria including human pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and other invasive bacteria. The development of novel antimicrobial compounds is required to treat the growing number of infections where antibiotic resistance is a serious threat, especially in situation where biofilms are involved. Research over the last two decades has revealed that bacteria in biofilms exhibit a higher tolerance to antimicrobial treatments [116]. Bacterial control through the inhibition of bacterial cell communication systems, which are involved in the regulation of virulence factor production, host colonization, and biofilm formation instead of inhibiting growth, could serve as an alternative to conventional ways of combating bacterial infections [117–119]. Thus molecules that interfere with QS promise new therapeutic strategies or prophylactic measures in infectious diseases.

In Gram-negative bacteria the cell-to-cell communication is carried out by AHLs, which are produced by the Lux1 family. The signal molecules differ with respect to the length of their side chains (C_4–C_{16}) and with various degrees of substitution and saturation [120]. Short-chain AHLs are freely diffusible over the cell membrane whereas long-chain AHLs are the substrate for efflux pumps, such as mexAB-oprM [121]. The AHLs are sensed by proteins belonging to the Lux family of response regulators. LuxR homologs contain two domains, an AHL-binding domain and a DNA-binding domain. When AHL is bound, it alters the configuration of the LuxR homolog, enabling it to interact with DNA and act as a transcriptional activator [122]. Some LuxR homologs act as repressors, blocking transcription in the absence of AHL and depressing the target genes when sufficient AHL is present [123]. The two key components of the QS system, Lux1 and LuxR homologs, are often linked genes, whereas the QS target genes are localized elsewhere on the genome. It has been reported that QS target genes are not merely activated at certain threshold concentrations but become activated as a continuum at different concentrations of AHL in the cell [124, 125]. This strategy would be based on small molecules with variation in their chemical composition that would allow them to block the AHL receptor site of the LuxR homologs or alternatively block the formation of active dimers that are required for binding to and expression of target genes.

A number of studies have identified several molecules that function as QS inhibitors (QSI) [119, 126–128]. Much effort has been spent on synthesis of AHL analogs, which antagonize the cognate signal molecules. Varying the length of the acyl side chain was found to be important; for example AHLs with extended side chains generally caused inhibition of the LuxR homologs [129–132]. Other modifications to the AHLs included alteration of the acyl chain by introducing ramified alkyl, cycloalkyl, or aryl/phenyl substituents at the C-4 position, resulting in both inducers (analogs with nonaromatic substitutions) and antagonists (analogs with phenyl substitutions) [127]. Modification of the lactone ring of AHLs by adding substituents to C-3 or C-4 did not give rise to strong QSI activity [128]. However, exchang-

ing the homoserine ring with a five- or six-membered alchohol or ketone ring generated a number of activators and inhibitors, some of which blocked *Ps. aeruginosa* QS *in vitro* [126, 133]. Their target specificity for QS regulation was not verified by transcriptomics.

In nature, eukaryotes live closely associated with virulent prokaryotes. This has forced mammals to evolve different defense systems. Plants and fungi, however, do not possess active immune systems, instead they have to rely on physical and chemical defenses. A well-studied example of this is the production of halogenated furanone compounds by the Australian alga *Delisea pulchra* [134]. This species produces furanones in the central vesicles of gland cells, from which they are released to the surface of the plant [135]. There they prevent extensive surface growth of bacteria and higher fouling organisms [136, 137]. The halogenated furanones have been shown to inhibit several QS-controlled phenotypes, including swarming motility of *Serratia liquefaciens*, toxin production by *Vibrio harveyi*, and bioluminescence of *Vibrio fischeri* [134, 138–140]. In a more clinical context, a synthetic derivative of the furanones (C_{30}) was found to downregulate expression of more than 80% of the QS-regulated genes found in *Ps. aeruginosa*, many of them encoding known virulence factors.

This effect is not limited to planktonic bacteria. It also applies to biofilm-dwelling *Ps. aeruginosa*. Biofilms developed in the presence of furanone compounds become more susceptible to treatment with antibiotics and disinfectants [124, 141]. This is highly interesting given that *Ps. aeruginosa* is an opportunistic pathogen often found in people with compromised immune systems, such as cystic fibrosis patients, where it is responsible for persistent, chronic infections probably caused by biofilm formation within the host [142–144]. Attenuating this bacterium with respect to virulence and persistence is undoubtedly desirable [141]. In recent years several workers have provided evidence of QSI efficacy and potential therapeutic value against one or other pathogenic bacteria. These QSI are either from AHLs or natural products either from plants or microorganisms [145–147].

Persson and co-workers [147] reported the rational design and synthesis of new QSIs derived from AHLs from garlic. Design and biological screening was based on targeted inhibition of QS comprising the competitive inhibitors of transcriptional regulation LuxR and LasR. The design was based on critical interactions within the binding sites and structural motifs in molecular component isolated from garlic and found QSIs but not antibiotics. A potent QSI *N*-(heptylsulfanylacetyl)-1-homoserine lactone was identified.

Bjarnsholt and co-workers [148] provided evidence that *Ps. aeruginosa* controls the expression of many of its virulence factors by means of QS. The biofilm bacteria in which QS is blocked either by mutation or by administration of QSI drugs are sensitive to treatment with tobramycin and H_2O_2 and are readily phagocytosed by polymorphic neutrophils, in contrast to bacteria with a functional QS system. They further suggested that a combination of the action of polymorphic neutrophils and QS inhibitors along with conventional antibiotics would eliminate the biofilm-forming bacteria before a chronic infection is established. Similarly Jones et al. [149] demonstrated inhibition of *Bacillus anthracis* growth and virulence gene

expression by the QS inhibitor (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2 (5H)-furanone, obtained from marine alga (*Delisea pulchra*).

Rasmussen and co-workers [146] screened 100 extracts from 50 *Penicillium* species, and found that 33 contained QSI compounds. In two cases patulin and penicillin acid were identified as being biologically active QSI compounds. Their effect on QS-controlled gene expression in *Ps. aeruginosa* was verified by DNA microarray transcriptomics. In a mouse pulmonary infection model *Ps. aeruginosa* was more rapidly cleared from the mice that were treated with patulin compared with the placebo group.

Screening of several plant extracts and compounds for QSI by use of a novel genetic system, the QSI selector, has been reported [146]. Of the 23 plant extracts looked at, eight (bean sprout, chamomile, carrot, garlic, habanera, propilis, water lily, and yellow pepper) showed QSI activity. The two most active were garlic extract and 4-nitro-pyridine-*N*-oxide (4-NPO). GeneCip-based transcriptose analysis revealed that garlic extract and 4-NPO has specificity for QS-controlled virulence genes in *Ps. aeruginosa*. These two QSIs also significantly reduced biofilm tolerance to tobramycin treatment as well as virulence in a *Caenorhabditis degans* model.

9.3 Other Potential Approaches

9.3.1 Targeting Gene Transfer Mechanisms

Both conjugative and nonconjugative plasmids are equally well transferable by conjugation and transformation processes to a wide variety of Gram-positive and Gram-negative bacteria. Horizontal gene transfer is a principal source of evolution, leading to change in the ecological character of bacterial species [156, 158].

Antibiotics themselves induce resistance in microorganisms via the transfer of horizontal mobile elements [157]. The process of bacterial conjugation is complex and involves many enzymes that could be potential targets for new antibiotics. The best-studied conjugation system is that of F-plasmid. The F transfer region contains about 40 genes spread over 33 kb that are involved in a variety of processes, including sex pilus formation, mating pair stabilization, surface exclusion, DNA nicking, and transfer [158].

One potential target is the integrases that facilitate the insertion of antibiotic resistance cassettes into integrons and bacterial genome, as similar integrases of HIV-1 have been the target for new drug development [159]. However other processes in conjugation are also potential targets for the development of new drugs. Antibiotics such as norfloxacin and ciprofloxacin are known to interfere with gene transfer in the conjugation process [14, 15].

Some workers have demonstrated that several antimicrobial agents, including mitomycin and molecules belonging to the 4-quinolone, aminoglycoside, and β-lactam groups, inhibit plasmid transfer to a varying extent in actively growing

E. coli. The results indicated that the drugs inhibited plasmid transfer by interfering with bacterial host functions rather than by recognizing a specific plasmid-mediated target [160, 161]. Hooper and co-workers [162] studied the antagonism of the DNA gyrase B subunit in the donor bacterium by coumermycin or thermal inactivation that inhibited transfer of plasmid R64 drd-11. Coumermycin also inhibited Hfr transfer, with kinetics after drug removal suggesting that transfer resumed from the point of inhibition, in contrast to inhibition with nalidixic acid, after which transfer reinitiated from the origin of transfer.

Phytoextracts/compounds may also be screened for such properties. In a preliminary study we have tested few plant extracts that influence (decrease) the transfer frequency of Rp4 plasmid from *E. coli* to *E. coli* [163]. However, more effort in this direction is needed to develop more effective screening assays.

9.3.2 Targeting R-Plasmid Elimination

Novel targets for combating drug-resistant bacteria may be found through interfering with the stability of R-plasmids. The acquisition and dissemination of antibiotic resistance genes from plasmids is a common mechanism in bacteria. Many bacteria become resistant to multiple antibiotics through the uptake of a plasmid that codes for resistance-mediating proteins. This lateral DNA transfer confers resistance to one or more antibiotics. As a consequence, significant plasmid-encoded resistance is observed clinically for many major classes of antibiotic such as β-lactams, macrolides, tetracyclines, aminoglycosides, and glycopeptides; even 4-quinolone is mentioned in a recent report [161, 164–166].

One approach is to eliminate these R-plasmids from bacteria and thus resensitize the bacteria to antibiotics. Various experimental approaches have been used to eliminate R-plasmids from bacterial cells by physical and chemical agents. Elimination by chemicals involves the inhibition of vital proteins, plasmid DNA and cell surface charges and plasmid compatibility, etc. [167–169].

As a result of extensive studies, particularly on the mechanism of plasmid DNA replication, various approaches to the elimination of plasmid DNA have been described, such as:

- Direct inhibition of DNA synthesis by intercalating dyes [162, 166, 170].
- Inhibition of DNA replication by alkylating agents or inhibition of synthesis of functional proteins.
- Dissolution of cell surface by surface acting agents.
- Elevation of temperature [170, 171]
- Nutritional starvation [172, 173].
- Ultraviolet irradiation [174, 175].
- Incompatibility grouping [176].
- Protoplast formation and regeneration [177].

The antibacterial drugs most effective in eliminating R-plasmids from their host cells *in vitro* were found to be novobiocin and 4-quinolone. However the curing was

found to be concentration dependent. The most effective concentration was found in the sub-MIC range [164–166]. However, antibiotics at such sub-MIC values *in vivo* may result in the selection and development of mutants. Therefore any compound having plasmid curing activity should be handled carefully *in vitro* and *in vivo*.

Phytocompounds and certain plant extracts (*Plumbago zeylanica*, *Camellia sinensis*) and naphthoquinone are reported to eliminate R-plasmids from *E. coli* and *Acinetobacter* [178, 179].

DeNap and co-workers [180] have used a novel compound apramycin, which binds SL1 in the important regulatory region that dictates plasmid replication control and incompatibility. *In vitro* studies demonstrated that this compound causes significant plasmid loss and resensitized bacteria to conventional antibiotics. They concluded that the discovery of small molecules that can mimic incompatibility, cause plasmid elimination, and resensitize bacteria to antibiotics opens up new arenas for research into antibacterial drugs.

In vitro elimination of the Rp4 plasmid from *E. coli* have been demonstrated by alcoholic extracts of *Holarrhena antidysenterica* and *Plumbago zeylanica* [163].

9.4 Conclusions and Future Directions

Many scientists from different fields are investigating plants with the hope of discovering novel bioactive chemotherapeutic compounds. Extensive screening programs of plants used mainly in traditional medicine have resulted in the discovery of thousands of phytochemicals with inhibitory effects on different types of microorganisms *in vitro*. Studies conducted in India and elsewhere have indicated that several plant extracts/phytocompounds have broad-spectrum activity against problematic MDR bacteria. Such bioactive extracts/compounds might be exploited in combating MDR bacteria in a synergistic manner with other phytocompounds (e.g. MDR inhibitors) and/or antibiotics. Furthermore, alternative mechanisms of infection prevention, such as decreasing the virulence and pathogenicity of bacteria, antiadherance activity, quorum sensing inhibition, and minimizing genetic transfer of drug resistance and elimination of R-plasmids, should be included in initial activity screening for a more holistic approach to targeting MDR bacteria. Here we propose a screening approach integrating various bioassays which could detect novel activity of plants against MDR bacteria (Fig. 9.2). It would be advantageous to standardize methods of extraction, activity-guided fractionation, and *in vitro* testing so that the search could be more systematic and reproducible, and the interpretation of results would be facilitated.

The bioactive compounds showing promising *in vitro* activity should be subjected to animal and human studies to determine their efficacy, stability, and bioavailability in whole organisms systems, including in particular toxicity studies as well as their effects on beneficial normal microbiota.

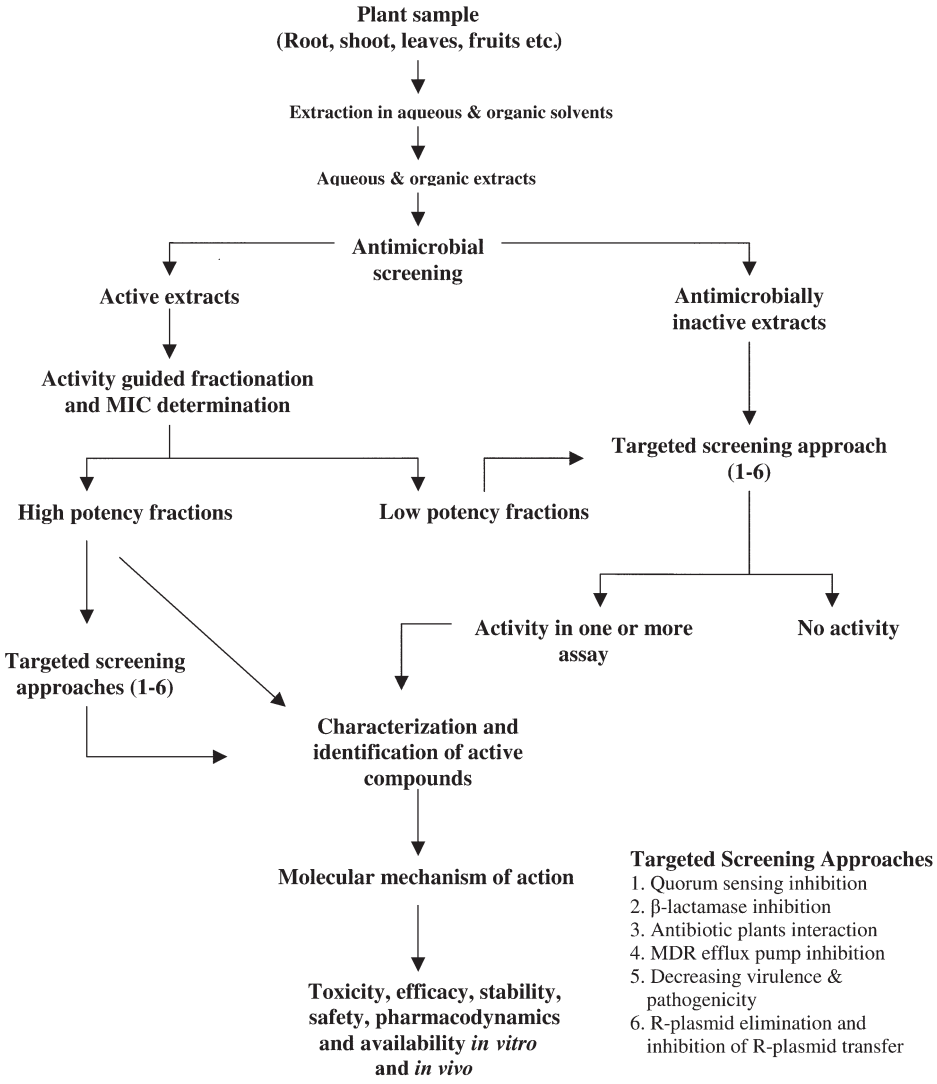


Fig. 9.2 Schematic representation of targeted screening of plant extracts/phytocompounds.

Acknowledgments

We are grateful to University Grant Commission, New Delhi for financial assistance in the form of UGC-Major Research Project no. F.3-58/ 2002 (SR-II) on medicinal plants.

References

1 WHO. *TB: A Global Emergency. WHO Report on the TB Epidemic*. World Health Organization, Geneva, **1994**.
2 Tait, S. *J. Med. Microbiol*. **1993**, *38*, 157–159.
3 Davies, J. *Science* **1994**, *264*, 375–382.
4 Nikaido, H. *J. Bacteriol*. **1996**, *178*, 3853–3859.
5 Paulson, I.T., Brown, M.H., Skurray, R.A. *Microbiol Rev*. **1996**, *60*, 575–608.
6 Goldman, J.D., White, D.G., Levy, S.B. *Antimicrob. Agents Chemother*. **1996**, *40*, 1266–1269.
7 Miller, P.F., Sulavik, M. *Mol. Microbiol*. **1996**, *21*, 441–448.
8 Kariuki, S., Hart, A. *Curr. Opin. Infect. Dis*. **2001**, *14*, 579–586
9 Dennesen, P.J.W., Bonten, M.J.M., Weinstein, R.A. *Trends Clin. Pract*. **1998**, *30*, 176–185.
10 Bachi, B.B., Rohrer, S. *Arch. Microbiol*. **2002**, *178*, 165–171.
11 Hiramatsu, K., Hanaki, H., Ito, T., Yabuta, K., Ogura, T., Tenova, F.C. *J. Antimicrob. Chemother*. **1997**, *41*, 135–146.
12 Livermore, D.M. *Clin. Microbiol. Rev*. **1995**, *8*, 557–584.
13 Bush, K., George, A., Jacoby, Medeiros, A.A. *Antimicrob. Agents Chemother*. **1995**, *39*, 1211–1233.
14 Wang, M., Tran, J.H., Jacoby, G.A., Zhang, Y., Wang, F., Hooper, D.C. *Antimicrob. Agents Chemother*. **2003**, *47*, 2242–2248.
15 Cheung, T.K., Chu, Y.W., Chu, M.Y., Ma, C.H., Yung, R.W., Kam, K.M. *J. Antimicrob. Chemother*. **2005**, *56*, 586–589.
16 Pelczar, M.J., Chan, E.C., Krieg, N.R. *Microbiology*, 5th edn. McGraw-Hill, New Delhi, **1998**.
17 De Pasquale, A. *J. Ethnopharmacol*. **1984**, *11*, 1–16.
18 Shu, Y.-Z. *J. Nat. Prod*. **1998**, *61*, 1053–1071.
19 Raskin, I., Ribnicky, D.M., Komarnystky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D.A., Ripoll, C., Yakoby, N., O'Niel, J.M., Cornwell, T., Pastor, I., Fridlender, B. *Trends Biotechnol*. **2002**, *20*, 1–10.
20 Rates, S.M.K. *Toxicon* **2001**, *39*, 603–613.
21 Chan, K. *Chemosphere* **2003**, *52*, 1361–1371.
22 Cordell, G.A. *Phytochemistry* **2000**, *55*, 463–480.
23 Hamburger, H., Hostettmann, K. *Phytochemistry* **1991**, *30*, 3864–3874.
24 Verpoorte R. Plant secondary metabolism. In *Metabolic Engineering of Plant Secondary Metabolism*, Verpoorte, R., Alfermann, A.W. (eds). Kluwer Academic Publishers, Dordrecht, **2000**, pp. 1–29.
25 Cowan, M.M. *Clin. Microbiol. Rev*. **1999**, *12*, 564–582.
26 Atta-ur-Rahman, Choudhary, M.I. *Nat. Prod. Rep*. **1995**, *12*, 361–379.
27 Burdick, E.M. *Econ. Bot*. **1971**, *25*, 363–365.
28 Harborne, S.B., Baxter, H. *Phytochmical Dictionary. A Handbook of Bioactive Compounds from Plants*. Taylor and Francis, London, **1995**.
29 Perrett, S., Whitfield, P.J., Sanderson, L., Bartlett, A. *J. Ethnopharmacol*. **1995**, *47*, 49–54.
30 Rojas, A., Hernandez, L., Pereda-Miranda, R., Mata, R. *J. Ethnopharmacol*. **1992**, *35*, 275–283.
31 Brinkworth, R.I., Stoermer, M.J., Fairlie, D.P. *Biochem. Biophys. Res. Commun*. **1992**, *188*, 631–637.
32 Taniguchi, M., Kubo, I. *J. Nat. Prod*. **1993**, *56*, 1539–1546.
33 Ono, K., Nakane, H., Fukishima, M., Chermann, J.-C., Barre-Sinoussi, F. *Biochem. Biophys. Res. Commun*. **1989**, *3*, 982–987.
34 Cushnie, T.P., Lamb, A.J. *J. Ethnopharmacol*. **2005**, *101*, 243–248.
35 Toda, M., Okubo, S., Ikigai, H., Suzuki, T., Suzuki, Y., Hara, Y., Shimamura, T. *Microbiol. Immunol*. **1992**, *36*, 999–1001.
36 Fernandez, M.A., Garcia, M.D., Saenz, M.T. *J. Ethnopharmacol*. **1996**, *53*, 11–14.
37 Duke, J.A. *Handbook of Medicinal Herbs*. CRC Press, Boca Raton, FL, **1985**.
38 King, S.R., Tempesta, M.S. *Ciba Found. Symp*. **1994**, *185*, 197–206.
39 Peres, M.T.L.P., Monache, F.D., Cruz, A.B., Pizzolatti, M.G., Yunes, R.A. *J. Ethnopharmacol*. **1997**, *56*, 223–226.

40 Schultz, J.C. Tannin-insect interactions. In *Chemistry and Significance of Condensed Tannins*, Hemingway, R.W., Karchesy, J.J. (eds). Plenum Press, New York, **1988**, pp. 553.

41 Stern, J.L., Hagerman, A.E., Steinberg, P.D., Mason, P.K. *J. Chem. Ecol*. **1996**, *22*, 1887–1899.

42 Scalbert, A. *Phytochemistry* **1991**, *30*, 3875–3883.

43 Haslam, E. *J. Nat. Prod*. **1996**, *59*, 205–215.

44 Brownlee, H.E., McEuen, A.R., Hedger, J., Scott, I.M. *Physiol. Mol. Plant Pathol*. **1990**, *36*, 39–48.

45 Butler, L.G. Effects of condensed tannin on animal nutrition. In *Chemistry and Significance of Condensed Tannins*, Hemingway, R.W., Karchesy, J.J. (eds). Plenum Press, New York, **1988**, p. 553.

46 Cichewicz, R.H., Thorpe, P.A. *J. Ethnopharmacol*. **1996**, *52*, 61–70.

47 Lewis, K. *Curr. Biol*. **1999**, *9*, R403–R407.

48 Nikaido, H. *Curr. Opin. Microbiol*. **1998**, *1*, 516–523.

49 Amin, A.H.,Subbaiah, T.V., Abbasi, K.M. *Can. J. Microbiol*. **1969**, *15*, 1067–1076.

50 Lolkema, J.S., Poolman, B., Konings, W.N. *Curr. Opin. Microbiol*. **1998**, *1*, 248–253.

51 Hsieh, P.C., Siegel, S.A., Rogers, B., Davis, D., Lewis, K. *Proc. Natl Acad. Sci. USA* **1998**, *95*, 6602–6606.

52 Lewis, K. In *Transport of Molecules Across Microbial Membranes*, Vol. 58, Broome-Smith, J.K., Baumberg, S., Stirling, C.J., Ward, F.B. (eds). Cambridge University Press, Cambridge, **1999**, pp. 15–40.

53 Dixon, R.A. *Nature* **2001**, *411*, 843–847.

54 Lewis, K. *J. Mol. Microbiol. Biotechnol*. **2001**, *3*, 247–254.

55 Lewis, K., Lomovskaya, O. Drug efflux. In *Bacterial Resistance to Antimicrobials, Mechanisms, Genetics, Medical Practice and Public Health*. Lewis, K., Salyers, A., Taber, H., Wax R. (eds). Marcel Dekker, New York, **2001**, pp. 61–90.

56 Lomovskaya, O., Lewis, K. *Proc. Natl Acad. Sci. USA* **1992**, *89*, 8938–8942.

57 Nikaido, H. *J. Bacteriol*. **1999**, *181*, 4–8.

58 Zgurskaya, H. I., H. Nikaido. *Proc. Natl Acad. Sci. USA* **1999**, *96*, 7190–7195.

59 Stermitz, F.R., Tawara-Matsuda, J., Lorenz, P., Mueller, P., Zenewicz, L., Lewis, K. *J. Nat. Prod*. **2000**, *63*, 1146–1149.

60 Renau, T.E., Leger, R., Flamme, E.M., Sangalang, J., She, M.W., Yen, R., Gannon, C.L., Griffith, D., Chamberland, S., Lomovskaya, O., Hecker, S. J., Lee V.J., Ohta, T., Nakayama, K. *J. Med. Chem*. **1999**, *42*, 4928–4931.

61 Markham, P.N., Westhaus, E., Klyachko, K., Johnson, M.E., Neyfakh, A.A. *Antimicrob. Agents Chemother*. **1999**, *43*, 2404–2408.

62 Tegos, G., Stermitz, F.R., Lomovskaya, O., Lewis K. *Antimicrob. Agents Chemother*. **2002**, 46 (10), 3133–3141.

63 Spencer, C.M., Wilde, M.I. *Drugs* **1997**, *53*, 98–106.

64 Musumeci, R., Speciale, A., Costanzo, R., Annino, A., Ragusa, S., Rapisarda, A., Pappalardo, M.S., Iauk, L. *Int. J. Antimicrob. Agents* **2003**, *22*, 48–53.

65 Smith, E., Williamson, E., Zloh, M., Gibbons, S. *Phytother Res*. **2005**, *19*, 538–542.

66 Oluwatuyi, M., Kaatz, G.W., Gibbons, S. *Phytochemistry* **2004**, *65*, 3249–3254.

67 Aoyagi, T., Yagisawa, M., Kumagai, M., Hamada, M., Okami, Y., Takeuchi T., Umezawa, H. *J. Antibiot*. **1971**, *24*, 860.

68 Liu, W.C., Astle, G., Wells, Jr. J.S., Treju, W.H., Principe, P.A., Rathnum, M.L., Parker, W.L., Kocy, R., Sykes, R.B. *J. Antibiot*. **1980**, *33*, 1256.

69 Song, Y., Sawa, T., Tsuchia, M., Horiuchi, Y., Kondo, S., Hamada, M. Umezawa, H. *J. Antibiot*. **1981**, *34*, 980.

70 Ikeda, Y., Kondo, S., Sawa, T., Tsuchia, M., Ikeda, D., Hamada, M., Takeuchi, T., Umezawa, H. *J. Antibiot*. **1981**, *34*, 1628.

71 Maddux, M. *Pharmacotherapy* **1991**, *11*, 40–50.

72 Blasquez, J., Baquero, M-R., Canton, I., Alos, I., Baquero, F. *Antimicrob. Agents Chemother*. **1993**, *37*, 2059–2063.

73 Chaibi, E.B., Sirot, D., Paul, G., Labia, R. *J. Antimicrob. Chemother*. **1999**, *43*, 447–458.

74 Vedel, G., Belaaouaj, A., Lilly, G., Labia, R., Philippon, A., Nevot, P., Paul, G. *J. Antimicrob. Chemother*. **1992**, *30*, 449–462.

75 Heritage, J.M., Zali, F.H., Gascoyne-Binzi, D., Hawkey, P.M. *J. Antimicrob. Chemother*. **1999**, *44*, 309–318.

76 Ito, H., Arakawa, Y., Ohsuka, S., Wacharotayankun, R., Kato, N., Ohta, M. *Antimicrob. Agents Chemother.* **1995**, *39*, 824–829.

77 Watanabe, M., Iyobe, S., Inoue, M., Mitsuhashi, S. *Antimicrob. Agents Chemother.* **1991**, *35*, 147–151.

78 Jiminez-Valera M., Ruiz-Bravo, A., Ramos-Cormenzana, R. *J. Antimicrob. Chemother.* **1987**, *19*, 31–37.

79 Zhao, W.H., Hu, Z.Q., Hara, Y., Shimamura T. *Antimicrob. Agents Chemother.* **2002**, *46*, 2266–2268.

80 Coates, N.J., Gilpin, M.L., Gwynn, M.N., Lewis, D.E., Milner, P.H., Spear, S.R., Tyler, J.W. *J. Nat. Prod.* **1994**, *57*, 654–657.

81 Liang, W.L., Huang, H.M., Lin, R.D., Hou, W.C. *Bot. Bull. Acad Sin.* **2003**, *44*, 187–191.

82 Bouttier, S., Fourniat, J., Garofalo, C., Gleye, C., Laurens, A., Hocquemiller, R. *Pharmaceut. Biol.* **2002**, *40*, 231–234.

83 Cole, M. Inhibition of beta lactamases. In *Beta Lacatamases*, Hamilton-Miller, J.M.T., Smith, J.T. (eds). Academic Press, London, **1979**, pp. 205–290.

84 Yam, T.S., Hamilton-Miller, J.M., Shah, S. *J. Antimicrob. Chemother.* **1998**, *42*, 211–216.

85 Didry, N., Dubreuil, L., Pinkas, M. *Phytother. Res.* **1993**, *7*, 21–24.

86 Iinuma, M., Tosa, H., Tanake, T., Miyauchi, K.I. *J. Pharm. Pharmacol.* **1996**, *48*, 861–865.

87 Eo, S.K., Kum, Y.S., Lee, C.K., Lee, D.I., Hun, S.S. *Yahhak Hoeji* **1996**, *40*, 653–559.

88 Sato, M., Tanaka, H., Yamaguchi, R., Kato, K., Etoh, H. *Int. J. Antimicrob. Agents* **2004**, *24*, 43–48.

89 Hill R.L.R., Duckworth, G.J., Casewell, M.W. *J. Antimicrob. Chemother.* **1988**, *22*, 377–384.

90 Cederna, J.E., Terpenning, M.S., Ensberg, M., Bradley, S.F., Kauffman, C.A. *Infect. Control Hosp. Epidemiol.* **1990**, *11*, 13–16.

91 Reagan, D, Doebbeling, B.N., Pfaller, M.A. et al. *Ann. Intern. Med.* **1991**, *114*, 101–106.

92 Casewell, M.W., Hill, R.L.R. *J. Antimicrob. Chemother.* **1987**, 19, 1–5.

93 Sato, M., Tsuchiya, H., Takase, I., Kureshiro, H., Tanigaki, S., Iinuma, M. *Phytother. Res.* **1995**, *9*, 509–12.

94 Nascimento, G.G.F., Lacatelli, J., Freitas, P.C., Silva, G. *Braz. J. Microbiol.* **2000**, *31*, 247–256.

95 Chattopadhyay, D., Dastidar, S.G., Chakrabarty, A.N. *Arzneimittelforschung* **1988**, *38*, 869–872.

96 Ahmad, I., Aqil, F. *Microbiol. Res.* **2006** (in press).

97 Aqil, F., Khan, M.S.A., Owais, M., Ahmad I. *J. Basic Microbiol.* **2005**, *45*, 114–123.

98 Sakagami, Y., Iinuma, Piyasena, K.G.N.P., Dharmaratne. H.R. *Phytomedicine* **2005**, *12*, 203–208.

99 Sakagami, Y., Kajimura, K., Wijesinghe, W.M., Dharmaratne, H.R. *Planta Med.* **2002**, *68*, 541–543.

100 Tiwari, R.P., Bharti, S.K., Kaur, H.D., Dikshit R.P., Hoondal, G.S. *Indian J. Med. Res.* **2005**, *122*, 80–84.

101 Zhao, W-H., Hu, Z-Q., Okubo, S., Hara, Y., Shimamura, T. *Antimicrob. Agents Chemother.* **2001**, *45*, 1737–1742.

102 Isogai, E., Isogai, H., Hirose, K., Hayashi, S., Oguma, K. *Curr. Microbiol.* **2001** *42*, 248–251.

103 Yu, H.H., Kim, K-J., Cha, J-D., Kim, H.-K., Lee, Y.-E., Choi, N-Y., You, Y-O., *J. Med. Food* **2005**, *8*, 454–461.

104 Mossa, J.S., El-Feraly, F.S., Muhammad, I. *Phytother. Res.* **2004**, *18*, 934–937.

105 Aburjai, T. Darwish, R.M., Al-Khalil, S., Mahafzah and A. Al-Abbadi J. *Ethnopharmacology* **2001**, *76*, 39–44.

106 Fernandes Jr. A., Balestrin, E.C., Betoni, J.E., Orsi, Rde. O., da Cunha, Mde. L., Montelli, A.C. *Mem. Inst. Oswaldo Cruz* **2005**, *100*, 563–566.

107 Stepanvoic, S., Antic, N., Dakic, I., Svabic-Vlahovic, M. *Microbiol. Res.* **2003**, *158*, 353–357.

108 Liu, X.I., Durham, D.G., Richards, R.M.E. *J. Pharm. Pharmacol.* **2000**, *52*, 361.

109 Arora, S., Dhillon, S., Rani, G., Nagpal, A. *Fitoterapia* **2004**, *75*, 385–388.

110 Ahmad, I. Studies on plasmid encoded virulence factors in the strains of *Escherichia coli* of man and animals: compatibility with R-plasmids. PhD thesis, Aligarh Muslim University, Aligarh, **1994**.

111 Rosenberg, M., Rotten, S., Rosenberg, E. *FEMS Microbiol. Lett.* **1982**, *13*, 167–169.

112 Balaban N, Goldkorn T, Nhan RT, Dang LB, Scott S, Ridgley RM, Rasooly A, Wright SC, Larrick JW, Rasooly R, Carlson JR. *Science* **1998**, *280*, 438–440.

113 Rozguyi, F. Ljungh, A., mamo, V., Hjerten, S., Wadstrom, T. Bacterial cell-surface hydrophobicity. In *Pathogenesis of Wound and Biomaterial Associated Infections*, Waadstrom T., Eliasson, I., Holder I., Ljungh, A. (eds). Springer-Verlag, London, **1990**, pp. 233–244.

114 Annuk, H., Hirmo, S., Turi, E., Mikelsaar, M., Arak, E., Wadstrom, T. *FEMS Microbiol. Lett.* **1999**, *172*, 41–45.

115 Turi, M., Turi, E., Koljalg, S., Mikelsaar, M. *APMIS* **1997**, *105*, 956–962.

116 Anwar, H., Dasgupta, M.K., Costerton, J.W. *Antimicrob. Agents Chemother.* **1990**, *34*, 2043–2046.

117 Finch, R.G., Pritchard, D.I., Bycroft, B.W., Williams, P., Stewart, G.S. *J. Antimicrob. Chemother.* **1998**, *42*, 569–571.

118 Habeck, M. *Drug Discov. Today* **2003**, *8*, 279–280.

119 Hentzer, M., Givskov, M. *J. Clin. Invest.* **2003**, *112*, 1300–1307.

120 Parsek, M.R., Val, D.L., Hanzelka, B.L., Cronan, J.E., Greenberg, E.P. *Proc. Natl Acad. Sci. USA* **1999**, *96*, 4360–4365.

121 Pearson, J.P., Van-Delden, C., Iglewski, B.H. *J. Bacteriol.* **1999**, *181*, 1203–1210.

122 Fuqua, W.C., Winans, S.C., Greenberg, E.P. *J. Bacteriol.* **1994**, *176*, 269–275.

123 Christensen, A.B., Riedel, K., Eberl, L., Flodgaard, L.R., Molin, S., Gram, L., Givskov. M. *Microbiology* **2003**, *149*, 471–483.

124 Hentzer, M., Riedel, K., Rasmussen, T.B., Heydorn, A., Andersen, J.B., Parsek, M.R., Rice, S.A., Eberl, L., Molin, S., Hoiby, N., Kjelleberg, S., Givskov, M. *Microbiology* **2002**, *148*, 87–102.

125 Schuster, M., Lostroh, C.P., Ogi, T., Greenberg, E.P. *J. Bacteriol.* **2003**, *185*, 2066–2079.

126 Smith, K.M., Bu, Y., Suga, H. *Chem. Biol.* **2003**, *10*, 81–89.

127 Reverchon, S., Chantegrel, B., Deshayes, C., Doutheau, A., Cotte-Pattat, N. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1153–1157.

128 Olsen, J.A., Severinsen, R., Rasmussen, T.B., Hentzer, M., Givskov, M., Nielsen, *J. Bioorg. Med. Chem. Lett.* **2002**, *12*, 325–328.

129 Chhabra, S.R., Stead, P., Bainton, N.J., Salmond, G.P., Stewart, G.S., Williams, P., Bycroft, B.W. *J Antibiot.* **1993**, *46*, 441–454.

130 Passador, L., Tucker, K.D., Guertin, K.R., Journet, M.P., Kende, A.S., Iglewski, B.H. *J. Bacteriol.* **1996**, *178*, 5995–6000.

131 Schaefer, A.L., Hanzelka, B.L., Eberhard, A., Greenberg, E.P. *J. Bacteriol.* **1996**, *178*, 2897–2901.

132 Zhu, J., Beaber, J.W., More, M.I., Fuqua, C., Eberhard, A., Winans, S. C. *J. Bacteriol.* **1998**, *180*, 5398–5405.

133 Smith, K. M., Bu, Y., Suga, H. *Chem. Biol.* **2003**, *10*, 563–571.

134 Givskov, M., de Nys, R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P.D., Kjelleberg, S. *J. Bacteriol.* **1996**, *178*, 6618–6622.

135 Dworjanyn, S.A., Steinberg, P. *Mar. Biol.* **1999**, *133*, 727–736.

136 Steinberg, P.D., Schneider, R., Kjelleberg, S. *Biodegradation* **1997**, *8*, 211–220.

137 Maximilien, R., de Nys, R., Holmstrom, C., Gram, L., Givskov, M., Kjelleberg, S., Steinberg, P. *Aquat. Microb. Ecol.* **1998**, *15*, 233–246.

138 Kjelleberg, S., Steinberg, P., Givskov, M., Gram, L., Manefield, M., de Nys, R. *Aquat. Microb. Ecol.* **1997**, *13*, 85–93.

139 Manefield, M., Harris, L., Rice, S.A., de Nys, R., Kjelleberg, S. *Appl. Environ. Microbiol.* **2000**, *66*, 2079–2084.

140 Rasmussen, T.B., Manfield, M., Anderson, B., Eberl, L., Antoni, C., Christophersen, P., Steinberg, S., Kjelleberg, S., Givskov, M. *Microbiology* **2000**, *187*, 3237–3244.

141 Hentzer, M., Wu, H., Anderson, B., Riededl, K., Rasmussen, T.B., Bagge, N., Kumar, N., Schembri, M.M., Song, Z., Kristoffersen, Manfield, M., Costerton, J.W., Moli, S., Eberl, L., Steinberg, P., Kjelleberg, S., Hoibi, N., Givskov, M. *EMBO J.* **2003**, *22*, 3803–3815.

142 Hoiby, N., Koch, C. *Thorax* **2000**, *55*, 349–350.

143 Hoiby, N. *Paediatr. Drugs* **2000**, *2*, 451–463.

144 Van Delden, C., Iglewski, B.H. *Emerg. Infect. Dis.* **1998**, *4*, 551–560.

145 Castang, S., Chantegrl, B., Deshayes, C., Dolmazon, R., Gouet, P., Haser, R., Reverchon, S., Nasser, W., Huguviex-Cotte-Pattat, N., Doutheau, A. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5145–5149.

146 Rasmussen, T.B., Bjarnsholt, T., Skindersoe, M.E., Hentzer, M., Kristoffersen, P., Kote, M., Nielsen, J., Eberl, L., Givskov, M. *J. Bacteriol.* **2005**, *187*, 1799–1814.

147 Persson, T., Hansen, T.H., Rasmussen, T.B., Skinderso, M.E., Givskov, M., Nielsen, J. *Org. Biomol. Chem.* **2005**, *21*, 253–262.

148 Bjarnsholt, T., Jensen, P.O., Burmolle, M., Hentzer, M., Haagensen, J.A., Hougen, H.P., Calum, H., Madsen, K.G., Moser, C., Molin, S., Holby, N., Givskov, M. *Microbiology* **2005**, *151*, 373–383.

149 Jones, M. B., Jani, R., Ren, D., Wood, T. K., Blaser, M.J. *J. Infect. Dis.* **2005**, *191*, 1881–1888.

150 Limsong, J., Benjavongkulchai, E., Kuvatanasuchati, J. *J. Ethnopharmacol.* **2004**, *92*, 281–289.

151 Koo, H., Gomes, B.P., Rosalen, P.L., Ambrosano, G.M., Park, Y.K., Cury, J. A. *Arch. Oral Biol.* **2000**, *45*, 141–148.

152 Nostro, A., Cannatelli, M.A., Crisafi, G., Musolino, A.D., Procopio, F., Alonzo, V. *Lett. Appl. Microbiol.* **2004**, *38*, 423–427.

153 Jagtap, A.G., Karkera, S.G. *J. Pharm. Pharmacol.* **2000**, *52*, 235–242.

154 Yatsuda, R., Rosalen, P.L., Cury, J.A., Murata, R.M., Rehder, V.L., Melo, L.V., Koo, H. *J. Ethnopharmacol.* **2005**, *97*, 183–189.

155 Mclean, R.J.C., Pierson, L.S., Fuqua, C. *J. Microbiol. Methods* **2004**, *58*, 351–360.

156 Dzidic, S., Bedekovic, V. *Acta Pharmacol. Sin.* **2003**, *24*, 519–526.

157 Heinmann, J.A. *Drug Discov. Today* **1999**, *4*, 72–79.

158 Tan Yu-Ting, Tillett, D.J., McKay, I.A. *Mol. Med. Today* **2000**, *6*, 309–314.

159 Goldgur, Y., Craigie, R., Cohen, G.H., Fujiwara, T., Yoshinaga, T., Fujishita, T., Sugimoto, H., Endo, T., Murai, H., Davies, D.R. *Proc. Natl Acad. Sci. USA* **1999**, *96*, 13040–13043.

160 Debbia, E.A., Massaro, S., Campora, U., Schito, G.C. *New Microbiol.* **1994**, *17*, 65–68.

161 Weisser, J., Wiedemann, B. *Antimicrob. Agents Chemother.* **1987**, *30*, 531–534.

162 Hooper, D.C., Wolfson, J.S., Tung, C., Souza, K.S., Swartz, M.N. *J. Bacteriol.* **1989**, 2235–2237.

163 Zahin, M. MSc dissertation, Department of Agricultural Microbiology, AMU, Aligarh. **2005**.

164 Weisser, J., Wiedemann, B. *Antimicrob. Agents Chemother.* **1985**, *28*, 700–702.

165 Weisser, J., Wiedemann, B. *J. Antimicrob. Chemother.* **1986**, *18*, 575–583.

166 Ahmad I, Ahmad, S., Yadava, J.N.S. *Indian Vet. Med. J.* **1993**, *17*, 115–122.

167 Hirota, Y., Lijima, T. *Nature* **1957**, *180*, 655–656.

168 Hirota Y. *Proc. Natl Acad. Sci. USA* **1960**, *46*, 57–64.

169 Jacob, F., Brenner, S., Cuzin, F. *Cold Spring Harb. Symp. Quant. Biol.* **1963**, *28*, 329.

170 Singh, M., Yadava, J.N.S. *Indian J. Exp. Biol.* **1988**, *26*, 668–670.

171 May, J.W., Houghton, R.H. Perret, C.J. *J. Gen. Microbiol.* **1964**, *37*, 157–169.

172 Pinney, R.J., Smith, J.T. *Genetic Res. Commun.* **1971**, *18*, 193–199.

173 Yadava, J.N.S., Ahmad, I., Ahmad, S., Bhradwaj, K.R. *Indian Vet. Med. J.* **1992**, *16*, 260–267.

174 Rownd, R., Nakaya, R., Nakamura, A. *J. Mol. Biol.* **1966**, *17*, 376–393.

175 Wamburkar, M.N., Panse, M.V. *Hind. Ant. Bull.* **1982**, *64*, 29–33.

176 Toh-E, A., Wickner, R. B. *J. Bacteriol.* **1981**, *145*, 1421–1424.

177 Novie, R.P., Sanchez-Rivas, C., Gruss, A., Edelman, J. *Plasmids* **1980**, *3*, 348–358.

178 Beg, A.Z., Ahmad, I. *World J. Microbiol. Biotechnol.* **2000**, *16*, 841–844.

179 Chopade, B.A, Patwardhan, R.B., Vaidy, V.C., Khairnar, S., Dhakephalkar, P.K., Padhye, S.B. Elimination of antibiotic and metal resistance plasmids in human pathogenic acinetobacter species. In *Tropical Diseases, Molecular Biology and Control Strategies*, Kumar, S., Sen, A.K., Dutta, G.P., Sharma, R.N. (eds). CSIR India, **1994**, pp. 695–703.

180 DeNap, J.C.B., Thomas, J.R., Musk, D.J., Hergenrother, P.J. *J. Am. Chem. Soc.* ASAP **2004**.

10
Activity of Plant Extracts and Plant-Derived Compounds against Drug-Resistant Microorganisms

Antonia Nostro

Summary

The inappropriate and indiscriminate use of antibiotics exerts a selective pressure among bacteria, encouraging the appearance of drug-resistant strains. This is an issue of major concern, especially in medical microbiology, because of the increasing incidence of multiresistant bacterial infections caused by Gram-positive bacteria (such as *Staphylococcus*, *Enterococcus*, and *Streptococcus* species) and Gram-negative bacteria (such as *Pseudomonas*, the *Enterobacteriaceae*, and *Helicobacter pylori*). The severity of this problem is further potentiated by the high prevalence of other drug-resistant organisms such as *Mycobacterium tuberculosis*, the main organism responsible for tuberculosis, and fungi also an important cause of morbidity, particularly in patients with an impaired immunological system.

In this context, it is necessary to find alternative strategies or more effective agents exhibiting activity against drug-resistant pathogens. Natural drugs could represent an interesting approach to limit the emergence and spread of these organisms, which are currently difficult to treat. Recently, scientific interest in the study of plant materials as sources of new compounds for processing into therapeutic agents has increased considerably. Medicinal plants have been used for centuries in folk medicines as remedies for human diseases, because they contain components of therapeutic value. Much attention has been focussed on the study of plant extracts and a large number of papers on their *in vitro* antimicrobial properties have been published. This review reports the findings from an extensive literature search of plant extracts/phytochemicals that have have been tested for activity against drug-resistant strains or that can act as antibiotic resistance inhibitors.

10.1
Introduction

The discovery of antibiotics was a great advance in modern medicine, leading to a considerable reduction of the morbidity and mortality from infectious diseases. In

spite of this, widespread overprescribing and inappropriate use of antibiotics have led to the development of resistance in previously susceptible organisms. Other factors contributing to the emergence of resistance include the uncontrolled use of antibiotics in animal husbandry and agriculture or within farm animals [1]. The major mechanisms by which bacteria overcome drug action include intrinsic impermeability or alterations in the bacterial outer membrane, extrusion of drugs from cells by multidrug resistance (MDR) pumps, the production of drug-inactivating enzymes, and modification of target [1]. Many of these mechanisms result from genetic mutations, acquisition of genes from other microorganisms and combinations of these two types of events.

Antibiotic resistance is a cause of major concern, especially in medical microbiology, because of the increasing incidence of multiresistant bacterial infections caused by Gram-positive bacteria (e.g. *Staphylococcus*, *Enterococcus*, and *Streptococcus* species), Gram-negative bacteria (e.g. *Pseudomonas*, the *Enterobacteriaceae*, and *Helicobacter pylori*), mycobacteria, and fungi.

In this context, it is necessary to find alternative strategies or more effective agents exhibiting activity against drug-resistant pathogens. Natural drugs could represent an interesting approach to limit the emergence and the spread of these organisms, which are currently difficult to treat. Recently, the scientific interest in the study of plant materials as sources of new compounds for their processing into therapeutic agents has increased considerably. Medicinal plants have been used for centuries in folk medicines as remedies for human diseases, and many studies have been carried out in order to find out the scientific basis for their effectiveness [2, 3]. The main active antimicrobial agents isolated include alkaloids, phenolic acids, quinones, tannins, coumarins, flavonoids, terpenoids, and essential oils. However, only a small proportion of plant species have been thoroughly investigated for their medicinal properties and undoubtedly there are other many biologically active new compounds to be discovered.

This review reports the findings from an extensive literature search of plant extracts/phytochemicals that have been tested for activity against drug-resistant strains or can act as antibiotic resistance inhibitors. They are divided into three categories: (1) plant materials with general antimicrobial activity against different microorganisms including some drug-resistant strain, (2) plant materials with specific antimicrobial activity against drug-resistant strains, (3) plant materials which restore the effectiveness of antimicrobial agents and/or inhibit drug resistance mechanisms.

10.2 Plant Materials with General Antimicrobial Activity Including some Drug-Resistant Strains

Many papers have been published on the study of plant materials with general antimicrobial activity against various microorganisms, including some drug-resistant strains. Some papers focus on a specific plant, and report the nature of the constit-

uents likely to be responsible for the activity [4–18]. Others screen several plant species chosen because of their peculiar characteristics, such as traditional medicinal use, native area location, or source. Examples are studies of plants from Argentina [19], northern Argentina [20], British Columbia [21], Palestine [22], Scotland [23], the island of Soqotra [24], as well as essential oils from commercial sources [25, 26]. Most of these papers report the activity of crude extracts evaluated by disk diffusion and/or minimum inhibitory concentration (MIC) methods. Only a few describe the effects of pure compounds isolated from the active plants or choose one particular plant with significant antibacterial activity and study its activity in combination with antibiotics [20].

10.3 Plant Materials with Specific Antimicrobial Activity Against Drug-Resistant Strains

Much attention has been focussed on the study of plant materials in order to find molecules with activity against strains having a particular significance for pathogenesis, such as drug-resistant microorganisms. However, the papers published are far too many to be dealt with and therefore they have been subdivided according to drug-resistant organisms for more detailed analysis.

10.3.1 Drug-Resistant Gram-Positive Bacteria

Antibiotic resistance is a cause of major concern, especially in hospitals where patients are vulnerable to infection. Methicillin-resistant *Staphylococcus aureus* (MRSA) as well as various vancomycin-resistant enterococci bacteria (VRE) and *Streptococcus pneumoniae* with intermediate or high-level resistance to penicillin or third-generation cephalosporins are responsible for one-third of nosocomial infections [27]. In particular, the emergence of MRSA strains has become a global health problem because it has been observed worldwide in hospitalized patients [28] and in children and adults in their daily life [29]. Infections caused by MRSA have become a therapeutic problem because these organisms are resistant not only to β-lactams but also to many other antimicrobial agents, including vancomycin (one of the powerful antibiotics available for severe MRSA infections) [30] and the recently developed oxazolidinone- and streptogramin-type antibiotics.

Plant extracts/phytochemicals with antimicrobial activity against drug-resistant Gram-positive bacteria could potentially be a way of controlling these strains that are currently difficult to treat. Table 10.1 summarizes the literature data and the major information about active plants and plant-derived products against these specific drug-resistant strains.

One essential oil that is particularly well known because it has been widely explored as an alternative agent against MRSA, is tea tree oil [31]. Carson [31] reported MIC values and minimum bactericidal concentration (MBC) values equal to 0.25% and 0.50% respectively for 64 MRSA strains. Tea tree oil is derived by steam

Table 10.1 Activity of plants extracts/phytochemicals against drug-resistant Gram-positive strains.

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity[a]*	*Method[b]*	*Reference*
Acacia aroma Gill.	Leaf, flower	Aqueous and ethanol extracts	MRSA ($n=12$), MRCNS strain, ampicillin-resistant *Enterococcus* sp. ($n=6$)	D, MIC, MBC, PS	42
Acacia kempeana	Leaf	Extract	VRE	D, TKA	43
Acalypha wilkesiana Muell. Arg.	Leaf	Aqueous and ethanol extracts	MRSA ($n=23$)	D, MIC, PS	44
Acorus calamus L.	Rhizome	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Allium sativum L.	Bulb	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
		Aqueous extract	Multidrug resistant *S. aureus*, *S. epidermidis*, *S. pneumoniae*, *S. pyogenes*	D, MIC	46
		Mashed, filtered and freeze-dried, allicin	VRE:*E. faecium* F346, BM4147, KH5V, KH16V, KS19V, KS31V, KS32V, F7, F16, F29, F52, F163, F199; *E. faecalis* V583; *E. durans* KH2V, KH32V	MIC, SY	47
		Water extract, diallyl sulfide diallyl disulfide	MRSA ($n=16$)	AS	48,
		Essential oil, diallyl sulfides: diallyl sulfide, diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide	MRSA ($n=60$)	MIC	49
Aloysia triphylla Royle	Leaf	Methanol extract	MRSA ($n=2$)	MIC, S	50
Amyema quandong	Leaf	Extract	MRSA, VRE	D, TKA	43
Angelica dahurica Benth. & Hook. f.	Root	Polyacetylenic product: falcarindiol	*S. aureus* EMRSA-15 and multi-drug resistant *S. aureus*: XU-212, RN-4220, SA-1199B	MIC, BF, S	51

Table 10.1 Activity of plants extracts/phytochemicals against drug-resistant Gram-positive strains. (Continued)

Plant/natural product	Parts used	Active extract(s)/compound(s)	Activity[a]	Method[b]	Reference
Anogeissus leiocarpa Guill. & Perr.	Root	Aqueous, methanol, chloroform, ether, butanol extracts	MRSA ($n=2$), VRE ($n=1$)	D	52
Artemisia gilvescens	Aerial	Sesquiterpenoids	MRSA	MIC, CA, HPLC, S	53
Azadirachta indica A. Juss.	Bark	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Beta vulgaris L.	Root	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Bixa orellana L.	Seed	Methanol extract	MRSA ($n=2$)	MIC, S	50
Bobgunnia madagascariensis	Root	Ethanol extract	Drug-resistant: MRSA ($n=10$), *E. faecalis* ($n=7$)	MBC	54
Bursera simaruba Serg.	Leaf, stem	Methanol extract	MRSA ($n=2$)	MIC, S	50
Caesalpinia sappan L.		Methanol, chloroform, butanol, aqueous extracts	MRSA ($n=13$)	D, MIC, CTA, PS	55
	Wood	Brasilin	MRSA ($n=2$) and VRE ($n=2$)	MIC, TKA, IRS, BF	56
Calophyllum brasiliense Camb.	Leaf, wood	Acetone, hexane, methanol extracts. Coumarins and xanthones	MRSA ($n=2$)	MIC, S	50
Calophyllum moonii	Bark	Calozeyloxanthone	VRE ($n=2$)	MIC, SY	57
Calophyllum species	Plant	Calozeyloxanthone	MRSA ($n=17$)	MIC, C, S	58
Camellia sinensis L.	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
		Ethanol extract and fractions	MRSA ($n=4$)	D, MIC, TLC, B, SY	59
		Compound P	MRSA	EM	39
		Extract, EGCg, theaflavin digallate	MRSA	TKA	60
		Aqueous extract and fractions, pure compounds	MRSA ($n=18$), penicillin resistant *S. pneumoniae*	MIC, MBC	61
Carpobrotus edulis L.	Leaf	Methanol extract	MRSA ($n=2$)	IPB	62
Casuarina equisetifolia Forst. f	Leaf, bark	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Cinnamomum verum		Essential oil	Drug-resistant *Staphylococcus coagulase* negative, *S. aureus*, *S. pneumoniae*, *Enterococcus* spp.	D	63

Table 10.1 Activity of plants extracts/phytochemicals against drug-resistant Gram-positive strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Cissus populnea Guill. & Perr.	Root	Ethanol extract	Drug-resistant: MRSA ($n=9$), *E. faecalis* ($n=8$)	MBC	54
Citrus sinensis L.	Rind	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Cordia dichotoma L.	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Croton draco Schlecht.	Leaf	Methanol extract	MRSA ($n=2$)	MIC, S	50
Cudrania cochinchinensis	Root	Xanthones: gerontoxanthone H, 1,3,7-trihydroxy-2-prenylxanthone, gerontoxanthone I, alvaxanthone, isoalvaxanthone	VRE (VanA, VanB, and VanC phenotypes)	MIC	64
Dalea scandens Miller	Root	Flavonoids: tetrahydroxy-flavanone, trihydroxyflavanone and tetrahydroxyflavone	MRSA	I, BF, S	65
Delonix regia Raf.	Flower	Ethanol extract and fractions	MRSA ($n=4$)	D, MIC, TLC, B	59
Emblica officinalis Gaertn.	Fruit	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Eremophila alternifolia	Leaf	Extract	MRSA	D, TKA	43
Eremophila duttonii	Leaf	Extract	MRSA, VRE	D, TKA	43
Erythrina poeppigiana O.F. Cook	Root	Isoflavonoids (erypoegin A, demethylmedicarpin, sandwicensin), ethyldeoxybenzoin (angolesin), cinnamylphenol (erypostyrene)	MRSA ($n=13$)	MIC, VC, S	66
		Isoflavonoid: 3,9-dihyroxy-10-γ,γ-dimethylallyl-6a,11a-dehydropterocarpan	MRSA ($n=13$)	MIC, MBC, LAS, IRS, S	67

Table 10.1 Activity of plants extracts/phytochemicals against drug-resistant Gram-positive strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Erythrina senegalensis DC.	Root	Ethanol extract	Drug-resistant: MRSA ($n=10$), *E. faecalis* ($n=7$)	MBC	54
Erythrina variegata L.	Root	Isoflavanone: bidwillon B	MRSA ($n=12$)	MIC, MBC, SY, IRS, S	68
	Root	Isoflavonoids: erycristagallin, orientanol B, orientanol C, orientanol F, 2-(γ,γ-dimethyl-allyl)-6a-hydroxyphaseollidin	MRSA	MIC, S	69
Erythrina zeyheri	Root	Isoflavonoids erybraedin A and eryzerin C	MRSA ($n=13$), VRE ($n=4$)	MIC, SY	70
		Isoflavonoids eryzerin A,C, D, and E	MRSA ($n=13$)	MIC, S	71
Eucalyptus sp.	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Ficus religiosa L.	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Ficus thonningii A. Rich.	Leaf	Ethanol extract	Drug-resistant *E. faecalis* ($n=8$)	MBC	54
Garcinia dioica		Rubraxanthone	MRSA	MIC, BF	72
Garcinia kola Heckel	Root	Aqueous, methanol, ether extracts	MRSA ($n=2$), VRE ($n=3$)	D	52
Garcinia mangostana L.	Fruit, bark	Xanthone: α-mangostin	MRSA	MIC BF	72
		α-Mangostin	MRSA, VRE	MIC, SY	73
		Ethanol extracts	MRSA ($n=35$)	D, MIC	74
Glycyrrhiza glabra L.		Flavonoids: glabridin, glabrene	MRSA	MIC	75
Glycyrrhiza inflata		Flavonoids: licochalcones A	MRSA	MIC	75
Glycyrrhiza uralensis DC.		Flavonoids: licoisoflavone B, licoricidin, isolicoflavonol, glyasperin D, gancaonin I	MRSA	MIC	75
Haematoxylum brasiletto Karst.	Stem	Methanol extract	MRSA ($n=2$)	MIC, S	50

Table 10.1 Activity of plants extracts/phytochemicals against drug-resistant Gram-positive strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Helichrysum italicum G. Don	Flower	Diethyl ether extract	MRSA ($n=9$)	MIC, TKA, IE	41
Hemidesmus indicus R. Br.	Root	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Holarrhena anti-dysenterica R.	Bark	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
			MRSA ($n=4$)	D, MIC, TLC, B	59
Hypericum species: *acmosepalum, addingtonii, androsaemum* L., *arnoldianum, beanii, bellum, calycinum* L., *curvisepalum, dummeri, foliosum, forrestii, frondosum, hidecote, hircinum, hookerianum, kouytchense, lagarocladum, lancasteri, maclarenii, maculatum, moserianum, olympicum, patulum, prolificum, pseudohenryi, reptans, revolutum, stellatum, subsessile, xylosteifolium*	Aerial	Chloroform and methanol extracts	MRSA XU212	D, MIC	76
Hyptis pectinata Poit.	Aerial	Pyrones; pectinolides A–C (1–3), pectinolide H (4): 2(5H)-furanone	*S. aureus* EMRSA-15 and multidrug resistant *S. aureus*: XU-212, 1199B	MIC, CA, BF	77
Juniperus communis		Essential oil	MRSA	D, MIC, GC	78
			MRSA ($n=15$), VRE ($n=5$)	MIC, MBC	79
Keetia hispida Bridson	Leaf	Ethanol extract	Drug-resistant *E. faecalis* ($n=8$)	MBC	54
Khaja senegalensis A. Juss.	Stem, bark	Ethanol extract	Drug-resistant *E. faecalis* ($n=8$)	MBC	54
Lannea acida A. Rich.	Root	Ethanol extract	Drug-resistant: MRSA ($n=9$), E. *faecalis* ($n=8$)	MBC	54
Lantana camara L.	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Lavandula angustifolia Mill.		Essential oil	MRSA ($n=15$), VRE ($n=5$)	MIC, MBC	79
Lawsonia inermis L.	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
			MRSA ($n=4$)	D, MIC, TLC, B, SY	59

Table 10.1 Activity of plants extracts/phytochemicals against drug-resistant Gram-positive strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Lepidosperma viscidum	Stem	Extract	MRSA, VRE	D, TKA	43
Mammea americana L.	Fruit, seed	Acetone, ethyl acetate, hexane, methanol extracts. Coumarin	MRSA ($n=2$)	MIC, S	50
Melaleuca alternifolia Cheel		Essential oil	MRSA	MIC, MBC	80
			MRSA ($n=64$)	MIC, MBC	31
			MRSA		81
			MRSA	D, VA	37
			MRSA ($n=20$)	MIC	82
			MRSA ($n=15$), VRE ($n=5$)	MIC, MBC	79
			MRSA ($n=2$), vancomycin-resistant *E. faecium* ($n=3$) and *E. faecalis* ($n=1$)	TKA	40
Mentha × *piperita* L.		Essential oil	MRSA ($n=15$), VRE ($n=5$)	MIC, MBC	79
Morus alba L.	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Nelumbo nucifera Gaertn.	Flower	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Nepeta cataria L.	Plant	Diethyl ether extract	MRSA ($n=12$)	IE , IA	83
Nigella sativa L.	Seed	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
		Alkaloid and aqueous extracts	Multidrug resistant Gram-positive bacteria	I	84
Nyctanthes arbor-tristis	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Ochna macrocalyx	Bark	Biflavonoids calodenin B, dihydrocalodenin B	Multidrug resistant (MDR) strain of *S. aureus*: RN4220, XU-212, 1199B	MIC	85
Ocimum basilicum L.	Aerial	Essential oil	Multidrug-resistant *S. aureus* ($n=3$), *S. epidermidis* ($n=2$), *E. faecalis* ($n=2$)	MIC, TKA, GC	86
Ocimum gratissimum L.	Leaf	Aqueous and ethanol extracts	MRSA ($n=23$)	D, MIC, PS	44
Ocimum sanctum L.	Plant	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45

Table 10.1 Activity of plants extracts/phytochemicals against drug-resistant Gram-positive strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Olea europaea L.		α,β-Unsaturated aldehydes: (*E*)-2-eptenal, (*E*)-2-nonenal, (*E*)-2-decenal, (*E,E*)-2,4-decadienal	MRSA ($n=11$), erythromycin-resistant *S. pyogenes* ($n=15$)	D, MIC	87
Origanum vulgare L.		Essential oil	Drug-resistant *Staphylococcus coagulase* negative, *S. aureus*, *S. pneumoniae*, *Enterococcus* spp.	D	63
			MRSA ($n=9$)	MIC	38
Phillantus discoideus Muell.-Arg.	Bark	Aqueous and ethanol extracts	MRSA ($n=23$)	D, MIC, PS	44
Pinus nigra J.F. Arnold	Cone	Diterpene isopimaric acid	*S. aureus* EMRSA-15, EMRSA-15 and multidrug-resistant strain of *S. aureus*: XU-212, 1199B, RN4220	MIC, S	88
Plant-derived compounds		2-Arylbenzofurans and isoflavone	VRE (VanA, VanB, and VanC phenotypes), MRSA	MIC	89
		Flavanones	MRSA	MIC	90
		Flavonols: myricetin, datiscetin, kaempferol, quercetin. Flavones: flavone, luteolin	MRSA ($n=2$), VRE	D, MIC	91
Plumbago zeylanica L.	Root	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Propolis		Ethanol extracts	Multidrug-resistant *S. aureus* ($n=10$), *Enterococcus* spp. ($n=17$)	D, MIC, MBC, TLC, B, HPLC	92
		Flavonoid galangin	Multidrug-resistant *S. aureus* ($n=4$), *S. sciuri*, *S. epidermidis*, *S. xylosus*, *E. faecalis*	D, MIC, SY	93
Psidium guajava L.	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Punica granatum L.	Rind	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
	Fruit	Ethanol extract and fractions	MRSA ($n=4$)	D, MIC, TLC, B, SY	59
		Ethanol, hexane, dichloro-methane, chloroform, ethyl acetate, butanoland water extracts. Ellagitannins	MRSA ($n=16$)	D, MIC	94
		Aqueous and ethanol extracts	MRSA ($n=35$)	D, MIC	74

Table 10.1 Activity of plants extracts/phytochemicals against drug-resistant Gram-positive strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Quecus infectoria Oliv.		Aqueous and ethanol extracts	MRSA ($n=35$)	D, MIC	74
Rosmarinus officinalis L.	Herb	Carnosic acid, carnosol, 4′,7-dimethoxy-5-hydroxyflavone, 12-methoxytranscarnosic acid	Multidrug-resistant (MDR) strain of *S. aureus*: RN4220, XU-212, 1199B	MIC, BF	95
Sapindus sp.	Fruit	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Satureja cuneifolia	Plant	Essential oil	Multidrug-resistant: VRE MB 5571, MRSA MB 5393	MIC, GC/MS	96
Satureja montana L.	Plant	Essential oil	Multidrug-resistant: MRSA MB 5393, VRE MB 5571,	MIC, GC/MS	96
Saussurea lappa C.B. Clarke	Root	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Scutellaria barbata D. Don	Aerial	Diethyl ether extract. Flavonoids: apigenin, luteolin	MRSA ($n=15$)	D, MIC, TLC, HPLC	97
		Essential oil	MRSA	MIC, MBC, GC/MS	17
Sophora alopecuroides L.	Root	Flavanostilbenes	MRSA ($n=21$)	MIC	98
Sophora esigua		Chromatographic fractions, exiguaflavanone B and D	MRSA	MIC	99
Sorindeia warneckei	Stem	Aqueous extract	MRSA ($n=2$)	D	52
Syrgium aromaticum L.	Bud Oil	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Syzygium cumini L.	Bark, Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Tabebuia avellanedae	Wood	Ethanol, hexane, dichloro-methane, chloroform, ethyl acetate, butanoland water extracts. Naphthoquinones α-lapachone I	MRSA ($n=16$)	D, MIC	94
Terminalia arjuna W. & A.	Bark	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Terminalia avicennioides Guill.	Bark	Aqueous and ethanol extracts	MRSA ($n=23$)	D, MIC, PS	44
Terminalia bellerica Roxb.	Fruit	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
			MRSA ($n=4$)	D, MIC, TLC, B, SY	59
Terminalia chebula Retz.	Fruit	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
		Gallic acid and its ethyl ester	MRSA ($n=4$)	D, MIC, TLC, B, SY	59
			MRSA	I, S	100

Table 10.1 Activity of plants extracts/phytochemicals against drug-resistant Gram-positive strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Terminalia glaucescens	Root	Aqueous extract	MRSA ($n=2$), VRE ($n=1$)	D	52
Thymus vulgaris L.		Essential oil	Drug-resistant *Staphylococcus coagulase* negative, *S. aureus*, *S. pneumoniae*, *Enterococcus* spp.	D	63
			MRSA ($n=15$), VRE ($n=5$)	MIC, MBC	79
Uapaca togoensis Pax.	Leaf	Ethanol extract	Drug-resistant *E. faecalis* ($n=8$)	MBC	54
Vitex doniana Sweet.	Root	Aqueous, methanol, ether, butanol extracts	MRSA ($n=2$)	D	52
Vitex rotundifolia	Plant	Vtrofolal C, D, detetrahydro-conidendrin	MRSA ($n=8$)	D, MIC, S	101
Vitis vinifera L.	Leaf	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45
Waltheria lanceolata R. Br. Ex Mast.	Root	Ethanol extract	Drug-resistant: MRSA ($n=10$), *E. faecalis* ($n=8$)	MBC	54
Xanthium sibiricum Patr er Widd	Leaf	Sesquiterpene lactone: xanthatin	MRSA		102
Ximenia americana L.	Root	Ethanol extract	Drug-resistant: MRSA ($n=9$), *E. faecalis* ($n=8$)	MBC	54
Zanthoxylum clava-herculis L.	Bark	Benzo[c]phenanthridine alkaloid chelerythrine	Multidrug-resistant *S. aureus*: RN-4220, XU-212, 1199B	MIC, BF	103
Ziziphus jujuba L.	Leaf Bark	Ethanol extract	Multidrug-resistant *S. aureus*	D, TLC, B	45

[a] *E. durans*, *Enterococcus durans*; *E. faecalis*, *Enterococcus faecalis*; *E. faecium*, *Enterococcus faecium*; MRCNS, methicillin-resistant coagulase negative staphylococci; MRSA, methicillin-resistant *Staphylococcus aureus*; *S. aureus*, *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*; *S. pneumoniae*, *Streptococcus pneumoniae*; *S. pyogenes*, *Streptococcus pyogenes*; *S. sciuri*, *Staphylococcus sciuri*; *S. xylosus*, *Staphylococcus xylosus*; VRE, vancomycin-resistant enterococci.

