Medicinal and Aromatic Plants of the World

Dinesh Chandra Agrawal
Hsin-Sheng Tsay
Lie-Fen Shyur
Yang-Chang Wu
Sheng-Yang Wang *Editors*

# Medicinal Plants and Fungi: Recent Advances in Research and Development

Springer

# Medicinal and Aromatic Plants of the World

Volume 4

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Medicinal and Aromatic Plants (MAPs) have been utilized in various forms since the earliest days of mankind. They have maintained their traditional basic curative role even in our modern societies. Apart from their traditional culinary and food industry uses, MAPs are intensively consumed as food supplements (food additives) and in animal husbandry, where feed additives are used to replace synthetic chemicals and production-increasing hormones. Importantly medicinal plants and their chemical ingredients can serve as starting and/or model materials for pharmaceutical research and medicine production. Current areas of utilization constitute powerful drivers for the exploitation of these natural resources. Today's demands, coupled with the already rather limited availability and potential exhaustion of these natural resources, make it necessary to take stock of them and our knowledge regarding research and development, production, trade and utilization, and especially from the viewpoint of sustainability. The series Medicinal and Aromatic Plants of the World is aimed to look carefully at our present knowledge of this vast interdisciplinary domain on a global scale. In the era of global climatic change, the series is expected to make an important contribution to the better knowledge and understanding of MAPs. The Editor of the series is indebted for all of the support and encouragement received in the course of international collaborations started with his ISHS involvement, in 1977. Special thanks are due to Professor D. Fritz, Germany for making it possible. The encouragement and assistance of Springer Editor, Mrs. Melanie van Overbeek, has been essential in realizing this challenging book project. Thanks are due to the publisher - Springer Science+Business Media, The Netherlands - for supporting this global collaboration in the domain of medicinal and aromatic plants. We sincerely hope this book series can contribute and give further impetus to the exploration and utilization of our mutual global, natural treasure of medicinal and aromatic plants. Budapest, Prof. Dr. Ákos Máthé.

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*With profound gratitude, lead editor Dinesh Agrawal dedicates this book to the memory of his "late parents" for their untiring support and for creating a fulfilling life.*

# Preface

This volume is the continuation of our previous Springer book *Medicinal Plants – Recent Advances in Research and Development* (Springer link: http://www.springer.com/in/book/9789811010842). In this book, chapters on medicinal fungi (mushrooms) have been included considering their importance in the human health. Once considered toxic and a cause of contamination, now mushrooms have become an important source of nutrition and remedial measures. Due to its immense nutritional and therapeutic advantages, consumption of mushrooms has significantly increased throughout the world. Medicinal mushrooms are now gaining worldwide attention because of its pharmacologically bioactive compounds which have demonstrated potent and unique clinical properties. Scientific studies carried out during the last decade have validated evidences of their efficacy in a wide range of diseases. Extracts and bioactive compounds obtained from different mushrooms have been used medicinally as immunomodulator, anticancer, antibacterial, antiviral, anti-inflammatory, anti-atherosclerotic, neuroprotectant, cardioprotectant, antioxidant, and anti-hypoglycemic agents. There are ongoing research efforts on various aspects of medicinal plants and fungi in different parts of the world. The editors wish to bring their recent research and development works into light in the form of this book.

The chapters, mostly review articles, have been contributed by eminent researchers working with different disciplines of medicinal plants and fungi in different countries across the globe. Sixteen chapters have been divided into four major themes (i) medicinal properties/therapeutic effects of medicinal plants and fungi, (ii) bioactive compounds of medicinal plants and fungal endophytes, (iii) production systems and biotechnology of medicinal plants and fungi, and (iv) resources/techniques in medicinal plants and fungi.

Chapter 1 reviews the therapeutic potential of *Centella asiatica* in relation to its neuroprotective properties. Chapter 2 summarizes the latest research on medicinal oyster mushrooms (*Pleurotus* species) growing in the Mediterranean environment. Chapter 3 mainly focuses on *Cordyceps* (*Ophiocordyceps sinensis*) and other related species, their bioactive constituents, and traditional and current medicinal usages as agents against cancer, viral infections, and male and female sexual dysfunction. Chapter 4 covers an overview about mushrooms with antiallergic activities and describes the challenges for the exploration of the antiallergic potential of mushrooms. Chapter 5 describes traditional and modern medicinal usages of the "tiger

milk mushroom" (*Lignosus rhinocerotis*) and its immunomodulatory, antiproliferative, neuritogenesis, antiviral, and antimicrobial activities. Chapter 6 reviews the current taxonomic position of *Antrodia cinnamomea*, an extremely rare Formosan medicinal mushroom. Also, this chapter deals with the ethnomedical value of the species and its chemical constituents and medicinal properties, especially antioxidant and Nrf2-mediated cytoprotective effects. Chapter 7 presents a comprehensive overview on fungal endobiome as a source of bioactive metabolites having anticancer, antioxidant, antimicrobial, immunomodulatory, antidiabetic, acetylcholinesterase inhibition, antihelminthic, antiplasmodial, antileishmanial, and antitubercular properties. Chapter 8 constitutes a review on the studies on fungal alkaloids of *Claviceps purpurea* having poly-therapeutic applications. Chapter 9 reviews the studies conducted by Chinese scientists after 2007 on isolation, structural elucidation, and biological activities of the bioactive compounds derived from edible and medicinal fungi. Chapter 10 describes the endophytes from Malaysian medicinal plants, highlighting the diversity, metabolites produced, the current methods used in the biosourcing, and the prospects of anticancer agents. The primary focus of Chap. 11 is on mushrooms used traditionally for the treatment of cardiovascular diseases (CVD). In Chap. 12, the enzymatic mechanisms behind lignocellulose degradation by fungi are summarized, and several of the most important cultivated mushrooms are presented on their production requirements and medicinal properties. Chapter 13 presents examples of recent identification of genes and gene clusters for a variety of bioactive compounds in edible and medicinal fungi. Chapter 14 focuses on the application of various in vitro culture systems in the production of bioactive secondary metabolites in medicinal herbs and fungi in Taiwan. Chapter 15 presents the findings on cholinesterase inhibitory and memory enhancing potentials of selected species of the Nigerian flora. Chapter 16 reviews several fungi as a promising source of bioactive metabolites for drug discovery processes. Also, it has outlined isolation and identification strategies of known and novel metabolites from fungal endophytes, fungi isolated from marine sources, and extreme environments.

The editors hope that this compendium of review articles will be very useful as a reference book for advanced students, researchers, academics, business houses, and all individuals concerned with medicinal plants and fungi (mushrooms).

Taichung, Taiwan — Dinesh Chandra Agrawal
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Taipei, Taiwan — Lie-Fen Shyur
Kaohsiung, Taiwan — Yang-Chang Wu
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May, 2017

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The editors thank all the contributors to this book who took their valuable time to prepare their manuscripts. Without their contributions, this book would not have been possible.

The editors wish to place on record special thanks to the lead editor of this book Professor Agrawal for initiating the book proposal, handling the entire correspondence with the authors and Springer, dealing with editing and revision process of all the manuscripts and managing them from start to finish. Without his untiring efforts, this book would not have become a reality.

Editors Professor Agrawal and Professor Tsay thank Professor Tao-Ming Cheng, President of the Chaoyang University of Technology (CYUT), and Professor Chia-Chi Cheng, Dean, College of Science and Engineering, CYUT, Taichung, Taiwan for their constant support and encouragement during the progress of the book.

The editors sincerely thank the entire Springer team concerned with the book. Our especial thanks to Ms. Aakanksha Tyagi, Associate Editor – Life Sciences, Springer, who always promptly answered our queries regarding publication of the book.

Lead editor Professor Agrawal thanks his spouse Mrs. Manju Agrawal and daughters Ms. Somya and Ms. Neha for their encouragement and great support during the course of this book. Also, he expresses profound gratitude towards 'God, The Infinite Being' for providing necessary intelligence and strength to accomplish the arduous task of handling this book.

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# About the Editors

**Professor/Dr. Dinesh Chandra Agrawal,** Ph.D., graduated in 1976 from Aligarh Muslim University (national university) and obtained his Ph.D. degree in 1982. Professor Agrawal has more than 35 years of research experience in plant biotechnology of diverse species including medicinal plants and fungi. After serving for more than 31 years, in 2013, he superannuated as a chief scientist and professor of biological sciences at the CSIR-National Chemical Laboratory, Pune, the top ranking institute in chemical sciences under the umbrella of the Council of Scientific and Industrial Research (CSIR), Ministry of Science and Technology, Govt. of India. Currently, he is working as a professor in the Department of Applied Chemistry, Chaoyang University of Technology (CYUT), Taiwan. While in CSIR-NCL, Prof. Agrawal worked as a coordinator and project leader of several research projects funded by the Govt. of India. He has more than 165 publications including three books to his credit on different aspects of plant biotechnology including medicinal plants and fungi. More than 35 M.Tech./M.Sc. and 7 Ph.D. students have completed their thesis work under his guidance. Professor Agrawal has been bestowed several prestigious awards and fellowships such as the Alexander von Humboldt Fellowship (Germany), DBT Overseas Associateship (USA), British Council Scholar (UK), European Research Fellow (UK), and INSA Visiting Scientist. During these fellowships, he had opportunities to work in the USA, Germany, and the UK. Also, he had a research collaboration with UMR Vigne et Vins, INRA, Centre de Recherché Colmar, France. For more than 10 years, he has been a member of the executive committee of the Humboldt Academy, Pune Chapter, and held the position of treasurer.

Professor Agrawal has reviewed a large number of research papers for several SCI journals on plant biotechnology and served as a member of the editorial board of *Medicinal and Aromatic Plant Abstracts*, NISCAIR, Govt. of India. Presently, he is on the editorial board of the *International Journal of Applied Science and Engineering* (Scopus), serving as associate editor in chief of the journal.

**Professor/Dr. Hsin-Sheng Tsay,** Ph.D., is a renowned researcher and teacher. He completed his Ph.D. in agronomy from National Taiwan University about 40 years ago. He worked with Prof. Toshio Murashige, University of California, Riverside, for his Ph.D. on anther culture of tobacco. Later, he served at the Taiwan Agricultural Research Institute (TARI) for about 25 years and became head of the Agronomy Department. In TARI, his research pertained to anther culture of rice. There, he also worked on asparagus, papaya, sweet potato, and bamboo. For the last 25 years, Prof. Tsay has been working with medicinal plants indigenous to Taiwan and China. For the last 14 years, he has been working at Chaoyang University of Technology. There he served as dean of the College of Science and Engineering and director and chair professor of the Graduate Institute of Biotechnology. Professor Tsay has transferred about 20 technologies pertaining to functional foods for commercialization. He has published 265 research papers and has guided 23 Ph.D. and more than 100 master's students. Professor Tsay has made a significant contribution to the biotechnology of medicinal plants. During his career, he has organized about 80 plant tissue culture training workshops for international and national researchers. Of these, about 45 tissue culture workshops were conducted for local researchers, professors, vocational school teachers, students, and farmers. These were sponsored by the National Science Council and the Council of Agriculture, Taiwan. About 35 workshops were supported by the International Cooperation and Development Fund (ICDF) and the National Science Council, Taiwan. Participants (more than 500) in these workshops came from about 40 countries. Professor Tsay was invited by 13 countries to conduct plant tissue culture training workshops. He has a galaxy of students across the globe.

During his career, Prof. Tsay has won several national and international awards, including the "National Science Council Outstanding Research Award" for three times. He is on the editorial boards of several international journals and serves as a reviewer for several SCI journals of plant biotechnology.

**Professor/Dr. Lie-Fen Shyur** is a research fellow at the Agricultural Biotechnology Research Center, Academia Sinica, Taiwan, and also holds adjunct or joint professorships at five academic institutions in Taiwan. She has participated as editorial board member and invited referee for many international scientific journals and governmental and academic committees. Dr. Shyur's lab research foci include (1) research and development of phytomedicines and their derived phyto-agents for prevention or therapy of inflammatory diseases, including cancers, septic shock, and hepatitis, (2) elucidating biosynthesis pathway of pharmacologically bioactive compounds in medicinal plants, and (3) industrial enzyme biotechnology. Her research achievements include publication of

lab results in high-caliber and reputed journals, such as *Pharmacology & Therapeutics*, *Cancer Research*, *Oncotarget*, *Molecular Cancer Therapeutics*, *Molecular Oncology*, *Current Opinion in Chemical Biology*, *Molecular Medicine*, *Journal of Biological Chemistry*, *Environmental Science & Technology*, and *Journal of Medicinal Chemistry*, among others. Dr. Shyur has obtained more than 25 international patents and 2 national awards, namely, the 2014 Silver Award of the National Invention and Creation and the 10th National Innovation Award (2013). A number of technology licensing and cooperative research and development agreement (CRADA) projects were/are proceeded with local biotech or pharmaceutical companies. In March 2017, Shyur's lab research in collaboration with a research team at the Development Center for Biotechnology, Taiwan, received the approval from US Food and Drug Administration (FDA)'s Investigational New Drug (IND) for a clinical trial of an anticancer botanical drug. The ultimate goal of Dr. Shyur's lab research is to develop agricultural or plant-derived agents for human or animal health-care or bio-industrial usages.

**Professor/Dr. Yang-Chang Wu,** Ph.D., was born in Chiayi, Taiwan, in 1951. He obtained his Ph.D. in pharmacognosy from the College of Pharmacy, Kaohsiung Medical University (KMU), Taiwan, in 1986. After that, he joined the group of Prof. Yoshimasa Hirata at Meijo University, Japan, as a postdoctoral researcher from 1986 to 1987. Later, he joined the laboratory of Prof. Kuo-Hsiung Lee for further postdoctoral research at the University of North Carolina (UNC), Chapel Hill, USA. There he worked on the various synthetic approaches toward natural products and medicinal chemistry.

In 1990, he became a professor at the College of Pharmacy at KMU and director of the Graduate Institute of Natural Products (GINP) in 1992. Later, he served as the dean of the Office of Research and Development at KMU, from 2006 to 2009. Attributed to his significant contribution to research on natural products, he was selected as the chair professor and vice-president of the Graduate Institute of Integrated Medicine and College of Chinese Medicine at China Medical University (CMU), Taiwan, from 2010 to 2012. Since 2012, he was appointed as the chair professor and vice-president as well as dean of the School of Pharmacy, CMU, Taiwan. In 2017, he returned to KMU and served as the chair professor of the GINP and director of the Research Center for Natural Products and Drug Development (RCNPDD).

In 2007, he was awarded by the Wang Ming-Ning Foundation for outstanding merit and high scholastic achievement to medical and pharmaceutical research. In 2009, he received the National Science Council Outstanding Research Award in Taiwan. He also received the Outstanding Medical and Pharmaceutical Technology Award in 2010 by the TienTe Lee Biomedical Foundation, Taiwan. Professor Wu is known for his expertise in the area of translational research on Chinese herbal medicine, functional food, and new drug development.

Professor Wu has served as an editorial board member of six journals and as a referee for about 30 journals. Also, he is an outstanding member of the American Society of Pharmacognosy (ASP) and ten more other associations. Currently, he has also served as a member of the National Standards Technology Committee, Bureau of Standards, Metrology and Inspection, Ministry of Economic Affairs, Taiwan; executive director of Academia-Industry Consortium for Agricultural Biotechnology Park, Taiwan; director of Academia-Industry Consortium for Southern Taiwan Science Park, Taiwan; executive director of Niu-Chang-Chih (*Antrodia cinnamomea)* Industry Association, Taiwan; and the chairman of the National Standards Technical Committee of Niu-Chang-Chih Industry Association, Taiwan.

Professor Wu has published more than 556 research articles in SCI journals along with the authorship in several book chapters. He has been granted more than 40 patents and is in cooperation with more than 20 industry-academic organizations. Professor Wu has transferred six patent/technologies (including one new drug R&D tech transfer) to industry.

**Professor/Dr. Sheng-Yang Wang,** Ph.D., is a lifetime distinguished professor at the Department of Forestry, National Chung Hsing University, Taiwan. Dr. Wang obtained his Ph.D. degree from National Taiwan University. He is one of the well-known phytochemists in Taiwan and has expertise in the qualitative and quantitative determination of natural products by chromatography and spectroscopy. He has published more than 140 scientific articles so far. Dr. Wang has obtained several important scientific awards in his academic career, including "Excellent Research Award" for Young Faculty of National Chung Hsing University (2004); Dr. Ta-You Wu Memorial Award established by the National Science Council (2004), Taiwan; Academia Award of the Chinese Forestry Association and Top 10 Outstanding Agricultural Specialists of Taiwan in 2011; Academic Award, Forest Products Association of ROC; Academician Shang-Fa Academy Award for Distinguished Young Scholars in 2012; Development Program of Industrialization for Agricultural Biotechnology (DPIAB) Award for Outstanding Industry-University Collaboration Projects in 2013; Silver Award of the National Invention and Creation 2014; and Gold Award in "Seoul International Invention Fair 2014." The research areas of interest in Dr. Wang's laboratory are (1) development of new methodology in the isolation and structural elucidation of natural products, (2) phytomedicine investment of indigenous plants of Taiwan, and (3) functional genomic study of woody plants.

# Part I

# Medicinal Properties/Therapeutic Effects: Medicinal Plants and Fungi

# 1 *Centella asiatica*, an Ayurvedic Medicinal Plant, Prevents the Major Neurodegenerative and Neurotoxic Mechanisms Associated with Cognitive Impairment

Manuj Ahuja, Mansi Patel, Mohammed Majrashi, Vanisree Mulabagal, and Muralikrishnan Dhanasekaran

**Abstract**

Ayurveda is one of the ancient traditional healthcare systems that originated in India. A number of herbal-based medicinal preparations have been used for the treatment of health disorders associated with the nervous system. According to Alzheimer's disease Facts and Figures, millions of people around the world are suffering with cognitive impairment. Cognitive ailments and diseases are a group of disorders associated with mental health. The cognitive disorders mainly comprise of acute and chronic or reversible or irreversible conditions such as amnesia, delirium, and various types of dementia. These disorders primarily cause deficits in cognitive tasks associated with awareness, insight, knowledge, memory, and problem-solving skills. Alzheimer's disease is the most common type of dementia. It is a chronic neurodegenerative disorder that occurs due to excessive protein deposition inside and outside the neuron, oxidative stress, apoptosis, mitochondrial dysfunction, inflammation, and excitotoxicity. These neurotoxic mechanisms cause synaptic disturbance, alteration of neurotransmission leading

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to neurodegeneration. *Centella asiatica* is a well-known medicinal herb used in Ayurveda to improve cognitive functions since ancient times. In this article, we review the therapeutic potential of *Centella asiatica* in relation to its neuroprotective properties.