[b] AS, animal studies; B, bioautography; BF, bioassay fractionation; C, chromatography; CA, cytotoxic assay; CTA, chequerboard titration assay; D, diffusion; EM, electron microscopy; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; I, inhibition; IA, inhibition adherence; IE, inhibition enzymes; IPB, inhibition phagogytosed bacteria ; IRS, inhibition radiolabeled substances; LAS, leakage absorbing substances; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; PS, phytochemical screening; S, spectroscopy; SY, synergism; TKA, time kill assay; TLC, thin-layer chromatography; VA, vapor assay; VC, viable count.

distillation from the leaves of *Melaleuca alternifolia* and contains approximatively 100 terpenes and their related alcohols. The popularity of tea tree oil has come mainly from its biological activities, such as its antibacterial, antifungal, antiviral, anti-inflammatory, and analgesic properties [32]. Interestingly, tea tree oil is active both in eliminating the flora associated with transient carriage and in maintaining commensal skin organisms [33]. Although the data for well-designed randomized clinical trials of tea tree oil are lacking [34], many papers have suggested its role in the treatment of cutaneous infection and in the decolonization of MRSA carriers [35, 36].

In vitro evidence indicates that other essential oils can act as antimicrobial agents against MRSA. Interestingly, the effects of patchouli, geranium, lavender, and again tea tree oil were studied either by contact or in the vapor phase, and the most inhibitory combinations of oils were used in a dressing model [37]. *Origanum vulgare* essential oil is another very versatile plant oil, known for a long time as a popular remedy, but only recently recognized for its potential therapeutic roles such as diaphoretic, antispasmodic, and antiseptic. Although its composition can differ even within plants of the same species, the chemical constituents are mainly phenolic derivatives such as carvacrol, thymol, and their precursors *p*-cymene and γ-terpinene. We confirmed a relevant and broad spectrum of antimicrobial activity of oregano oil against standard organisms and staphylococci isolates, characterized as methicillin-sensitive or methicillin-resistant by polymerase chain reaction (PCR) for *mec*A gene detection [38]. The antibacterial activity of the oregano oil can be attributed to a considerable degree to the existence of carvacrol and thymol, which appear to possess similar activities against all the tested bacteria (MIC values 0.015–0.125%, v/v).

Only a small proportion of studies delineate the possible mechanisms of action or study the time kill rates [39, 40] or reveal the effects of plant extracts on virulence factors. Our research team has selected *Helichrysum italicum* G. Don (Compositae), a plant native to Europe and widespread in the Mediterranean regions, rich in flavonoids and terpenes. More specifically, we demonstrated that *H. italicum* has antimicrobial activity mostly on *S. aureus* isolates, including methicillin-resistant strains (MIC values for 50% of the strains tested were 0.25 mg L^{-1}). Moreover, at sub-minimum inhibitory concentrations (sub-MICs) it interferes with some of the virulence factors of MRSA strains, such as coagulase, DNase, lipase, and thermonuclease enzymes [41]. The components responsible for the observed activity are flavonoids and terpenes which showed bioautographic well-defined inhibition bands.

10.3.2
Drug-Resistant Gram-Negative Bacteria

Multiple reports have revealed the prevalence of antibiotic resistance among Gram-negative isolates, which may become particularly dangerous pathogens because of the emergence of extended-spectrum β-lactamase and carbapenemase-producing organisms [104]. Among these are *Pseudomonas aeruginosa*, the most notorious bacterium for its intrinsic resistance to multiple classes of antibiotics

and its ability to acquire adaptive resistance, and bacteria with the potential for causing epidemic diseases as *Salmonella typhi* and *Shigella* spp. *Helicobacter pylori*, moreover, is the most common organism associated with gastrointestinal bacterial diseases and with gastric malignancies, and is difficult to eradicate because of its increasing antibiotic resistance.

Table 10.2 summarizes the literature data on the activity of plant extracts and plant-derived products against drug-resistant Gram-negative bacteria. Plant materials with activity specifically targeted against antibiotic-resistant strains and with other biological activities that could potentiate their therapeutic role provide examples of natural products that are worthy of further investigation. In this context, we studied propolis and *Zingiber officinale* extracts with multiple biological properties such as anti-inflammatory, antioxidant, hepatoprotective, antitumoral, and antimicrobial activities. Propolis and *Z. officinale* have shown specific antibacterial activity against rifabutine-, tinidazole-, and clarithromycin-resistant *H. pylori* strains (isolates from antral mucosal biopsies of patients with chronic gastritis or duodenal ulcer) with MIC values equal to 0.075–0.3 and 0.075–0.6 mg mL^{-1} respectively [105]. Equally interesting are plants that have activity against bacteria belonging to different genera. Examples are plants with traditional medicinal roles that have been scientifically documented such as *Allium sativum* (garlic), *Glycyrrhiza* sp. and *Melaleuca alternifolia* (Tables 10.1 and 10.2). Water and alcoholic extracts of *A. sativum* or its components, such as allicin, possess antimicrobial activity against a wide range of Gram-negative and Gram-positive bacteria, mycobacteria, and fungi. The main mechanism of its antibacterial activity is assumed to be the inhibition of thiol-containing enzymes [106]. However, clinical trials focussed on the eradication of *H. pylori* recorded failure [107].

10.3.3
Other Drug-Resistant Microorganisms

The severity of the antibiotic-resistance problem is further potentiated by a high prevalence of other drug-resistant organisms such as *Mycobacterium tuberculosis*, the main organism responsible for tuberculosis, fungi and also an important cause of morbidity, particularly in patients with an impaired immunological system.

Multidrug-resistant (MDR) tuberculosis is another example in which antibiotic options are nearly exhausted and is a further problem because it is one of the most frequent opportunistic infections in people with human immunodeficiency virus (HIV) infection. For fungal infections, in addition to the problem of drug resistance, the available drugs, especially polyenes and azoles, have a number of limitations such as toxicity and side effects. Consequently, the demand for safe and effective therapeutic alternatives has dramatically increased. The antimycobacterial and antifungal effects of plant extracts have been described in several studies [2, 118–121]. There are definitely fewer studies on activity against drug-resistant mycobacteria and mycetes, even if there appear to be a number of plants listed in Table 10.3. This is because the author's studies have been mainly based on the screening of several plants. These studies enable us to discover new plants for their

Table 10.2 Activity of plants extracts/phytochemicals against drug-resistant Gram-negative strains.

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Acacia aroma Gill.	Leaf, flower	Aqueous and ethanol extracts	Cefotaxime-resistant *K. pneumoniae* and *S. marcescens*, ceftazidime-resistant *P. aeruginosa* and *A. baumanii*	D, MIC, MBC, PS	42
Acorus calamus L.	Rhizome	Ethanol extract	Multidrug-resistant *E. coli*, *S. dysenteriae*	D, TLC, B	45
Aegle marmelos	Fruit pulp	Aqueous and methanol extracts	Multidrug-resistant *S. typhi* strain B330	D, MIC	
	Leaf	Ethanol extract	Multidrug-resistant *E. coli*	D, TLC, B	45
Allium sativum L.	Leaf	Ethanol extract	Multidrug-resistant *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45, 46
	Bulb	Aqueous extract	Multidrug-resistant *H. influenzae*, *S. typhi*, *P. aeruginosa*, *E. coli*, *Shigella* spp., *Proteus* spp.	D, MIC	
Anogeissus leiocarpa Guill. & Perrr.	Root	Aqueous extract, methanol, ether, butanol extracts	Mmultidrug-resistant *B. cepacia* ($n=2$)	D	52
Azadirachta indica A. Juss.	Bark	Ethanol extract	Multidrug-resistant *E. coli*	D, TLC, B	45
Beta vulgaris L.	Root	Ethanol extract	Multidrug-resistant *E. coli*	D, TLC, B	45
Caesalpina sappan L.		Brasilin	Multidrug-resistant *B. cepacia*	MIC, BF	56
Camellia sinensis L.	Leaf	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
		Catechins: epicatechin gallate, epigallocatechin gallate	Metronidazole- and clarithromycin-resistant *H. pylori* ($n=19$)	MIC, TKA, SY	109
Casuarina equistifolia L.	Leaf, Bark	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Cinnamomum verum		Essential oil	Drug-resistant *E. coli*, *S. marcescens*, *E. cloacae*, *K. pneumoniae*, *S. enteridis*, *S. sonnei*	D	63
Cinnamomum zeylanicum Bl.	Bark	Essential oil	Ampicillin- and metronidazole-resistant *H. pylori*	D, MBC, AS	110

Table 10.2 Activity of plants extracts/phytochemicals against drug-resistant Gram-negative strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Citrus paradisi Macfad.		Essential oil	Ampicillin- and metronidazole-resistant *H. pylori*	D, MBC, AS	110
Citrus sinensis L.	Rind	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Combretum paniculatum		Aqueous and ethanol extracts	Multi-drug-resistant *S. typhi*	MIC, MBC	111
Cordia dichotoma L.	Leaf	Ethanol extract	Drug-resistant *S. dysenteriae*	D, TLC, B	45
Cuminum cyminum L.	Seed	Ethanol extract	Drug-resistant *H. pylori* ($n=4$)	D, MIC	105
Cynara scolymus L.	Leaf	Ethanol extract	Drug-resistant *H. pylori* ($n=4$)	D, MIC	105
Daucus carota L.	Seed	Essential oil	Ampicillin- and metronidazole-resistant *H. pylori*	D, MBC, AS	110
Emblica officinalis Gaertn.	Fruit	Ethanol extract	Multidrug-resistant *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Emblica officinalis – *Terminalia chebula* Retz. – *Terminalia belerica* Roxb. (1:1:1)	Fruit	Aqueous and methanol extracts	Multidrug-resistant *S. typhi* strain *B330*	D, MIC	105
Eucalyptus sp.	Leaf	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Ficus carica L.	Leaf	Ethanol extract	Multidrug-resistant *S. dysenteriae*	D, TLC, B	45
Ficus religiosa L.	Leaf	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Glycyrrhiza aspera	Aerial	Aqueous extract	Drug-resistant *H. pylori* ($n=70$)	D, MIC, TLC, S	112
Glycyrrhyza glabra L.		Flavonoids with strong activity: formononetin, glabridin, glabrene; weak activity – glycirrhetic acid, liquiritigenin	Amoxicillin- and clarithromycin-resistant *H. pylori* GP98	D, MIC	113
		Glycyrrhetinic acid	Clarithromycin- and metronidazole-resistant *H. pylori*	HPLC, S, MIC, TKA	114
Glycyrrhyza inflata		Flavonoids: licochalcone A	Amoxicillin- and clarithromycin resistant *H. pylori* GP98	D, MIC, S, HPLC	113-

Table 10.2 Activity of plants extracts/phytochemicals against drug-resistant Gram-negative strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Glycyrrhiza uralensis DC.	Root	Flavonoids with strong activity: licoricidin, licoisoflavone B, vestitol, licoricone, gancaonol B, glyasperin D, 1-methoxyphaselidin, gancaonol C; weak activity – glycyrin, isolicoflavonol, 6,8-diprenylorobol, gancaonin I, dihydrolicoisoflavone A	Amoxicillin- and clarithromycin-resistant *H. pylori* GP98	D, MIC, S, HPLC	113
Hemidesmus indicus R. Br.	Root	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Holarrhena anti-dysenterica R.	Bark	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
	Seed	Aqueous and methanol extracts	Multidrug-resistant *S. typhi* strain B330	D, MIC	108
Juniperus communis L.		Essential oil	Resistant bacteria: *S. marcescens*, *E. cloacae*, *Kl. pneumoniae*, *P. aeruginosa*, *A. baumanii*	D, MIC, GC	78
Lantana camara L.	Leaf	Ethanol extract	Multidrug-resistant *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Lawsonia inermis L.	Leaf	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Leptospermum scoparium Forst. & Forst.		Essential oil	Ampicillin- and metronidazole-resistant *H. pylori* strain	D, MBC, AS TKA	110 40
Melaleuca alternifolia Cheel		Essential oil	Gentamycin-resistant *P. aeruginosa* ($n=2$) and *K. pneumoniae* ($n=2$)		
Mentha aquatica L.		Essential oil	Multiresistant *S. sonei*	MIC, RSC, TLC, GC/MS	115
Mentha longifolia L.		Essential oil	Multiresistant *S. sonei*	MIC, RSC, TLC, GC/MS	115
Mentha × *piperita* L.		Essential oil	Multiresistant *S. sonei*	MIC, RSC, TLC, GC/MS	115

Table 10.2 Activity of plants extracts/phytochemicals against drug-resistant Gram-negative strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Momordica balsamina L.		Aqueous and ethanol extracts	Multi-drug-resistant *S. typhi*	MIC, MBC	111
Morinda lucida Benth.		Aqueous extract	Multi-drug-resistant *S. typhi*	MIC, MBC	111
Morus alba L.	Leaf	Ethanol extract	Multidrug-resistant *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Myristica fragrans Houtt.	Fruit	Aqueous and methanol extracts	Multi-drug-resistant *S. typhi* strain B330	D, MIC	108
Nelumbo nucifera Gaertn.	Flower	Ethanol extract	Multidrug-resistant *E. coli*, *S. dysenteriae*	D, TLC, B	45
Nigella sativa L.	Seed	Ethanol extract	Multidrug-resistant *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
		Alkaloid and aqueous extracts	Multidrug-resistant Gram-negative bacteria	I	84
Nyctanthes arbor-tristis L.	Leaf	Ethanol extract	Multidrug-resistant *S. paratyphi*,	D, TLC, B	45
Ocimum basilicum L.	Aerial	Essential oil	Multidrug-resistant *P. aeruginosa*	MIC, TKA, GC	86
Ocimum gratissimum L.		Aqueous extract	Multidrug-resistant *S. typhi* strains	MIC, MBC	111
Ocimum sanctum L.	Plant	Ethanol extract	Multidrug-resistant *E. coli*, *S. dysenteriae*	D, TLC, B	45
Olea europea L.		α,β-Unsaturated aldehydes: (*E*)-2-eptenal, (*E*)-2-nonenal, (*E*)-2-decenal, (*E*,*E*)-2,4-decadienal	*M. catarrhalis* β-lattamase$^+$ ($n=5$), *H. influenzae* β-lattamase$^+$ ($n=4$)	D, MIC	87
Origanum vulgare L.		Essential oil	Drug-resistant,*E. coli*, *S. marcescens*, *E. cloacae*, *K. pneumoniae*, *S. enteridis*, *S. sonnei*	D	63
Ostericum koreanum		Essential oil	Streptomycin-resistant *S. enteritidis and S. typhimurium* ($n=2$)	D, MIC, CTA, GC/MS	116
Plant-derived flavonoid		Flavonol, myricetin,	Multidrug-resistant *B. cepacia*	D, MIC, VC, IRS	91
Plumbago zeylanica L.	Root	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Portulaca quadrifolia L.	Stem, leaf	Ethanol extract	Multidrug-resistant *S. dysenteriae*	D, TLC, B	45

Table 10.2 Activity of plants extracts/phytochemicals against drug-resistant Gram-negative strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Propolis		Ethanol extract	Drug-resistant *H. pylori* ($n=4$)	D, MIC	105
		Ethanol extract, flavonoid galangin	Multidrug-resistant *P. aeruginosa* ($n=10$)	D, MIC, MBC, TLC-B	92
Punica granatum L.	Rind	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
	Fruit peel	Aqueous and methanol extracts	Multidrug-resistant *S. typhi* strain B330	D, MIC	108
Salmalia malabarica	Bark	Methanol extracts	Multidrug-resistant *S. typhi* strain B330	D, MIC	108
Satureja cuneifolia	Plant	Essential oil	Penicillin, cephalosporins and macrolides-resistant *S. marcescens* MB 979	MIC, GC/MS	96
Satureja montana L.	Plant	Essential oil	Ampicillin- and metronidazole-resistant *H. pylori*	D, MBC, AS	110
			Penicillin, cephalosporins and macrolides-resistant *S. marcescens* MB 979	MIC, GC/MS	96
Satureja lappa C.B. Clarke	Root	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Saussurea warneckei	Stem	Aqueous extract	Multidrug-resistant *B. cepacia* ($n=2$)	D	52
Syrgium aromaticum L.	Bud, oil	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Syzgium cumini L.	Bark, leaf	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Terminalia arjuna W. & A.	Bark	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
		Aqueous and methanol extracts	Multi-drug-resistant *S. typhi* strain B330	D, MIC	108
Terminalia avicennioides Guill.		Aqueous and ethano extracts	Multi-drug-resistant *S. typhi* strains	MIC, MBC	111
Terminalia belerica Roxb.	Fruit	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45

Table 10.2 Activity of plants extracts/phytochemicals against drug-resistant Gram-negative strains. (Continued)

Plant/natural product	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Terminalia chebula Retz.	Fruit	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*	D, TLC, B	45
Terminalia glaucescens	Root	Aqueous extract	Multidrug-resistant *B. cepacia* ($n=2$)	D	52
Terminalia macroptera Guill. & Perr.	Leaf	Ethanol extract, diethyl ether fraction	Penicillin-resistant *N. gonorrhoeae* ($n=3$), penicillin and tetracycline-resistant *N. gonorrhoeae* ($n=2$)	MIC	117
Thymus kotschyanus	Root	Aqueous extract	Drug-resistant *H. pylori* ($n=70$)	D, MIC, TLC, S	112
Thymus vulgaris L.		Essential oil	Drug-resistant *E. coli*, *S. marcescens*, *E. cloacae*, *K. pneumoniae*, *S. enteridis*, *S. sonnei*	D	63
Trachyspermum copticum	Aerial	Aqueous, methanol, methanol: diethyl ether: petroleum benzene extracts	Drug-resistant *H. pylori* ($n=70$)	D, MIC, TLC, S	112
Trema guineensis Fic.		Aqueous and ethano extracts	Multi-drug-resistant *S. typhi*	MIC, MBC	111
Vitex doniana Sweet.	Root	Aqueous extract, methanol, ether, butanol extracts	Multidrug-resistant *B. cepacia* ($n=2$)	D	52
Vitis vinifera L.	Leaf	Ethanol extract	Multidrug-resistant *E. coli*, *S. dysenteriae*	D, TLC, B	45
Xanthium brasilicum	Aerial	Aqueous, methanol, methanol: diethyl ether: petroleum benzene extracts, flavonoid and xanthanolide	Drug-resistant *H. pylori* ($n=70$)	D, MIC, TLC, S	112
Zingiber officinale Roscoe	Rhizome	Ethanol extract	Drug-resistant *H. pylori* ($n=4$)	D, MIC	105
Ziziphus jujube L.	Leaf, bark	Ethanol extract	Multidrug-resistant *E. coli*, *S. paratyphi*, *S. dysenteriae*, drug-resistant *S. paratyphi*	D, TLC, B	45

[a] *A. baumanii, Acinetobacter baumanii; B. cepacia, Burkholderia cepacia; E. cloacae, Enterobacter cloacae; E. coli, Escherichia coli; H. influenzae, Hemophilus influenzae; H. pylori, Helicobacter pylori; K. pneumoniae, Klebsiella pneumoniae; M. catarrhalis, Moraxella catarrhalis; N. gonorrhoeae, Neisseria gonorrhoeae ; P. aeruginosa, Pseudomonas aeruginosa; S. typhi, Salmonella typhi; S. dysenteriae, Shigella dysenteriae; S. enteridis, Salmonella enteridis; S. marcescens, Serratia marcescens; S. paratyphi, Salmonella paratyphi; S. sonnei, Shigella sonnei.*

[b] AS, animal studies; B, bioautography; BF, bioassay fractionation; CTA, chequerboard titration assay; D, diffusion; GC, gas chromatography; GC/MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; I, inhibition; IA, inhibition adherence; IRS, inhibition radiolabeled substances; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; PS, phytochemical screening; RSC, free radical scavenging capacity; S, spectroscopy; TKA, time kill assay; TLC, thin-layer chromatography; VC, viable count.

Table 10.3 Activity of plants extracts/phytochemicals against other drug-resistant strains.

Plant	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Acacia xanthophloea Bentn.	Bark	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Acorus calamus L.	Rhizome	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Allium sativum L.	Bulb, leaf	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Alpinia galanga Willd.	Stalk, rhizome	Ethanol extract	Ketoconazole and/or amphotericin-resistant yeast and filamentous fungi ($n=6$)	D	123
Alpinia officinarum Hence	Rhizome	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Artemisia ludoviciana Nutt.	Aerial	Hexane and methanol extracts	Drug-resistant *M. tuberculosis* ($n=12$)	ABA	125
Berberis vulgaris L.	Fruit	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Borago officinalis L.	Flower	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Carpobrotus edulis l.	Leaves	Methanol extract	Multidrug-resistant *M. tuberculosis* ($n=2$)	IPB	65
Cassia alata	Bark	Aqueous and ethanol extracts	Fluconazole and ketoconazole *C. albicans*	D	126
Casuarina equisetifolia Forst. f.	Leaf, bark	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Chamaedorea tepejilote liebm.	Leaves	Hexane extract	Drug-resistant *M. tuberculosis* ($n=12$)	ABA	125
Chenopodium ambrosioides L.	Aerial	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Chrozophora verbasafalia L.	Leaf	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Cinnamomum zeylanicum Bl.	Stem, bark	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
	Bark		Fluconazole-resistant *Candida* strains	MIC, HS	127
Citrus sinensis L.	Rind	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Combretum molle R. Br. Ex G. Don	Bark	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Costus globosus Bl.	Rhizome	Ethanol extract	Ketoconazole and/or amphotericin-resistant filamentous fungi ($n=5$)	D	123
Croton pseudopulchellus Pax	Aerial	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Cryptocarya latifoglia Sond.	Bark	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122

Table 10.3 Activity of plants extracts/phytochemicals against other drug-resistant strains. (Continued)

Plant	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Curcuma zedoaria Roscoe	Rhizome	Ethanol extract	Ketoconazole and/or amphotericin-resistant yeast ($n=3$) and filamentous fungi ($n=3$)	D	123
Dianthus caryophyllus L.	Flower	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Dyospiros montana Roxb.	Bark	Aminoacetate derivative of diospyrin	Multidrug-resistant *M. tuberculosis:* CCKO28469V, C84, CGT1237617	MIC, RM	128
Ekebergia capensis Sparrm.	Bark	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Emblica officinalis Gaertn.	Fruit	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Ephedra intermedia Schrenk & Mey	Stem	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Eucalyptus sp.	Leaf	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Euclea natalensis DC.	Root	Binaphthoquinoid, diospyrin	Multidrug-resistant *M. tuberculosis:* CCK028469V, C9, C84, CGT1296429, CCK070370H' CGT1330497	MIC	129
		Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Ferula communis L.		Ferulenol	Multidrug-resistant *M. tuberculosis*	MIC, SY, BF	130
Ficus religiosa L.	Leaf	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Helichrysum melanacme DC.	Plant	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Helichrysum odoratissimum Sweet	Plant	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Helicteres isora L.	Fruit	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Helleborus niger L.	Root	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Hemidesmus indicus R. Br.	Root	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Holarrhena anti-dysenterica R.	Bark	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Hyoscyamus niger L.	Flower, seed	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Impatiens balsamina L.	Aerial	Methoxy-1,4-naphthoquinone (MNQ)	Amphotericin B and fluconozale *C. albicans* ($n=2$)	D, MIC, HPLC	131

Table 10.3 Activity of plants extracts/phytochemicals against other drug-resistant strains. (Continued)

Plant	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Juniperus communis L.	Leaves	Hexane and methanol extracts	Drug-resistant *M. tuberculosis* ($n=12$)	ABA	125
Juniperus procera Hochst. ex Endl.		Totarol	Multidrug-resistant *M. tuberculosis*	MIC, SY, BF	130
Lantana hispida	Aerial	Hexane and methanol extracts, chromatographic fractions	Drug-resistant *M. tuberculosis* ($n=12$)	ABA	125
Lawsonia inermis L.	Leaf	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Maytenus senegalensis Exell	Aerial	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Melaleuca alternifolia Cheel		Essential oil	Fluconazole-resistant *Candida* strains	D, MIC	132
			Fluconazole and/or itraconazole-resistant *C. albicans* ($n=14$)	MIC, TKA, AS	133
Myrtus communis L.	Leaf, stem	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Nelumbo nucifera Gaertn.	Flower	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Nidorella anomala Steetz	Plant	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Nidorella auriculata DC.	Plant	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Ocimum sanctum L.	Plant	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Pimpinella anisum L.	Fruit	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Plumbago zeylanica L.	Root	Plumbagin	Multidrug-resistant *M. tuberculosis*	MIC, SY, BF	130
		Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Polygala myrtifolia L.	Aerial	Acetone extract	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
Punica granatum L.	Rind	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Rubus idaeus L.	Leaf	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Salvia officinalis L.	Flower	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Sapindus sp.	Fruit	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Saussurea lappa C.B. Clarke	Root	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Semecarpus anacardium L.	Stem bark	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Syrgium aromaticum L.	Bud, oil	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45

Table 10.3 Activity of plants extracts/phytochemicals against other drug-resistant strains. (Continued)

Plant	*Parts used*	*Active extract(s)/compound(s)*	*Activity*[a]	*Method*[b]	*Reference*
Terminalia belerica Roxb.	Fruit	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
Terminalia chebula retz.	Fruit	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45
	Seed	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Thymus mastichina		Essential oil, thymol, carvacrol, *p*-cymene, 1,8-cineole	Fluconazole-resistant *C. albicans*, *C. tropicalis*, *C.glabrata*, *C. kruzei*	MIC, MLC, SY, GC, GC/MS	134
Thymus vulgaris L.	Aerial	Acetone extracts	Multidrug-resistant *M. tuberculosis* CCKO28469V	MIC, RM	122
	Plant	Essential oil, thymol, carvacrol, *p*-cymene, 1,8-cineole	Fluconazole-resistant *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. kruzei*	MIC, MLC, SY, GC, GC/MS	134
		Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Thymus zygis L.		Essential oil, thymol, carvacrol, *p*-cymene, 1,8-cineole	Fluconazole-resistant *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. kruzei*	MIC, MLC, SY, GC, GC/MS	134
Trachyspermum copticum Link	Fruit	Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
	Rhizome	Ethanol extract	Ketoconazole and/or amphotericin-resistant yeast and filamentous fungi ($n=4$)	D	123
Zingiber officinale Roscoe		Methanol extract	Clotrimazole-resistant *C. albicans*	MIC	124
Zingiber purpureum	Rhizome	Ethanol extract	Ketoconazole and/or amphotericin-resistant yeast ($n=3$) and filamentous fungi ($n=6$)	D	123
Ziziphus jujube L.	Leaf, bark	Ethanol extract	Multidrug-resistant *C. albicans*	D, TLC, B	45

[a] *C. albicans*, *Candida albicans*; *C. kruzei*, *Candida kruzei*; *C. tropicalis*, *Candida tropicalis*; *C. glabrata*, *Candida glabrata*; *M. tuberculosis*, *Mycobacterium tuberculosis*.
[b] ABA, alamar blue assay; B, bioautography; BF, bioassay fractionation; D, diffusion; GC, gas hromatography; GC/MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; HS, human studies; IPB, inhibition phagogytosed bacteria; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; RM, radiometric method; SY, synergism; TKA, time kill assay; TLC, thin-layer chromatography.

activity against drug-resistant mycobacteria and/or mycetes, or to confirm the potential role of others. Among these, *A. sativum*, *M. alternifolia*, and *Thymus vulgaris*, plants already previously considered for their antibacterial activity, are here shown also as antifungal agents with *in vitro* activity against *Candida albicans*. Moreover *T. vulgaris* and *Plumbago zeylanica* are plants with activity both against drug-resistant *M. tuberculosis* and yeasts (Table 10.3).

10.4 Plant Materials that Restore the Effectiveness of Antimicrobial Agents and/or Inhibit Drug Resistance Mechanisms

The discovery of natural agents that restore the effectiveness of antimicrobial agents and/or inhibit antibiotic resistance mechanisms could be useful for the treatment of bacteria for which the majority of antibiotics are of no further clinical use. The combination therapies based upon the administration of reduced concentrations of antibiotics and natural extracts could have the advantage of extending the usefulness and effectiveness of antibiotics of known pharmacology and toxicology, also against antibiotic-resistant strains, and could potentially control the resistance development. In this context, different ethanolic extracts of propolis (13 samples), at concentrations equal to 0.078% and 0.039%, showed synergism with ampicillin, ceftriaxone, and doxycycline for multidrug-resistant *S. aureus* [93].

Interestingly, several papers report that tea (*Camellia sinensis*) extract and/or its components (tea catechins) reverse the resistance to β-lactams. In particular, catechins [135], compound P [136], epicatechin gallate [137], theasinensin A [138], and gallotannin [139] markedly reduced the MIC values of β-lactams against MRSA. This effect may be due to compound P, which prevents PBP2′ synthesis and inhibits secretion of β-lactamase [136] or to the interference of epigallocatechin gallate (EGCg, the main constituent of green tea) with the integrity of the cell wall through direct binding to the peptidoglycan [140]. According to Zhao et al. [141], besides the effect of EGCg on the cell wall, the direct inhibition of penicillinase activity was responsible for the synergism between EGCg and penicillin. However, the combinations of cefotaxime or imipenem and EGCg against β-lactamase-producing Gram-negative rods only showed slight synergy. The different effects of the combinations on different β-lactamase-producing species were confirmed to be related to the cellular locations of β-lactamases [142]. Other studies reported the potent synergy between EGCg and ampicillin/sulbactam or relatively new β-lactams (carbapenemes) against 28 and 24 MRSA isolates respectively, with MIC values of antibiotics restored to the susceptible breakpoint [143, 144]. In contrast to the synergy between EGCg and β-lactams, EGCg may also affect the activities of antibiotics negatively with additive, indifferent, and antagonistic effects. Additive or indifferent effects between EGCg and non-β-lactams (inhibitors of either protein or nucleic acid synthesis) and antagonism between EGCg and glycopeptides (vancomycin, teicoplanin, and polymyxin B) have been reported [145]. Probably, this was due to a direct binding of EGCg with the peptide structure of the antibiotics [145].

Several studies have shown that other plant compounds such as tellimagrandin I and rugosin B from red roses (*Rosa canina* L.) [146, 147], totarol from the totara tree [148], baicalin from *Scutellaria amoena* C.H. Wright (inhibits β-lactamase) [149], corilagin (inhibits the activity of the PBP2′) from *Arctostaphylos uva-ursi* [147, 150], sophoraflavanone G [151, 152], and methanol extract of *Caesalpinia sappan* [55] reduced the MIC values of β-lactams in MRSA strains. Flavone and its derivatives that had weak antibacterial activity but dramatically intensified the susceptibility of β-lactams in MRSA, have been named ILSMRs (intensifiers of β-lactam-susceptibility in MRSA) [153]. The methicillin MIC decreased from ≥1024 to 2–512 μg mL^{-1} for 12/20 MRSA strains. Sato et al. [154, 155] found that flavone and 6,7-dihydroxyflavone decreased the number of intermediates of *N*-acetylmuramyl-pentapeptide in MRSA and stated that this could be a plausible explanation for the reduction in β-lactam MIC observed. Recently, Shibata et al. [156] described the ILSMR effects of ethyl gallate from *Caesalpinia spinosa* and other alkyl gallates, providing evidence that the length of the alkyl chain was associated with the activity observed (an optimum length was C5 to C6). Such an activity appeared to be specific for β-lactams, because no changes were observed in the MIC values of other classes of antibiotics. The evidence of no ILSMR effect of *n*-amyl or isoamyl 4-hydroxybenzoate was due to the key role of the galloyl moiety of the molecules. On the other hand, tellimagrandin I, epicathechin gallate, EGCg, and corilagin, which all contain this moiety and have been reported to be ILSMR active, support this findings.

Interestingly, garlic and allicin [47], calozeyloxanthone isolated from *Calophyllum moonii* [57], isoflavonoids from *Erythrina zeyheri* (erybraedin A and eryzerin C) [70], galangin, and 3,7-dihydroxyflavone [157] were active against VRE and showed marked synergism with vancomycin. Besides, α-mangostin from *Garcinia mangostana* L. was effective alone or in combination with gentamicin against VRE [73].

The use of plant materials in combination therapy could be promising also in the treatment of Gram-negative infections. Shahverdi et al. [158] reported that piperitone, a component of the essential oil from *Mentha longifolia* (L.) var. *chlorodictya* Rech F., enhanced the antimicrobial activity of nitrofrantoin against nitrofurantoin-resistant *E. cloacae*. *Ocimum gratissimum* L. essential oil has been demonstrated to interfere with virulence factors of multidrug-resistant *Shigella* and to reduce the MIC values of antibiotics to which *Shigella* showed resistance [159]. An additive effect (FIC index between 0.5 and 1) was observed when EGCg was combined with metronidazole or clarithromycin against 9/12 and 9/14 clinical isolates of metronidazole- and clarithromycin-resistant *H. pylori*, respectively [109].

The difficulty in treating drug-resistant infections is often due to the fact that many strains also have efflux pumps, such as in MRSA the specific TetK and MsrA transporters, which export certain tetracyclines and macrolides, and the multidrug resistance (MDR) proteins NorA and QacA, which confer resistance to a wide range of structurally unrelated antibiotics and antiseptics. Thus, the availability of efflux pumps inhibitors (MDR inhibitory) could be another way to cope with the antibiotic resistance problem and could also be used to extend the effectiveness of plant compounds [160]. The alkaloid reserpine, the first inhibitor found, has been

shown to inhibit multidrug transporters such as NorA, increasing the intracellular concentration of fluoroquinolones and TetK, reducing significantly the MIC of tetracycline [161, 162]. More results have been achieved thanks to studies carried out by some research teams who found that a wide variety of *Berberis* plants produce berberine, a weak antimicrobial cationic alkaloid and substrate of MDR pumps, and also produce the MDR inhibitors flavonolignan (5′-methoxyhydnocarpin D, 5′-MHC-D) and porphyrin (pheophorbide A), which facilitate the penetration of berberine into *S. aureus* [163–165] and could lead to a striking increase in the antimicrobial activity of antibiotics such as ciprofloxacin [166]. Subsequently, it was found that both synthetic and natural flavones (chrysoplenol-D and chrysoplenetin from *Artemisia annua*) were MDR inhibitors [167, 168] and that isoflavones isolated from *Lupinus argenteus* potentiated the antibacterial activity of α-linoleic acid, berberine and the antibiotic norfloxacin [169].

Other modulators of MDR in *S. aureus* have been discovered and include acylated neohesperidosides from *Geranium caespitosum* [170], the diterpenes carnosic acid and carnosol from *Rosmarinus officinalis* [95], epicatechin gallate and epigallocatechin gallate [171, 172], and bergamottin epoxide from grapefruit oil [173]. Moreover, resistance inhibitory activities have been reported for Jordanian plants on *Ps. aeruginosa* and *S. aureus* [174, 175] and for Korean plants on multidrug-resistant *S. aureus* [176, 177].

10.4.1 Other Mechanisms

Microorganisms in biofilms are known to be less susceptible to conventional antibiotic treatment than their planktonic counterparts. The mechanisms of lowered susceptibility in biofilms depend on many factors, including poor antibiotic penetration, nutrient limitation, slow growth, and adaptive stress responses [178]. The discovery of more effective antimicrobial agents that are active on biofilms and able to prevent, or at least interfere with biofilm formation would be a considerable achievement. The antibiofilm activity of nonantibiotic compounds such as allicin and carvacrol, and formulations containing essential oils have been reported [179–181]. Essential oils that can penetrate plaque biofilms and kill plaque-forming microorganisms by disrupting their cell walls, could be used as plaque control agents [182].

The recent studies on the antimicrobial activities of plant extracts encourage us to take into account new therapy schemes including phytotherapy as an alternative. Plant-based diets with intake and consumption of natural substances may have an important role in the control of resistance gene dissemination by the inhibition of conjugal R-plasmid transfer. Papaya seed macerate and EGCg have been studied as inhibitors of conjugative R-plasmid transfer in enteric bacteria [183, 184]. Papaya caused a reduction of R-plasmid transfer by conjugation from *Salmonella typhimurium* to *Escherichia coli*, both *in vitro* and *in vivo* [183]. The inhibition rates of conjugative transfer of R-plasmid between *E. coli* C600 and *E coli* K^+12 RC85 with EGCg were 42–67% at 50–200 μg mL^{-1} and up to 99% at 800 μg mL^{-1} [184].

A further interesting way to attack the antibiotic resistance problem is based on the curing ability of plant extracts. *Plumbago zeylanica*, already studied for its antimicrobial activity against multidrug-resistant strains, has been reported to cure the plasmid from 14% *E. coli* x^+ (pUK 651)-treated cells, probably because of the DNA-intercalating effect of its active constituent plumbagin [185].

10.5 Conclusions

The increasing development of microbial resistance is a problem that should be solved as soon as possible. The data reported in this review emphasize the potential role of plant extracts/phytocompounds in developing new antimicrobial agents. However, even if plenty has been published already, there is still much more to be done so that these substances can really be used in the future. In particular, more detailed safety investigations to determine the degree of toxicity and *in vivo* clinical trials to validate the *in vitro* results should be carried out. Furthermore, we strongly recommend that standard criteria should be found for the evaluation of plant activity in order to make comparison between the different studies possible.

References

1 Sharma, R., Sharma, C.L., Kapoor, B. *Indian J. Med. Sci.* **2005**, *59*, 120–129.
2 Cowan, M.M. *Clin. Microbiol. Rev.* **1999**, *12*, 564–582.
3 Rios, J.L., Recio, M.C. *J. Ethnopharmacol.* **2005**, *100*, 80–84.
4 Ahmad, R., Ali, A.M., Israf, D.A., Ismail N.H., Shaari, K., Lajis, N.H. *Life Sci.* **2005**, *76*, 1953–1964.
5 Chang, S.T., Chen, P.F., Chang S.C. *J. Ethnopharmacol.* **2001**, *77*, 123–127.
6 Didry, N., Seidel, V., Dubreuil, L., Tillequin, F., Bailleul, F. *J. Ethnopharmacol.* **1999**, *67*, 197–202.
7 Feresin, G.E., Tapia, A., Gimenez, A., Ravelo, A.G., Zacchino, S., Sortino, M., Schmeda-Hirschmann, G. *J. Ethnopharmacol.* **2003**, *89*, 73–80.
8 Feresin, G.E., Tapia, A., Sortino, M., Zacchino, S., de Arias A.R., Inchausti, A., Yaluff, G., Rodriguez, J., Theoduloz, C., Schmeda-Hirschmann, G. *J. Ethnopharmacol.* **2003**, *88*, 241–247.
9 Imai, H., Osawa, K., Yasuda, H., Hamashima, H., Arai, T., Sasatsu, M. *Microbios* **2001**, *106* (Suppl 1), 31–39.
10 Kolodziej, H., Kayser, O., Radtke, O.A., Kiderlen, A.F., Koch, E. *Phytomedicine* **2003**, *10* (Suppl 4), 18–24.
11 Perez, C., Agnese, A.M., Cabrera, J.L. *J. Ethnopharmacol.* **1999**, *66*, 91–96.
12 Reichling, J., Weseler, A., Saller, R. *Pharmacopsychiatry* **2001**, *34*, S116–S118.
13 Rukachaisirikul, V., Phainuphong, P., Sukpondma, Y., Phongpaichit, S., Taylor, W.C. *Planta Med.* **2005**, *71*, 165–170.
14 Skočibušič, M., Bezič, N., Dunkič, V., Radonič, A. *Fitoterapia* **2004**, *75*, 733–736.
15 Wilkinson, J.M., Hipwell, M., Ryan, T., Cavanagh, H.M.A. *J. Agric. Food Chem.* **2003**, *51*, 76–81.
16 Woldemichael, G.M., Singh, M.P., Maiese, W.M., Timmerman, B.N. *Z. Naturforsch.* **2003**, *58*, 70–75.
17 Yu, J., Lei, J., Yu, H., Cai, X., Zou, G. *Phytochemistry* **2004**, *65*, 881–884.
18 Zgoda-Pols, J.R., Freyer, A.J., Killmer, L.B., Porter, J.R. *Fitoterapia* **2002**, *73*, 434–438.
19 Feresin, G.E., Tapia, A., López, S.N., Zacchino, S.A. *J. Ethnopharmacol.* **2001**, *78*, 103–107.

20 Salvat, A., Antonnacci, L., Fortunato, R.H., Suarez, E.Y., Godoy, H.M. *Lett. Appl. Microbiol.* **2001**, *32*, 293–297.
21 McCutcheon, A.R., Ellis, S.M., Hancock, R.E., Towers, G.H. *J. Ethnopharmacol.* **1992**, *37*, 213–223.
22 Essawi, T., Srour M. *J. Ethnopharmacol.* **2000**, *70*, 343–349.
23 Kumarasamy, Y., Cox, P.J., Jaspars, M., Nahar, L., Sarker, S.D. *J. Ethnopharmacol.* **2002**, *83*, 73–77.
24 Mothana, R.A.A., Lindequist, U. *J. Ethnopharmacol.* **2005**, *96*, 177–181.
25 Inouye, S., Takizawa, T., Yamaguchi, H. *J. Antimicrob. Chemother.* **2001**, *47*, 565–573.
26 Harkenthal, M., Reichling, J., Geiss, H.K., Saller, R. *Pharmazie* **1999**, *54*, 460–463.
27 Cookson, B., Morrison, D., Marples, R. *J. Med. Microbiol.* **1997**, *46*, 439–442.
28 Chambers, H.F. *Clin. Microbiol. Rev.* **1997**, *10*, 781–791.
29 Moreno, F., Crisp, C., Jorgensen, J.H., Patterson, J.E. *Clin. Infect. Dis.* **1995**, *21*, 1308–1312.
30 Centers for Disease Control and Prevention. *Morb. Mortal. Wkly Rep.* **2002**, *51*, 565–567.
31 Carson, C.F., Cookson, B.D., Farrelly, H.D., Riley, T.V. *J. Antimicrob. Chemother.* **1995**, *35*, 421–424.
32 Markham, J.L. Biological activity of tea tree oil. In *Tea Tree, the Genus Melaleuca*, Southwell, I., Lowe, R. (eds). Harwood Academic Publishers, Amsterdam, **1999**.
33 Hammer, K.A., Carson, C.F., Riley, T.V. *Am. J. Infect. Control* **1996**, *24*, 186–189.
34 Martin, K.W., Ernst, E. *J. Antimicrob. Chemother.* **2003**, *51*, 241–246.
35 Caelli, M., Porteous, J., Carson, C.F., Heller, R., Riley, T.V. *J. Hosp. Infect.* **2000**, *46*, 236–237.
36 Dryden, M.S., Dailly, S., Crouch, M. *J. Hosp. Infect.* **2004**, *56*, 283–286.
37 Edwards-Jones, V., Buck, R., Shawcross, S.G., Dawson, M.M., Dunn, K. *Burns* **2004**, *30*, 772–777.
38 Nostro, A., Blanco, A.R., Cannatelli, M.A., Enea, V., Flamini, G., Morelli, I., Sudano Roccaro, A., Alonzo, V. *FEMS Microbiol. Lett.* **2004**, *230*, 191–195.
39 Hamilton-Miller J.M.T., Shah, S. *FEMS Microbiol. Lett.* **1999**, *176*, 463–469.
40 May, J. Chan, C.H., King, A., Williams, L., French, G.L. *J. Antimicrob. Chemother.* **2000**, *45*, 639–643.
41 Nostro, A., Bisignano, G., Cannatelli, M.A., Crisafi, G., Germano, M.P., Alonzo, V. *Int. J. Antimicrob. Agents* **2001**, *17*, 517–520.
42 Arias, ME., Gomez, J.D., Cudmani, N.M., Vattuone, M.A., Isla, M.I. *Life Sci.* **2004**, *75*, 191–202.
43 Palombo, E.A., Semple, S.J. *J. Basic Microbiol.* **2002**, *42*, 444–448.
44 Akinyemi, K.O., Oladapo, O., Clement, O.E., Ibe, C.E., Fasure, K.A. *BMC Complement. Altern. Med.* **2005**, *5*, 6.
45 Ahmad, I., Beg, A.Z. *J. Ethnopharmacol.* **2001**, *74*, 113–123.
46 Iwalokun, B.A., Ogunledun, A., Ogbolu, D.O., Bamiro, S.B., Jimi-Omojola, J. *J. Med. Food* **2004**, *7*, 327–333.
47 Jonkers, D., Sluimer, J., Stobberingh, E. *Antimicrob. Agents Chemother.* **1999**, *43*, 3045.
48 Tsao, S., Hsu, C., Yin, M. *J. Antimicrob. Chemother.* **2003**, *52*, 974–980.
49 Tsao, S.M., Ying, M.C. *J. Med. Microbiol.* **2001**, *50*, 646–649.
50 Yasunaka, K., Abe, F., Nagayama, A., Okabe, H., Lozada-Pérez, L., López-Villafranco, E., Muňiz, E.E., Aguilar, A., Reyes–Chilpa, R. *J. Ethnopharmacol.* **2005**, *97*, 293–299.
51 Lechner, D., Stavri, M., Oluwatuyi, M., Pereda–Miranda, R., Gibbons, S. *Phytochemistry* **2004**, *65*, 331–335.
52 Taiwo, O., Xu, H.X., Lee, S.F. *Phytother. Res.* **1999**, *13*, 675–679.
53 Kawazoe, K., Tsubouchi, Y., Abdullah, N., Takaishi, Y., Shibata, H. Higuti, T., Hori, H., Ogawa, M. *J. Nat. Prod.* **2003**, *66*, 538–539.
54 Koné, W.M., Atindehou, K.K., Terreaux, C., Hostettmann, K., Traoré, D., Dosso, M. *J. Ethnopharmacol.* **2004**, *93*, 43–49.
55 Kim, K.J., Yu, H.H., Jeong, S.I., Cha, J.D., Kim, S.M., You, Y.O. *J. Ethnopharmacol.* **2004**, *91*, 81–87.
56 Xu, H.X., Lee, S.F. *Phytother. Res.* **2004**, *18*, 647–651.
57 Sakagami, Y., Kajimura, K., Wijesinghe, W.M.N.M., Dharmaratne, H.R.W. *Planta Med.* **2002**, *68*, 541–543.

58 Dharmaratne, H.R.W., Wijesinghe, W.M.N.M., Thevanasem V. *J. Ethnopharmacol.* **1999**, *66*, 339–342.

59 Aqil, F., Khan, M.S.A., Owais, M., Ahmad, I. *J. Basic Microbiol.* **2005**, *45*, 106–114.

60 Toda, M., Okubo, S., Hara, Y., Shimamura, T. *Nippon Saikingaku Zasshi* **1991**, *46*, 839–845.

61 Yam, T.S., Shah, S., Hamilton-Miller, J.M.T. *FEMS Microbiol. Lett.* **1997**, *152*, 169–174.

62 Martins, M., Ordway, D., Kristiansen, M., Viveiros, M., Leandro, C., Molnar, J., Amaral, L. *Fitoterapia* **2005**, *76*, 96–99.

63 Hersch-Martínez, P., Leaños-Miranda, B.E., Solórzano-Santos, F. *Fitoterapia* **2005**, *76*, 453–457.

64 Fukai, T., Oku, Y., Hou, A.J., Yonekawa, M., Terada, S. *Phytomedicine* **2005**, *12*, 510–513.

65 Nanayakkara, N.P., Burandt, C.L. Jr., Jacob, M.R. *Planta Med.* **2002**, *68*, 519–522.

66 Sato, M., Tanaka, H. Yamaguchi, R., Oh-Uchi, T., Etoh, H. *Lett. Appl. Microbiol.* **2003**, *37*, 81–85.

67 Tanaka, H., Sato, M., Oh-Uchi, T., Yamaguchi, R., Etoh, H., Shimizu, H., Sako, M., Takeuchi, H. *Phytomedicine* **2004**, *11*, 331–337.

68 Sato, M., Tanaka, H., Yamaguchi, R., Kato, K., Etoh, H. *Int. J. Antimicrob. Agents* **2004**, *24*, 43–48.

69 Tanaka, H, Sato, M., Fujiwara, S., Hirata, M., Etoh, H., Takeuchi, H. *Lett. Appl. Microbiol.* **2002**, *35*, 494–498.

70 Sato, M., Tanaka, H., Oh-Uchi, T., Fukai, T., Etoh, H., Yamaguchi R. *Phytother. Res.* **2004**, *18*, 906–910.

71 Tanaka, H., Oh–Uchi, T., Etoh, H., Sako, M., Asai, F., Fukai, T., Sato, M., Murata, J., Tateishi, Y. *Phytochemistry* **2003**, *64*, 753–758.

72 Iinuma, M., Tosc, H., Tanaka, T., Asai, F., Kobayashi, Y., Stimano, R., Miyauchi, K. *J. Pharm. Pharmacol.* **1996**, *48*, 861–865.

73 Sakagami, Y., Iinuma, M., Piyasena, K.G., Dharmaratne, H.R. *Phytomedicine* **2005**, *12*, 203–208.

74 Voravuthikunchai, S.P., Kitpipit, L. *Clin. Microbiol. Infect.* **2005**, *11*, 510–512.

75 Fukai, T., Marumo, A., Kaitou, K., Kanda, T., Terada, S., Nomura, T. *Fitoterapia* **2002**, *73*, 536–539.

76 Gibbons, S., Ohlendorf, B., Johnsen, I. *Fitoterapia* **2002**, *73*, 300–304.

77 Fragoso-Serrano, M., Gibbons, S., Pereda-Miranda, R. *Planta Med.* **2005**, *71*, 278–280.

78 Filipowicz, N., Kamiński, M., Kurlenda, J., Asztemborska, M., Ochocka, J.R. *Phytother. Res.* **2003**, *17*, 227–231.

79 Nelson, R.R.S. *J. Antimicrob. Chemother.* **1997**, *40*, 305–306.

80 Banes-Mashall, L., Cawley, P., Phillips, C.A. *Br. J. Biomed. Sci.* **2001**, *58*, 139–145.

81 Chan, C.H., Loudon, K.W. *J. Hosp. Infect.* **1998**, *39*, 244–245.

82 Hada, T., Furuse, S., Matsumoto, Y., Hamashima, H., Masuda, K., Shiojima, K., Arai, T., Sasatsu, M. *Microbios* **2001**, *106* (Suppl 2), 133–141.

83 Nostro, A., Cannatelli, M.A., Crisafi, G., Alonzo, V. *Int. J. Antimicrob. Agents* **2001**, *18*, 583–585.

84 Morsi, N.M. *Acta Microbiol. Pol.* **2000**, *49*, 63–74.

85 Tang, S., Bremner, P., Kortenkamp, A., Schlage, C., Gray, A.I., Gibbons, S., Heinrich, M. *Planta Med.* **2003**, *69*, 247–253.

86 Opalchenova, G., Obreshkova D. *J. Microbiol. Methods* **2003**, *54*, 105–110.

87 Bisignano, G., Laganà, M.G., Trombetta, D., Arena, S., Nostro, A., Uccella, N., Mazzanti, G., and Saija, A. *FEMS Microbiol. Lett.* **2001**, *198*, 9–13.

88 Smith, E., Williamson, E., Zloh, M., Gibbons, S. *Phytother. Res.* **2005**, *19*, 538–542.

89 Fukai, T., Oku, Y., Hano, Y., Terada, S. *Planta Med.* **2004**, *70*, 685–687.

90 Tsuchiya, H., Sato, M., Miyazaki, T., Fujiwara, S., Tanigaki, S., Ohyama, M., Tanaka, T., Iinuma, M. *J. Ethnopharmacol.* **1996**, *50*, 27–34.

91 Xu, H.X., Lee S.F. *Phytother. Res.* **2001**, *15*, 39–43.

92 Pepeljnjak, S., Kosalec, I. *FEMS Microbiol. Lett.* **2004**, *240*, 111–116.

93 Stepanović, S., Antić, N., Dakić, I., Švabic-Vlahović, M. *Microbiol. Res.* **2003**, *158*, 353–357.

94 Machado, T.B., Pinto, A.V.,. Pinto, M.C.F.R., Leal, I.C.R., Silva, M.G., Amaral, A.C.F., Kuster, R.M., Netto–dosSantos, K.R. *Int. J. Antimicrob. Agents* **2003**, *21*, 279–284.

95 Oluwatuyi, M., Kaatz, G.W., Gibbons, S. *Phytochemistry* **2004**, *65*, 3249–3254.

96 Skočibušič M., Bezič, N. *Phytother. Res.* **2004**, *18*, 967–970.

97 Sato, Y., Suzaki, S., Nishikawa, T., Kihara, M., Shibata, H., Higuti, T. *J. Ethnopharmacol.* **2000**, *72*, 483–488.

98 Sato, M., Tsuchiya, H., Miyazaki, T., Ohyama, M., Tanaka, T., Iinuma, M. *Lett. Appl. Microbiol.* **1995**, *21*, 219–222.

99 Iinuma, M., Tsuchiya, H., Sato, M., Yokoyama, J., Ohyama, M., Ohkawa, Y., Tanaka, T., Fujiwara, S., Fujii, T. *J. Pharm. Pharmacol.* **1994**, *46*, 892–895.

100 Sato, Y., Oketani, H., Singyouchi, K., Ohtsubo, T., Kihara, M., Shibata, H., Higuti, T. *Biol. Pharm. Bull.* **1997**, *20*, 401–404.

101 Kawazoe, K., Yutani, A., Tamemoto, K., Yuasa, S., Shibata, H., Higuti, T., Takaishi, Y. *J. Nat. Prod.* **2001**, *64*, 588–591.

102 Sato, Y., Oketani, H., Yamada, T., Singyouchi, K., Ohtsubo, T., Kihara, M., Shibata, H., Higuti, T. *J. Pharm. Pharmacol.* **1997**, *49*, 1042–1044.

103 Gibbons, S., Leimkugel, J., Oluwatuyi, M., Heinrich, M. *Phytother. Res.* **2003**, *17*, 274–275.

104 Gniadkowski, M. *Clin. Microbiol. Infect.* **2001**, *7*, 597–608.

105 Nostro, A., Cellini, L., Di Bartolomeo, S., Di Campli, E. Grande, R., Cannatelli, M.A., Marzio, L., Alonzo, V. *Phytother. Res.* **2005**, *19*, 198–202.

106 Anki, S., Mirelman, D. Microbes Infect. **1999**, *1*, 125–129.

107 Martin, K.W., Ernst, E. *J. Antimicrob. Chemother.* **2003**, *51*, 241–246.

108 Rani, P., and Khullar N. *Phytother. Res.* **2004**, *18*, 670–673.

109 Yanagawa, Y., Yamamoto, Y., Hara, Y., Shimamura, T. *Curr. Microbiol.* **2003**, *47*, 244–249.

110 Bergonzelli, G.E., Donnicola, D., Porta, N., Corthésy-Theulaz, I.E. *Antimicrob. Agents Chemother.* **2003**, *47*, 3240–3246.

111 Akinyemi, K.O., Mendie, U.E., Smith, S.T., Oyefolu, A.O., Coker, A.O. *J. Herb. Pharmacother.* **2004**, *5*, 45–60.

112 Nariman, F., Eftekhar, F., Habibi, Z., Falsafi, T. *Helicobacter* **2004**, *9*, 146–151.

113 Fukai,T., Marumo, A., Kaitou, K., Kanda, T., Terada, S., Nomura, T. *Life Sci.* **2002**, *71*, 1449–1463.

114 Krausse, R., Bielenberg, J., Blaschek, W., Ullmann, U. *J. Antimicrob. Chemother.* **2004**, *54*, 243–246.

115 Mimica-Dukic, N., Bozin, B., Sokovic, M., Mihajlovic, B., Matavulj, M. *Planta Med.* **2003**, *69*, 413–419.

116 Shin, S. *Arch. Pharm. Res.* **2005**, *28*, 765–769.

117 Silva, O., Ferreira, E., Van Pato, M., Caniça, M., Gomes, E.T. *FEMS Microbiol. Lett.* **2002**, *217*, 271–274.

118 Cantrell, C.L., Franzblau, S.G., Fisher, N.H. *Planta Med.* **2001**, *67*, 685–694.

119 Martin, K.W., Ernst, E. *Mycoses* **2004**, *47*, 87–92.

120 Newton, S.M., Lau, C., Wright, C.W. *Phytother. Res.* **2000**, *14*, 303–322.

121 Okunade, AL., Elvin-Lewis, M.P., Lewis, W.H. *Phytochemistry* **2004**, *65*, 1017–1032.

122 Lall, N., Meyer J.J.M *J. Ethnopharmacol.* **1999**, *66*, 347–354.

123 Ficker, C.E., Smith, M.L., Susiarti S., Leaman, D.J, Irawati, Ç., Arnason, J.T. *J. Ethnopharmacol.* **2003**, *85*, 289–293.

124 Shahidi Bonjar, G.H. *Fitoterapia* **2004**, *75*, 74–76.

125 Jimenez-Arellanes, A., Meckes, M., Ramirez, R., Torres, J., Luna-Herrera, J. *Phytother. Res.* **2003**, *17*, 903–908.

126 Somchit, M.N., Reezal, I., Elisha Nur, I., Mutalib, A.R. *J. Ethnopharmacol.* **2003**, *84*, 1–4.

127 Quale, J.M., Landman, D., Zaman, M.M., Burney, S., Sathe, S.S. *Am. J. Chin. Med.* **1996**, *24*, 103–109.

128 Lall, N., Das Sarma, M., Hazra, B., Meyer, J. J. M. *J. Antimicrob. Chemother.* **2003**, *51*, 435–438.

129 Lall, N., Meyer J.J.M. *J. Ethnopharmacol.* **2001**, *78*, 213–216.

130 Mossa, J.S., El-Feraly, F.S., Muhammad, I. *Phytother. Res.* **2004**, *18*, 934–937.

131 Yang, X., Summerhurst, D.K., Koval, S.F., Ficker, C., Smith, M.L., Bernards, M.A. *Phytother. Res.* **2001**, *15*, 676–680.

132 Ergin, A., Arikan, S. *J. Chemother.* **2002**, *14*, 465–472.

133 Mondello, F., De Bernardis, F., Girolamo, A., Salvatore, G., Cassone, A. *J. Antimicrob. Chemother.* **2003**, *51*, 1223–1229.

134 Pina-Vaz, C., Gonçalves Rodrigues, A., Pinto, E., Costa-de-Oliveira, S., Tavares, C., Salgueiro, L., Cavaleiro, C., Gonçalves, M.J., Martinez-de-Oliveira, J. *J. Eur. Acad. Dermatol. Venereol.* **2004**, *18*, 73–78.

135 Takahashi, O., Cai, Z., Toda, M., Hara, Y., Shimamura, T. *Kansenshogaku Zasshi* **1995**, *69*, 1126–1134.

136 Yam, Y. S., Hamilton-Miller, J. M., Shah. S. *J. Antimicrob. Chemother.* **1998**, *42*, 211–216.

137 Shiota, S., Shimizu, M., Mizushima, T., Ito, H., Hatano, T., Yoshida, T., Tsuchiya, T. *Biol. Pharm. Bull.* **1999**, *22*, 1388–1390.

138 Hatano, T., Kusuda, M., Hori, M., Shiota, S., Tsuchiya, T., Yoshida, T. *Planta Med.* **2003**, *69*, 984–989.

139 Akiyama, H., Fujii, K., Yamasaki, O., Oono, T., Iwatsuki, K. *J. Antimicrob. Chemother.* **2001**, *48*, 487–491.

140 Zhao, W.H., Hu, Z.Q., Okubo, S., Hara, Y., Shimamura, T. *Antimicrob. Agents Chemother.* **2001**, *45*, 1737–1742.

141 Zhao, W.H., Hu, Z.Q., Hara, Y., Shimamura, T. *Antimicrob. Agents Chemother.* **2002**, *46*, 2266–2268.

142 Zhao, W.H., Asano, N., Hu, Z.Q., Shimamura, T. *J. Pharm. Pharmacol.* **2003**, *55*, 735–740.

143 Hu, Z.Q., Zhao, W.H., Hara, Y., Shimamura, T. *J. Antimicrob. Chemother.* **2001**, *48*, 361–364.

144 Hu, Z.Q., Zhao, W.H., Asano, N., Yoda, Y., Hara, Y., Shimamura, T. *Antimicrob. Agents Chemother.* **2002**, *46*, 558–560.

145 Hu, Z.Q., Zhao, W.H., Yoda, Y., Asano, N., Hara, Y., Shimamura, T. *J. Antimicrob. Chemother.* **2002**, *50*, 1051–1054.

146 Shiota, S., Shimizu, M., Mizushima, T., Ito, H., Hatano, T., Yoshida, T., Tsuchiya, T. *FEMS Microbiol. Lett.* **2000**, *15*, 135–138.

147 Shiota, S., Shimizu, M., Sugiyama, J., Morita, Y., Mizushima, T., Tsuchiya, T. *Microbiol. Immunol.* **2004**, *48*, 67–73.

148 Nicolson, K., Evans, G., O'Toole P. W. *FEMS Microbiol. Lett.* **1999**, *179*, 233–239.

149 Liu, I.X., Durham, D.G., Richards, R.M. *J. Pharm. Pharmacol.* **2000**, *52*, 361–366.

150 Shimizu, M., Shiota, S., Mizushima, T., Ito, H., Hatano, T., Yoshida, T., Tsuchiya, T. *Antimicrob. Agents Chemother.* **2001**, *45*, 3198–3201.

151 Sakagami, Y., Mimura, M., Kajimura, K., Yokoyama, H., Linuma, M., Tanaka, T., Ohyama, M. *Lett. Appl. Microbiol.* **1998**, *27*, 98–100.

152 Sato, M., Tsuchiya, H., Takase, I., Kureshiro, H., Tanigaki, S., Iinuma, M. *Phytother. Res.* **1995**, *9*, 509–512.

153 Shibata, H., Shirakata, C., Kawasaki, H., Sato, Y., Kuwahara, T., Ohnishi, Y., Arakaki, N., Higuti, T. *Biol. Pharm. Bull.* **2003**, *26*, 1478–1483.

154 Sato, Y., Shibata, H., Arai, T., Yamamoto, A., Okimura, Y., Arakaki, N., Higuti, T. *Int. J. Antimicrob. Agents* **2004**, *24*, 28–35.

155 Sato, Y., Shibata, H., Arakaki, N., Higuti, T. *Antimicrob. Agents Chemother.* **2004**, *48*, 1357–1360.

156 Shibata, H., Kondo, K., Katsuyama, R., Kawazoe, K., Sato, Y., Marakami, K., Takaishi, Y., Arakaki, N., Higuti, T. *Antimicrob. Agents Chemother.* **2005**, *49*, 549–555.

157 Liu, L.X., Durham, D.G., Richards, R.M. *J. Pharm. Pharmacol.* **2001**, *53*, 129–132.

158 Shahverdi, A.R., Rafii, F., Tavassoli, F., Bagheri, M., Attar, F., Ghahraman A. *Phytother. Res.* **2004**, *18*, 911–914.

159 Iwalokun, B. A., Gbenle, G. O., Adewole, T. A., Smith, S. I., Akinsinde, K. A., Omonigbehin E.O. *Acta Pathol. Microbiol. Immunol. Scand.* **2003**, *111*, 477–482.

160 Tegos, G., Stermitz, F.R., Lomovskaya, O., Lewis, K. *Antimicrob. Agents Chemother.* **2002**, *46*, 3133–3141.

161 Gibbons, S., Udo, E.E. *Phytother. Res.* **2000**, *14*, 139–140.

162 Schmitz, F.J., Fluit, A.C., Lückefahr, M., Engler, B., Hofmann, B., Verhoef, J., Heinz, H.P., Hadding, U., Jones, M.E. *J. Antimicrob. Chemother.* **1998**, *42*, 807–810.

163 Stermitz, F.R., Lorenz, P., Tawara, J.N., Zenewicz, L.A., Lewis, K. *Proc. Natl Acad. Sci. USA* **2000**, *97*, 1433–1437.

164 Stermitz, F.R., Tawara-Matsuda, J., Lorenz, P., Mueller, P., Zenewicz, L., Lewis, K. *J. Nat. Prod.* **2000**, *63*, 1146–1149.

165 Stermitz, F.R., Beeson, T.D., Mueller, P.J., Hsiang, J., Lewis, K. *Biochem. Syst. Ecol.* **2001**, *29*, 793–798.

166 Musumeci, R., Speciale, A., Costanzo, R., Annino, A., Ragusa, S., Rapisarda, A., Pappalardo, M.S., Iauk, L. *Int. J. Antimicrob. Agents* **2003**, *22*, 48–53.

167 Guz, N.R., Stermitz, F.R., Johnson, J.B., Beeson, T.D., Willen, S., Hsiang, J.F., Lewis, K. *J. Med. Chem.* **2001**, *44*, 261–268.

168 Stermitz, F.R., Scriven, L.N., Tegos, G., Lewis, K. *Planta Med.* **2002**, *68*, 1140–1141.

169 Morel, C., Stermitz, F.R., Tegos, G., Lewis, K. *J. Agric. Food Chem.* **2003**, *51*, 5677–5679.

170 Stermitz, F.R., Cashman, K.K., Halligan, K.M., Morel, C., Tegos, G.P., Lewis, K. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1915–1918.

171 Gibbons, S., Moser, E., Kaatz, G.W. *Planta Med.* **2004**, *70*, 1240–1242.

172 Sudano Roccaro, A., Blanco, A.R., Giuliano, F., Rusciano, D., Enea, V. *Antimicrob. Agents Chemother.* **2004**, *48*, 1968–1973.

173 Abulrob, A.N., Suller, M.T.E., Gumbleton, M., Simons, C., Russell, A.D. *Phytochemistry* **2004**, *65*, 3021–3027.

174 Aburjai, T., Darwish, R.M., Al–Khalil, S., Mahafzah, A., Al–Abbadi, A. *J. Ethnopharmacol.* **2001**, *76*, 39–44.

175 Darwish, R.M., Aburjai, T., Al-Khalil, S., Mahafzah, A. *J. Ethnopharmacol.* **2002**, *79*, 359–364.

176 Kim, H., Moon, K.H., Ryu, S.Y., Moon, D.C., Lee, C.K. *Arch. Pharm. Res.* **1998**, *21*, 734–737.

177 Lee, C.K., Kim, H., Moon, K.H., Shin, K.H. *Arch. Pharm. Res.* **1998**, *21*, 62–66.

178 Stewart, P. S. Int. *J. Med. Microbiol.* **2002**, *292*, 107–113.

179 Al-Shuneigat, J., Cox, S.D., Markham, J.L. *Lett. Appl. Microbiol.* **2005**, *41*, 52–55.

180 Knowles, J.R., Roller, S., Murray, D.B., Naidu, A.S. *Appl. Environ. Microbiol.* **2005**, *71*, 797–803.

181 Pérez-Giraldo, C., Cruz-Villalón, G., Sánchez-Silos, R., Martinéz-Rubio, R., Blanco, M.T., Gómez-García, A.C. *J. Appl. Microbiol.* **2003**, *95*, 709–711.

182 Ouhayoun, J.P. *J. Clin. Periodontol.* **2003**, *30* (Suppl 5), 10–12.

183 Leite, A.A.M., Nardi, R.M.D., Nicoli, J.R., Chartone-Souza, E., Nascimento, A.M.A. *J. Gen. Appl. Microbiol.* **2005**, *51*, 21–26.

184 Zhao, W.H., Hu, Z.Q., Hara, Y., Shimamura, T. *J. Infect. Chemother.* **2001**, *7*, 195–197.

185 Beg, A.Z., Ahmad, I. *J. Microbiol. Biotechnol.* **2000**, *16*, 841–844.

11
An Alternative Holistic Medicinal Approach to the Total Management of Hepatic Disorders: A Novel Polyherbal Formulation

Mohammad Owais, Iqbal Ahmad, Shazia Khan, Umber Khan, and Nadeem Ahmad

Summary

The liver, the largest internal organ, plays a major role in metabolic processes, performing a number of physiological functions. The liver functions as a great metabolic factory and is particularly concerned with metabolizing drugs, especially those given orally. It plays a key role in the metabolism of lipids, proteins, and carbohydrates, as well as in immunomodulation. The sheer complexity and varied nature of its interactions continually exposes it to a variety of toxins, therapeutic agents, etc., making it susceptible to literally hundreds of diseases. Some of these diseases are rare; others are more common, such as hepatitis, cirrhosis, pediatric liver disorders, alcohol-related disorders, liver cancer, and weakened liver function in older people. Cirrhosis is the third leading cause of death in adults aged between 25 and 59, and the seventh leading cause of death overall. With lack of effective treatment for liver diseases, researchers are turning towards ethnic drugs of herbal origin used traditionally, especially in the light of new findings.

11.1
Introduction

The fact that the liver plays a significant role in physiological processes has been known to physicians from ancient times, as is evident from descriptions in the earliest medical treatises [1]. All substances absorbed by the gastrointestinal tract pass through the hepatic system before entering the circulation system, making the liver a focal point of biological faculty – a key player in metabolism. Many chronic, irreversible, and acute hepatic diseases and disorders culminate in untimely death due to lack of adequate remedies in modern medicines. Over the past few decades there has been considerable improvement in our understanding of the cellular mechanisms and pathophysiology underlying liver diseases. In spite of remarkable breakthrough made in mainstream modern medicine to unravel complicated

metabolic processes of liver, no single therapeutic agent has been found to date that can provide a lasting remedy for patients with hepatic disorders. This clearly highlights the relevance of alternative therapies to offer effective remedy for various hepatic diseases.

The liver as a regulator of metabolism also plays a role in longevity as well as in general health. There has been widespread recognition that indigenous drugs used traditionally by ethnic tribes or societies across the globe can provide respite to patients with hepatic disorders. As a result we are witnessing a conscious effort to screen indigenous drugs used conventionally in different parts and regions of the world, especially China and India [2, 3]. Tapping the pool of knowledge generated by this collective effort for holistic treatments with low side-effects by formulating a new polyherbal drug is a more desirable and sought-after goal now than ever before. Harvesting a drug, its active ingredient, as well as polymers and other excipients from natural herbal sources is not only feasible but also technically and commercially viable. The absence of or very low incidence of adverse effects and side-effects in the case of herbal resources have made them very popular for the treatment of hepatic disorders.

The liver is the largest gland of the body, comprising up to 3.5% of the body weight of an adult rat [4] or 2% of the body weight of an adult human. A miniature refinery, the liver processes many chemicals necessary for the body's overall functioning. It converts carbohydrates, fats, and proteins into chemicals essential for life and growth. It manufactures and exports to other organs some of the substances they need to function properly, such as the bile used by the intestine during digestion. It modifies drugs taken to treat disease into forms that are more easily used by the body. It cleanses the blood of toxic substances either ingested or produced by the body itself.

The liver also has other important functions, such as the storage of extra vitamins, minerals, and sugars to prevent shortages or to produce energy quickly as needed. The liver controls the production and excretion of cholesterol and maintains hormonal balance. It monitors and maintains the right level of numerous chemical and drugs in the blood, as well as storing iron. It helps the body resist infection by producing immune factors and removing bacteria from the blood. It regulates the blood's ability to clot and governs the transport of fat stores as well as breaking down alcohol.

Nevertheless, the liver's susceptibility to damage is far greater than that of any other internal organ. This probably explains its remarkable power of regeneration. It is able to regenerate itself after being injured or diseased. If, however, a disease progresses beyond the liver tissue's capacity to generate new cells, an imbalance between liver cell death and regeneration may occur, leading to hepatic injury and subsequently to its failure, thus severely affecting the body's entire metabolism. A large number of disorders can affect the liver and interfere with the blood supply, the hepatic and Kupffer cells and the bile ducts.

The ineffectiveness of modern therapeutic agents in completely curing hepatic disorders has been lamented. The remedies available in modern medicine provide

only symptomatic relief, without any significant changes to the etiological causes of the disease process. Their usage, especially if prolonged, is associated with severe side-effects and the chances of relapses if the therapy is discontinued are high. Moreover, as many therapeutic agents are known to cause conditions such as liver cirrhosis and fulminant hepatic failure, the development or identification of new molecules effective in treating or preventing hepatic damage remains a challenge in the field of drug development.

The commonly occurring liver diseases include cirrhosis, hepatitis, liver abscesses, and pediatric liver diseases. Other serious diseases of the liver include fatty liver, hepatic coma, and liver cancer [6, 7]. Causes of liver damage include drug overdose, metabolic and autoimmune disorders, many chemical drugs and trauma [8, 9]. Liver failure can progress extremely rapidly as in fulminant hepatic failure (FHF) [10], or slowly, as with chronic liver diseases [11].

Viral hepatitis (A, B, C, D, E, and G) is a contagious infection of liver usually caused by one of three different organisms. Hepatitis A, formerly known as infectious hepatitis, can be contracted by consuming contaminated water or food, most notably shellfish. The virus is eliminated in stool and though seldom serious it can cause severe liver failure and death. It does not cause chronic hepatitis and does not lead to cirrhosis or other long-term liver problems. Hepatitis B, formerly known as serum hepatitis, is found in blood and other body fluids such as urine, tears, semen, breast milk, and vaginal secretions. It is usually transmitted in blood, via transfusion or through illicit injectable-drug use. Type C hepatitis virus is the cause of a disease known as "non-A, non-B hepatitis" which is also contracted through contact with infected person. Viral hepatitis may produce no symptoms at all. Symptoms of viral hepatitis mimic flu. While patients recover from hepatitis A and develop a lifelong immunity, hepatitis B patients may become chronic carriers for an indefinite period. Cirrhosis and primary cancer of the liver can also be long-term consequences.

In cirrhosis, liver cells are damaged and replaced by scar tissue which as it accumulates, hardens the liver, diminishes blood flow, and causes even more cells to die. The loss of liver function that accompanies this degenerative condition results in gastrointestinal disturbances, jaundice, enlargement of liver and spleen, maceration and accumulation of fluid in the abdomen and other tissues. Alcohol abuse, hepatitis, chemical poisoning, excess of iron or copper, other viruses, and blockages of the bile duct can cause the disease.

Liver abscesses are caused by bacteria such as *Escherichia coli*, *Staphylococcus*, or *Entamoeba histolytica* and result in destruction of liver tissue leaving a cavity that fills with other infectious organisms, white blood cells, and liquefied liver cells. Symptoms include pain, fever, jaundice, and anemia.

There are about 100 pediatric liver diseases affecting children, most of which are genetic. They include biliary atresia, which is characterized by an inadequate bile duct and often fatal, chronic active hepatitis; Wilson's disease, characterized by and abnormally large build-up of copper in the liver; and Reye's syndrome, in which fat accumulates in the liver and the patient lapses into coma.

In general, the effects induced by hepatotoxic compounds are reversible if the causative substance is withdrawn, but in many cases this is not possible. The causative substance may not always be discernible, for example, in which case exposure cannot be stopped or prevented. Moreover, the administration of anticancer, antiepileptic, or antituberculosis drugs, which are mildly hepatotoxic, has to be continued for prolonged periods because of the lack of alternative therapies. Likewise, stopping treatment with interferon alpha, a biotech medicine derived from the human immune system, and ribovarin is not easy, as side-effects tend to be maximal in the first two weeks.

11.2
Conventional Medicines for Liver Disorders

Immunoglobulin (Ig) is quite effective against hepatitis A when administered to anyone exposed to the virus as soon as possible or within two weeks after jaundice appears. Vaccines for hepatitis are now a common feature of immunization programs the world over. Treatment for acute hepatitis consists of rest and small, nourishing meals, fluids, and sometimes antinausea drugs such as trimethobenzamide (Tigan). Chronic cases of hepatitis B and C are treated with interferon. The problem of gallstones is usually solved by surgical operation. Chenodiol, a recently available drug that dissolves gallstones is an alternative to surgery, but troublesome side-effects have been reported.

In the treatment of cirrhosis elimination of the underlying cause is emphasized, if possible, to avoid further damage, and to prevent or treat complications. Diuretics, vitamins, and abstinence from alcohol are supportive measures. For extreme cases a liver transplant is an option, though risky. If the offending organism cannot be determined, liver abscesses are treated with long-term administration of antibiotics such as aminoglycosides, cephalosporins, clindamycin, or chloramphemicol. If *E. coli* is the cause of infection, treatment includes ampicillin. For *Entamoeba histolytica*, chloroquine (aralen) or metronidazole (flazyl) are included. Biliary atresia is sometimes relieved by surgery. Vitamin B6 and *d*-penicillamine as well as corticosteroids such as prednisone are administered in cases of Wilson's disease.

Despite advancements in modern medicine, no hepatoprotective medicine is available. Treatment options for cirrhosis, fatty liver, and chronic hepatitis are limited as well as problematic. The conventional drugs used in such treatments are corticosteroids, interferon, colchicine, penicillamine, and antiviral and immune suppressant drugs. These are inadequate and inconsistent at best. Paradoxically, these drugs may themselves cause damage (e.g. azathioprine can cause cholestatic jaundice [12], while interferons and virazole can cause elevation of serum transaminase [13, 14]). Alternative treatments for liver diseases to replace the currently used drugs need to be given impetus in the light of current findings from research studies and publications in the field of herbal treatment of liver diseases, especially during the last quarter of the twentieth century.

11.3
Herbal Medicines – Potential Therapeutic Agents with Minimal Side-Effects

Indigenous medicines, especially of plant origin, are used extensively for the treatment of various diseases. With lack of safe and effective treatment for liver diseases, researchers have been looking for alternative therapies that curb symptoms with minimum adverse effects on patients. *Silybum marianum* (milk-thistle) [15] and its extracts have been used since the times of ancient Greece for medicinal purposes. It is now currently used widely in Europe for liver disease, and is readily available in the United States from alternative medicine outlets and outdoor markets. Studies on effect of silymarin, an extract of milk-thistle, in preventing complications of chronic hepatitis virus infection at a dose of 140 mg three times daily suggest there is a need for optimization (e.g. single dosage, dose doubling), as efficacy could not be established. Silymarin may benefit the liver by promoting the growth of certain types of liver cells, demonstrating a protective effect, inhibiting inflammation and fighting oxidation. Similar studies have been reported from China, Africa, Arabia and India.

As well as milk-thistle, several hundred other plants are reported to have hepatoprotective properties [18], and a number of studies have been conducted taking into consideration valid scientific, clinical, and research parameters. These plants include *Cochlospurmum planchonii* [17], *Zingiber officinale* [19], *Nardostachys jatamansi* (jatamansi) [20], *Swertia chirata* (chirayata) [21, 22], *Cichorium intybus*. (chicory) [23], *Hyprophilia auriculata* (talamakhana] [24], *Apium graveolens* (celery), *Tephrosia purpurea* (sharpunkha) [25], *Plumbazo zeylanica* (chitrak) [25], *Solanum nigrum* (makove) [26], *Tinospora cardifolia* (guduchi) [27] *Terminalia belerica* (bibhitake) [28], *Boerhavia diffusa* (punarnava) [29], *Eclipta alba* (bhringraj) [30], *Andrographis peninculata* (kalmegh) [31], *Allium sativa* (garlic) [32], *Glycyrrhiza uralensis* (liquorice) [33], *Camellia sinensis* (green tea) [34], *Curcuma longa* (turmeric) [35], *Picrorhiza kurroa* (katuki) [36], *Oldenlandia corymbasa*, *Asteracantha longifolia*, *Cassia occidentalis*, *Embelia ribes*, *Trachyspermum ammi*, and *Capparis spinosa*.

Some of the constituents isolated from these hepatoprotective plants and reported to have antihepatotoxic activity include kaempferol, caffeic acid, ferulic acid, and *p*-cumaric acid (*Capparis spinosa*), azelaic acid, alpha-amyfrin, taraxerone, baurenyl acetate, beta-sitosterol, and daucosterol (*Cichorium intybus*), nigrumnins I and II (*Solanum nigrum*), arjunetoside, oleanolic acid, arjunic acid, and arjunaphthanoloside (*Terminalia arjuna*), andrographolide (*Andrographis paninculata*), silybin and silymarin (*Silybum marianum*), kutkoside and picroside I and II (*Picrorhiza kurroa*), gomishins (*Schizandra achinensis*), wuweizisuc and schisandrin A (*Schizandra chinensis*), glycyrrhizin and glycyrrhizinic acid (*Glycyrrhiza glabra*), saikosaponins (*Bupleurum falcatum*), sarmantosins (*Sedum sarmentosum*), catechin (*Anacardium occidentalis*), ursolic acid (*Eucalyptus* spp.), curcumin (*Curcuma longa*), and fumaric acid (*Sida cardifolia*).

In India hundreds of medicinal plants are used alone or in different combinations in the preparation of around three dozen patented herbal formulations [37]. A large number of plants have been studied in last couple of years for their antihe-

patotoxic potential. However in most cases, the mechanism of their hepatoprotective effect still remains to be ascertained. Most of the plants have been shown to stimulate secretion of bile fluid (choleretic) and salt (chologogue) in experimental animals [37]. Potent hepatoprotective plants such as *Andrographis paniculata* and *Trichopus zeylanicus* also stimulate biliary function in normal rats [38, 39]. In general, the therapeutic values of drugs are evaluated in model animals by inducing the disease and comparing the parameters of model drugs with those of active ingredients or extracts. Formulations may also be prepared using active ingredient or excipients from natural sources in the preparation of drug delivery system for studies on efficacy. Sometimes simple powdered forms of parts of herbs are also used in clinical studies.

Detailed efficacy and toxicity studies in experimental animals should be followed by clinical trials. Biochemical and other *in vitro* assays are required to determine the mechanism of action of these herbal products. To assess any toxic activity, *in vivo* and *in vitro* test systems are used. Identifying the hepatoprotective efficacy of drugs is not easy as this activity for a given drug may be different against different toxins [40]. Thus the efficacy of each drug has to be tested against hepatotoxins that act by different methods. Currently available data show that a few plants are promising hepatoprotective agents. These include *Capparis spinosa* (kaempferol), *Picrorhiza kurroa* (picroliv), *Andrographis paninculata* (andrographolide), and *Silybum marianum* (silymarin). Kumars and Mishra have documented the hepatoprotective activity of fumaric acid from *Sida cardifolia* [41]. Ursolic acid, which occurs in many plants, also shows hepatoprotective properties [42, 43].

Although some herbal medicines are effective in the treatment of diseases against which modern medicines are inefficient, very often these drugs are unscientifically exploited and improperly used. Numerous plants and polyherbal formulations are used for the treatment of liver diseases. However, in most of the severe cases, the treatments are not satisfactory. Experimental evaluation in most cases has been incomplete and insufficient and the therapeutic values have been tested against chemically induced subclinical levels of damage in rodents. Even common dietary antioxidant and micronutrients such as tocopherol [44], ascorbic acid [45], beta-carotene [45], glutathione, uric acid, and bilirubin, and proteins such as ceruloplasmin can provide protection from liver damage.

The synergistic action of various ingredients of a polyherbal formulation for holistic and long-lasting cure of hepatic disorder might help in regulating the metabolism, which is one of the factors responsible for longevity. Various experimental and clinical studies by different researchers have been well documented in this subject field. Khanfar et al. isolated and identified the active ingredient of *Capparis spinosa* as "beta 3-methyl-2-butenyl-beta-glucoside" [47]. "*p*-Methoxy benzoic acid" isolated from *Capparis spinosa* was found to possess potent hepatoprotective activity against CCl_4-, paracetamol- (*in vivo*), and thiacetamide galactosamine- (*in vitro*) induced hepatotoxicity [48]. Al-Said et al. demonstrated the strong anti-inflammatory activity of *Capparis spinosa*, which was comparable to that of oxyphenbutazone [49, 50]. Bonina et al. documented a significant antioxidant activity of *Capparis spinosa* and also identified flavonols (kaempferol and quercetin derivatives) and hy-

droxycinnamic acids (caffeic acid, ferulic acid, *p*-cumaric acid, and cinnamic acid) as major antioxidants from *Capparis spinosa* [51]. Mahasneh observed potent antimicrobial and antifungal activity of *Capparis spinosa* [52, 53].

He and co-workers isolated 2,3,4,9-tetrahydro-14-pyrido [3,4-b] indole-3-carboxylic acid, azelaic acid, and daucosterol as the major constituents of *Cichorium intybus* [54], and Du et al. identified the other constituents as alpha-amyrin, taraxerone, baurenyl acetate, and beta-sitosterol [55]. Aktay et al. and Zafar et al. observed the hepatoprotective effect (confirmed by histopathological examination) of *Cichorium intybus* against CCl_4-induced hepatotoxicity and reported significant prevention of the elevation of malondialdehyde formation (plasma and hepatic) and enzyme levels (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) [23, 56]. Ahmed et al. screened *Cichorium intybus* for antihepatotoxic activity and measured the degree of protection using biochemical parameters (AST, ALT, alkaline phosphatase (ALP), and total protein (TP)). Potent antihepatotoxic activity comparable to silymarin was observed with almost complete normalization of the tissues (as neither fatty accumulation nor necrosis was observed on histopathological studies) [57]. Mun et al. studied the effects of *Cichorium intybus* on the immunotoxicity of ethanol and reported a significant increase in the number of circulating leukocytes, the weight of concerned organs (liver, spleen, and thymus), number of splenic plaque-forming cells, hemagglutination titers, and the secondary IgG antibody response. A significant increase in delayed-type hypersensitivity reaction, phagocytic activity, natural killer cell activity, cell proliferation, and interferon-gamma secretion was also observed [58]. Sultana et al. reported that the presence of *Cichorium intybus* in the reaction mixture containing calf thymus DNA and a free radical-generating system protects DNA against oxidative damage to its sugar moiety.

All these studies suggest that the observed hepatoprotective effects might be due to the ability to suppress the oxidative degradation of DNA in the tissue debris [59]. Gurbuz et al. observed significant cytoprotection against ethanol-induced damage and these results were further confirmed by using histopathological techniques [60]. Aminghofran et al. reported the capacity of *Cichorium intybus* to enhance the proliferation of lymphocytes after stimulation with allogenic cells [61]. Kim et al. investigated the effect of *Cichorium intybus* on mast cell-mediated immediate-type allergic reactions and observed inhibition of the systemic anaphylactic reaction and a reduction of plasma histamine levels [62].

Ikeda et al. identified saponin (nigrumnins I and II) as the active ingredients of *Solanum nigrum* [63]. *Solanum nigrum* was investigated for its hepatoprotective activity against CCl_4-induced hepatic damage and Raju et al. observed remarkable hepatoprotective activity confirmed by evaluated biochemical parameters (AST, ALT, ALP, and TP) [64]. Moundipa et al. studied the effects of *Solanum nigrum* on hepatotoxicity and reported increased level of activity of aminopyrine, *N*-dimethylase, uridine diphosphate, glucuronyl transferase and glutathione-*S*-transferase, without any alteration in levels of ALP, ALT, and gamma-glutamyltransferase levels in the serum [65]. Prasant Kumar et al. tested *Solanum nigrum in vitro* for its cytoprotective activity against gentamicin-induced toxicity and observed significant

inhibition of cytotoxicity, along with hydroxyl radical scavenging potential, which might be the mechanism of cytoprotection [66]. Qureshi et al. reported the antifungal activity of *Solanum nigrum* [67]. Perumal Samy et al. demonstrated the potent antibacterial activity of *Terminalia arjuna* [68].

Ali et al. demonstrated that arjunaphthanoloside from *Terminalia arjuna* decreases inducible nitric oxide synthase levels in lipopolysaccharide-stimulated peritoneal macrophages [69]. Jafri et al. reported significant hepatoprotective effects of *Cassia occidentalis* in chemically induced liver damage [70]. Bin-Hafeez et al. showed that *Cassia occidentalis* modulated hepatic enzymes and provided hepatoprotection against induced immunosuppression [71]. Harnyk et al.. demonstrated the clinically beneficial effects of *Achillea millefolium* in the treatment of chronic hepatitis [72]. Kriverko et al. reported clinical improvements in chronic hepatocholecystitis and angiocholitis with *Achillea millefolium* [73]. Lin et al. observed antihepatoma activity of *Achillea millefolium* [74]. Devarshi et al. studied *Mandura bhasma* for its hepatoprotective properties in hepatitis induced by CCl_4 and observed prevention of CCl_4-mediated changes in enzyme activities, which suggest the hepatoprotective role of the plant [75].

The synergistic action of a polyherbal formulation (hepatoprotective, antimicrobial, antioxidant, and anti-inflammatory) could bring about holistic cure and treatment.

11.4 Contributions of Elementology to Potential Treatments for Hepatic Disorders

Elementology is a new branch of the natural sciences, based on the scientific study of metals and other trace elements for their therapeutic value. The search for alternative therapies in hepatic disorders provides immense opportunity for the field. The liver plays an important role in element metabolism, both in normal and pathological conditions. Medicines taken through the oral route on reaching the gastrointestinal system are first released from the various formulations in order to be absorbed before becoming bioavailable. The liver plays a regulatory role in metabolism, as it is the very first organ perfused by the hepatic portal system containing newly absorbed materials. In case of minerals, the liver acts as a sink for excess absorbed materials or metal ions. Minerals released from the liver are usually bound by plasma proteins that are mostly synthesized in the liver, such as albumin, ceruloplasmin, etc. [76].

The significance of trace elements in biological systems is widely recognized, since they are components of many metalloproteins and metal enzymes. The properties of trace elements, which feature in their therapeutic activity, are in binding to macromolecules (enzymes, nucleic acids). This is far from specific, as is reflected in the fact that a number of diseases involve trace elements. Interactions with other elements are another such property [77]. The role of elements in the treatment of liver disease is very well documented [78].

Cascales and coworkers investigated altered liver function induced by chronic administration of thioacetamide (TAA), which was partially restored by rhodium complex [79]. Schwartz reported the importance of selenium in the treatment of liver necrosis [80]. The biochemical role of selenium as a component of glutathione peroxidase was studied by Rotruck et al. [81]. The antioxidant activity of this enzyme serves to maintain the integrity of cellular and subcellular structures. Selenium is a natural antioxidant and appears to preserve tissue elasticity by delaying the oxidation of polyunsaturated fatty acids. Selenium participates in the lipooxygenase pathway along with catalase, superoxide dismutase, vitamin E, vitamin C, carotenoids etc., whose principal function is to eliminate the free radicals involved in the pathogenesis of liver disorders [82]. Zinc, as a component of metalloenzymes, protects against hydroxyl radicals and inhibits apoptosis induced by glucocorticoids. It is also effective against cirrhosis induced by thioacetamide [83, 84]. Boron hydrides are also inhibitors of pyridoxal-dependent enzymes and aspartate aminotransferase activity and interact with cytochrome P-450 enzyme system of liver. Boron has recently been reported to protect against liver injury [85, 86].

Copper is a component of a variety of oxidative enzymes including ceruloplasmin, cytochrome oxidase, monoamine oxidase, and superoxide dismutase. It is also important in liver disorders, as liver is the organ responsible for storage of copper, its incorporation into ceruloplasmin, and its secretion in bile. Small amounts of copper are stored in liver in its parenchymal cells. Copper, added generally as copper sulfate to the diets of experimental animals, resulted in a decrease in hepatoma formation in response to carcinogenic azodyes and ethionine. Copper may also exert an effect through its role in the complex with a tripeptide, glycyl-histidyl-lysine, which may function to regulate growth and adhesiveness of both normal liver and cultured hepatoma cell [87–90].

The hepatoprotective effect of the organic germanium compound propagermanium is seen against concanavalin A and lipopolysaccharide-induced liver injury in mice [91]. The anticarcinogenic activity of manganese was also studied and it was found to antagonize the carcinogenic effects of simultaneously applied nickel sulfide in rats. The carcinoma incidence was reduced from 77% in rats not given manganese to 70% in those given manganese. This was found to be due to its effect on superoxide dismutase, which prevents accumulation of the manganese superoxide radical [92].

The hepatoprotective effect of nickel was linked to an increase in the erythrocyte activity of Cu-Zn superoxide dismutase by $NiCl_2$, which catalyzes the dismutation of the superoxide free radical and protects cells against superoxide damage [93]. Tin, as $SnCl_2$, acts as reducing agent and can remove superoxide by reduction. Sn^{4+} protoporphyrin IX is a potent competitive inhibitor of heme oxygenase and thus of heme oxidation in liver, spleen, and kidney. This indicates the possibility that tin protoporphyrin IX may be useful in the chemoprevention of neonatal jaundice or hyperbilirubinemia [94].

11.5 Other Alternatives in Liver Therapy

Apart from the use of herbal medicines and trace metal elements to treat liver disorders there are other alternative approaches currently in use. Some of these therapies are effective in treating liver diseases, as has been shown from a few cases reported in reviews. For example "thymosin therapy" involves using hormones normally secreted by the thymus gland, such as thymosin, thymopoietin, and serum thymic factor. These hormones appear to stimulate the body's production of interferon. People with low levels of these hormones are susceptible to infections of the liver. Replenishment of hormone level according to biological demand might explain the disease alleviation that has been noted in these types of cases [95]. Metabolic therapies involve the use of very high doses of vitamins and restricted diets, the latter to relieve the liver from extra toiling. "Megadose vitamin therapy" is based on the theory that the higher the dose of vitamins, the faster the cure. However, a consistent low dosage of vitamins is a much more effective preventive measure. "Alpha-lipoic acid therapy," which uses the antioxidant enzyme helper alpha-lipoic acid, may just have some benefit in protecting the liver if it is administered soon after an incidence of poisoning, such as from mushroom or acetaminophen overdose [96].

11.6 Conclusions

Because liver diseases can be fatal and because of the susceptibility of liver to damage, we need to be vigilant throughout life in caring for the liver, more so than for any other body organ. Moreover, as there are many gaps in our knowledge of liver functions, we still do not know much about some of the less common liver diseases. Indeed, many domino effects lead to liver diseases and nonprimary factors are always crucial in hepatic disorders. A holistic approach, at least till we have some substantial advances in modern medicine, leads us to a polyherbal formulation for synergistic effects. Prophylactic measures in the absence of toxicity are suggested to be effective. Reducing the rate of metabolism might lead to longevity, so regulation of the hepatic system, which plays coordinating role in metabolism, is an added advantage of an integrated and holistic medicine for total management of a biological system. Plant–drug combinations have been shown to be more useful than individual drugs. Active ingredients may also be studied in combination for efficacy and effectiveness by using different compositions and dosages. Qualitative measures used at present should be supplemented and complimented by indigenous polyherbal formulation to obtain the desired result. Even excipients and trace elements should be obtained from natural sources.

References

1 Ibn-e-Sina. *Tarjuma Qanoon Tibbe Islami ka encylopedia*. Urdu translation by Syed Gulam Husnain Kantoori. Mian Chameeraz-3, Temple Road, Lahore, India, **1992**.
2 Anonymous, *The Wealth of India*, PID, CSIR, New Delhi, **1950**.
3 Satyavati, G.V., Gupta, A.K. *Medicinal Plants of India*, Vols I and II. Indian Council of Medical Research, New Delhi, **1987**.
4 Stribble, J.H., Steffens, A.B. *Ann. Endocr. (Paris)* **1988**, 49 (2), 105–112.
5 Tokunaga, Y., Matsugzwa, Y., Kotani, K., Keno, Y., Kobatake, T., Fujioka, S., Tarui, S. *Int. J. Obes.* **1991**, 15(1), 1–5.
6 Youssef, W.I., Tavill, A.S. *J. Clin. Gastroenterol.* **2002**, 35(4), 345–349.
7 Evans, C.D., Oien K.A., Mac-Sween, R.N., Mills, P.R. *J. Clin. Pathol.* **2002**, 55(9), 689–692.
8 Lee, W.R. *N. Engl. J. Med.* **1993**, 329, 1865–1872.
9 O'Grady, J.G. Schalman, S.W., Williams, R. *Lancet* **1993**, 342, 273–275.
10 Ferlan, Marolt, V., Stepes, S. *Dig. Dis. Sci.* **1999**, 44(5), 1054–1058.
11 Mishio, A., Bass, N.M., Luketic, V.A., Coppel, R.L., Gershwin, M.E. *Gastrointest. Dir.* **2001**, 12(2), 89–102.
12 Romaguolo, J, Sadowski, D.C., Lator, E., Jewell, L., Thomson, A.B. *Can. J. Gastroenterol.* **1998**, 12(7), 479–482.
13 Fujimori, K., Mochida, S., Matsui, A., Ohno, A., Fujiwara, K. *J. Gastroenterol.* **2002**, 37(1), 40–46.
14 Cattral, M.S., Hemming, A.W., Wanless, I.R, Al-Ashgar, H. Krajden, M., Lilly, L., Greig, P.D., Levy, G.A. *Transplantation* **1999**, 67(9), 1277–1280.
15 Muriel, P., Mourelle, M. *J. Appl. Toxicol.* **1990**, 10, 275–279.
16 Potter, W.Z., Thorgeisson, S.S., Jollow, D.J. *Pharmacology* **1974**, 12, 129–143.
17 Aliyn, R., Okoye, Z.S.C., Sir, E.T. *J. Ethmopharmacol.* **1995**, 48(2), 89–97.
18 Anonymous, *Pharmacognosy of Indigenous Drugs*. CCRAS Publications, New Delhi **1987**.
19 Agarwal, S., Prakash, S., Rose, J. Paper presented at *International Seminar on Recent Trends in Pharmaceutical Science*, Ootacmund, 18–20 February **1995**, abstract no. D21.
20 Ali, S. Ansari, K.A., Jafry, M.A., Kabeer, H., Diwakar, G. *J. Ethnophamacol.* **2000**, 71, 359–363.
21 Chakravarty, A.K., Mukhopadhyay, S.S., Maitra, S.K., Das, B. *Ind. J. Chem.* **1994**, 33B(4), 405.
22 Mukherjee, S., Sur, A., Maiti, B.R. *Ind. J. Exp. Biol.* **1997**, 35(4), 384–388.
23 Aktay, G., Deliorman, D., Ergun, E., Ergun, F., Yesilada, E. *J. Ethnopharmacol.* **2000**, 73(1–2), 121–129.
24 Singh, A. Handa, S.S. *J. Ethnopharmacol.* **1995**, 49, 119–126.
25 Sharma, A., Singh, R.T., Sehgal, V., Handa, S.S. *Fitoterapia* **1991**, 62, 131–138.
26 Nadeem, M., Dandiya, P.C., Pasha, K.V., Imran, M., Balani, D.K., Vohra, S.B. *Fitoterapia* **1997**, 3, 245–251.
27 Nagarkatti, D.S., Rege, N.N., Desai, N.K. Dahanukar, S.A. *J. Postgrad. Med.* **1994**, 40(2), 65–67.
28 Anand, K.K, Singh, B., Saxena, A.K, Chandan, B.K. Gupta, V.K., Bhardwaj, V. *Pharmacol. Pharmacol. Res.* **1997**, 36(4), 315–321.
29 Rawat, A.K.S., Mehrotra, S. Tripathi, S.C., Shome, U. *J. Ethnopharmacol.* **1997**, 56, 61–66.
30 Saxena, A.K., Singh, B., Anand, K.K. *J. Ethnopharmacol.* **1993**, 40(3), 155–161.
31 Visen, P.K. Shukla, B., Patnaik, G.K., Dhawan, B.N. *J. Ethnopharmacol.* **1993**, 40(2), 131–136.
32 Kim, S.G., Nam, S.Y., Chung, H.C., Hong, S.Y., Jung, K.H. *J. Pharm. Pharmacol.* **1995**, 47(8), 678–682.
33 Shim, S.B., Kim, N.J., Kim, D.H. *Planta Med.* **2000**, 66(1), 40–43.
34 He, P., Moda, Y., Sugiyama, K. *J. Nutr.* **2001**, 131(5), 1560–1567.
35 Sang, E.K., Cho, H., Kim, J.S., Kim, N.Y. An, N.H., Kim, J.A., Lee S.H., Kim, Y.C. *Plant Med.* **2001**, 67(9), 876–877.
36 Floersheim, G.L., Bieri, A., Koenig, R., Pletscher, A. *Agents Actions* **1990**, 29, 386–387.
37 Handa, S.S., Sharma, A., Chakravarty, K.K. *Fitoterapia* **1989**, 57, 307–51.

38 Subramaniam, A. Evans, D.A., Rajashekharan, S., Pushpangadan, P. *Ind. J. Exp. Biol.* **1998**, 36, 385–389.
39 Chaudhary, S.K. *Ind. J. Exp. Biol.* **1978**, 16, 830–836.
40 Chrungo, V.J., Singh K, Singh, J. *Ind. J. Exp. Biol.* **1997**, 35, 603–610.
41 Kumar, S.R., Mishra, S.H. *Ind. Drugs* **1997**, 34, 702–706.
42 Shukla, B., Visen, P.K.S., Patnaik, G.K., Tripathi, S.C., Srimal, R.C., Dayal, R., Dobhal, P.C. *Phytother. Res.* **1992**, 6, 74–79.
43 Saraswat, B., Visen, P.K.S., Dayal, R., Agarwal, D.P., Patnaik, G.K. *Ind. J. Pharmacol.* **1996**, 28, 32–39.
44 Bendich, A., Garbriel, E., Machlin, L.J. *J. Nutr.* **1986**, 116(4), 675–681.
45 Kunert, K.J., Tappel, L. *Lipids* **1983**, 18(4), 27–24.
46 Burton, G.W., Ingold, K.U. *Science* **1984**, 224(4649), 569–573.
47 Khanfar, M.A., Sabri, S.S., Zarga, M.H., Zeller, K.P. *Nat. Prod. Res.* **2003**, 17(1), 9–14.
48 Gadgoli, C., Mishra, S.H. *J. Ethnopharmacol.* **1999**, 66(2), 187–192.
49 Al-Said, M.S., Abdel-Sattar, E.A., Khalifa, S.I., el-Feraly, F.S. *Pharmazie* **1988**, 43(9), 640–641.
50 Azeel, A.M., Parman, N.S., Mossa, J.S., Al-Yahya, M.A., Al-Said M.S., Tariq, M. *Agents Action* **1986**, 17(3–4), 383–384.
51 Bonina, F., Puglia, C., Ventura, D. Aquino, R., Tortora, S., Sacchi, A. et al. *J. Cosmet. Sci.* **2002**, 53(6), 321–335.
52 Mahasneh, A.M. *Phytother. Res.* **2002**, 16(8), 751–753.
53 Ali-Shatayeh, M.S., Abu-Ghdeib, S.I. *Mycoses* **1999**, 42(11–12), 665–672.
54 He, Y., Guo, Y.J., Gao, Y.Y. *Zhongguo Zhong Yao Za Zhi* **2002**, 27(3), 209–210.
55 Du, H., Yuan, S., Jiang, P. *Zhongguo Zhong Yao Za Zhi* **1998**, 23(11), 682–683, 704.
56 Zafar, R., Mujahid, Ali, S. *J. Ethnopharmacol.* **1998**, 63(3), 227–231.
57 Ahmed, B, Al-Howising, T.A. Siddiqui AB. *J. Ethnopharmacol.* **2003**, 87(2–3), 237–240.
58 Mun, J.N., Woo, Y.J., Jeon, W.N., An, N.H., Park, J.S. *Int. Immunopharmacol.* **2002**, 2(6), 733–744.
59 Sultana, S., Parwaiz, S., Iqbal, M., Athar, M. *J. Ethnopharmacol.* **1995**, 45(3), 189–192.
60 Gurbuz, I., Ustun, O., Yesilada, E., Sezik, E., Ekyurek, N. *J. Ethnopharmacol.* **2002**, 83(3), 241–244.
61 Amirghofran, Z., Azadbakht, M., Karimi, M.H. *J. Ethnopharmacol.* **2000**,72(1–2), 167–172.
62 Kim, H.M., Kim, H.W., Lyn, Y.S., Won, J.H., Kim, D.K., Lee, Y.M. *Pharmacol. Res.* **1999**, 40(1), 61–65.
63 Ikeda, T., Tsumagari, H., Mohara, T. *Chem. Pharm. Bull. (Tokyo)* **2000**, 48(2), 1062–1064.
64 Raju, K., Anbuganapathi, G., Gokul, Krishnan, V., Raj Kapoor, B., Jayakar, B., Manian, S. *Biol. Pharm. Bull.* **2003**, 26(11), 1618–1619.
65 Moundipa, P.F., Domngang, F.M. *Br. J. Nutr.* **1991**, 65(1), 89–91.
66 Kumar, V.P., Shashidhara, S., Kumar, M.M., Sridhara, B.Y. *Fitoterapia* **2001**, 72(5), 481–486.
67 Qureshi, S., Rai, M.K., Agarwal, S.C. *Hindustan Antibiotic Bull.* **1997**, 39(1–4), 56–60.
68 Perumal Samy, R., Ignacimuthu, S., Sen, A. *J. Ethnopharmacol.* **1988**, 62(2), 173–182.
69 Ali, A., Kaur, G., Hamid, H., Abdullah, T., Ali, M., Niwa, M. Terminoside, A. *J. Asian Nat. Prod. Res.* **2003**, 5(2) , 137–142.
70 Jafri, M.A., Jalees Subhani, M., Javed, K., Singh, S. *J. Ethnopharmacol.* **1999**, 66(3), 355–361.
71 Bin-Hafeez, B, Ahmad, I., Haque, R., Raisuddin, S. *J. Ethnopharmacol.* **2001**, 75(1), 13–18.
72 Harnyk, T.P. *Lik Sprava* **1999**, (7–8), 168–170.
73 Krivenko, V.V., Potebnia, G.P., Loika, V.V. *Vrach Delo* **1989**, 3, 76–78.
74 Lin, L.T., Liu, L.T., Chiang, L.C., Lin, C.C. *Phytother. Res.* **2002**, 16(5), 440–440.
75 Devarshi, P., Kanase, R., Mane, S., Patil, S., Varute, A.T. *J. Biosci.* **1986**, 10(2), 227–234.
76 Sakumoto, I., Kikuchi, N., Takane, T., Yoshida. H., Hosaka, H., *Gastroenterol. Jpn* **1976**, 11(3) , 224–236.
77 Sarkar, B. *Biol. Trace Elem. Res.* **1989**, 21, 137–144.

78 Antipenko, A.Y., Antipenko, Y.N. *Adv. Enzyme Regul.* **1994**, 34, 173–198.

79 Cascales, M., Martin, Sanz, P., Craciunescu, D.G., Mayo, I., Aquilar, A., Robles, Chillida, E.M., Cascales, C. *Carcinogenesis* **1991**, 12(2), 233–240.

80 Schwartz, K., Foltz, C.M. *J. Am. Chem. Soc.* **1957**, 79, 3292–3293.

81 Rotruck, J.T., Pope, A.L., Ganther, H.E. Swanson W.G. *Science* **1973**, 179(73), 588–590.

82 Batcioglu, K., Karagozler, A.A., Genc, M., Celik, S. *Eur J. Cancer Prev.* **2002**, 11(1), 57–61.

83 Dashti, H.M., Mathew, T.C., Jadaon, M.M., Ashkanani, E. *Nutrition* **1997**, 13(3), 206–212.

84 Telford, W.G., Franker, P.J. *Biochem. Biophys. Res. Commun.* **1997**, 238(1), 86–89.

85 Ali, S., Diwakar, G., Pawa, S., Jain, S.K. *Trace Elem. Exp. Med.* **2002**, 15, 47–55.

86 Valerino, D.M., Soliman, M.R., Aurori, K.C., Tripp, S.L., Wykes, A.A., Vesell, E.S. *Toxicol. Appl. Pharmacol.* **1974**, 29(3), 358–366.

87 Goldblatt, P.J. Gunning WT III. *Ann. Clin. Lab. Sci.* **1984**, 14(2), 159–167.

88 Imai, K., Sato, M., Kojima, N., Miura, M., Safo, T., Sugiyama, T., Enomoto, K., Senoo, H. *Anat. Rec.* **2000**, 258(4), 338–348.

89 Elo, H.O., Lumme, P.O. *Cancer Treat. Rep.* **1985**, 69(9), 1021–1022.

90 Conato, C. et al. *Biochim. Biophys. Acta* **2001**, 1526(2), 199–210.

91 Ishiwata, Y., Yakochi, S., Hashimoto, H., Ninomiya, F., Suzuki, T. *Scand. J. Immunol.* **1998**, 48(6), 605–614.

92 Pani, G. et al. *Cancer Res.* **2000**, 60(16) , 4654–4660.

93 Novelli, E.L. et al, *Braz. J. Med. Biol. Res.* **1990**, 23(8), 643– 645.

94 Steffenrud, S. *Netest* **1998**, 17(5), 11–17.

95 Mutchnick, M.G., Appelman, H.D., Chung, H.T., Aragona, E., Gupta, T.P., Cummings, G.D., Waggoner, J.G., Hoofnagle, J.H., Shafritz, D.A. *Hepatology* **1991**, 14(3), 409–615.

96 Lykkesfeldt, J., Hagen, T.M., Vinarsky, V., Ames, BN. Age-associated decline in ascorbic and concentration, recycling and biosynthesis in rat hepatocytes-reversal with (R)-alpha-lipoic acid supplementation. *FASEB J.* **1998**, 12, 1183–1189.

12
Traditional Plants and Herbal Remedies Used in the Treatment of Diarrheal Disease: Mode of Action, Quality, Efficacy, and Safety Considerations

Enzo A. Palombo

Summary

Medicinal plants and herbs have proven to be an abundant source of biologically active compounds, many of which have been the basis for new pharmaceuticals. Diarrheal disease continues to be a major cause of morbidity and mortality throughout the world, particularly among children in developing countries, often as a result of infection by bacteria, viruses, and protozoal parasites. Given the increasing resistance in many common pathogens to currently used chemotherapeutic agents, there is renewed interest in the discovery of novel compounds that can be used to fight infectious diseases. There have been numerous studies that have served to validate the traditional use of medicinal plants used to treat or prevent diarrhea. Many plant extracts have been screened for antimicrobial activity, while others have been investigated for their antidiarrheal properties. Extracts can exhibit antispasmodic effects, delay intestinal transit, suppress gut motility, stimulate water adsorption, or reduce electrolyte secretion. These activities, coupled with antimicrobial activity, may help to explain the benefits of using particular plants in the treatment of diarrheal disease.

Phytochemical screening of bioactive plants extracts has revealed the presence of alkaloids, tannins, flavonoids, sterols, terpenes, carbohydrates, lactones, proteins, amino acids, glycosides, and saponins. Of these, flavonoids have been linked to antibacterial activity, while tannins and flavonoids are thought to be responsible for antidiarrheal activity. Different phytochemicals display various mechanism of action such as increasing colonic water and electrolyte reabsorption and inhibiting intestinal motility, while some components have been shown to inhibit specific pathogens. As some of the active ingredients are potentially toxic, there is a need to evaluate the safety of plants preparations.

There is limited information about the safety of traditional plant extracts, although some clinical trials have evaluated the safety and tolerability of herbal medicine preparations used to treat diarrhea and generally indicate that minimal side-effects are observed. However, with the increased popularity of plant-derived and

herbal medicines, particularly in Western society, the quality, efficacy, benefits and potential dangers of these medicines must be considered.

This chapter will present recent examples of studies that have provided scientific or clinical evidence that support the traditional use of medicinal plants. Specifically, plant extracts or phytochemicals that have been shown to inhibit infectious diarrheal agents or reduce the symptoms of diarrhea will be discussed. Studies that have investigated the mode of antidiarrheal action and the safety of plant-derived medicines will also be described.

12.1 Introduction

Diarrheal diseases continue to be a major cause of morbidity and mortality throughout the world, especially in developing countries. Despite advances in the understanding of the causes, treatment, and prevention of diarrhea, many millions of people, including 2.5 million children, die from diarrhea every year [1, 2]. The World Health Organization defines diarrhea as three or more loose of watery stools in a period of 24 hours [3], although changes in consistency are as important as changes in the frequency of stools. Diarrhea can be classified as acute or chronic, with acute diarrhea being the most common form. Acute diarrhea has an abrupt onset, resolves within about 14 days and is usually caused by an infectious agent, although drugs, poisons (including bacterial toxins), or acute inflammatory reactions can also contribute [2].

Worldwide, rotavirus is the major cause of infectious diarrhea, particularly among young children, however, other viral (adenovirus, enterovirus, and norovirus), bacterial (*Escherichia coli*, *Salmonella*, *Shigella*, *Campylobacter*, and *Vibrio cholerae*), and parasitic (*Cryptosporidium* and *Giardia*) agents are important contributors [3]. While oral rehydration therapy (ORT) remains the major treatment for diarrhea, it does not reduce the volume or duration of diarrhea [4]. Other options include antibiotics and gut motility-suppressing agents, all of which aim to reverse dehydration, shorten the length of illness and reduce the period of time an individual is infectious [3]. Where patients are suffering from prolonged diarrhea, treatment with pharmacological agents that are pathogen-specific or that suppress severe symptoms would be of benefit [5, 6].

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world, particularly in the rural areas of developing countries, where they continue to be used as the primary source of medicine [7]. About 80% of people in developing countries use traditional medicines for their health care [8]. The natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals. Given the increasing resistance in many common pathogens to currently used therapeutic agents, such as antibiotics and antivirals, there is re-

newed interest in the discovery of novel compounds that can be used to fight infectious diseases. As there are approximately 500 000 plant species occurring worldwide, of which only 1% has been phytochemically investigated, there is great potential for discovering novel bioactive compounds. However, according to the United Nations Environment Programme World Conservation Monitoring Centre, at current extinction rates of plants and animals, the world is losing one major drug every two years [9].

There have been numerous reports of the use of traditional plants for the treatment of diarrheal diseases. Many plant-derived medicines used in traditional African, American, Asian, European, and other indigenous medicinal systems have been recorded in pharmacopeias as agents used to treat diarrhea. The purpose of this chapter is not to document and categorize such plants. Instead, the aim is to present some recent examples of studies that have served to validate the traditional use of medicinal plants with specific biological activity. In particular, traditional medicinal plant extracts or phytochemicals that have been shown to inhibit infectious diarrheal agents or reduce the symptoms of diarrhea will be discussed. In addition, studies that have investigated the mode of antidiarrheal action and the safety of plant-derived medicines will be described.

12.2 Methods Used in the Evaluation of Bioactivity of Medicinal Plants

12.2.1 Antibacterial Activity

Many plants have been used to treat or prevent diarrheal diseases and screening of extracts of these plants for antimicrobial activity is relatively uncomplicated. In particular, screening for antibacterial activity is carried out using conventional assays used to test antibiotics that detect inhibition of bacterial growth in liquid or solid growth media [10]. The most commonly used method used to evaluate antimicrobial activity of plant extracts is the agar diffusion method. It is reliable, precise, and inexpensive, although it yields only semi-quantitative results. The methods involves inoculation of the surface of an agar plate with the test microorganism or pouring molten agar inoculated with the test organism into a Petri dish. The compound to be evaluated can be applied on a paper disk or into a well made in the agar. After appropriate incubation, the appearance of zones of growth inhibition around the disc or well indicates antimicrobial activity (Fig. 12.1). The type of medium used, incubation conditions, the diameter of the paper disk or the well, in addition to the chemical nature of the test compound (size and polarity), will affect the diffusibility of the antimicrobial agent and hence the sizes of the zones of inhibition that develop. Agar or broth dilution methods are able to yield quantitative results by determining growth inhibition indices, minimal inhibitory concentrations, or minimal lethal concentrations [10].

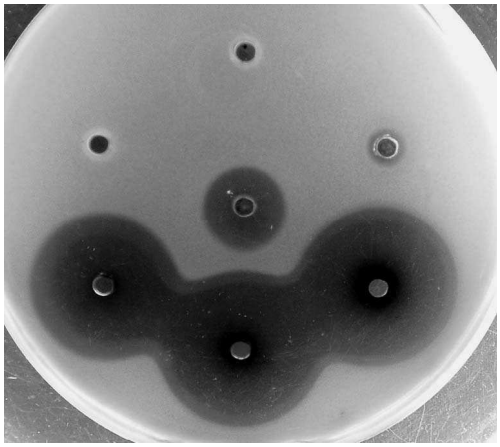


Fig. 12.1 Agar diffusion assay showing zones of bacterial growth inhibition in agar inoculated with test microorganism. This experiment depicts a plate-hole diffusion assay where plant extracts were added to wells made in the agar. Photo courtesy of J. McRae.

12.2.2 Antiprotozoal Activity

Antiprotozoal screening has been carried out using methods analogous to those used for antibacterial assessment. Typically, *Entamoeba histolytica* or *Giardia lamblia* trophozoites are inoculated into test-tubes containing medicinal plant extracts. After incubation, samples of the tubes are taken and tested for cell viability using trypan blue dye exclusion or tetrazolium salt metabolism assay methods [11, 12].

12.2.3 Antihelminthic Activity

Testing for antihelminthic (nematicidal and larvicidal) activity is carried out by assessing the ability of plant extracts to inhibit the hatching and development of nematode eggs [13]. Eggs of *Haemochonus contortus*, a nematode of veterinary importance, are produced by infecting sheep orally with third stage (infectious) larvae and collecting eggs from feces after three weeks. Testing of plant extracts involves addition of the extracts to multiwell plates, followed by overlaying with agar and adding culture media to favor the growth of bacteria which are used as nutrients by free larvae. The degree of successful hatching of larvae in wells containing plant extract is observed microscopically. The number of nematodes present and their development into different larval stages is evaluated. High nematicidal activity is defined as 95–100% total larval mortality, intermediate activity is 80–95% mortality, while 60–80% mortality is seen as low activity.

12.2.4 Antiviral Activity

Antiviral screening assays are complicated by the fact that they employ cell culture methods. Nevertheless, a number of studies have sought to investigate activity

against these types of pathogens. Antiviral testing aims to determine the inhibition of virus induced cytotoxicity of appropriate host cells. Confluent monolayers of cells are infected with virus in combination with various concentrations of the plant extract and incubated for an appropriate period of time. The number of viable cells is determined colorimetrically and the 50% effective concentration (EC_{50}) of extract is determined as the reciprocal dilution required to prevent virus induced cytolysis by 50% [5, 14]. Alternatively, the reduction in viral titer (expressed as $TCID_{50}$) can be determined [15] or the absence of microscopically visible cytopathic effect is observed [16]. A modification of the above methods involves measuring the reduction in the number of viral plaques formed on cell monolayers [5]. To determine the mode of antiviral activity, the reduction in virus binding can be assayed by calculating the percentage of radioactively labeled virus that binds to cell monolayers [5].

12.2.5 Antidiarrheal Activity

Many animal-based studies have investigated the bioactivity and effects on intestinal function of plants traditionally used as treatments for diarrhea where no particular etiologic agent is identified. These plant extracts can have antispasmodic effects, delay gastrointestinal transit, suppress gut motility, stimulate water adsorption, or reduce electrolyte secretion. These activities, coupled with antimicrobial activity, may help to explain the benefits of using particular plants in the treatment of diarrheal disease. To determine the antidiarrheal activity of an extract, diarrhea is induced by an agent such as castor oil, arachidonic acid, prostaglandin E_2, or magnesium sulfate and the ability of the extract under investigation to confer protection is determined by measuring fecal output. As these agents have different mechanisms of action (for example, castor oil increases peristaltic activity and alters the permeability of the intestinal mucosa to water and electrolytes, while magnesium sulfate is an osmotic active agent), the nature of the antidiarrheal activity can be determined [17, 18].

Gastrointestinal transit is usually determined by measuring the transit of a charcoal plug (a 5% charcoal suspension in 10% aqueous solution of tragacanth powder administered orally). The distance traveled by the plug is expressed as a percentage of the total length of the small intestine [18–20]. The effect on gut motility is determined by measuring the ability of an extract to block contractions evoked by agonists (e.g. acetylcholine, histamine, and nicotine) [19, 21]. The ability of an extract to stimulate water adsorption or reduce electrolyte secretion is measured using ligated intestinal loop or colon assays [6, 18]. In these experiments, sections of gut are ligated and plant extracts are introduced into the isolated sections. At the end of the experimental period, the contents of the ligated gut are evaluated (by measuring the amount of fluid accumulated and the concentration of Na^+, K^+, and Cl^- ions) and the net absorption of water and electrolytes with and without extract is determined. For the determination of specific bioactivity, the sections can also be treated with an agent known to result in fluid accumulation and electrolyte secre-

tion into the intestinal lumen, such as cholera toxin, to determine if the extract can inhibit the effects of these agents.

12.3 Traditional Medicinal Plants Used in the Treatment of Diarrhea that Display Antimicrobial Activity

This section will describe some recent studies that have sought to validate the use of particular plants as traditional treatments for diarrhea. Finding experimental evidence for activity against pathogens known to cause diarrheal disease was the major purpose of such studies, although some have also included phytochemical analysis of plant preparations.

Nigella satavia (Ranunculaceae), commonly known as black seed or black cumin, is used in Europe, Arabian countries, and the Indian subcontinent for culinary and medicinal purposes [22]. It is used to treat numerous ailments, including diarrhea, and its essential oil has been shown to exhibit activity against *Staphylococcus aureus*, *Salmonella*, *Shigella*, *V. cholerae*, and *E. coli*. The major constituents of the essential oil are thymoquinone, *p*-cymene, carvacrol, *t*-anethole, 4-terpineol, and longifoline.

Swertia corymbose (Gentianaceae) is traditionally used in Indican medicine as an antidote for poisoning, diarrhea, and as a stomach wash in cattle [23]. Hexane, chloroform, and methanol extracts show antibacterial activity against a wide range of microorganisms, including a number that cause diarrhea (*E. coli*, *Salmonella*, *V. cholerae*, and *Staphylococcus aureus*). Alkaloids, flavones, lignins, phenols, proteins, quinine, saponins, starch, steroids, tannins, and triterpenes have been identified in the solvent extracts.

Cocos nucifera (Palmae) is widely distributed on the coast of north-eastern Brazil. The husk fibre decoction is used in the traditional medicine of north-eastern Brazil for treating diarrhea and arthritis [24]. A crude water extract and four out of five fractions of this extract showed selective activity against *S. aureus*, with catechins and B-type procyanidins thought to be responsible for this activity. Methanol and water extracts of this plant were also found to have significant activity against enteropathogens in a recent study of traditional Mexican plants used to treat diarrhea and dysentery [25]. Other Mexican plants with significant antibacterial activity included *Caesalpinia pulcherria* (Leguminoceae), *Geranium mexicanum* (Geraniaceae), *Hippocratea excelsa* (Hippocrateaceae), and *Punica granatum* (Puniaceae).

Extracts of guava, *Psidium guajava* (Myrtaceae), and paw paw, *Carica papaya* (Caricaceae), which are both used in Brazilian traditional medicine, have been tested for their ability to inhibit enterotoxigenic *E. coli* (ETEC) and *S. aureus* [26]. While ethanol, acetone, and water extracts of guava were able to inhibit both bacteria, papaya extracts showed no activity. Similarly, extracts of papaya were not found to have significant activity in the study by Alanís et al. [25].

A study of 10 plants used in Indian traditional medicine to treat dysentery and diarrhea showed that some displayed high antibacterial activity, while little activity

was detected in others [27]. Those that were highly active included garlic (*Allium sativum*), svet kanchan (*Bauhinia racemosa*), tea (*Camellia sinensis*), garden spurge (*Chamaesyce* [*Euphorbia*] *hirta*), and velvet leaf (*Cissampelos pareira*). Sweet flag (*Acorus calamus*), guava (*Psidium guajava*), and globe thistle (*Sphaeranthus indicus*) were moderately active, while neem (*Azadirachta indica*) and sweet indrajao (*Wrightia tinctoria*) were only weakly active or inactive. *Vibrio cholerae* was the most susceptible organism, followed by a number of *Shigella* spp., ETEC, and *Klebsiella*. It is interesting to note that the study by Alanís et al. [25] did not find significant antibacterial activity with *Allium sativum*.

Methanol and water extracts of a number of medicinal plants used to treat dysentery and diarrhea in the Democratic Republic of Congo showed activity against one or more enteropathogens, including *Shigella*, *Salmonella*, *E. coli*, *Vibrio*, and *Campylobacter* [28]. The active plants were *Roureopsis obliquifolialata* (Connaraceae), *Cissus rubiginosa* (Vitaceae), and *Epinetrum villosum* (Menispermaceae) and it was proposed that the antibacterial action might be attributed to the presence of alkaloids in *Epinetrum villosum*, and tannins and saponins in the other two plants.

Paulo et al. [29] investigated the activity of *Cryptolepsis sanguinolenta* (Asclepiadaceae), a shrub indigenous to West Africa, against *Campylobacter jejuni*, *C. coli*, and *V. cholerae*. Although this plant is not used in traditional diarrheal treatment, the authors wished to determine if the medicinal uses of this endemic plant could be extended to include antidiarrheal therapy. Ethanol extracts of the roots and the main phytochemical, cryptolepine (Fig. 12.2), displayed activity against the bacteria that was sometimes greater than antibiotics used to treat infections caused by these pathogens, suggesting that the roots could be useful as an alternative therapy for diarrhea.

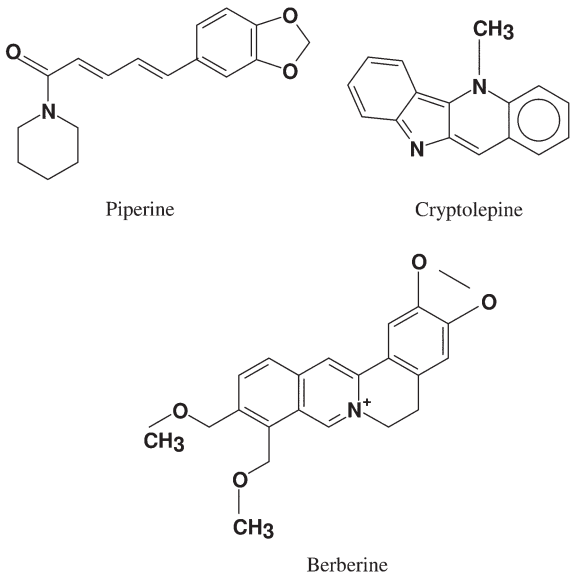


Fig. 12.2 Piperine, an alkaloidal constituent of black and long peppers has antidiarrheal activity [30], but is also able to inhibit cytochrome P450 enzymes [31]. Other alkaloids, such as cryptolepine [29] and berberine [32], display antibacterial activity.

Diehl et al. [13] evaluated 60 plants collected in the Ivory Coast used traditionally in human or veterinary medicine to treat worm infections (worms in general, round worms, Guinea worms, or flatworms), diarrhea and dysentery or abdominal pain for their antihelminthic activity. Fifty per cent of the selected plants showed activity against eggs of *Haemochonus contortus*, with 32% showing high activity.

Considering that rotavirus is one of the major diarrheal pathogens, there has been surprisingly little research on the activity of traditional plants against this virus. While ORT remains the main treatment for rotavirus diarrhea and there has been considerable progress in the development of a human rotavirus vaccine, virus-specific therapy will still be required for individuals with persistent diarrhea [5, 15].

An extensive investigation of 100 British Columbian medicinal plant extracts found only one, derived from the roots of *Lomatium dissectum* (Umbelliferae), which was active against bovine rotavirus [16]. This extract completely inhibited viral cytopathic effects on African green monkey kidney cells, MA104. Clark et al. [14] investigated the ability of crude theaflavins extracted from black tea, *Camellia sinensis* (Theaceae), to inhibit bovine rotavirus. Theaflavin and gallate derivatives were able to inactivate rotavirus, although the crude extract had greater activity than purified theaflavin or any of the high-performance liquid chromatography (HPLC)-purified fractions. Some of the fractions showed synergism, having greater activity when combined than when tested individually. This study supports the anecdotal data from Egypt and India and Japanese folklore that black tea is a cure for gastroenteritis. *Stevia rebaudiana* (Asteraceae) originates from Paraguay and has been used as a medicinal plant for a long time [5]. The hot water extract has been shown to display antibacterial activity against *E. coli* and other food-borne pathogens and is able to inhibit the replication of human rotavirus. The extract is not inactivated by exposure to acid at pH 2, suggesting it may be clinically useful as it can survive passage through the stomach. Anti-rotavirus activity appears to be the result of blocking of virus binding by a specific anionic polysaccharide fraction.

Tormentil root, *Potentilla tormentilla* (Roseaceae) has been used as a folk medicine in many parts of Europe for the treatment of diarrhea. While several manufacturers market tormentil root extract and it is considered safe and nontoxic, only a single clinical study has been conducted to test its efficacy in treating diarrhea. The study by Subbotina et al. [4] showed that the extract was effective in reducing the duration of rotavirus diarrhea in children admitted to hospital from five days in the untreated group to three days in the treated children ($P < 0.0001$). Stool output was reduced ($P < 0.029$), stool consistency was normalized earlier ($P < 0.0001$), less parenteral rehydration was required ($P = 0.0009$) and length of hospitalization was reduced ($P < 0.0001$) in treated children compared with controls. The study concluded that tormentil root is a safe and effective treatment that reduces fluid loss and shortens the length of rotavirus diarrhea.

Recently, 12 medicinal plants used in Brazil to treat diarrhea were evaluated for their ability to inhibit the growth of simian and human rotavirus [15]. Hot water extracts of the seeds of *Myristica fragrans* (Myristiaceae) were able to inhibit human rotavirus, the leaves of *Anacardium occidentale* (Anacardiaceae) and *Psidium guaja-*

va (Myrtaceae) inhibited simian rotavirus, while the bark of *Artocarpus integrifolia* (Moraceae) and the leaves of *Spongias lutea* (Anacardiaceae) inhibited both. The level of inhibition considered to be anti-rotaviral was greater than 80%. Of interest was the finding that a number of tested plant extracts were weakly active or ineffective, suggesting that the plants act on pathogens other than rotavirus or that the plants might only be useful against diarrhea caused by pathophysiological disturbances.

Decoctions of the roots and leaves of *Helianthemum glomeratum* (Cistaceae) are used by the Maya people of southern Mexico to treat diarrheal pain, particularly in cases of bloody diarrhea [11]. Crude extracts and isolated compounds were evaluated for activity against *E. histolytica* and *G. lamblia*. Methanol extracts obtained from the aerial parts and roots were active against trophozoites of *E. histolytica* but not *G. lamblia*. However, the flavonoids kaempferol and tiliroside present in the aerial parts were active against both protozoa. Polyphenols, found in the aerial parts and roots, were also antiprotozoal. Fractionation identified the flavan-3-ol, (–)-epigallocatechin as an active component of this plant. Previously, the polyphenols of this plant were shown to have antibacterial activity against *Shigella* spp. and *V. cholerae* [33]. Moundipa et al. [12] investigated the activity of 55 medicinal plants from Cameroon against *E. histolytica*. The plants selected for investigation have been used to cure jaundice and other liver disease, given that invasion of the liver by parasites can lead to the development of hepatic amoebiasis. Many plants showed activity, with the best being *Codiaeum variegatum*, which displayed activity greater than that of metronidazole, the reference antiprotozoal drug.

Many herbs have been used as traditional treatments for diarrhea. The uses of bayberry (see below), clove, *Syzygium aromaticum* (Myrtaceae), peppermint, *Mentha piperita* (Lamiaceae), and yarrow, *Achillea clavennae* (Asteraceae), are supported by laboratory studies indicating that plant components and essential oils are active against diarrheal pathogens [10, 34, 35].