**Keywords**

Alzheimer's disease • Ayurveda • Botanicals • *Centella asiatica* • Cognitive disorders • Dementia • Herbal medicine • Neurotoxic mechanisms

## Contents



## Abbreviations

| | |
|---|---|
| 15-LOX | 15- Lipoxygenase |
| 3-NPA | 3-Nitropropionic acid |
| 5-HT | Serotonin |
| Ach | Acetylcholine |
| AChE | Acetylcholinesterase |
| AD | Alzheimer's disease |
| ADDLs | Aβ-derived diffusible ligands |
| AICD | APP intracellular domain |
| AMPARs | 2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propionic acid receptors |
| APH-1 | Anterior pharynx-defective 1 |
| APP | Amyloid β precursor protein |
| ATP | Adenosine triphosphate |
| Aβ | Amyloid beta |
| BACE | Beta-site APP-cleaving enzyme |
| BBB | Blood-brain barrier |
| BDNF | Brain-derived neurotrophic factor |
| C83 | Carboxyl-terminal 83-aa fragment |
| $Ca^{2+}$ | Calcium |
| cAMP | Cyclic adenosine monophosphate |

| | |
|---|---|
| CNS | Central nervous system |
| COX-2 | Cyclooxygenase 2 |
| CREB | Cyclic adenosine monophosphate response element-binding protein |
| CTLs | T-cell lymphocytes |
| FAD | Familial Alzheimer's disease |
| GABA | Gamma-aminobutyric acid |
| GAD | Glutamate decarboxylase enzyme |
| GLT | Glutamate transporter |
| IFN | Interferon |
| IL | Interleukin |
| $iPLA_2$ | $Ca^{2+}$-independent phospholipase $A_2$ |
| LTP | Long-term potentiation |
| MAPK | Mitogen-activated protein kinase |
| mGluRs | Metabotropic glutamate receptors |
| MPTP | 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| NADPH | Nicotinamide adenine dinucleotide phosphate oxidase |
| NE | Norepinephrine |
| NFTs | The neurofibrillary tangles |
| NMDARs | N-Methyl-D-aspartate receptors |
| NO | Nitric oxide |
| NSAIDs | Nonsteroidal anti-inflammatory drugs |
| $O^{2-}$ | Superoxide radical |
| PEN-1 | Presenilin 1 |
| PEN-2 | Presenilin 2 |
| PKA | Protein kinase A |
| $PLA_2$ | Phospholipase $A_2$ |
| PS | Presenilin |
| SAD | Sporadic Alzheimer's disease |
| SPs | Senile plaques |
| TNF | Tumor necrosis factors |
| β-CTF | Carboxyl-terminal 99-aa fragment |

## 1.1 Introduction

For centuries, substantial extent of the world population (80%) has mainly relied on traditional medicinal practices involving herbal medicine for their routine healthcare needs. Herbal medicines are also referred as botanicals or phytomedicines. These medicines refer to herbs, herbal materials, herbal preparations, botanical formulations, and finished herbal products. The active ingredients of the herbal medicines are made of different plant parts, such as seeds, berries, roots, leaves, bark, or flowers (Sharma and Chaudhary 2015). Herbal medicines are exponentially gaining attention for its use in preventing and treating broad spectrum of health-related disorders (Kumar et al. 2012). Majority of the medical prescriptions used today for the treatment of innumerable chronic disorders are plant-based formulations or

botanical-derived synthetic analogues. In the eastern part of the world, medicinal herbs have been used prophylactically and therapeutically for eras. The herbal medicines are presently practiced as a primary source of medicine for prevention and treatment of various health disorders. However, the herbal medicine practices are actively pursued in the rural areas mostly as compared to the urban places. Eastern countries have the unique distinction of having various medicinal practices associated with herbal medicine. With regard to the Indian herbal medicines, Ayurveda, Siddha, and Unani are well-known ancient medicinal practices in the healthcare system (Pandey et al. 2013).

Ayurveda is considered as a unique and distinct medicinal practice, which takes into consideration the physical, psychological, philosophical, ethical, and spiritual well-being of mankind. Ayurveda literally means "science of life" and has been used since folklore times for both preventive and curative measures. Around 25,000 effective plant-based formulations are used in ethnopharmacological approach and folklore Ayurvedic medicine in India (Alvari et al. 2012). This medicinal system has a holistic approach and thus has different approach in its therapeutic treatment as compared to the modern drug therapy. Instead of adopting the organ-oriented anatomy and physiology theory of the conventional medical science, Ayurveda has its own science which is known as the *Panchamahabhuta.* This is based on the five rudimentary elements: *akasha* (sky), *vayu* (air), *agni* (fire), *prithvi* (earth), and *jala* (water). Ayurveda, through reverse pharmacology, has made a pivotal contribution to the drug discovery processes along with new ways of identifying active compounds, reduction of drug-induced adverse reactions, and development costs. Plant components can act alone or along with other constituents from the same plant that may boost the activity of compounds or counter the toxic effects of compounds. Interestingly, an herbal medicine might have an additive, potentiating, synergistic, or antagonistic effect. This is the main path of action of Ayurvedic medicine where a combination of herbs is often prescribed. A significant number of traditional Ayurvedic medicine has been used to enhance cognitive functions and to ease symptoms that are correlated with cognitive disorders (Howes et al. 2003). The major Ayurvedic botanicals that have been evaluated for their potential to treat cognitive disorders are as follows: *Withania somnifera* (ashwagandha), *Bacopa monnieri* (brahmi), *Curcuma longa* (Indian ginseng), *Convolvulus pluricaulis* (shankapushpi), *Clitoria ternatea* (butterfly pea), *Centella asiatica* (Gotu kola), *Celastrus paniculatus* (jyotishmati), *Terminalia chebula* (yellow myrobalan or chebulic myrobalan), and *Nardostachys jatamansi* (jatamansi) (Ven Murthy et al. 2010; Solanki et al. 2015). The seeds and the seed oil of *Celastrus paniculatus* (Celastraceae) have been used for "stimulating intellectual ability and sharpening the memory" (Warrier et al. 1995; Howes and Houghton 2003). The roots of *Clitoria ternatea* (Leguminosae) have been shown to promote intelligence and enhance memory retention by affecting the cholinergic activity (Warrier et al. 1995; Misra 1998). *Terminalia chebula*'s (Combretaceae) ripe fruit is regarded as a brain power booster and memory promoter (Misra 1998; Manyam 1999; Naik et al. 2002). In the current article, we describe the cognitive neurodegenerative disorders and the therapeutic potential of *Centella asiatica* in relation to its neuroprotective properties.

## 1.2 Cognitive Disorders

Cognitive disorders are a group of mental disorders that can affect mood, functional activities, and behavior of an individual by impairing perception, memory, communication, reasoning, and judgment. Among cognitive impairments, dementia has attracted global attention for the past few decades. A slow and progressive neuronal dysfunction in the central nervous system (CNS) is the root cause of dementia, which ultimately leads to sensory dysfunction (Solanki et al. 2015). According to the definition given by the physician in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (*DSM-IV),* dementia has symptoms such as decline of memory along with at least loss of one of the cognitive abilities such as coherent speech/language, perception, judgment, execution of motor functions, and/or reasoning. A definitive diagnosis of the loss of the cognitive abilities must be present for at least 6 months. There are various types of dementia such as mild cognitive impairment, vascular dementia, mixed dementia, Lewy body dementia, Alzheimer's disease, Parkinson's disease, frontotemporal dementia, Creutzfeldt-Jakob disease, normal pressure hydrocephalus, Huntington's disease, and Wernicke-Korsakoff syndrome (Mori et al. 2012). Dementia is an age-related disorder and affects 10–15% of adults aged above 65 years, and its occurrence increases by 35–50% in adults of ages more than 85 years (Mori et al. 2012). It was estimated that in 2010, about 36 million people were suffering from some form of dementia worldwide. If left unchecked, it is expected to rise to 66 million by 2030 and 115 million by 2050 (Label 2009). Various types of dementias are as given in Table 1.1.

Alzheimer's disease is a chronic progressive neurodegenerative cognitive disorder and is the most common type of age-related dementia (Hage et al. 2010). Alzheimer's disease mainly affects the elderly people. Since the life expectancy in the United States (from 55 years to over 75 years of age) and around the world has increased, a strong correlation with a widespread of age-related cognitive disorders has been established (Gray et al. 2016). In the United States, Alzheimer's disease is one of the most predominant irreversible neurodegenerative disorders, which gradually leads to the overall attenuation of higher cognitive abilities (Alzheimer's Disease International 2009). Amnesic cognitive impairment (mild memory loss) is the early sign of Alzheimer's disease (Howes et al. 2003). Mild memory loss usually leads to the decline in other cognitive abilities such as word finding, vision/spatial issues, and impaired reasoning. Based on time of the onset of disease, Alzheimer's disease can be classified into two categories: familial Alzheimer's disease (FAD) and sporadic Alzheimer's disease (SAD). FAD occurs due to the uncontrolled genetic cause such as genetic mutations in the amyloid β precursor protein (APP) and presenilin (PS) genes which accounts for almost 3% of the cases and usually affects patients at an early age of less than 60 years (Mori et al. 2012). However, SAD is caused by the unknown pathological factors and represents 97% of the cases. SAD affects people of age 65 years or above and accounts for 50–60% of dementia cases (Howes et al. 2003; Label 2009). Irrespective of the types, both FAD and SAD have severe loss of memory, language, visuospatial skills, and emotion followed by inability to walk and swallow. Alzheimer's disease patients with early

**Table 1.1** Characteristic features of different forms of dementia

| Types of dementia | Characteristic features |
|---|---|
| Mild cognitive impairment | Memory loss, language impairment, other mental malfunction |
| Vascular dementia | Elevated cholesterol levels, high blood pressure, hardening of the blood vessels (arterial walls), hyperglycemia, disrupted blood flow to the brain |
| Mixed dementia | Vascular dementia and Alzheimer's disease occur simultaneously |
| Lewy body dementia | Alpha-synuclein composed of Lewy body deposition, progressive cognitive decline, memory impairment deterioration in visuospatial ability, visual hallucinations, rapid eye movement, sleep behavior disorder, severe neuroleptic sensitivity |
| Parkinson's disease | Bradykinesia, rigidity, postural instability, tremor or shaking of limbs, speech impairment, muscle stiffness, loss of autonomic movements. Lewy body deposition, drooling, seborrhea, constipation, sexual dysfunction, olfactory problem |
| Frontotemporal dementia | Tremor, rigidity, memory impairment, loss of speech |
| Creutzfeldt-Jakob disease | Progresses rapidly, depression, deposition of prion protein, memory deficit, movement disorders, mood swings |
| Normal pressure hydrocephalus | Accumulation of cerebrospinal fluid in the ventricles of the brain and spinal cord, seizures, memory deficit, impaired vision |
| Huntington's disease | Degeneration of striatal neurons, movement, cognitive and psychiatric dysfunction, inherited changes in a single gene |
| Wernicke-Korsakoff syndrome | Thiamine deficiency, ataxia, memory loss, alcoholism |
| *Other cognitive impairments* | |
| Down syndrome | Due to genetic impairment chromosome 21 |
| Prenatal alcohol/nicotine exposure | Excessive alcohol intake or nicotine exposure |
| Homocysteinemia | Increased levels of homocysteine |

onset tend to have more brain irregularities as compared to the late-onset disease. As this neurodegenerative disease progresses, it is further distinguished and classified depending on the severity of symptoms, such as mild Alzheimer's disease, moderate Alzheimer's disease, and severe Alzheimer's disease. The stages of Alzheimer's disease and their symptoms have been summarized in Table 1.2. However, it is not just the genetic and symptomatic features that are vital, but the prevention, therapy, drug interaction, patient care, and economic aspects also play a critical role in Alzheimer's disease "care." Hence, in the next section, we shall look into the prevalence and economic impacts of Alzheimer's disease.

### 1.2.1 Prevalence of Alzheimer's Disease

Universally, nearly 44 million people have been diagnosed with Alzheimer's disease or a related dementia. Alzheimer's disease is most common in Western Europe (North America is close behind) and is least prevalent in sub-Saharan Africa (Alzheimer's Disease International). Alzheimer's disease is the sixth leading cause

**Table 1.2** Different stages and symptoms of Alzheimer's disease

| Stages of Alzheimer's disease | Symptoms |
|---|---|
| Mild | Mild coordination issues |
| | Mood swings |
| | Recurrent recent memory loss |
| | Repeating questions |
| Moderate | Confusions, misapprehensions, aggression, and paranoia |
| | Difficultly in recognizing family and friends |
| | Disturbances in sleep |
| | Feeling withdrawn and forgetfulness |
| | Perpetual memory loss |
| | Tremors and rigidity |
| Severe | Completely dependent on support giver for day-to-day activity |
| | Confusion between past and present |
| | Immobility |
| | Problems with swallowing and incontinence |
| | Severe impairment of speech |

of mortality in the United States. Based on Alzheimer's Association statistics in 2015, an estimated 5.3 million Americans of all ages had Alzheimer's disease and other dementia (Alzheimer's Association 2015). These numbers are projected to rise to 15 million by the year 2050 (Jadidi-Niaragh et al. 2012). About 35.6 million people were diagnosed with dementia in 2010 worldwide (World Alzheimer's report 2009). It has been predicted that the number will double every 20 years and rise to 65.7 million in 2030 and 115.4 million in 2050. Men have fewer incidences of Alzheimer's disease and other dementia than women. It is postulated that almost two-thirds of Alzheimer's diagnosed American population are women. It is estimated that in the United States, one in eight people of 65 years and older and nearly half of people of 85 years and older are Alzheimer's patients. About 7.7 million new cases of dementia have been reported each year, thereby implying the prevalence of new case every 4 s in the world.

### 1.2.2 Etiology and Pathophysiology of Alzheimer's Disease

Even though Alzheimer's disease is an old disease, the specific etiological causes of this disease are still to be precisely elucidated and thus lead to the subject of continuous research. From the various studies carried on so far, it can be concluded that majority of the pathological features observed in the CNS are senile plaques (SPs), neurofibrillary tangles, oxidative stress, apoptosis, inflammation, excitotoxicity, and neurotransmitter disturbances (Howes et al. 2003). Based on the morphology and histology, the Alzheimer's disease occurrence was hypothesized as cerebrovascular accumulation of amyloid beta (Aβ), predominant formation of neurofibrillary tangles in the cortex and hippocampus, followed by neuronal and synaptic loss, brain

atrophy, and enlargement of cerebral ventricles (Khachaturian 1985; Cummings et al. 1998; Imbimbo et al. 2005). Diminutive neuronal loss or reactive gliosis is related with diffused Aβ plaques, whereas the Congo red and thioflavin S-positive plaques made up of fibrillary Aβ are related with neuronal loss, dystrophic neurites, and reactive astrocytes (Rozemuller et al. 1989; Itagaki et al. 1989). The neurofibrillary tangles (NFTs) are intracellular lesions consisting of twisted filaments of a cytoskeleton protein called tau protein (Castellani et al. 2010). The neuritic plaques, also known as senile plaques, are extracellular lesions composed of the 40–42 amino acid-long peptide Aβ fragments derived from amyloid precursor protein (APP) (Gomez-Isla et al. 1996; Selkoe 1998). Biochemically, the SPs are amyloid protein deposits mainly composed of beta-convoluted sheet of amyloid peptide of length 1–40 and 1–42 ($A\beta_{1-40}$ and $A\beta_{1-42}$) that gets cleaved from APP found in the neuronal cell membranes. NFTs are hyperphosphorylated tau proteins in the neuronal cells. Postmortem brain autopsy of Alzheimer's disease expresses SP, NFTs, and atrophy of the cortical and temporal lobes of the brain. Such pathogenic hallmarks also co-localize with activated microglia, astrocytes, inflammatory cytokines, and proteins surrounding the dystrophic neuritis (Akiyama et al. 2000). Most of the cases of genetically inherited Alzheimer's disease (FAD) are due to the mutations in one of the gene that encodes for the APP or in genes encoding APP (presenilin1-PSEN1 and presenilin 2-PSEN2) processing compound cellular machinery (Imbimbo et al. 2005; Piaceri et al. 2013). Conversely, SAD has been associated with several genetic and environmental factors. Despite numerous researches being carried out for almost a century, the etiology of SAD still remains vague (Piaceri et al. 2013). Out of several existing theories that have been put forth for understanding the pathogenesis of Alzheimer's disease, Aβ and tau hypothesis are the widely accepted scientific theories currently. Nevertheless, the certainty of these theories still needs to be further elucidated.

#### 1.2.2.1 Amyloid Beta (Aβ)-Induced Neurotoxicity in Alzheimer's Disease

The main basis of amyloid cascade hypothesis is the characteristic extracellular senile plaques, one of the histological hallmarks of Alzheimer's disease (Imbimbo et al. 2005). According to the Aβ toxicity or amyloid hypothesis, the primary causative agent for Alzheimer's disease is Aβ ($A\beta_{40}$ and $A\beta_{42}$) peptides deposition (Cummings et al. 1998; Farlow 1998; Imbimbo et al. 2005; Karantzoulis and Galvin 2011; Piaceri et al. 2013). The Aβ peptides are derived by proteolysis of a type I transmembrane glycoprotein produced in many cells called the APP. In short, APP is a type I transmembrane protein processed by two competing catabolic pathways, non-amyloidogenic and amyloidogenic pathway (Hage et al. 2010). In non-amyloidogenic pathway, the initial proteolysis with α-secretase enzyme results in the generation of sAPPα and carboxyl-terminal 83-aa fragment (C83 or carboxyl-terminal fragments, CTFα), anchored at the membrane, followed by further proteolysis of C83 fragment with γ-secretase subsequently results in formation of N-terminal peptides, p3, and APP intracellular domain (AICD). Conversely, in the amyloidogenic pathway, the initial proteolysis of APP is performed by APP-cleaving

enzyme, β-secretase that results in the formation of sAPPβ and carboxyl-terminal 99-aa fragment (C99 or β-CTF). The γ-secretase further processes C99 fragment to secrete Aβ peptides and APP intracellular domain (Hage et al. 2010). According to amyloid Alzheimer's disease theory, these Aβ peptides of varying lengths oligomerize to form soluble oligomers and accumulate in the brain (Suh and Checler 2002). This is the significant initiating event in the development of Alzheimer's disease. However, the specific species of Aβ that results in neurotoxicity is one of the many uncertainties that remain to be determined.