12.4 Traditional Medicinal Plants Used in the Treatment of Diarrhea that Display Antidiarrheal Activity

In contrast to the studies described in the previous section, the plants investigated below have been validated as treatments for diarrhea on the basis of their ability to prevent or ameliorate diarrheal symptoms induced in experimental animals or tested in clinical trials. As in the previous section, phytochemical analysis of plant preparations and identification of active components has helped to explain the mechanism of antidiarrheal activity.

The roots of *Jatropha curcus* (Euphorbiaceae) are used traditionally in the western coastal areas of India to control dysentery and diarrhea [36]. Methanol extracts showed dose-dependent inhibition of castor oil-induced diarrhea and intraluminal fluid accumulation, as well as small intestinal transit. This extract may act by inhibiting prostaglandin and reducing small intestinal propulsive movement. In a sim-

ilar manner, Chitme et al. [7] have investigated the medicinal plant *Calotropis gigantea* (Asclepiadaceae). A water:ethanol (50:50) extract produced a statistically significant reduction in severity and frequency of diarrhea produced by castor oil. In addition, both castor oil-induced intestinal fluid accumulation and intestinal volume content were significantly inhibited. Numerous phytochemicals, including sugars, flavonoids, flavonol glycosides, and terpenes, which have been identified in this plant, may mediate the antidiarrheal properties, although the active component has not been defined.

Black and long peppers are used as components of antidiarrheal herbal formulations [30]. Piperine, the alkaloid constituent that is reported to have numerous pharmacological actions (Fig. 12.2), has been shown to have dose-dependent inhibitory activity against castor oil, $MgSO_4$, and arachidonic acid-induced diarrhea, gastrointestinal transit and castor oil-induced enteropooling in mice. It is thus thought to affect the actions of these gut function modulators and act by normalizing the permeability changes of water and electrolytes.

Sangre do grado, also known as dragon's blood, is the viscous red tree sap derived from several *Croton* species [37]. It is used extensively by people in the Amazon River basin to treat skin disorders such as abrasions, cuts, scratches, blisters, bites, and stings, but can also taken orally, in dilute form, to treat gastrointestinal illness, including infections and diarrhea. Sangre de grado is available commercially as "Zangrado" (Rainforest Phytoceuticals, Delmar, New York, USA). Miller et al. [37] found that the action of Zangrado as a therapy for diarrhea is caused by its effect on sensory afferent neurons, as shown in assays in which guinea-pig ileum was mounted in Ussing chambers and chloride secretion was evoked by capsaicin. The authors found that Zangrado was able to attenuate the response, suggesting that the medicine acts by suppression of nonmyelinated sensory nerves, leading to selective suppression of epithelial electrolyte secretion.

Jussiaea suffruticosa (Onagraceae) is a well-known traditional medicine India, where the whole plant is reduced to pulp and steeped in buttermilk as a treatment for dysentery and diarrhea [20]. An extract of this plant has been shown to inhibit castor oil-induced diarrhea, enteropooling, and gastrointestinal motility. The incidence and severity of diarrhea, as well as the frequency of defecation and wetness of fecal droppings were reduced and the effects were comparable to those seen for standard antidiarrheal drugs. Tannins present in plant extracts may be responsible for the observed effects. Similarly, an extract of the Nigerian antidiarrheal plant, *Pentaclethra macrophylla* (Mimosaceae), significantly reduced fecal output of castor oil-induced diarrhea in rats, significantly reduced gastrointestinal motility in mice, and blocked contractions of guinea-pig ileum evoked by various drugs [19]. In addition, the extract exhibited antibacterial activity against *E. coli*, but not *S. aureus*.

The leaves and stem bark of *Alchornea cordifolia* (Euphorbiaceae) are used in African folk medicine to treat urinary, respiratory, and gastrointestinal disorders [18]. A leaf extract has been shown to ameliorate the symptoms of castor oil-induced diarrhea in rats and reduce gastrointestinal transit of a charcoal meal in mice. Measurement of the fluid volume and Na^+, K^+, and Cl^- concentrations in tied-off rat colon indicated that the extract stimulated net water absorption and reduced electro-

lyte secretion. An extract of the roots of *Terminalia avicennoides* (Combretraceae), a traditional African medicine, produced a dose-dependent reduction of spontaneous and acetylcholine-induced contraction of rabbit jejunum, reduced gastrointestinal transit and protected mice against castor oil-induced diarrhea [21].

Black tea has antiviral activity (see above) but has also been shown to affect gastrointestinal function [38]. Hot water black tea extracts (BTE) increased upper gastrointestinal tract transit, but inhibited castor oil-induced diarrhea and intestinal fluid accumulation, and normal defecation in mice. The inhibitory effects could be prevented by naloxone, an opioid antagonist, suggesting a role of the opioid system in the antidiarrheal activity of BTE. The rhizomes of ginger, *Zingiber officinale* (Zingiberaceae), are widely used for treating numerous diseases, including diarrhea. Borrelli et al. [39] investigated the effect of this herbal remedy on contractions induced by electrical field stimulation (EFS) and acetylcholine on isolated rat ileum. Ginger produced concentration-dependent inhibition of both stimulants, starting at 1 μg mL^{-1} for acetylcholine-induced contractions and 300 μg mL^{-1} for EFS-induded contractions. These observations indicated an antispasmodic effect by reducing enteric excitatory transmission and direct inhibition of smooth muscle activity.

Baccharis teindalensis (Asteraceae) is commonly used in Ecuador as an anti-inflammatory, analgesic, and antimicrobial remedy [40]. An ethanol extract showed antidiarrheal activity against castor oil-induced diarrhea in mice, at doses of 50 and 100 mg kg^{-1}, by extending the time before the first diarrheic feces, decreasing the percentage of wet feces and decreasing the total weight of excreted feces. A number of flavonoids have been identified in the extract (Fig. 12.3) which could be responsible for the observed effects.

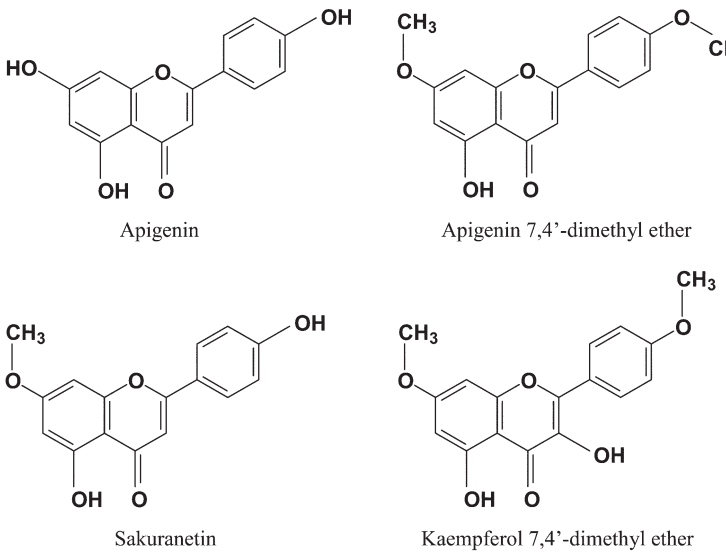


Fig. 12.3 Flavonoids isolated from the traditional medicinal plant, Baccharis teindalensis [40].

A large study by Atta and Mouneir [41] reported on the antidiarrheal properties of six Egyptian medicinal plants. At a dose of 200 mg kg^{-1}, only some of the plant extracts showed a significant effect on castor oil-induced diarrhea in rats, while the effects were better with an increased dose of 400 mg kg^{-1}. Some of the extracts induced a dose-dependent relaxation of rabbit duodenal smooth muscle while others increased the contractile force in contractions. Various phytochemicals were identified, including tannins, flavonoids, unsaturated sterols, triterpenes, carbohydrates, lactones, proteins/amino acids, and saponins, although the active ingredients were not confirmed. Similarly, several medicinal plants used by people in the Democratic Republic of Congo [42], India [43], and Zulu traditional healers [44] to treat diarrhea have been evaluated. These plants showed activity that supported their traditional use, including activity against enteric pathogens, and activity in experimental models of diarrhea in mice or rats.

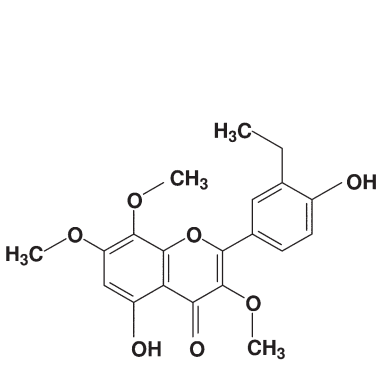
A number of plants that have been used as traditional medicines in Africa for the treatment of diarrhea and dysentery have been recently described in detail by Mueller and Mechler [45], but only those for which experimental or clinical studies support the traditional use are summarized here:

- The flesh of the fruit of the baobab tree, *Adansonia digitata* (Bombacaeae), is eaten raw as a treatment for diarrhea and dysentery. A clinical study in Senegal compared *Adansonia* fruit with ORT (82 children in each group) and found no significant difference between the two treatments in terms of duration of diarrhea and increase in weight, thus confirming the efficacy of the traditional medicine. The astringent constituents of the fruit may explain the medicinal properties, although the high levels of tartaric acid can lead to gastrointestinal irritation if large quantities of fruit are consumed.

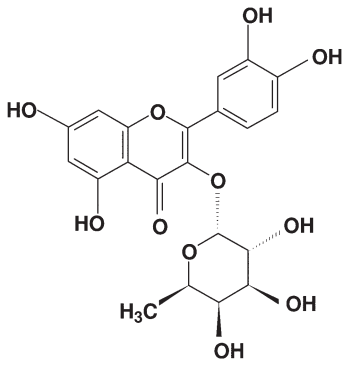
- *Euphorbia hirta* (Euphorbiaceae) is used widely in Western Africa for the treatment of diarrhea. The active constituent, quercitrin, is able to reduce diarrhea induced by castor oil and prostaglandin E_2 in mice (see below). Clinical studies have supported the use of *E. hirta* extracts for the treatment of amoebic dysentery, where 83.3% of patients in one study and 92.5% in another treated with an ethanol extract of the plant were cured.

- The bark, root, or leaves of the mango tree, *Mangifera indica* (Anacardiaceae), are macerated or made into decoctions or teas. The preparations are drunk or used as enemas to treat diarrhea. The high tannin content of the leaves and bark may explain the relief provided for the condition due to astringent and anti-inflammatory effects. However, there are no clinical studies to support this.

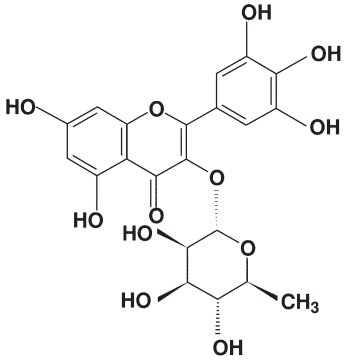
- Teas, decoctions, or macerations of the leaves of the guava tree, *Psidium guajava* (Myrtaceae), are well known as treatments for diarrhea in tropical countries (see above). Independent experimental studies in mice support the use of decoctions of dried leaves and aqueous extracts of leaves to treat diarrhea, although no clinical studies have confirmed these observations. The active component is believed to be quercitrin (Fig. 12.4).



Ternatin
(5,4'-dihydroxy-3,7,8,3'-tetramethoxyflavone)



Quercitrin
(3-rhamnosyl-3,3',4',5,7-pentahydroxyflavone)



Myricitrin
(Flavone,3,3',4',5,5',7-hexahydroxy-,3-rhamnoside)

Fig. 12.4 The flavonoids ternatin [46] and quercitrin [17] have antidiarrheal activity, while myricitrin is antibacterial [47].

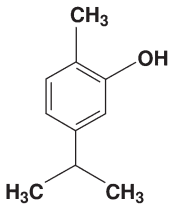
- Decoctions or extracts of the leaves of pomegranate, *Punica granatum* (Punicaceae), are used in many countries to treat diarrhea. In the Chinese pharmacopeia, the skins of fruit are recommended. The high tannin and alkaloid content of the skins are possibly responsible for the antidiarrheal effects. An orally administered methanol extract of the seeds, containing steroids, flavonoids, and tannins, has been shown to significantly reduce the frequency of stools and reduce gastrointestinal motility in mice. No clinical studies are available to support the experimental studies. The alkaloids found in all parts of the plant mean that high doses of *P. granatum* are toxic. Methanol and water extracts of this plant have recently been shown to have significant antimicrobial activity against enteropathogens [25].

- The leaves, roots, and seeds of paw paw, *Carica papaya* (Caricaceae), are used to treat bloody diarrhea, although no experimental studies or evidence of efficacy are available. In support of this, the studies by Alanís et al. [25] and dos Fernandes Vieira et al. [26] mentioned above indicated that paw paw extracts were not effective against common diarrheal pathogens. Similarly, although used in Uganda and Congo, there is no experimental or clinical evidence for the efficacy of the leaves of the passion flower, *Passiflora incarnata* (Passifloraceae), for the treatment of diarrhea.

12.5 Phytochemical Analysis, Identification of Active Plant Components, and Mechanism of Action of Medicinal Plants Used in the Treatment of Diarrhea

Phytochemical screening of plants extracts (made in organic solvents or water) has revealed the presence of numerous chemicals including alkaloids, tannins, flavonoids, sterols, terpenes, carbohydrates, lactones, proteins, amino acids, glycosides, and saponins (Table 12.1). Of these, tannins, pheolics, saponins, alkaloids, and flavonoids have been linked or suggested to be involved with antibacterial and antiviral activity, while tannins and flavonoids are thought to be responsible for antidiarrheal activity. Investigations of the mode of action indicate that tannins and flavonoids increase colonic water and electrolyte reabsorption and other phytochemicals act by inhibiting intestinal motility, while some components have been shown to inhibit particular enteropathogens.

The essential oil of *Satureja hortensis* (Laminaceae), an Iranian traditional medicine, is thought to act as an antispasmodic due to its high content of the phenolic carvacrol [48] (Fig. 12.5, Table 12.1). In contrast, analysis of the antidiarrheal constituents of *Eglete viscose* (Compositae), a traditional Brazilian medicine, and *Euphorbia hirta* (Euphorbiaceae), used widely in Africa and Asia, has identified the flavonoids ternatin and quercitrin, respectively, as the active constituents [17, 46] (Fig. 12.4, Table 12.1). Phytochemical analysis of a number of medicinal plants commonly found along the Mediterranean coast and used to treat diarrhea identified tannins and flavonoids as the likely active antidiarrheal constituents as these were found in all plants tested [49]. Yavada and Jain [50] have recently identified a new flavone glycoside, 5,7,4′-trihydroxy-6,3′-dimethoxy flavone-7-*O*-α-L-arabinopy-



Carvacrol (2-methyl-5-(1-methylethyl) phenol)

Fig. 12.5 Carvacrol, a major component of the essential oil of *Satureja hortensis*, is a phenolic with antispasmolytic activity.

Table 12.1 Phytochemical components and mechanism of action of selected medicinal plants used to treat diarrhea.[a]

Plant(s)	*Phytochemicals identified*	*Phytochemical(s) with bioactivity*	*Mechanism of action*
Alchornea cordifolia	Alkoloids, tannins, saponins, flavonoids, phenols	Not known, possibly tannins and flavonoids	Antidiarrheal; stimulation of net water absorption and reduction in electrolyte secretion
Anacardium occidentale	Tannins, flavonoids, terpenes, saponins	Possibly flavonoids, as these are common components	Antiviral; inhibition of rotavirus propagation
Artocarpus integrifolia	Flavonoids, terpenes, nitrogen compounds		
Myristica fragrans	Flavonoids		
Psidium guajava	Tannins, flavonoids		
Spondias lutea	Flavonoids		
Spongias lutea	Tannins, flavonoids		
Calotropis gigantea	Sugars, flavonoids, flavonol glycosides, oxypregnane-oligoglycosides, terpenes and terpene derivatives	Not known	Antidiarrheal; altered activity of Na^+K^+ATPase or activation of chloride channels and reversal of chloride secretion?
Cissus rubiginosa	Flavonoids, tannins	Probably tannins	Antimicrobial; mechanism unknown
Cocos nucifera	Tannins	Catechin, epicatechin, B-type procyanidins	Antibacterial; mechanism unknown
Egletes viscosa		Ternatin (flavonoid)	Antidiarrheal; inhibition of intestinal transit, secretion and motility; interference with cellular enzyme and neurotransmitter systems or interaction with calcium channels?
Epinetrum villosum	Alkaloids, saponins	Probably alkaloids	Antimicrobial; mechanism unknown
Euphorbia hirta		Quercitrin (flavonoid)	Antidiarrheal; modulation of arachidonic metabolism via inhibition of cyclo-oxygenase and lipoygenase?
Pentaclethra macrophylla	Flavonoids, reducing sugar, tannins, glycosides	Not known	Antidiarrheal; musculotropic; limits availability of Ca^{2+} at steps involved in excitation–contraction coupling?
Roureopsis obliquifolia	Flavonoids, saponins, tannins	Probably tannins and saponins	Antimicrobial; mechanism unknown
Satureja hortensis essential oil	Major constituents are carvacrol (33.7%) and γ-terpinene (31.8%)	Probably carvacrol (phenolic)	Antispasmolytic; inhibition of contractile overactivity of the ileum
Terminalia avicennoides	Saponins, tannins, flavonoids	Not known	Antidiarrheal; inhibition of spontaneous and agonist-induced contractions of jejunum

[a] Other plants are described in the text.

ranosyl-(1→6)-*O*-β-D-galactopyranoside from *Melilotus indica* (Leguminosae), a medicinal plant used in various applications, including the treatment of infantile diarrhea, found in India, the Middle East, and Europe. This compound was found to exhibit antibacterial activity against pathogens that caused diarrhea.

Herbs with astringent properties, such as meadowsweet, *Filipendula ulmaria* (Rosaceae), agrimony, *Agrimonia eupatoria* (Rosaceae), shepherd's purse, *Capsella*

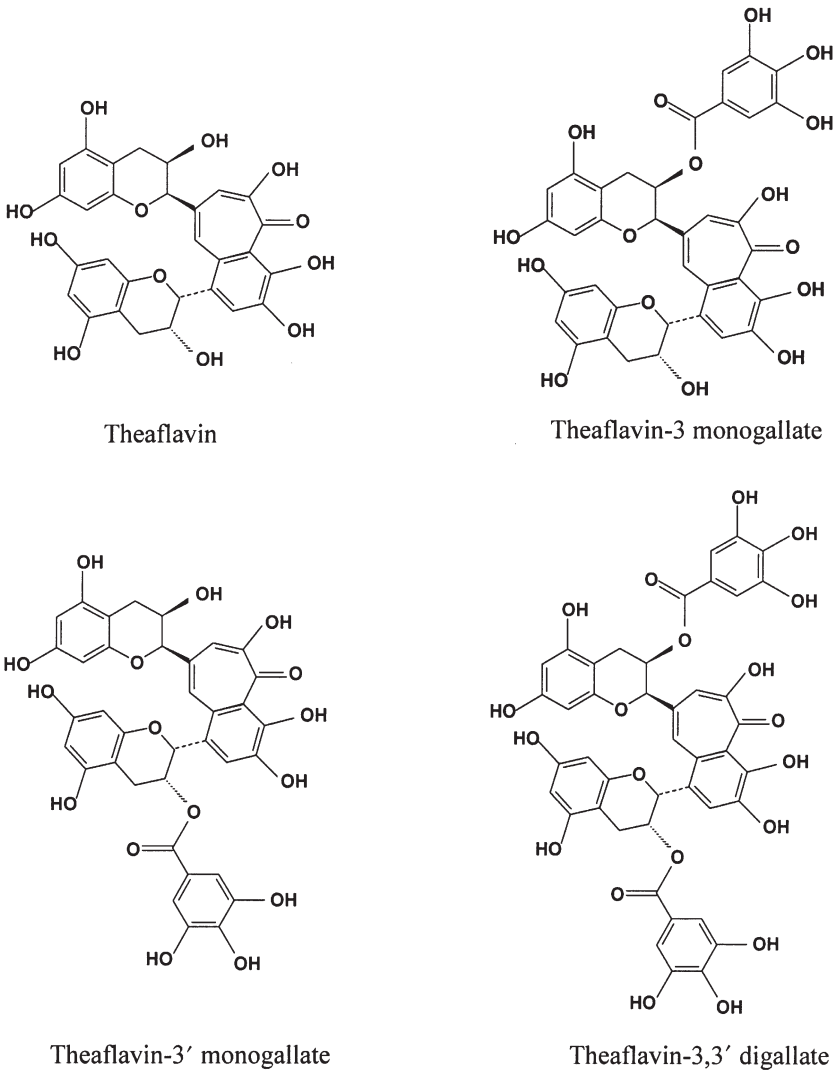


Fig. 12.6 Theaflavin and gallate derivatives are polyphenolic compounds extracted from black tea which neutralize bovine rotavirus [14].

bursa-pastoris (Cruciferae), and cranesbill, *Geranium maculatum* (Geraniaceae), are suggested to be useful as they bind to the mucosal lining of the small intestine [32]. Herbs that contain the alkaloid berberine (Fig. 12.2), for example goldenseal, *Hydrastis canadensis* (Ranunculaceae), and barberry, *Berberis vulgaris* (Berberidaceae), have an antimicrobial effect [51] and may also be helpful. Bayberry, *Myrica cerifera* (Myricaceae), contains the antibacterial compound myricitrin (Fig. 12.4), which may explain why it is a useful treatment for diarrhea [47]. As mentioned earlier, crude black tea extracts, theaflavins, and theaflavin gallate derivatives are able to neutralize rotavirus *in vitro* [14]. The structures of these polyphenolic compounds are shown in Fig. 12.6.

Numerous phytochemicals have demonstrated antibacterial activity and the various mechanisms of action have been described by Cowan [51]. Phenolics are a broad class of compounds that have a variety of antibacterial mechanisms. The following subclasses have specific mechanisms of action. Simple phenols such as catechol and epicatechin work by substrate deprivation and membrane disruption, respectively; phenolic acids and quinones bind to adhesins, complex with the cell wall and inactivate enzymes; flavonoids bind to adhesins; flavones complex with the cell wall; and tannins bind to proteins and adhesins, inhibit enzymes, complex with the cell wall, disrupt membranes, complex metal ions, and work by substrate deprivation. The mechanism of action of flavonols in unknown. Other classes of antibacterial phytochemicals include terpenoids, such as capsaicin, and essential oils which act by membrane disruption and alkaloids, such as berberine and piperine, which intercalate into the cell wall and/or DNA.

12.6 Quality, Efficacy, and Safety Considerations

Issues about the quality, efficacy, and safety of medicinal plants and herbals are of concern to all forms of these medicines, not only those used to treat diarrhea. This has been highlighted by recent examples of herbal medicines that have been linked to serious adverse effects [52, 53], including herbal preparations derived from comfrey which have been used to treat diarrhea [54]. The use of comfrey leaves has been identified as a health hazard, leading to hepatic toxicity (veno-occlusive disease) in humans. This toxicity appears to result from the conversion of pyrrolizidine alkaloids into reactive pyrroles or alkaloid-*N*-oxides by hepatic enzymes. The toxicity leads to necrosis of hepatocytes and mesenchymal cells and eventually results in liver damage in the form of portal hypertension.

Public perceptions are that traditional or complementary and alternative medicines (CAM) are safer than conventional drugs. However, quality, efficacy, and safety are guided by the regulatory environment of the country in which the medicines are manufactured or distributed. The regulation of CAM is a new and evolving area, although some countries have made major efforts to develop guidelines for the safe use and quality assurance of CAM [55]. For example, Canada, Germany, France, Sweden, and Australia have implemented strategies for the licencing of

herbal remedies [52, 55]. Since plant and herbal medicines can be classified as drugs or foods, the stringency of regulations governing the latter means that herbal medicines can avoid the need to carry warning labels about possible side-effects. Drug regulations require that safety, efficacy, and quality of the product are defined, whereas food regulations are less rigorous. Herbal medications may be produced without compliance to the standards of Good Manufacturing Practise [52]. This was illustrated recently in Australia where the Therapeutic Goods Administration recalled over 15 000 products manufactured by the country's largest producer of CAM because of substandard manufacturing practises and many reports of adverse reaction to one of its products [56].

The quality of plant-based remedies can be difficult to assure because herbs and plants contain complex mixtures and because the active constituents are often unknown. This makes standardization of the medicinal product difficult [52]. Plant and herbal remedies should be controlled to ensure that the products have the expected effects and do not contain adulterants or contaminants, such as other botanicals, microorganisms, toxins, pesticides, fumigation agents, toxic metals, or drugs. As the use of herbal remedies can pose serious health risks, absolute establishment of safety is critical. While advocates of herbal medicines claim the use of a plant in traditional medicine as evidence of safety, it is relatively easy to recognize acute toxic reactions compared to adverse effects that may develop over extended use of a product [52]. Another concern is that Western use of a traditional plant may not reflect the manner in which the plant was used in traditional medicine. The combined use of CAM with conventional medicine is an obvious concern (see below). Efficacy is also an important issue, hence the purpose of the current review. However, experiments *in vitro* or in laboratory animals cannot predict the behavior of medicines in appropriately designed trials or in the general community once they are licenced. Only a small fraction of the thousands of medicinal plants have been tested rigorously in randomized, controlled trials. Even fewer trials have been carried out where combinations of plant medicines and conventional medicines are tested.

Some of the active ingredients of medicinal plants and herbs used in the treatment of diarrhea are potentially toxic. In some cases, chemicals besides those that constitute the active ingredients can be responsible for toxicity. For example, preparations of the leaves and roots of *Maytenus senegalensis* (confetti tree) are used in various countries of Africa to treat diarrhea [45]. However, the plant is acutely and highly toxic and is not recommended for any purpose. Instead of the active antidiarrheal component being toxic, some of the toxicological effects are due to a constituent found in the leaves, maytansine. In response to the need to evaluate the safety of plant preparations, a limited number of clinical trials have evaluated the safety and tolerability of herbal medicine preparations used to treat diarrhea and generally indicate that minimal side-effects are observed. However, with the increased popularity of plant-derived and herbal medicines, particularly in Western society, the benefits and potential dangers of these medicines must be considered.

A plant whose toxicological properties have been investigated in detail is *Nigella satvia* [22]. The seed extract and its constituents appear to have low levels of toxic-

ity. Administration of seed extract or oil to mice and rats did not significantly affect the function of hepatic or renal enzymes, cause mortality, or show other signs of toxicity.

Plant-derived antidiarrheal medicines that are available commercially include Seirogan [57], tormentil root [4], Zangrado [37], and Kampo [6]. Seirogan, which has been used throughout Asia for more than a century, has been assessed for safety in numerous studies. The active ingredient of this medication is wood creosote, an oily liquid obtained by the fractional distillation of beechwood tar that consists of many simple phenolic compounds (Fig. 12.7). Oral doses (five doses of 45–225 mg every 2 h) of wood creosote were found to be safe and well tolerated with minimal side-effects, including altered taste and somnolence [57]. In addition, doses of up to 200 mg kg^{-1} body weight per day did not show evidence of oncogenicity in rats, further supporting the safety of this product [60].

As herbal medicines are often used in conjunction with prescription drugs, a relevant health and safety concern is the potential interaction between plant extracts and drugs. Given that herbal medicines contain many active ingredients, the large number of pharmacologically active compounds increases the likelihood of inter-

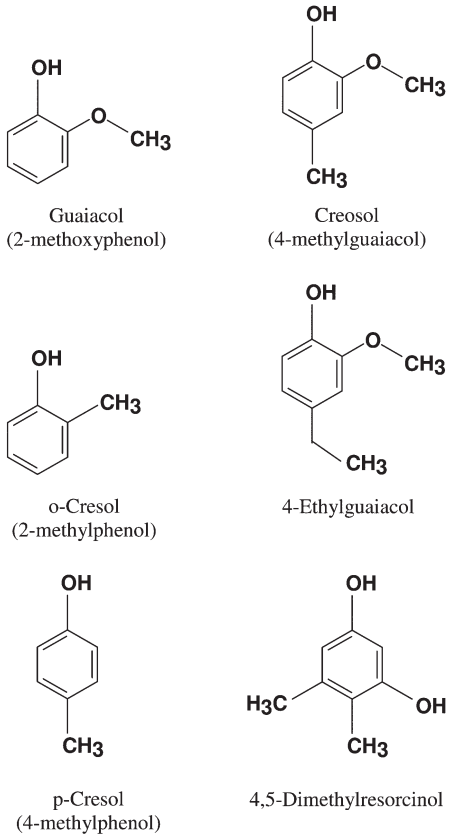


Fig. 12.7 Simple phenolics are some of the major components of wood creosote [57–59]. 4,5-Dimethylresorcinol has been shown to inhibit Cl^- secretion [59].

actions taking place. For example, flavonoids exhibit a range of biological activities and have the ability to modulate several enzymes or cell receptors, mainly as a result of their antioxidant properties. By comparison, synthetic drugs usually contain single chemical entities so that drug–drug interactions are less likely [61]. This highlights the need to identify and purify active components from medicinal plant preparations as the potential for adverse interactions with purified compounds is less likely.

Various phytochemicals, including piperine, flavonoids, triterpenoids, anthraquinones, polyphenols, and alkaloids, some of which are present in antidiarrheal preparations, interact with and inhibit cytochrome P450 systems and can impact on the pharmacokinetics of any co-administered drugs metabolized by these systems [31]. For example, piperine (Fig. 12.2) has been shown to inhibit arylhydrocarbon hydroxylase and 7-ethoxycourmarin deethylase (CYP2A) by a noncompetitive mechanism. Inhibition or induction of specific cytochrome P450 enzymes can lead to adverse drug interactions, including some fatal interactions [31]. Herb–cytochrome P450 interactions may have important clinical and toxicological implications and rigorous testing for possible interactions is needed.

Another important issue is the safe use of herbal medicines in children. Given that a major target group of antidiarrheal preparations will be children, and the long-term use of herbal medicines in children is not recommended because of the potential effect on developing cells and tissue, the safety of such preparations must be fully examined. As children differ from adults in their adsorption, distribution, metabolism, and excretion of certain substances, they may be more vulnerable to the adverse affects of herbal medicines [62]. A study of the mutagenic potential of an extract of *Stachitarpheta jamaicensis* (Verbenaceae), a plant commonly used in Cuba as a vermifuge and treatment for diarrhea, showed no positive responses in an Ames mutagenicity assay, no induction of micronuclei and absence of toxicity to bone marrow in mice [63]. While this study indicated that this extract was safe and did not induce genetic damage, further such studies of plant-derived medicines are needed. In general, the use of plant and herbal therapies is not recommended for children [53] or should be used with caution after consultation between parents and a clinician, especially if the child is being treated with conventional medication [62].

12.7 Conclusions

Modern scientific evaluation of medicinal plants and herbs is concerned with validating the traditional use of plants as well as identifying the active components of extracts and preparations. While this may be important in situations where the plant in question also produces potentially toxic compounds, there is the possibility that a number of components act synergistically to produce the therapeutic effects. Separating the individual components may lead to a loss of the desired activity. As a result, further examination of traditional plant medicines is required to es-

tablish the scientific basis for activity and enable the quality, efficacy, and safety of such preparations to be more precisely defined. With respect to traditional medicines used to treat diarrheal diseases, such medicines will continue to be used as long as there are communities with limited access to modern therapies. In the future, it may be possible to supplement conventional ORT treatment with plant extracts resulting in complementary treatments that may lead to a reduction in the length of disease symptoms. Certainly, the evidence provided by recent studies of traditional plant-based therapies encourages further investigation in the expectation that alternative treatments for diarrheal diseases will be developed.

Acknowledgments

Financial support for the author's research on medical plants has been generously provided by the Sunshine Foundation and the Swinburne University Alumni & Development Office.

References

1 Kosek, M., Bern, C., Guerrant, R.L. *Bull. World Health Org.* **2003**, *81*, 197–204.
2 Thapar, N., Sanderson, I.R., *Lancet* **2004**,*363*, 641–653.
3 Allen, S.J., Okoko, B., Martinez, E., Gregorio, G., Dans L.F. *Cochrane Database Syst. Rev*. Issue 4. **2003**, CD003048.pub2. DOI, 10.1002/14651858.CD003048.pub2.
4 Subbotina, M.D., Timchenko, V.N., Vorobyov, M.M., Konunova, Y.S, Aleksandrovih, Y.S., Shushunov, S. *Pediatr. Infect. Dis. J*. **2003**, *22*, 706–711.
5 Takahashi, K., Matsuda, M., Ohashi, K., Taniguchi, K., Nakagomi, O., Abe, Y., Mori, S., Sato, N., Okutani, K., Shigeta, S. *Antiviral Res*. **2001**, *49*, 15–24.
6 Oi, H., Matsuura, D., Miyake, M., Ueno, M., Takai, I., Yamamoto, T., Kubo, M., Moss, J., Noda, M. *Proc. Natl Acad. Sci. USA* **2002**, *99*, 3042–3046.
7 Chitme, H.R., Chandra, R., Kaushik, S. *J. Pharm. Pharm. Sci*. **2003**, *7*, 70–75.
8 Kim, H–S. *J. Ethnopharmacol*. **2005**, *100*, 37–39.
9 Groombridge, B., Jenkins, M.D. *World Atlas of Biodiversity, Earth's Living Resources in the 21st Century*. University of California Press, California, USA. **2002**.
10 Kalemba, D., Kunicka, A. *Curr. Med. Chem*. **2003**, *10*, 813–829.
11 Meckes, M., Calzada, F., Tapia-Contreras, A., Cedillo-Rivera, R. *Phytother. Res*. **1999**, *13*, 102–105.
12 Moundipa, P.F., Flore, K.G.M., Bilong Bilong, C.F., Bruchhaus, I. *Afr. J. Trad. Complement. Alternat. Med*. **2005**, *2*, 113–121.
13 Diehl, M.S., Kamanzi Atindehou, K., Téré, H. Betschart, B. *J. Ethnopharmacol*. **2004**, 95, 277–284.
14 Clark, K.J., Grant, P.G., Sarr, A.B., Belakere, J.R., Swaggerty, C.L., Phillips, T.D., Woode, G.N. *Vet. Microbiol*. **1998**, 63, 147–157.
15 Gonçalves J.L.S., Lopes, R.C. Oliveira, D.B. Costa, S.S. Miranda, M.M.F.S. Romanos, M.T.V. Santos, N.S.O., Wigg, M.D. *J. Ethnopharmacol*. **2005**, *99*, 403–407.
16 McCutcheon, A.R., Roberts, T.E., Gibbons, E., Ellis, S.M., Babiuk, L.A., Hancock, R.E., Towers, G.H. *J. Ethnopharmacol*. **1995**, *49*, 101–110.
17 Galvez, J., Zarzuelo, A., Crespo, M.E., Lorente, M.D., Ocete, M.A., Jiménez, J. *Planta Med*. **1993**, *59*, 333–336.

18 Agbor, G.A., Léopold. T., Jeanne, N.Y. *Phytother. Res.* **2004**, *18*, 873–876.
19 Akah, P.A., Aguwa, C.N., Agu, R.U. *Phytother. Res.* **1999**, *13*, 292–295.
20 Murugesan, T., Ghosh, L., Mukherjee, K., Das, J., M. Pal, M., Saha, B. P. *Phytother. Res.* **2000**, *14*, 381–383.
21 Abdullahi, A.L., Agho, M.O., Amos, S., Gamaniel, K.S., Wambebe, C. *Phytother. Res.* **2001**, *15*, 431–434.
22 Ali, B.H., Blunden, G. *Phytother. Res.* **2003**, *17*, 299–305.
23 Ramesh, N., Viswanathan, M.B., Saraswathy, A., Balakrishna, K., Brindha, P. Lakshmanaperumalsamy, P. *Fitoterapia* **2002**, *73*, 160–164.
24 Esquenazi, D, Wigg, M.D., Miranda, M.M.F.S., Rodrigues, H.M., Tostes, J.B.F., Rozental, S., da Silva, A.J.R., Alviano, C.S. *Res. Microbiol.* **2002**, *153*, 647–652.
25 Alanís, A.D., Calzada, F., Cervantes, J.A., Torres, J., Ceballos, G.M. *J. Ethnopharmacol.* **2005**, *100*, 153–157.
26 dos Fernandes Vieira, R.H.S., dos Prazeres Rodrigues, D., Gonçalves, F.A., de Menzes, F.G.R., Arargão, J.S., Sousa, O.V. *Rev. Inst. Med. Trop. Sao Paulo* **2001**, *43*, 145–148.
27 Vijaya, K., Ananthan, S. *J. Alternat. Complement. Med.* **1997**, *3*, 13–20.
28 Longanga Otshudi, A., Foriers, A., Vercruysse, A., Van Zeebroeck, A., Lauwers, S. *Phytomedicine* **1999**, *7*, 167–172.
29 Paulo, A., Pimentel, M., Viegas, S., Pires, I., Duarte, A., Cabrita, J., Gomes, E.T. *J. Ethnopharmacol.* **1994**, *44*, 73–77.
30 Bajad, S., Bedi, K.L., Singla, A.K., Johri, R.K. *Planta Med.* **2001**, *67*, 284–287.
31 Zhou, S. Gao, Y., Jiang, W., Huang, M., Xu, A., Paxton, J.W. *Drug Metab. Rev.* **2000**, *35*, 35–98.
32 Hoffman, D. *An Elders' Herbal. Natural Techniques for Promoting Health and Vitality*. Healing Arts Press, Rochester, Vermont, USA, **1993**.
33 Meckes, M, Torres, J., Calzada, Rivera, F.J., Camorlinga, M., Lemus, H., Rodríguez, G. *Phytother. Res.* **1997**, *11*, 128–131.
34 Imai, H., Osawa, K., Yasuda, H., Hamashima, H., Arai, T., Sasatsu, M. *Microbios* **2001**, *106* (Suppl 1), 31–39.
35 Bezic, N., Skocibusic, M., Dunkic, V., Radonic, A. *Phytother. Res.* **2003**, *17*, 1037–1040.
36 Mujumdar, A.M., Upadhye, A.S., Misar, A.V. *J. Ethnopharmacol.* **2000**, *70*, 183–187.
37 Miller, M.J.S., MacNaughton, W.K., Zhang, X.-J., Thompson, J.H., Charbonnet, R.M., Bobrowski, P., Lao, J., Tentacosti, A.M., Sandoval, M. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2000**, *279*, G192–G200.
38 Besra, S.E., Gomes, A., Ganguly, D.K., Vedasiromoni, J.R. *Phytother. Res.* **2003**, *17*, 380–384.
39 Borrelli, F., Capasso, R., Pinto, A., Izzo, A.A. *Life Sci.* **2004**, *74*, 2889–2896.
40 Vidari, G., Vita Finzi, P., Zaruelo, A., Gálvez, J., Zafra, C., Chiroboga, X., Berenguer, B., La Casa, C., Alarcón de la Lastra, C., Motilva, V., Martin, M.J. *Pharm. Biol.* **2003**, *41*, 405–411.
41 Atta, A.H., Mouneir. S.M. *J. Ethnopharmacol.* **2004**, *92*, 303–309.
42 Longanga Otshudi, A., Vercruysse, A., Foriers, A. *J. Ethnopharmacol.* **2000**, *71*, 411–423.
43 Gricilda Shoba, F., Thomas, M. *J. Ethnopharmacol.* **2001**, *76*, 73–76.
44 Lin, J., Puckree, T., Mvelase, T.P. *J. Ethnopharmacol.* **2002**, *79*, 53–56.
45 Mueller, M.S., Mechler, E. *Medicinal Plants in Tropical Countries, Traditional Use, Experience, Facts*. Georg Thieme Verlag, Stuttgart, **2005**.
46 Rao, V.S.N., Santos, F.A., Sobreira, T.T., Souza, M.F., Melo, C.L., Silveira, E.R. *Planta Med.* **1997**, *63*, 146–149.
47 Compton, M.S. *Herbal Gold*. Llewellyn Publications, St. Paul, Minnesota, **2000**.
48 Hajhashemi, V., Sadraei, H., Ghannadi, A.R., Mohseni, M. *J. Ethnopharmacol.* **2000**, *71*, 187–192.
49 Atta, A.H., Mouneir, S.M. *Phytother. Res.* **2005**, *19*, 481–485.
50 Yavada, R.N. Jain, S. *J. Asian Nat. Prod. Res.* **2005**, *7*, 595–599.
51 Cowan. M.M. *Clin. Microbiol. Rev.* **1999**, *12*, 564–582.
52 de Smet, P.A.G.M. *N. Engl. J. Med.* **2002**, *347*, 2046–2056.
53 Philip, R.B. *J. Complement. Integr. Med.* **2004**, *1*, 4 (www.bepress.com/jcim/vol1/iss1/4)

54 Stickel, F., Seitz, H.K. *Public Health Nutr.* **2000**, 3, 501–508.
55 Rousseaux, C.G., Schachter, H. *Birth Defects Res.* **2003**, *68*, 505–510.
56 McCabe, P. *Complement. Ther. Clin. Pract.* **2005**, *11*, 28–31.
57 Kuge, T., Shibata, T., Willett, M.S. *J. Clin. Pharmacol.* **2003**, *43*, 284–290.
58 Morino, H., Ataka, K., Ito, M., Kuge, T. *Biol. Pharm. Bull.* **2004**, *27*, 1046–1051.
59 Ogata, N., Shibata, T. *Pharmacology* **2004**, *72*, 247–253.
60 Kuge, T., Shibata, T., Willett, M.S., Turck, P., Traul, K.A. *Int. J. Toxicol.* **2001**, *20*, 297–305.
61 Izzo, A.A. *Fundam. Clin. Pharmacol.* **2004**, *19*, 1–16.
62 Woolf, A.D. *Pediatrics* **2003**, *112*, 240–246.
63 Ramos, A., Piloto, J., Visozo, A., García, A., Lastra, H. Ponce de León, H. *Phytother. Res.* **2001**, *15*, 360–363.

13
Mutagenicity and Antimutagenicity of Medicinal Plants

Javed Musarrat, Farrukh Aqil, and Iqbal Ahmad

Summary

Plants synthesize an array of structurally and functionally diverse bioactive secondary metabolites. These phytocompounds are subject to wide experimental scrutiny for their pharmacological and therapeutic potential. Substantial work has been reported on the screening of medicinal and edible plants for their mutagenicity and/or antimutagenic properties. Most of these natural products are regarded as potential sources of novel therapeutic agents against mutational disorders in humans. For a successful screening program, it is essential to evaluate critically the biological activity of plant extracts through appropriate assay systems. This chapter presents information related to traditionally used medicinal and edible plants endowed with a variety of phytochemicals conferring mutagenicity and antimutagenicity.

13.1
Introduction

Mutations are the cause of innate metabolic defects in cellular systems, triggering morbidity and mortality in living organisms. A plethora of synthetic and natural substances, apart from various genotoxic physical and biological agents, are known to act as mutagenic, co-carcinogenic, and/or carcinogenic agents. Since the mutagens are involved in the initiation and promotion of several human diseases, including cancer, the significance of novel bioactive phytocompounds in counteracting these promutagenic and carcinogenic effects is now gaining credence. Indeed, the chemicals that reduce the mutagenicity of physical and chemical mutagens are referred to as antimutagens [1]. The existence of antimutagens was first reported almost four decades ago, and since then numerous studies have been carried out in order to identify compounds that might protect humans against DNA damage and its consequences. In the last 10 years, a number of laboratories have reported the antimutagenic and anticarcinogenic properties of a wide variety of dietary constit-

uents [2]. There are continued efforts all over the world to explore the rich biodiversity of edible as well as medicinal herbs and other nontoxic plants in search of the most effective phytoantimutagens.

Large-scale screening trials with plant extracts have led to the identification of numerous protective phytocompounds [3–5]. Systematic carcinogenicity studies with rodents suggested the protective effects of *Brassica* and other vegetables and their constituents [6–9]. This has led to the possibility of developing dietary strategies to protect humans against DNA damage and cancer. This assumption is also supported by epidemiological studies, which suggested that around 20–60% of all cancers are diet related [10, 11] and that the intake of vegetables and fruits is inversely related to the incidence of various forms of cancer [12]. Computer-aided literature search revealed that out of more than 25 000 articles published on antimutagens and anticarcinogens in the last 25 years, about 80% are on plant constituents used as foods or for medical purposes. These "bioactive" compounds belong to a variety of different chemical groups such as phenolics, pigments, allylsulfides, glucosinolates, tannins, anthocyans, flavonoids, phytosterols, protease inhibitors, and phytoestrogens. Many of these substances elicit, apart from their antimutagenic and anticarcinogenic properties, additional beneficial effects such as activation of the immune system and/or protection against cardiovascular diseases [13].

13.2 Plants as Protective Agents Against DNA Damage

The use of natural products or their active components for prevention and/or treatment of chronic diseases is based primarily on traditional medicine from various ethnic societies and on epidemiological data. The chemopreventive role of edible plants – mainly vegetables and fruits against cancer – have convincing epidemiological evidence. For instance, the legumes are an important food crop both economically and nutritionally, being cultivated and consumed worldwide. Many leguminous micronutrients such as anthocyanins, lecithin, and trypsin inhibitors have been suggested to have protective and therapeutic effects against cancer [14, 15].

The mutagenicity/genotoxicity and antimutagenicity/antigenotoxicity of cooked and dehydrated black beans have been investigated in mouse bone marrow and peripheral blood cells by the micronucleus test and comet assay, respectively. The two end-points (micronucleus and primary DNA lesions) as expressed in two different cell types (erythrocytes and leukocytes) corroborate the protective activity of black beans in the maintenance of genomic stability.

Similarly, crude extracts of propolis, a natural composite balsam produced by honeybees from gum of various plants, have been used as folk medicine. Recently, these extracts have gained popularity both as a medicine with antibacterial, antiviral, anti-inflammatory, and antioxidant properties, and as a food to improve health and prevent disease [16–18]. Analyses of chemical composition have identified at

least 200 compounds in extracts of propolis, including fatty and phenolic acids as well as esters, flavonoids, terpenes, aromatics aldehydes, alcohols, sesquiterpenes, β-steroids, and naphthalene [17]. Various studies have indicated that propolis and some of its components, such as the caffeic acid phenyl esters (CAPE) and artepellin C, have antimutagenic and anticarcinogenic effects [19, 20]. Matsuno et al. [21] reported the cytotoxic effects of an isolated compound (PRF-1) from propolis on human hepatocellular carcinoma. Later, Varanda and co-workers [22] showed the inhibitory effect of a propolis extract on daunomycin, benzo(a)pyrene, and aflatoxin B1-induced mutagenicity in the *Salmonella*/microsome assay. More recently, Kimoto et al. [23] have also reported a protective effect of propolis against renal adenocarcinoma in CD-1 and ddY mice that were treated with FeNTA (ferric nitrilotriacetate). It has also been demonstrated that a hydroalcoholic extract of propolis may have protective activity on colon carcinogenesis, suppressing the development of preneoplastic lesions.

Another edible item with proven nutritional and therapeutic values throughout the world since ancient times is mushrooms [24–26]. Numerous kinds of mushrooms are utilized as foods and traditional medicines in many countries and there have been investigations of the biological activities of mushroom extracts. The activities of various mushroom extracts include anticarcinogenic effects, [27–31], antimutagenic effects [32–36], and protection from blocks to gap junction-based intercellular communication [37]. At the molecular level, researchers have found that antigenotoxic factors in mushrooms include polysaccharides, such as beta- and alpha-glucan. In *Agrocybe cylindracea* (yanagimatsutake), the anticarcinogenic substances detected in the mushroom have been identified as alpha-D-glucan-*O*-carboxy methylated derivatives [38]. Infusion of the dried fruiting bodies has been used as a stimulant and as auxiliary treatment of various diseases, including cancer [39, 40]. Many isolated polysaccharides and protein-bound polysaccharides from *Agaricus blazei* have shown potential direct antitumor activity or through specific and nonspecific immune response activation [41, 42]. In contrast to the investigations of the established antitumor activity of *A. blazei* and its components observed in tumor-transplantable models, few studies have been performed on chemical carcinogenesis models.

A recent study suggested that aqueous extracts of *A. blazei* exert a hepatoprotective effect on liver toxicity and on the initiation step of hepatocarcinogenesis in an environment of moderate toxicity. An aqueous solution obtained from a mixture of lineages (AB 96/07, AB 96/09, and AB 97/11) of the mushroom reduced the frequency of micronuclei induced by methyl methanesulfonate in cultured Chinese hamster V79 cells [43] and by cyclophosphamide in mouse bone marrow polychromatic erythrocytes and reticulocytes [44].

Studies have pointed out that the mushroom *Lentinula edodes* and some of its active substances exert a protective effect against mutagenesis and carcinogenesis [45–47]. *L. edodes* also was observed to be effective in protecting against DNA damage, which can be responsible for the initiation of carcinogenesis.

13.3 Antimutagenic Properties of Edible and Medicinal Plants

Natural antimutagens from edible and medicinal plants are of particular importance because they may be useful for human cancer prevention and have no undesirable xenobiotic effects on living organisms [48, 49]. Encouraging reports on the antimutagenic properties of edible plants have led to increased interest in the search for natural phytoantimutagens from medicinal plants from different parts of world. An extensive literature survey on phytoantimutagens has been carried out and is presented in Table 13.1. Edible plants with antimutagenic activity and chemopreventive potential have been documented from several plants groups, including vegetables such as *Solanum melongena* (fruit), *Raphanus sativus* (root), *Allium sativum* (bulb), *Allium cepa* (bulb), *Brassica oleraceae* (curds), *Lycopersicon esculentum* (fruit) and spices such as *Zingiber officinalis* (rhizome), *Syzygium aromaticum* (bud), *Curcuma domestica*, *Cuminum cyminum*, *Carum carvi* (seed), *Coriandrum sativum* (seed), and *Piper nigram* (seed) [50–55]. Similarly, four Nigerian common edible vegetables extracts (*Bryophyllum pinnatum*, *Dialium guincense*, *Ocimum gratissium*, and *Vernonia amygdalina*) showed antimutagenic effects against reverse mutation induced by ethyl methane sulfonate (EMS) and 4-nitrophenylenediamine and 2-aminofluorine [50].

In addition, several other plants such as *Coffee arabica*, *Camellia sinensis*, *Piper betle*, *Glycyrrhiza glabra*, and *Eucommia ulmoides* exhibit antimutagenic properties [1, 56–58]. The chemopreventive importance led to increased use of vegetables and vegetable plants in many countries. Newly emerging edible Taiwanese plants such as bas (*Basella alba*), bou (*Boussingaulia gracilis*), cen (*Centella asiatica*), cor (*Corchorus olitorius*), cra (*Crassocephalum creidioides*), por (*Portulaca oleraceae*), sec (*Sechium edule*), and sol (*Solanum nigrum*) have demonstrated moderate to strong antimutagenic activity against one or other mutagen in the Ames *Salmonella* test [59].

Yoshikawa and co-workers [60] investigated the antimutagenic effects of specific components of extracts from eggplant fruits using the *Salmonella*/microsome assay. Eggplant fruit juice exhibited antimutagenic activity against 3-amino-1-methyl-5H-pyrido(4,3b)indole (Trp-P-2)-induced mutagenicity. Krizkova et al. [61] examined the possible protective effect of a suberin extract from *Quercus* cork on acridine orange (AO), ofloxacin and UV radiation-induced mutagenicity (bleaching activity in *Euglena gracilis*). These results were the first attempt to analyze suberin in relation to mutagenicity of some chemicals. Suberin exhibited a significant dose-dependent protective effect against AO-induced mutagenicity and the concentration of 500 μg mL^{-1} completely eliminated the *Euglena* bleaching activity of AO. The mutagenicity of ofloxacin was also significantly reduced in the presence of suberin (125, 250, and 500 μg mL^{-1}).

A fraction isolated from *Terminalia arjuna* was studied for its antimutagenic effect against 4-nitro-*o*-phenylenediamine (NOP) in TA98 and TA100 tester strains of *Salmonella typhimurium* using the Ames assay. The fraction inhibited the mutagenicity of 2AF very significantly in both strains while the revertant colonies

Table 13.1 Antimutagenicity of medicinal and edible plants.

Name of plant (Family)	*Active extracts/isolated phytocompounds*	*Active against mutagen*	*Reference*
Allium cepa (Liliaceae)	Ethyl acetate extract	IQ, MNNG	72
Allium sativum (Liliaceae)	Ajoene	B[a]P, NPD	107
Aloe arborescens (Liliaceae)	Aloe-emodin	Trp-P-1	103
Aloe vera (Liliaceae)	Di (2- ethyl hexyl) phthalate	2-AF	110
Aplysia dactylomela (Fabaceae)	Elatol and obtusol	2-AN	111
Aquilaria aqallocha (Thymelaeaceae)	Erythoxydiol	2-AN	105
Areca catechu (Arecaceae)	Catechin, epicatechin	IQ	113
Asiasarum heterotopoides (Aristolochiaceae)	Methanol extract/methyleugenol, elemicin, gamma-asron	2 Amino-3,4-dimethyl-imidazole (4,5-f) quinoline	114
Azadirachta indica (Meliaceae)	Flavonoid, baicalein, methanol extract of flower/flavonones	Trp-P-1, heterocyclic amines	64, 65
Berry (strawberry, blueberry, and raspberry)	Hydrolyzed tannin containing extract	MMS, B[a]P	73
Brassica oleracea (Cruciferae)	Chlorophyll, chlorophyllin, Methyl methanethiosulphonate	MNU, 2-Aminoanthracene, UV-induced mutation	53, 115, 116
Brophyllum pinnatum (Crassulaceae)	Ethylacetate and petroleum ether fraction	4-Nitrophenylenediamine, 2-AF, EMS	50
Campomanesia xanthocarpa (Myrtaceae)	Aqueous extracts	2-AF	79
Cacao liquor poly phenol	Cacao liquor	Mytomicin C	117
Caesalpinia pulcherrima (Caesalpiniaceae)	Pulcherrimins A, B, C, D	–	106
Camellia sinensis (Theaceae)	(–)-Epicatechingallate, (–)-epigallocatechin	4-NQO	118
Camellia sinensis (Theaceae)	Persicarin, kaempferol, morin, fisetin, hesperatin	AFB1	95
Camellia sinensis (Theaceae)	Catechins, epigallocatechin	Trp-P-1	119, 120
Capsicum annuum (Solanaceae)	Capsaicin	Cyclophosphamide, NNK	121, 122
Castela texana (Simarubaceae)	Extract	2-AAF	123
Citrus species (Rutaceae)	*d*-Limonene	DMBA	124
Coffea arabica (Rubiaceae)	Chlorogenic acid	Trp-P-1	108
Crocus sativus L. (Iridaceae)	Carotenoid	2-AA, B[a]P	70
Curcuma longa (Zingiberaceae)	Ethanol extract	NaN_3 (sodium azide)	74
Curcuma longa (Zingiberaceae)	Diferuloylmethane (curcumin I), feruloyl (curcumin II), *p*-hydroxycinnamoyl methane (curcumin III)	AAF	125, 126
Cuscuta chinensis (Convolvulaceae)	Flavonoid, baicalein	Trp-P-1	64
Cymopolia barbata (Chlorophyceae)	Cymopol, cyclocymopol, cymobarbatol and 4-isocymobarbatol	2-AN, EMS	127, 128

Table 13.1 Antimutagenicity of medicinal and edible plants. (Continued)

Name of plant (Family)	*Active extracts/isolated phytocompounds*	*Active against mutagen*	*Reference*
Dialium guineense (Leguminosae)	Methanol extract	EMS, 4-nitrophenylene diamine	50
Dioscorea japonica (Dioscoreaceae)	Piperine	Trp-P-1, furylfuramide	129
Emblica officinalis (Euphorbiaceae)	Flavonoid, baicalein	Trp-P-1	64
Glycyrrhiza inflata (Fabaceae)	G 9315 (a complex of six flavonoids	Cytoxan	130
Glycyrrhiza glabra (Fabaceae)	Glabrene	EMS	1, 128
Hibiscus sabdariffa (Malvaceae)	Ethanol extracts	Trp-P-1, Trp-P-2	131
Hoffmanseggia intricata (Caesalpiniaceae)	Intricatol, intricatinol	2-AN, AAF, EMS	128, 132
Litsea petiolata (Lauraceae)	Flavonoid, baicalein	Trp-P-1	64
Lupinus campestris (Leguminosae)	Phenolic compounds/alkaloids, catechins, quinolizidine	1-Nitropyrene	133
Mikania laevigata (Asteraceae)	Aqueous extract	2-AF	79
Mahonia aquifolium (Berberidaceae)	Berberine	Acridine orange	134
Mentha piperita (Lamiaceae)	Luteolin	Trp-P-2	135
Mesona procumbens Hemsl. Hsian-tsao (Lamiaceae)	Water, methanol and ethyl acetate/polyphenolic compounds and ascorbic acid	B[a]P, 2-amino 3-methyl imidazole (4,5-f) quinoline	59
Micromelum minutum (Rutaceae)	Flavonoid, baicalein	Trp-P-1	64
Murdannia loriformis (Commelinaceae)	Ethanolic extract	B[a]P and many more	136
Muscari racemosum L. (Hyacinthaceae)	Homoisoflavonoids (3-benzylidine-4-chromanones)	9-Aminoacridine, 4- nitroquinoline-*N*-oxide, natrium azide, MNNG	68.
Myrtus communis (Myrtaceae)	Aqueous, methanol, ethyl acetate, chloroform, hexane and essential oils	AFB1	112
Ocimum gratissimum (Labiateae)	Methanol fraction	NOP	50
Oenanthe javanica (Apiaceae)	Isorhamnetin	AFB1	137
Onion, licorice, garlic, green pepper, carrot, pineapple	Extracts of veg., ethanolic extracts	NDMA: N-nitrosodimethylamine, NDBA: N-nitrosodibutylamine, NPIP: N-nitrosopiperidine	138
Oroxylum indicum (Bignoniaceae)	Flavonoid, baicalein	Trp-P-1	64
Phoenix dactylifera L. (Arecacaceae)	Fruits, aqueous extract	B[a]P	139
Phyllanthus amarus (Euphorbiaceae)	Methanolic and aqueous extracts	2AAF/aflatoxin, NaN_3, MNNG	140
Phyllanthus orbicularis (Euphorbiaceae)	Aqueous extract (leaves and stem)	Aromatic amines, hydrogen peroxide	67
Piper betle (Piperaceae)	Hydroxychavicol	Arecoline	141
Psoralea corylifolia (Fabaceae)	Umbelliferone, 8-methoxy-psoralin (xanthotoxin)	Trp-P-1, Trp-P-2	5, 142
Psorothamnus fremontii (Fabaceae)	Fremontin, fremontone	EMS, AN	143

Table 13.1 Antimutagenicity of medicinal and edible plants. (Continued)

Name of plant (Family)	*Active extracts/isolated phytocompounds*	*Active against mutagen*	*Reference*
Quercus suber (Fagaceae)	Cork extract, suberin	Acridine orange, ofloxacin and UV in *Euglena gracilis*	61
Rheum officinale (Polygonaceae)	Anthraquinones	Trp-P-2	144
Rhoeo discolor (Commelinaceae)	Ethanolic crude extract	Norfloxacin	145
Rhus verniciflua (Anacardiaceae)	Methanol extract of heartwood/flavonoids (sulfuretin)	AFB1	146
Salvia officinalis (Lamiaceae)	Luteolin	Trp-P-2	109, 135
Scindapsus officinalis (Araceae)	Trigonelline, caffeine	2-AN	109
Selenium monnier (Apaceae)	Imperatorin, osthol	2 AN, B[a]P	128, 142
Several plant species (number of families)	Catechin (epigallocatechin)	NOP	56, 148, 149
Solanum melongena (Solanaceae)	Pheophytin 'a'	Trp-P-2	60
Solanum melongena L. (Solanaceae)	Acetone, petroleum ether methanol, ethyl acetate/ Pheophytin	Trp-P2	60
Soy bean	Saponin 2,3-dihydro, 2,5-dihydroxy-6 methyl 4H-pyrone-4-one	2-AAAF	150
Soybean paste	Water extract	Aflotoxin	75
Strawberries, raspberries, grapes, blackcurrants, and walnut	Ellagic acid	1-Nitropyrene	97
Terminalia bellerica (Combretaceae)	Phenolics	NOP and 2-AF	71
Terminalia catappa (Combretaceae)	Leaves	MNNG, B[a]P	151
Terminalia arjuna (Combretaceae)	Bark extracts various fractions	NOP and 2-AF	69, 152
Thymus vulgaris (Lamiaceae)	Luteolin	Trp-P-2	147
Trifolium pratanse (Fabaceae)	Biochanin A	B[a]P	153
Vernonia amygdalina (Compositae)	Petroleum ether fraction	2-AF	50
Vismia amazonica (Clusiaceae)	Euxanthone and 1,5-dihydroxyxanthone	2-AN and EMS	104, 128
Vitex rotundiforia (Verbenaceae)	(+)-Polyalthic acid	Trp-P-1	154
Yucca schidigera (Yuccaceae)	3,4,5-trihydroxystilbene	Trp-P-1	155

2-AF, 2-aminofluorene; 2-AAAF, 2-acetoxyacetylaminofluorene; 2-AAF, 2-acetyl aminofluorene; 2-AN, 2-aminoanthracene; 4-NQO, 4-nitroquinoline-*N*-oxide; 6TG, 6-thioguanine; AFB1, aflatoxin B1; B[a]P, benzo[a]pyrene; DMBA, 7,12-dimethyl benz(a)anthracene; EMS, ethyl methane sulfonate; Glu-P-1, 2-amino-6-methyldipyrido (1,2-a: 3,2-d)-imidazole; IQ, 2-amino-3-methyl-imidazo (4,5-f) quinoline; MMS, methyl methane sulfonate; MNNG, *N*-methyl-*N*_-nitro-*N*-nitroguanidine; MNU, *N*-methyl-*N*-nitrosourea; NNK, Nitrosamine-4- (methyl nitrosamino), NOP, 4-nitro-*o*-phenylene-diamine; NPD, 4-nitro-1,2-phenylenediamine; 4-NQO, 4-nitroquinoline-*N*-oxide; Trp-P1, 3-amino-1,4-dimethyl-5H-pyrido(4,3-b)indole; Trp-P-2, 3-amino-1-methyl-5 H-pyrido(4,3b)indole.

induced by NOP and sodium azide were reduced moderately. ^{1}H-NMR, ^{13}C-NMR, IR, and UV spectroscopic data of the fraction revealed tannins as active constituents [62]. Shankel et al. [63] described the antimutagenic potential of Glabrene analogs against EMS-induced mutations utilizing modified Ames tests. Nakahara et al. [64] have shown that a methanolic extract of *Oroxylum indicum* strongly inhibits the mutagenicity of 3-amino-1,4-dimethyl-5H-pyrido(4,3-b)indole (Trp-P-1) by Ames test. Later, Nakahara and workers [65] demonstrated the antimutagenic activity of methanolic extracts of 118 samples (108 species) of edible Thai plants against Trp-P-1. The major antimutagenic constituent has been identified as baicalein with an IC_{50} value of 2.78 ± 0.1 μmol L^{-1}. The potent antimutagenicity of the extract has been correlated with the high content of baicalein, which also acts as a desmutagen and inhibits the *N*-hydroxylation of Trp-P-2.

Sharma et al. [66] evaluated the antimutagenic effect of *Cinnamomum cassia* against two mutagens: benzo[a]pyrene (B[a]P) and cyclophosphamide (CP). Ohe et al. [100] studied the antigenotoxic properties of tea leaf extracts in a *Salmonella* umu-test. Seven nonfermented teas (green tea), one semi-fermented tea (oolong tea), also fermented teas (black tea and Chinese pur er tea) and two other teas were examined for their antigenotoxic abilities and for their catechins contents relationship. The antigenotoxic effect of 12 tea leaf extracts reportedly decreased in the order: oolong tea (semi-fermented tea) > black tea (fermented tea) > sencha (nonfermented tea, an ordinary grade green tea) > tocyucya (other tea) > Chinese pure tea (fermented tea).

Yen and colleagues [59] determined the antimutagenic activity of various solvent extracts from a herb *Mesona procumbens* Hemsl, normally called hsian tsao in China. The antimutagenicity of water extract of Hsian tsao (*Mesona precumbens*) has been attributed mainly to their polyphenolic compounds and ascorbic acid. Ferrer et al. [67] demonstrated the antimutagenicity of *Phyllanthus orbicularis* against hydrogen peroxide using *Salmonella* assay. Similarly, Miadokova and co-workers [68] evaluated the potential antimutagenic effect of a plant extract of *Muscari racemosum* bulbs, rich in 3-benzylidene-4-chromanones, on three genetic model organisms. The mixture of three homoisoflavonoids has been tested together with diagnostic mutagens in the Ames assay on four bacterial strains: *Salmonella typhimurium* TA97, TA98, TA100, TA102, in the toxicity and mutagenicity/antimutagenicity assay on the yeast strain *Saccharomyces cerevisiae* D7, and in the simultaneous phytotoxicity and clastogenicity/anticlastogenicity assay on *Vicia sativa*.

Pasquini et al. [69] determined the antimutagenic potential of chloroform, acetone, methanol, methanol plus HCl, diethyl ether and ethyl acetate extracts of *Terminalia arjuna* (bark) against the model mutagen 4-nitroquinoline-*N*-oxide (4-NQO) using the *Salmonella*/microsome, comet, and micronucleus tests. Also, the antioxidant and antimutagenic properties of an aqueous extract of date fruit (*Phoenix dactylifera*) has been demonstrated. The aqueous extract of date fruit exhibited dose-dependent inhibition of superoxide and hydroxyl radicals and B[a]P-induced mutagenicity on *Salmonella* tester strains TA98 and TA100 with metabolic activation.

Abdullaev et al. [70] assessed the antimutagenic, co-mutagenic, and cytotoxic effects of saffron and its main ingredients using the Ames/*Salmonella* test system.

The saffron component responsible for this unusual co-mutagenic effect is safranal. In the *in vitro* colony formation test system, saffron exhibits a dose-dependent inhibitory effect only against human malignant cells.

In search for novel polyphenolic antimutagenic agents from Indian medicinal plants, Kaur et al. [71] examined the water, acetone, and chloroform extracts of *Terminalia bellerica* for their antimutagenic potency using the Ames *Salmonella*/microsome assay. Acetone extract exhibited variable inhibitory activity of 65.6%, and 69.7% with 4-*O*-nitrophenylenediamine (NOP) and sodium azide respectively (as direct acting mutagens), and 81.4% with 2-aminofluorene (2-AF) (an S9-dependent mutagen). Studies demonstrated that polyphenolic compounds from acetone extract could be used as effective chemopreventive agents in the future.

Shon et al. [72] assessed the antioxidant and antimutagenic activities of red, yellow, and white onion extracts. Smith et al. [73] evaluated the fresh juices and organic solvent extracts from the fruits of strawberry, blueberry, and raspberry for their ability to inhibit the production of mutations by the direct acting mutagen methyl methanosulfonate and the metabolically activated carcinogen B[a]P.