Beta-site APP-cleaving enzyme (BACE) is a single protein that is associated with β-secretase. β-site amyloid precursor protein-cleaving enzyme 1 (BACE-1) is a type 1 transmembrane aspartic protease related to the pepsin and retroviral aspartic protease families. It is mainly present in the acidic intracellular compartments (e.g., endosomes, trans-Golgi) with its active site in the lumen of the vesicles. The maximum activity and amount of BACE are found in the CNS (neurons), which is similar to β-secretase. BACE transfection (cDNA) and its antisense oligonucleotide exposure increased or decreased Aβ levels and β-secretase-cleaved APP fragments in APP-overexpressing cells. BACE2 is expressed in neurons (low levels), and it does not have the same cleavage activity on APP as β-secretase. Reformation of γ-secretase activity in yeast has discovered the presence of four components: presenilin, nicastrin, anterior pharynx-defective 1, and presenilin 2 (Gotz et al. 2004). FAD patients with mutations at APP, PSEN-1, and BACE protein confirmed the genetic framework to this theory, in turn confirming the pathological hallmarks of Aβ deposition in the brain of such patients less than 65 years (Citron et al. 1992; Piaceri et al. 2013). Increased BACE activity and linkage to the apolipoprotein E polymorphism found in sporadic Alzheimer's disease patients have further confirmed that these are the key risk factors for developing late-onset Alzheimer's disease (Bales et al. 1997; Farlow 1998). Various other biochemical studies also strengthen the Aβ theory associated with neuronal insult in cholinergic neurodegeneration. Aβ deposition prior to the formation of NFTs, tau fibril formation in the presence of Aβ, neuronal loss, and clinical symptoms of Alzheimer's disease are the notable biochemical processes seen in the early stages of Alzheimer's disease (Lewis et al. 2001; Mayeux et al. 2003; Hurtado et al. 2010; Chiu et al. 2012).

Neuronal toxicity is induced by Aβ via various mechanisms such as oxidative stress (Butterfield et al. 2001), disruption of mitochondrial activity (Schapira and Reichmann 1995), energy imbalance (Carvalho et al. 2012), stimulation of neuroinflammation (Verri et al. 2012), disturbance in calcium ($Ca^{2+}$) homeostasis (Resende et al. 2007), perturbation of axonal transport (Decker et al. 2010), and activation of apoptotic signaling (Imaizumi et al. 1999; Kudo et al. 2012). These mechanisms eventually lead to the disturbance in neuronal cell integrity and CNS function, thereby resulting in accelerated neuronal cell death and Alzheimer's disease pathogenesis. Excitotoxicity also plays a crucial role in neuronal cell death. This is distinctly evident in several chronic progressive neurodegenerative disorders (Thellung et al. 2013). Glutamate can act on various types of receptors (AMPA, NMDA, and kainate). Glutamate-like acetylcholine is also involved in cognitive functions such as learning and memory in the brain. The form of synaptic plasticity known as

long-term potentiation takes place at glutamatergic synapses in the hippocampus, neocortex, and other parts of the brain. Excitotoxicity is mainly caused by the imbalance of $Ca^{2+}$ homeostasis mediated by slow N-methyl-D-aspartate receptors ion channels resulting in the stimulation of various oxidative stress pathways. One of the theories is that the Aβ peptides induce synaptic discrepancies such as inhibition of long-term potentiation (LTP) in the hippocampal perforant pathway (Rowan et al. 2004; Parameshwaran et al. 2008; Ferreira et al. 2012). Aβ peptides also decrease surface expression and function of 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propionic acid receptors (AMPARs) and NMDARs, thereby impairing glutamatergic transmission (Parameshwaran et al. 2008). This Aβ deposition through induction of these harmful effects may lead to neurotoxicity, thereby resulting in the progression of Alzheimer's disease.

#### 1.2.2.2 Oxidative Stress: A Pathogenic Factor

Oxidative stress is a vital pathogenic factor underlying various neurodegenerative disorders (Karpińska and Gromadzka 2013). Oxidative stress is one of the well-studied neurotoxic mechanisms for the study of Alzheimer's disease and has been investigated both as secondary and primary incident in Alzheimer's disease pathogenesis (Markesbery 1997; Butterfield et al. 2001; Moreira et al. 2008). It is also contemplated that free radical generation and depletion of antioxidants may play a pivotal role in the pathogenesis of Alzheimer's disease, similar to the oxidative stress theory of aging (Choi et al. 2012). In support of this theory, there are direct evidences which suggests that there is an increase in the level of metals ($Al^{3+}$ and $Mn^{2+}$) in the brains that are capable of stimulating free radical generation (Leskovjan et al. 2011). Remarkable amount and variety of reactive oxygen species (ROS) are generated in the early stages which decline with the disease progression (Nunomura et al. 2001). Studies have also found a significant increase in the lipid peroxidation and its product 4-hydroxynonenal in the ventricular fluids of Alzheimer's disease patients and also in transgenic animals (Siegel et al. 2007). Lipid peroxidation is a product of the direct effect of free radicals on lipids. Lipids are highly vulnerable to oxidative stress. As stated by the amyloid theory, deposition of Aβ is the key initiating factor resulting in the ROS generation via several mechanisms such as interaction with metals. This results in the release of hydrogen peroxide that further induces the lipid peroxidation and consequent production of extremely reactive neurotoxic oxygen species, aldehyde, 4-hydroxynonenal (Abdul et al. 2008). Increased ROS leads to an altered activity of key enzyme complexes involved in energy metabolism and electron transport chain, thereby resulting in superoxide radical ($O^{2-}$) formation. Such reactive species oxidize other molecules like nitric oxide (NO), leading to the formation of highly reactive peroxynitrite radical, a cytotoxic agent that induces peroxidation of lipid membranes, and other hydroxyl radical/free radicals causing neurotoxicity (Subathra et al. 2005; Chen et al. 2013). Furthermore, the oxidative stress causes an increase in the levels of damaged proteins and DNA in the brains of Alzheimer's disease patients (Markesbery 1997; Moreira et al. 2008). Additionally, mitochondrial dysfunction associated with disrupted energy metabolism, notable Aβ release, and its deposition leads to programmed cell death (Siegel

et al. 2007; Abdul et al. 2008; Chami and Checler 2012; Verri et al. 2012). Free radicals also attack DNA, resulting in DNA cleavage and damage, thereby leading to apoptotic changes in neurons (Kumar and Gupta 2002; Boland and Campbell 2004; Tabner et al. 2005) and, consequently, cell death (Sultana et al. 2006; Moreira et al. 2008; Abdul et al. 2008; Verri et al. 2012). It is known that an elevated ROS production in mitochondria can inhibit adenosine triphosphate (ATP) synthesis, release cytochrome c, and induce mitochondrial permeability transition (Ichas and Mazat 1998; Rizzuto et al. 2000; Tewari et al. 2016). Dysfunction in the powerhouse of the cell, mitochondria, can result in the release of pro-inflammatory and proapoptotic factors which initiate, intensify, and implement various signals resulting in apoptotic cell death (Kroemer and Reed 2000). Moreover, mitochondrial dysfunction and associated bioenergetic breakdown leads to aberrant cellular ion homeostasis, which can result in cellular disruption and swelling of the cells, ultimately causing necrotic cell death (Nieminen 2003). In conclusion, oxidative stress is known to be the earliest, most detrimental event and could be one of the targets for therapeutic interventions for Alzheimer's disease.

#### 1.2.2.3 Neurochemical Alterations

Noticeable change in the levels of several neurotransmitters has been observed in Alzheimer's disease patients, which closely correlates with its different cognitive and noncognitive symptoms (Reinikainen et al. 1990). Various studies have associated the neuropathology resulting in memory loss due to the substantial deficits in cholinergic neurotransmission, one of the severities seen in Alzheimer's disease (Bowen et al. 1976; Bierer et al. 1995; Francis et al. 1999; Gottwald and Rozanski 1999; Howes et al. 2003). Initially, various histological and pathological studies have discovered substantial degeneration of cholinergic nucleus basalis of Meynert in neocortical and hippocampal regions of the brains of Alzheimer's disease patients. Drugs that stimulate cholinergic receptors prolong the availability of acetylcholine (Ach) by inhibiting the hydrolysis of Ach by acetylcholinesterase (AChE), and increased Ach release into the synaptic cleft can increase cholinergic neurotransmission. Cholinesterase inhibitors significantly improved cognitive functions, thereby supporting the pathogenic role of cholinergic deficiency (Gottwald and Rozanski 1999; Howes et al. 2003; Atri 2011; Hong-Qi et al. 2012). However, such treatments did not show permanent improvement in Alzheimer's disease patients, because of neurodegenerative nature. The cortical regions of the brain of patients suffering from Alzheimer's disease also showed variations in norepinephrine (NE) and serotonin (5-HT) levels, thereby resulting in secondary symptoms, such as language deficit, depression, and behavioral problems like agitation, disturbance in mood, aggression, and psychosis (Engelborghs and De Deyn 1997). Likewise, levels of the most abundant excitatory and inhibitory neurotransmitters of the brain, glutamate and gamma-aminobutyric acid (GABA), respectively, were also altered in patients suffering from Alzheimer's disease. However, the glutamatergic system was significantly affected more than the GABAergic system (Hyman et al. 1987; Reinikainen et al. 1990; Engelborghs and De Deyn 1997; Butterfield and Pocernich 2003; Schwab et al. 2013). As mentioned earlier, the surface expression and

function of NMDA and AMPA receptors play a major role in glutamatergic mode of neurotransmission. In neurodegeneration, observation of the synaptic levels of glutamate is imperative for neuronal physiology and survival. Varied levels of glutamate have been seen in different regions of the brains of Alzheimer's disease patients. Glutamatergic transmission in neocortical and hippocampal regions is known to be severely affected in Alzheimer's disease (Hyman et al. 1987; Butterfield and Pocernich 2003). Significantly high level of glutamate is also observed in the occipital lobe, and remarkable decreased content is seen in frontal and temporal lobes (Ernst et al. 1997; Fayed et al. 2011). Additionally, various APP transgenic mice model studies have confirmed the noticeable increase in the level of extracellular glutamate levels (Schallier et al. 2011). It has been hypothesized that the glutamate cytotoxicity is mainly mediated by NMDA subtypes of glutamate receptors. As mentioned earlier, NMDA receptors also mediate glutamate-induced processing of APP, which in turn results in enhanced Aβ secretion. Further increase in the level of Aβ in the brain results in oxidative stress leading to the oxidation of various lipids, sugars, and protein molecules in the neuronal cells. Glutamine synthetase is an enzyme involved in the conversion of glutamate to glutamine. In Alzheimer's disease patients, reduced brain activity was noticed because of oxidized glutamine synthetase (Bowen et al. 1976). Similarly, both clinical and experimental cases of Alzheimer's disease have observed reduction in activity and protein expression of glial glutamate transporter (GLT-1) (Sheldon and Robinson 2007). Due to the significant reduction in the glutamine synthetase activity and decreased GLT-1 levels in Alzheimer's disease patients, there is a remarkable decrease in synaptic clearance of glutamate, which in turn causes NMDA-mediated neuronal injury. Additionally, NO production in response to the excitatory NMDA receptors activation causes extensive damage to cerebral neurocytes (Wang et al. 2010). Glutamate decarboxylase enzyme (GAD) converts glutamate to GABA. Recent studies show that 50% increase in the mRNA of GAD in the brains of Alzheimer's disease patients indicates an increase in the GABAergic stimulation in the dorsal striatum (Reinikainen et al. 1990; Schwab et al. 2013). Increase in the level of GABA via presynaptic $GABA_A$ receptors results in prolonged inhibition of CNS neurons, which eventually leads to neuronal dystrophy, deafferentation, and increased neuronal degeneration (Schwab et al. 2013). All the above studies suggest that alteration in glutamate and GABA levels may play critical role in the pathogenesis of Alzheimer's disease. Nevertheless, due to their narrow pathogenic potential and analgesic therapeutic effects, these neurotransmitter alterations are considered to be the indicators of collateral damage occurring as a result of severe neuronal degeneration in Alzheimer's disease (Robichaud 2006). Changes in the cholinergic and glutamatergic system are considered to be the key element in the current treatment of Alzheimer's disease.

#### 1.2.2.4 Synaptic Deficits and Cognitive Dysfunction

Based on the electron microscopy and immunohistochemical staining in the cortical and hippocampal areas of the brains of Alzheimer's disease patients, a significant decrease in synaptic density and loss of presynaptic and postsynaptic markers were observed (Masliah et al. 2001; Reddy et al. 2005). The loss of synapses is an early

and consistent characteristic feature of Alzheimer's disease that strongly associates with the severity of cognitive impairment (Masliah et al. 2001; Scheff et al. 2007). Various studies have proposed that synaptic dysfunction and neuronal loss are caused by Aβ peptides and hyperphosphorylated tau, respectively (Frautschy and Cole 2010). Soluble non-fibrillar Aβ assemblies [also known as Aβ-derived diffusible ligands (ADDLs)], oligomers, paranuclei, and protofibrils emerge long before the Aβ deposits and are thought to be the main culprit. Aβ-induced synaptic deficits are caused by three basic mechanisms: (1) toxic gain of function (due to novel interactions by new conformations of Aβ), (2) loss of usual physiological function, and (3) precipitation of physiological dysfunction by excessive Aβ. Under normal conditions, Aβ peptides play a vital role in homoeostatic plasticity by altering the excitatory transmission through AMPA and NMDA subtypes of glutamatergic receptors. Moreover, Aβ can also hinder LTP in the hippocampal perforant pathway (Chen et al. 2000; Wang et al. 2002; Rowan et al. 2004). It also decreases AMPAR and NMDAR surface expression and function. Dysfunctions caused by Aβ are internalization of NMDA receptors through calcineurin-dependent pathway (Dewachter et al. 2009) and weakening of synaptic transmission and plasticity via activation of group I metabotropic glutamate receptors (mGluRs) with p38 mitogen-activated protein kinase (MAPK) and calcineurin as downstream effectors (Hsieh et al. 2006; Li et al. 2009). Excitotoxicity and the resultant cell death caused by Aβ are primarily because of its interaction with NMDA receptors and disruption of the $Ca^{2+}$ balance inside the cell. In several in vivo and in vitro studies, it has been observed that ADDLs readily bind to the dendritic arbors of a specific type of cultured neurons (Lacor et al. 2004; Laurén et al. 2009). In such studies, it has also been observed that ADDLs facilitate dendritic spine loss (Lacor et al. 2007), increase ROS generation, and disrupt excitatory and inhibitory neurotransmission balance, which in turn leads to epileptic behavior in transgenic mice. In various transgenic animal studies, synaptic deficits are the common disorders that are introduced for observing various pathophysiological aspects of Alzheimer's disease. Therefore, damage to the synaptic connections and impairment of synaptic plasticity are elementary in pathogenesis of memory impairment in Alzheimer's disease. Thus, further research in elucidating the underlying mechanisms of synaptic toxicity may help in the development of novel therapeutic medications.

#### 1.2.2.5 Inflammation and Neuronal Cell Death

Inflammation also plays a pivotal role in various neurodegenerative and neurological disorders such as multiple sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease, and traumatic brain injury (Blasko et al. 2004). According to the inflammatory hypothesis of Alzheimer's disease, chronic inflammation developing independently (due to several unknown factors) or as a result of Aβ pathology, plays a pivotal role in neurodegeneration. Along with oxidative stress, Aβ plaques, and NFTs, brain inflammation is an early pathological indication of Alzheimer's disease (McGeer et al. 1989; Eikelenboom et al. 2010). Neuroinflammation is a chronic and self-sustaining pathogenic process which is specific to Alzheimer's disease and is extremely capable of neuronal injury and neurodegeneration. Various direct and

indirect evidences confirm the pathophysiological significance of neuroinflammation in Alzheimer's disease (Akiyama et al. 2000). One of the confirmations is the co-localization of activated microglia, astroglia, and monocytes around the Aβ plaques and dystrophic neurites, specifically in the frontal neocortex and limbic cortex; however, no signs of inflammation are observed in the cerebellum (Rogers et al. 1988; Dickson et al. 1988). Furthermore, prudent but remarkable inflammation is observed in patients with low Braak scores for Alzheimer's disease pathology (i.e., patients without any history of dementia but ample amount of Aβ and neurofibrillary tangles at autopsy). There is an upregulation of the pro-inflammatory genes (gene encoding for complement factors; major histocompatibility complex proteins II, cell adhesion molecules) and pro-inflammatory enzymes resulting in the enhanced synthesis of prostaglandin and various other pro-inflammatory cytokines (Lue et al. 1996; Blalock et al. 2004; Parachikova et al. 2007). Likewise, in numerous in vitro and in vivo studies, Aβ has demonstrated to activate complement cascade and stimulate secretion of pro-inflammatory cytokines, chemokines, and other inflammatory markers from the activated monocytes (O'Barr and Cooper 2000), microglia (Del Bo et al. 1995; Chong 1997), astrocytes (Hu et al. 1998), endothelial cells (Suo et al. 1998), and neurons (Del Bo et al. 1995; Du Yan et al. 1997). Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and myeloperoxidase pathway are induced by Aβ, resulting in the ROS and cytokines generation via stimulated monocytes, microglia, and neurons (Bianca et al. 1999).

Recent studies have demonstrated that the pro-inflammatory mediators such as interleukins 1 (IL-1) and 6 IL-6 and interferon-γ (IFN-γ) induce APP secretion and processing and also annihilate the production of soluble APP (sAPP; a well-known neuroprotectant) leading to Aβ deposition and neurotoxicity (Blasko et al. 1999). Patients suffering from Alzheimer's disease have shown increased levels of gliosis, pro-inflammatory markers like IL-1, and conspicuous complement activation in postmortem brain (Rozemuller et al. 2005). Due to pathogenic role, anti-inflammatory therapies are known to be effective in delaying the onset of inflammation in Alzheimer's disease or slowing the progression of Alzheimer's disease (Jaturapatporn et al. 2012). Clinical trials have shown that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) can decrease the risk of developing Alzheimer's disease. This hypothesis is supported further when rheumatoid arthritis patients, who frequently use NSAIDs, have a lower incidence of Alzheimer's disease (Breitner and Welsh 1995; Breitner 1996; McGeer et al. 1996; Howes et al. 2003). In addition to this, it is found that passive immunization therapy with Aβ antibodies have been proven beneficial to the Alzheimer's disease suffering individuals (Aisen and Vellas 2013). All the above indications establish the pathogenic significance of chronic neuro-inflammatory processes in pathophysiology and progression of Alzheimer's disease.