Kuttan et al. [74] showed the antimutagenicity of herbal detoxification formula smoke shield against environmental mutagens. Smoke shield contains a dual extract of turmeric (*Curcuma longa*) obtained by supercritical CO_2 gas extraction and post supercritical hydroethanolic extraction together with extracts of green tea and other spices. The presence of these synergistically increases the activity of turmeric smoke shield and it was found to produce significant inhibition of mutagenicity to *Salmonella typhimurium* induced by sodium azide and NOP at a concentration of 2 mg per plate, while inhibition of mutagenicity induced by *N*-methyl-*N*-nitro-*N*′-nitrosoguanidine (MNNG) was less significant. It also inhibited the mutagenicity induced by tobacco extract to TA102. Similarly Kim [75] demonstrated the antigenotoxic effects of water extract of Korean fermented soybean paste (doen-jang).

13.4 Mutagenicity of Plant Extracts and Phytocompounds

Research into the plants used in folk medicines in the form of beverages and other formulations, and their specific potential efficacy, safety, and toxicity has been the subject of intense investigation. Specific attention is focussed on the mutagenicity of plant extracts, herbal formulations, and specific phytocompounds. Considerable amounts of data have been generated on medicinal and edible plants. In a few cases mutagenic compounds have been postulated or identified.

Considerable work has been done on screening of Brazilian plants for mutagenicity in the extracts of *Achyrocline satureoids*, *Baccharis amomola*, *Luchea divaricata*, *Myriciaria tenella*, *Similax compestris*, *Tripodanthus acutifolius*, *Cassia corymbosa*, and *Campomanesia xanthocarpa* in Ames *Salmonella* assay with or without S9 and in few cases with SOS chromotest microscreen phage induction assay [76–79]. It has been suggested that the mutagenicity might be due to flavonoids, tannins, and anthraquinones, quercetin and caffeic acid. Schimmer and co-workers [80] evaluat-

ed 55 commercial phytopharmaceuticals (extract and tinctures) from 44 plant species. The extracts of the plants (e.g. Alchemillae tinctura, Centaurii extractum, Hippocastani extractum, Myrtilli extractum, Hyperici tinctura, Trifolii fibrini extractum and Trifolii fibrini tinctura) showed signs of mutagenicity in TA98 and TA100 *Salmonella* strains with S9. Sandnes et al. [147] reported mutagenic potential of extracts of senna folium and senna fructus in TA98 strain with S9 in *Salmonella* test. Rubiolo et al. [81] evaluated the mutagenicity of a series of pyrrolizidine alkaloids and extracts of several Italian *Senecio* species containing pyrrolizidine alkaloids including *Senecio inaeguidens*, *S. fuchii*, and *S. cacaliastes*. Also, the mutagenicity potential of eight plants including *Combretum erythrophyllum*, *Gnidia kraussiana*, and *Barlerii randii*, traditionally used in Zimbabwe has been demonstrated. The mutagenicity of extract from *Ruta graveolens* in *Salmonella* tester strain TA98, TA100 has been showed in the presence and absence of S9 mix due to the presence of furoquinoline alkaloids [82].

Medicinal herbs from Poland, such as *Erigeron canadensis*, *Anthyllis vulnararia*, and *Pyrola chloranta* have been used for isolation of quercetin, rhamnetin, isohamnetin, apigenin, and luteoline flavonoids. Of the above flavonoids only quercetin and rhamnetin revealed mutagenic activity in the test using TA97a, TA98, TA100, and TA102 tester strains *Salmonella typhimurium*. The presence of S9 rat liver microsome fraction markedly enhances the mutagenic activity of quercetin. Rhamnetin appeared to be much weaker mutagen in the Ames test [83]. Moreover, the aqueous extracts of the plants *Lannea edulis* and *Monotes glaber* used in traditional medical practice of Zimbabwe and other part of Africa also showed signs of mutagenicity in TA97a, TA98, and TA100 *Salmonella typhimurium* [84].

Mutagenicity testing of the plant essential oils and their monoterpenoid constituents such as citral, citronellol (+/–), camphor compound, 1,8-cineole (eucalyptol), terpineol, and C-1-menthol revealed terpineol to be mutagenic in TA102 tester strains both in the presence and absence of S9 mix. Other monoterpanoids have been reported to be nonmutagenic in TA97a, TA98, TA100, and TA102 tester strains in Ames test [85].

13.5 "Janus Carcinogens and Mutagens"

Many substances reported to be antimutagens or anticarcinogens have, themselves, been shown to be promutagenic or carcinogenic. Chemicals belonging to such a category are termed "Janus carcinogens and mutagens" after the ancient Roman god "Janus," who is depicted as having one head with two faces, one looking forward and one looking backward [86]. Several other recent reports have also addressed or emphasized the biphasic nature of many active substances reported to "modulate" the mutagenicity and/or carcinogenicity of heterocyclic amines. The majority of these modulating substances are plant products or extracts. A compendium of the antimutagenicity literature by Waters et al. [87] showed that a number of chemicals have both antimutagenic and mutagenic effects. For instance, β-caro-

tene was the first presumptive anticarcinogen to be included in large-scale, clinical intervention trials, but the trials were terminated prematurely upon revelation that β-carotene treatment was associated with an increased cancer incidence rather than the expected decrease [88, 89].

Fahrig [90] showed that three substances, testosterone, β-estradiol, and diethylstilbestrol, were antimutagens and co-recombinogens in yeast in the absence of S9 but became co-mutagenic and anti-recombinogenic in the presence of rat liver S9. Also, vanillin, which was antimutagenic in mice *in vivo*, was co-mutagenic in yeast *in vitro* in the absence of S9. The antioxidant ascorbic acid, which is not mutagenic in the *Drosophila* wing spot test, has been reported to be clastogenic in mammalian cells [91, 92]. Indeed, many authors reporting on the antimutagenicity of a substance, failed to cite articles showing the mutagenicity and/or carcinogenicity of the same substance. It is evident from available literature searches that the majority of these "protective" substances have not been tested adequately, or tested at all, for mutagenicity or carcinogenicity.

An additional concern is that many published reports of the antimutagenicity of a substance have not addressed rigorously test protocol factors that could have reduced the levels of mutated cells, or adequately examined the substance's potential mutagenicity. Thus, the study of antimutagenesis and anticarcinogenesis is not as simple as it appears from many of the publications. Indeed, in the multitude of antimutagenicity and anticarcinogenicity studies, the modulating responses seen are highly dependent on the (1) test systems, (2) protocols used, (3) interactions among the specific test chemical(s), (4) the cell or organism's physiology, and (5) stages of the life cycle. The biphasic properties of many test substances have led to situations where documented mutagenic chemicals, and others that have not been tested for carcinogenicity, are being recommended for human use as anticarcinogens.

13.6
Chemical Nature of Phytoantimutagenic Compounds

Extensive research in the last few decades on the detection and characterization of antimutagenic compounds from edible, nonedible, and medicinal plants/herbs has demonstrated a great diversity. Several authors have suggested that phytoantimutagens may belong to any of the following major class of phytocompounds. Major emphasis has been laid on the flavonoids, phenolics, coumarins, anthraquinone, tannins, terpenoides, diterpenes, and several others as specified in Table 13.1.

More than 500 compounds belonging to at least 25 chemical classes have been recognized as possessing antimutagenic/protective effects [93]. In recent years, there has been an increased interest in identifying the antimutagenic and anticarcinogenic constituents of both dietary and medicinal plants all over the world. The major classes of antimutagenic compounds are briefly described.

13.6.1 Flavonoids

Flavonoids are polyphenolic compounds are ubiquitously present in plants. More than 4000 different flavonoids have been isolated and identified so far. This class of phytocompounds received attention because they possess several biological activities, including antimutagenic and anticancer properties [94]. Different flavonoids from a variety of plants have been reported (Table 13.1). Some common flavonoids are glabridine (isoflavanone), quercetin, myricetin, kaempferol, fisetin, morin, and hesperetin [95, 96].

13.6.2 Phenolic Compounds

Phenolic compounds are a widely studied group of compounds from natural food and medicinal plants and are also implicated in various biological activities. Certain phenolic compounds such as ellagic acid found in strawberries, raspberries, grapes, walnuts, etc. have been found to be antimutagenic [97]. Also, the compounds such as epicatechin, (–)-epicatechin gallate, (–)-epigallocatechins, (–)-epigallocatechin gallate have been reported to be responsible for the antimutagenic activity of green tea and black tea [98, 99]. Ohe et al. [100] studied the antigenotoxic properties of tea leaf extracts in a *Salmonella* umu-test. Geetha and workers [101] demonstrated the antimutagenic activity of green tea catechins against oxidative mutagens such as tertiary butyl hydroxide, hydrogen peroxide using *Salmonella typhimurium* 102 tester strains.

13.6.3 Coumarins

Coumarins are 2H-1-benzopyran-2-ones, widely distributed in the vegetable kingdom. A wide range of structures with varying complexity occurs in angiosperms. Coumarins have been shown to behave both as antimutagens as well as anticarcinogen [94, 102]. For instance, umbelliferone, 8-methoxysoralin, imperatorin, and osthol have been described to have antimutagenic activity.

The antimutagenic activity of a wide array of phytochemicals, including anthraquinone (aloe-emodin-anthraquinone isolated from *Aloe barborescence*), has been reported [103]. Xanthones such as euxanthone and 1,5 dihydroxy-8-methoxyxanthone isolated from *Visma amazonica* display considerable antimutagenic activity against 2-aminoanthracene and EMS [104] (Table 13.1).

13.6.4 Diterpenoids

Diterpenoid-like erythroxydiol isolated from *Aquillaria agallocha* demonstrated antimutagenic as well as antitumor activity [105]. Four novel dibenzoate diter-

penes, pulcherrimins A, B, C, and D obtained from roots of *Caesalpinia pulcherrima*, were found to be active in DNA repair-deficient yeast mutant [106]. Eugenol, commonly found in clove oil, has been reported to possess significant antimutagenic activity [54].

13.6.5 Organosulfur Compounds

Ajoene and one of the derivatives of allicin have been found in garlic extract with significant antimutagenic activity [107]. Various other miscellaneous groups of phytocompounds, such as caffeine, trigonelline, and piperine, have been demonstrated to possess antimutagenic properties [108, 109]. The antimutagenic activities of various plant extracts and phytocomponds and plant extractrs are summarized in Table 13.1.

13.7 Assays for Mutagenicity and Antimutagenicity

Several short-term and long-term assays for the assessment of mutagenicity and antimutagenicity of a variety of compounds involving microbial, viral, plant cell and cell lines as well as animal systems have been developed. Their short lifespan and information available on genomes, mutation, and recombination processes make several viruses, bacteria (*E. coli*, *Bacillus*, *Salmonella typhimurium*), yeast (*Saccharomyces cerevisiae*), plant cells (*Allium cepa*, *Vicia sativa*), and plant and animal cell cultures suitable systems for studying mutagenesis and antimutagenesis [156]. Above all, the assay for mutagenicity testing developed by Ames et al. [157] employing *Salmonella typhimurium* has been extensively used in the identification of mutagenic and antimutagenic effects of variety of physical, chemical, and natural compounds, including plant extracts. The *S. typhimurium* TA97a, TA98, TA100, TA102, TA104, TA1535, 1537, 1538 and some other mutant strains have been commonly employed in mutagen and antimutagens screening programmes [157, 158]. To make the system more meaningful, a metabolic activation step has been included to mimic the biotransformation that can occur in animals when chemicals are ingested.

There has been considerable development of other methods for the genotoxicity testing of chemicals, because some of the genotoxic mechanisms are not be detected by nutritional reversion assays such as the *Salmonella* His^- reversion test. In particular chromosomal interchanges, DNA strand breaks, and larger chromosome deletions are not efficiently detected in the Ames assay. Thus, other *in vitro* and *in vivo* tests have been recommended for the genotoxic assessment of chemicals, including the *in vitro* micronucleus test, *Saccharomyces cerevisiae*, and *Vibrio harveyi* systems [68, 159]. Similarly clastogenicity and anticlastogenicity properties of plant extracts were evaluated by *Vicia sativa*, aberration assays, and prokaryotic murine mammary tumor FM3A cell lines [160].

Recently, some new *in vitro* models have been developed that have a better predictive value for the identification of protective compounds. For example, the use of genetically engineered cells that express individual phase I and phase II enzymes offer the possibility of carrying out mechanistic studies [161, 162]. Also, genes encoding for human enzymes have been successfully transfected. However, one of the major disadvantages of these cell lines for antimutagenicity studies is that the enzymes are not represented in an inducible form. Eckl and Raffelsberger [163] improved the culture medium for primary hepatocytes by adding growth factors and changing the salt concentrations in such a way that the cells divide and can be used for sister chromatid exchange (SCE) and micronucleus assay.

Another promising approach is the use of human-derived hepatoma cells that have maintained the activities of phase I and phase II enzymes that are usually lost during cultivation [164]. Some of the drug-metabolizing enzymes, for example CYP1A1, CYP1A2, CYP2E1, aryl hydrocarbon hydroxylase (AHH), UPDGT, and GST, are represented in an inducible form, therefore these are useful tools for the identification of protective compounds [165, 166]. Natarajan and Darroudi [167] established a method for micronucleus assay using HepG2 cells; also, a protocol for single-cell gel electrophoresis (SCGE) assay has been developed and validated [168, 169]. These models enable the detection of genotoxic effects of problematic compounds viz. safrole, hexamethylphosphoramide (HMPA), isatidine, and certain mycotoxins, which give false negative results in other *in vitro* assays. *In vivo* mutagenicity studies with rodents, mainly bone marrow micronucleus assays and chromosomal aberration tests with peripheral lymphocytes, have been used. Moreover, in some studies, the unscheduled DNA synthesis (UDS) assays and alkaline elution method have been used. The use of the former two methods is hampered by the fact that they are quite insensitive towards the effects of dietary carcinogens such as nitrosamines and heterocyclic aromatic amines (HAAs), and do not enable measurements in organs that are targets for tumor induction [170–172]. The newly developed approaches are DNA-adduct measurements, the use of transgenic animals, and *in vivo* SCGE assays with a variety of inner organs.

Furthermore, transgenic rodent mutation systems have been developed. The first report of a transgenic assay for mutation in mammals has been designed by incorporating the bacterial lacZ gene, encoding beta-galactosidase, in a lambda gt10 vector [173]. Transgenic lacZ mice have been produced by stable integration of the lambda gt10 vector into the chromosome of CD2F1 mice. Mutation analysis was carried out by extracting high molecular weight genomic DNA from the tissue of interest, packaging the lambda shuttle vector *in vitro* into lambda phage heads, and testing for mutations that arise in the transgene sequences following infection of an appropriate strain of *Escherichia coli*. A variety of transgenic rodent models have subsequently been developed, of which MutaTM mouse, Big Blue mouse and rat, the LacZ plasmid mouse, and the gpt delta mouse have a sufficient quantity of experimental data associated with them to allow evaluation of overall performance [174].

13.8
Paradigms in Antimutagenicity Research

The paradigm in antimutagenicity research is that any method that can be used for the detection of mutagens can also be used for the detection of antimutagens. This assumption is wrong, as important DNA-protective mechanisms are not represented in most of the conventional experimental models. Therefore, the use of such experimental systems may lead to false positive and false negative results. In most of the studies aimed at identifying antimutagens, conventional *in vitro* mutagenicity assays with bacteria or stable cell lines such as CHO or V-79 have been used. The evaluation of the current database on compounds that protect against HAAs revealed that out of a total of 301 studies published, about 279 are based on *in vitro* mutagenicity tests [175]. In these studies the model used in the routine testing of chemicals [176] employs indicator cells that are devoid of enzymes involved in the biotransformation of xenobiotics. Therefore, exogenous liver enzyme mixtures are added to reflect the metabolic activation processes in mammals [177]. It is assumed that the most important mechanisms of chemoprotection towards DNA-reactive carcinogens are inactivation of the parent compounds or their metabolites by direct binding, inhibition of enzymatic activation, and induction of detoxifying (phase II) enzymes [178, 179].

13.9
Conclusions

The plants exhibiting mutagenicity are distinct from those that display antimutagenic activity. Potentially antimutagenic plants include a number of common or ethnic group restricted edible plants, including cereals, pulses, vegetables, and spices and medicinal herb and health tonic plants. It is realized that the general practice of identifying antimutagens and anticarcinogens by their activities against specific chemicals in specific test systems is not sufficient to sustain a conclusion that the same substance will be similarly active in other systems. The report on β-carotene provides a model for the types of information that must be gathered before proclaiming a substance to be a potential anticarcinogen and recommending its use.

The concepts of antimutagenicity and anticarcinogenicity are not simple and one-sided, and the reports of antimutagenicity and anticarcinogenicity, similar to those of mutagenicity or carcinogenicity, should be interpreted with caution. The understanding that many substances are not inherently mutagens or antimutagens (or carcinogens or anticarcinogens) may help define the issues and will also aid in designing laboratory experiments and epidemiology studies to determine the health effects of specific chemicals, dietary regimens, or lifestyles. The basic assumption over the years has been that any test system that can be used for the detection of mutagens is appropriate for the detection of antimutagens as well. Moreover, the active phytochemicals such as flavonoids, tannins, and anthocyanins

need to be carefully evaluated as both mutagenic and antimutagenic compounds. The search for nontoxic and broad-spectrum phytoantimutagens should be extended through systematic screening of the unexplored rich diversity of plants. Potential antimutagenic compounds should then be adequately evaluated before propounding their mechanism of action.

So far, the vast majority of antimutagenicity studies have been performed under *in vitro* conditions, in particular with bacterial indicators. Thus, it is advisable that the antimutagens identified in cost- and time-effective *in vitro* experiments with exogenous metabolic activation systems should be further evaluated in animal studies.

References

1 Mitscher, L.A., Drake, S., Gollapuri, S.R., Harris, J.A., Shankel, D.M. In *Antimutagenesis and Anticarcinogenesis Mechanisms* Shankel, D.M., Hartmen, P.E., Kada, T., Hollaender, A. (eds). Plenum Press, New York, **1986**, pp. 153–165.

2 Stavric, B. *Food Chem. Toxicol.* **1994**, *32*, 79–90.

3 Kada, T., Morita, K., Inoue, T. *Mutat. Res.* **1978**, *53*, 351–353.

4 Kada, T., Inoue, T., Namiki, M. Environmentaldes mutagens and antimutagens. In *Environmental Mutagenesis and Plant Biology*, Klekowski, E.J. (ed.). Praeger Scientific, New York, **1982**.

5 Kada, T., Inoue, T., Morita, K., Namiki, M. *Prog. Clin. Biol. Res.* **1986**, *20*, 6245–6253.

6 Wattenberg, L.W. *J. Natl Cancer Inst.* **1971**, *52*, 1583–1587.

7 Wattenberg, L.W. *Cancer Res.* **1981**, *41*, 2991–2994.

8 Wattenberg, L.W., Harley, A.B., Barany, G., Sparnins, V.L., Lam, L.K.T., Ferwi, G.R., Hayashi, Y. (ed.), *Diet Nutrition and Cancer*. Japan Science Society Press, Tokyo, **1986**, pp. 1993–2003.

9 Wattenberg, L.W. *Carcinogenesis* **1987**, *8*, 1971–1973.

10 Doll, R., Peto, R. *J. Natl Cancer Inst.* **1981**, *66*, 1191–1193.

11 Doll, R. *Cancer Res.* **1992**, *52*, 2024–2029.

12 Steinmetz, K.A., Potter, J. D. *J. Am. Diet. Assoc.* **1996**, *96*, 1027–1039.

13 Middelton, E., Kandaswami, C. Plant flavonoid modulation of immune and inflammatory cell functions. In: *Human Nutrition. A Comprehensive Treatise*, Vol. 8, *Nutrition and Immunology*. Klurfeld, D.M. (ed.). Plenum Press, New York, **1993**, pp. 239–266.

14 Wang, H., Murphy, P.A. *J. Agric. Food Chem.* **1994**, *42*, 1666–1673.

15 Lazze, M.C., Pizzala, R., Savio, M., Stivala, L.A., Prosperi, E., Branchi, L. *Mutat. Res.* **2003**, *535*, 103–115.

16 Marcucci, M.C. *Apidologie* **1995**, *26*, 83–99.

17 Burdock, G.A. *Food Chem. Toxicol.* **1995**, *36*, 347–363.

18 Banskota, A.R., Tezuka, Y., Adnyana, K., Midorikawa, K., Matsushige, K., Message, D., Huertas, A.A.G., Kadota, S. *J. Ethnopharmacol.* **2000**, *72*, 239–246.

19 Rao, C.V., Desai, D., Kaul, B., Amin, S., Reddy, B.S. *Chem. Biol. Interac.* **1992**, *84*, 277–290.

20 Frenkel, K., Wei, H., Bhimani, R., Ye, J., Zadunaisky, J.A., Huang, M.T., Ferraro, T., Conney, A.H. Grunberger, D. *Cancer Res.* **1993**, *53*, 1255–1261.

21 Matsuno, T., Chen, C., Basnet, P.V. *Med. Sci. Res.* **1997** *25*, 583–584.

22 Varanda, E.A., Monti, R., Tavares, D.C. *Teratog. Carcinog. Mutagen.* **1999**, *19*, 403–413.

23 Kimoto, T., Koya, S., Hino, K., Yamamoto, Y., Nomura, Y., Micallef, M.J., Hanaya, T., Arai, S., Ikeda, M., Kurimoto, M. *Pathol. Int.* **2000**, *50*, 679–689.

24 Chang, R. *Nutr. Rev.* **1996**, *54*, 591–593.

25 Chang, S.T., Hayes, W.A. *The Biology and Cultivation of Edible Mushrooms*. Academic Press, New York, **1980**.

26 Wuest, D.J., Royse, D.J., Beelman, R.B. *Cultivating Edible Fungi*. Elsevier, Amsterdam, **1987**.

27 Fullerton, S.A., Samadi, A.A., Tortorells, D.G., Choudhury, M.S., Mallouh, C., Tazaki, H., Konno, S. *Mol. Urol*. **2000**, *4*, 7–13.

28 Kidd, P.M. *Altern. Med. Rev*. **2000**, *5*, 4–27.

29 Lee, I.S., Nishizawa, A. *Life Sci*. **2003**, *73*, 3225–3234.

30 Pinheiro, F. Faria, R.R., de Camargo, Spinardi-Barbisan, A.L.T., Da Eira, A.F., Barbisan, L.F. *Food Chem. Toxicol*. **2003**, *41*, 1543–1550.

31 Sliva, D. *Int. Cancer Ther*. **2003**, *2*, 358–364.

32 Shon, Y.H., Nam, K.S. *J. Ethnopharmacol*. **2001**, *77*, 103–109.

33 Lakshmi, B., Ajith, T.A., Sheena, N., Gunapalan, N., Janardhanan, K.K. *Teratog. Carcinog. Mutagen*. **2003**, *1*, 85–97.

34 Luiz, R.C., Jordao, B.Q., da Eira, A.F., Ribeiro, L.R., Mantovani, M.S. *Mutat Res*. **2003**, *528*, 75–79.

35 De Lima, P.L.A., Delmanto, R.D., Sugui, M.M., da Eira, A.F., Salvadori, D.M.F., Speit, G., Ribeiro, L .R. *Mutat. Res*. **2001**, *496*, 23–32.

36 De Oliveira, J.M., Jord˜ ao, B.Q., Ribeiro, L.R., Da Eira, A.F., Mantovani, M.S. *Food Chem. Toxicol*. **2002**, *40*, 1775–1780.

37 Cho, J.H., Cho, S.D., Hu, H., Kim, S.H., Lee, S.K., Lee, Y.S., Kang, K.S. *Carcinogenesis*, **2002**, *23*, 1163–1169.

38 Kiho, T., Yoshida, I., Nagai, K., Ukai, S., Hara, C. *Carbohydr. Res*. **1989**, *189*, 273–279.

39 Fujimiya, Y., Suzuki, Y., Oshiman, K., Kobori, H., Moriguchi, K., Nakashima, H., Matumoto, Y., Takahara, S., Ebina, T., Katakura, R. *Cancer Immunol. Immunother*. **1998**, *46*, 147–159.

40 Takaku, T., Kimura, Y., Okuda, H. *J. Nutr*. **2001**, *131*, 1409–1413.

41 Kawagishi, H.R., Inagaki, T., Kanao, T., Mizuno, K., Shimura, H., Itos, T., Hagiwara, T., Nakamura.*Carbohydr. Res*. **1989**, *186*, 273–276.

42 Ito, H., Shimura, K., Itoh, H., Kawade, M. *Anticancer Res*. **1997**, *17*, 277–284.

43 Menoli, R.C.R.N., Mantovani, M.S., Ribeiro, L.R., Speit, G., Jordão, B.Q. *Mutat. Res*. **2001**, *496*, 5–13.

44 Delmanto, R.D., Alves de Lima, P.L., Sugui, M.M., Eira, A.F., Salvadori, D.M.F., Speit, G., Ribeiro, L.R. *Mutat. Res*. **2001**, *496*, 15–21.

45 Chiraha, G., Hamuro, J., Maeda,Y.Y., Arai,Y., Fukuoka, F. *Cancer Res*. **1970**, *30*, 2776–2781.

46 Nanba, H., Kuroda, M. *Chem.Pharm. Bull*. **1987** *35*, 2453–2458.

47 Hasegawa, J., Hosokawa, M., Okada, F., Kobayashi, H. *Mutat. Res*. **1989**, *226*, 9–12.

48 Ferguson, L.R. *Mutat. Res*. **1994**, *307*, 395–410.

49 Flora, S.D. *Mutat. Res*. **1998**, *402*, 151–158.

50 Obaseiki-Ebor, E., Emmanuel, E., Odukoya, K., Telikepalli, H., Mitscher, L.A., Shankel, D.M. *Mutat. Res*. **1993**, *302*, 109–117.

51 Higashimoto, M., Purintrapiban, J., Kataoka, K, Kinouchi, T., Vinitketkumnuen, U., Akimoto, S., Matsumoto, H., Ohnishi, Y. *Mutat. Res*. **1993**, *303*, 135–142.

52 Soudamini, K.K., Unnikrishnan, M.C., Sukumaran, K., Kuttan, R. *Indian J. Physiol. Pharmacol*. **1995**, *39*, 347–354.

53 Nakamura, Y.K., Atsuo, T., Shimoi, K., Namakura, Y. Tomita, I. *Biosci. Biotechnol. Biochem*. **1996**, *60*, 1439–1443.

54 Noriega-Ponce, P., Moriega-Ballon, L., Pedro, D., Miguel, A. *Cient. Cult. (Sao Paulo)* **1996**, *48*, 364–365.

55 Sangwan, S., Shanker, S., Sangwan, R.S., Kumar, S. *Phytother. Res*. **1998**, *12*, 389–399.

56 Imaida, K., Hirose, M., Shirai, T., Ito, H. *Proc. Am. Assoc. Cancer Res*. **1992**, *33*, 160.

57 Sasaki, Y.F., Chiba, A., Murakami, M. Sekihashi, K., Tanaka, M., Takahoko, M., Moribayashi, S., Kudou, C., Hara, Y., Nakazawa, Y., Nakamurab, N., Onizukab, S. *Mutat. Res*. **1996**, *371*, 203–214.

58 Yamad, J., Tomita, Y. *Biosci. Biotecnolh. Biochem*. **1996**, *60*, 328–329.

59 Yen, G.C., Chen, H.Y., Peng, H.H. *Food Chem. Toxicol*. **2001**, *39*, 1045–1053.

60 Yoshikawa, K., Inagaki, K., Terashita, T., Shishiyama, J., Kuo, S., Shankel, D.M. *Mutat. Res*. **1996**, *371*, 65–71.

61 Krizkova, L., Lopes, M.H., Polonyi, J., Belicova, A., Dobias, J., Ebringer, L. *Mutat. Res*. **1999**, *446*, 225–230.

62 Kaur, S. J., Grover, I. S., Kumar, S. *Food Chem. Toxicol.* **2000**, *38*, 1113–1119.
63 Shankel, D.M., Pillai, S.P., Telikepalli, H, Menon, S.R., Pillai, C.A., Mistcher, L.A. *Biofactor* **2000**, *12*, 113–121.
64 Nakahara, K., Kameyama, O.M., Ono, H., Yoshida, M., Trakoontivakorn, G. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2358–2360.
65 Nakahara, K., Trakoontivakorn, G., Alzoreky. N.S., Ono, N, Kameyama, O.M., Yoshida, M. *J. Agric. Food Chem.* **2002**, *50*, 4796–4802.
66 Sharma, N., Trikha, P., Athar, M., Raisuddin, S. *Mutat. Res.* **2001**, *480/481*, 179–188.
67 Ferrer, M., Lamar, S.A., Fuentes, L.J., Barbe, J., Llagostera, M. *Mutat. Res.* **2002**, 517, 251–254.
68 Miadokova, E., Masterova, I., Vlckova, V., Duhova, V., Toth, J. *J. Ethnopharmacol.* **2002**, *81*, 381–386.
69 Pasquini, R., Sforzdini, G.S., Villarini, M., Moretti, M., Marcarelli, M., Fatigoni, C., Kaur, S., Kumar, S., Grover, I.S. *J. Environ. Pathol. Toxicol. Oncol.* **2002**, *21*, 33–44.
70 Abdullaev, F. I., Negrete, R.L., Ortega, C. H., Hernandez, M.J., Lopez, P.I., Miranda, P.R., Aguirre, E.J. *J. Toxicol. In vitro*. **2003**, *17*, 731–736.
71 Kaur, S., Arora, S., Kaur, S., Kumar, S. *J. Environ. Pathol. Toxicol. Oncol.* **2003**, *22*, 69–76.
72 Shon, M.Y., Choi, S.D., Kahng, G.G., Nam, S.H., Sung, N.J. *Food Chem. Toxicol.* **2004**, *42*, 659–666.
73 Smith, H.S., Tate, P.L., Huang, G., Magee, J.B., Meepagala, K.M., Wedge, D.E., Larcom, L.L. *J. Med. Food*. **2004**, *7*, 450–455.
74 Kuttan, R., Kuttan, G., Joseph, S., Ajith, T.A., Mohan, M., Srimal, R.C. *J. Exp. Clin. Cancer Res.* **2004**, *23*, 61–68.
75 Kim, J.G. *J. Food Protec.* **2004**, *67*, 156–161.
76 Vargas, V.M., Motta, V.E., Leitao, A.C., Henriques, J.A. *Mutat. Res.* **1990**, *240*, 13–18.
77 Vargas, V.M., Guidobono, R.R., Henriques, J.A. *Mem. Inst. Oswaldo Cruz* **1991**, *86*, 67–70.
78 De Saferreira, I., Ferrao, C., Vargas, V.M. *Phytother. Res.* **1999**, *13*, 397–400.
79 Fernandes, J.B., Vargas, V.M. *Phyto. Res.* **2003**, *17*, 269–273.
80 Schimmer, O., Kruger, A., Paulini, H., Haefele, F. *Pharmazie* **1994** *49*, 448–451.
81 Rubiolo, P., Pieters, L., Calomme, M., Bicchi, C., Vlietinck, A., Vanden Berghe, D. *Mutat. Res.* **1992**, *281*, 143–147.
82 Paulini, H., Eilert, U., Schimmer, O. *Mutagenesis*, **1987**, *2*, 271–273.
83 Czeczot, H., Tudek, B., Kusztelak, J., Szymczyk, T., Dobrowolska, B., Glinkowska, G., Malinowski, J., Strzelecka, H. *Mutat. Res.* **1990**, *240*, 209–216.
84 Sohni, Y.R., Davis, C.L., DesChamps, A.B., Kale, P.G. *Environ. Mol. Mutagen* **1995**, *25*, 77–82.
85 Gomes-Carneiro, M.R., Felzenszwalb, I., Paumgartten, F.J. *Mutat. Res.* **1998**, *416*, 129–136.
86 Von Borstal, R.C., Higgins, J.A. *Mutat. Res.* **1998**, *402*, 321–329.
87 Waters, M.D., Stack, H.F., Jackson, M.A., Brockman, H.E., De Flora, S. *Mutat. Res.* **1996**, *350*, 109–129.
88 Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Meyskens, F.L., Valanis, B., Williams, J.H., Barnhart, S., Cherniack, M.G., Brodkin, C.A., Hammar, S. *J. Natl Cancer Inst.* **1996**, *88*, 1550–1559.
89 Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Meyskens, F.L., Valanis, B., Williams, J.H., Barnhart, S., Hammar, S. *N. Engl. J. Med.* **1996**, *234*, 1150–1155.
90 Fahrig, R. *Mutat. Res.* **1996**, *350*, 59–67.
91 Stich, H.F., Karim, J., Koropatrick, J., Lo, L. *Nature* **1976**, *260*, 722–724.
92 Galloway, S.M., Painter, R.B. *Mutat. Res.* **1979**, *60*, 321–327.
93 Boone C.W., Kelloff, G.J., Malone, W. E. *Cancer Res.* **1990**, *50*, 2–9.
94 Harborne, J.B., Baxter, H. *Phytochemical Dictionary: A Handbook of Bioactive Compounds from Plants*. Taylor & Francis, London, **1995**.
95 Choi, J.S., Park, K.Y., Moon, S.H., Rhee, S.H., Young, H.S. *Arch. Pharmacol. Res.* **1994**, *179*, 71–75.
96 Geetha, Malhotra, V., Chopra, K., Kaur, I.P. *Ind. J. Exp. Biol.* **2005**, *43*, 61–67.

97 Loarca-Pina, G., Kuzmicky, P.A., de Mejia, E.J., Koda, N.Y., Hsieh, Y.D.H. *Mutat. Res.* **1996**, *360*, 15–21.

98 Hour, T.C., Liang, Y.C., Chu, I.S., Lin, J.K. *Food Chem. Toxicol.* **1999**, *37*, 569–579.

99 Weisburger, J.H., Hara, Y., Dolan, L., Luo, F.Q., Pittman, B., Zang, E. *Mutat. Res.* **1996**, *371*, 5763.

100 Ohe, Takeshi., Marutani, K., Nakase, S. *Mutat. Res.* **2001**, *496*, 75–81.

101 Geetha, T., Garg, A., Chopra, K., Pal-Kaur, I. *Mutat Res.* **2004**, *556*, 65–74.

102 Wattenberg, L.W. *Cancer Res.* **1983**, *43*, 2448–2453.

103 Nakarugi, T., Komai, K. *Kinki Daiguku Nogakubu Kiyo* **1994**, *27*, 47–54.

104 Monache, F.D., Mac-Quhae, M.M. Monache, G.D., Bettolo, G.B.M., Delima, R.A. *Phytochemistry* **1983**, *22*, 227–232.

105 Conolly, J.D. McCrindle, R., Murray, R.D.H., Renfrew, A.J., Overton, K.H., Melera, A. *J. Chem. Soc.* **1966**, *7*, 268–274.

106 Patil, A.D., Freyer A.J. Webb R.L. et al. *Tetrahedron*, **1997**, *53*, 1583–1592.

107 Ishikawa, K., Naganawa, R. Oshida H. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 2086–2088.

108 Nguyen, V.C., Araki, Y., Jinnouchi, T. Murai, H. On the antimutagenicity mechanism of coffee. Paper presented at the 16th Colloque Scientifique International Sur Le Café, **1995**, pp. 88–93.

109 DeCleyn, A., Verzele, M. *Bull. Soc. Chim. Belgium*, **1975**, *84*, 435.

110 Lee, K.H., Kim, J.H., Lim, D.S., Kim, C.H. *J. Pharm. Pharmacol.* **2000** *52*, 593–598.

111 Gonzalez, A. G., Martin J. D., Martinez-Ripoli, M. Fayos, J. *Tetrahedron Lett.* **1979**, *20*, 2717–2718.

112 Hayder, N., Abdelwahed, A., Kilani, S., Ammar, R.B., Mahmoud, A., Ghedira, K., Chekir-Ghedira, L. *Mutat. Res.* **2004**, *564*, 89–95

113 Wang, C.K. Lee, W.H. *J. Agric. Food Chem.* **1996**, *44*, 2014–2019.

114 Miyazawa, M., Kohno, G. *Nat. Prod. Res.* **2005**, *19*, 26–36.

115 Lawsone, T., Nunnally, J., Walker, B., Bresnick, E., Wheeler, D., Wheeler M. *J. Agric. Food Chem.* **1989**, *37*, 1363–1367.

116 Roderick, D. Dixin G. *Environ. Mol. Mutagen* **1993**, *22*, 164–171.

117 Yamagishi, M., Natsume, M., Nagaki, A., Dachi, T., Osakabe, N., Takizawa, T., Kumon, H., Osawa, T. *J. Agric. Food Chem.* **2000**, *48*, 1574–1578.

118 Kuroda, Y. *Mutat. Res.* **1990**, *361*, 179–186.

119 Wang, Z.Y., Cheng, S.J., Zhou, Z.C., Athar, M., Khan, W.A., Bickers, D.R., Mukhtar, H. *Mutat. Res.* **1989**, *223*, 273–285.

120 Kada, T., Kaneko, K., Matsuzaki, S., Matsuzaki, T., Hara, Y. *Mutat. Res.* **1985**, *150*, 127–132.

121 De, A. K. Chilli and its therapeutic prospects. International Conference on Progress in Medicinal and Aromatic Plant Research, Calcutta, India, **1995**, pp. 105.

122 Pelkonen, O., Nebert, D.W. *Pharmacol. Rev.* **1982**, *37*, 189–222.

123 Lopez, R.M., Trevino, V.S., Alba, A.M., Lazarini, A.L., Mendiola, R.M., Castro, A.C., Fazenda, F.S., Garza de la M. *Toxicol In vitro* **2005**, *19*, 91–97.

124 Elson, C.E., Maltzman, T.H., Boston, J.L., Tabber, M.A., Gould, M.N. *Carcinogenesis* **1988**, *9*, 331–332.

125 Ruby, J.A., George, J., Dinesh Babu, K.V., Rajeshkharan, K.N., Kultan, R. *Amala Res. Bull.* **1995**, *15*, 57–61.

126 Nagabhushan, M., Amonkar, A.J., Bhide, S.V. *Food Chem. Toxicol.* **1987**, *25*, 545–547.

127 McConnell, O.J., Hughes, P.A., Targette, N.M. *Phytochemistry* **1986**, *2*, 2139–2141.

128 Wall, M.E. *J Nat. Prod.* **1992**, *55*, 1561.

129 Miyazawa, M., Shimamura, H., Nakamura, S.I., Kameoka, H. *J. Agric. Food Chem.* **1996**, *44*, 1647–1650.

130 Fu, N., Liu, Z., Zhang, R. *Zhongguo Yixue Kexueyuan Xuebao* **1995**, *17*, 349–352.

131 Chewonarin, T., Kinouchi, T., Kataoka, K., Arimochi, H., Kuwahara, T., Vinitketkumnuen, U., Ohnishi, Y. *Food Chem Toxicol.* **1999**, *37*, 591–601.

132 Wall, M.E. Wani, M.C., Manikumar, G., Taylor H., Hughes T.J. *J. Nat. Prod.* **1989**, *52*, 1092–1099.

133 Martinez, J.M.R., Pascual, A., Garcia, I., Martinez, L.M. *J. Antimicrob. Chemother.* **2003**, *52*, 703–706.

134 Cernakova, M., Kostalova, D., Kettmann, V., Plodova, M., Toth, J., Drimal, J. *BMC Complement Altern. Med.* **2002**, *2*, 2.

135 Samejima, K., Kanazawa, K., Ashida, H., Danno G. I. *J. Agric. Food Chem.* **1995**, *43*, 400–414.

136 Intiyot, Y., Kinouchi, T., Kataoka, K., Arimochi, H., Kuwahara, T., Vinitketkumnuen, U., Ohnishi, Y. *J. Med. Invest.* **2002**, *49*, 25–34.

137 Park, J.C., Ha, J.O., Park, K.Y. *Hoechi.* **1996**, *25*, 588–592.

138 Ikken, Y, Morales, O., Martinez, A., Martin, M.L., Haza, A.I., Cambero, M.I. *J. Agric. Food Chem.* **1999**, *47*, 3257–3264.

139 Vayalil, P.K. *J. Agric. Food Chem.* **2002**, *50*, 610–617.

140 Raphael, K.R., Ajith, T.A., Joseph, S., Kuttan, R. *Teratog. Carcinog. Mutag.* **2002**, *22*, 285–291.

141 Wang, C.K. Wu, M. J. *Zhongguo Nongye Huaxue Huizhi*, **1996**, *34*, 638–647.

142 Wall, M.E., Wani, M.C., Hughes, T.J., Taylor, H. *J. Nat. Prod.* **1988**, *51*, 866–873.

143 ManiKumar, G., Gaetano, K., Wani, M.C. *et al. J. Nat. Prod.* **1989**, *52*, 769.

144 Sun M, Sakakibara, H, Ashida, H., Donno, G., Kanazawa, K. *Biosci. Biochem. Biotechnol.* **2000**, *64*, 1373–1378.

145 Avila, G.M., Alba, A.M., Garzade, Lam., Pretelin, del. C.H.M., Ortiz, D.M.A., Fazenda, F.S., Trevino, V.S. *Toxicology In vitro.* **2003**, *17*, 77–83.

146 Park, K.Y., Jung, G.O., Lee, K.T., Choi, J., Choi, M.Y., Kim, G.T., Jung, H.J., Park H.J. *J. Ethnopharmacol.* **2004**, *90*, 73–79.

147 Sandnes, D., Johansen, T., Teien, G., Ulsaker, G. *Pharmacol. Toxicol.* **1992**, *71*, 165–172.

148 Toering, S.J., Gentile, G.J., Gentile J.M. *Mutat. Res.* **1996**, *361*, 81–87.

149 Nagabhushan, M., Bhide, S.V. *Mutagenesis*, **1988**, *3*, 293–296.

150 Berhow, M.A., Wagner, E.D., Vaughn, S.F., Plewa, M.J. *Mutat. Res.* **2000**, *448*, 11–22.

151 Ko, T. F., Weng, Y.M., Lin, S.B., Chiou, R.Y. *J. Agric. Food Chem.* **2003**, *51*, 3564–3567.

152 Kaur, K., Arora, S., Kumar, S., Nagpal, A. *Food Chem. Toxicol.* **2002**, *40*, 1475–1482.

153 Cassady J.M. *J. Nat. Prod.* **1990**, *53*, 23–41.

154 Miyazawa, M., Shimamura, H., Nakamura, S.I., Kameoka, H. *J. Agric. Food Chem.* **1995**, *43*, 3012–3015.

155 Uenebo, F., Nakamura, S.-I., Miyazawa, M. *Mutat. Res.* **1997**, *373*, 197–200.

156 Zhang, X.B., Otha, Y. *Mutat. Res.* **1993**, *300*, 301–306.

157 Ames, B.N., McCann, J., Yamasaki, E. *Mutat. Res.* **1975**, *31*, 347–364.

158 Maron, D.M., Ames, B.N. *Mutat. Res.* **1983**, *113*, 173–215.

159 Podgorska, B., Chec, E., Ulanowska Wegrzyn, G. *J. App. Gen.* **2005**, *46*, 241–246.

160 Mitscher, L.A., Telikepalli, H., McGhee, E. Shankel, D.M. *Mutat. Res.* **1996**, *350*, 143–152.

161 Doehmer, J., Seidel, A., Glatt, H.R., Molders, H.P., Oesch, F. *Mol. Pharmacol.* **1990**, *37*, 608–612.

162 Crespi, C.L. In: Philips, D.H., Venitt, S. (eds). *Use of Genetically Engineered Cells for Genetic Toxicology Testing.* Bio Press, Eynsham, **1995**, pp. 233–248.

163 Eckl, P.M., Raffelsberger, I. *Mutat. Res.* **1997**, *392*, 117–120.

164 Knasmuller, S., Zeilmaker, M., Kassie, F., Schulte-Hermann, R., Mohn, G.R. *Environ. Mutagen. Carcinog.* **1998**, *3*, 18–32.

165 Knasmüller, S., Parzefall, W., Sanyal, R., Ecker, S., Schwab, C., Uhl, M., Mersch-Sundermann, V., Williamson, G., Hietsch, G., Langer, Darroudi, F., Natarajan, A.T. *Mutat. Res.* **1998**, *402*, 185–202.

166 Laky, B., Knasmüller, S., Gminski, R., Mersch-Sundermann, V., Scharf, G., Freywald, C., Lutz, W.K., Schlatter. *J. Carcinog.* **1992**, *13*, 2211–2216.

167 Natarajan, A.T., Darroudi, F. *Mutagenesis* **1991**, *6*, 399–403.

168 Uhl, M., Helma, C., Knasmüller, S. *Mutat. Res.* **1999**, *441*, 215–224.

169 Uhl, M., Helma, C., Knasmüller, S. *Mutat. Res.* **2000**, *468*, 213–225.

170 Heddle, J.A., Hite, M., Kirkart, B., Mavournin, K., Macgregor, J.T., Newell, G.W., Salamone, M.F. *Mutat. Res.* **1983**, *123*, 61–118.

171 IARC *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.*Vol. 56. *Some Naturally Occurring Substances: Food Items, and Constituents, Heterocyclic Aromatic Amines and Mycotoxins.* International Agency for Research on Cancer, Lyon, **1993**.

172 IARC. In: *The Relevance of N-Nitroso Compounds to Human Cancer. Exposure and Mechanisms*. Bartsch, H., O'Neill, I., Schulte-Hermann, R. (eds). IARC Scientific Publications no. 84. International Agency for Research on Cancer, Lyon, **1987**.

173 Gossen, J.A., de Leeuw, W.J., Tan, C.H., Zwarthoff, E.C., Berends, F., Lohman, P.H., Knook, D.L., Vijg, J. *Proc. Natl Acad. Sci. USA* **1989**, *86*, 7971–7975.

174 Lambert, I.B., Singer, T.M., Boucher, S.E., Douglas, G.R. *Mutat. Res.* **2005**, *590*, 1.280.

175 Schwab, C.E., Huber, W.W., Parzefall, W., Hietsch, G., Kassie, F., Schulte-Hermann, R., Knasmüller, S. *Crit. Rev. Toxicol.* **2000** *301*, 69.

176 OECD, *Guidelines for Testing of Chemicals*. OECD, **1997**, 471–486.

177 Wiebel, F.G., Schwarz, L.R., Goto, T. In short term test systems for detecting carcinogens. Norpoth, K.H., Garner, R.C. (eds). Springer Verlag, Berlin, **1980**, pp. 209–218.

178 Stoner, G.D., Morse, M.A., Kellho, G.J. *Environ. Health Perspect.* **1977**, *105*, 945–954.

179 DeFlora, S., Ramel, S.C. *Mutat. Res.* **1988**, *2*, 285–306.

14
Potential of Plant-Derived Products in the Treatment of Mycobacterial Infections

Deepa Bisht, Mohammad Owais, and Krishnamurthy Venkatesan

Summary

Emerging and re-emerging infections and the spread of drug-resistant strains of microorganisms are posing a challenge to global public health in terms of treatment. The solution perhaps lies in the indigenous systems of medicine and plant-based drugs, which could provide a concept of therapy and therapeutic agents to complement modern medicine in the management of communicable diseases such as tuberculosis (TB) and leprosy. Medicinal plant products may also prove useful in reducing or minimizing the adverse effects of various chemotherapeutic agents already in use for these diseases.

India has a rich heritage of using medicinal plants in traditional medicines such as the Ayurveda, Siddha, and Unani systems, besides folklore practises. Many plants have been successfully used in the treatment of various diseases. There is a need to develop second-line therapeutic agents, both natural and synthetic, in view of the twin problems of resistance and persistence. Such chemotherapeutic agents may have antimycobacterial potential or may function as immunomodulators, thereby enhancing the immune status of the affected host, enabling it to combat the disease better. Chaulmoogra oil was used for the treatment of leprosy long before the introduction of modern chemotherapy. Levamisole has been used as an immunomodulator in leprosy. Allicin, tuberosin, tryptanthrisis, various crude plant extracts, etc. have shown antimycobacterial activity against *Mycobacterium tuberculosis*.

In this chapter, various plant-derived products that have been tested for the treatment of mycobacterial infections will be discussed.

14.1
Introduction

The genus *Mycobacterium* is responsible for more misery and suffering than any other genus of bacteria. Mycobacteria are Gram-positive, nonmotile, aerobic, rod-

shaped, saprophytic, or parasitic organisms that belong to the order Actinomycetales, family *Mycobacteriaceae*. *Mycobacterium tuberculosis*, *M. leprae*, *M. bovis*, *M. africanum*, *M. microti*, and *M. avium* are important intracellular pathogens of higher vertebrates, and infection can lead to death in animals and humans [1]. Tuberculosis remains a major public health problem, both in developing countries and in many industrialized countries, with 8–10 million new cases and 2 million deaths yearly in the world. It is estimated that one-third of the world's population is latently infected with *M. tuberculosis* [2]. The situation has been further worsened due to emergence of multidrug resistant strains and the AIDS pandemic. Protection with bacillus Calmette–Guérin (BCG), the only vaccine available, has been disappointing, as it has shown a wide range of protection from 0 to 80% in trials carried out around the world [3].

Leprosy (also called Hansen's disease) is an infectious disease caused by *M. leprae* that usually affects the skin, peripheral nervous system, and some other organs. It is a disease of great antiquity, having been recognized from Vedic times in India and from Biblical times in the Middle East. It has acquired a distinct position among communicable diseases because of long duration of illness, frequency of impairment, deformities, disabilities, and socioeconomic consequences. The implementation of multidrug treatment resulted in a significant decrease in the number of leprosy cases in the world to one million, but there has not been any change in the incidence since, indicating that the transmission is still going on in the population. At the beginning of 2005, the global registered prevalence of leprosy was 286 063 cases and the number of new cases detected during 2004 was 407 791 [4].

Mycobacterium avium complex and *M. kansasii* (usually associated with pneumonia or disseminated infection) are the leading causes of nontuberculous mycobacterial infections in humans. Other causes include *M. malmoense*, *M. simiae*, *M. szulgai*, and *M. xenopi* (associated with pneumonia); *M. scrofulaceum* (associated with lymphadenitis); *M. abscessus*, *M. chelonae*, *M. haemophilum*, and *M. ulcerans* (associated with skin and soft tissue infections). In some areas of the tropics, Buruli ulcer disease caused by infection with *M. ulcerans* is a common cause of severe morbidity and disability.

14.2 Current Therapy of Tuberculosis and Leprosy

The drugs that have been used to fight TB include isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin, p-aminosalicylic acid, ethionamide, cycloserine, rifabutin, aminoglycosides, ciprofloxacin, and ofloxacin, amithiozone, capreomycin, kanamycin, and thioacetazone. However, the important first-line anti-TB drugs are streptomycin, isoniazid, rifampicin, ethambutol, and pyrazinamide [5]. For leprosy, the World Health Organization (WHO) advocates multidrug treatment comprising of rifampicin, clofazimine and dapsone [6, 7], while other drugs such as ofloxacin, clarythromycin, and minocycline have been tried by isolated groups as additional agents [8]. Thalidomide and levamisole are two immunomo-

dulatory drugs, of which thalidomide is administered to treat the leprosy reaction and levamisole to make chemotherapy more effective [9].

14.3 Need for Newer Antimycobacterial Drugs

The present recommended treatment regimen is highly effective and rates of severe adverse reactions are low. However, unpleasant side-effects and a relatively long course of treatment are the drawbacks that increase the rate of noncompliance to treatment regimen. Such nonadherence with the course of treatment leads to treatment failure and the development of drug resistance. The second-line drugs used for multidrug-resistant TB are more expensive, less effective, and more toxic than the four-drug standard regimen. This has led to increased pressure on current chemotherapy regimes and necessitated the need to look into new therapeutic and prophylactic measures. Efforts are being made all over the world to explore the potential of natural products as antimycobacterial drugs.

A suitable drug would need to be cost effective, have low side-effects, and have favorable pharmacokinetic properties. Considering the seriousness of the diseases, the cost and side-effects of the available drugs, several attempts have been made to discover antimycobacterial drugs from natural products. There is an increasing interest in natural products, including plant extracts, as potential therapeutic agents as evidenced by the extensive reviews on this topic [10–12].

14.4 Plant Extracts

Plants have been used as medicines since time immemorial. Herbal medicines form an integral part of healing practiced by the traditional healers. India has a rich heritage of using medicinal plants in traditional medicines such as the Ayurveda, Siddha, and Unani systems, besides folklore practises. The earliest mention of the medicinal uses of plants is found in the Rigveda, which is one of the oldest repositories of human knowledge [13]. Fairly comprehensive information on the curative properties of some herbs has been recorded in "Charaka Samhita" and "Sushrutha Samhita." The plant kingdom is a virtual goldmine of biologically active compounds and it is estimated that only 10–15% of 250 000–750 000 of existing species of higher plants have been surveyed. Many plants have been successfully used in the treatment of various diseases. The list of natural products having therapeutic value is ever growing and a plethora of new compounds are being isolated every day.

In the late nineteenth century, the main treatment for leprosy was chaulmoogra, extracted from *Hydnocarpus* seeds. Chaulmoogra was a traditional treatment for skin diseases in Ayurvedic and Chinese medicine and, although once used as the treatment for leprosy worldwide, is now nearly forgotten [14]. Gotu kola (*Centella asiatica*) is an important herb in Ayurvedic medicine, often mentioned in combina-

tion with the related European marsh pennywort (*Hydrocotyle vulgaris*). About 20 species related to gotu kola grow in most parts of the tropic or wet pantropical areas such as rice paddies, and also in rocky, higher elevations. A perennial plant, goto kola is known by many names, including Indian pennywort, Brahmi, Chihsueh, Ts'ao, and Talepetraco. In India, where it is known as Brahmi (bringing knowledge of Brahman), it is widely used as a blood purifier as well as for treating a variety of other illnesses. In Ayurveda, Brahmi is one of the chief herbs for revitalizing the nerves and brain cells.

In Western medicine during the middle of the twentieth century, gotu kola and its alcoholic extract showed positive results in the treatment of leprosy [15]. The plant and its extract contain asiaticoside – an active principle of *C. asiatica*, in which a trisaccharide moiety is linked to the aglycone asiatic acid.

Twenty South African medicinal plants used to treat pulmonary diseases were screened for activity against drug-resistant and drug-sensitive strains of *M. tuberculosis*. A preliminary screening of acetone and water extracts of the plant against a drug-sensitive strain of *M. tuberculosis* $H_{37}R_v$ was done by the agar plate method. Fourteen of the 20 acetone extracts showed inhibitory activity at a concentration of 0.5 mg mL^{-1} against this strain. Acetone as well as water extracts of *Cryptocarya latifolia*, *Euclea natalensis*, *Helichrysum malanacme*, *Nidorella anomala* and *Thymus vulgaris* inhibited the growth of *M. tuberculosis*. Given the activity of 14 acetone extracts at 0.5 mg mL^{-1} against the drug-sensitive strain by the agar plate method, a further study was done employing a rapid radiometric method to confirm the inhibitory activity. These active acetone extracts were screened against the $H_{37}R_v$ strain as well as a strain resistant to the drugs isoniazid (INH) and rifampicin (RMP). The minimum inhibitory concentration (MIC) of *Croton pseudopulchellus*, *Ekebergia capensis*, *Euclea natalensis*, *N. anomala* and *P. myrtifolia* was 0.1 mg mL^{-1} against the *M. tuberculosis* H_{37} R_v strain by the radiometric method. Extracts of *Chenopodium ambrosioides*, *E. capensis*, *E. natalensis*, *H. melanacme*, *N. anomala* and *P. mytrifolia* were active against the resistant strain at 0.1 mg mL^{-1}. Eight plants showed activity against both strains at a concentration of 1.0 mg mL^{-1} [16].

The activity of an ethanolic extract of *Galipea officinalis* bark against *M. tuberculosis* was shown to reside mainly in the basic alkaloidal fraction, although the major part of the alkaloids present were in the neutral fraction. Six alkaloids were isolated from the bark, including two other alkaloids not previously reported from *G. officinalis* and a new quinoline named allocuspareine. Isolation and testing of fractions and individual alkaloids against 10 strains of *M. tuberculosis* showed that all the alkaloids possessed some activity but that the unidentified most polar basic fraction exhibited the greatest effect [17]. The aqueous and organic extracts of *Withania somnifera*, *Euphorbia pilufifera*, *Azadirachta indica*, *Emblica officinalis*, *Ocinum sanctum* and *Allium sativum* have bactericidal activity against *M. tuberculosis in vitro* [18].

Crude chloroform extract of the plant *Physalis angulata* and physalin-containing fractions displayed antimycobacterial activity against *M. tuberculosis*, *M. avium*, *M. kansasii*, *M. malmoense*, and *M. intracellulare*, through bioassay-guided fractionation by *in vitro* determination of MIC using the microdilution method with Alamar blue oxidation-reduction dye [19]. In a study in which aqueous extracts of plants

A. indica, *Zingiber officinalis*, *O. sanctum*, and *A. sativum* were added to sputum of tuberculosis patients and organisms were isolated on LJ (Lowenstein Jensen) slopes, it was found that all the plants extracts inhibited or arrested the growth of *M. tuberculosis* [20]. The dichloromethane extract of *Amorphophallus bequaertii* inhibited the growth of *M. tuberculosis* with an MIC of 100 μg mL^{-1} [21].

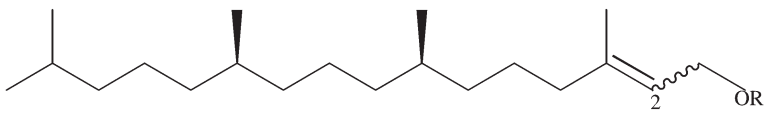
A new preussomerin isomer, 3′-*O*-demethyl-preussomerin-I, five known preussomerins, and two known deoxypreussomerins – deoxypreussomerin A and bipendensin (palmarumycin) – were isolated from a lichenicolous fungus *Microphaeropsis* sp. BCC 3050 which possessed a good antimycobacterial potency [22].

14.5
Well-Characterized Plant-Derived Compounds

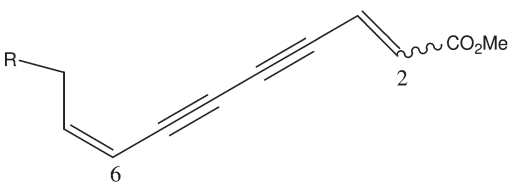
Effort has been made cover various plant-derived secondary metabolites that have been evaluated for their antimycobacterial activity using several methodologies, including classical disk diffusion and broth (micro) dilution assay format, to radiorespirometric (BACTEC), and fluorescent/luminescence/reporter assays. Structures are presented only for few structures. This chapter gives a compilation of available data on antimycobacterial plant products studied prior to mid-2005 and structures are presented for only a few selective compounds. Although the compilation of data on various phytic products has been presented under selective groups/classes of compounds, the compounds cited may overlap classwise.

14.5.1
Alkanes, Alkenes, Alkynes, Fatty Acids and their Esters and Simple Aromatics

Investigation of a methanol extract of the Kenyan shrub *Leucas volkensii* afforded (*E*)-phytol (**1**) (Fig. 14.1) [23] as the principal antimycobacterial constituent, with an MIC of 2 μg mL^{-1}. The simple di-α,β-unsaturated ketone, yoshixol, isolated from



1 E-Phytol, R= H, (2E)



2 and **3** Matricaria esters **2** R= H, (2Z) **3** R= H, (2E)

Fig. 14.1 Structures of (*E*)-phytol and two matricaria esters.

wood oil of he Japanese tree *Chamacyparis obtusa* exhibited antibiotic activity towards a range of organisms including *M. chelonei* at unspecified potency [24]. Two matricaria esters (**2** and **3**) were isolated from the roots of *Chrysoma pauciflosculosa*, three C-10 *O*-acylated derivatives and a triyne from *Solidago canadensis*, and two known matricaria lactones from *Erigeron philadelphicus*. These eight components exhibited MIC values of 50, 50, >100, 25, >50, 25, 12.5, and 50 μg mL^{-1} respectively against *M. tuberculosis* $H_{37}R_v$ on radiometric bioassay.

Similar trends were seen towards *M. avium* in this study. The results indicate the usefulness of the angelate group for ester potency, and the importance of the double bond geometry in the case of matricaria lactones. It was also speculated by the authors that the overall lipophilicity of the natural product was an important determination of potency and that the α,β-unsaturated δ-lactone moiety in the two compounds isolated from *Erigeron philadelphicus* may act as a Michael acceptor, leading to irreversible inhibition of mycobacterial enzymes.

Some species of lichen are reputed to be effective in the treatment of pulmonary tuberculosis [25]. The polyacetylene carboxylic acids 13,14-dihydrooropheic acid and oropheic acid were isolated from the stem bark of *Mitrephora celebica*, a plant native to North Sulawesi [26]. Both these compounds inhibited the growth of *M. smegmatis* with an MIC of 12.5 μg mL^{-1}. A range of diynes, including the known antibiotics falcarinol and falcaridiol and two more new analogs were isolated in a bioassay-directed fractionation of the extracts of the inner bark and roots of *Oplopanax horridus* (devil's club), which are used by the First Nations peoples of North America for a number ailments including tuberculosis. All these compounds inhibited the growth of *M. tuberculosis* and *M. avium* at 10 μg per disk loading in a disk diffusion assay [27].

Bioassay-guided chromatographic separation of the antimycobacterial extract of the leaves of *Piper sanctum* afforded 14 new compounds while the anti-TB stem extract of the plant afforded 10 additional known compounds. Of the 24 compounds, 3′,4′-methylenedioxyphenyl) tetradecane, 2-oxo-16-(3′,4′-methylene-dioxyphenyl)-hexadecane, 2-oxo-16-(3′,4′-methylenedioxyphenyl)-*trans*-15-hexadecene, demethoxyyangonin, 5,6-dehydro-7,8-dihydromethysticin and cepharanone B, piperolactam A inhibited the growth of *M. tuberculosis* when tested by the microplate alamar blue assay (MABA) assay, with MIC values ranging from 4 to 64 μg mL^{-1}. GC-MS and HPLC analyses of the essential oils of the leaves and stem revealed that safrol was the major component of the oils [28].

Diospyrin, a binaphthoquinoid compound, was isolated from *Euclea natalensis*, and evaluated for its activity against drug-sensitive and drug-resistant strains of *M. tuberculosis*. The MIC of diospyrin was found to be 100 μg mL^{-1} for all the *M. tuberculosis* strains [29].

Dried roots extracts of *Pelargonium reniforme* and *P. sidoides* (Geraniaceae family) have been examined for antibacterial activity against rapidly growing mycobacteria. Active mixtures varied in the relative abundance of their components, although major constituents like palmitic, oleic, and linoleic acids were found in all. When tested against rapidly growing mycobacteria (*M. aurum*, *M. smegmatis*, *M. fortutium*, *M. abscessus*, and *M. phlei*), unsaturated compounds exhibited antimyco-

bacterial activity depending on degree of unsaturation, chain length, and the bacterial species tested, whereas saturated compounds except 12:0 were found to lack any antimycobacterial activity. Linoleic acid, with an MIC of 2 μg mL^{-1} against *M. aurum*, was found to be the most novel compound [30].

Three phorbol esters, 12-(2-*N*-methylaminobenzoyl)-4β,5,20-trideoxyphorobol-13-acetate, 12-(2-*N*-methylaminobenzoyl)-4α,5,20-trideoxyphorobol-13-acetate, 12-(2-*N*-methylaminobenzoyl)-4α,20-dideoxy-5-hydroxyphorobol-13-acetate, along with six other known compounds were isolated from the fruits of *Sapium indicum*. The first two compounds showed antimycobacterial activity with an MIC between 3.12 and 200 μg mL^{-1}, whereas the third compound was inactive (MIC >200 μg mL^{-1}). Some compounds did not exhibit any activity [31].

14.5.2 Alkaloids

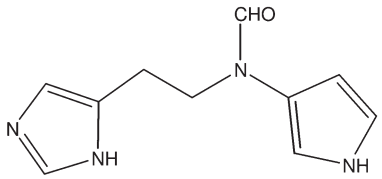
The pyrrole alkaloid solsodomine A (**4**) was isolated from the plant *Solanum sodomaeum*, collected in Libya (Fig. 14.2). The alkaloid inhibited the growth of *M. intracellulare* with an MIC of 10 μg mL^{-1} [32]. Vasicine (**5**), isolated from the Indian shrub *Adhatoda vasica* and related semi-synthetic derivatives bromhexine (**6**) and ambroxol (**7**) were found to inhibit growth of *M. tuberculosis* [33]. Bromhexine and ambroxol, widely used as mucolytics, have a pH-dependent growth-inhibitory effect on *M. tuberculosis*. As these compounds are concentrated in macrophages, they might exert a clinically useful effect on intracellular tubercle bacilli. This, combined with indirect effects including enhancement of lysozyme levels in bronchial secretions and levels of rifampicin in lung tissue and sputum, and possibly clearance of bacilli-laden mucus from cavities and bronchi, suggests a potentially useful adjunctive function for these agents in the therapy of tuberculosis.

Investigation of the ethanolic extract of angostura bark, derived from *Galipea officinalis*, afforded more antimycobacterial alkaloids (MIC of 6.25–50 μg mL^{-1} towards *M. tuberculosis*) [17]. Two structurally related compounds, azaanthraquinone (**8**) isolated from the plant *Mitracarpus scaber* [34] and cleistopholine (**9**) originally isolated from the plant *Cleistopholis patens* [35], inhibited the growth of *M. intracellulare* with MIC values of 6.25 and 12.5 μg mL^{-1} respectively. Tryptanthrin (**10**), an indoloquinazolinone alkaloid isolated from the Chinese medicinal plant *Strobilanthes cusia*, exhibited growth inhibition of *M. tuberculosis*, *M. avium* complex, and *M. smegmatis* with MIC values of 1, 4, and 6 μg μL^{-1}, respectively [36, 37]. Cryptolepine (**11**), neocryptolepine (**12**), and the dimer biscryptolepine (**13**), indoloquinoline alkaloids isolated from the African climbing liana *Cryptolapis sanguinolenta*, inhibited the growth of *M. fortuitum* (MIC 25, 31, and 6.25 μg mL^{-1}, respectively [38]). The tetracyclic oxazole alkaloid texalin (**14**), isolated from *Amyris elemifera*, inhibited *M. tuberculosis*, *M. avium*, and *M. kansasii* with an MIC of 25 μg mL^{-1} [39].

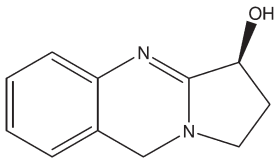
The antimycobacterial activity of a well-known antimicrobial alkaloid berberine (**15**) has been reported in several studies. The alkaloid inhibited the growth of *M. intracellulare* with an MIC of 0.78–1.56 μg mL^{-1} [40] and *M. smegmatis* and *M. tuberculosis* with an MIC of 25 μg mL^{-1}. The methylenedioxybenzene-containing al-

kaloid chabamide, isolated from the stems of the *Piper chaba*, exhibited an MIC of 12.5 μg mL^{-1} towards *M. tuberculosis* $H_{37}R_a$ [41].