Even though T-helper cells' role in Alzheimer's disease is controversial, recent studies have shown that the T-cell-regulated inflammatory response plays a role in either pathophysiology, progression, or both in the most common dementia-related neurodegenerative disorder (Togo et al. 2002; Panossian et al. 2003; Magnus et al. 2005). It has also been observed that the blood-brain barrier (BBB) is permeable to

activated T cells, which is intensely increased during systemic infection or other neurodegenerative diseases (Ransohoff et al. 2003). However, the number of T cells affecting the brains of Alzheimer's disease patients is far less as compared to other neurodegenerative disorders like multiple sclerosis. A study by Itagaki et al. (1988) showed that the CD4 (T-helper-inducer) and CD8 (T-cytotoxic-suppressor) T-cell trafficking was altered in significantly large amounts in the hippocampus and temporal cortex of the brains of Alzheimer's disease patients when compared to the normal brain tissues. Recent investigations are indicative of the involvement of dendritic and microglia cells in orchestrating the T-cell response in the brains of Alzheimer's disease patients (Rogers et al. 1988; Magnus et al. 2005; Fisher et al. 2011). Microglia protects and supports the neurons as well as act as an immunoprotectant in the CNS. It is able to express major histocompatibility complex II, release cytokines, complement proteins, and in an activated states also possess scavenger and phagocytic properties (Moore and O'Banion 2002). Activated microglia expresses co-stimulatory molecules like CD80, CD86, and CD40 during CNS inflammation in neurodegenerative disorders. These molecules interact with T cells and modulate T-cell-mediated response, thereby promoting either their proliferation, T-effector functions (cytokine secretion), or both (Aloisi 2001; Magnus et al. 2005; Wirenfeldt et al. 2011). Microglia activation and T-cell-induced cytotoxic inflammatory response is mediated by the release of pro-inflammatory cytokines. Cytokines, IL-1, IL-2, IL-17a, and other secreted cellular products also play a vital role in the neuronal immune response by regulating the neuronal cell viability or BBB permeability (Huppert et al. 2010). These cytokines also play a role in the development and differentiation of T cell in a given disease state. In case of multiple sclerosis, an autoimmune disorder, the integrity of BBB is impaired by the IL-17 producing T-helper cells, which leads to the increase in its permeability to other lymphocytes. Increase in the tumor necrosis factors (TNF)-α protein expression is directly related with inflammatory response in Alzheimer's disease (Fillit et al. 1991; Blasko et al. 1999; Perry et al. 2001). Tumor necrosis factor-alpha (TNF-alpha) is a potent central regulator of inflammation. The expression of TNF-α and IFN-γ helps Aβ peptides production, which leads to the deposition of Aβ. This leads to the disruption of homeostatic balance and plaque formation (Blasko et al. 1999). Additionally, production and release of prostaglandins are also stimulated by these cytokines, which may result in neurotoxic effects associated with Alzheimer's disease pathology (Prasad et al. 1998).

## 1.3 Therapeutic Efficacy of *Centella asiatica*

*Centella asiatica* is a slender creeping perennial herb belonging to Umbelliferae (also known as Apiaceae) family. This plant has delicately scalloped hairy leaves with barely visible flowers. It is commonly found in tropical swampy areas. *Centella* species are widespread throughout tropical and subtropical countries worldwide (Awang 1998; Wattanathorn et al. 2008; Vasavi et al. 2014). *Centella asiatica* has numerous common names depending on the geographical location of origin. It is

known as *Gotu kola* in Sinhala, *mandukaparni* in Sanskrit, *kodakan* in Malayalam, *pegaga* in Malayasia, Indian pennywort and Indian water navelwort in India, *tsubokusa* in Japan, and *tungchian* or *luei gong gen* in China. Botanically it is known as *Hydrocotyle asiatica* Linn and *Trisanthus cochinchinensis* Lour (Kalshetty et al. 2012). As described earlier, *Centella asiatica* is an ancient medical herbal drug and has been traditionally used for its plethora of therapeutic purposes in Indian Ayurvedic medicinal practices for eras. According to the *Charaka Chikitsa I*, an ancient Indian literature, the juice of *Centella asiatica* promotes longevity and cures a variety of diseases. *Centella asiatica* is an active component of "Medhya Rasayana" (Bhavna and Jyoti 2011) and considered as crucial herb for the treatment of various health-related disorders (Thomas et al. 2010; Ermertcan et al. 2008; Somboonwong et al. 2012; Subathra et al. 2005; Bian et al. 2013; Di Tomo et al. 2015; Abas et al. 2015; Jayathirtha and Mishra 2004; Belcaro et al. 2011; Shukla et al. 1999; Cesarone et al. 2001a, b; De Sanctis et al. 2001; Incandela et al. 2001; Paocharoen 2010).

Therapeutically, the whole aerial part of *Centella asiatica* is useful as it is very rich in numerous bioactive compounds (Kumar and Gupta 2002). Various in vitro and in vivo (animals and human clinical) studies have been undertaken to evaluate its bioactive components and the medicinal value of *Centella asiatica.* The basic molecular mechanisms related to the therapeutic efficacy are being investigated. *Centella asiatica* has been used extensively for the dermatological pathologies. It has been widely used in the treatment of wounds, burns, and ulcerous skin ailments and for the prevention of keloid and hypertrophic scars (Belcaro et al. 2011; Brinkhaus et al. 2000; Gohil et al. 2010; Paocharoen 2010). This botanical is also used to treat second- and third-degree burns and topically to accelerate healing, mainly in cases of chronic postsurgical and post-trauma wounds (Belcaro et al. 2011). Details of medicinal applications of *Centella asiatica* are summarized in Table 1.3.

### 1.3.1 Active Components of *Centella asiatica*

The major active chemical components of *Centella asiatica* are identified as triterpenes, flavonoids, polyphenols, and other compounds (Bhattachryya and Lythgoe 1949; Singh and Rastogi 1969; Zainol et al. 2003; Mangas et al. 2006; Zheng and Qin 2007; Shinomol and Muralidhara 2008a; Zhang et al. 2008; James and Dubery 2009; Thomas et al. 2010; Gohil et al. 2010; Kalshetty et al. 2012; Long et al. 2012; Tiwari et al. 2013). The triterpenoids exist in the forms of saponins and glycosides, such as asiaticoside and madecassoside with its respective ursane-type sapogenins (Fig. 1.1). Other compounds reported in *Centella asiatica* with therapeutic efficacy are amino acids (alanine, serine, aminobutyrate, aspartate, glutamate, histidine, lysine, and threonine), fatty acids (linoleic acids, linolenic acid, lignocene, oleic acid, palmitic acid, and stearic acid), phytosterols (campesterol, sitosterol, and stigmasterol), resin, tannin, and various other terpenoids (beta-caryophyllene, trans-beta-farnesene and germacrene D, alpha-pinene, and beta-pinene) (George

**Table 1.3** Medicinal use of *Centella asiatica* in cognitive disorders

| Plant part used | Medicinal use | References |
|---|---|---|
| Whole plant | Enhances learning and memory | Rao et al. (2005) |
| Whole plant | Effective against MPTP-induced Parkinsonism | Ittiyavirah and Hameed (2014) |
| Whole plant | Enhances cognitive functions | Gray et al. (2016) |
| Whole plant | Significantly prevents the cognitive impairments | Gupta et al. (2003) |
| Whole plant | Memory enhancer in Alzheimer's disease | Hage et al. (2010) |
| Whole plant | Prevents Alzheimer's disease development | Chen et al. (2015) |
| Whole plant | Enhances cognitive function | Kumar and Gupta (2002) |
| | Improves learning and memory | |
| Whole plant | Neuroprotective and beneficial in Alzheimer's disease | Soumyanath et al. (2012) |
| Whole plant | Prevents memory deficits | Kumar et al. (2011) |
| | Improves the memory impairment | |
| Whole plant | Significant neuroprotective effects | Subathra et al. (2005) |
| | Protects brain against age-related diseases | |
| Whole plant | Enhances the cognitive function | Omar et al. (2011) |
| | Neuroprotective actions | |
| Whole plant | Anti-Parkinson's effect | Khotimah et al. (2015) |
| | Improves learning and memory | |
| | Protects the brain from age-related oxidative damage | |
| Whole plant | Neuroprotective properties | Shinomol et al. (2011) |
| | Improves memory | |
| | Prevents cognitive impairment | |
| Whole plant | Restores memory | Howes and Houghton (2012) |
| | Prevents dementia | |
| | Improves cognitive function | |
| Whole plant | Cognitive enhancement | Iqbal et al. (2011) |
| Whole plant | Increase memory | Krishna (2013) |
| Whole plant | Enhances learning | Mohd Salim et al. (2013) |
| Whole plant | Neuroprotective effect against Parkinson's disease and Alzheimer's disease | Orhan (2012) |
| Whole plant | Memory-enhancing effects | Singh et al. (2008) |
| Whole plant | Improves memory retention | Kumar and Gupta (2003) |
| | Prevents cognitive impairment | |
| Whole plant | Enhances cognitive performances | Xu et al. (2008) |
| Whole plant | Possesses neuroprotective properties against Alzheimer's disease | Dhanasekaran et al. (2009) |
| Whole plant | Improves cognitive function Neuroprotective effects | Gray et al. (2015) |

(continued)

**Table 1.3** (continued)

| Plant part used | Medicinal use | References |
|---|---|---|
| Whole plant | Improves both speed and accuracy of working memory | Wattanathorn et al. (2008) |
| | Increases accuracy of word recognition | |
| Whole plant | Improves symptoms of Parkinson's disease | Barbosa et al. (2008) |
| | Enhances memory | |
| Leaves | Improves memory and cognitive function | Howes and Houghton (2003) |
| | Prevent dementia | |
| Leaves | Alternatives for the treatment of Alzheimer's disease | Akagi et al. (2015) |
| Leaves | Increases memory power in children | Sarkar et al. (2015) |
| Leaves | Memory-enhancing properties | Seevaratnam (2012) |
| | Protection against age-related changes in brain | |
| Leaves | Augments memory power | Tiwari et al. 2016) |
| Leaves | Potent in reducing the Parkinsonian symptoms | Siddique et al. (2014) |
| Leaves | Enhances memory | Mohandas Rao et al. (2012) |
| Leaves | Neuroprotective properties | Kasture et al. (2014) |
| Leaves | Protects against age-related changes in brain | Sugunabai and Karpagam (2015) |
| Leaves | Memory improvement and improves symptoms of Alzheimer's disease | Patel et al. (2014) |
| Leaves | Neuroprotective effect | Bhavna and Jyoti (2011) |
| Leaves | Improves memory against dementia and aging | Stafford et al. (2008) |
| Leaves | Prophylactic neuroprotective action | Shinomol and Muralidhara (2008a, b) |
| | Brain stimulant | |
| Leaves | Neuroprotective effects against neurodegenerative disorders including Alzheimer's disease and Parkinson's disease | Zhang et al. (2012) |
| Leaves | Improves memory | Ashalatha and Shenoy (2015) |
| | Brain tonic | |
| Leaves | Brain booster for improving memory | Wanakhachornkrai et al. (2013) |
| Leaves | Enhances memory retention | Rasoanaivo (2011) |
| Leaves and stem | Improves symptoms of Alzheimer's disease | Khatun et al. (2011) |
| | Neuroprotective effect in Parkinsonism | |
| Pure compound (asiatic acid) | Neuroprotective effects | Xu et al. (2012) |
| | Improves cognitive function against glutamate-induced dementia | |

(continued)

**Table 1.3** (continued)

| Plant part used | Medicinal use | References |
|---|---|---|
| Pure compound (asiatic acid) | Enhances working memory | Sirichoat et al. (2015) |
| Pure compound (asiaticoside) | Improves memory | Chen et al. (2014) |
| | Delays the onset and progression of neurological disorders | |
| Pure compound (madecassoside) | Improves cognitive function in Alzheimer's disease | Du et al. (2014) |

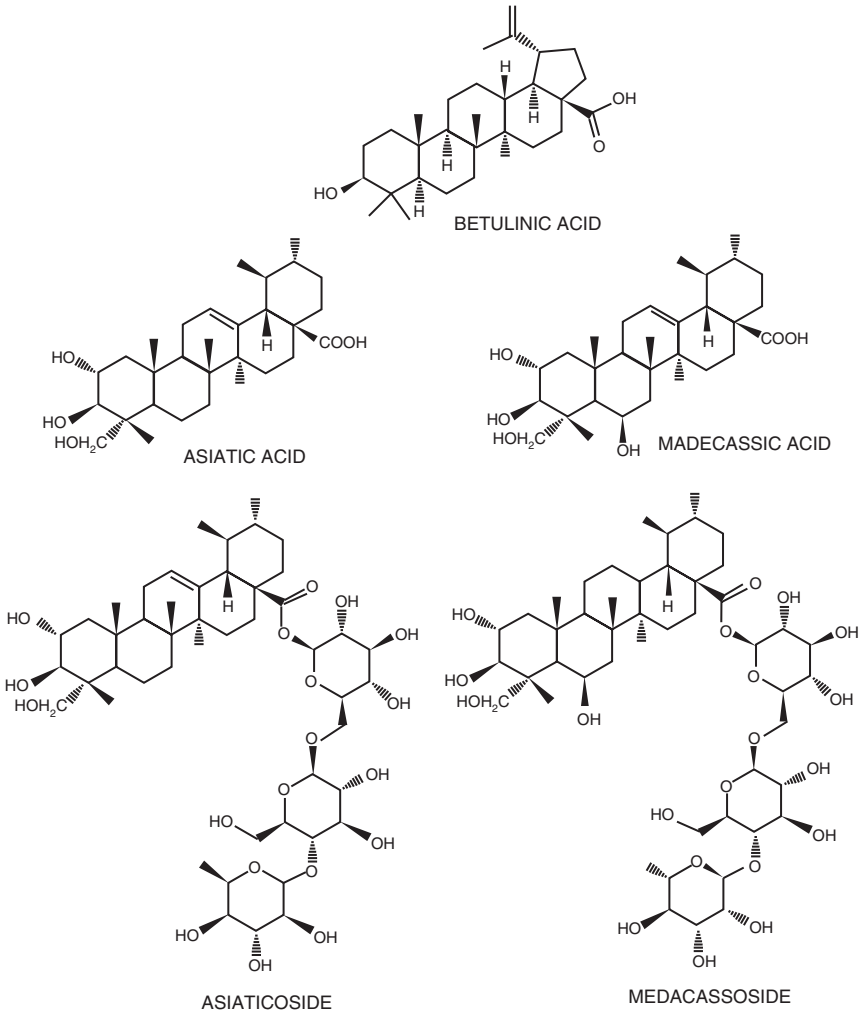


**Fig. 1.1** Bioactive triterpenoids isolated from *Centella asiatica*

and Gnanarethinam 1975). Madecassoside, a pentacyclic triterpenoid derivative saponin, is reported to possess anti-inflammatory (Li et al. 2009; Liu et al. 2008; Wu et al. 2012), antioxidant (Luo et al. 2014), and cardioprotective (anti-myocardial infraction) activities (Bian et al. 2008). Main compounds responsible for the antioxidant activity of *Centella asiatica* are reported to be flavonoids and phenolic compounds (Hussin et al. 2005).

### 1.3.2 Learning and Memory-Enhancing Capability in Alzheimer's Disease

*Centella asiatica* extract was well enumerated and may have pro-cognitive effects in humans and rodents (Dhanasekaran et al. 2009; Kumar and Gupta 2002; Wattanathorn et al. 2008; Gohil et al. 2010; Shinomol et al. 2011; Xu et al. 2012b). Pharmacological studies have established the cognitive enhancing capability of *Centella asiatica* extract in a series of behavioral tests and memory retention tasks such as active and passive avoidance, object recognition, and water maze in rodents (Nalini et al. 1992; Kumar and Gupta 2002; Gupta and Pansari 2003). In a clinical study, *Centella asiatica* extract positively modulated cognition and mood-enhancing quality of life in healthy elderly volunteer (Wattanathorn et al. 2008; Mato et al. 2011; Nasir et al. 2011). Additionally, a few human clinical studies have reported that *Centella asiatica* extract substantially improved cognitive ability of mentally retarded children (Appa Rao et al. 1973).

The activation of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) leads to the increase in BDNF which in turn modulates the NMDA receptor activity and its expression on the pre- and post- synaptic sites (Madara and Levine 2008). It is also observed that the synaptic localization of pre- and postsynaptic proteins such as synaptobrevin is enhanced by BDNF through activation of TrkB receptor. Synaptobrevin plays an important role in maintaining synaptic plasticity (Minichiello et al. 2002; Huang and Reichardt 2003). Several pathways such as protein kinase A (PKA), NO signaling and CaMKII activation lead to the phosphorylation of CREB. As mentioned in previous sections, Aβ initiates numerous cytotoxic pathways in the CNS which leads to neurodegeneration and progression of Alzheimer's disease. Besides neuronal plaques formation, Aβ also stimulates the immune system of the CNS, which leads to the inflammatory-mediated response (Lue et al. 1996; Gupta and Pansari 2003). Additionally, it has been proven that the inflammatory mediators increase the APP processing in the neuronal cells, thereby accelerating and augmenting the secretion and deposition of Aβ (Blasko et al. 1999). It can thus be concluded that chronic inflammation may indirectly act as the main pathogenic agent in the formation of Aβ plaques in the brains of Alzheimer's disease patients. Therefore, studies were conducted with *Centella asiatica* to evaluate its neuroprotective properties against Aβ toxicity in association with BDNF and inflammation (Xu et al. 2008).

*Centella asiatica* plays a key role in neuronal regeneration by stimulating the dendrites of rat brains, neurite elongations in human SH-SY5Y cells, and increasing

axonal regeneration in rats. Synaptic strengthening and cognitive enhancement activities of *Centella* are by increasing CREB phosphorylation and thereby aiding the neuronal dendritic arborization and axonal regeneration in rats (Rao et al. 2005; Soumyanath et al. 2005). One of the physiological processes of memory formation is generally induced by CREB (a transcription factor) during LTP. The important early genes like c-fos and proteins such as brain-derived neurotrophic factor (BDNF) responsible for neuronal survival and protein synthesis are regulated by CREB (Malinow et al. 2000). Phosphorylation of CREB leads to the activation of factors such as several protein kinases, MAPK, calcium-/calmodulin-dependent protein kinase II (CaMKII), protein kinase C, and signaling factors like Wnt, which play an important role in neuronal survival and arborization (Wayman et al. 2006). Numerous studies have demonstrated BDNF to robustly stimulate the dendrite outgrowth by triggering TrKB receptors, resulting in the stimulation of various proteins including the activation of CAMKII (Minichiello et al. 2002; Huang and Reichardt 2003). Overall, these studies have concluded that one of the mechanisms mediating cognitive enhancement activity of *Centella asiatica* can be via increasing CREB signaling (Xu et al. 2008, Fig. 1.2).

### 1.3.3 Neuroprotective Activity in Alzheimer's Disease

Numerous experiments have established that *Centella asiatica* has neuroprotective mechanisms of action significant to the therapeutic treatment of Alzheimer's disease. As pointed out earlier, excessive levels of the neurotransmitter, glutamate, is found in the brains of Alzheimer's disease patients. It has been demonstrated that *Centella asiatica* may have a neuroprotective effect when cultured neurons are exposed to glutamate (Lee et al. 2000; Ramanathan et al. 2007). Some studies have proven that *Centella asiatica* extract influences neurotransmitter systems such as dopamine and serotonin (5-HT) that regulate the production of Aβ peptide (Sakina and Dandiya 1990; Nalini et al. 1992; Nitsch et al. 1996), which consequently may serve to limit the production of Aβ peptide.

*Centella asiatica* alters fabrication of extracellular matrix molecules (Maquart et al. 1999). Deposition of fibrillar Aβ is known to promote extracellular matrix molecule, perlecan, in rodent brain (Snow et al. 1994; Holcomb et al. 2000). Whereas, laminin, an extracellular matrix molecule, has recently been demonstrated to hinder Aβ fibril formation (Castillo et al. 2000). *Centella asiatica* may function as an anti-fibrillogenic agent, limiting the formation of amyloid deposits through alterations in the production of key extracellular matrix proteins. *Centella asiatica* has shown to exhibit preventive effects on cognitive deficits in streptozotocin-treated animal model of memory deficit (Kumar and Gupta 2003). Furthermore, reduction in the protein carbonyl production was seen in the brains of aged rats when treated with *Centella asiatica* (Subathra et al. 2005). Overall, these studies have demonstrated that *Centella asiatica* may reduce neuropathologies of Alzheimer's disease.

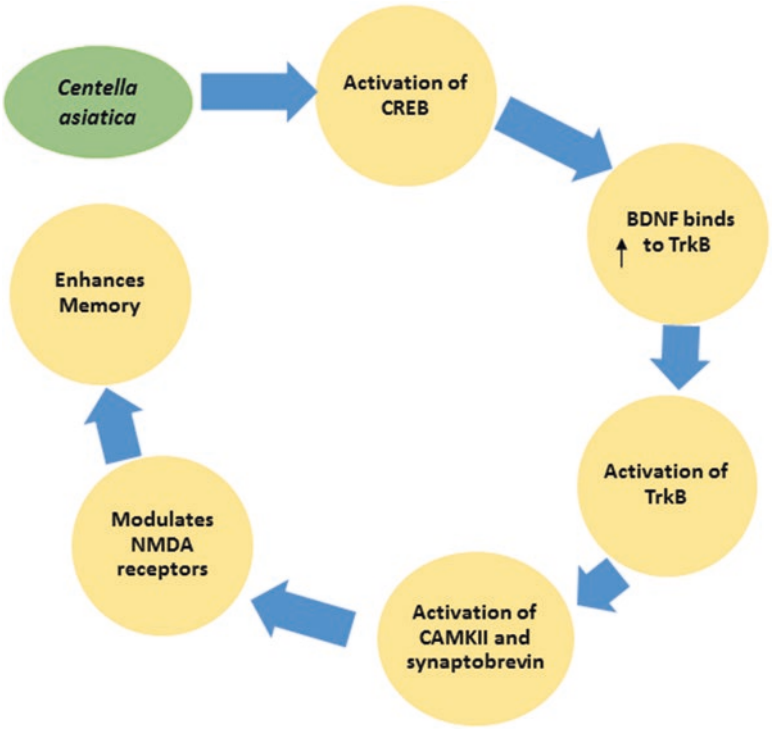


**Fig. 1.2** Mechanisms of action

Researchers have found that prior to the increase of Aβ 1–40 and 1–42 levels or noticeable Aβ deposition in the Alzheimer's disease mouse model, augmented lipid peroxidation takes place (Praticò et al. 2001). Studies conducted in our research laboratory (Dhanasekaran et al. 2009) have found that *Centella asiatica* could block lipid peroxidation, thereby suggesting that the primary action could be hindering the hydroxyl radical-generated membrane damage. This study further documented that *Centella asiatica* may also help in reducing Aβ deposition and elicited protection to proteins, sugars, and nucleic acids from oxidative and Aβ damage.

Asiatic acid and asiaticoside present in *Centella asiatica* provoke the acceleration of learning and memory performance (Kumar and Gupta 2003; Gupta et al. 2003; Rao et al. 2005). Asiatic acid from *Centella* also exhibits neuroprotective properties and has shown to protect cortical neurons from glutamate-induced excitotoxicity in vitro (Mook-Jung et al. 1999; Lee et al. 2000; Xiong et al. 2009; Krishnamurthy et al. 2009).

Ceramides derived from sphingosine are known to cause mitochondrial dysfunction which results in neuronal cell death (Arboleda et al. 2009; Zhang et al. 2012b). Several researchers have found that ceramides may play a role in various neurological disorders such as Alzheimer's disease (Cutler et al. 2004; Jana et al. 2009), Parkinson's disease (France-Lanord et al. 1997; Bras et al. 2008), and cerebral

ischemia (Novgorodov and Gudz 2009). Specifically, C2-ceramide (short chain active analog of ceramide) causes mitochondrial disruptions and apoptosis. Zhang et al. (2012) demonstrated that *Centella asiatica* exhibited protective activity against C2-ceramide-induced mitochondria-dependent apoptosis may be by regulating the ERK1/2 signaling pathway (Movsesyan et al. 2002; Stoica et al. 2005).

Apoptosis is seen in peripheral chronic disorders and in various neurological disorders mainly in Alzheimer's disease and Parkinson's disease. Neurodegeneration is mainly caused by apoptosis that can be triggered by various pathways (LeBlanc 2005). In a recent study, it has been hypothesized that early induction of caspase-6 leads to the activation of downstream apoptotic mediators such as the caspase-3. Induction of caspase-6 results in the disruption of the cytoskeleton of neurites and also leads to the damage of proper trafficking of proteins and organelles, thereby resulting in neurodegeneration and synaptic loss in Alzheimer's disease (LeBlanc 2005). Prakash and Kumar (2012) showed that *Centella asiatica* exhibited anti-apoptotic activity by effectively inhibiting the caspase activity induced by D-galactose neurotoxicity in rats.

In the double transgenic mice model of Alzheimer's disease, Aβ plaque accumulation occurs in the frontal cortex and has DNA fragmentation (Feng et al. 2004; Wang et al. 2005; Migliore et al. 2005). A single-strand break in DNA may lead to the double-strand breaks, which may result in apoptosis, or may inactivate vital genes, or may also cause chromosomal aberrations (Barzilai and Yamamoto 2004). Disturbance of the supercoiled form of the plasmid DNA caused by exposure to hydrogen peroxide and UV light was inhibited by *Centella asiatica*, thereby preventing the single-strand or double-strand breaks formation of DNA.

In addition to improving cognition, *Centella asiatica* may also be used to treat the motor agitation and mood changes of people suffering from Alzheimer's disease. It is postulated that *Centella* also helps in promoting a deep state of relaxation and mental calmness during meditation practices (Brown 1995). Formulations of *Centella asiatica* in combination with other herbs are used to alleviate depression and anxiety (Bradwejn et al. 2000). The antidepressant and anxiolytic activities of *Centella asiatica* are thought to be mediated through the hypothalamic-pituitary-adrenocortical axis, which relieves stress and alters the components of the monoaminergic neurotransmitters (Chen et al. 2005; Bobade et al. 2015 Fig. 1.3). In addition, *Centella asiatica* has been shown to possess antioxidant activity in the hippocampus and corpus striatum (Ittiyavirah and Hameed 2014).

### 1.3.4 Antioxidant and Anti-inflammatory Activities in Alzheimer's Disease

Phenolic compounds exhibit potent antioxidant activity and also act as mitochondrial energizers. Free radicals are responsible to initiate cell injury (Spiteller 1993; Maxwell 1995; Howes et al. 2003). Thus, the use of antioxidants has been considered to slow the neuronal degeneration and progression of Alzheimer's disease (Howes et al. 2003). *Centella asiatica* is known to have an effective antioxidant and

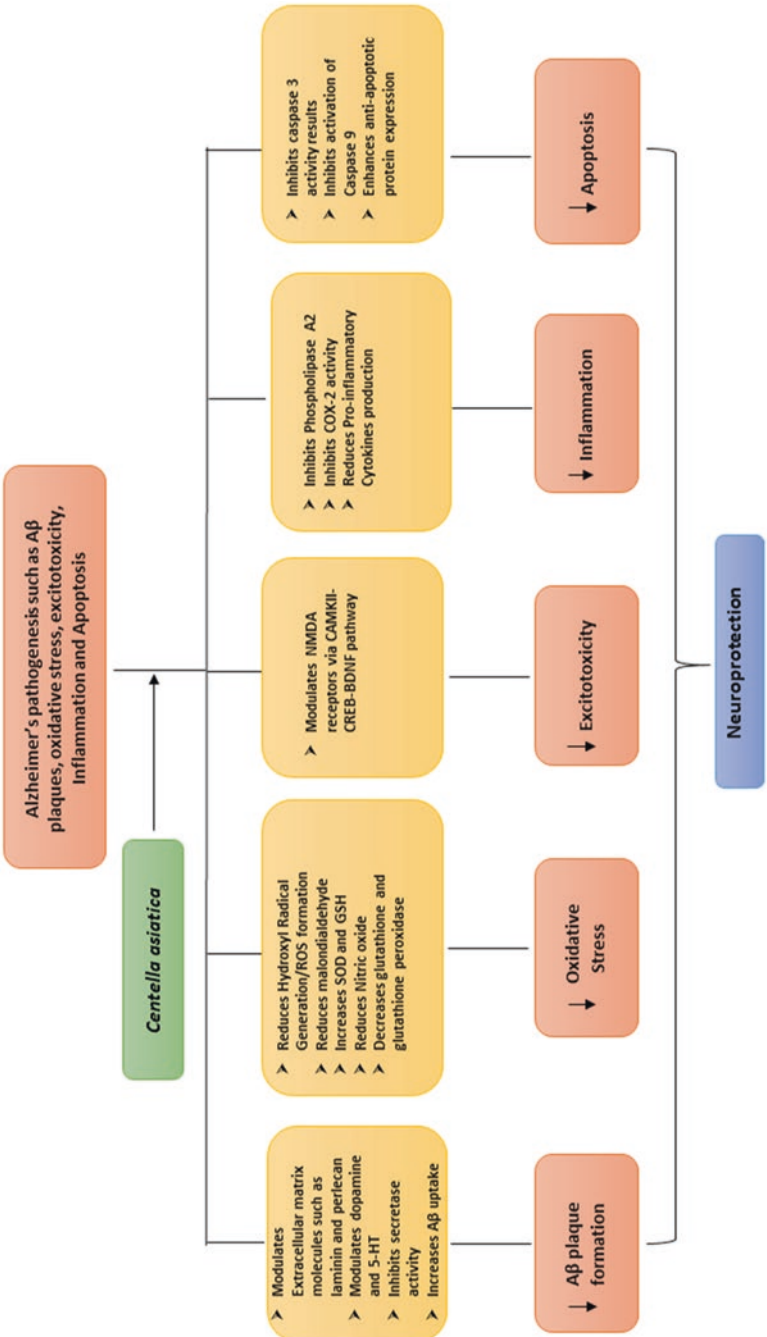


**Fig. 1.3** Mode of action of *Centella asiatica* in the pathogenesis of Alzheimer's disease

anti-inflammatory capabilities since it has shown to attenuate cyclooxygenase, lipoxygenase, and phospholipase expression. A study by Shinomol and Muralishara demonstrated that *Centella asiatica* reduces endogenous oxidative markers, enhances antioxidant defenses in cytosol and other brain regions of prepubertal mice (Shinomol and Muralidhara 2008a), and has neuroprotective properties (Shinomol and Muralidhara 2008b). *Centella* inhibits phospholipase 2, thereby highlighting its significant role in anti-inflammatory activities (Defillipo et al. 2012). The two major components of arachidonic pathway are COX-2 and 15-lipoxygenase (15-LOX) enzymes. The arachidonic pathway leads to the formation of pro-inflammatory prostaglandins, leukotrienes, and other mediators, which are associated with numerous chronic neurological disorders (Hoozemans et al. 2004). *Centella asiatica* significantly inhibits the activity of $Ca^{2+}$-independent phospholipase $A_2$ ($iPLA_2$) and cytosolic phospholipase $A_2$ ($PLA_2$) in the rat brain. Drugs affecting either of the phospholipase will hinder the profound inflammatory reaction and reduce neurodegeneration (Kolko et al. 2002, Burnett et al. 2011, Fig. 1.3).

### 1.3.5 Study in Double Transgenic Alzheimer's Disease Mice Model

Based on the extensive scientific literature, it is clear that *Centella asiatica* has been used for memory enhancement in Ayurvedic medicine for years. In our laboratory, we had undertaken a chronic study to evaluate the neuroprotective effects of *C. asiatica* extract on amyloid pathology in the PSAPP mouse model of Alzheimer's disease (Dhanasekaran et al. 2009). PSAPP mice expressing the "Swedish" amyloid precursor protein and the M146L presenilin 1 mutations are a well-characterized model for spontaneous Aβ plaque formation. PSAPP doubly transgenic mice were obtained by crossbreeding Tg2576 mouse line with "Swedish" APP mutation (APPK670NM671L) with mutant presenilin 1 mouse line (PS- 1M146L6.2). The resultant breed was due to these two mutations that caused and elevated the production of Aβ peptide with a measureable deposition of plaques as early as 3–4 months of age (Holcomb et al. 1998, 1999; McGowan et al. 1999; Emilien et al. 2000). In a chronic therapeutic approach (8 month treatment), we had demonstrated that *C. asiatica* extracts decline the Aβ 1–40 and 1–42 levels in the hippocampus and cortex. Extracts of *C. asiatica* decreased the amyloid load due to fibrillar Aβ. Dense-core plaque formation has recently been shown to occur predominantly and is associated with the vasculature in PSAPP mice, suggesting a malfunction of the clearance of Aβ through the neurovascular system that may impact amyloid deposition (Kumar-Singh et al. 2005). It has been reported that *C. asiatica* effects in regulating the Aβ clearance or deposition as noticeable plaques are due to the alteration of extracellular matrix composition, perlecan or fibril formation subdued by other extracellular matrix molecule, laminin (Holcomb et al. 2000; Castillo et al. 2000). Asiaticoside upregulates biglycan, another heparin sulfate proteoglycan in other amyloidosis, which is also known to bind Aβ (Snow et al. 1994). *Centella asiatica* extract-based treatment thus may cause the regional alterations of extracellular

matrix molecules and influence the fibrillar amyloid load detected in PSAPP mouse brain. In Tg2576 mouse, one of the PSAPP parental lines, previous researches have concluded that there is a significant elevation in lipid peroxidation before the increase of Aβ 1–40 and 1–42 levels or noticeable amyloid deposition (Praticò et al. 2001). *Centella asiatica* blocked lipid peroxidation, which in turn infers that the basic mechanism is by inhibiting the membrane damage caused by the hydroxyl free radical, thereby reducing the oxidative stress. It can thereby be concluded that the continuous and chronic treatment with *C. asiatica* may alter the Aβ pathology in the PSAPP Alzheimer's disease mouse model. Based on our research data, it is clear that the antioxidant and anti-inflammatory properties of *C. asiatica* may prevent neurodegeneration (Table 1.4).

### 1.3.6 Other Neurological Disorders

3-Nitropropionic acid (3-NPA) is an irreversible complex II inhibitor of the electron transport chain and Krebs cycle in the mouse brain (Shinomol and Muralidhara 2008b). 3-NPA is a fungal neurotoxin known to induce selective striatal pathology that is encountered in Huntington's disease. It was observed that upon administration of 3-NPA, there was a significant increase in the level of ROS and malondialdehyde (MDA) indicating the oxidative stress in cytoplasmic and mitochondrial fractions of striatum and other brain regions of prepubertal mice (Binienda et al. 1998; Hussin et al. 2005). Upon treatment, it has been demonstrated that the prophylactic activity of *C. asiatica* resulted in the protection against the glutathione depletion, protein oxidation damage, and diminishing of $Na^+$, $K^+$ ATPase activity caused by 3-NPA (Shinomol and Muralidhara 2008b). The study thereby concluded that *Centella asiatica* helps in the protection of oxidative stress and mitochondrial dysfunction which is usually observed in the neurodegenerative disorders (Shinomol and Muralidhara 2008b).