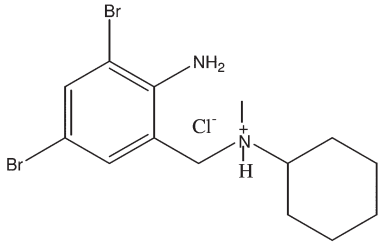
Anti-TB bioassay-directed fractionation of CH_2Cl_2 extract of the bark of *Micromelum hirsutum* led to the isolation of six carbazole alkaloids as well as a γ-lactone derivative of oleic acid. The carbazoles included the new alkaloid micromeline and five known alkaloids, lansine, 3-methylcarbazole, methylcarbazole-3-carboxylate,



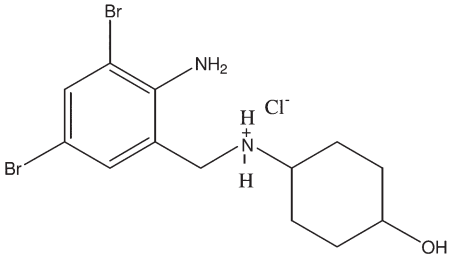
4 Solsodomine A



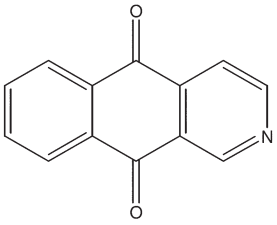
5 Vasicine



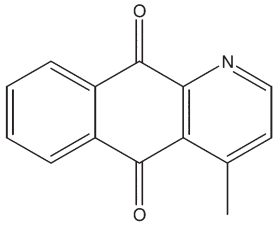
6 Bromhexine



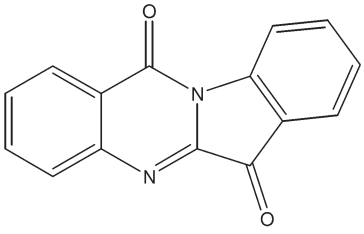
7 Ambroxol



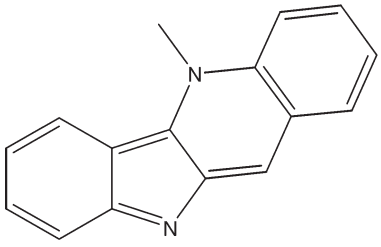
8 Azaanthraquinone



9 Cleistopholine



10 Tryptanthrin

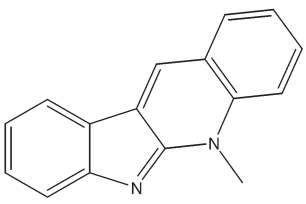


11 Cryptolepine

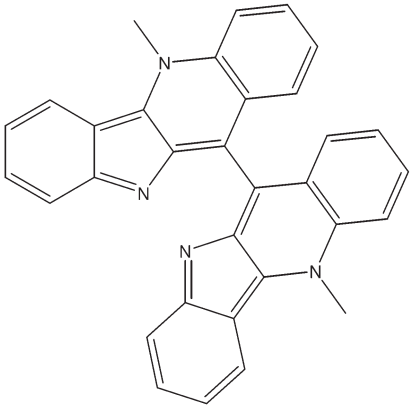
Fig. 14.2 Structures of some alkaloids. (Part 1)

3-formylcarbazole, and 3-formyl-6-methoxy carbazole. The first component, micromeline, was identified as the γ-lactone derivative of oleic acid, (–)-2,9-octadecene-4-olide, for which the trivial name micromolide is suggested. It showed potent *in vitro* anti-TB activity against $H_{37}R_v$ (MIC of 1.5 μg mL^{-1}), selectivity index (SI) of 63 and exhibited activity against the Erdman strain of *M. tuberculosis* in a J774 mouse macrophage model (EC_{90} 5.6 μg mL^{-1}). These findings suggest that further evaluation of the γ-lactone derivative of oleic acid, (–)-2,9-octadecene-4-olide, as a potential new anti-TB agent should be carried out [42].

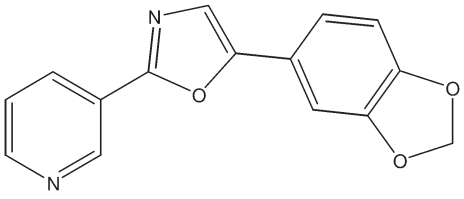
Carbazole alkaloids possessing an aldehyde group at the C3 position exhibit significantly greater activity than those lacking this functionality. Kanokmedhakul et al. [43] reported the isolation of a compound, chaetomanone (anthraquinone-chromanone), along with seven known compounds, ergosterol, ergosteryl palmitate, chrysophanol, chaetoglobosin C, alternariol monomethyl ether, echinuline, and isochaetoglobosin D, from a fungus called *Chaetomium globosum* KMITL-N0802. It was found that chaetomanone and echinuline showed activity towards *M. tuberculosis*. An antimycobacterial coumarin (6-geranyl-7-hydroxycoumarin, os-



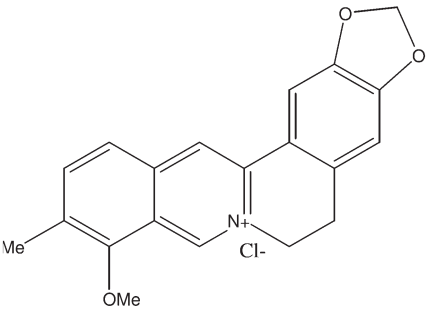
12 Neocryptolepine



13 Biscryptolepine



14 Texalin



15 Berberine

Fig. 14.2 Structures of some alkaloids. (Part 2)

truthin), isolated from the roots of *Peucedanum ostruthium* Koch (Apiaceae) following a bioassay-guided fractionation showed pronounced activity against several species of rapidly growing mycobacteria, namely *M. abscessus*, *M. aurum*, *M. fortuitum*, *M. phlei*, and *M. smegmatis*, with MIC values ranging from 3.4 to 107.4 μmol L^{-1} and were comparable to those of ethambutol and isoniazid [44]. However, the other component, imperatorin (8-isopent-2-eneloxy-6,7-furanocoumarin) showed no activity at concentrations up to 1.9 mmol L^{-1} and umbelliferone (7-hydroxycoumarin) was only weakly active with an MIC of 0.79 mmol L^{-1}.

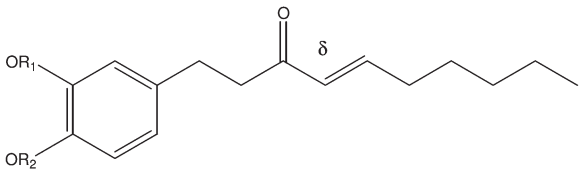
The aerial parts of *Ducrosia anethifolia* afforded the monoterpene glucoside 8-debenzoylpaeoniflorin and the prenylated furanocoumarin pangelin [5-[2″(*R*)-hydroxy-3″-methyl-3″-butenyloxy]furocoumarin]. Their structures were determined by extensive one- and two-dimensional NMR studies. The latter compound demonstrated activity against a panel of fast-growing mycobacteria, namely *M. fortuitum*, *M. aurum*, *M. phlei*, and *M. smegmatis* and MIC values ranged from 64 to 128 μg mL^{-1} [45].

Crude methanolic extracts from three of the plants, *Psoralea corylifolia*, *Sanguinaria canadensis*, and *Commiphora mukul* were found to have significant antimycobacterial activity against *M. avium* only (MIC of 62.5 μg mL^{-1}). Bioassay-guided fractionation led to the isolation of two known benzophenanthridine alkaloids, sanguinarine and chelerythrine, from the roots of *S. canadensis* and the known phenolic meroterpene bakuchiol from the seeds of *P. corylifolia*. The compound chelerythrine was most active against *M. avium* and *M. smegmatis*, with MIC values of 7.30 μg mL^{-1} (19.02 μmol L^{-1}) and 29.0 μg mL^{-1} (75.56 μmol L^{-1}) respectively. These results support the use of these plants in traditional medicine [46].

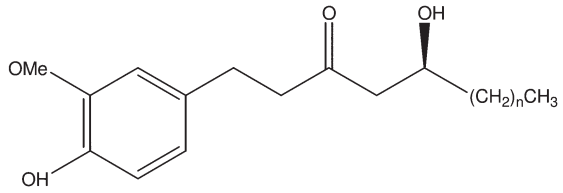
14.5.3
Phenolics and Acetogenic Quinones

The seeds of *Aframomum melegueta* K. Schum. (Zingiberaceae) yielded 6-paradol (**16**) and 6-shogaol (**17**) as the major antimycobacterium agents, based on a bioactivity-guided fractionation (Fig. 14.3). These isolates were found to be active against *M. chelonei*, *M. intracellulare*, *M. smegmatis*, and *M. xenopi* (MIC 10–15 μg mL^{-1}). The desmethyl derivative of 6-paradol retained the antimycobacterial activity and was found to be more active against *Candida albicans* than 6-paradol and 6-shogaol [47].

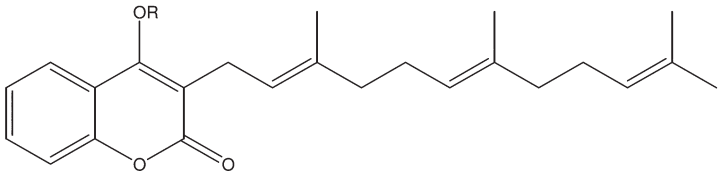
Investigations on ginger rhizome (*Zingiber officinale*) afforded three gingerol analogs, 6-gingerol, 8-gingerol, and 10-gingerol. The lipophilic analogs 8-gingerol (**18**) and 10-gingerol (**19**) were more active, with MIC values of 25–50 μg mL^{-1} towards *M. tuberculosis* $H_{37}R_v$ [48]. Bakuchiol, a lipophilic phenol isolated from the seeds of *Psoralea corylifolia*, inhibited the growth of *M. aurum* (MIC 15.8 μg mL^{-1}) and *M. bovis* BCG (MIC 21.4 μg mL^{-1}) but was found to be inactive towards *M. smegmatis* (MIC >500 μg mL^{-1}) [46]. Out of number of homologs of 2-(4′–hydroxyphenyl) ethanol isolated from the stem bark extract of *Buddleia cordata* subsp. *cordata* the long-chain fatty ester of 2-(4′-hydroxyphenyl) ethanol only exhibited antimycobacterial activity in a radiorespirometric assay against *M. tuberculosis* $H_{37}R_v$



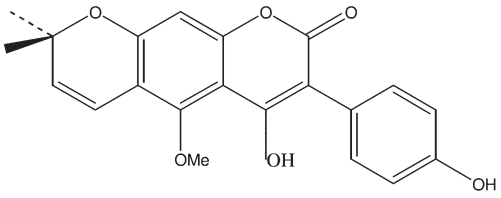
16 6-paradol R_1= methyl R_2 = H, δ saturated **17** 6-shogaol R_1=methyl R_2=H



18 8-Gingerol n=6 **19** 10-Gingerol n=8



20 Ferulenol R=H



21 Indicanine B

Fig. 14.3 Structures of some phenolics and acetogenic quinones.

with an MIC of 64 μg mL^{-1} [49]. The structurally related *O*-pentenyl stearate derivative of 2-(4′-hydroxyphenyl) ethanol, isolated as an antimycobacterial component of extracts of the stem and leaves of *Stemodia foliosa*, inhibited the growth of *M. fortuitum*, but not *M. phlei*, at a loading of 10 μg per disk in a disk diffusion assay [50].

The rhizomes of *Ferula communis* yielded a range of antibacterial phenolic functionalized antibiotics including ferulenol (**20**), originally initiated in 1987 from *F. communis* var. *genuina* [51], and ferchromone [52]. While ferulenol exhibited strong growth inhibitory activity towards a range of mycobacteria including *M. intracellulare*, *M. xenopi*, *M. chelonei*, and *M. smegmatis*, with an MIC of 1.25 μg mL^{-1}, ferchromone was found to be less active with an MIC of 50 μg mL^{-1} against the same range of mycobacteria. Licochalcone A, isolated from Chinese licorice root, exhib-

ited growth inhibition of a range of clinical isolates of mycobacteria including *M. tuberculosis* (MIC 5–10 μg /ml), *M. bovis* (MIC 10–20 μg mL^{-1}), and *M. bovis* BCG (MIC 5–10 μg mL^{-1}), whereas it was less active towards *M. avium* (MIC >80 μg mL^{-1}) and *M. intracellulare* (MIC 20–80 μg mL^{-1}) [53].

While plants of the genus *Erythrina* are known to produce a diverse array of alkaloid secondary metabolites which have been used as medicinal plants in many cultures worldwide, nonalkaloidal bioactive components including phenolic derivatives such as isoflavones, flavanones, and chalcones have also been found in the same genus. Investigation of the chemistry of the root bark of *Erythrina indica* afforded the new 3-phenylcoumarin indicanine B, the isoflavone indicanine and its methyl ester, and cajanin. Of these four compounds only indicanine B (**21**) inhibited the growth of *M. smegmatis*, with an MIC of 18.5 μg mL^{-1} [54]. Licoisoflavanone isolated from the medicinal plant *Glycyrrhiza glabra* inhibited the growth of *M. tuberculosis* with an MIC of 25 μg mL^{-1} [36, 37]. The Panamanian plant *Erythrina gibbosa* afforded the flavonoids phaseollidin and erythrabyssin II as antimycobacterial components, both inhibiting the growth of *M. tuberculosis* with MIC values of 8–25 μg mL^{-1} [37].

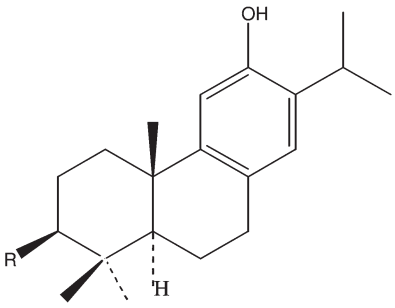
Recently about 200 species of Taiwanese plants were screened for antitubercular activity and *Engelhardia roxburghiana* Wall. (*E. chrysolepis* Hance; *E. formosana* (Hay) Hayata; *E. spicata* BI. var. *formosana* Hayata) (Juglandaceae) was shown to be an active species. *E. roxburghiana* is a deciduous tree growing in India, Indonesia, China, and Taiwan. Three new compounds, engelhardione, (–)-5-hydroxy-4-methoxy-1-tetralone and 3-methoxycarboxyl-1,4-dihydroxyanthraquinone, together with 12 known compounds have been isolated from the roots of *E. roxburghiana*. Of these, engelhardione, 3-methoxyjuglone and (–)-4-hydroxy 1-tetralone showed anti-TB activities with MIC values of 3.125, 3.125, and 6.25 μg mL^{-1} against *M. tuberculosis* 90-221387 and MIC values of 0.2, 0.2, and 4.0 μg mL^{-1} against *M. tuberculosis* $H_{37}R_v$ individually [55].

14.5.4 Terpenes

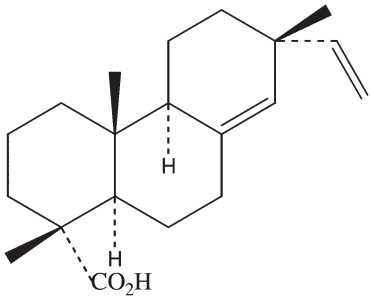
Terpenes are widely distributed secondary metabolism products with low boiling points and, thus, a strong aroma. The name terpene is properly reserved for hydrocarbons made up from isoprene units, but is frequently extended to derivatives of these (alcohols, ethers, carboxylic acids, esters, etc.), which should be called terpene derivatives. Furthermore, benzoid dehydration products of terpenes appear in plants (e.g. the phenol thymol). Depending on molecule size, terpenes can be classified as mono-, sesqui-, di-, and triterpenes, having 10, 15, 20, and 30 carbon atoms, respectively. Of these, monoterpenes are of most importance; 90% of all spices owe their fragrance to them.

Secondary metabolites of terpenoid origin dominate the number of natural products reported with antimycobacterial potential. The terpene derivatives are typically of moderate lipophilicity which would aid in their penetration of mycobacterial cell wall [56].

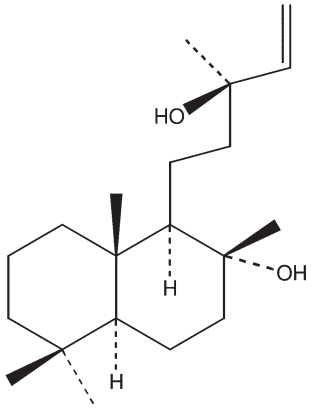
Extracts of *Juniperus excelsa* are used in Saudi Arabia, Yemen, and Oman as a traditional remedy for tuberculosis and jaundice. Bioassay-directed fractionation of an extract of the leaves of the *J. excelsa* showed the diterpenes ferruginol (**22**) and sandararacopimaric acid (**23**) to be antimycobacterial constituents [57] (Fig. 14.4).



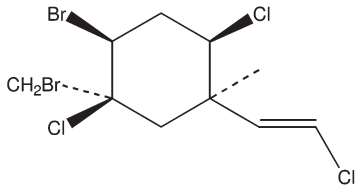
22 Ferruginol R=H



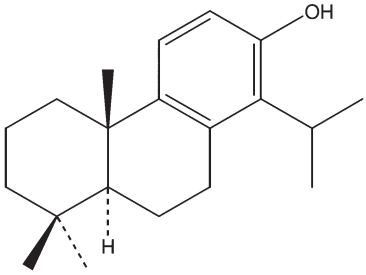
23 Sandararacopimaric acid



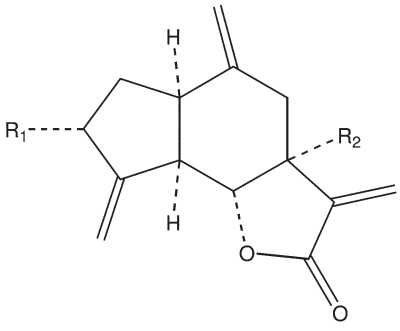
24 Sclareol



25 Halogenated monoterpene



26 (+)- Totarol



27 Dehydrocostus lactone (sesquiterpene)

Fig. 14.4 Structures of some terpenes.

Ferruginol exhibited an MIC of 5 μg mL^{-1} towards *M. smegmatis*, *M. intracellulare*, *M. xenopei*, and *M. chelonei*, and sandararacopimaric acid exhibited an MIC of 36 μg mL^{-1} towards *M. smegmatis*. Sandararacopimaric acid and two more compounds – juniperexcelsic acid and sclareol (**24**) – isolated from this plant by another group in a subsequent study showed antitubercular activity, with MIC values of 15.0, 14.4, and 6.0 μg mL^{-1} respectively towards *M. tuberculosis* $H_{37}R_v$ [58].

Insecticidal monoterpene esters belonging to the pyrethrin class are commonly isolated from flowers of *Chrysanthemum* species. Bioassay-guided fractionation of a Kenyan collection of *Chrysanthemum cinerariaefolium* yielded pyrethrin I and II, which showed mild growth inhibition of *M. tuberculosis* $H_{37}R_v$ with MIC values of 64 and 32 μg mL^{-1}, respectively [59]. A halogenated monoterpene (**25**), originally isolated from the marine red alga *Plocamium cartilagineum*, exhibited antimycobacterial activity towards *M. tuberculosis* $H_{37}R_v$ (MIC 32 μg mL^{-1}) and *M. avium* (MIC 64 μg mL^{-1}) [60].

In a study of Rwandese medicinal plants, diterpenediol was identified as an active principle of *Tetradenia riparia*, exhibiting an MIC of 25–100 μg mL^{-1} against *M. tuberculosis* [61]. (+)-Totarol (**26**), an isomer of ferruginol, was isolated from *Chamaecyparis nootkatensia* as an antimycobacterial component (*M. tuberculosis* $H_{37}R_v$, MIC 16 μg mL^{-1}) [62].

Mossa et al. [63] isolated antimycobacterial constituents from Saudi Arabian plants – *Juniperus procera* (totarol), *Ferula communis* (ferulenol), and *Plumbago zeylanica* (plumbagin) – and found synergistic activity of these constituents in their evaluation in combination with INH against four atypical organisms, namely *M. intracellulare*, *M. smegmatis*, *M. xenopei*, and *M. chelonei*. The potency of INH was found increased four-fold, using an *in vitro* checkerboard method against each mycobacteria when tested with a subtoxic concentration of these constituents. The MIC values of these components were lowered from 1.25–2.5 to 0.15–0.3 μg mL^{-1} due to synergism with INH. When tested against the resistant strain of *M. tuberculosis* $H_{37}R_v$, plumbagin and 7-hydroxyabieta-8,13-dien-11,12-dione showed an inhibitory effect at 12.5 μg mL^{-1}, while others failed to show activity at this concentration.

The Central American medicinal plant *Azorella madreporica* afforded the new mulinane skeleton diterpene which inhibited the growth of *M. tuberculosis* $H_{37}R_v$ with an MIC of 20 μg mL^{-1} [64].

Salvia species plants are used in folk medicine for the treatment of a range of diseases. A range of new norditerpenoids and diterpenoids were isolated from the roots of a Turkish collection of *Salvia multicaulis* [65] which showed growth inhibition of *M. tuberculosis* $H_{37}R_v$ with MIC values of 0.46–7.3 μg mL^{-1}. In a subsequent study by Thangadurai et al. [66] one of these compounds was re-isolated from *Indigofera longeracemosa* and this component exhibited an MIC of 0.38 μg mL^{-1} towards *M. tuberculosis*. A new noricetexane diterpene, salvimultine, has been isolated from the polar fractions of *S. multicaulis* (roots) [67]. Furanoid labdane diterpenes isolated from the salt-tolerant plant *Potamogeton malaianus* collected in Thailand, exhibited weak growth inhibition of *M. tuberculosis* $H_{37}R_a$ with MIC values of 100 and 50 μg mL^{-1} [68]. Extracts of the stem bark of *Mitrephora celebica*, collected in North

Sulawesi, Indonesia, afforded a range of metabolites including the antibiotic diterpenes ent-trachyloban-19-oic acid and ent-kaur-16-en-19-oic acid. In addition to exhibiting activity towards methicillin-resistant *Staphylococcus aureus*, the former also inhibited the growth of *M. smegmatis* with an MIC of 6.25 μg mL^{-1} [69].

Among the diterpenes isolated from *Calceolaria pinnifolia*, 19-malonyloxydehydroabietinol and 19-methylmaloyloxy-ent-isopimara-8,15-diene were found most active against *M. tuberculosis*, each with an MIC of 4 μg mL^{-1}. The MIC values for the terpenes from this plant, 3-epi-ursolic acid and 3-epi-olenolic acid, were 8 and 16 μg mL^{-1}, respectively [70]. Bioassay-guided fractionation of the crude extracts of Mexican medicinal plants *Rumex hymanosepalus* (Polygonaceae), *Larrea divaricata* (Zygophyllaceae), *Phoradendron robinsonii* (Loranthaceae), and *Amphipteryngium adstringens* (Julianiaceae) led to the isolation of several antimycobacterial compounds. Four stilbenoids, two flavon-3-ols, and three anthraquinones were isolated from *R. hymenosepalus*. Two flavonols and nordihydrogualaretic acid were isolated from *L. divaricata*. Sakuranetin was isolated from *P. robinsonii* and two known triterpenoids and the novel natural product 3-dodecyl-1,8-dihydroxy-2-naphthoic acid were obtained from *A. adstringens*. The MIC values of all the compounds ranged from 16 to 128 μg mL^{-1}. Among the tested compounds, the glycolipids, sesquiterpenoids, and triterpenoids showed the best antimycobacterial activity [71]. This is the first time the antimycobacterial activity of the glycolipids has been reported.

Screening of plants from South America for anti-TB activity and subsequent bioassay-guided fractionation resulted in the isolation and characterization of several pentacyclic triterpenoids. The MIC values of 22 triterpenoids were determined using the radiorespiratory BACTEC assay. The MIC value ranged from 8 μmol L^{-1} to >128 μmol L^{-1} [72]. Fifteen crude extracts prepared from seven Ethiopian medicinal plants used to treat various infectious diseases were assessed for their ability to inhibit the growth of *M. tuberculosis*. A preliminary screening of the crude extracts against *M. tuberculosis* typus humanus (ATCC 27294) was done by dilution assay using LJ medium. None of the tested extracts except the acetone fraction obtained from the stem bark of *Combretum molle* showed significant inhibitory activity against this strain. The acetone fraction of the stem bark of *C. molle* caused complete inhibition at concentrations higher than 1 mg mL^{-1}. Further phytochemical analysis of the bioactive fraction led to the isolation of a major tannin and two oleanane-type pentacyclic triterpene glycosides. The tannin was identified as the ellagitannin punicalagin while the saponins were characterized as arjunglucoside (4-epi-sericoside) and sericoside. All the compounds were further tested against the ATCC strain, punicalagin was found to inhibit totally the growth of the ATCC strain and also of a patient strain, which was fully sensitive to the standard anti-TB drugs, at concentrations higher than 600 μg mL^{-1} and 1200 μg mL^{-1}, respectively. It was the first report of tannins exhibiting antimycobacterial activity [73].

With a view to discovering new chemotypes with antimycobacterial activity, a range of sesquiterpenes lactones were prepared using sesquiterpene, dehydrocostus lactone (**27**) as starting material [74]. However, only the parent compound exhibited significant activity in radiorespirometric bioassays against *M. tuberculosis*

$H_{37}R_v$ (MIC 2 μg mL^{-1}) and *M. avium* (MIC 16 μg mL^{-1}). The α-methylene-δ-lactone-containing plant-derived germacranolide sesquiterpenes – parthenolide, costunolide, 1,10-epoxycostunolide, santamarine, and reynosin – exhibited antimycobacterial activity towards *M. tuberculosis* $H_{37}R_v$ (MIC 16–64 μg mL^{-1}) and *M. avium* (MIC 64–128 μg mL^{-1}) [75]. Evaluation of the antimycobacterial activity of a wide array of eudesmanolides sesquiterpene lactones isolated from the North American plants, *Inula helenium*, *Rudbeckia subtomentosa*, *R. mollis*, *Iva imbricata*, and *Montanoa speciosa* confirmed that the α-methylene-δ-lactone ring is an essential but not sufficient structural requirement for antimycobcterial activity [76, 77]. Root extracts of *Inula helenicum*, used by North American natives as a treatment for lung disorders and tuberculosis, provided alantolactone and isoalantolactone, which showed antitubercular activity (MIC towards *M. tuberculosis* $H_{37}R_v$ 32 μg mL^{-1}) [76].

14.5.5 Steroids

Ergosterol-5,8-endoperoxide has been isolated as an antimycobacterial component of the plant *Ajuga remota* [78]. Investigation of the antimycobacterial components of the Brazilian shrub *Physalis angulata* has afforded the known highly oxygenated *seco*-steroid physalin D as the active constituent with an MIC of 32 μg mL^{-1} towards *M. tuberculosis* $H_{37}R_v$ [79]. Bioactivity-guided fractionation of the CH_2Cl_2/CH_3OH extract of the aerial part of *Ruprechtia triflora* Griseb led to the identification of several sterols as the active components against *M. tuberculosis* [70]. The novel acylated sterol, 5α,8β-epidioxyergosta-6,22-dien-3β-yl stearate was isolated. In a microplate alamar blue assay the sterols from this plant were found to be active with MIC values ranging from 2 to 128 μg mL^{-1}, with 5α,8α-epidioxyergost-6,22-den-3β-ol, 5α,8α-epidioxystigmasta-6,22-dien-3β-ol, and stigmast-4-en-6β-ol-3-one being the most active, each with an MIC of 2 μg mL^{-1}.

14.6 Conclusion

The compulsion to find novel agents with antimycobacterial activity has led to exhaustive research in the area of the isolation, characterization, and evaluation of natural products covering a wide diversity of chemical structures. The array of phytic products covered in this chapter is only a partial account of those reported in the literature. Among the compounds with antimycobacterial activity included in this article, especially those showing activity with lower MIC values (less than 5–10 μg mL^{-1}), there may be useful effective chemotherapeutic agents. Some of the compounds may have limitations as effective antimycobacterial drugs on account of their cytotoxicity, poor solubility, and pharmacokinetic limitations, but knowledge of the structural skeletons of these compounds could provide a useful base for the development of new-generation antimycobacterial drugs.

With the development of better *in vitro* and *in vivo* screening/evaluating methods in recent times, the prospects of developing new drugs based on natural products appear to be bright. It is hoped that by the end of this decade a range of more effective new-generation antimycobacterial drugs will be developed.

Acknowledgements

The authors are grateful to the Director, NJIL & OMD, Agra for his constant support and encouragement and to Mrs Nirmal Deo, research assistant, and Mr Gaurav Pratap Singh and Mr Prashant Sharma, research scholars, for helping in preparation of the manuscript.

References

1 T. Parish, N.G. Stoker. In *Mycobacteria Protocols, Methods in Molecular Biology*, Parish, T. Stoker, N.G. (eds). Humana Press, Totowa, NJ, **1998**, pp. 1–13.
2 World Health Organization. Fact Sheet 104, dated August **2002**, available at www.who.int.
3 Roche, P.W., Triccas, J.A., Winter, N. *Trends Microbiol*. **1995**, *3*, 397–401.
4 Weekly epidemiological record-WHO, **2005**, *80*, 289–296 (http://www.who.int/wer)
5 Rattan, A. In *Tuberculosis, Round Table Conference Series*, Gupta, S., Sood, O.P. (eds). Ranbaxy Science Foundation, New Delhi, India, **2000**, pp. 113–122.
6 World Health Organization. *Tech Rep Ser*, *675*. **1982**.
7 Venkatesan, K. *Clin. Pharmacokinet*. **1989**, *16*, 365–386.
8 Venkatesan, K. In *Leprosy Elimination, Critical Issues, Round Table Conference Series*, Sood, O.P., Rattan, A. (eds). Ranbaxy Science Foundation, New Delhi, India, **2002**, pp. 139–146.
9 Katoch, K. In *Leprosy Elimination,Critical Issues, Round Table Conference Series*. Sood, O.P., Rattan, A. (eds). Ranbaxy Science Foundation, New Delhi, India, **2002**, pp. 79–87.
10 Newton, S.M., Lau, C., Wright, C.W. *Phytother. Res*. **2000**, *14*, 303–322.
11 Copp, B.R. *Nat. Prod. Rep*. **2003**, *20*, 535–557.
12 Tripathi, R.P. Tewari, N., Dwivedi, N. Tiwari, V.K. *Med. Res. Rev*. **2005**, *25*, 93–131.
13 Chopra, R.N., Chopra, I.C., Handa, K.L., Kapur, L.D. *Chopra's Indigenous Drugs of India*, 2nd edn. U.N. Dhur & Sons, Calcutta, **1958**.
14 Norton, S.A., *Hawaii Med. J*. **1998**, *57*, 382–386.
15 Baily, E. *Nature* **1945**, *55*, 601.
16 Lall, N., Meyer, J.J.M. *J. Ethnopharmacol*. **1999**, *66*, 347–354
17 Houghton, P.J., Woldemariam, T.Z., Watanabe, Y., Yates, M. *Planta Med*. **1999**, *65*, 250–254.
18 Saroja, S., Usha, K., Shoba, P., Meenakumari, V. *Indian J. Nutr. Diet*. **1997**, *34*, 193–198.
19 Pietro, R.C., Kashima, S., Sato, D.N., Januario, A.H., Franca, S.C. *Phytomedicine* **2000**, *7*, 335–338.
20 Usha, K. Saroja, S. *J. Med. Aromat. Plant Sci*. **2001**, *22*, 182–184.
21 Tshibangu, J.N., Chifundera, K., Kaminsky, R., Wright, A.D., Konig, G.M. *J. Ethnopharmacol*. **2002**, *80*, 25–35.
22 Seephonkai, P., Isaka, M., Kittakoop, P., Palittapongarnpim, P., Kamchonwongpaisan, S., Tanticharoen, M., Thebtaranonth, Y. *Planta Med*. **2002**, *68*, 45–48.
23 Rajah, M.S., Cantrell, C.L., Franzblau, S.G., Fischer, N.H., *Planta Med*. **1998**, *64*, 2–4.

24 Koyama, S., Yamaguchi, Y., Tanaka, S., Motoyoshiya, J. *Gen. Pharmacol.* **1997**, *28*, 797–804.

25 Lu, T., Cantrell, C.L., Robbs, S.L., Franzblau, S.G., Fischer, N.H. *Planta Med.* **1998**, *64*, 665–667.

26 Zgoda, J.R., Freyer, A.J., Killmer, L.S., Porter, J.R. *J. Nat. Prod.* **2001**, *64*, 1348–1349.

27 Kobaisy, M., Abramowski, Z., Lermer, Z.L., Saxena G., Hancock, G.R.E.W., Towers, G.H.N., Doxsee, D., Stokes, R.W. *J. Nat. Prod.* **1997**, *60* , 1210–1213.

28 Mata R., MoralesI, I ., Perez, O., Rivero-Cruz , I ., Acevedo, L., Enriquez-Mendoza, I., Bye, R., Franzblau, S.G, Timmermann, B. *J. Nat. Prod.* **2004**, *67*, 1961–1968.

29 Lall, N., Meyer, J.J.M. *J. Ethnopharmacol.* **2001**, *78*, 213–216.

30 Seidel, V., Taylor, P.W., *Int. J. Antimicrob. Agents* **2004**, *23*, 613–619.

31 Chumkaew, P., Karalai, C. Ponglimanont, C., Chantrapromma, K. *J. Nat. Prod.* **2003**, *66*, 540–543.

32 Sayed, K.A.E.I., Hamann, M.T., Abd-EI Rehman, H.A., Zagloul, A.M. *J. Nat. Prod.* **1998**, *61*, 848–850.

33 Grange, J.M. Snell, N.J.C., *J. Ethnopharmacol.* **1996**, *50*, 49–53.

34 Peterson, J.R.J., Zjawiony, K., Liu, S., Hufford, C.D., Clark, A.M., Rogers, R.D. *J. Med. Chem.* **1992**, *35*, 4069–4077.

35 Rao, J. U. M., Giri, G. S., Hanumaiah, T., Rao, K. V. J. J. *J. Nat. Prod.* **1986**, *49*, 346–347.

36 Mitscher, L.A., Baker, W.R., *Pure Appl. Chem.* **1998**, *70*, 365–370.

37 Mitscher, L.A., Baker, W.R. *Med. Res. Rev.* **1998**,*18*, 363– 374.

38 Cimanga, K., de Bruyne, T.T., Pieters, L., Totte, J., Tona, L., Kambu, K., vanden Gerghe, D., Vlietinck, A.J. *Phytomedicine* **1998**, *5*, 209–214.

39 Rastogi, N., Abaul, J., Goh, K.S., Devallois, A., Philogene, E., Bourgeois, P. *FEMS Immunol. Med. Microbiol.*, **1998**, *20*, 267–279.

40 Okunade, A.L., Hufford, C.D., Richardson, M.D., Peterson, J.R., Clark., A.M. *J.Pharm. Sci.* **1994**, *83*, 404–406.

41 Rukachaisirikul, T., Prabpai, S., Champung, P., Suksamram, A. *Planta Med.* **2002**, *68*, 853–855.

42 Ma, C., Case, R.J., Wang, Y., Zhang, H–J., Tan, G.T., Hung, N.V., Cuong, N.M., Franzblau, S.G., Sejarto, D.D., Fong, H.H.S., Pauli, G.F. *Planta Med.* **2005**, *71*, 261–267.

43 Kanokmedhakul, S., Kanokmedhakul, K., Phonkerd, N., Soytong, K., Kongsaeree, P., Suksamrarn, A. *Planta Med.* **2002**, *68*, 834–836.

44 Schinkovitz, A., Gibbons, S., Starvi, M., Cocksedge, M.J., Bucar, F. *Planta Med.* **2003**, *69*, 369–371.

45 Stavri, M., Mathew, K.T., Bucar, F., Gibbons, F.S. *Planta Med.* **2003**, 69, 956–959.

46 Newton, S. M., Lau, C., Gurcha, S.S., Besra, G.S., Wright, C.W. *J. Ethnopharmacol.* **2002**, *79*, 57–67.

47 Galal, A.M. *Int. J. Pharmacol.* **1996**, *34*, 64–69 .

48 Hiserodt, R.D., Franzblau, S.G., Rosen, R.T.J. *Agric. Food Chem.* **1998**, *46*, 2504–2508.

49 Acevedo, L., Martinez, E., Castaneda, P., Franzblau, S.G., Timmermann, B.N., Linares, E., Bye, R., Mata, R. *Planta Med.* **2000**, *66*, 257–261.

50 da Silva, L.L.D., Nascimento, M., Silva, D.H.S., Furlan, M., da Silva V. Bolzani, *Planta Med.* **2002**, *68*, 1137–1139.

51 Lamnaouer, D., Bodo, B., Martin, M.T., Molho, D. *Phytochemistry* **1987**, *26*, 1613–1616.

52 Al-Yahya, M. A., Muhammad, I., Mirza, H.H., EI-Feraly, F.S. *Phytother. Res.* **1998**, *12*, 335–339.

53 Friis-Moller, A., Chen, M., Fuursted, K., Christensen, S.B., Kharazmi, A. *Planta Med.* **2002**, *68*, 416–419 .

54 Waffo, A.K., Zabase, G.A., Nkengfack, A.E., Fomum, Z.T., Meyer, M., Bodo B., van Heerden, F.R., *Phytochemistry* **2000**, *53*, 981–985.

55 Lin, W-Y., Peng, C-F., Tsai, I-L., Chen, J-J., Cheng, M-J., Chen, I-S. *Planta Med.* **2005**, *71*, 171–175.

56 Sayed, K.A.E.I., Bartyzel, P., Shen, X., Perry, T.L., Zjawiony, J.K., Hamann. M.T. *Tetrahedron* **2000**, *56*, 949–953.

57 Muhammad, I., Mossa, J.S., EI. Feraly, F.S. *Phytother. Res.* **1992**, *6*, 261–264.

58 Topcu, G., Erenler, R., Cakmak, O., Johanson, C.D., Celik, C., Chai, H.-B., Pazzato, J.M. *Phytochemistry* **1999**, *50*, 1195–1197.

59 Rµgutt, J.K., Henry III, C.W., Franzblau, S.G., Warner, I.M. *J. Agric. Food Chem.* **1999**, *47*, 3402–3410.

60 Higgs, M.D., Vanderah, D.J., Faulkner, D.J. *Tetrahedron* **1977**, *33*, 2775–2780.

61 van Puyvelde, L., Ntawukiliyayo, J. D., Portaels, F., Hakizamungu, E. *Phytother. Res.* **1994**, *50*, 1195–1119.

62 Constantine, G.H., Karchesy, J.J., Franzblau, S.G., LaFleur, L.E. *Fitoterapia* **2001**, *72*, 572–574.

63 Mossa, J.S., EI. Feraly, F.S., Muhammad I., *Phytother Res.* **2004**, *18*, 934–937.

64 Wachter, G.A., Franzblau, S.G., Montenegro, G., Suarez, E., Fortunato, R.H., Saavedra, E., Timmermann, B.N. *J. Nat. Prod.* **1998**, 61, 965–968.

65 Ulubelen, A., Topcu, G., Johansson, C.B. *J. Nat. Prod.* **1998**, *60*, 1275–1280.

66 Thangadurai, D., Viswanathan, M.B., Ramesh, N. *Pharmazie* **2002**, 57, 714–715.

67 Ulubelen, A., Topcu, G. *J. Nat. Prod.* **2000**, *63*, 879–880.

68 Kittakoop, P., Wanasith, S., Watts, P., Kramyu, J., Tantichareon, M., Thebtaranonth, Y. *J. Nat. Prod.* **2001**, *64*, 385–388 .

69 Zgoda-Pols, J.R., Freyer, A.J., Killmer, L.B., Porter, J.R. *Fitoterapia* **2002**, *73*, 434–438.

70 Woldenmichael, G.H.W., Franzblau, S.G., Zhang, F., Wang, Y., Timmermann B.N. *Planta Med.* **2003**, *69*, 628–631.

71 Rivero-Cruz, I., Acevedo, L., Guerrero J.A., Martinez, S., Bye, R., Pereda-Miranda, R., Franzblau, S.G., Timmermann, B.N., Mata, R. *J. Pharm. Pharmacol.* **2005**, *57*, 1117–1126.

72 Wachter, G.A., Valcic, S., Flagg, M.L., Franzblau, S.G., Montenegro, G., Suarez, E., Timmermann, B.N., *Phytomedicine* **1999**, *6*, 341–345.

73 Asres, K., Bucar, F., Edlsbrunner, S., Kartnig, T., Hoger, G., Thiel, W., *Phytother. Res.* **2001**, *15*, 323–326.

74 Cantrell, C.L., Nunez, I.S., Castaneda-Acosta J., Foroozesh, M., Fronczek, F.R., Fischer, N.H., Franzblau, S.G. *J. Nat. Prod.* **1998**, *61*, 1181–1186.

75 Fischer, N.H., Lu, T., Cantrell, C.L., Castaneda-Acosta, J., Quijano, L., Franzblau, S.G. *Phytochemistry* **1998**, *49*, 559–564.

76 Cantrell, C.L., Abate, L., Fronczek, F.R., Franzblau, S.G., Quijano, L., Fischer, N.H., *Planta Med.* **1999**, *65*, 351–355.

77 Cantrell, C.L., Franzblau, S.G., Fischer, N.H., *Planta Med* , **2001**, *67*, 685–689.

78 Cantrell ,C.L., Rajab, M.S., Fronczek, S.R., Fischer, N.H. *Planta Med.* **1999**, *65*, 732–734.

79 Januario, A.H., Filho, E.R., Pietro, R.C.L.R., Koshima, S., Sato, D.N., Franca, S.C. *Phytother. Res.* **2002**, *16*, 445–448.

15
Ethnomedicinal Antivirals: Scope and Opportunity

Debprasad Chattopadhyay

Summary

Viral diseases, including emerging, reemerging, and chronic infections, are an increasing health concern throughout the world. As a consequence, the development of new antivirals from plants, particularly from ethnomedicinal practices, has assumed more urgency today. Ethnomedicines provide a diverse range of natural products with antimicrobial and immunomodulating potential. A wide variety of active phytochemicals such as alkaloids, coumarins, essential oils, flavonoids, phytosterols, polysaccharides, polyphenols, tannins, saponins, proteins, and peptides from hundreds of plants, culinary herbs, spices and teas have complementary and overlapping mechanisms of action, including inhibition of viral reproduction or genome formation. Immune-related conditions with a high unmet clinical need still exist, along with the problem of increasing antiviral resistance in many viral infections. The ethnomedicinal phytochemicals might be able to provide an alternative to the costly antivirals and immunotherapeutics.

Assay methods to determine *in vitro* and *in vivo* antiviral activity are needed to link antiviral efficacy or potency and laboratory-based research. The relative success achieved in the last two decades with such plant extracts which are capable of acting therapeutically in various viral infections, has raised optimism about the future of phytoantiviral agents. This chapter reviews some potentially useful ethnomedicinal plants and compounds, evaluated and exploited for therapeutic applications against genetically and functionally diverse virus families including Retroviridae, Hepadnaviridae, and Herpesviridae causing sexually transmitted viral infections.

15.1
Introduction

The search for healing power in plants is an age-old idea and throughout the history man has relied on nature for their food, clothing, shelter, transportation and

medicines. People of all continents have long applied poultices and imbibed infusions of hundreds or thousands of indigenous plants. Historically, the therapeutic results of such traditional medicaments are mixed; quite often they cure or relieve symptoms but sometimes poisonings occurred. The oldest record of ethnomedicine, dating back to 2600 BC from Mesopotamia, describes the use of thousands of phytomedicines including oils of cedar wood (*Cedrus* sp.) and cypress (*Cupressus sempevirens*), licorice (*Glycyrrhiza glabra*), myrrh (*Commiphora* sp.), and poppy (*Papaver somniferum*) juice, which are still used today for the treatment of ailments from coughs and colds to parasitic infections and inflammation [1]. The Egyptian *Ebers Papyrus* (1500 BC) documented over 700 medicaments and formulas, such as gargles, poultices, infusions, pills and ointments. The Chinese materia medica, extensively documented over the centuries, contained the *Shennong Herbal* (first century AD) and the *Tang Herbal* (AD 659), while the Indian Ayurvedic system includes 857 drugs [2] from the Charaka, Sushruta, and Samhitas traditions.

Perhaps the most substantial contribution to the rational development and the use of herbal medicine was made by the Greeks. Theophrastus (~300 BC) in his *History of Plants* describes many medicinal herbs and their chemical variation through cultivation, while Galen (AD 130–200) published 30 books on prescriptions and formulas. During the fifth to twelfth centuries, the monasteries in England, Ireland, France, and Germany preserved some of this knowledge. The Arabs, meanwhile, combined the Greco-Roman expertise with Chinese and Indian formulations into their own resources. The *Canon Medicinae* compiled by Avicenna, a Persian physician, was superseded by the *Corpus of Simples* of Ibn al-Baitar of Spain, and all these formulations were compiled into the *London Pharmacopoeia* in 1618. The idea of "pure" compounds as drugs originates from the isolation of the active principles strychnine, morphine, atropine, and colchicine from common plants in the early 1800s, which led to the isolation of the first pure natural product, morphine, by E. Merck in 1826 and the first semi-synthetic drug, aspirin, by Bayer in 1899 [3].

This review is an attempt to summarize the current state of knowledge of antiviral extracts from ethnomedicinal plants of different community, including compounds prospected and tested against viral infections caused by genetically and functionally diverse virus families. The structure and antiviral properties of some potentially useful phytomedicines will also be addressed. Discussion will be focused on how the ethnic phytomedicines have led to the development of some useful antiviral drugs that are currently in preclincal or clinical evaluation.

15.1.1
Ethnomedicines and Drug Discovery

Earth is estimated to contain about 250 000–500 000 plant species, of which nearly 10% are used as food and 10–15% as drugs [4]. Phytomedicines have always formed the basis of traditional medicaments in China [5], India [2] Africa, and in many other cultures [6] over the centuries. Approximately 80% of the world's population still relies mainly on phytomedicines for primary health care and the re-

maining 20% use plant products as ingredients for several drugs [7]. Currently, 119 drugs of modern medicines are derived from 90 plants, of which 74% are of ethnomedicinal origin. The search for drugs and dietary supplements from ethnomedicinal sources has accelerated in recent times therefore, the ethnopharmacologists and natural products chemists are combing the earth surface for "lead" compound of therapeutic potential.

Although about 40% of modern pharmaceuticals are derived from plants, none are used against viruses. In contrast, traditional healers have long used phytomedicines to prevent or cure infectious conditions. Clinical microbiologists are interested in antimicrobial plant extracts because (i) the effective lifespan of any antibiotic is limited; (ii) increasing public awareness of overprescription and misuse of antibiotics; (iii) public preferences for natural products in treating and preventing medical problems; and (iv) viral diseases remain intractable to most orthodox antibiotics. Another factor is the rapid rate of species extinction [8] leading to irretrievable loss of structurally diverse and potentially useful phytochemicals [4]. In addition, the rapid spread of human immunodeficiency virus (HIV), the emergence of severe acute respiratory syndrome (SARS), and the reemergence of many diseases have spurred intensive investigation into phytomedicines, especially for people who have little access to expensive Western medicines [9].

Plants can produce far more compounds than are necessary for their survival and propagation. These secondary metabolites are species/strain-specific with diverse structures and bioactivities (like flavors, colors, dyes, fragrances, insecticides and drugs), synthesized mainly for defense against predators. These toxic, foul-tasting chemicals are the natural version of chemical warfare. The plant metabolites can be broadly grouped into phenolics (anthocyanins, coumarins, flavonoids, quinones, and tannins), terpenoids (essential oils, saponins, sterols, and cucurbitacins), alkaloids, proteins, and peptides.

15.1.2 Viruses: The Acellular Parasite of Cellular Hosts

Viruses are ultramicroscopic, acellular, metabolically inert nucleoprotein particles containing bundles of gene strands of either RNA or DNA, with or without a lipid-containing envelope [10]. Unlike free-living bacteria, viruses are obligate intracellular parasites. They utilize the host cell machinery to propagate new viruses and can cause ailments as benign as a common wart, as irritating as a cold, or as deadly as the bloody African fever. The viruses that cause Lassa and Ebola fever and AIDS spread easily, kill swiftly, and have no cure or vaccine. Viruses have numerous invasion strategies and each strain has its own unique configuration of surface molecules [10, 11], enabling them to enter into host cells by precisely fitting their surface molecules with the molecules of target cell. The genetic variation, variety of transmission, efficient replication and the ability to persist within the host are the major evolutionary advantages of viruses. As a consequence viruses have adapted to all forms of life and have occupied numerous ecological niches resulting in widespread diseases in humans, livestock and plants [11].

15.1.2.1 Viral Infection Control

Viral infections can be controlled either through prophylactic (protective) or therapeutic measures. Being metabolically inert viruses require living cells to replicate, and as most steps in their replication involve cellular metabolic pathways it is difficult to design a treatment to attack the virion or its replication without affecting the infected host [10, 11]. Although a vast number of natural or synthetic compounds have been tested on different viruses, the development of antivirals has been less explored, probably because of the simplicity and nature of viruses. When a virus effectively takes over the control of infected cell there are very few specific viral targets for small molecules to interact with. Fortunately, many viruses have unique features in their structure or replication cycles that can be the potential targets. For example, acycloguanosine (acyclovir) acts against herpesviruses by interfering with certain key viral enzymes having distinctive affinities for nucleotide analogs [11]. As viral enzymes play a key role in triggering disease, therefore, when these enzymes are neutralized, viral replication does not take place.

15.2 Antiviral Ethnomedicines Against Common Virus Families

Many ethnomedicinal plants are reported to possess strong antiviral activity and some are already used in the treatment of viral infections in different parts of the world [12–16]. The antiviral activities of some of these important ethnomedicinal plants are presented in Table 15.1.

Herpes simplex viruses (HSV) are reported to be a high risk factor for HIV infection and scientists are looking towards ethnomedicines as a source of novel antiherpes and antiretroviral drugs. A large number of phytophores, such as phenols, polyphenols, flavonoids, terpenoids, and sugar-containing compounds, have promising antiherpetic [17] and antiretroviral [1] activities.

The search for plant-based antivirals was initiated by the Boots Drug Company, England in 1952 and they found that 12 plant extracts can suppress the amplification of influenza A virus [18]. During the last 50 years numerous broad-based screening programmes have been conducted throughout the world to evaluate the *in vitro* and *in vivo* antiviral activity of hundreds of ethnomedicinal plants. Canadian researchers reported the antiviral activities of grape, apple, and strawberry juices against HSV, poliovirus 1, coxsackievirus B5, and echovirus 7. While the leaf extract of *Azadirachta indica* (neem) was found to inhibit many DNA viruses such as smallpox, chickenpox, poxvirus and HSV and the RNA virus poliomyelitis [15, 19]. The British Columbian ethnomedicines *Potentilla arguta* and *Sambucus racemosa* inhibited respiratory syncytial virus (RSV), *Lomatium dissectum* inhibited rotavirus, while *Cardamine angulata*, *Conocephalum conicum*, and *Polypodium glycyrrhiza* had anti-HSV-1 activity [20]. Strong anti-HSV activity was found in *Byrsonima verbascifolia* extract, a remedy of skin infections in Colombia [21]. *Eleutherococcus senticosus* root extracts inhibited human rotavirus, RSV, and influenza A, while *Sanicula europaea* inhibited influenza virus by blocking RNA-dependent enzymes

Table 15.1 Antiviral activities of some important ethnomedicinal plants.

Virus	*Name of plants*	*Compound (class)*	*References*
HSV-1	*Agrimonia pilosa, Punica granatum, Moringa oleifera, Aglaia odorata, Ventilago enticulata*	Polyphenols	51, 30
	Solanum torvum	Torvanol, torvoside (flavonoid)	67
	Morus alba	Mulberoside (flavonoid)	81
	Maesa lanceolata	Maesasaponin (saponin)	97
	Rheum officinale, Aloe barbadensis, Cassia angustifolia	Anthroquinone, flavones	46
	Santalum album, lemon grass	Essential oil	15
	Artemisia douglasiana, Eupatorium patens,Tessaria absinthioides	Essential oil	100
	Melia azedarach	Meliacine (peptide)	24
	Bupleurum nigidum	Iridoids (saponins)	96
	Aloe-emodin	Rosmarinic acids (phenolics)	46
	Minthostachys verticillata	Pulegone (essential oil)	98
HSV-1, HSV-2	*Eupatorium patens*	Essential oil	100
	Geum japonicum	Eugeniin (tannin)	17
	Alstonia macrophylla	Ursolic acid (triterpene)	110
HSV-2	*Rhus javanica*	Morin (triterpene)	17
	Terminalia arjuna	Casuarinin (tannin)	88
	Melissa officinalis	Essential oil	101
VSV	*Bupleurum nigidum, Scrophularia scorodonia*	Saikosaponin iridoid (saponins)	96
VZV, Influenza, PRV	*Aloe emodin, A. barbadensis*	Rosmarinic, chlorogenic, caffeic acids (phenolics)	46
HSV, ADV 8	*Boussingaultia gracilis, Serissa japonica*	Essential oil	31
Dengue-2	*Artemisia douglasiana, Eupatorium patens*	Essential oil	100
Pseudorabies	*Minthostachys verticillata*	Pulegone (essential oil)	98
RSV	*Barleria prionitis*	Luteoside (flavonol)	76
	Blumea laciniata, Markhamia lutea, Elephantopus scaber, Mussaenda pubescens, Scutellaria indica	Polyphenols	51
RSV, Influenza	*Aesculus chinensis*	Flavonoid	82
Influenza	*Bergenia ligulata*	Tannins	86
	Geranium sanguineum	Polyphenol	35
Parainfluenza 3	*Caesalpinia minax*	Caesalmin (diterpenoid)	15
Measles	*Zanthoxylum chalybeum*	Skimmianine (alkaloid)	105

Table 15.1 Antiviral activities of some important ethnomedicinal plants. (Continued)

Virus	*Name of plants*	*Compound (class)*	*References*
HBV	*Rheum palmatum*	Essential oil	15
	Phyllanthus niruri, Phyllanthus urinaria	Chebulic acid (tannin), Niruriside	128
	Phyllanthus spp.	Niruriside	13
	Sophorae flavescentis		130
HCV	*Amebia euchroma, Thlaspi arvens, Poncirus trifoliata*	Flavonoids	37
HCV Protease	*Stylogne cauliflora*	Oligophenol	50
HIV	*Drymaria diandra*	Drymaritin (alkaloid)	109
	Brazilian propolis	Moronic acid (triterpenoid)	124
	Glycyrrhiza lepidota, Glycyrrhiza glabra	Diprenyl bibenzyl, glycyrrhizin (flavonoid)	16, 124
	Maesa lancolata	Maesasaponin (saponin)	97
	Desmos spp.	Cinnamoylbenzaldehyde (flavone)	80
	Ailanthus altissima	Flavonoids	43
	Begonia nantoensis	Oleanoic, catechin (flavonoid)	82
	Momordica charantia L.	Mannose-specific lectin MAP30	16, 113
	Cymbidium spp., *Hippeastrum, Epipactis helleborine, Listeria ovata Gelonium multiflorum*	Ribosome-inactivating proteins (RIP), mannose-specific lectins GAP 31 (lectin)	113
	Urtica dioca	*N*-Acetyl glucosamine, lectins	83
HIV entry	*Tieghemella heckelii*	Arganine (saponin)	95
HIV-1	*Stephania cepharantha*	Cepharanthine (alkaloid)	106
	Prangos tschimganica	Coumarine	55
	Vatica cinerea	Vaticinone (triterpenes)	94
	Leucojum vernum	Alkaloid	108
HIV replication	*Scutellaria baicalensis*	Baicalein, baicalin (Flavonoid)	78
HIV-1 RT	*Phaseolus vulgaris* and *P. coccineus*	Polypeptide	114
	Callophyllum lanigerum	Calonides (coumarins)	16
	Dryopteris crassirhizoma	Kaempferol	120
	Momordica charantia	MRK 29 (polypeptide)	115
	Panax notoginseng	Xylanase (Protein)	16, 114
	Shepherdia argentea	Shephagenin, strictinin (tannin)	87
	Phyllanthus amarus	Geraniin (gallotannins)	87
HIV-1 protease	*Geum japonicum*	Ursolic acid (triterpene)	75
	Camellia japonica	Camelliatannin (tannin)	89
HIV fusion	*Prunella vulgaris*	Polyphenol	90
	Rhizoma cibolte	Tannin	90

Table 15.1 Antiviral activities of some important ethnomedicinal plants. (Continued)

Virus	*Name of plants*	*Compound (class)*	*References*
HIV-1 integrase and protease	*Curcuma linga* L.	Curcumin (phenolics)	16, 83
	Larrea tridentata L.	Lignan	
HIV replication	*Homolanthus mutans*	Prostratin (phorbol ester)	
HIV gene expression	*Euphorbia poissonii*	Phorbol ester	
Coxsackie B3	*Loranthus yadoriki*	Camp B, C (polyphenol)	25
Poliovirus 2, 3 (picorna and rhinoviruses)	*Dianella longifolia, Pterocaulas sphaedatum*	Chrysophenate (anthraquinone), chrysophenol	61
	Psiadia dentata	Kaempferol (flavonoids)	69
Junin virus	*Lippia junelliana, L. turbinata, Heterotheca latifolia, Tessaria absinthides*	Essential oil	100
Epstein–Barr	*Syzygium aromaticum*	Ellagitannin (tannin)	15
SARS-CoV	*Stephania cepharantha, Glycyrrhiza glabra*	Isoquinoline (alkaloid), glycyrrhizin (flavonoid)	51
Rotavirus, coronavirus	*Camellia sinensis (Thea sinensis), Eleutherococcus senticosus*	Theaflavin, catechin (flavonoid)	75, 22

HSV, herpes simplex virus; VSV, vesicular stomatitis virus; VZV, varicella zoster virus; PRV, pseudorabies virus; PV, poliovirus; ADV, adenovirus; RSV, respiratory syncytial virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; SARS-CoV, severe acute respiratory syndrome coronavirus.

[22]. In contrast, aqueous extracts of *Nepeta nepetella*, *Dittrichia viscosa*, and *Sanguisorba minor magnolii* of the Iberian Peninsula inhibited HSV-1 and vesicular stomatitis virus (VSV) [23]. A combination of *Verbascum thapsiforme* flower infusion and amantadine resulted in a marked inhibition of influenza H7N7, while meliacine from *Melia azedarach* leaf extract was found to control HSV-1-induced ocular disease [15, 24]. The virucidal activity of *Loranthus yadoriki* extracts against coxsackievirus B3 is reported to be better than that of antiviral drug ribavirin [25]. The Nepalese ethnomedicine *Nerium indicum* inhibited influenza A and HSV [26] while the antipyretic and anti-inflammatory plant *Rheum officinale* and *Paeonia suffruticosa* block HSV attachment and penetration [27]. The La Reunion Island plant *Senecio ambavilla* was found to have anti-HSV-1 and anti-poliovirus 2 activities [28]. It has been reported that the oral administration of *Thuja occidentalis*, *Baptisia tinctoria*, and *Echinacea purpurea* extract to influenza A-infected Balb/c mice significantly increased the survival rate and mean survival time with reduced virus titer [29]. On the other hand, extracts of *Aglaia odorata*, *Moringa oleifera* and *Ventilago denticulata* of Thailand inhibited thymidine kinase-deficient and phosphonoacetate-resist-

ant HSV-1 and delayed the development of skin lesions, prolonged the mean survival times and reduced mortality of infected mice like antiviral drug acyclovir [30]. Extracts of *Boussingaultia gracilis* and *Serissa japonica* of Taiwan inhibited HSV and adenoviruses (ADV 3, 8 and 11), while the extracts of *Ardisia squamulosa* and *Artemisia princeps* were most effective against ADV-8 replication [31]. Interestingly the adsorption, replication, and transcription of HSV-1 were inhibited by *Ceratostigma willmottianum*, a folk medicine of China [32], but Radix Glycyrrhizae inhibited the replication of RSV in a dose-dependent manner [33]. Extracts of *Senna petersiana*, a folk remedy for sexually transmitted diseases, have strong anti-HSV activity [34], while the polyphenol-rich fraction of *Geranium sanguineum* extract showed strong activity against influenza virus [35] (Table 15.1).

Hepatitis B virus (HBV) is known to be associated with hepatitis, cirrhosis, chronic liver disease, and primary hepatocellular carcinoma. There are about 450 million chronic carriers of HBV and 200 million carriers of HCV worldwide, but many of them are asymptomatic [36]. Although a safe and effective HBV vaccine exists there is no effective therapy for the carriers. Traditionally, the genus *Phyllanthus* has been used globally against liver disease, such as jaundice, retrospectively caused by HBV; hence several *Phyllanthus* species have been screened for anti-HBV activity (Table 15.1). About 300 ethnobotanical reports have shown that aqueous extracts of *P. amarus*, *P. debilis*, *P. fraternus*, *P. niruri*, *P. urinaria*, and *P. mimicus* inhibit the DNA polymerase of hepadnaviruses in vitro. Aqueous extracts of *P. amarus* (*P. niruri*) collected from Madras, India, inhibited viral DNA polymerase in vitro and eliminated detectable virus from the sera of woodchucks (*Marmota monax*) acutely or chronically infected with the woodchuck hepatitis virus [12].

Extracts of the Chinese ethnomedicinal plants *Arnebia euchroma*, *Thlaspi arvense*, and *Poncirus trifoliata* (Table 15.1) displayed strong anti-HCV activities [37], while the *Prunella vulgaris* spike can inhibit HIV-1 adsorption and replication by blocking the reverse transcriptase enzyme and reducing the copies of proviral DNA [38]. The Korean ethnomedicine *Agrimonia pilosa* and *Mallotus japonicus* significantly inhibited HIV-1 reverse transcriptase and RNase H enzymes [39]. On the other hand, the Ethiopian anti-infective plant *Combretum paniculatum* strongly inhibited replication of both HIV-1 and HIV-2 [40], while *Hyssop officinalis* and *Dittrichia viscosa* inhibited HIV-1 induced infections [41]. The Rwandan folklore plants *Aspilia pluriseta* and *Rumex bequaertii*, used for infections and rheumatoid diseases, showed strong anti-HIV-1 activity (Table 15.1). Interestingly, further fractionation of an antivirally inactive extract of *Tithonia diversifolia* yielded an aqueous fraction with high anti-HIV-1 activity [16], indicating that the cytotoxicity of some phytomedicines may mask the antiviral properties of the active compounds in crude extracts [16]. The Indian ethnomedicinal plants *Cinnamomum cassia* and *Cardiospermum helicacabum* inhibited HIV-1 and 2 [42], while the stem bark extract of the Korean ethnomedicine *Ailanthus altissima* inhibited HIV-1 fusion [43]. Extracts of *Ocimum gratissimum* and *Alchornea cordifolia* inhibited cytopathicity of HSV-2 by blocking reverse transcriptase activity and proviral DNA copying, like zidovudine [44]. Ishikawa et al. [45] reported that tremulacin and cochinchiside B extracted from the root bark of *Homalium cochinchinensis* inhibited HIV-1 [45].

A review of promising anti-HIV phytomedicines reported that most of these plant-derived compounds interfere with early steps of virus life cycle, such as virus entry, reverse transcriptase, and integrase [16]. Since several phytochemicals can modulate cellular factors such as NFκB and tumor necrosis factor α (TNFα) involved in viral replications, their role as potential antiviral compounds is important. Hence ethnomedicinal antivirals are good candidates for therapeutic and/or prophylactic use, probably in combination with other antiviral drugs.

15.3 Major Groups of Antivirals from Plants

Because of their amazing structural diversity and broad range of bioactivities, traditional phytomedicines can also be explored as a source of complementary antivirals. Ethnomedicinal plants with diverse chemical constituents can inhibit the replication cycle and cellular factors of various DNA and or RNA viruses. A list of some potential plant derived compounds, their chemical class and mechanism of antiviral actions are summarized in Table 15.2.

Table 15.2 Antiviral targets of plant-derived compounds.

Class	*Subclass*	*Example(s)*	*Antiviral mechanism*
Phenolics	Simple phenols and phenolic acids	Caffeic acid, rosmarinic and chlorogenic acid	Virus clumping, inhibition of adsorption, RT, RNA polymerase
	Anthocyanins	Proanthocyanidins	HIV-RT inhibition
	Coumarins	Warfarin, calanolide	Inhibit entry, RT, integrase
	Flavones, flavonols	Taxifolin, torvanol, amentoflavone	Inactivate enzymes: protease, RT, gp120 interaction, protein binding
	Quinones, fluoroquinone	Hypericin, chicoric acid, Chrysophenol C	Bind to integrase, protein inactivation, replication inhibition
	Tannins	Ellagitannin (eugeniin), shephagenin, strictinin, geraniin, casuarinin, camelliatannin	Inhibit adsorption, RT, protease, DNA polymerase, transport protein, polysaccharide, attachment and penetration
	Flavonoids	Chrysin, quercetin, morin, myricetin, catechin, glycyrrhizin, baicalin	Inhibit adsorption, entry, binding, RT, integrase, protease, DNA and RNA polymerase, complex with proteins
Terpenoid	Terpenoid and essential oils	Caesalmin, capsaicin, pulegone, terpinen-4-ol	Inhibit adsorption, cell-to-cell transmission, multiplication
	Triterpenoids	Betulinic acid, arginine, vaticonine, ursolic acid	Inhibit virus entry, protease, replication
	Other terpenoids	Faicalein, swertifrancheside	Protein binding

Table 15.2 Antiviral targets of plant-derived compounds. (Continued

Class	*Subclass*	*Example(s)*	*Antiviral mechanism*
Alkaloids		Cepharanthine, michellamine, solamargine, harman, skimmianine, triptonine	Inhibit, viral gene expression, replication, protein synthesis, interfere with cellular factors
Sulfated polysaccharides/ polypeptides	Mannose-specific lectins	MAP 30, GAP 31, MRK 29, DAP 30, MAR 10, fabatin	Block fusion, adsorption, RT, form disulfide bridges
	Polypeptides	Meliacin, xylanase, trichosanthin	Fusion, RT, cellular factors
	Polysaccharide	Jacalin, prunellin, RAP, RMP	Replication, budding blocker

RT, reverse transcriptase; MAP30, a 30 kDa protein of *Momordica charantia*, GAP31, a 31 kDa protein of *Gelonium multiform*; RAP, *Rhizophora apiculata* polysaccharide; RMP, *Rhizophora mucronata* polysaccharide.

15.3.1 Phenolics and Polyphenols

The simplest bioactive phytochemicals containing a single substituted phenolic ring, like cinnamic and caffeic acids, belong to a wide group of phenylpropanes that are in the highest oxidation state and have wide range of antiviral activities. For example, purified aloe emodin inactivates HSV-1, varicella zoster (VZV), pseudorabies, and influenza virus by the polyphenols rosmarinic, chlorogenic, and caffeic acid (Fig. 15.1) derivatives [46]. Polyphenols and proanthocyanidins (Fig. 15.2) of *Hamamelis virginiana* are reported to have remarkable anti-HSV-1 and anti-HIV-1 reverse transcriptase activity [15, 17], while proanthocyanidin A-1 of *Vaccinium vitis-idaea* can block HSV-2 attachment and penetration [47]. Cadman [48] suggested that the antiviral activity of *Rubus idaeus* leaf is due to the clumping of the virus particles by polyphenols, while Hudson [14] concluded that polyphenols preferential bind to the protein coat of the virus. Sakagami et al. [49] suggested that polyphenols arrest viral adsorption or binding to the host cell protein and inhibit viral enzymes such as reverse transcriptase of HIV and RNA polymerase of influenza.