Cerebral ischemia which can result in cerebral infarction or stroke is the leading cause of disability and mortality in the world (Lo et al. 2003) and can only be cured by the restoration of adequate blood flow in brain. However, the reperfusion can cause tissue damage that is well past the ischemia, via is excitotoxicity, oxidative stress, inflammation, and apoptosis (Luo et al. 2014). Madecassic acid from *Centella asiatica* is reported to reduce oxidative stress by diminishing 3,4-methylenedioxyamphetamine level, increasing glutathione and superoxide dismutase activity, and reducing COX-2 expression and production of prostaglandin E2. It also reduces the plasma levels of TNF-α and IL-6 (Won et al. 2010). Generally, oxidative stress regulates anti-inflammatory activity by triggering toll-like receptors and NF-κB. Activation of NF-κB is known to mediate inflammation and apoptosis and augment the expression of various inflammation markers (TNF-α, IL-1β, COX-2), inducible nitric oxide synthase, and additional mediators such as adhesion molecules (Tak and Firestein 2001). Madacasic acid reduces activation of NF-κB, thereby suppressing the IL-1β, IL-6, and TNF-α levels, which in turn suppresses neuronal apoptosis. Madacasic acid found in *Centella asiatica* is protected against

**Table 1.4** Various studies on *Centella asiatica* to validate its efficacy

| Model used | Toxin/task | Mechanism of action (MOA) | References |
|---|---|---|---|
| B103 cells | $H_2O_2$ | Inhibition of Aβ and free radical-induced cell death | Mook-Jung et al. (1999) |
| | Staurosporine | | |
| Mice | Passive avoidance task | Modulate dopamine, 5-HT and noradrenaline systems | Sakina and Dandiya (1990) and Nalini et al. (1992) |
| Albino rats | | | |
| Male Wistar rats | $H_2O_2$ | Antioxidant, reduce the oxidative stress by decreasing the lipid peroxidation | Kumar and Gupta (2002) |
| Rat cortical neurons cells | Glutamate | Increase the levels of glutathione and glutathione peroxidase and reduced the overproduction of NO | Lee et al. (2000) |
| Mice | Nootropic, radial arm maze | Increase release of acetylcholine in the hippocampus | Rao et al. (2005) |
| | | Increase dendritic arborization of hippocampal CA3 neurons | |
| Neonatal mice in vivo | Glutamate | Restores lipid peroxidation and glutathione content | Xu et al. (2012b) |
| Human neuroblastoma SH-SY5Y cells in vitro | | Reinstates superoxide dismutase activity in the hippocampus and cortex | |
| | | Regulates NMDA receptors | |
| | | Reduced reactive oxygen species | |
| | | Stabilizes the mitochondrial membrane potential | |
| | | Promotes the expression of PGC-1α and Sirt1 | |

(continued)

**Table 1.4** (continued)

| Model used | Toxin/task | Mechanism of action (MOA) | References |
|---|---|---|---|
| Male Sprague-Dawley rats<br>Human SH-SY5Y cells | Aβ | Increasephosphorylation of cyclic AMP response element-binding protein and thereby aiding the neuronal dendritic arborization and axonal regeneration | Soumyanath et al. (2005) |
| Neonatal Wistar rat pups | Aβ | Increase phosphorylation of cyclic AMP response element-binding protein and thereby aiding the neuronal dendritic arborization and axonal regeneration | Mohandas Rao et al. (2006) |
| N2a neuroblastoma cells expressing Aβ1–42<br>Rat embryonic cortical cells | Aβ<br>$H_2O_2$ | Increase phosphorylation of cyclic AMP response element-binding protein that leads to the increase in BDNF<br>Modulates the NMDA receptor | Xu et al. (2008) |
| Wistar rats | $FeCl_2$ | Antioxidant action<br>Reduces lipid peroxidation and protein carbonyl production | Subathra et al. (2005) |
| PSAPP mice | Aβ<br>$H_2O_2$ | Decreases amyloid beta deposition<br>Antioxidant effect<br>Scavenged free radicals, reduced lipid peroxidation, and protected against DNA damage | Dhanasekaran et al. (2009) |
| Rat embryonic cortical cells | C2-ceramide | Inhibits caspase 3 and the dephosphorylation of ERK1/2 | Zhang et al. (2012a) |
| Rat embryonic cortical cells | Arachidonic acid | Inhibits cytosolic phospholipase A2 and secretory phospholipases A2 | Defillipo et al. (2012) |

| | | | |
|---|---|---|---|
| Swiss albino mice | D-galactose | Anti-apoptotic activity by effectively controlling the caspase-3 activity | Prakash and Kumar (2012) |
| Prepubertal male CFT-Swiss mice | 3-Nitropropionic acid (3-NPA) | Antioxidant activity | Shinomol and Muralidhara (2008a) |
| | | Enhances GSH and thiols levels | |
| RAW 264.7 murine macrophage cells | Lipopolysaccharide | Inhibits inducible nitric oxide, COX-2, tumor necrosis factor-alpha, interleukin-1 beta, and IL-6 via the downregulation of nuclear factor-kappaB activation which in turn suppresses neuronal apoptosis | Won et al. (2010) |
| Male Wistar rats | Pentylenetetrazol | Inhibits oxidative stress | Gupta et al. (2003) |
| | | Reduce malondialdehyde levels and increase in the glutathione levels | |
| Adult male Wistar rats | Arachidonic acid | Inhibits the activity of $Ca^{2+}$-independent phospholipase A2 and cytosolic phospholipase A2 | Barbosa et al. (2008) |
| Female Sprague-Dawley rats | Glutamate | Antioxidant effects | Ramanathan et al. (2007) |
| | | Reduces catalase, superoxide dismutase, and lipid peroxides | |
| C57Bl/6 mice | Glutamate | Modulates antioxidant and mitochondrial pathways | Gray et al. (2016) |
| | | Increased the expression of synaptic genes | |
| MC65 | Tetracycline | Modulates antioxidant and mitochondrial pathway | Gray et al. (2015) |
| SH-SY5Y neuroblastoma cells | | | |

(continued)

**Table 1.4** (continued)

| Model used | Toxin/task | Mechanism of action (MOA) | References |
|---|---|---|---|
| Human neuroblastoma SH-SY5Y cells | Buthionine sulfoximine (BSO) | Inhibits the activation of caspase-9 pathway | Omar et al. (2011) |
| Male Sprague-Dawley rats | Ischemia reperfusion injury | Reduce the levels of malondialdehyde, nitric oxide, pro-inflammatory cytokines and nuclear factor kappa-light-chain-enhancer of activated B cells | Luo et al. (2014) |
| Mouse NG108-15 neural cell line | Aβ25–35 | Blocks the conversion of light chain3-I to light chain3-II (protects microtubule-associated proteins) | Du et al. (2014) |
| Human neuroblastoma cell SH-SY5Y | | Reduce Beclin-1 | |
| | | Increase anti-apoptotic protein Bcl-2 level | |
| Adult zebra fish | Rotenone | Decreases reactive oxygen species probability of radical attack to a-synuclein protein | Khotimah et al. (2015) |
| | | Increase dopamine level | |
| Rat PC12 pheochromocytoma cells | Aβ1–40 | Modulates the activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase and alters the levels of glutathione and glutathione disulfide | Chen et al. (2015) |
| Human IMR32 neuroblastoma cells | | | |
| Male Wistar rats | Colchicine | Reduces AChE activity | Kumar et al. (2009) |
| IMR-32 human neuroblastoma cells | Aβ | Upregulates the level of activated ERK1/2 and protein kinase B (Akt) | Wanakhachornkrai et al. (2013) |
| Tg2576 mouse | Glutamate | Modulates the toxic effects of Aβ by inhibiting Aβ-induced nitric oxide (NO) | Soumyanath et al. (2012) |
| SH-SY5Y cells and MC65 human neuroblastoma cells | Aβ | | |

| | | | |
|---|---|---|---|
| PD model *Drosophila* flies | Transgenic *Drosophila* fly lines that expresses wild-type human synuclein | Reduces lipid peroxidation, protein carbonyl, GST, and MDA content | Siddique et al. (2014) |
| | | Increase GSH content | |
| Male Laca mice | D-Galactose | Upregulates NADH dehydrogenase, succinate dehydrogenase activity, | Kumar et al. (2011) |
| | | Increased neuronal viability | |
| | | Reduces acetylcholine esterase | |
| Adult Wistar rats | Silver nitrate | BDNF-like action | Mohandas Rao et al. (2012) |
| | | Activate the expression of the early genes such as activity-regulated cytoskeleton-associated protein (arc) leading to enhancement of the dendritic arborization | |
| In vitro | Markers of oxidative stress | Antioxidants prevent chain initiation, binding of transition metal ion, decomposition of peroxides in addition to free radical scavenging | Sugunabai and Karpagam (2015) |
| Male Wistar rats | Streptozotocin | Decreases MDA and increase in glutathione and catalase levels | Kumar and Gupta (2003) |
| | | Improves acetylcholine synthesis | |
| Male Sprague-Dawley rats | Behavioral cognitive | Increase doublecortin (DCX) and Notch1 protein levels in the hippocampus | Sirichoat et al. (2015) |
| Male ICR mice | Transient cerebral ischemia-reperfusion | Reduced the microglial overactivation and the phosphorylation of p38 mitogen-activated protein kinases | Chen et al. (2014) |
| Adult male Wistar rats | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) | Antioxidant maintain the metabolic balance of dopamine and increasing ratio of apoptosis regulator Bcl-2/apoptosis regulator Bax | Xu et al. (2012a) |

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity.

It is postulated that Aβ peptide increases vasoactivity in a single transgenic mouse model of Alzheimer's disease (Paris et al. 2000). *C. asiatica* is also known to exert a positive effect in the treatment of venous insufficiency (Cataldi et al. 2001), wound healing by stimulating type 1 collagen synthesis in fibroblast cells, and has been shown to help normalize the vasculature (Widgerow et al. 2000; Brinkhaus et al. 2000; Lee et al. 2006).

## 1.4 Conclusions

An old Sinhalese proverb righteously depicts about the therapeutic effect of *Centella asiatica,* "Two leaves (of *Centella asiatica*) a day keeps old age away." True to the above, *Centella asiatica* has shown to possess significant neuroprotective phytochemicals and nutrients. These neuroprotectants exhibit multiple pharmacological effects which can prevent cell injury, attenuate toxic insults, and protect against endogenous and exogenous lethality. *Centella asiatica* has significantly shown to protect against dermal and CNS disorders. Without any uncertainties, it is definite to conclude that *Centella asiatica* possesses significant memory-enhancing effects with very minimal adverse effects. Hence, *Centella asiatica* and its active components can be a potent botanical with memory-enhancing properties, prevent neurodegeneration, and additionally have therapeutic value in preventing cognitive impairment.

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# Medicinal Properties of Mediterranean Oyster Mushrooms: Species of Genus *Pleurotus* (Higher *Basidiomycetes*)

2

Giuseppe Venturella and Maria Letizia Gargano

**Abstract**

The term "Mediterranean area," applied in this chapter, refers to the definition reported in Med-Checklist and particularly to all countries bordering the Mediterranean Sea plus Portugal, Bulgaria, the Crimea (Ukraine), and Jordan. The "Mediterranean oyster mushrooms" is a geographically and ecologically well-defined group of *Basidiomycetes*. The medicinal properties of some widely investigated species such as *Pleurotus ostreatus* and *P. eryngii* are recognized worldwide, while in the case of some other Mediterranean *Pleurotus* taxa, there is still a lack of knowledge. A substantial increase in knowledge about the anticancer and antibacterial properties of the group of *Pleurotus* species growing as saprophytes on dead roots of plants of family *Apiaceae* (*P. nebrodensis*, *P. eryngii* var. *elaeoselini*, *P. eryngii* var. *ferulae* in particular) has been recorded in recent years, thanks to research carried out at the University of Palermo (Italy). This chapter summarizes the latest research on medicinal oyster mushrooms growing in the Mediterranean environment.

**Keywords**

Mediterranean area • Medicinal mushrooms • Antibacterial activity • Oyster mushrooms • *Pleurotus*

**Disclosure** The authors contributed equally to this work.

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## Contents



## Abbreviations

| | |
|---|---|
| CWE | Cold water extracts |
| HIV | Antihuman immunodeficiency virus |
| MIC | Minimum inhibitory concentrations |

## 2.1 Introduction

The genus *Pleurotus* (Fr.) P. Kumm is one of the largest and the most diverse genus among the class *Basidiomycetes*. In a recent taxonomic assessment, the genus *Pleurotus* is included in the kingdom of *Fungi*, phylum *Basidiomycota*, subdivision *Agaricomycotina*, class *Agaricomycetes*, subclass *Agaricomycetidae*, order *Agaricales,* and family *Pleurotaceae* (Kirk et al. 2010).

Under the term "oyster mushrooms," we include basidiomata (fruit bodies) with an eccentric stalk (sometimes absent) together with a gilled hymenium, a firm flesh, and a wide cap of different color shaped like an oyster shell (Fig. 2.1a–e) (Rajarathnam and Bano 1987).

According to Zervakis and Polemis (2013), the genus comprises ca. 30 species and subspecific taxa of edible mushrooms with a worldwide distribution. Several species are widely consumed due to their high nutritional and potential medicinal value (Khan and Tania 2012). Literature data reported a large number of therapeutic values for *Pleurotus* species such as antimicrobial, antiviral, antihuman immunodeficiency virus (HIV), antineoplastic, antitumor, antimutagenic, antioxidant, antilipidemic, hyperglycemic, hypotensive, anti-inflammatory, hepatoprotective, hypocholesterolemic, immunomodulatory, and antiaging (Patel et al. 2012).

In this chapter, we want to focus the reader's attention on the medicinal properties of species of oyster mushrooms growing in the Mediterranean area. The term "Mediterranean area" refers to the definition reported in Med-Checklist (Greuter 2008) and particularly to all countries bordering the Mediterranean Sea plus Portugal, Bulgaria, the Crimea (Ukraine), and Jordan.


**Fig. 2.1** Mediterranean oyster mushrooms: (**a**) *Pleurotus eryngii* var. *elaeoselini*, (**b**) *Pleurotus nebrodensis*, (**c**) *Pleurotus eryngii* var. *eryngii*, (**d**) *Pleurotus eryngii* var. *ferulae*, (**e**) *Pleurotus eryngii* var. *thapsiae*, (**f**) *Pleurotus ostreatus*

## 2.2 Medicinal Properties of Mediterranean Oyster Mushrooms

In the Mediterranean area, species of the genus *Pleurotus* grows as weak parasites on different broad-leaved and conifer trees or as saprotrophs on roots of herbaceous plants of the family *Apiaceae*. We analyzed literature data from the countries included in the Mediterranean area (*sensu* Greuter 2008) : East Aegean Islands, Algeria, Albania, Asiatic Turkey, Balearic Islands, Bulgaria, Corsica, Crete and Karpathos, Cyprus, Egypt, Crimea, Italy, Sinai, Tunisia, and Turkey. Some of

*Pleurotus* species are widely cultivated for food use and/or their medicinal properties. Literature data are mostly available for the most common species such as *P. ostreatus* and *P. eryngii*, while little or nothing is known about the potential therapeutic properties of some taxa (i.e., *P. opuntiae*). The medicinal value of *Pleurotus* taxa is still under-investigated in most of the Mediterranean countries.

### 2.2.1 *Pleurotus cornucopiae* (Paulet) Rolland

*P. cornucopiae* is one of the potential mushrooms that contain antioxidants or increase antioxidant enzyme activity. In particular, *P. cornucopiae* possesses antigenotoxic and bio-antimutagenic activities when tested on *Salmonella typhimurium* and *Escherichia coli* (Filipic et al. 2002). In addition, Hagiwara et al. (2005) have reported D-mannitol content and antihypertensive activity in *P. cornucopiae*.

### 2.2.2 *Pleurotus eryngii* (DC.) Quél. var. *eryngii*

The protein eryngeolysin, isolated from *P. eryngii* basidiomata, exhibited cytotoxicity against leukemia cells and inhibited the stimulated mitogenic response of murine splenocytes (Ngai and Ng 2006). Sano et al. (2002) have reported antiallergic activity of *P. eryngii* extract.

The antifungal peptide eryngin is active against *Fusarium oxysporum* Schltdl. and *Cercospora arachidicola* Hori (sub: *Mycosphaerella arachidicola* W. A. Jenkins), while the hemolysin designated as eryngeolysin shows antimicrobial activity against *Bacillus* spp. (Gregori et al. 2007).

Extracts of *P. eryngii* var. *eryngii* were tested for their in vitro growth inhibitory activity against *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The extracts were able to inhibit all tested microorganisms (Schillaci et al. 2013).

### 2.2.3 *Pleurotus eryngii* (DC.) Quél. var. *elaeoselini* Venturella, Zervakis, & La Rocca

Schillaci et al. (2013) recently reported the antibacterial activity of extracts obtained from this taxon. The extracts were tested in vitro against a group of bacteria of medical relevance. The extracts of *P. eryngii* var. *elaeoselini* inhibited the tested microorganisms with activity expressed as minimum inhibitory concentrations (MIC) in the amount of 0.05 MIC (% v.v.) for *P. aeruginosa* and *S. epidermidis* and 0.1 MIC (% v.v.) for *S. aureus* and *E. coli*.

### 2.2.4 *Pleurotus eryngii* (DC.) Quél. var. *ferulae* (Lanzi) Sacc

The methyl alcohol extracts of basidiomata of *P. eryngii* var. *ferulae* cultivated on various agro-wastes in Turkey show an antimicrobial activity against some bacteria, yeasts, and dermatophytes (Akyuz and Kirbag 2009).

Cold water extracts (CWE) from basidomata of *P. eryngii* var. *ferulae*, collected in Sicily (southern Italy), was tested in vitro on human colon cancer cells. The results demonstrated that the extracts are able to inhibit cell migration and to affect homotypic and heterotypic cell-cell adhesion (Fontana et al. 2014).

The growth inhibitory activity of extracts of *P. eryngii* var. *ferulae* was tested in vitro against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *E. coli*. The extracts were able to inhibit all tested microorganisms (Schillaci et al. 2013).

### 2.2.5 *Pleurotus nebrodensis* (Inzenga) Quél

Venturella et al. (2016) have recently clarified the proper taxonomic identity of this taxon in comparison to Asian populations.

In vitro antitumor effects of CWE from basidiomata of *P. nebrodensis*, collected in Sicily (southern Italy) and tested on human colon cancer cells, underline that they can be considered as possible sources for new alternative therapeutic agents for cancer treatment (Fontana et al. 2014).

Schillaci et al. (2013) recently evaluated the antibacterial activity of *P. nebrodensis*. The extracts were tested in vitro against a group of bacteria of medical relevance. The extracts of *P. nebrodensis* inhibited the tested microorganisms with activity expressed as MIC (minimum inhibitory concentrations) in the amount of 0.05 MIC (% v.v.) for *P. aeruginosa*, ≤0.025 MIC (% v.v.) for *S. epidermidis*, and 0.1 MIC (% v.v.) for *S. aureus* and *E. coli*.

### 2.2.6 *Pleurotus ostreatus* (Jacq.) P. Kumm

*P. ostreatus* represents one of the most popular mushroom species in the Mediterranean area. Pleuran is an insoluble polysaccharide [β-(1,3/1,6)-D-glucan] isolated from the oyster mushroom (Karácsonyi and Kuniak 1994) and one of the most famous bioactive polysaccharides from the mushroom origin (El Enshasy et al. 2013).

*P. ostreatus* contains lovastatin, a naturally occurring compound and the hypolipidemic agent used for lowering cholesterol and prevention of cardiovascular diseases (Gunde-Cimerman 1999; Wasser and Weis 1999). A high production of lovastatin was detected from strains of *P. ostreatus* collected in Turkey (Atli and Yamac 2012).

The immunostimulatory effect of B-glucans from *P. ostreatus* was also demonstrated in daily intake in individuals debilitated by extreme conditions such as

prolonged wars and subsequent famine in Bosnia and Herzegovina (western Balkan) (Redzic et al. 2010).

In Egypt the production of *P. ostreatus* by submerged fermentation, using glucose as a medium, has been positively tested by Daba et al. (2008): These authors demonstrated the potential of oyster mushrooms as fungal protein and as a source of medicinal compounds.