The most pronounced *in vitro* anti-influenza and antiherpes activity of polyphenol was reported with the Bulgarian folk medicine *Geranium sanguineum* (Table 15.1), but broad *in vitro* antiviral activities of polyphenols do not correspond to their *in vivo* activities [49]. The structure–activity analyses indicate that the site(s) and number of hydroxyl groups on phenols are responsible for their antiviral activity as evident with the catechol (Fig. 15.3) and pyrogallol. The polyphenols isolated from the extracts of *Agrimonia pilosa*, *Pithecellobium clypearia*, and *Punica granatum*,

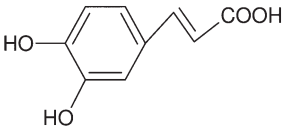


Fig. 15.1 Caffeic acid.

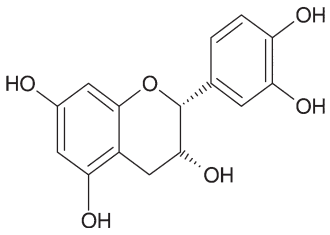


Fig. 15.2 Procyanidin B2.

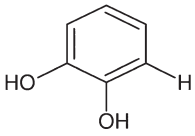


Fig. 15.3 Catechol.

plants of southern mainland China, showed anti-HSV-1 activity, while the Peruvian plant *Stylogne cauliflora* had anti-HCV NS3 protease activity due to oligophenols [50]. On the other hand, polyphenols extracted from *Blumea laciniata*, *Elephantopus scaber*, and *Scutellaria indica* exhibited anti-RSV activity [51].

15.3.2 Coumarins

Coumarins are phenolics with fused benzene (Fig. 15.4) and α-pyrone rings, responsible for the characteristic odor of hay. They have a "species-dependent metabolism" and toxic coumarin derivatives may be safely excreted in human urine [52]. It is reported that the coumarins can stimulate macrophages [53] and thereby exert an indirect effect on viral infections, as found with the oral anticoagulant warfarin (Fig. 15.5), which prevents recurrences of cold sores caused by HSV-1 in humans [54]. Specific antiviral properties of coumarins are scarce (Table 15.2), though some, like the coumarins of *Prangos tschimganica* have anti-HIV activity [55].

The most exciting natural reverse transcriptase inhibitors are 4-propyldipyranocoumarins, or calanolides, isolated from the tropical rainforest trees *Calophyllum lanigerum* var. *austrocoriaceum* and *C. inophyllum* of Sarawak in Borneo Island of Malaysia [56]. *Calophyllum* coumarins are classified as calanolides, inophyllums, and cordatolides, according to the C-4 substituent on the lactone ring. Structure–activity relationship studies revealed that methyl groups at C-10 and C-11 and a hydrogen bond at C-12 are responsible for anti-HIV-1 activity. Because of its potent activity on HIV-1 reverse transcriptase, calanolide A represent a novel natural non-nucleotide reverse transcriptase inhibitor and may be useful in combination with other antiretroviral drugs [16].

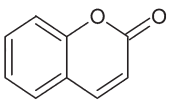


Fig. 15.4 Coumarin.

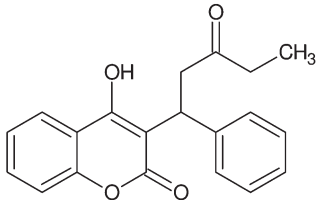


Fig. 15.5 Warfarin.

15.3.3
Quinones

Quinones have aromatic rings with two ketone substitutions (Fig. 15.6), and are highly reactive. These colored compounds are responsible for the browning reaction in cut or injured fruits and vegetables, dying of henna and are an intermediate of the melanin synthesis pathway [57]. The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxido-reduction reactions and hence, the individual redox potential of a particular quinone–hydroquinone pair is very important in biological systems. Quinones provide a source of stable free radicals, and also irreversibly bind with nucleophilic amino acids in proteins [58] leading to the inactivation and loss of protein function. Hence, the range of antiviral effects of quinone is considerable, and the probable target of quinones is the virus attachment site and some viral enzymes (Table 15.2).

Hypericin (Fig. 15.7), an antidepressant anthraquinone from *Hypericum perforatum* (St. John's wort), is reported to have antiviral activity [59], while chrysosplenol C is a potent and specific inhibitor of picornaviruses and rhinoviruses, the common cold viruses [60]. Chrysophenol C isolated from the Australian plant *Dianella longifolia* and chrysophanic acid from *Pterocaulon sphacelatum* inhibits the replication of poliovirus 2 and 3 due to hydrophobic C-6, and the methyl group at C-3 of chrysophanate molecule [61]. It is evident that the introduction of an aryl group at the piperazine moiety of fluoroquinolone is responsible for its antiviral activity, with a specific action on HIV by inhibiting transcription and *tat* functions [62]. Substitution of the fluorine at position 6 with an amine group yielded aryl-piperazinyl-6-amino-quinolone, which is a selective and potent inhibitor of HIV-1 replication by interfering with *tat-TAR* interactions [62], and can be useful for rational drug design with optimized antiviral activity.

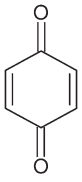


Fig. 15.6 Quinone.

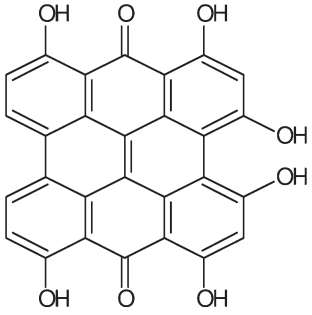


Fig. 15.7 Hypericin.

15.3.4
Flavones, Flavonoids, and Flavonols

Flavones are hydroxylated phenolics (Fig. 15.8) containing one carbonyl groups (two in quinones), while the addition of a 3-hydroxyl group yields a flavonol [63]. Flavonoids occur as a C_6–C_3 unit linked to an aromatic ring and are synthesized in

response to microbial infection of plants [64], and hence have broad spectrum of antimicrobial activity. The antiviral activity of flavones is due to their ability to form a complex with extracellular and soluble proteins, and partly due to interference with virus–cell binding, as found in glycyrrhizin [65]. Flavonoids can inhibit several critical steps of the viral life cycle, such as viral protease, integrase, and reverse transcriptase. Flavanones with an OH group at C-3 (taxifolin) inhibit protease, reverse transcriptase, and CD4/gp120 interactions; while flavanones lacking an OH group at C-3, such as aromadendrin, are more specific inhibitors of CD4/gp120 interaction [15, 16].

The nonspecific anti-HIV-1 activity of (–)-epigallocatechin 3-*O*-gallate is due to its ability to disrupt post-adsorption entry and inhibition of viral protease and reverse transcriptase [66] (Table 15.2). The C-4 sulfated isoflavonoid torvanol A and the steroidal glycoside torvoside H of *Solanum torvum* fruits are found to have strong anti-HSV-1 activity [67]. Galangin, a 3,5,7-trihydroxyflavone, isolated from *Helichrysum aureonitens* inhibited HSV-1 and coxsackie B virus, while isoquercitrin of *Waldsteinia fragarioides* had anti-HSV activity; but swertifrancheside, glycyrrhizin, and chrysin (Fig. 15.9) had anti-HIV activities [15, 16]. Kaul et al. [68] reported that hesperetin inhibited replication of HSV, poliovirus 1, parainfluenza 3, and RSV, but catechin (Fig. 15.10) inhibited infectivity of RSV and HSV-1, while quercetin inhibited all the four viruses, as the small structural differences of these compounds are critical to their activity. Robin et al. [69] found that 3-methylkaempferol of *Psiadia dentata* is the most potent inhibitor of genomic RNA synthesis of poliovirus, while dihydroxyoleanoic acid, indole-3-carboxylic acid, and (–)-catechin isolated from *Begonia nantoensis* inhibited HIV replication [70], but oxyresveratrol from *Millettia erythrocalyx* and *Artocarpus lakoocha* inhibited both HSV and HIV-1 [71]. Similarly the flavone glycoside dihydroxy-trimethoxyflavone-β-D-xylopyranosyl-β-D-glucopyranoside isolated from *Butea monosperma* seed had broad-spectrum antiviral activity [72]. Wogonin, a natural antioxidant monoflavonoid, with rapid tissue distribution and prolonged plasma elimination rate, had anti-inflammatory,

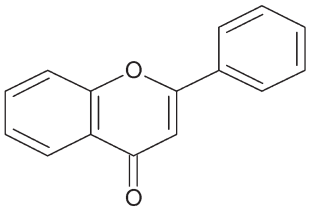


Fig. 15.8 Flavone.

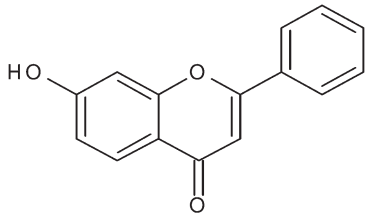


Fig. 15.9 Chrysin.

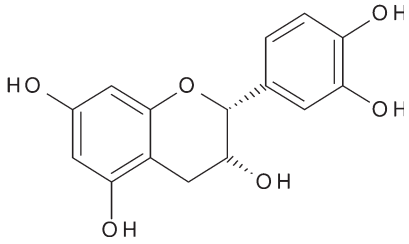


Fig. 15.10 Catechin.

anticancer, neuroprotective, and antiviral activities [73], and thus may be helpful in designing antirabies and antiencephalitis drugs.

A considerable amount of evidence suggests that bioflavonoids are health-promoting, disease-preventing dietary compounds and are the basis of many traditional folk remedies. Some are applied in therapy or used as prototypes for the development of specific drugs [15, 16]. The antiviral activities of plant bioflavonoids have been evaluated [74] and reviewed [15] earlier. The black tea flavonoid theaflavin is an antioxidant and has been shown to neutralize bovine rotavirus and coronavirus [75], while the flavonol iridoid glycosides luteoside isolated from *Barleria prionitis* and *Markhamia lutea* root have potent anti-RSV activity [76]. Flavones isolated from *Rhus succedanea* and *Garcinia multiflora* showed various antiviral activities: amentoflavone and robustaflavone inhibited HSV; amentoflavone, robustaflavone, and agathisflavone inhibited influenza virus; while measles and VZV were inhibited by rhusflavanone and succedaneflavanone [77].

The anti-inflammatory flavonoids baicalein and baicalin, obtained from *Scutellaria baicalensis* of China, interacted with HIV envelope glycoproteins and chemokine coreceptors to block virus entry to CD4 cells [78] and inhibited HIV-1 replication. The bioflavonoids arctiin, phillyrin, liquiritin, genistein (Fig. 15.11), daidzein, glycitrin, and chlorogenic acid inhibited influenza virus [79], while cinnamoylbenzaldehyde and lawinal of *Desmos* spp. blocked HIV-1 replication [80] and mulberroside C and leachianone G from *Morus alba* root inhibited HSV-1 [81] (Table 15.2). The flavonoids of *Aesculus chinensis* seed extract inhibited RSV and influenza A [82], while morin, another flavonoid group, isolated from *Maclura cochinchinensis*, is a powerful anti-HSV-2 agent. The structure– activity relationship studies indicated that most of these flavonoids (baicalein, quercentin, and myricetin) not only block the virus-associated reverse transcriptase but also the cellular DNA or RNA polymerases of HIV [16], and the degree of inhibition depends on the structure and side-chain. The triterpene ursolic acid isolated from *Geum japonicum* inhibits HIV-1 protease activity [16, 83], while oleanolic acid, betulinic acid (Fig. 15.12), ursolic acid, and their derivatives inhibit HIV protease and the stability of gp120/gp41 complex [83].

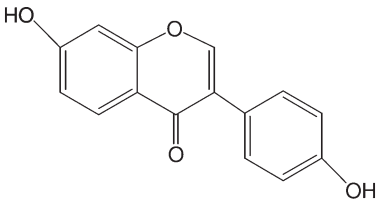


Fig. 15.11 Genistein.

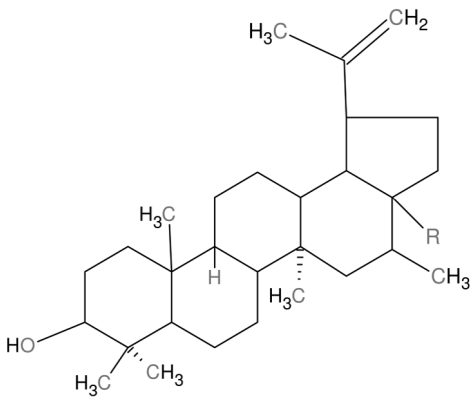


Fig. 15.12 Betulinic acid (R = COOH).

15.3.5
Tannins

Tannins are a group of polymeric phenolics with molecular weights of 500–3000 found in almost every plant part that are capable of tanning leather or precipitating gelatin from solution (astringency) (Fig. 15.13). They are classified as hydrolyzable or condensed tannins. Hydrolyzable tannins are based on gallic acid, while the condensed tannins (proanthocyanidins) are derived from flavonoid monomers. The consumption of tannin-containing beverages, such as green teas and red wines, is reported to cure or prevent a variety of illnesses because tannins can stimulate phagocytic cells, inhibit tumors and a wide range of microbes by forming complexes with microbial proteins through hydrophobicity, and hydrogen and covalent bonding [84]. Thus, their mode of antiviral action is to inactivate adsorption, transport proteins, polysaccharides and reverse transcriptase enzyme [15, 68], as found with the anti-HSV-1 and HIV-1 reverse transcriptase inhibitors shephagenin, strictinin (Table 15.2), and hippophaenin of *Shepherdia argentea* [16, 17].

The phenolic eugeniin from *Geum japonicum* and *Syzygium aromaticum* can block viral DNA polymerase and thereby inhibit acyclovir-resistant and thymidine kinase-deficient HSV-1, wild HSV-2, and Epstein–Barr virus [17, 85]. Extracts of *Bergenia ligulata* rhizomes of Nepal inhibit influenza virus replication by blocking RNA and protein synthesis in a dose-dependent manner [86]. Similarly, the gallotannin geraniin isolated from *Phyllanthus amarus* inhibited HIV-1 replication by inhibiting reverse transcriptase in a dose-dependent manner [87]. The hydrolyzable tannin casuarinin obtained from *Terminalia arjuna* bark is virucidal and inhibits HSV-2 attachment and penetration [88], but camelliatannin H from *Camellia japonica* pericarp inhibits HIV-1 protease [89]. Tannin from *Prunella vulgaris* and *Rhizoma cibotte* inhibit HIV-1 entry to CD4 cells by blocking gp41 six-helix bundle formation, a critical step of membrane fusion between HIV and the target cell [90].

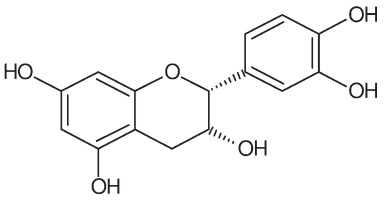


Fig. 15.13 Tannin.

15.3.6
Lignans

Lignans are widely distributed in plants and very few of them have antiviral activities. The extracts of *Larrea tridentates*, *Rhinacanthus nasutus*, and *Kadsura matsudai*, showed anti-HIV, anti-influenza, and antiherpes activities [91, 92], while the extracts of *Rhus javanica* exhibited anti-HSV-2 activity similar to acyclovir [93].

15.3.7
Terpenoids and Essential Oils

The essential oil or *quinta essentia* is responsible for the fragrance of plants. Phenolic compounds with a C-3 side-chain and at a lower level of oxidation without any oxygen are classified as essential oils. The oils that are highly enriched in the isoprene structure (Fig. 15.14) are called terpenes, having the general chemical formula $C_{10}H_{16}$. They occur as di (C_{20}), tri (C_{30}), and tetraterpenes (C_{40}), as well as hemi (C_5) and sesquiterpenes (C_{15}). When they contain additional elements such as oxygen, they are called as terpenoids, which are active against many viruses. The furanoditerpene caesalmin, isolated from *Caesalpinia minax* seeds, has been shown to inhibit parainfluenza 3, while ovatodiolide from *Anisomeles indica* had anti-HIV activity and the triterpenoid betulinic acid can inhibit HIV [83]. The tetracyclic furanoditerpenoid caesalmin is more potent than the furanoditerpenoid lactone. The triterpene vaticinone isolated from *Vatica cinerea* of Vietnam inhibited HIV-1 replication [94], while arganine C, a triterpene saponin from *Tieghemella heckelii* fruits, inhibited HIV entry into host cells, suggesting their usefulness as potential antiviral agents [95].

The inhibition of VSV by saikosaponins, iridoids, and glycosides isolated from *Bupleurum rigidum* and *Scrophularia scorodonia* [96], as well as the virucidal activity of the iridoid maesasaponin of *Maesa lanceolata* against HSV-1, are due to diacylation [97]. The sandalwood oil of *Santalum album* showed a dose-dependent anti-HSV-1 activity, but the essential oil of the Italian food plant *Santolina insularis* inhibited cell-to-cell transmission of herpesviruses, while pulegone of *Minthostachys verticillata* inhibited HSV-1 and pseudorabies virus multiplication [15, 98]. The terpinen-4-ol, the essential oil of tree tea *Melaleuca alternifolia*, used as an antimicrobial preservative in many pharmaceutical cosmetics, exhibited strong virucidal activity against HSV-1 and HSV-2, while *Eucalyptus* oil reduced HSV titers from 57.9 to 75.4%, as both the oils affect HSV before or during adsorption [99]. The essential oil of Argentinean plant *Lippia junelliana* and *L. turbinata* is virucidal against Junin virus, while essential oils of *Artemisia douglasiana* and *Eupatorium patens* inhibit HSV-1 and dengue 2 viruses [100], but *Melissa officinalis* oil inhibits HSV-2 replication [101] (Table 15.2). Although the active antiherpes components of tea tree and eucalyptus oil are not very clear, their application in recurrent herpes infection is promising.

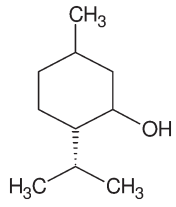


Fig. 15.14 Terpenoid (menthol).

15.3.8
Alkaloids

Alkaloids are heterocyclic nitrogen compounds. The morphine (from the Greek *Morpheus*, god of dreams), isolated in 1805 from the opium poppy *Papaver somniferum*, were the first alkaloid used in medicine. Solamargine and michellamine B, the glycoalkaloids of *Solanum khasianum* berries, has been shown to inhibit HIV, while berberine inhibited intestinal infections associated with AIDS [102]. Duan et al. [103] reported that the pyridine alkaloid triptonine from *Tripterygium hypoglaucum* and a clinically used extract of *T. wilfordii* have potent anti-HIV activity [104], but matairesinol and harman from *Symplocos setchuensis* inhibited HIV replication [16]. The skimmianine isolated from the extract of *Zanthoxylum chalybeum* seed inhibited Edmonston and Swartz strains of measles virus [105]. On the other hand, the aromoline alkaloid of *Stephania cepharantha* root tuber, used in folklore medicine of China and Mongolia, inhibited HIV-1 [106], while the isoquinoline alkaloid thalimonine of *Thalictrum simplex* inhibited influenza A replication by reducing the expression of viral neuraminidase, hemagglutinin, nucleoprotein, and virus-specific protein synthesis [107]. Szlavik et al. [108] reported that lycorine, homolycorine, and acetyllycorine hemanthamine isolated from *Leucojum vernum* possessed high antiretroviral activities with low therapeutic indices, while drymaritin isolated from *Drymaria diandra* had anti-HIV activity [109]. Interestingly the whole extract and harman alkaloid fraction of *Ophiorrhiza nicobarica*, a folklore plant of the Little Andaman Islands, completely inhibited the plaque formation and delayed the eclipse phase of HSV replication [110].

Cepharanthine (Fig. 15.15), a biscoclaurine alkaloid isolated from a Chinese folklore plant *Stephania cepharantha*, inhibited HIV-1 replication by inhibiting kappa B, a potent inducer of HIV-1 gene expression [104] and displayed potent activity against SARS coronavirus, HSV-1, and coxsackie B3 (Table 15.1) along with antitumor and immunomodulating activity [111]. As cepharanthine had strong activity against both RNA and DNA viruses it may be a source of potential lead compounds for developing new antivirals.

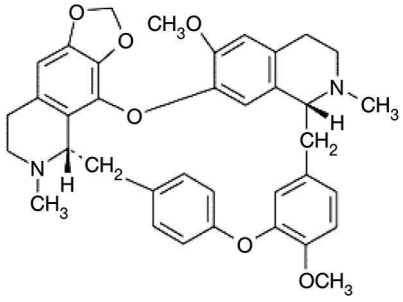


Fig. 15.15 Cepharanthine.

15.3.9
Lectins, Polypeptides and Sugar-Containing Compounds

Antimicrobial peptides are often positively charged and contain disulfide bonds. Meliacine isolated from *Melia azedarach* leaves of Argentina, and many common plants [14], has strong activity against HSV-1-induced ocular disease [24]. Meliacine is also reported to inhibit multiplication of Junin virus and foot-and-mouth disease virus by blocking virus fusion (i.e. uncoating and budding) [15, 112] and spread of the virus. The larger mannose-specific lectins MAP30 (a 30 kDa protein of *Momordica charantia*), GAP31 (31 kDa protein of *Gelonium multiflorum*), and jacalin inhibited proliferation of HIV and cytomegalovirus (Table 15.2) by inhibiting host–viral interaction [16, 113]. Xylanase, a 15-kDa protein from *Panax notoginseng*, and 5-kDa peptides isolated from pinto and red beans inhibited HIV reverse transcriptase [114]. Thai bitter gourd protein MRK29 inhibited HIV-1 reverse transcriptase and its salt-precipitated fraction strongly reduced viral p24 expression in HIV-infected cells but increased TNF activity [115], indicating its immunomodulatory role. The mannose-specific lectins of *Cymbidium* hybrid, *Epipactis helleborine*, *Hippeastrum*, and *Listeria ovata*; and the *N*-acetylglucosamine (NAG)-specific lectins of *Urtica dioica* inhibited the HIV-1 fusion process by interacting with specific glycosylation sites within the viral gp120 and/or gp41 [16, 83].

Polysaccharides from *Rhizophora apiculata* leaf and *R. mucronata* bark prevent HIV budding by blocking capsid protein p24 expression [16] to stop the infection cycle. On the other hand, aloe polymannose (AP) of *Aloe barbadensis* potentiated antibody production against capsid protein epitopes of nonenveloped picornaviruses and enhanced antibody concentrations against enteroviruses and poliovirus vaccine strains [116]. The heterogeneous anionic polysaccharide with different ionic charges stevian isolated from *Stevia rebaudiana* and *Achyrocline flaccida*, inhibited the replication of HSV-1 and four serotypes of human rotavirus by blocking the virus binding [117], while the acidic polysaccharides of *Cedrela tubiflora* inhibited HSV-2 and VSV replication [118] (Table 15.2), indicating that the antiviral activity of polysaccharides correlates with their molecular weight and sulfate content.

15.4
Mixtures and Other Compounds

Ayurvedic medicine, Chinese traditional medicine, and many other ethnomedicinal systems rely on both "pure" single-plant preparations and mixed formulations with many plants. Propolis, a crude extract of the balsam of various trees, contains terpenoids, flavonoids, benzoic acids and esters, phenolic acids and esters, and was found to inhibit hemagglutination activity of influenza virus, acyclovir-resistant HSV-1, adenovirus 2, VSV, and poliovirus, because the compounds in the mixture act synergistically, while flavone and flavonol were active in isolation against HSV-1 [119]. On the other hand, the kaempferol crassirhizomoside and sutchuenoside of *Dryopteris crassirhizoma* inhibited reverse transcriptase-associated DNA polyme-

rase and RNase H activities [120]. Similarly the flavonoids, triterpenoids, and their glycosides isolated from *Azadirachta indica* leaf inhibited plaque formation in six antigenic types of Coxsackie virus B by interfering with the early steps of replication [121], while *Artemisia capillaris* inhibited HIV replication due to a mixture of three compounds [122].

Reports on the antiviral activity of other phytochemicals are very scarce. The methyl esters dehydrochebulic acid and methyl brevifolin carboxylate isolated from *Phyllanthus urinaria* have anti-HBV activity [123], while diprenylated bibenzyl of *Glycyrrhiza lepidota* leaf extract inhibited HIV-1 (Table 15.1) replication [124]. An extract of fresh garlic, called ajoene, can protect CD4 cells from HIV attack early in the viral life cycle and its anti-HIV activity is 45 times more powerful than that of dextran sulfate. This is because the garlic impairs the activities of liver enzymes that process protease inhibitors and thereby raises the protease inhibitor levels [16]. The free hydroxyl group of galloyl residues of tertagalloyl-glucopyranose obtained from *Juglans mandshurica* inhibited reverse transcriptase and RNase H activity; while asiaticoside of *Centella asiatica* and mangiferin of *Mangifera indica*, used as a herpesvirus remedy in Thailand, have anti-HSV activities. Interestingly, combinations of any of these extracts with acyclovir resulted in additive or synergistic inhibition of HSV-2 [125].

15.5 Experimental Approaches

15.5.1 *In Vitro* Efficacy

The antiviral or virucidal efficacy of plant extracts can be detected by several methods. Usually cytopathic effects or plaque formation or transformation or proliferative effects on cell lines [68, 126] are the markers of antiviral testing. Viral replication can be assayed by detection of viral DNA, RNA, or polypeptides. The assay method for antiviral substances used in various laboratories is not yet standardized and, therefore, the results are often not comparable. Hence, researchers have to distinguish between merely toxic effects and true antiviral properties of phytomedicines. Table 15.3 lists the major *in vitro* antiviral screening assays. However, it is

Table 15.3 Major in vitro antiviral assays.

Virus nature	*Assay type*	*Assay methods*
Viruses that forms plaques in suitable cell line	Plaque inhibition	Titer determination in presence of nontoxic dose of a single compound
	Plaque reduction	Titer determination of viral infectivity after extracellular action of a single or mixture of compounds

Table 15.3 Major in vitro antiviral assays. (Continued)

Virus nature	*Assay type*	*Assay methods*
Viruses that induce CPE in cell culture	Inhibition of virus-induced CPE	Determination of CPE in monolayer infected with limited dose of virus in liquid media for single compound or mixture
	Virus yield reduction	Determination of virus yield in cell line infected with a given amount of virus and treated with single or mixture of test compounds. Carried out after plaque reduction test or 50% TCI end-point.
	End-point titer determination	Determination of virus titer reduction in 2-fold dilutions of a single compound or mixture
Viruses which do not form plaque or induce CPE	Virus-specific function determination	Hemagglutination test
		Hemadsorption test (Myxoviruses)
		Inhibition of cell transformation (EBV)
		Immunological tests: Detection of antiviral antigens in cell culture (HSV, CMV, EBV, HIV)
Special tests	Nucleic acid/ polypeptide inhibition	Reduction or Inhibition of viral specific nucleic acid/polypeptides synthesis in infected culture
	Radioisotope uptake study	Determination of uptake of radioisotope-labeled precursors
	Genome number determination	Viral genome copy (numbers) with single compound or with mixtures

CPE, cytopathic effect; TCI, tissue culture infective dose;
HSV, herpes simplex virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus;
HIV, human immunodeficiency virus.

important to note that the antiviral assays that prevent virus adsorption to host cells are usually overlooked in screening procedures, though a large body of literature indicated that phytochemicals should be evaluated for anti-fusion or anti-adsorption action in addition to their killing and inhibitory activities.

15.5.2 Clinical Trials in Humans

Traditional phytomedicines have been used for centuries to treat infections in aboriginal or ethnic communities, but controlled clinical studies are scarce. Sometimes traditional healers working with trained scientists keep records of the safety

and effectiveness of treatments, but these are usually uncontrolled and unrandomized studies. Some randomized clinical trials of plant antivirals have been reported to date [15, 126]. Two proprietary compounds prepared from tropical plants are Provir and Virend. Provir is used to treat respiratory viral infections, while Virend is a topical antiherpes agent. Both are tested, but only the efficacy and safety of Virend have been established [126]. A trial with andrographolide from *Andrographis paniculata* on 13 HIV patients and 5 uninfected healthy volunteers showed a significant rise in the mean CD4+ lymphocyte level with 10 mg kg^{-1} andrographolide. However, no significant change in mean plasma HIV-1 RNA levels was found as andrographolide dysregulate the HIV-induced cell cycle, leading to a rise in CD4+ levels [127].

In a hepatitis B clinical trial *Phyllanthus amarus* extract eliminated detectable HBV antigen from the sera of 59% treated human carriers as compared to 4% of placebo controls [13, 128]. A review of randomized trials on *Phyllanthus* in chronic HBV patients by Liu et al. [129] showed *Phyllanthus* species had a positive effect on the clearance of serum HBsAg compared with placebo or no intervention, and is better than nonspecific or other herbal treatment. It appears from these studies that some *Phyllanthus* species have positive effects on HBV and liver biochemistry in chronic hepatitis infection. Another review on randomized clinical trials showed that the aqueous extract of the Chinese herb *Sophorae flavescentis* (matrine) had anti-HBV activity with protective effects on liver function in chronic HBV patients. However, *Phyllanthus* or matrine cannot be recommended for routine clinical use due to low methodological quality and variations in the herbal preparations [130].

Martin and Ernst [131] found that hundreds of herbal preparations had antiviral activity, but extracts from only 11 species met the inclusion criteria. Out of 33 randomized and 8 nonrandomized trials 14 used *Phyllanthus* spp. of which seven showed positive results. On the other hand, 27 trials with 10 other herbs yielded six positive results. Tani et al. [132] conducted a long-term (1992–2000) treatment of pediatric AIDS patients with Romanian folk medicine and found that 92 months of treatment helped to decrease HIV-RNA levels below measurable levels in 90% cases. However, at least 1–3 years of treatment was required to get the beneficial effects without any side effects and the emergence of drug-resistant strains. A controlled study with the Japanese Oriental medicine Mao-to in 18 chronic hepatitis C patients showed that the treatment relieves the side effects of interferon (IFN) [133]. In another study 12 chronic HCV patients were treated with a combination of IFN-beta and Mao-to or Dai-seiryu (groups A and B), and 16 with IFN alone (group C). Mao-to was administered to eight patients and Dai-seiryu-to four, in A and B groups respectively. In all patients, HCV-RNA was negative and serum alanine aminotransferase levels were normal at the end of the treatment, indicating that these phytomedicines reduced the adverse effects of IFN treatment in chronic HCV patients [133].

A placebo-controlled double blind pilot study on 30 HCV patients with a phenol-rich antioxidant grain food showed that antioxidant food considerably improved defense. In 11 of the 15 patients (who received 6 g food powder three times daily for three months) liver enzymes were decreased and the viral load remained unchanged,

compared to placebo (15 patients who received an herbal extract with no antioxidant). After three months of treatment a sustained response was observed in 5 of 9 antioxidant-pretreated patients when they received interferon and ribavirin treatment even six months after discontinuation of the 12-month antioxidant therapy [134].

15.6 Future Prospects

Considerable evidence of the antiviral activity of plant medicines against herpes and retroviruses has been documented [15–17, 40], but there are many parameters to be considered for the evaluation of antiviral activity, such as the methods of extraction, the plant parts to be used, season(s) for collection, and administration. The pokeweed antiviral protein (PAP) of *Phytolacca americana*, which cause depurination of HIV genomic RNA, and ribosome-inactivating proteins (RIPs) that inhibit viral protein synthesis, alter ribosomal function, depurinate rRNAs and nucleic acid [15, 16] should also be considered. Trichobitacin, an RIP isolated from *Trichosanthes kirilowii* root reduced the p24 antigen expression in HIV-1. While PAP29 of *Phytolacca americana*, a single-chain RIP similar to MAP30 and GAP31, can be used as a prophylactic anti-HIV agent, as they inactivate viruses and virus-infected cells in semen [16], but further investigation on these potential agents is essential.

Similarly, niruriside isolated from *Phyllanthus niruri* inhibited HBV, and reverse transcriptase of HIV can block the binding of a regulator gene that expresses virion protein REV to stop viral propagation [135] in HIV infected individuals. These phytomedicine can help to stop viral propagation. Clearly, there are areas of natural product research that are "blooming" in-so-far as derivation of unusual or "unnatural" structures are concerned, and thus, in the coming days they will become a major source of novel antivirals with pharmacological promise. The identified structure of bioactive extracts will also help to synthesize new agents with improved pharmacokinetics and toxicology. The molecules that are used by ethnic or aboriginal groups can also act as a template for combinatorial approaches or as sources of simplified molecules, as has been shown with bryostatins and ecteinascidins, where simpler but biologically active molecules have been made as a result of synthetic endeavors.

15.7 Conclusions

Many of the viral diseases are still fatal and or are not yet curable, although some can be kept under control with life-prolonging drugs, but those expensive drugs are beyond the reach of the majority of the global population. Therefore, the development of safe, effective, and inexpensive antivirals is among the top global priorities of drug development. Furthermore, the long-term combination therapies for

retroviruses may yield drug-resistant mutants. Therefore, scientists from divergent fields are investigating ethnomedicinal plants, with an eye to their antiviral usefulness. A sense of urgency accompanies the search as the pace of species extinction continues.

Different laboratories have found hundreds of phytochemicals with inhibitory effects on different types of viruses *in vitro*. Many of these have been subjected to animal and human studies to determine their effectiveness in whole-organism systems, including toxicity studies. It would be advantageous to standardize methods of extraction and *in vitro* testing to make the search more systematic. Also, alternative mechanisms of infection prevention and treatment, such as prevention of virus entry into host cells and blocking of specific cellular enzymes, should be included in screening programmes. As a significant number of plant extracts display antiviral activities, it seems reasonable to conclude that there are probably numerous antiviral compounds from a wide range of different structural classes in these extracts and further characterization of bioactive extracts will help to elucidate the exact antivirals and their mechanism of action. Hence, the traditional use of these phytomedicines for viral disease treatment is justified.

Finally, the development of new phytomedicines is vital to control the threats posed by emerging, re-emerging, and drug-resistant viruses, because most viral diseases are difficult to eliminate using the available antivirals.

Acknowledgments

The authors wish to acknowledge Dr Sujit K. Bhattacharya, the Additional Director General, Indian Council of Medical Research, New Delhi, Director, National Institute of Cholera and Enteric Diseases (NICED), and the Officer-in Charge, ICMR Virus Unit, Kolkata and Dr T.N. Naïk, Senior Deputy Director, NICED, Kolkata, for their constant help and encouragement during the preparation of this manuscript.

References

1 Newman, D.J., Cragg, G.M., Snader, K.M. *Nat. Prod. Rep.* **2000**,*17*, 215–234.
2 Dev, S. *Environ. Health Perspect.* **1999**, *107*, 783–789.
3 Grabley, S., Thiericke, R. *Adv. Biochem. Eng. Biotechnol.* **1999**, *64*, 104.
4 Borris, R.P. *J. Ethnopharmacol.* **1996**, *5*, 29–38.
5 Chang, H.M., But, P.P.H. *Pharmacology and Applications of Chinese Materia Medica*, Vols 1 and 2, World Scientific Inc., Singapore, Philadelphia, PA, **1986**.
6 Schultes, R.E., Raffauf, R.F. *The Healing Forest*. Dioscorides Press, Portland, **1990**.
7 Farnsworth, N.R. The role of ethnopharmacology in drug development. In *Bioactive Compounds from Plants*, Chadwick, D.J., Marsh, J. (eds). Ciba Foundation Symposium 154. John Wiley and Sons, Chichester, **1990**, pp. 2–21.
8 Lewis, W.H., Elvin–Lewis, M.P. *Ann. Missouri Bot. Garden* **1995**, *82*, 16–24.
9 De Clercq, E. *Clin. Microbiol. Rev.* **1995**, *8*, 200–239.
10 Chattopadhyay, D., Chakraborty, M.S., Saha G.C. *Ind. J. Sexual Transmitted Dis.* **1999**, *20*, 54–60.

11 Wagner, E.K., Hewlett, M.J. *Basic Virology*. Blackwell Science, Malden, MA, **1999**.
12 Venkateswaran, P.S., Millman, I., Blumberg, B.S. *Proc. Natl Acad. Sci. USA* **1987**, *84*, 274–278.
13 Thyagarajan, S.P., Jayaram, S., Valliammai, T. *Lancet* **1990**, *336*, 49–50.
14 Hudson, J.B. *Antiviral Compounds from Plants*. CRC Press, Boca Raton, Boston, **1990**.
15 Jassim, S.A., Naji, M.A. *J. Appl. Microbiol*. **2003**, *95*, 412–427.
16 Cos, P., Maes, L., Vanden Berghe, D., Hermans, N., Pieters, L., Vlietinck, A. *J. Nat. Prod*. **2004**, *67*, 284–293.
17 Khan, M.T., Ather, A., Thompson, K.D., Gambari, R. *Antivir. Res*. **2005**, *67*, 107–119.
18 Chantrill, B.H., Coulthard, C.E., Dickinson, L., Inkley, G.W., Morris, W., Pyle, A. H. *J. Gen. Microbiol*. **1952**, *6*, 74–84.
19 Rao, A.R., Kumar, S.S.V., Paramasivam, T.B., Kamalakshi, S., Parashuraman, A.R., Shanta, B. *Ind. J. Med. Res*. **1969**, *57*, 495–502.
20 McCutcheon, A.R., Roberts, T.E., Gibbons, E., Ellis, S.M., Babiuk, L.A., Hancock, R.E., Towers, G.H. *J. Ethnopharmacol*. **1995**, *49*, 101–110.
21 Glatthaar-Saalmuller, B., Sacher, F., Esperester, A. *Antivir. Res*. **2001**, *50*, 223–228.
22 Turan, K., Nagata, K., Kuru, A. *Biochem. Biophys. Res. Commun*. **1996**, *225*, 22–26.
23 Abad, M.J., Guerra, J.A., Bermejom P., Irurzunm A., Carrascom L. *Phytother. Res*. **2000**, *14*, 604–607.
24 Alche, L.E., Berra, A., Veloso, M.J., Coto, C.E. *J. Med. Virol*. **2000**, *61*, 474–480.
25 Wang, Z.J., Yang, Z.Q., Huang, T.N., Wen, L., Liu, Y.W. *Zhongguo Zhong Yao Za Zhi* **2000**, *25*, 685–687.
26 Rajbhandari, M., Wegner, U., Julich, M., Schopke, T., Mentel, R. *J. Ethnopharmacol*. **2001**, *74*, 251–255.
27 Hsiang, C.Y., Hsieh, C.L., Wu, S.L., Lai, I.L., Ho, T.Y. *Am. J. Chin. Med*. **2001**, *29*, 459–467.
28 Fortin, H., Vigor, C., Lohezic–Le Devehat, F., Robinm V., Le Bossem B., Boustiem J., Amoros, M. *Fitoterapia* **2002**, *73*, 346–350.
29 Bodinet, C., Mentel, R., Wegner, U., Lindequist, U., Teuscher, E., Freudenstein, J. *Medicine* **2002**, *68*, 896–900.
30 Lipipun, V., Kurokawa, M., Suttisri, R., Taweechotipatr, P., Pramyothin, P., Hattori, M., Shiraki, K. *Antiviral Res*. **2003**, *60*, 175–180.
31 Chiang, L.C., Cheng, H.Y., Liu, M.C., Chiang, W., Lin, C.C. *Biol. Pharmac. Bull*. **2003**, *26*, 1600–1604.
32 Chen, T., Jai, W.X., Yang, F.L., Xie, Y., Yang, W.Q., Zeng, W., Zhang, Z.R., Li, H., Jiang, S.P., Yang, Z., Chen, J.R. *Zhonggou Zhong Yao Za Zhi* **2004**, *29*, 882–886.
33 Dong, Y., Li, H., Yao, Z., Tian, W., Han, Z., Qiu, H., Piao, Y. *Zhong. Yao Cai*. 2004, *27*, 425–427.
34 Tshikalange, T.E., Meyer, J.J., Hussein, A.A. *J. Ethnopharmacol*. **2005**, *96*, 515–519.
35 Sokmen, M., Angelova, M., Krumova, E., Pashova, S., Ivancheva, S., Sokmen, A., Serkedjieva, J. *Life Sci*. **2005**, *76*, 2981–2993.
36 Wands, J.R. *The New England J Med*. **2004**, *35* (15), 1567–1570.
37 Ho, T.Y., Wu, S.L., Lai, I.L., Cheng, K.S., Kao, S.T., Hsiang, C.Y. *Antivir. Res*. **2003**, *58*, 199–208.
38 Kageyama, S., Kurokawa, M., Shiraki, K. *Antivir. Chem. Chemother*. **2000**, *11*, 157–164.
39 Min, B.S., Kim, Y.H., Tomiyama, M., Nakamura, N., Miyashiro, H., Otake, T., Hattori, M. *Phytother. Res*. **2001**, *15*, 481–486.
40 Asres, K., Bucar, F., Kartnig, T., Witvrouw, M., Pannecouque, C., De Clercq, E. *Phytother. Res*. **2001**, *15*, 62–69.
41 Bedoya, L.M., Palomino, S.S., Abad, M.J., Bermejom P., Alcami, J. *Phytother. Res*. **2002**, *16*, 550–554.
42 Premanathan, M., Rajendran, S., Ramanathan, T., Kathiresan, K., Nakashima, H., Yamamoto, N. *Ind. J. Med. Res*. **2000**, *112*, 73–77.
43 Chang, Y.S., Woo, E.R. *Phytother. Res*. **2003**, *17*, 426–429.
44 Ayisi, N.K., Nyadedzor, C. *Antivir. Res*. **2003**, *58*, 25–33.
45 Ishikawa, T., Nishigaya, K., Takami, K., Uchikoshi, H., Chen, I.S., Tsai, I.L. *J. Nat. Prod*. **2004**, *67*, 659–663.

46 Sydiskis, R.J., Owen, D.G., Lohr, J.L., Rosler, K.H., Blomster, R.N. *Antimicrob. Agents Chemother.* **1991**, *35*, 2463–2466.

47 Cheng, H.Y., Lin, T.C., Yang, C.M., Shieh, D.E., Lin, C.C. *J. Sci. Food Agric.* **2005**, *85*, 10–15.

48 Cadman, C.H. In *Phenolics in Plants in Health and Disease*, Pridham, J.B. (ed.). Pergamon Press, Oxford, **1960**, pp. 101–105.

49 Sakagami, H., Sakagami, T., Takeda, M. *Polyphenol Actual.* **1995**, *12*, 30–32.

50 Hegde, V.R., Pu, H., Patel, M., Das, P.R., Butkiewicz, N., Arreaza, G., Gullo, V.P., Chan, T.M. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2925–2928.

51 Li, Y., Ooi, L.S., Wang, H., But, P.P., Ooi, V.E *Phytother. Res.* **2004**, *18*, 718–722.

52 Weinmann, I. History of the development and applications of coumarin and coumarin-related compounds. In *Coumarins: Biology, Applications and Mode of Action*, Kennedy, R.O., Thornes, R.D. (eds). John Wiley and Sons, New York, **1997**.

53 Casley-Smith, J.R., Casley-Smith, J.R. Coumarin in the treatment of lymphoedema and other high–protein oedemas. In *Coumarins: Biology, Applications and Mode of Action*, Kennedy, R.O., Thornes, R.D. (eds). John Wiley and Sons, New York, **1997**.

54 Berkada, B. *J. Irish Colleges Phys. Surg.* **1978**, *22* (Suppl.), 56.

55 Shikishima, Y., Takaishi, Y., Honda, G., Ito, M., Takfda, Y., Kodzhimatov, O.K., Ashurmetov, O., Lee, K.H. *Chem. Pharm. Bull.* **2001**, *49*, 877–880.

56 Currens, M.J., Gulakowski, R.J., Mariner, J.M., Moran, R.A., Buckheit, R.W.Jr, Gustafson, K.R., McMahon, J.B., Boyd, M.R. *J. Pharmacol. Exp. Ther.* **1996**, *279*, 645–651.

57 Schmidt, H. Phenol oxidase (E.I.14.18.1), a marker enzyme for defense cells. In *Progress in Histochemistry and Cytochemistry*, Schmidt H. (ed.), Vol. 17. Gustav Fischer, New York, **1988**.

58 Stern, J.L., Hagerman, A.E., Steinberg, P.D., Mason, P.K. *J. Chem. Ecol.* **1996**, *22*, 1887–1899.

59 Duke, J.A. *Handbook of Medicinal Herbs.* CRC Press, Inc., Boca Raton, **1985**.

60 Semple, S.J., Nobbs, S.F., Pyke, S.M., Reynolds, G.D., Flower, R.L. *J. Ethnopharmacol.* **1999**, *68*, 283–288.

61 Semple, S.J., Pyke, S.M., Reynolds, G.D., Flower, R.L. *Antivir. Res.* **2001**, *49*, 169–178.

62 Richter, S., Parolin, C., Palumbo, M., Palu, G. *Curr. Drug. Targets Infect. Disord.* **2004**, *4*, 111–116.

63 Fessenden, R.J., Fessenden, J.S. *Organic Chemistry*, 2nd edn. Willard Grant Press, Boston, MA, **1982**.

64 Dixon, R.A., Dey, P.M., Lamb, C.J. *Adv. Enzymol. Related Mol. Biol.* **1983**, *55*, 1–69.

65 De Clercq, E. *Med. Res. Rev.* **2000**, *20*, 323–349.

66 Yamaguchi, K., Honda, M., Ikigai, H., Hara, Y., Shimamura, T. *Antivir. Res.* **2002**, *53*, 19–34.

67 Arthan, D., Svasti, J., Kittakoop, P., Pittayakhachonwut, D., Tanticharoen, M., Thebtaranonth, Y. *Phytochemistry* **2002**, *59*, 459–463.

68 Kaul, T.N., Jr. Middletown, E., Ogra, P.L. *J. Med. Virol.* **1985**, *15*, 71–79.

69 Robin, V., Irurzun, A., Amoros, M., Boustie, J., Carrasco, L. *Antivir. Chem. Chemother.* **2001**, *12*, 283–291.

70 Wu, P.L., Lin, F.W., Wu, T.S., Kuoh, C.S., Lee, K.H., Lee, S.J. *Chem. Pharm. Bull.* **2004**, *52*, 345–349.

71 Likhitwitayawuid, K., Sritularak, B., Benchanak, K., Lipipun, V., Mathew, J., Schinazi, R.F. *Nat. Prod. Res.* **2005**, *19*, 177–182.

72 Yadava, R.N., Tiwari, L. *J. Asia. Nat. Prod. Res.* **2005**, *7*, 185–188.

73 Tai, M.C., Tsang, S.Y., Chang, L.Y., Xue, H. *CNS Drug Rev.* **2005**, *11*, 141–150.

74 Tsuchiya, Y., Shimizu, M., Hiyama, Y., Itoh, K., Hashimoto, Y., Nakayama, M., Horie, T., Morita, N. *Chem. Pharm. Bull. (Tokyo)* **1985**, *33*, 3881–3886.

75 Clark, K.J., Grant, P.G., Sarr, A.B., Belakere, J.R., Swaggerty, C.L., Phillips, T.D., Woode, G. N. *Vet. Microbiol.* **1998**, *63*, 147–157.

76 Kernan, M.R., Amarquaye, A., Chen, J.L., Chan, J., Sesin, D.F., Parkinson, N., Ye, Z., Barrett, M. *J. Nat. Prod.* **1998**, *61*, 564–570.

77 Lin, Y.M., Flavin, M.T., Schure, R., Chen, F.C., Sidwell, R., Barnard, D.L., Huffman, J.H., Kern, E.R. *Planta Med.* **1999**, *65*, 120–125.

78 Li, B.Q., Fu, T., Dongyan, Y., Mikovits, J.A., Ruscetti, F.W., Wang, J.M. *Biochem. Biophys. Res. Commun.* **2000**, *276*, 534–538.

79 Shi, Y., Shi, R.B., Liu, B., Lu, Y.R., Du, L.J. *Zhongguo Zhong Yao Za Zhi*. 2003, *28*, 43–47.

80 Wu, J.H., Wang, X.H., Yi, Y.H., Lee, K.H. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1813–1815.

81 Du, J., He, Z.D., Jiang, R.W., Ye, W.C., Xu, H.X., But, P.P. *Phytochemistry* **2003**, *62*, 1235–1238.

82 Wei, F., Ma, S.C., Ma, L.Y., But, P.P., Lin, R.C., Khan, I.A. *J. Nat. Prod.* **2004**, *67*, 650–653.

83 Yogeeswari, P., Sriram, D. *Curr. Med. Chem.* **2005**, *12*, 657–666.

84 Haslam, E. *J. Nat. Prod.* **1996**, *59*, 205–215.

85 Kurokawa, M., Hozumi, T., Basnet, P., Nakano, M., Kadota, S., Namba, T., Kawana, T., Shiraki, K. *J. Pharmacol. Exp. Ther.* **1998**, *284*, 728–735.

86 Rajbhandari, M., Wegner, U., Schopke, T., Lindequist, U., Mentel, R. *Die Pharmaz*. **2003**, *58*, 268–271.

87 Notka, F., Meier, G.R., Wagner, R. *Antivir. Res.* **2003**, *58*, 175–186.

88 Cheng, H.Y., Lin, C.C., Lin, T.C. *Antivir. Res.* **2002**, *55*, 447–455.

89 Park, J.C., Hur, J.M., Park, J.G., Hatano, T., Yoshida, T., Miyashiro, H., Min, B.S., Hattori, M. *Phytother. Res.* **2002**, *16*, 422–426.

90 Liu, S., Jiang, S., Wu, Z., Lv, L., Zhang, J., Zhu, Z., Wu, S. *Life Sci.* **2002**, *71*, 1779–1791.

91 Kernan, M.R., Sendl, A., Chen, J.L., Jolad, S.D., Blanc, P., Murphy, J.T., Stoddart, C.A., Nanakorn, W. *J. Nat. Prod.* **1997**, *60*, 635–637.

92 Kuo, Y.H., Li, S.Y., Huang, R.L., Wu, M.D., Huang, H.C., Lee, K.H. *J. Nat. Prod.* **2001**, *64*, 487–490.

93 Nakano, M., Kurokawa, M., Hozumi, T., Saito, A., Ida, M., Morohashi, M., Namba, T., Kawana, T. *Antivir. Res.* **1999**, *41*, 153–154.

94 Zhang, H.J., Tan, G.T., Hoang, V.D., Hung, N.V., Cuong, N.M., Soejarto, D.D., Pezzuto, J.M., Fong, H.H. *J. Nat. Prod.* **2003**, *66*, 263–268.

95 Gosse, B., Gnabre, J., Bates, R.B., Dicus, C.W., Nakkiew, P., Huang, R.C. *J. Nat. Prod.* **2002**, *65*, 1942–1944.

96 Bermejo, P., Abad, M.J., Diaz, A.M., Fernandez, L., Santos, J.D., Sanchez, S., Villaescusa, L., Carrasco, L., Irurzun, A. *Planta Med.* **2002**, *68*, 106–110.

97 Apers, S., Baronikova, S., Sindambiwe, J.B., Witvrouwm, M., De Clercqm E., Vanden Berghe, D., Van Marck, E. *Planta Med.* **2001**, *67*, 528–532.

98 Primo, V., Rovera, M., Zanon, S., Oliva, M., Demo, M., Daghero, J., Sabini, L. *Rev. Argent. Microbiol.* **2001**, *33*, 113–117.

99 Schnitzler, P., Schon, K., Reichling, J. *Die Pharmaz*. **2001**, *56*, 343–347.

100 Garcia, C.C., Talarico, L., Almeida, N., Colombres, S., Duschatzky, C., Damonte, E.B. *Phytother. Res.* **2003**, *17*, 1073–1075.

101 Allahverdiyev, A., Duran, N., Ozguven, M., Koltas, S. *Phytomedicine* **2004**, *11*, 657–661.

102 McDevitt, J.T., Schneider, D.M., Katiyar, S.K., Edlind, T.D. Berberine: a candidate for the treatment of diarrhoea in AIDS patients, abstract 175. In *Program and Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy*. American Society for Microbiology, Washington, D.C., **1996**.

103 Duan, H., Takaishi, Y., Imakura, Y., Jia, Y., Li, D., Cosentino, L.M., Lee, K.H. *J. Nat. Prod.* **2000**, *63*, 357–361.

104 Baba, M., Okamoto, M., Kashiwaba, N., Ono, M. *Antivir. Chem. Chemother.* **2001**, *2*, 307–312.

105 Olila D, Olwa–Odyek, A., Opuda–Asibo, J. *African Health Sci.* **2002**, *2*, 2–10.

106 Ma, C.M., Nakamura, N., Miyashiro, H., Hattori, M., Komatsu, K., Kawahata, T., Otake, T. *Phytother. Res.* **2002**, *16*, 186–189.

107 Serkedjieva, J., Velcheva, M. *Antivir. Chem. Chemother.* **2003**, *14*, 75–80.

108 Szlavik, L., Gyuris, A., Minarovits, J., Forgo, P., Molnar, J., Hohmann, J. *Planta Med.* **2004**, *70*, 871–873.

109 Hsieh, P.W., Chang, F.R., Lee, K.H., Hwang, T.L., Chang, S.M., Wu, Y.C. *J. Nat. Prod.* **2004**, *67*, 1175–1177.

110 Chattopadhyay, D., Arunachalam, G., Mandal A.B., Bhattacharya S.K. *Chemotherapy* **2006**, *52(3)*, 151–157.

111 Liu, X.J., Wang, Y.F., Zhang, M.Y. *J. Chinese Med. Mater.* **2004**, *27*, 107–110.

112 Castilla, V., Barquero, A.A., Mersich, S.E., Coto, C.E. *Int. J. Antimicrob. Agents* **1998**, *10*, 67–75.

113 Bourinbaiar, A.S., Lee Huang, S. *Biochem. Biophys. Res. Commun.* **1996**, *219*, 923–929.

114 Ye, X.Y., Ng, T.B. *Biochem. Biophys. Res. Commun.* **2001**, *285*, 424–429.

115 Jiratchariyakul, W., Wiwat, C., Vongsakul, M., Somanabandhu, A., Leelamanit, W., Fujii, I., Suwannaroj, N., Ebizuka, Y. *Planta Med.* **2001**, *67*, 350–353.

116 Gauntt, C.J., Wood, H.J., McDaniel, H.R., McAnalley, B.H. *Phytother. Res.* **2000**, *14*, 261–266.

117 Takahashi, K., Matsuda, M., Ohashi, K., Taniguchi, K., Nakagomi, O., Abe, Y., Mori, S., Sato, N., Okutani, K., Shigeta, S. *Antivir. Res.* **2001**, *49*, 15–24.

118 Craig, M.I., Benencia, F., Coulombie, F.C. *Fitoterapia* **2001**, *72*, 113–119.

119 Amoros, M., Simoes, C.M.O., Girre, L. *J. Nat. Prod.* **1992**, *55*, 1732–1740.

120 Min, B.S., Tomiyama M., Ma, C.M., Nakamura, N., Hattori, M. *Chem. Pharm. Bull.* **2001**, *49*, 546–550.

121 Badam, L., Joshi, S.P., Bedekar, S.S. *J. Commun. Dis.* **1999**, *31*, 79–90.

122 Wu, T.S., Tsang, Z.J., Wu, P.L., Lin, F.W., Li, C.Y., Teng, C.M., Lee, K.H. *Bioorg. Med. Chem.* **2001**, *9*, 77–83.

123 Zhong, Y., Zuo, C., Li, F., Ding, X., Yao, Q., Wu. K,, Zhang, Q., Wang, Z., Zhou, L.W., Lan, J., Wang, X. *Zhongguo Zhong Yao Za Zhi* **1998**, *23*, 363–384.

124 Manfredi, K.P., Vallurupalli, V., Demidova, M., Kindscher, K., Pannell, L.K. *Phytochem.* **2001**, *58*, 153–157.

125 Yoosook, C., Bunyapraphatsara, N., Boonyakiat, Y., Kantasuk, C. *Phytomed.* **2000**, *6:* 411–419.

126 Cowan, M.M. *Clin. Microbiol. Rev.* **1999**, *12*, 564–582.

127 Calabrese, C., Berman, S.H., Babish, J.G., Ma, X., Shinto, L., Dorr, M., Wells, K., Wenner, C.A., Standish, L.J. *Phytother. Res.* **2000**, *14*, 333–338.

128 Thyagarajan, S.P., Subremanian, S., Thirunalasundari, T., Venkateswaran, P.S., Blumberg, B.S. *Lancet* **1988**, *ii*, 764–766.

129 Liu, J., Lin, H., McIntosh, H. *J. Viral Hepatitis* **2001**, *8*, 358–366.

130 Liu, J., Zhu, M., Shi, R., Yang, M. *Am. J. Chin. Med.* **2003**, *31*, 337–354.

131 Martin, K.W., Ernst. E. *Antivir. Ther.* **2003**, *8*, 77–90.

132 Tani, M., Ngase, M., Nishiyama, T., Yamamoto, T., Matsua, R. *Am. J. Chin. Med.* **2002**, *30*, 51–64.

133 Kainuma, M., Sakai, S., Sekiya, N., Mantani, N., Ogata, N., Shimada, Y., Terasawa, K. *Phytomedicine* **2004**, *11*, 5–10.

134 Emerit, I., Huang, C.Y., Serejo, F., Filipe, P., Fernandes, A., Costa, A., Freitas, J., Baptista, A., Carneiro de Moura, M. *Hepatogastroenterology* **2005**, *52*, 530–536.

135 von Gegerfelt, A.S., Liska, V., Li, P.L., McClurem H.M., Horie, K., Nappi, F., Montefiori, D.C., Pavlakis, G.N., Marthas, M.L., Ruprecht, R.M., Felber, B.K. *J. Virol.* **2002**, *76*, 96–104.

16
Immunomodulatory Effects of Phytocompounds

Buket Cicioğlu Arıdoğan

Summary

Inflammation is the local response of a tissue to damage or infection and represents most of the physiological and immunological reactions. The first stage is a cellular response to harmful stimuli in an effort to localize pathogens, toxic materials, or to prevent tissue injury. Several cell types and serum molecules such as neutrophils, mononuclear phagocytes, activated lymphocytes, antibodies, complement adhesion molecules, proinflammatory and inflammatory cytokines play important roles in the pathogenesis of both acute and chronic inflammatory diseases.

Echinacea is frequently used for medicinal purposes or as a food supplement to stimulate immune function in many American and European countries. Three major species from the Asteraceae family have also been studied for candidate bioactivity in pharmacological and immunological effects on macrophages or other immune cells: *Bidens pilosa* L. var. *minor*, *B. pilosa* L., and *B. chilensis*. The Taiwanese folk medicine known as "ham-hong-chho" derived from these plants has been traditionally used for medicinal purposes. *Bidens pilosa* is widely distributed in tropical regions, where it is used to treat inflammatory processes triggered by bacteria, fungi, and helminths. Bioactive compounds isolated from *B. pilosa* have been reported to have antibiotic, anti-inflammatory, antimalarial, and hepatoprotective activities.

The traditional use of plants such as *B. pilosa* might confer beneficial effects by increasing nonspecific defense mechanisms. Bioactive compounds that stimulate the immune system are useful as adjuvants in the treatment of certain bacterial, fungal, and parasitic diseases; in addition immunosuppressive compounds may be useful in situations in which immune responses are undesirable, such as transplant rejection, autoimmune diseases, or allergies.

Naphthoquinone compounds present in root extracts of a traditional Chinese medicinal herb, *Lithospermum erythrorhizon*, have been shown to have many medicinal properties such as antibacterial, wound-healing, anti-inflammatory, antithrombotic, and antitumor effects, etc.

Guarana (*Paullinia cupana*) is a Brazilian native plant with aphrodisiac properties that also has a stimulant effect on the cardiovascular and nervous system. Most of its properties are due to its caffeine content and related compounds such as theophylline, which are also presented as medicinal products for enhancing analgesic and bronchodilatory effects. However their potential risks to human health are not known.

In this chapter immunomodulatory and other related activities of above plant extracts or phytocompounds and their multifactorial interactions or genomic responses with human immune cell receptors and plant ligand molecules are considered briefly. Adverse effects of certain phytocompounds/herbal medicine have also been discussed.

16.1 Introduction

Immunology is an experimental science and the concept of immunomodulation arose in 1796 when Edward Jenner recorded the first successful vaccination against smallpox. Our environment contains a great variety of infectious agents. Many attempts have been made to help the immune system against external (bacteria, viruses, etc.) or internal (cancer and autoimmunity) attacks. New therapeutic strategies have been made possible by about a century of fundamental discoveries and the recognition of immunology and microbiology as distinct scientific disciplines [1–3].

In 1928, Alexander Fleming discovered the potent lytic effect of a mold contaminant, *Penicillium notatum*, on a staphylococcal culture; this marked the official birth of chemotherapy. Florey and Chain then demonstrated the therapeutic activity of penicillin G, taking almost 10 years to do so.

The golden age of antibiotics, stimulated by the needs of the Second World War, began in the early twentieth century. Selman Waksman defined the term "antibiotics" as "compounds produced by microorganisms that can inhibit the growth of other microorganisms or even destroy them." Streptomycin was discovered in 1944, and gramicidin S was found in 1942. Chloramphenicol, erythromycin, neomycin, cephalosporins, and many others were found in the 1940s and 1950s.

However, microorganisms have an extreme capacity to develop resistance strategies and that necessitates the creation of new antibacterial weapons. Could the sentence of Sir Almroth Wright, "The physician of the future will be the immunisator," be a premonition? [3, 4].

16.1.1 General Properties and Classification of Phytocompounds

In recent years, there has been an increasing interest worldwide in the use of medicines derived from plants (phytomedicines) as health supplements or alternative therapies for treatment of diseases in both developing and developed countries

[5–7]. Plants have become increasingly important as a source of biologically active natural products. It is estimated that 25% of all medicines contain plant derivatives, and plant components have been used as the starting material for many semi-synthetic drugs [8, 9]. Herbs are often considered to be safer, gentler, and of lower cost than conventional pharmaceutical drugs. Plants are efficient chemical factories that produce a wide variety of chemical compounds called phytochemicals. Phytopharmaceuticals are complex products, and their inherent biological variation is due to different growth, harvest, drying, and storage conditions [7]. There was a big upsurge in the popularity of complementary and alternative medicine (CAM) in the United States in the 1990s. A survey showed that use of herbal medicines increased from 2.5% in 1990 to 12.1% in 1997. The usage of CAM is generally more frequent among women and people in the 35–49 age groups [5, 10]. In the western United States research results showed that the prevalence of herbal use among racially/ethnically diverse primary care patients varies [5, 10–17], ranging from 30% among primary care patients residing in urban settings on the west coast of the United States [10–13] to 77% among primary care patients residing in the largest United States–Mexico border city [14]. Kuo et al. [10] showed that herbal use is common (36%) among urban multi-ethnic primary care patients, but has a wide variability among racial/ethnic groups. Hispanic and Asian groups reported the highest rates of herbal use (50%), and African Americans reported the lowest (22%) rates.

16.2
Effect of Specific Medicinal Herbs on Immune System and Immune Cells

Systematic studies on the effect of specific medicinal herbs on immune system are designed to obtain evidence-based scientific knowledge on the appropriate use of traditional medicinal herbs. The development of immunology has resulted in further complexity by combining external (environment and pathogens) and internal (neuroendocrine-immune system) factors in the pathogenesis of infectious diseases. The most important thing is to learn how to modulate the immune response to external conditions with powerful new techniques and drugs. Immunopharmacology is still a young science, and the molecular complexity of the immune system is not yet fully understood. Nevertheless, along with Metchnikoff, we can say, “we therefore have the right to hope that in the future, medicine will find more than one way to bring phagocytes into play for the benefit of health” [1–3].

Traditional herbal medicine provides several remedies for strengthening the body’s resistance to illness through effects on immune system components such as dendritic cells, T cells, macrophages, etc. [18].

Inflammation is the body’s protective reaction to controlling infections and promoting tissue repair. However, uncontrolled and excessive inflammation results in tissue damage and diseases including rheumatoid arthritis, inflammatory bowel disease, psoriasis, cancer, etc. Recently many laboratories have focused on the identification of immunomodulatory phytocompounds from herbal medicines

that are reported to modulate immunity. Several biochemical, cellular immunological, and molecular biological techniques and mouse models have been used to investigate the immunomodulatory function of phytocompounds (e.g. *Echinacea*, *Bidens*) in regulating immunity, and in modulating human immune cells including T cells, macrophages, and dendritic cell functions. Several plant compounds are known to be able to bind T cell components and to regulate T cell function. For example, ConA, a plant lectin, can activate T cells by cross linking glycoproteins such as the TCR/CD3 complex. The identification of genes involved in T cell function is also very important. In T cell differentiation several genes play very important roles. These gene products can be important for screening the phytocompounds to which the gene products can bind. Also an understanding of important signaling molecules in T cells helps us to screen their interaction partners from plants [1, 2, 19–23].

16.3 General Properties of *Echinacea* Species

The genus *Echinacea*, known as the purple coneflower, is represented by nine species found in the United States and in south central Canada. Three species, *Echinacea purpurea*, *Echinacea angustifolia*, and *Echinacea pallida*, are found in common herbal preparations and are used medicinally [24, 25]. Each shows different medicinal properties; however, little has been done to compare the effectiveness of these species. The composition of each species of herb is similar; with slight variations in the amount of each active component. The roots have more volatile oils and pyrrolizidene alkaloids, such as tussilagine and isotussilagine, than the parts above ground. The active components of the upper plant are thought to be caffeic and ferulic acid derivatives (such as cichoric acid and echinacoside) and complex polysaccharides (such as acidic arabinogalactan, rhamnoarabinogalactans, and 4-*O*-methylglucuronylarabinoxylans). Many other active components in *Echinacea* have been identified [26].

Echinacea species have been described as the most important plants used by the Native Americans for treatment of many diseases. In many American and European countries *Echinacea* is widely used as a medicinal herb or food supplement for stimulating immune function. *Echinacea* species have been used for treatment of many diseases, including colds, tonsillitis, bowel pain, toothaches, snake bites, seizures, cancer, septic conditions, and wound infections. At the beginning of the twentieth century, *Echinacea* was the best-selling American medical plant in the United States, although its use decreased after the introduction of antibiotic drugs [24, 27, 28].

Fluid extracts of *Echinacea purpurea* are widely used for the prevention and treatment of colds and respiratory infections, although the clinical efficacy of this agent has not been proven. It has been used in high doses for short period of time for treatment of the common cold, coughs, flu or acute cold, bronchitis, upper respiratory infections, and in low doses over an extended time to build immune function

and some inflammatory conditions. They are also used as immunostimulants and anti-infective or topically as wound-healing agents. Some of the results of studies showed that the incidence, duration, or severity of colds and respiratory infections did not significantly decrease with treatment with fluid extract of *E. purpurea* when compared with placebo [24].