In a study of the action of cyclophosphamide and extract of the mycelium of *P. ostreatus* in vivo on mice bearing melanoma, Meerovich et al. (2005) demonstrated that the mushroom mycelium extract combined with the chemotherapeutic agent cyclophosphamide decreased the degree of leukopenia. Water-soluble proteins and/or polypeptides in the water extract of *P. ostreatus* exhibited significant cytotoxicity by inducing apoptosis of human carcinoma cells (Gu and Sivam 2006). The α-glucans from *P. ostreatus* induced apoptosis of colon cancer cells in vitro (Lavi et al. 2006), lectin inhibited growth of sarcoma and hepatoma in mice (Wang et al. 2000), the *P. ostreatus* DNA stimulated natural mouse killer cytotoxic activity in vitro (Shlyakhovenko et al. 2006), and anti-HIV activity was also demonstrated (Wang and Ng 2000).

Various extracts of *P. ostreatus* possess antimicrobial activities against Gram-positive (*Bacillus subtilis*), Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa, Vibrio cholerae*), *Salmonella typhi*, bacteria, black mold (*Aspergillus niger* Tiegh.), and *F. oxysporum* (Gregori et al. 2007).

### 2.2.7 *Pleurotus pulmonarius* (Fr.) Quél

Jose et al. (2002) have demonstrated the therapeutic potential of methanol extract of *P. pulmonarius* as an antitumor and anti-inflammatory agent. The hot water extracts of *P. pulmonarius* demonstrate inhibitory activity against human immunodeficiency virus (HIV) (Wang et al. 2007) (Table 2.1).

## 2.3 Conclusions

The importance of a Mediterranean diet and its benefits on human health mainly refer to its anti-inflammatory effects (Djuric 2011). The Mediterranean diet ensures lower risk for cardiovascular disease, for several forms of cancer, and for Alzheimer's disease (Scarmeas et al. 2006). Fruits and vegetables are a key part of an overall healthy life (Slavin and Lloyd 2012).

Mushrooms have a unique nutrient profile, and they are biologically distinct from the plant- and animal-derived foods (Feeney et al. 2014). Europe is the largest market for cultivated mushrooms, accounting for more than 35% of the global market. On the contrary, the industry of medicinal mushrooms is less developed than in Eastern countries. Most of the companies that have headquarters in the Mediterranean countries and which sell food supplements based on medicinal mushrooms are directly dependent on foreign companies, and most of the fungi are cultivated

**Table 2.1** *Pleurotus* Mediterranean taxa and corresponding medicinal properties

| Taxa | Active compounds | Medicinal properties | References |
|---|---|---|---|
| *Pleurotus cornucopiae* | β-Glucans | Antioxidant; antigenotoxic; bio-antimutagenic; antihypertensive | Filipic et al. (2002) and Hagiwara et al. (2005) |
| *Pleurotus eryngii* var. *eryngii* | Eryngeolysin, β-Glucans, Eryngin | Anticancer (leukemia); antiallergic; antimicrobial; antibacterial | Gregori et al. (2007), Ngai and Ng (2006), Sano et al. (2002), and Schillaci et al. (2013) |
| *Pleurotus eryngii* var. *elaeoselini* | β-Glucans | Antibacterial | Schillaci et al. (2013) |
| *Pleurotus eryngii* var. *ferulae* | β-Glucans | Antimicrobial; anticancer | Akyuz and Kirbag (2009), Fontana et al. (2014), and Schillaci et al. (2013) |
| *Pleurotus nebrodensis* | β-Glucans | Antimicrobial; anticancer | Fontana et al. (2014), Schillaci et al. (2013), and Venturella et al. (2016) |
| *Pleurotus ostreatus* | Pleuran, Lovastatin, α-Glucans | Hypolipidemic; prevention of cardiovascular diseases; immunostimulatory; anticancer (melanoma, sarcoma, hepatoma); antimicrobial; antibacterial; antifungal | Atli and Yamac (2012), Daba et al. (2008), El Enshasy et al. (2013), Gregori et al. (2007), Gu and Sivam (2006), Gunde-Cimerman (1999), Karácsonyi and Kuniak (1994), Lavi et al. (2006), Meerovich et al. (2005), Redzic et al. 2010, Shlyakhovenko et al. (2006), Wang et al. (2000), Wasser and Weis (1999), and Wang and Ng (2000) |
| *Pleurotus pulmonarius* | β-Glucans | Anticancer; anti-inflammatory; anti-HIV | Jose et al. (2002) and Wang et al. (2007) |

abroad, while the extracts they buy are mainly sourced from China. This induces considerable confusion for the consumers that buy food supplements only apparently labeled in Europe. In addition, the certification of fungal extracts is often not responsive to the standards established by the European Community.

There is an increasing request by the pharmaceutical companies of Mediterranean countries to the research laboratories in the Universities to activate scientific cooperation in order to identify new fungal extracts and stimulate entrepreneurs to start large-scale cultivation of edible and medicinal mushrooms to allow those companies a greater autonomy from the market of Eastern countries.

For the above reasons, the recent preliminary in vivo experiments (still in progress) based on *Pleurotus* species and carried out at the University of Palermo (Italy) on humans, pets, and livestock are very encouraging.

In particular, attention is paid on some new varieties of *P. eryngii* (i.e., *P. eryngii* var. *elaeoselini* and *P. eryngii* var. *thapsiae* Venturella, Zervakis, & Saitta) and on *P. nebrodensis*, creating the conditions for the production of a new line of mushroom-based supplements.

The specificity of the *Pleurotus* species growing in the Mediterranean area, compared to the same wild edible species of oyster mushrooms growing in Asiatic countries, lies mainly in their high nutritional value (La Guardia et al. 2005). For this reason, the possibility of combining quality food and medicinal value is the true challenge for the near future for a better enhancement of the Mediterranean oyster mushrooms.

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# *Cordyceps*: A Highly Coveted Medicinal Mushroom

3

John Holliday

**Abstract**

Traditional Chinese medicine (TCM) has a long history of using plant, animal, and fungal materials for their medicinal values. One of the better known and by far the most expensive of the Oriental medicines is the family of fungal-insect pairings known as *Cordyceps*. Different *Cordyceps* species, most notably *Ophiocordyceps sinensis*, have a long history of use and have been found growing only from the head of a subterranean caterpillar above 3000 m altitude on the Qinghai-Tibetan plateau. Environmental and ecological factors have driven *O. sinensis* to the status of an endangered species, with annual harvests steadily declining while at the same time the worldwide demand is ever increasing. This has driven the prices for *O. sinensis* into an ever-increasing spiral over the last decade, with top-quality *Cordyceps* currently valued at more than one hundred thousand US dollars per kilogram (100,000 USD) in the major Chinese cities. Its value today is literally higher than the price of gold. Such fantastic prices are driving research into cultivating *O. sinensis* and other *Cordyceps* species with an eye to making them more affordable for commercial trade. Aloha Medicinals in the United States is currently the largest cultivator of *Cordyceps* in the world producing an estimated half of all the *Cordyceps* annually consumed, but in addition to cultivating this rare fungus for the pharmaceutical trade, they are also involved in researching new species of *Cordyceps* from around the world with similar or identical medicinal properties as the classic species, *O. sinensis*. Recently, Aloha Medicinals expeditions into the Tingo Maria Valley in Peru have found that this area has the greatest diversity of *Cordyceps* species of any known location. More than 1000 types of *Cordyceps* have been found in Tingo Maria Valley so far, the vast majority of which have yet to be described or named.

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Currently, biotechnology companies around the world are cultivating about a dozen different species of *Cordyceps* for use in the pharmaceutical and nutraceutical sectors. With the discovery of the incredible diversity of entomogenous fungi in Peru, many new candidate species are now being researched for their medicinal properties and the potential for cultivation, in an effort to commercialization as substitutes for increasingly rare *O. sinensis*. As research continues to prove the wide range of medicinal properties claimed for *Cordyceps*, the potential for developing new medicinal *Cordyceps* species to replace the rare and endangered wild-collected *O. sinensis* has great potential.

**Keywords**

*Cordyceps* • *Ophiocordyceps sinensis* • *Cordyceps* sensu lato • *Cordyceps militaris* • Cordycepin • 2′ deoxyadenosine • Cordycepic acid

## Contents



## Abbreviations

| | |
|---|---|
| *Cordyceps* s.lat. | *Cordyceps* sensu lato, meaning the broad group of insect-inhabiting fungi classed together as medicinally important species of fungi growing from the bodies of insects or occasionally truffles of the genus *Elaphomyces*. |
| TAR | Tibetan Autonomous Region |

**A Note Concerning the Usage of Species Names in This Chapter**

For many decades, the genus name *Cordyceps* was used when referring to any and all of the insect-inhabiting ascomycetes fungi used in medicine or associated with this class of fungi. Specifically, the name *Cordyceps sinensis* was used extensively to describe both the naturally occurring wild-collected insect larva and fungi complex and the cultivated anamorph (asexual mycelial form) varieties. Today, the term "*Cordyceps*" is still in nearly universal use by the public, when referring to all forms of dietary supplements manufactured from this class of fungi, regardless of the actual genus and species used. However, the huge grouping including these fungi is basically a mega genus, containing many hundreds of species. No one is even certain of the exact number of species included in his group. To try to clarify this taxonomy (naming) nightmare, in 2007 a complete and comprehensive phylogenetic study of the *Cordyceps* group was performed and published under the title *Phylogenetic Classification of Cordyceps and the Clavicipitaceous Fungi* by Gi-Ho Sung and associates (Sung et al. 2007). In this landmark study, they were able to break the *Cordyceps* group into different genera and subgenera based upon their molecular ID, by using modern methods of multigene DNA sequencing rather than depending on the traditional morphology-based identification. One of the major results of this work was to reclassify *Cordyceps* species into a number of different genera: with most species falling into the genera *Cordyceps*, *Metacordyceps*, *Elaphocordyceps*, and *Ophiocordyceps*, as well as a few species falling into other genera and subgenera, based primarily on their DNA but also on the spore characteristics. However, this renaming of popularly used species has also led to quite a bit of confusion when dealing with the commercial products on the market today but also when looking at the research done on this fascinating group of fungi over the last four or five decades. In order to help clarify this and to make the naming issue a bit more understandable for those readers who are not professional mycology taxonomists, several names have been used in this chapter for these fungi: The term *Cordyceps* sensu lato, which in Latin means the entire or overall grouping of *Cordyceps*-type fungi, is used where appropriate, to refer to overall characteristics of the *Cordyceps* group of insect-inhabiting fungi. When the name *Cordyceps sinensis* was used in the research articles referred to in this chapter, that earlier name has been converted in this chapter to the currently correct name, *Ophiocordyceps sinensis*. When the species discussed in the research was not clearly identified, or when an unidentified anamorph species was used, the term "*Cordyceps*" is used here. When other species are mentioned, those correct species names are used, such as the name *Cordyceps militaris*, which is a species used commonly in commercial trade today. The author's intent was to clarify the names used while accurately reporting the data represented in the research articles referred to. It is my sincere hope we have accomplished this.

## 3.1 Introduction

Imagine a mushroom that only grows from the head of a buried caterpillar high in the rugged mountains of Tibet, only above 3000 m elevation. This is one of the most remote, inaccessible, and hostile environments on the planet, one not normally associated with an environment suitable for mushroom hunting. This is the natural habitat of the famed *Cordyceps* mushroom. In use for many centuries as a precious medicine in Chinese and Tibetan medicine practices, *Cordyceps* is and always has been one of the rarest and most mysterious of mushrooms. It was long thought to be a nearly mythical creature that was able to change from a plant in the summer into a worm in the winter and back again when the snow cleared and the weather warmed. The famous Tibetan *Cordyceps*, which today is properly named *Ophiocordyceps sinensis*, is an ascomycetes mushroom that grows only on the dead subterranean larva of several species of moths, known as Himalayan ghost moths (around 30 species of the genus *Thitarodes*) (Fig. 3.1a–e). There are a number of species of this group of entomogenous (insect-inhabiting) fungi used in medicine today, which overall group is known broadly as *Cordyceps* sensu lato – and this group of fungi is typified by the most well-known and historically important species, *Ophiocordyceps sinensis*. This name has only been in use since about 2007 and was formerly known as *Cordyceps sinensis*, which is still the most commonly used name in medicine and by most practitioners and consumers other than professional mycologists. These fungal species have been and still are important medicines in the traditional medical practices of Asia. They are also species of great economic importance in the regions where they grow. *O. sinensis* is known regionally by many names dōng chóng xià cǎo (冬虫夏草) in Chinese, the Chinese caterpillar fungus in English, tochukaso in Japanese (translation into Japanese of the Chinese characters 冬虫夏草), known as yartsa gunbu (དབྱར་རྩྭ་དགུན་འབུ) in Tibetan, and yarsagumba (यार्चागुन्बू) in Nepali. The term *Cordyceps* sensu lato (*Cordyceps* s.lat.) refers to and includes the entire broader grouping of ascomycetes fungi found growing on insect larvae and mature insects and in some cases found growing on the underground sporocarps of truffles of the genus *Elaphomyces.* Most of the species referred to as *Cordyceps* s.lat. belong to one of four genera: *Cordyceps*, *Ophiocordyceps*, *Elaphocordyceps*, and *Metacordyceps*. While *Cordyceps* s.lat. has been medicinally used for centuries, in-depth research into this group of fungi is all fairly recent. It was not until as late as 2007 that a full study was made of the phylogeny of *Cordyceps* s.lat., showing the relationship between the various genera and species (Sung et al. 2007). By far, the most well-known and most economically important species, as well as the most well-studied species of this group of fungi, is *Ophiocordyceps sinensis*, which has a long history of use as a rare and exotic medicinal fungus of great importance in TCM. While it is unclear from the written record exactly how long this has been used medicinally, we know that it has been regarded as one of the cornerstones of Tibetan and Chinese medicine for a very long time, at least since the fifteenth century (Boesi and Cardi 2005). *O. sinensis* is a medicinal herb that has long been

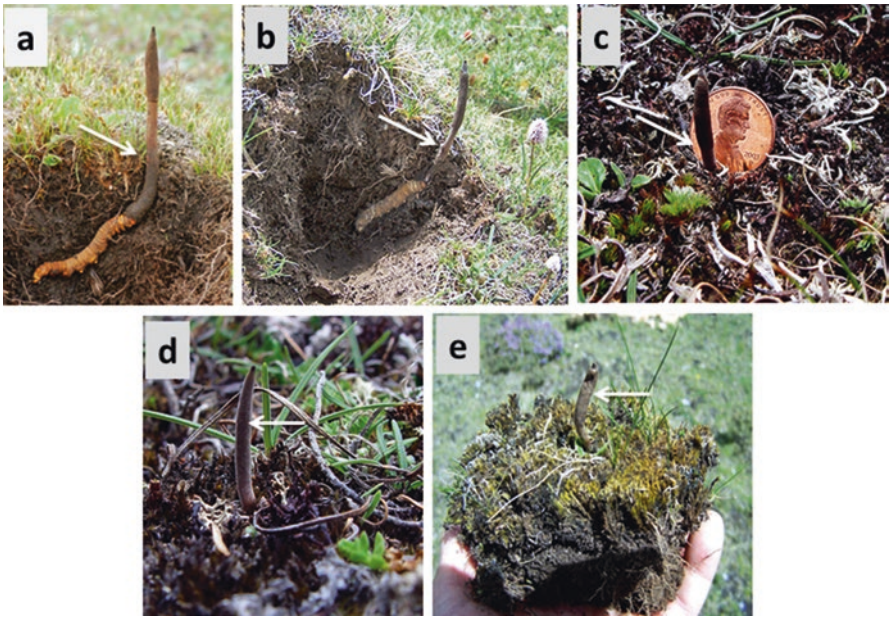


**Fig. 3.1** (**a**) *Ophiocordyceps sinensis* at 5600 m elevation, Tibet. (**b**) *Ophiocordyceps sinensis* at 4800 m elevation – Tibet. (**c**) *Ophiocordyceps sinensis* with American penny for size reference. (**d**) *Ophiocordyceps sinensis* found growing at 6000 m Serkim La pass, Tibet, June 2006. (**e**) Tibetan *Ophiocordyceps sinensis* from Mila, Tibet, 2006 (*Arrows* showing the *Cordyceps*)

believed to have a wide range of medicinal effects (Bensky et al. 2004; Zhu et al. 1998), many of which modern science has confirmed to be true. Up until very recently, the use of *O. sinensis* was restricted almost exclusively to the richer population in very limited areas of Asia due to its cost and rarity, but within the last 20 or 30 years, this medicinal fungus has become well known in Western countries. With all the claims of the wide-ranging therapeutic effects of *O. sinensis*, much research effort has been devoted to this group of fungi in recent years. Starting around 1980, modern scientific methods have been applied to investigating the wide range of claimed therapeutic effects in an attempt to validate what TCM practitioners have observed for centuries (Holliday and Cleaver 2008). While *O. sinensis* was the earliest used and is the most well known of the medicinal fungi of the group *Cordyceps* s.lat., in recent years many other fungal species from this broad group have been brought into medicinal usage, mainly through commercial cultivation, as other species of *Cordyceps* s.lat. are far easier to grow in laboratory conditions than *O. sinensis*. As science and technology move ahead, we are bound to see more and more *Cordyceps* products brought into the herbal medicine market and perhaps used as pharmaceutical raw material, so while this chapter will focus mainly on *O. sinensis*, the other fungi of the broader grouping *Cordyceps* s.lat. will be discussed as well.