In vitro, active components of *Echinacea* have been found to have protective effects on skin connective tissue. Caffeoyl derivatives, typical constituents of *Echinacea*, protected collagen from damage caused by the superoxide and hydroxyl radicals generated in a xanthine/xanthine oxidase system [26, 29]. The polyunsaturated alkamides from *E. angustifolia* were found to inhibit microsomal cyclooxygenase activity and leukocyte 5-lipoxygenase activities, suggesting an anti-inflammatory effect [26, 30]. Animal studies in the late 1980s also showed an anti-inflammatory effect from topical application of the polysaccharide fraction derived from *E. angustifolia* root. These studies showed that *Echinacea* reduced significantly both carrageenan paw edema and the inflammation associated with croton oil ear topically in animals [26, 31, 32].

It has been suggested that *Echinacea* is also a potentially useful therapeutic agent for infections with *Candida albicans* and *Listeria monocytogenes* [33]. During the past five decades, more than 200 papers have been published on the chemistry, pharmacology, and clinical use of *E. purpurea*, and to a lesser extent *E. angustifolia* and *E. pallida*

Although many of the active compounds of *Echinacea* have been identified, the mechanism of action, the bioavailability, relative potency, or synergistic effects of the active compounds is not known. Future studies need to clearly identify the species of *Echinacea* and distinguish between the efficacies of the different plant parts.

In addition, based these results, bioactivity guiding assays of specific phytocompounds from this medicinal herb are being systematically tested, by bioorganic partition fractionation, to identify the potential phytocompound groups, single compounds, or reconstitution formulation from the plant extracts. Interpretation of the results in the literature suggests that *Echinacea* is indeed effective in reducing the duration and severity of symptoms, but that this effect is noted only with certain preparations of *Echinacea* [24–36].

16.4 Effects of *Echinacea* Species on the Immune System and Various Immune Cells

Studies show that the plant and its active components affect the phagocytic immune system, but not the specifically acquired immune system. *Echinacea* is best known as an immunostimulant. A series of studies in mice using purified polysaccharides from *Echinacea* plant cell cultures showed a stimulatory effect when applied to immune cells in culture or injected intraperitoneally into mice. These effects include an increase in phagocytosis, chemotaxis, and oxidative burst of either neutrophils [26, 36] or macrophages [26, 34, 37]. Peritoneal macrophages produced more tumor necrosis factor (TNF), interleukin 1 (IL-1), IL-6, and IL-10 [38] and

were able to kill tumor cells (WEHI 164 cells) and cells infected either with the parasite *Leishmania enriettii* or with the yeast *Candida albicans* [33, 39].

Mice with suppressed immunity due to treatment with cyclophosphamide or cyclosporin also had an increase in these immune functions when given purified polysaccharides from *Echinacea* [39]. These studies suggest that *Echinacea* stimulates immune functions in healthy and in immunosuppressed animals.

These immunologically active polysaccharides did not stimulate all immune cells. For example B cells were not activated and they did not produce more antibodies to sheep red blood cells [26, 34]. Although in one study a slight increase in T-cell proliferation was showed, the T cells did not produce more IL-2, interferon (IFN)-β2, or IFN-γ and a common T-cell response, delayed type hypersensitivity, was not affected by *Echinacea* treatment [33]. These findings showed that purified polysaccharides from *E. purpurea* might act on the phagocytic cells, the nonspecific branch of immunity, rather than the specifically acquired branch [26].

Roesler et al. [35] studied the effect of *Echinacea* on human and *ex vivo* conditions. They showed that *Echinacea* increased neutrophil chemotaxis and bactericidal activity against staphylococcus in *ex vivo* conditions and they showed that monocytes produced more TNF, IL-6, and IL-1, but not TH2 cytokines. But when *Echinacea* was injected intravenously, there was a reduction in the number of neutrophils in the blood. They interpreted this as an increase in the adherence of the cells to the endothelium. They also observed the appearance of juvenile forms of cells in the periphery, an increase in C-reactive protein, and an increase in erythrocyte sedimentation rate. These results suggested that *Echinacea* could be enhancing the acute phase response.

Burger et al. [38] isolated peripheral blood macrophages from healthy humans and incubated them with freshly pressed *E. purpurea* juice, which were harvested and pressed immediately in 20% ethanol. They showed that the macrophages had an increased production of TNF, IL-10, IL-6, and IL-1.

In another study See et al. [40], isolated the peripheral blood mononuclear cells from healthy adults and from adults with chronic fatigue syndrome or AIDS. They homogenized dried and ground *E. purpurea* in cell culture medium and incubated it with the peripheral blood mononuclear cells. They found that natural killer cells had an increased ability to kill K562 human leukemic cells and an increased antibody-dependent cytotoxicity against H9 cells infected with herpes 6 virus also.

Several human clinical trials have been carried out related to measuring the effects of various species of *Echinacea* on the phagocytic activity of peripheral neutrophils. The phagocytic activity was measured using various methods such as the Brand t-Test method or the flow cytometry technique. Only the complex preparation of *E. angustifolia* that was taken as an injection and the ethanol extract of *E. purpurea* root taken orally showed an increase in phagocytic activity of peripheral blood neutrophils [41].

Many other studies have been done on *Echinacea*, but are published largely in the German language. Barrett et al. [42] recently reviewed seven German studies published between 1984 and 1997 and concluded that in general these studies

showed that *Echinacea* can be used to modify the severity and shorten the duration of cold symptoms, but is not useful as a prophylactic [26, 41].

Lüttig et al. [34] and Stimpel et al. [37] showed that purified polysaccharides from cell cultures of *E. purpurea* stimulate phagocyte activities *in vitro* and *in vivo* in mice and Roesler et al. [35] found that intravenous application of polysaccharides from cell cultures of *E. purpurea* induced acute phase reactions and activation of phagocytes in humans because monocytes were activated to secrete TNFα as well as interleukin 1 and 6. Roesler et al. [33] and Elsasser et al. [43] showed that these polysaccharides play an important role in the enhancement of the resistance of immunosuppressed mice against systemic infections with *Candida albicans* and *Listeria monocytogenes*. However a number of randomized controlled trials must be done to prove the clinical efficacy of various preparations of *Echinacea* species.

Echinacea is able to stimulate innate immune responses, including those regulated by macrophages and natural killer cells. Indeed, macrophages respond to purified polysaccharide and alkylamide preparations. However, the mechanisms for stimulation of cells responsible for adaptive immunity have not been fully elucidated for other molecules present in *E. purpurea* preparations.

Shen-An Hwang et al. [44] found that a water-soluble extract of *Echinacea* was able to stimulate IL-6, IL-10, MIP-1α, and TNFα from murine splenocytes, similar to that identified as produced from human peripheral blood cells and from CD3+ enriched populations[38, 44].

Other constituents of *Echinacea*, such as the polysaccharide arabinogalactan, have been identified as macrophage activators *in vitro*, causing macrophages to attack microorganisms and tumor cells. An increase of TNFα, IL-1β, and interferon B2 levels has also been determined [34]. The crude polysaccharide of *E. purpurea* also has an increased cytotoxicity towards tumor cells and causes increased IL-1β production [37], and augments natural killer cell function [40, 44, 45].

Senchina et al. [46] investigated whether the use of homemade preparations of several species of *Echinacea* extracts affected immunomodulatory efficiency with storage at 4 °C over a 4-day period or not. They prepared three extract types (50% ethanol tincture, cold water infusion, and hot water infusion) from five different species (*E. angustifolia*, *E. pallida*, *E. purpurea*, *E. sanguinea*, and *E. tennesseensis*). They used four *in vitro* immune assays (monocyte secretion of TNFα, IL-10, and IL-12; and peripheral blood mononuclear cell proliferation) to test extract efficiency on days 1 and 4 post extraction.

In response to viral infection, antigen-specific T cells proliferate, secrete cytokines, and destroy virally infected cells via T cell-mediated cytotoxicity. The effect of *Echinacea* extracts on T cell function has not been examined extensively. *Echinacea* extracts are commonly used by the public to enhance the clearance of viral infection and it is possible that the potential benefits of *Echinacea* involve T cells.

Macrophages are also important as a first line of defense against viral infection. *Echinacea* may alter macrophage and/or monocyte function, and as these cells become activated, TNFα secretion typically increases. TNFα secretion can be used as a marker of macrophage activation. The IL-10 secreted by monocyte/macrophages

may have anti-inflammatory properties and/or enhance B cell function, and IL-12 release can alter the TH1/TH2 cytokine balance in response to infection. However, our knowledge about the effect of *Echinacea* on IL-10 or IL-12 production in human cells is rather limited. Unlike laboratory scientists, lay herbalists are limited in the types of extracts they can prepare. Despite the increasing practice of lay herbalism, the overwhelming majority of research has focused on extracts prepared in scientific laboratories or large-scale commercial facilities [43, 46].

Endotoxin derives from the outer membrane of certain bacteria and has a direct immune stimulatory effect. The presence of endotoxin is not a product of the extraction process but derives from extract contamination – bacteria growing on the plant at the time of harvest become inadvertently incorporated into the extracts [43, 46].

Gertsch et al. [47] has recently demonstrated that *Echinacea* preparations may act on innate immune system cells mechanistically, similarly to lipopolysaccharides.

Many *in vitro* studies have not reported endotoxin levels from their extracts, but some investigators have considered the importance of endotoxin effects in *Echinacea* [37, 38]. Endotoxin is more of a concern for *in vitro* than for *in vivo* assays, because the gut typically stops endotoxins from entering the bloodstream.

T cells are components of the adaptive (specific) immune system [1, 2]. However, most studies have shown that *Echinacea* affects members of the innate (nonspecific) immune system more regularly. Senchina et al. [46] demonstrated that extracts from *Echinacea* species have minimal effect on T cell proliferation [46, 48, 49]. One study did show an enhancement of proliferation when *Echinacea*-derived arabinogalactan (a polysaccharide) was employed [34].

Experiments have demonstrated that *Echinacea* can modulate production of specific cytokines from human and rodent macrophages and peripheral blood mononuclear cells *in vitro* and *in vivo*. It has previously been demonstrated that TNFα production by monocytes or macrophages may be increased upon application of laboratory-derived *Echinacea* extracts in both human *in vitro* cultures [35, 38], rodent *in vitro* cultures [33, 50, 51], and rodent *in vivo* models [52, 53]. However, it is very difficult to interpret whether *Echinacea*-induced upregulation of TNFα has beneficial or harmful effects as this depends on the *in vivo* conditions.

IL-10 is a TH2 cytokine that has anti-inflammatory properties and may play a role in the clearance of viral or other infectious diseases. It is very important in the B cell response and subsequent antibody production [1, 2, 54]. Senchina et al. and other workers have shown that *Echinacea* extracts can promote IL-10 production in human and rodent lymphocyte cultures. *Echinacea*-induced augmentation of IL-10 could yield physiologically relevant benefits [38, 46, 51].

IL-12 promotes a strong TH1 (CD8+ or cytotoxic T cell) response in reaction to viral infection, and increased levels of this cytokine could lead to more efficient viral clearance [1, 2, 55]. Senchina et al. [46] showed that *Echinacea*-induced upregulation of IL-12 could yield physiologically relevant benefits. The only significant activity seen in cold and hot water infusion extracts on day 4 was promotion of IL-12 production.

Dendritic cells are very important target cells, mainly because they are professional antigen-presenting cells and play an important role in innate as well as adap-

tive immunities. Although the bioactivities of three major *Echinacea* species have been studied and they have some bioactivity on macrophages or other immune cells, the possible effects on dendritic cells are little known. Recent studies on the effect of *Echinacea* plant extracts and derived phytocompounds on human dendritic cells have revealed significant changes in specific gene and protein expression levels. *Echinacea* root extract can stimulate the expression of CD83 in dendritic cells with or without co-treatment with lipopolysaccharide, and *Echinacea* may be associated with anti-inflammatory activity. *Echinacea* can stimulate or suppress certain specific activities of dendritic cells at the gene and/or protein expression level [1–3]. Differentially expressed genes and proteins have bee identified using DNA microarray and MALDI-TOF mass spectrometry analyses. Bioinformatics analyses have been employed to understand the possible connections and involvement of these responsive genes in immune-related cell-signaling mechanisms. Flow cytometry and DNA microarray techniques have been used to analyze the surface proteins (CD markers) and the differential gene/genomic expression patterns of dendritic cells (nsyang@ gate.sinica.edu.tw).

Echinacea may be used therapeutically, rather than prophylactically [28, 56]. The beneficial effects of *Echinacea* in the treatment of infections appear to be a result of its ability to stimulate the immune system. After exposure to *Echinacea*, macrophages and T lymphocytes demonstrate increased phagocytic activity and release of immunomodulators such as tumor necrosis factors and interferons. Although the exact mechanism of the immunostimulatory effect of *Echinacea* is still unknown, controlled studies suggest that oral administration may be beneficial in the early treatment of upper respiratory infections [48, 57–60].

16.5 Asteraceae

The Asteraceae (Compositae) family is the largest flowering plant family in the world. It is also very important among medicinal plants. Polyacetylenes are present in all parts of the plant, which characterizes this family. Several compounds with biological actions have been isolated from different species of Asteraceae. *Bidens pilosa* L., for example, is distributed in tropical regions and is commonly used to treat various ailments in different countries, such as stomach disorders in South Africa and Taiwan, and malaria and liver disorders in Brazil. The habitual use of plants can stimulate the immune system and *B. pilosa* might confer beneficial effects by increasing nonspecific defense mechanisms. Bioactive compounds can stimulate the immune system as adjuvants in the treatment of certain bacterial, fungal, and parasitic diseases [9, 61–64].

Rachel et al. [9] examined the possible effect of *B. pilosa* on lymphocyte activation *in vitro* and *in vivo*. They reported that the methanolic extract has an immunosuppressive effect on lymphocyte activation that may be associated with the presence of the polyacetylene 2-*O*-β-D-glucosyltrideca-11*E*-en-3,5,7,9-tetrayn-1,2-diol. In 1994 Redl et al. [65] showed the presence of this compound in *Bidens camphyloteca*

and Rachel et al. [9] isolated it for the first time from *B. pilosa* in 1999. They reported that both the methanolic extract of *B. pilosa* and the polyacetylene PA-1 suppressed human lymphocyte proliferation. They added increasing concentrations of extract and concluded that the immunosuppressive effect is dose dependent. They also investigated the immunosuppressive effect on lymphocyte activation *in vivo* by testing the effect of *B. pilosa* preparations on inflammation induced by zymosan.

Rachel et al. showed for the first time that extracts of *B. pilosa* L. have an immunosuppressive effect and they suggest that this effect may be due to the presence of the polyactylene in this species. *B. pilosa* is widely used in tropical regions to treat inflammatory processes triggered by bacteria, fungi, and helminths [64, 66]. The proliferative responses of lymphocytes from different species to various stimuli were completely inhibited by a methanolic extract of *B. pilosa*. After stimulation, T lymphocytes undergo intracellular events that involve phosphorylation and lead to an autocrine growth in which the stimulated naïve lymphocytes proliferate in response to their own production of IL-2 and the receptor for IL-2 [67]. Among the substances that induce lymphocyte proliferation are the lectins that bind to carbohydrate residues. The activation of the T lymphocyte upon binding of a lectin triggers intracellular reactions that generate second messengers such as diacylglycerol; this molecule stimulates protein kinase C (PKC) activity. Thus, PKC activity is a common step in the proliferative pathway [9, 68]. The results obtained *in vitro* led workers to examine a possible *in vivo* effect of *B. pilosa*. The peripheral lymphoid organs, mainly the lymph nodes, are drain sites where the antigens are concentrated and also the place into which naïve lymphocytes preferentially migrate. In addition, accessory cells, which are required for lymphocyte activation, are abundant in these tissues [1]. The treatment of mice with *B. pilosa* for 5 days significantly reduced the increase in PLN weight, probably by inhibiting lymphocyte proliferation, as observed *in vitro*. These results demonstrated an anti-inflammatory action of extracts of *B. pilosa* [9, 61].

These results also indicated that the polyacetylene PA-1, isolated from *B. pilosa*, may be involved in the immunosuppressive effect found in the crude extract. The data indicated that there is a potent immunosuppressive action of components present in the species *B. pilosa*, suggesting a promising application as an anti-inflammatory drug [9].

Bidens pilosa L. var *minor*, *B. pilosa* L., and *B. chilensis* have been traditionally used for medicinal purposes. They are commonly known as "ham-hong-chho" in Taiwan. Chin et al. [69] investigated the hepatoprotective effects of ham-hong-chho in rats. They induced the liver damage by administering CCl_4 and acetaminophen. They then compared the pharmacological and pathological effects of these three crude groups with those of *Bupleurum chinensis*. They suggested that *B. pilosa* var. *minor*, *B. pilosa*, and *B. chilensis* can protect liver injuries from various hepatotoxins and have potential as broad-spectrum antihepatic agents. They found that the protective effects of the extract of *B. chilensis* were stronger than the extracts of *B. pilosa* var. *minor* and *B. pilosa*.

Bidens pilosa Linn. var. *radiata* is a tropical weed widely distributed in the countryside of Taiwan. This plant was originally found in tropical America and the Pacific region and parts of Asia. The whole plant or its aerial parts are used in various folk medicines and as a popular ingredient in herbal tea for its anti-inflammatory, antiseptic, liver-protective, blood-pressure lowering, and hypoglycemic effects [9, 70–72].

Wu et al. [70] is investigated the antiangiogenic effects of plant extracts and polyacetylenes isolated from *B. pilosa* Linn var. *radiata*. Angiogenesis, the formation of new blood vessels from preexisting endothelium, has been shown to play an important role both in animal development and pathologic conditions like tumor growth and metastasis or cardiovascular diseases [73]. Wu and co-workers [70] used anti-cell proliferation, anti-tube formation, and cell migration assays for the evaluation of bioactivities of target plant extracts and phytocompounds against angiogenesis. They used bioactivity-guided fractionation, high-performance liquid chromatography (HPLC), and various types of spectral analyses to identify active fractions and antiangiogenic phytocompounds. The results showed that the alcoholic fraction of *B. pilosa* extract and polyacetylene aglycones, namely 1,2-dihydroxytrideca-5,7,9,11-tetrayne and 1,3-dihydroxy-6(*E*)-tetradecene-8,10,12-triyne, exhibited significant and potent antiangiogenic activities. The ability of both compounds to block angiogenesis is possibly in part through induction of p27 (Kip1) and regulation of other cell cycle mediators including p21 (Cip1) and cyclin E. The relationship of apoptosis and the angiogenic inhibition mediated by both compounds **1** and **2** in HUVEC is under investigated. More studies are needed to determine the detailed mechanism of the action of compounds **1** and **2** on angiogenesis and the potential application of these compounds as antiangiogenic drugs in cancer therapy [70].

16.6 *Lithospermum erythrorhizon*

Naphthoquinone compounds present in root extracts of a traditional Chinese medicinal herb, *Lithospermum erythrorhizon*, have been reported to confer many medicinal properties such as antibacterial, wound-healing, anti-inflammatory, antithrombotic, and antitumor effects. Recent studies suggest that the anti-inflammatory effects of these shikonins may depend on several mechanisms of action, including inhibition of leukotriene B4 biosynthesis, suppression of mast cell degranulation, and protection of the vasculature blockade of chemokine ligands binding to CC chemokine receptor 1, etc. [74, 75].

Skin is an immune-competent organ that serves as a first line of defense to various agents, such as exogenous stress, environmental antigens, or pathogens. In skin, TNFα is one of the most important proinflammatory cytokines and seems to be important in allergic and irritant contact dermatitis and in other inflammatory conditions [2, 74, 76, 77]. Modulating TNFα expression in skin may provide therapeutic benefits for a variety of skin disorders. Inappropriate or overexpression of

TNFα is the hallmark of a number of inflammatory and autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, asthma, and AIDS [78–80].

Current studies on the inhibition of TNFα promoter activity by shikonins have provided insight into the molecular mechanism underlying the anti-inflammatory properties of these phytocompounds. Staniforth et al. [74] have evaluated the effects of shikonin and its derivatives on the transcriptional activation of human TNFα promoter in a gene gun-transfected mouse skin system by using a luciferase reporter gene assay.

Although the efficacy of shikonin and its derivatives has been demonstrated *in vitro* and *in vivo*, according to my opinion further *in vivo* studies must be done to demonstrate the molecular basis for the anti-inflammatory actions.

Recent studies have shown that mechanical stress can result in activation of distinct mitogen-activated protein (MAP) kinase signaling pathways in skeletal muscle fibers [81], and inflammation and injury have been reported to activate the NF-κB signal transduction pathway in keratinocytes [82]. Based on reporter gene activity in an *in vivo* mouse skin system, Staniforth et al. [74] found that gene gun particle-mediated injury/stress induced the activation of Erk1/2 and NF-κB. Their results show that a crude extract of *L. erythrorhizon* significantly inhibited the physical injury/stress-induced transcriptional activation of human TNFα promoter, and the level of inhibition was comparable to that of the commercially available topical anti-inflammatory corticosteroids hydrocortisone and betamethasone [74].

Further studies to determine whether shikonin and its derivatives also suppress the expression of other proinflammatory and immunoregulatory cytokine genes would provide greater insight into their potential therapeutic use as anti-inflammatory and immunosuppressive agents.

16.7
Guarana

Guarana (*Paullinia cupana* Mart.) is a Brazilian native plant, the seeds of which are the only part suitable for human consumption. Guarana seeds derivatives are used as ingredients of a variety of phytopharmaceutical products. Guarana has been shown to be an aphrodisiac and analgesic, and to produce bronchodilator effects over the cardiovascular, respiratory, and nervous systems. Guarana seeds contain high concentrations of caffeine (approximately 3–6%), very small amounts of theophylline and theobromine and large quantities of tannins [83, 84].

16.8
Side and Adverse Effects of Some Phytocompounds

Although herbal remedies are often perceived as being natural and consequently safe, many have toxic and detrimental side-effects. The most common problems

with medicinal herb are caused by adulteration, contamination, substitution, and lack of standardization, misidentification, incorrect preparation and/or dosage and inappropriate labeling [83, 85].

Substitution or adulterations with more toxic herbs or synthetic drugs may result in mistaken cases of clinical complications, and adverse effects resulting from the synthetic drugs in herbal medicines have been reported [83, 86, 87].

Cultivation of the plants under standardized conditions is necessary and desirable. The polarity of the solvent, the mode of extraction, and the instability of constituents may influence the composition and quality of the extracts. Depending on the type of preparation, sensoric features, moisture, ash, physical constants, solvents residues, and adulterations have to be checked to prove identity and purity.

To check and prove the identity, purity, and quality of herbal preparations, adequate analytical methods have to be applied to the quantitative determination of the constituents with known therapeutic activity. HPLC is one of the analytical methods widely used for routine analyses in the pharmaceutical industry.

Capillary electrophoresis (CE) is another suitable technique and has a number of advantages, including short analysis time, high separation efficiency and low sample and solvent consumption [88–93].

Efficacy studies of herbal supplements are on the rise, but most data published to date are preliminary and do not provide strong evidence for the clinical effectiveness of herbs.

Many people who use herbal remedies do not discuss this with their physicians or pharmacists and do not know about the potential adverse effects of drug–herb interactions. They may put themselves at increased risk of adverse drug–herb interactions and make it extremely difficult for health care professionals to monitor them for such interactions [5, 10, 12–14, 94].

Many adverse drug–herb interactions constitute a great danger for patients and might adversely affect the monitoring of certain drug therapies, such as an increase in bleeding time (garlic and ginseng both interact with warfarin) [95, 96]. Other examples include the action against standard immunosuppressants, life-threatening allergic reactions such as contact dermatitis and anaphylaxis (*Echinacea*), interference with the monitoring of digoxin (ginseng, hawthorn, licorice, etc.), and hepatotoxicity (kava-kava with alprazolam) [5, 44, 95, 97–109].

16.9 Conclusion

The commonly used herbal medicine and phytocompounds known for their significant immunomodulatory and related activities including *E. purpureae*, effectiveness in treating illness or in enhancing human health has not yet been proven beyond a reasonable doubt. In most of the cases no single agent or class of agents is solely responsible for all activities. Instead it appears to be contributory actions of various phytocompounds. Although published evidence to date support the safety and effectiveness of *Echinaceae*. However, better research is needed before definite

recommendation of herbal medicine. Since the toxicity problems in herbal medicine also arises due to several other factors as discussed above. Herbal medicines are not always safe and non-toxic. They have some life threatening side effects. Health-care professionals and patients must be educated about the detrimental effects and adverse drug-herb interactions of herbal supplements. Identity purity and quality of herbal preparations must be checked and proved by adequate analytical methods.

References

1 Abbas, A.K., Lichtman, A.H. (eds) *Cellular and Molecular Immunology*, 5th edn. Saunders, USA, **2003**.
2 Roitt, I., Brostoff, J., Male, D. (eds) *Immunology*, 6th edn. Mosby, London, **2001**.
3 Labro, M.T. *Clin. Microbiol. Rev.* **2000**, *13*, 615–650.
4 Crellin, JK. Antibiosis in the 19th century. In *The history of antibiotics. A symposium*. Parascandola, J. (ed.). American Institute of the History of Pharmacy, Madison, WI, **1980**, pp. 5–13.
5 Eisenberg, D.M., Davis, R.B., Ettner, S.L., Appel, S., Wilkey, S., Van Rompay, M., Kessler, RC. *JAMA* **1998**, *280*, 1569–1575.
6 Gilroy, C.M., Steiner, J.F., Byers, T., Shapiro, H., Georgian, W. *Arch. Intern. Med.* **2003**, *163*, 699–704.
7 Sombra, L.L., Gómez, M.R., Olsina, R., Martínez, L.D., Silva, M.F. *J. Pharmac. Biomed. Anal.* **2005**, *36*, 989–994.
8 Farnsworth, N.R. In *Natural Products and Drug Development*, Krogsgaard-Larsen, P., Christensen, S.G., Kofod, H. (eds). Munksgaard, Copenhagen, **1984**, pp. 17–30.
9 Rachel Pereira, L.C., Tereza, I., Leonardo, L., Jorge da Silva, R.A., Gonçalves de Moraes, V.L. *Immunopharmacology* **1999**, *43*, 31–37.
10 Kuo, G.M., Hawley, S.T., Weiss, L.T., Balkrishnan, 1.R., Volk, R.J. *BMC Complement. Altern. Med.* **2004**, *4*, 18.
11 Johnston. B.A. *HerbalGram.* **1997**, *40*, 49.
12 Elder N.C., Gillcrist, A., Minz, R. *Arch. Fam. Med.* **1997**, *6*, 181–184.
13 Palinkas, L.A., Kabongo, M.L. *J. Fam. Pract.* **2000**, *49*, 1121–1130.
14 J.O. Rivera, M. Ortiz, M.E. Lawson, K.M. Verma, J. *Pharmacotherapy* **2002**, *22*, 256–264.
15 Zeilmann, C.A., Dole, E.J., Skipper, B.J., McCabe, M., Dog, T.L., Rhyne, R.L. *Pharmacotherapy* **2003**, *23*, 526–532.
16 Dole, E.J., Rhyne, R.L., Zeilmann, C.A., Skipper, B.J., McCabe, M.L., Dog, T.L. *J. Am. Pharm. Assoc.* **2000**, *40*, 359–365.
17 Giveon, S.M., Liberman, N., Klang, S., Kahan, E. *Patient Educ. Couns.* **2004**, *53*, 5–11.
18 Block, K.I., Mead, M.N. *Integr. Cancer Ther.* **2003**, *2*, 247–267.
19 Yang, W.C., Ghiotto, M., Barbarat, B., Olive, D. *J. Biol. Chem.* **1999**, *274*, 607–617.
20 Yang, W.C., Olive, D. *Eur. J. Immunol.* **1999**, *29*, 1842–1849.
21 Yang, W.C., Ghiotto, M., Castellano, R., Collette, Y., Auphan, N., Nunes, J., Olive, D. *Int. Immunol.* **2000**, *12*, 1547–1552.
22 Yang, W.C., Collette, Y., Nunes, J., Olive, D. *Immunity* **2000**, *12*, 373–382.
23 Yang, W. C., Ching, K.A., Tsoukas, C.D., Berg, L.J. *J. Immunol.* **2001**, *166*, 387–395.
24 Grimm, W., Müller. H.H. *Am. J. Med.* **1999**, *106*, 138–143.
25 Mazza, G., Cottrell, T. *J. Agric. Food Chem.* **1999**, *47*, 3081–3085.
26 Percival, S.S. *Biochem. Pharmacol.* **2000**, *60*, 155–158.
27 Hobbs, C.R. *The Echinacea Handbook*. Eclectic Medical Publications, Portland, Oregon, **1989**.
28 Bauer, R. Echinacea: biologic effects and active principles. In *Phytomedicines of Europe, Chemistry and Biologic Activity*, Lawson LD, Bauer R. (eds). Washington, DC, American Chemists Society, **1998**, pp. 140–157.

29 Facino, R.M., Carini, M., Aldini, G., Saibene, L., Pietta, P., Mauri, P., *Planta Med*. **1995**, *61*, 510–514.
30 Muller-Jakic, B., Breu, W., Probstle, A., Redl, K., Greger, H., Bauer, R. *Planta Med*. **1994**, *60*, 37–40.
31 Tubaro, A., Tragni, E., Del Negro, P., Galli, C.L., Della Loggia, R. *J. Pharm. Pharmacol*. **1987**, *39*, 567–569.
32 Tragni, E., Galli, C.L., Tubaro, A., Del Negro, P., Della Loggia, R. *Pharmacol. Res. Commun*. **1988**, *20*, 87–90.
33 Roesler, J., Steinmüller, C., Kinderlen, A. et al. *Int. J. Immunopharmacol*. **1991**, *13*, 27–38.
34 Luettig, B., Steinmüller, C., Gifford, G.E. et al. *J. Natl Cancer Inst*. **1989**, *81*, 669–675.
35 Roesler, J., Emmendörfer, A., Steinmüller, C. et al. *Int. J. Immunopharmacol*. **1991**, *13*, 931–941.
36 Wagner, H., Stuppner, H., Schafer H., Zenk, M. *Phytochemistry* **1988**, *27*, 119–127.
37 Stimpel, M., Proksch, A., Wagner, H., Lohmann-Matthes, M-L. *Infect. Immun*. **1984**, *46*, 845–849.
38 Burger, R.A., Torres, A.R., Warren, R.P., Caldwell, V.D., Hughes, B.G. *Int. J. Immunopharmacol*. **1997**, *19*, 371–379.
39 Steinmüller, C., Roesler, J., Gröttrup, E., Franke, G., Wagner, H., Lohmann-Matthes, M-L. *Int. J. Immunopharmacol*. **1993**, *15*, 605–614.
40 See, D.M., Broumand, N., Sahl, L., Tilles, J.G. *Immunopharmacology* **1997**, *35*, 229–235.
41 Melchart, D., Linde, K., Worku, F., Sarkady, L., Holzmann, M., Jurcic, K., Wagner, H. *J. Altern. Complement. Med*. **1995**, *1*, 145–160.
42 Barrett, B., Kiefer, D., Rabago, D. *Altern. Ther*. **1999**, *5*, 40–50.
43 Elsasser, B.U., Willenbacher, W., Bartsch, H.H. et al. *J. Clin. Lab. Anal*. **1996**, *10*, 441–445.
44 Hwang, S., Dasgupta, A., Actor, J.K. *Clin. Chim. Acta* **2004**, *343*, 161–166.
45 Currier N.L., Miller, S.C. *J. Altern. Complement. Med*. **2001**, *7*, 241–251.
46 Senchina, D.S., McCann, D.A., Asp, J.M., Johnson, J.A., Cunnick, J.E., Kaiser, M.S., M.L. Kohut. *Clin. Chim. Acta* **2005**, *355*, 67–82.
47 Gertsch, J., Schoop, R., Kuenzle, U., Sutter, A., *FEBS Lett*. **2004**, *577*, 563–569.
48 Percival, S.S. *Biochem. Pharmacol*. **2000**, *60*, 155–158.
49 South, E.H., Exon, J.H. *Immunopharmacol. Immunotoxicol*. **2001**, *23*, 411–421.
50 Bodinet, C., Lindequist, U., Teuscher, E. Freudenstein, J. *Phytomedicine* **2002**, *9*, 606–613.
51 Hwang, S-A. Dasgupta A., Actor, J.K. *Clin. Chim. Acta* **2004**, *343*, 161–166.
52 Goel, V., Chang, C., Slama, J.V., Barton, R., Bauer, R., Gahler, R. et al. *Int. Immunopharmacol*. **2002**, *2*, 381–387.
53 Goel, V., Chang, C., Slama, J.V., Barton, R., Bauer, R., Gahler, R. et al. *J. Nutr. Biochem*. **2002**, *13*, 487–492.
54 Spits H., de Waal Malefyt, R. *Int. Arch. Allergy Immunol*. **1992**, *99*, 8–15.
55 Holscher, C. *Med. Microbiol. Immunol. (Berl.)*. **2004**, *193*, 1–17.
56 Schwarz, E., Metzler, J., Diedrich, J., Freudenstein, J., Bode, C., Bode, J.C. *J. Immunother*. **2002**, *25*, 413–420.
57 Gorsk, J.C., Huang, S., Pinto, A., Hamman, M. A., Hilligoss, J.K., Zaheer, N.A., Desai, M., Miller, M., Hall, S.D. *Clin. Pharmacol. Ther*. **2004**, *75*, 89–100.
58 Rininger, J.A., Kickner, S., Chigurupati, P., McLean, A., Franck, Z. *J. Leukoc. Biol*. **2000**, *68*, 503–510.
59 Barrett, B., Vohmann, M., Calabrese, C. *J. Fam. Pract*. **1999**, *48*, 628–635.
60 Lindenmuth, G.F., Lindenmuth, E.B. *J. Altern. Complement. Med*. **2000**, *6*, 327–334.
61 Geissberger, P., Sequin, U. *Acta Trop*. **1991**, *48*, 251–261.
62 Hudson, J.B., Graham, E.A., Rossi, R., Carpita, A., Neri, D., Towers, G.H.N. *Planta Med*. **1993**, *59*, 447–450.
63 Chin, H.W., Lin, C.C., Tang, K.S. *Am. J. Chin. Med*. **1995**, *23*, 273–278.
64 Brandão, M.G.L., Krettli, A.U., Soares, L.S.R., Nery C.G.C., Marinuzzi, H.C., *J. Ethnopharmacol*. **1997**, *57*, 131–138.
65 Redl, K., Breu, W., Davis B., Bauer, R. *Planta Med*. **1994**, *60*, 58–62.
66 Alvarez, L., Marquina, S., Villarreal, M.L., Alonso, D., Aranda, E., Delgado, G., *Planta Med*. **1996**, *62*, 355–357.
67 Schwartz , R.H. *Science*. **1990**, *248*, 1349–1356.

68 Mey, A., Revillard, J.P. *Infect. Immun.* **1998**, *66*, 2547–2552.
69 Chin, H.W., Lin, C.C., Tang, K.S. *Am. J. Chin. Med.* **1996**, *24*, 231–40.
70 Wu, L.W., Chiang, Y.M., Chuang, H.C., Wang, S.Y., Yang, G.W., Chen, Y.H., Lai, L.Y., Shyur, L.F. *Pharmac. Res.* **2004**, *21*.
71 Dimo, T., Rakotonirina, S.V., Tan, P.V., Azay, J., Dongo, E., Cros, G. *J. Ethnopharmacol.* **2002**, *83*, 183–191.
72 Ubillas, R.P., Mendez, C.D., Jolad, S.D., Luo, J., King, S.R., Carlson, T.J., Fort, M. *Planta Med.* **2000**, *66*, 82–83.
73 Folkman. J. *Nat. Med.* **1995**, *1*, 27–31.
74 Staniforth, V., Wang, S.Y., Shyur, L.F., Yang. N.S. *J. Biol. Chem.* **2004**, *279*, 5877–5885.
75 Chen, X., Oppenheim, J., Howard, O.M. *Int. Immunopharmacol.* **2001**, *1*, 229–236.
76 LaDuca, J. R., Gaspari, A.A. *Dermatol. Clin.* **2001**, *19*, 617–635.
77 Beutler, B. *J. Investig. Med.* **1995**, *43*, 227–235.
78 Ackermann, L., Harvima, I.T. *Arch. Dermatol. Res.* **1998**, *290*, 353–359.
79 Shah, A. Church, M.K., Holgate, S.T., *Clin. Exp. Allergy.* **1995**, *25*, 1038–1044.
80 Rieckmann, P., Albrecht, M., Kitze, B., Weber, T., Tumani, H., Broocks, A., Luer, W., Helwig, A., Poser, S. *Ann. Neurol.* **1995**, *37*, 82–88.
81 Kumar, A., Chaudhry, I., Reid, M.B., Boriek, A.M, *J. Biol. Chem.* **2002**, *277*, 46493–46503.
82 Haas, A.F., Wong, J.W., Iwahashi, C.K., Halliwell, B., Cross, C.E.,. Davis. P.A. *Free Radic. Biol. Med.* **1998**, *25*, 998–1005.
83 Sombra. L.L., Gomez. M.R., Olsina, R, Martinez, L.D., Silva, M.F. *J. Pharm. Biomed. Anal.* **2005**, *36*, 989–994.
84 Weckerle, C., Stutz, M., Baumann, T. *Phytochemistry.* **2003**, *64*, 735–742.
85 Chan, K. *Chemosphere* **2003**, *52*, 1361–1371.
86 Ernst, E. *Trends Pharmacol. Sci.* **2002**, *23*, 136–139.
87 Gertner, E., Marshall, P.S., Filandrinos, D., Potek, A.S., Smith, T.M. *Arthritis Rheum.* **1995**, *38*, 107–112.
88 Bauer, R., *Drug Inf. J.* **1998**, *32*, 101–110.
89 Wang, A., Li, L., Zanga, F., Fang, Y. *Anal. Chim. Acta.* **2000**, *419*, 235–242.
90 Richling, E., Höhn, C., Weckerle, B., Heckel F., Schreier, P. *Eur. Food Res. Technol.* **2003**, 544–548.
91 Schaneberg, B., Crockett, S., Bedir, E., Khan, I. *Phytochemistry* **2003**, *62*, 911–918.
92 Kunkel, A., Günter, S., Dette, C., Wätzig, H. *J. Chromatogr. A.* **1997**, *781*, 445–455.
93 Cameiro, M.C., Puignou, L., Galceran, M.T. *J. Chromatogr. A.* **1994**, *669*, 217.
94 Sorensen, J.M. *J. Altern. Complement. Med.* **2002**, *8*, 293–308.
95 Miller, L.G. *Arch. Intern. Med.* **1998**, *158*, 2200–2211.
96 Norred, C.L., Brinker, F. *Altern. Ther. Health Med* .**2001**, *7*, 58–67.
97 Parnham. M.J. *Phytomedicine* **1996**, *3*,95–102.
98 Escher, M.J, Desmeules, E., Giostra, G. *Mentha Drug points, BMJ* **2001**, *322*, 139.
99 Eliason, B.C., Kruger, J., Mark, D., Rasmann, D.N. *J. Am. Board. Fam. Pract.* **1997**, *10*, 265–271.
100 Fugh-Berman. A. *Arch. Intern. Med.* **1999**, *159*, 1957–1958.
101 Fugh-Berman, A., Ernst E. *Br. J. Clin. Pharmacol.* **2001**, *52*, 587–595.
102 Neal. R. *J. Altern. Complement. Med.* **2001**, *7*, S19–S21.
103 Kessler, R.C., Davis, R.B., Foster, D.F., Van Rompay, M.I., Walters, E.E., Wilkey, S.A., Kaptchuk, T.J., Eisenberg, D.M. *Ann. Intern. Med.* **2001**, *135*, 262–268.
104 Ernst, E. *Am. J. Med.* **1998**, *104*, 170–178.
105 Ko, R.J., Greenwald, M.S., Loscutoff, S.M., Au, A.M., Appel, B.R., Kreutzer R.A. et al. *West. J. Med.* **1996**, *164*, 71–75.
106 Dasgupta, A. *Am. J. Clin. Pathol.* **2003**, *120*, 127–137.
107 Boullata, J.I., Nace, A.M. *Pharmacotherapy* **2000**, *20*, 257–269.
108 Gordon, D.W., Rosenthal, G., Hart, J., Sirota, R., Baker, A.L. *JAMA* **1995**, *273*, 489–490.
109 Stickel, F., Egerer, G., Seitz, H.K. *Public Health Nutr.* **2000**, *3*, 113–124.

17
Use of a Liposomal Delivery System for Herbal-Based Therapeutics (with a Focus on Clove Oil)

Nadeem Ahmad, Maroof Alam, Iqbal Ahmad, and Mohammad Owais

Summary

Plant-derived medicines, used the world over since ancient times, appeal to the medical community and general populace because of their effectiveness in treating diseases with minimal toxicity. Plants are rich sources of all classes of chemicals, possessing a number of pharmacological actions that are widely exploited as therapeutics. Among them essential oils such as cinnamon oil, oregano oil, and clove oil are known for their promising antimicrobial properties. This chapter discusses our evaluation of the efficacy of clove oil in treating vulvovaginal candidiasis and the suitability and efficiency of its liposomized formulation. We found clove oil to possess strong antifungal activity against opportunistic fungal pathogens such as *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. It also displayed strong antibacterial activity. This inhibition of bacterial and fungal growth occurs in a concentration-dependent manner. On evaluating various formulations of clove oil, topical administration of the liposomized clove oil was found to be most effective against experimental murine vaginitis.

The development of modern delivery system-based formulations of plant-based medicines is paving the way for curing drug-resistant pathogens in the near future.

17.1
Introduction

Plant or herbal products are rich sources of nutrients and are good alternative medicines that are known to cure many diseases and disorders. Since the earliest days of recorded history, medicinal herbal products have been used effectively and safely for achieving various health benefits. Herbal medicine is based on the usage of a range of plant parts – seeds, berries, roots, leaves, bark, and flowers. Many countries in Asia, Africa, and South America possess a rich source of such medicinal plants, whose products have been used in traditional as well as folk medicine since

ancient times. In India, hundreds of medicinal plants are used alone or in different combinations in the preparation of around three dozen patented herbal formulations [1]. Herbal medicines are favored for their efficacy, minimal toxicity, and ability to cure even those diseases for which allopathic medicines fall short or are becoming less effective due to resistance, as in the case of antibiotics.

Over the years antibiotics have virtually eliminated certain diseases from the modern world and at one stage the scientific community started thinking it could cut back the development of newer chemotherapeutic agents. However, what we could not foresee was the ability of bacteria and other microorganisms to survive this onslaught by the development of drug resistance. Through mutation that takes place all the time at a low but dependable rate, pathogens that are resistant are constantly arising from the sensitive strains. Due to recent trend of globalization of resistance among different pathogens, we may shortly find ourselves back where we were before the Second World War era, when a simple infection in hospital often proved to be fatal.

The answer to this potential disaster could lie in finding some alternate way to combat the problem of resistance. Plant products found to possess antimicrobial properties have generated a mass of research aimed at bringing plant-based antimicrobial agents into use. Studies in the last decade have shown that garlic, onion, cinnamon, cloves, thyme, sage, and other spices can inhibit the growth of both Gram-positive and Gram-negative food-borne bacteria, yeasts, and molds [2]. The relatively long shelf life of spiced food products such as pickles, rice, and meat products clearly show the effects of the presence of these properties. Essential oils, the volatile fraction extracted from spices and herbs, are also recognized as containing many active antimicrobial compounds [3–6]. Several workers have evaluated the efficacy of essential oils in inhibiting various bacteria and fungi [7–10]. Table 17.1 lists the approximate essential oil content of some spices and herbs and their antimicrobial components.

In this chapter we focus on cinnamon oil, oregano oil, and clove oil, especially noted for their potential in alternative therapies to combat antibiotic-resistant microorganisms.

Table 17.1 Antimicrobial components of spices and herbs [11].

Spice/herb	*Approximate essential oil content (%)*	*Antimicrobial component(s)*
Garlic	0.3–0.5	Allicin
Mustard	0.5–1.0	Allyl isothiocyanate
Cinnamon	0.5–2.0	Cinnamaldehyde, eugenol
Cloves	16–18	Eugenol
Sage	0.7–2.0	Thymol, eugenol
Oregano	0.8–0.9	Thymol, carvacrol

17.1.1 Cinnamon Oil

Cinnamon oil is mainly extracted from the bark and leaves of the many species of the genus *Cinnamomum* of the Laurel family (Lauraceae), which is widely distributed throughout the tropical and subtropical Asia [12], and from waste products formed during the preparation of cinnamon spice. However, *Cinnamomum zeylanicum*, native to Sri Lanka, is the chief source of cinnamon spice and oil worldwide. Cinnamaldehyde and eugenol have been reported to be the main constituents in the oil and extract of *C. zeylanicum* [13], while others have also reported eugenyl acetate. However, the composition of cinnamon oil, and hence its potential use, depends very much on the species that is distilled as well as the part of the plant which is utilized. Cinnamon bark oil contains cinnamaldehyde as the major constituent, while cinnamon leaf oil's major constituent is eugenol rather than cinnamaldehyde. Cinnamon has been found to be effective in inhibiting the mycotoxigenic *Aspergillus* species *Aspergillus parasiticus* [14–16].

17.1.2 Oregano Oil

Carvacrol, the main antimicrobial component of oregano oil (obtained from *Origanum vulgare*), is a phenolic compound and possesses a broad sprectrum of antimicrobial properties. It has been found to have very effective antifungal activity against yeast [17], *Aspergillus ochraeus* [18], *Fusarium proliferatum* [19], and *Fusarium graminearum* [20]. Carvacrol also has good antibacterial properties against the food-borne pathogens *E. coli* 0157:H7 and *Salmonella enterica*. Carvacrol also exhibits strong activity against *H. pylori* [21].

17.1.3 Clove Oil

Clove oil extracted from the clove tree, *Syzygium aromaticum* (family Myrtaceae), also known as *Eugenia caryophyllata*, *Eugenia aromatica*, and *Eugenia carophyllus*, has been used as a stimulant, flavoring, and antiseptic agent since ancient times [22]. It derives its name form the Latin word *clavus*, which means "nail-shaped", referring to the clove tree bud, from which it used to be extracted. It can be extracted from the leaves, stem, or buds of the clove tree, the amount of the different constituents varying in each case.

Clove oil is a mixture of various essential oils and the constituents include 84–95% phenols comprising about 97% eugenol and 3% acetyl eugenol [22, 23]. Clove oil has a strong, spicy smell and the color varies from colorless to pale yellow with a medium to watery viscosity. Strong antimicrobial activities of clove oil against a range of bacterial and fungal pathogens have been reported by many workers [24, 25]. In spite of the fact that antibacterial as well as antifungal activity

has been reported for clove oil no serious efforts have been made to evaluate its potential in the treatment of fungal diseases such as vulvovaginal candidiasis and other ailments for which newer and better drugs need to be developed, mainly due to inefficiencies and/or toxic manifestations of the currently available drugs [8]. Based on studies conducted in our laboratory on a mouse, this chapter assesses the potential of clove oil as a future drug to treat vulvovaginal candidiasis.

We have also attempted to develop an effective and safe formulation of clove oil by incorporating it in liposomes. Our laboratory has been working on the use of liposomes as delivery system for various drugs as well as antigens [26].

Vulvovaginal candidiasis is a common mucosal fungal infection that is reported to affect at least three out every four women during their childbearing age [27]. It may never affect some women, while causing infrequent episodes in others, but yet it manifests in a third significant subpopulation, which comprises up to 5% of all adult women, as recurrent vulvovaginal candidiasis [28–30]. Though such infections may not usually be considered as life-threatening, they pose serious complications in the case of immunocompromised individuals such as patients with acquired immune deficiency syndrome (AIDS).

The causative organism in 85–90% of cases of vulvovaginal candidiasis is *Candida albicans* [28–30], which is also responsible for 60–80% of all types of *Candida* infections.

Oral and intravaginal treatments for vulvovaginal candidiasis are available, but still newer and better therapeutic agents need to be developed. Moreover, the resistance shown by fungal pathogens like *C. albicans* against most of the available antifungal drugs justifies the use of herbal medicines in the quest to develop a new strategy for the treatment of fungal infections [31–35].

In this chapter we present data on the use of clove oil in the treatment of vulvovaginal candidiasis from our laboratory studies, conducted on female Swiss mice. We have selected clove oil for the development of an effective and safe antifungal formulation on the basis of its already reported antifungal properties, its relatively easy availability, and its long and widespread use through traditional and folk medicine.

Our studies included the comparison of the antifungal properties of clove oil, either in free or liposomized form, with that of nystatin, a widely used antifungal drug. For antifungal studies we used *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* strains for comparison. We also tested antibacterial activity of clove oil against *Salmonella typhimurium*. We compared liposomized formulations of clove oil with liposomized nystatin and clove oil emulsion, all of which were applied by both topical and subcutaneous routes.

17.1.3.1 **Composition of the Clove Oil Used**

Before proceeding with our experiments we analyzed the batch of clove oil that we used in our studies by gas chromatography. The major constituents of the clove oil from Indian clove tree bud are listed in Table 17.2.

Table 17.2 Percentage composition of *Syzigium aromaticum* bud oil.

Compounds	**% *Composition***
Linalool	0.15
Methyl salicylate	0.34
Carvone	0.11
Eugenol	72.50
n-Butyl benzoate	01.50
iso-Eugenol-I	0.90
α-Copaene	0.10
β-Caryophyllene	16.50
(*E*)-α-Bergamotene	2.20
α-Humulene	1.80
Allo-aromadendrene	0.30
Eugenyl acetate	2.30
Calamenene	0.10
Cadinene	0.80
Caryophyllene oxide	0.40

17.2 Rationale for Using Liposomized Formulation of Clove Oil

Liposomes, first described by Alec D. Bangham [36], are essentially biodegradable, colloidal bilayered vesicular structures, formed when phospholipid molecules are dispersed in water. When such lipids are mixed in water under conditions of low shear, multilayered vesicles (MLV) are formed, usually ranging in size between 1 and 50 μm. Single bilayered liposomes are also formed, with a size range of 100–500 nm (large unilamellar vesicles, LUV) or 25–100 nm (small unilamellar vesicles, SUV).

Liposomes have been recognized as potentially effective delivery systems for various drugs and immunogenic molecules. They enclose an aqueous core in which molecules like hydrophilic drugs and peptides can be enclosed, while hydrophobic molecules are incorporated into the lipid bilayer during vesicle formation. The application of liposomes for drug delivery depends on physiochemical and colloidal properties such as composition, size, loading efficiency, and stability of the carrier molecules, as well as their biological interactions with cells. Recently a variety of other types of liposome structures have been developed. These include oligolamellar vesicles (OLV) and multivesicular liposomes (MVL). A variety of different techniques have been used for their preparation, including reverse phase evaporation vesicles (REV) and dehydration–rehydration vesicles (DRV).

Mezei and Gulasekharam [37] developed the first topical application of liposomes. Their studies revealed the increase in bioavailability within the skin of drugs incorporated in liposome. They also showed that there was a comparatively low level of drug in the blood, which indicates a preferable target site localization of the applied drug.

Since these initial studies, liposomes have been variously modified and improved for topical application, examples including niosomes (nonionic surfactant vesicles) [38], skin lipid liposomes [39], and transfersomes [40]. Biphasic delivery vesicles developed by Foldvari have been found to be very suitable for dermal and mucosal delivery of therapeutic agents, including proteins and DNA [41].

In view of these favorable properties and abilities of liposomes we have decided to use them for the development of an effective clove oil formulation.

17.2.1
Advantageous Properties of Liposomes

The properties of liposomes that make them useful as drug transporters are as follows:

- *Solubilization* – Using liposomes, hydrophobic compounds (e.g. those that constitute clove oil) can be delivered easily *in vivo*.
- *Flexibility in creating formulations* – Depending on the required route of administration, whether topical or *in vivo*, liposomes can be formulated as suspensions, aerosols or as gels, creams, or powders.
- *Longer, sustained release of drug* – Incorporation of clove oil in liposomes allows for its relatively constant release, resulting in longer lasting effects.
- *Internalization* – The ability of liposomes to be endocytosed/phagocytosed by cells allows delivery of clove oil to intracellular pathogens such as *C. albicans*.
- *Reduction of toxicity possibility* – Encapsulation of clove oil in liposomes prevents exposure of red blood corpuscles and the heart to clove oil.
- *Protection from enzymatic degradation* – The incorporated clove oil is protected from degradation by metabolizing enzymes.
- *Low dosage requirements* – The potential of delivery of clove oil to the desired site of action and prevention of its degradation allows relatively low amounts of clove oil to be used in the formulation.

17.3
Experiments Conducted to Develop Liposomal Clove Oil Formulation

Towards the sequential development of an effective clove oil formulation based on liposomes, we first conducted a series of *in vitro* evaluation tests followed by *in vivo* experiments. The *in vitro* experiments such as minimum inhibitory concentration (MIC) tests and growth-inhibition tests yielded results that showed clove oil to be quite effective against a range of bacterial and fungal pathogens. Clove oil inhibits bacterial and fungal growth in a dose-dependent manner. Next, to evaluate its *in*

vivo efficacy we tested it against vaginal candidiasis experimentally induced in female Swiss albino mice. The oil was found to be quite effective against vaginal candidiasis as evaluated by frequent monitoring of fungal loads in vaginal lavage fluid. A further encouraging factor was that the liposomized form of clove oil was found to be more effective than both its free form as well as emulsion form in suppression of experimental vaginal candidiasis.

17.3.1
Determination of MIC of Clove Oil against *Candida albicans*

The MIC for clove oil against *Candida albicans*, compared with that of the polyene antifungal drug nystatin, was determined by tube dilution method as described by National Committee for Clinical Laboratory Standards [42], with optical density readings measured at 580 nm. We found the MIC of clove oil against *Candida albicans* to be 0.051 mg mL^{-1} compared with 0.013 mg mL^{-1} for nystatin. This suggests that clove oil is at least four times less potent than nystatin.

17.3.2
Determination of MIC of Clove Oil against *Escherichia coli*

To test the antibacterial potency of clove oil, we determined its MIC and compared it with that of chloramphenicol, a widely used commercial antibiotic, against *E. coli* by tube dilution method as in the case of antifungal MIC determination. We found the MIC of clove oil against *E. coli* to be 0.102 mg mL^{-1} compared with 0.051 mg mL^{-1} for chloramphenicol. Thus clove oil has nearly half the antibacterial activity of chloramphenicol.

17.3.3
In Vitro Antibacterial Activity Test Results

The methanolic solution of clove oil was found to possess strong antibacterial activity as tested against *Escherichia coli*, *Salmonella typhimurium*, and *P. mirabilis*. In each case it was found to inhibit the bacterial growth in a dose-dependent manner. Neat methanol used as control showed no antibacterial activity (Table 17.3).

Table 17.3 Effect of clove oil on the growth of test bacteria.

	Amount (g per well)	*Zone of inhibition (cm) ± SD (cm)*		
		E. coli	*S. typhimurium*	*P. mirabilis*
Control	Neat methanol	0.00	0.00	0.00
Extract	0.010	2.60 ± 0.05	2.12 ± 0.05	2.10 ± 0.1

17.3.4
In Vitro Antifungal Activity Tests Results (Table 17.4)

The methanolic solution of clove oil displayed strong antifungal activity against *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*. In each case it inhibited the fungal growth in a dose-dependent manner. Strongest activity was shown against *A. fumigatus* followed by *C. albicans* and *C. neoformans*. Neat methanol showed no antifungal activity

Table 17.4 Effect of clove oil on the growth of fungal pathogens.

Sample	*Amount (g per well)*	*Zone of inhibition (cm) ± SD (cm)*		
		C. albicans	*C. neoformans*	*A. fumigatus*
C (Control)	Neat methanol	0.00	0.00	0.00
1	0.0025	0.00	0.00	1.00 ± 0.00
2	0.0050	1.25 ± 0.05	1.35 ± 0.05	2.00 ± 0.2
3	0.010	2.10 ± 0.1	1.35 ± 0.05	2.00 ± 0.2
4	0.020	2.50 ± 0.1	2.15 ± 0.15	2.95 ± 0.05

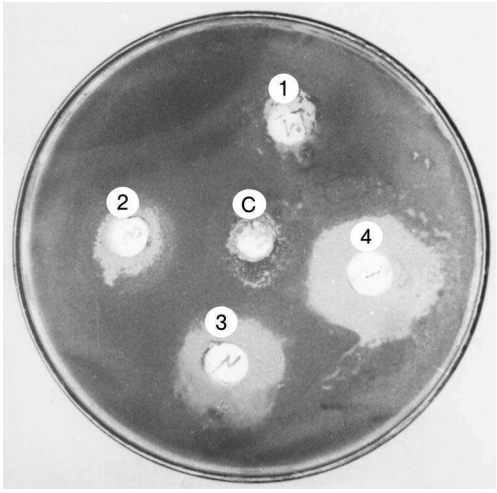


Fig. 17.1 *Candida albicans* culture plate showing growth inhibition zones caused by increasing amounts of clove oil.

17.3.5
In Vivo Antifungal Activity Test Results against Experimental Vaginal Candidiasis

17.3.5.1 Evaluation of Efficacy of Liposomized Clove Oil

In our quest to develop an efficient therapeutic formulation of clove oil we evaluated the role of its various formulations for their potential to suppress murine vagi-

nal candidiasis. Among various formulations, the liposomized clove oil was found to be more effective in suppression of fungal burden, measured as colony forming unit (cfu) at the end of 18 days, as compared to free form and an emulsion preparation of clove oil. Application of the formulation in all cases was subcutaneous (Table 17.5).

The data represent three different experiments. The viable cell number of *C. albicans* (cfu) was quantified by culturing serial dilution of vaginal lavage fluid collected from each mouse on day 18 post infection.

Table 17.5 Effect of clove oil and nystatin on *C. albicans* vaginal infection in mice.

Formulation	*cfu (18 days post infection)*
No treatment	55 000
Liposomized nystatin	1 000
Liposomized clove oil	8 000
Clove oil emulsion	31 000

Group of 10 estrogen-treated mice were inoculated intravaginally with 1.2×10^6 *C. albicans* blastoconidia. Drugs were administered on days 2, 4, 6, and 8 post challenge with infection.

17.3.5.2 Evaluation of Route of Administration

Next we evaluated the effect of route of administration and found that topical treatment with various clove oil formulations was most effective in decreasing fungal burden (cfu) followed by subcutaneous treatment (Table 17.6).

Table 17.6 Effect of route of administration of various clove oil formulations on *Candida albicans* vaginal infection in mice.

Days (post infection)	*Colony forming unit (cfu)*						
	No treatment	*Clove oil emulsion*		*Liposomized nystatin*		*Liposomized love oil*	
		Topical	*Subcutaneous*	*Topical*	*Subcutaneous*	*Topical*	*Subcutaneous*
2	55 000	53 000	56 000	52 000	59 000	55 000	56 000
4	59 000	55 000	59 000	49 000	50 000	50 000	51 000
8	54 000	38 000	49 000	15 000	35 000	23 000	39 000
16	57 000	34 000	41 000	1 000	19 000	2 000	22 000
18	54 000	33 000	40 000	1 000	18 000	1 000	22 000
20	57 000	34 000	40 000	1 000	19 000	1 000	21 000

Groups of estrogen-treated mice (10 mice per group) were intravaginally inoculated with 1.2×10^6 *C. albicans* blastoconidia. Drugs were administered on days 2, 4, 6, and 8 post challenge. The data are representative of three different experiments.

17.4 Conclusions

The emergence of drug resistance in pathogenic fungi as well as bacteria and the nonavailability of suitable antifungal drugs for certain infections such as systemic and mucosal mycoses (e.g. vaginal candidiasis) points out the urgent need for the discovery and development of alternative drugs sourced from natural sources. Biological resources such as plants hold a great promise as source of curative molecules. However these need thorough screening for their scientific and large-scale use as drugs. Numerous preliminary reports have demonstrated the antibacterial properties and antifungal properties of cinnamon oil, carvacrol, and clove oil. However these studies have been routine screening tests and there has been no concrete effort made to develop efficient drug formulations from these plant-derived molecules for the treatment of diseases such as vaginal candidiasis.

A large variety of formulations already exist for intravaginal therapy (tablets, creams, suppositories, pessaries, foams, solutions, ointments, and gels). However, their efficiency is often hindered by poor retention at the site of action due to the self-cleansing action of the vaginal tract. Liposomes offer an effective delivery option because of their ability to prolong contact of drug with the mucosal surface without inducing adverse local effects on the epithelium. The therapeutic potential of any drug depends on its bioavailability, retention time, and amount of drug at target site. Our experiments show that incorporating clove oil into liposomes can considerably increase its therapeutic efficacy. We have also demonstrated that in the case of vaginal candidiasis topical treatment may be most effective as it may lead to a systemic rather than a localized effect due to the remarkable absorption of drug from the vaginal wall [43].

Although the MIC of clove oil suggests it to be less potent than antifungal drugs such as nystatin, it can be safely said that the fungicidal potency of a liposomized formulation of the oil compares very well with that of nystatin, while providing for a less toxic, safe, and inexpensive alternative to commercial drugs without the risk of ever-increasing resistance shown by the target pathogens, toxicity problems at the increasing required doses, and problematic side-effects.

References

1 Handa, S.S., Sharma, A., Chakravarty, K.K. *Fitoterapia* **1989**, *57*, 307–351.

2 Nakatani, N. Antioxidant and antimicrobial constituents of herbs and spices. In *Spices, Herbs and Edible Fungi. Developments in Food Science*, 34th edn. Charalambous, G. (ed.). Elsevier, London, **1994**, pp. 251–271.

3 Maruzella, J.C. *Perfum. Essential Oil Records* **1963**, *68*, 40–44.

4 Deans, S.G., Ritchie, G. *Int. J. Food Microbiol.* **1987**, *5*, 165–180.

5 Dorman H.J.D., Deans, S.G. *J. Appl. Microbiol.* **2000**, *88*, 308–316.

6 Baratta, M.T., Dorman, H.J.D., Deans, S.G., Figueiredo, A.C., Barroso, J.G., Ruberto. G. *Flav. Fragr. J.* **1998**, *13*, 235–244.

7 Alice, D., Sivaprakasam, K. *J. Ecol.* **1996**, *62*, 182–193.

8 Hammer, K.A., Carson, C.F., Riley, T.V. *J. Appl. Microbiol.* **1999**, *86*, 985–990.
9 Kalembda, D., Kunicka, A. *Curr. Med. Chem.* **2003**, *10*, 813–829.
10 Mehmood, Z., Ahmad, I., Mohammed, F., Ahmad, S. *Pharmacol. Biol.* **1999**, *37*, 237–242.
11 Shelef, L.A. *J. Food Safety* **1983**, *6*, 29–44.
12 Zhu, L., Ding, D., Lawrence, B.M. *Perfum. Flav.* **1994**, *19*, 17–21.
13 Tateo, F., Chizzini, F. *J. Essent. Oil Res.* **1989**, *1*, 165–168
14 Azzouz, M.A., Bullerman, L.R. *J. Food Protect.* **1982**, *45*, 1248–1301.
15 Bullerman, L.B. *J. Food Sci.* **1974**, *39*, 1163–1165.
16 Bullerman, L. B., Lieu, F. Y., Seier, S. *J. Food Sci.* **1977**, *42*, 1107–1109, 1116.
17 Manohar, V., Ingram, C., Gray, J., Nadeem A. Talpur,. Echard, B.W., Bagchi, D., Preuss, H.G. *Mol. Cell Biochem.* **2001**, *228*, 111–117.
18 Basilico, MZ, Basílico J.C. *Lett. Appl. Microbiol.* **1999**, *29*, 238–241.
19 Velluti, A., Sanchis, V., Ramos, A. J., Egido, J., Marín, S. *Int. J. Food Microbiol.* **2003**, *89*, 145–154.
20 Velluti, A., Sanchis, V., Ramos, A.J., Turon, C., Marín, S. *J. Appl . Microbiol.* **2004**, *96*, 716–724.
21 Bergonzelli, G.E., Donnicola, D., Porta, N., Corthésy–Theulaz I.E. *Antimicrob. Agents Chemother.* **2003**, *47*, 3240–3246.
22 Trease, G.E., Evans, W.C. *Pharmacognosy*, 15th edn. Baillière Tindall, London, **1989**, pp. 460–462.
23 Myint, S., Daud, W.R.W., Mohmand, A.B., Kadhum, A.A.H. *J. Am. Oil. Chem. Soc.* **1995**, *72*, 1231–1233.
24 Beg, A. Z., Ahmad, I. *World J Microbiol. Biotech.* **2002**, *18*, 313–315.
25 Aqil, F., Beg, A.Z., Ahmad, I. *JMAPS* **2000**, *22*, 177–181.
26 Ahmad, N., Khan, M.A., Owais, M. *Eur. J. Biochem.* **2001**, *286*, 1–10.
27 Sobel, J.D. *Ann. N.Y. Acad. Sci.* **1998**, *544*, 547–557.
28 Fidel, P.L. Jr . *Am. J. Obstet. Gynecol.* **1985**, *152*, 924–935.
29 Sobel, J.D. *Am. J. Obstet. Gynaecol.* **1985**, *152*, 924–935.
30 Sobel, J.D. *Clin. Infect. Dis.* **1992**, *14*, S148–S153.
31 Sangland, D., Kuchler, K., Ischer, F., Pagani, J. L., Monod, M., Bille J. *Antimicrob. Agents Chemother.* **1995**, *99*, 2378–2386.
32 Laguna, F., Rodriguez-Tudela, J.L., Amriguez, A. *Clin. Infect. Dis.* **1994**, *19*, 542–543.
33 Vanden-Bossch, H., Dromer, F., Improvisi, I., Lozano-Chiu, M., Rex, J.H., Sanglard, D. *Med. Mycol.* **1999**, *36* , 119–128.
34 Davis, J. *Science* **1994**, *264*, 375–382.
35 Vromen, M., AJAM, Vander Ven, Knols, A., Stobberingh, E.E. *J. Antimicrob. Chemother.* **1994**, *44*, 113–116.
36 Bangham A.D., Standish, M.M., Watkins, J.C. *J. Mol. Biol.* **1965**, *13*, 328.
37 Mezei, M., Gulasekharan V, Liposome as selective new drug delivery system. Abstract, 39th International Congress in Pharmaceutical Sciences FIP, Brighton, UK, **1979**, p. 134.
38 Hofland, H.E.J., Bouwstra, J.A., Ponec, M., Bodde, H.E., Spies, F., Coos Verhoef, J., Junginger, H.E. *J. Controlled Release* **1991**, *16*, 155–168.
39 Abraham, W., Wertz, P.W., Landmann, L., Downing, D.T. *J. Invest. Dermatol.* **1987**, *88*.
40 Cevc, G., Blume, G., *Biochim. Biophys. Acta* **1992**, *1104*, 226–232.
41 Foldvari, M. Biphasic lipid vesicles. US Patent **1998**, 853 (5), 755.
42 National Committee for Chemical Laboratory Standards. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, M 7-A5, Vol. 20(2). Approved standard, 5th edn. NCCLS, Vayne, PA, **2000**.
43 Robinson Jr, Bolonga, W.J. *J. Control Release* **1994**, *28*, 87–94.

Subject Index