## 3.2 *Ophiocordyceps sinensis*: The Prototypical *Cordyceps* Species

*O. sinensis* is the species used for centuries in TCM and the one named in all the earlier texts. This is not simply a mushroom, but rather it is a complex consisting of the mushroom and the attached insect larvae, both of which are consumed together for their medicinal properties (Fig. 3.2). In fact, the mushroom portion alone is not considered to have any medicinal properties by itself; rather the caterpillar portion is the part that contains the majority of active ingredients. Recent studies by this author have confirmed this to be true, because while the mummified larvae may still look like a caterpillar, in fact, the only part of the original larvae remaining is the outermost chitinous shell, with the rest of the caterpillar having been consumed and replaced with mycelium from the fungus. This outer chitinous larval shell then acts as a reservoir, which contains all the fungal extracellular metabolites. These are the compounds that the fungus excretes out through the cell wall as it grows, in order to digest its food source in place, to act as transport molecules to bring the nutrients back across the cell wall, to stun or kill bacteria and other fungi (the antibiotic compounds), and to give itself an advantage in the food chain, and undoubtedly perform other functions as well. While it is true that the cell wall and cytoplasm of the fungus do contain some medicinal properties, in fungal metabolism, it is the extracellular compounds that contain the majority of the biologically active compounds. If we consider the classic fungal fermentation process – beer or wine production – it is easy to understand why this is the case. In making wine, for example, a substrate (fruit juice) is first sterilized and then inoculated with the target fungal species (the

**Fig. 3.2** Two types of *Ophiocordyceps sinensis* from Tibet

yeast *Saccharomyces cerveza* in the case of wine), and over the next few weeks as the fungus bioconverts the substrate into fungal tissue, the biological compounds of interest, things like the color, the flavor, and the alcohol, are not retained within the cell boundary, but rather they are excreted into the environment, into the liquid substrate which the fungus is growing in. The resulting wine is this mixture of the extracellular compounds and the remaining substrate. The wine is not the cell mass that collects at the bottom of the tank. This concept of extracellular activity is true for all fungi, *Cordyceps* included. Since fungal cells have no mouth with which to ingest their nutrients for growth, nearly all of their interactions with the surrounding environment take place by passing through the cell wall, with certain compounds traveling out from the cell and other nutrients passing the other way back into the cell. Understanding this concept has led to great advances in the cultivation process for medicinally important fungi today. Some medicinal mushrooms are cultivated in a liquid media, where the cells are grown in a liquid media, either to capture the compounds within the cells or to extract target compounds from the residual liquid. Other medicinal mushrooms are cultivated in a solid media, termed solid-state fermentation, where the entire suite of growth compounds and secondary metabolites are captured together by harvesting the entire mass of fungal tissue and residual substrate mass together. When grown in this manner, the commercial products are termed "Full Spectrum Mycoproducts" as they contain everything that was produced through the entire fungal life cycle. While the liquid fermentation process is great for producing specific isolated compounds, liquid fermentation cannot match the full profile of chemical complexity such as is found in the entire *O. sinensis* mushroom and insect complex. Solid-state fermentation can much more closely match the entire chemical complexity, often producing nearly an identical analytical signature as the wild variety, since both the cellular and the extracellular compounds produced by the fungus throughout the entire growth cycle are captured and harvested together (Fig. 3.3).

## 3.3 Historical Use of *O. sinensis* and Other *Cordyceps* spp.

The first written record believed to likely refer to *O. sinensis* comes from China in the year AD 620, at the time of the Tang Dynasty (Bensky et al. 2004). However, this record is not entirely clear and does not positively refer to *Cordyceps*, although many researchers believe that to be the case. The first written record that can be confirmed as a reference to the use of *O. sinensis* in medicine appeared in the Tibetan text *Man ngag bye ba ring bsrel* (*Oral Instructions on a Myriad of Medicines*) written in the fifteenth century by Zurkhar Nyamnyi Dorje [1439–1475]. The first confirmed written reference of its use in TCM comes from Wang Ang's 1694 compendium of materia medica, *Ben Cao Bei Yao* (Winkler 2008), and it was again mentioned in Wu Yiluo's *Ben Cao Cong Xin* (*New Compilation of Materia Medica*) written in the year 1757, during the Qing Dynasty (Zhu et al. 1998; Halpern 2007). These early writings all speak of a creature whose annual existence included a transformation from animal to plant during the summer and then again from a plant back into an animal during the winter (Bensky et al. 2004). This belief in the

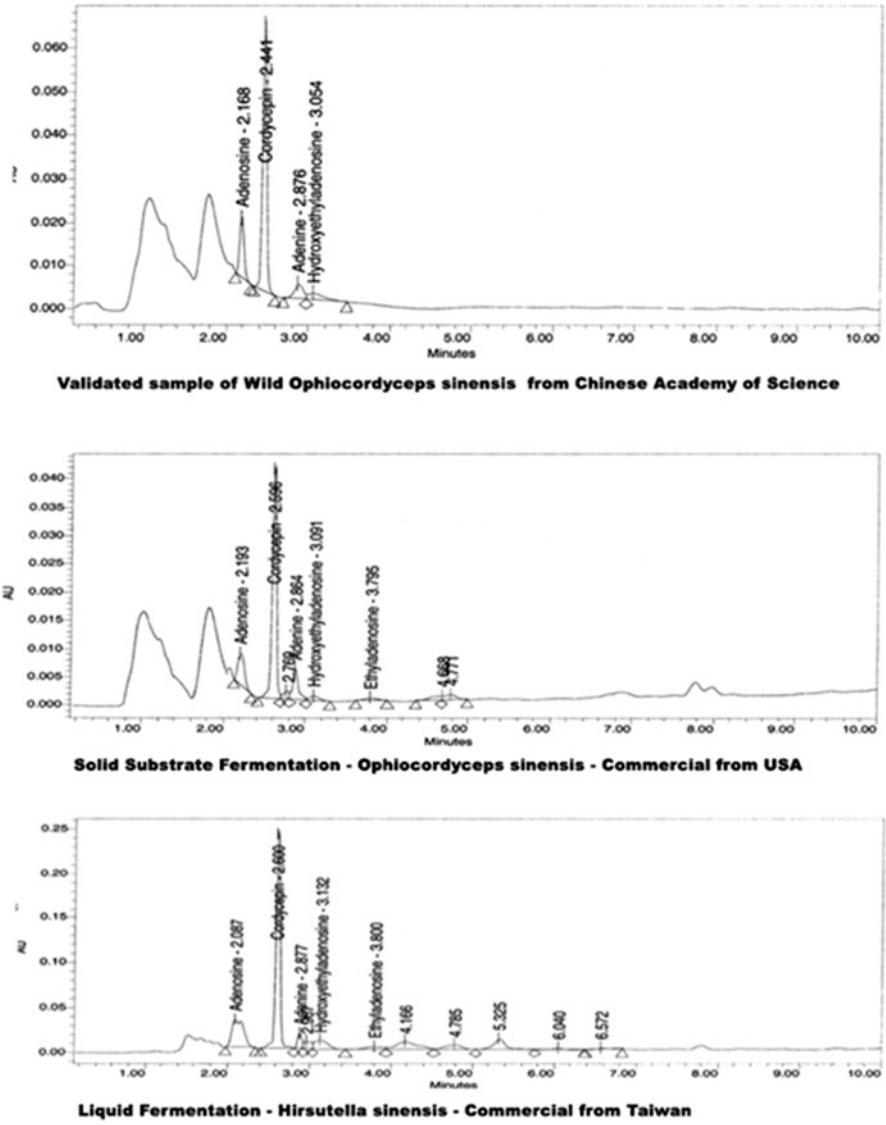


**Fig. 3.3** HPLC comparison of chemistry of solid fermentation of *O. sinensis* and liquid fermentation of *H. sinensis*

transformation leads to the common Tibetan name, yartsa gunbu, which translates literally as "winter worm, summer grass." This is also the literal translation of the common Chinese name, dōng chóng xià cǎo.

The species referred to in these early texts were all *O. sinensis*, as this is the only endemic Asian species of insect-inhabiting fungus that was known to be traditionally used in Asian medicine and one of only two species of *Cordyceps* with any

**Fig. 3.4** *Cordyceps robertsii* – known in New Zealand as "awheto"

known historical or traditional medicinal usage. The only other *Cordyceps* species known with a history of traditional use is *Cordyceps robertsii*, a species closely related to *O. sinensis* (Sung et al. 2007) *and* found only in New Zealand and traditionally used for tattooing. *C. robertsii* (Fig. 3.4) has been used by the Maori people to make the tattoo ink favored by traditional people in New Zealand, by burning the fungus and caterpillar complex to a fine ash and mixing the ash with oil or water. It was also used to prevent infection in the fresh tattoo by mashing the fresh fruit body/caterpillar complex and mixing with water and then applying this paste over the site of the fresh tattoo (Relph 1991; also the result of author's discussion with various New Zealand Maori historians and elders). It is interesting to note that *O. sinensis* and *C. robertsii* are the closest relatives on the phylogenetic tree as laid out in the phylogenetic study of *Cordyceps* s.lat. by Sung et al. (2007). Humans tend to be very good observers of nature, and when separate peoples in different parts of the world are found to use closely related mushroom species for similar or exact purposes, it supports the idea that there is some fact underlying the belief.

## 3.4 The Collection and Economic Value of Wild *O. sinensis*

In the wild, *O. sinensis* has a very small natural range. The natural habitat is restricted to high altitudes on the Himalayan Plateau above an elevation of approximately 3000 m. The primary collecting region is the Naqu district of Tibet, with an annual harvest reported at 7000 kg (Chen et al. 2000). The total *O. sinensis* harvest for the entire Tibet Autonomous Region (TAR) is estimated to be about 50 tons annually, with a total harvest of about 60 tons worldwide (Boesi and Cardi 2009; Winkler 2008). It is also collected in smaller volumes throughout the rest of Tibet, in the Gansu, Qinghai, Sichuan, and Yunnan provinces of China, in the countries of

Nepal and Bhutan, and a small region in northernmost India in Sikkim state. The value of wild-collected *O. sinensis* is literally higher than gold. The price in Beijing in recent years has been as high as $112,000 USD per kilogram (Lo et al. 2013). The annual harvest in Tibet, which takes place over just a 4- to 6-week period in May and June, is estimated to add approximately $225 million USD to the GDP of the TAR, accounting for fully 40% of the total rural cash income of Tibet. The collection of *O. sinensis* is understandably very popular throughout the TAR, where the average Tibetan nomad family earns the largest portion of its annual income from the collection of *O. sinensis*, representing up to 80–90% of the total annual income for many rural nomadic families (Winkler 2010). While the importance of the *O. sinensis* harvest to the economy of the TAR cannot be overstated, it is unknown how sustainable this harvest will prove to be, due to environmental destruction from overharvesting and the dearth of regulations controlling the harvest in a sustainable manner. This fantastic financial growth in TAR triggered by *Cordyceps* has led to other environmental problems as well, even outside of TAR itself. In Tibetan culture, there is a long tradition that tiger skins represent personal wealth and power. Since the trade in *Cordyceps* is an entirely cash-based economy, the ready availability of large quantities of unreported and untaxed cash from the trade has dramatically increased demand in TAR for tiger skins, which are being smuggled into TAR from India, despite strong international restrictions on such trade. This increased demand for tiger skins in Tibet is leading to a serious reduction in populations of tigers in India, perhaps presaging their eventual extinction (Moyl 2009; Wright 2012).

Due to the elevation, the remote location, the small geographical area where it is found and the subsequent difficulty in harvesting *O. sinensis*, it is understandable why it has always been one of the most expensive medicinal substances known. Historically, its high price meant its use was restricted almost exclusively the Emperor's court and others of the ranking Chinese nobility. Its high price tended to place it beyond the reach of the average Chinese and Tibetan population. Despite its cost and rarity, the unprecedented list of medicinal uses for *O. sinensis* made it a valued staple of TCM. In modern years with the increasing economic status and a growing middle class in Asia, *O. sinensis* has become more widely used. It is known today by most people in Asia and considered to be a favored medicine. It is no longer just in Asia that it is widely prescribed by TCM practitioners but more so throughout the world today. Since the opening of China to trade with the west in 1972, TCM was brought to the attention of people in the Western countries. This growing interest in TCM in the West has brought *O. sinensis* to the attention of people in more affluent areas, such as North America and Europe. In a study published in 2013, the annual harvest was valued between 5 billion and 11 billion US dollars (Shrestha and Bawa 2013). This growing popularity throughout the world has resulted in increased demand and increasing supply, both of which have created multiple problems in recent years: the ever-increasing price has meant the number of collectors is increasing each year, which is resulting in the species being dramatically overharvested, with more than a 50% reduction in the quantity harvested, just between the years 2009 and 2011 (Shrestha and Bawa 2013). This overharvesting is

considered to be the main reason for the declining yields every year. This is why the future remains uncertain as to the continuing availability of wild-collected *O. sinensis.* These reduced yields and inflated prices have led to many counterfeit and adulterated products reaching the consumer in recent years (Holliday and Cleaver 2008), although the development and application of new analytical techniques and a more discerning consumer are reducing these number of false *Cordyceps* products as time goes on.

## 3.5 *Cordyceps* Diversity and Artificial Cultivation

There are more than 600 species of *Cordyceps* s.lat. known to science, and many more species are known to exist that have not been described to science or named yet. In fact, there are *Cordyceps* species found on all continents except Antarctica and in most of the various climatic zones and habitats. This author alone has found more than 1000 types of *Cordyceps* in the Tingo Maria Valley in Peru, most of which are assumed to be new, as yet undescribed species. Tingo Maria Valley was used as an experimental area by the US Drug Enforcement Administration to aerially apply spores from a plant pathogen fungus, the species *Fusarium oxysporum*, in an effort to eradicate the coca crop and reduce Peru's involvement in the international cocaine trade. It is unknown whether this experiment was a success in reducing the number of coca bushes, but one thing became clear within a short time after application of the spores: the *F. oxysporum* spores certainly increased the number of *Cordyceps* fruiting, with a multitude of species growing in virtually every family of insects and arachnids of the Tingo Maria Valley (Fig. 3.5a–e).

The sheer number of specimens collected there has limited the description and naming of these specimens, although the work is ongoing and will be for years to come. These *Cordyceps* species all occur parasitically or commensally (perhaps in some cases symbiotically, as with termites in Africa) with a wide range of insect and arachnid hosts (Holliday and Cleaver 2008; Halpern 2007; Sung et al. 2007), with *Elaphocordyceps* species inhabiting truffles of the genus *Elaphomycetes*. Due to the ever-increasing rarity and high prices of wild-collected *O. sinensis*, much effort has been made to artificially cultivate *O. sinensis* or related species, many of which have similar chemical makeup and medicinal properties. In fact, *O. sinensis* has proven to be very difficult to cultivate in artificial conditions, perhaps due to the vast range of associated fungal species that are known to coinhabit the insect host from which the *O. sinensis* fruiting body erupts. Using modern high-throughput DNA sequencing, more than 600 species of fungi belonging to at least 65 genera have been found to coinhabit wild *O. sinensis* (Xia et al. 2016). It is unknown how these different fungi interact with the *O. sinensis* or with the insect host. Many researchers believe there is a symbiotic relationship between some or all of these fungi, and the cocultivation of many different species may be required to replicate the complex conditions required for fruiting the *O. sinensis* fungus. It also appears from this author's work in cultivating *O. sinensis* and other *Cordyceps* species that several different species need to be cocultivated together in order to replicate the complex chemical

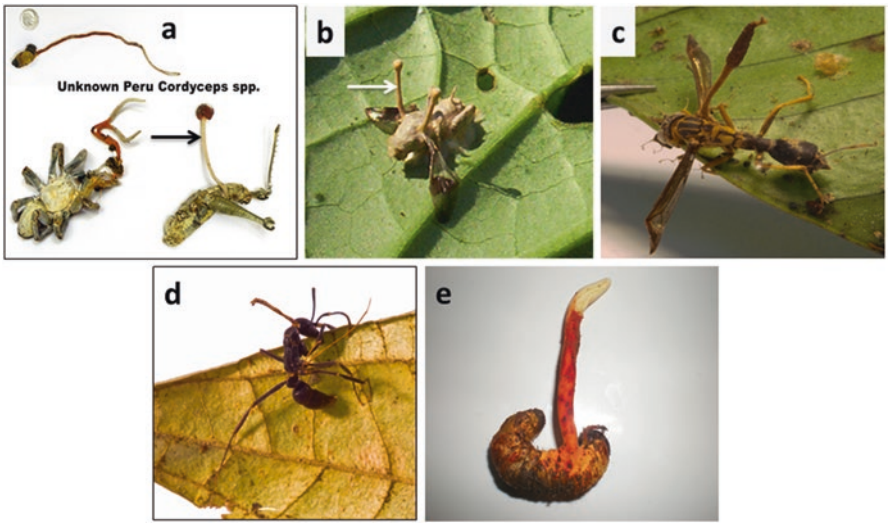


**Fig. 3.5** (**a**) *Cordyceps* spp. from Tingo Maria Valley, Peru. (**b**) Another unknown species from Peru. (**c**) *Cordyceps* fruit body growing from a wasp in Tingo Maria Valley, Peru. (**d**) A Peruvian ant species of *Cordyceps*. (**e**) The largest *Cordyceps* species found in Peru so far – up to 400 mm in length

signature of naturally occurring *O. sinensis*. In other words, even though the known medicinal species is *O. sinensis*, from a consideration of the active medicinal compounds present, things are not as simple as they seem. The myriad species complex found in the natural *O. sinensis* each adds their own unique compounds to the mix, and no single one of the species represents all of the known medicinal or chemical properties when they are cultivated alone as single species. With the escalating price of natural *O. sinensis*, as early as the mid-1980s, the majority of *Cordyceps* available in the world's marketplace was artificially cultivated (Mizuno 1999). In 1985, the first officially approved drug derived from *O. sinensis* cultivation was brought to market in China, under the name Jin Shui Bao. Although today it is known through DNA sequencing that Jin Shui Bao is not actually a *Cordyceps* species, certainly not *O. sinensis*, rather it is another of the commensal ascomycetes species found cohabiting *O. sinensis*, a species known as *Paecilomyces hepiali*. This species of fungus is fairly easy to cultivate and is usually passed off in the dietary supplement market under the name *Cordyceps sinensis* CS-4. When this species was first isolated from wild *O. sinensis* in the early 1980s, it was assumed to be the anamorph of *O. sinensis*. Later as DNA sequencing and molecular identification techniques became more widely applied, it was found to be *P. hepiali* rather than the *O. sinensis* anamorph as had been assumed (Halpern 2007). This has led to much confusion, as the original research from the 1980s refer to this as *Cordyceps sinensis*, so much of the medicinal properties thought to be from that species are now known to be from *P. hepiali* instead. Even though this misidentification has now been corrected, many commercial companies still sell this under the name "*Cordyceps* CS-4." Many companies

now produce artificially cultivated *Cordyceps* products, both from the mycelium and also from the fruit bodies of a few *Cordyceps* species, most notably *Cordyceps militaris*, a species that fruits readily in culture. *Cordyceps militaris* is usually passed off today as a direct analog of natural *O. sinensis*, especially in Asia, even though it is a different genus and species with no traditional medicinal usage. There are a few large biotech companies today, such as Aloha Medicinals in the United States and Chung Jing Biotechnology in Taiwan, which have invested huge sums into research in methods for commercially cultivating *Cordyceps*, with the result that today those companies specializing in cultivating *Cordyceps* produce herbal supplements with a virtually identical chemical profile to wild-collected *O. sinensis*. However, the increase in suppliers has also given rise to variations in purity and quality, creating a situation in which there are a large number of counterfeit and adulterated products being sold (Halpern 2007). However, that situation is changing as time progresses, as there have been new methods recently introduced for assaying the quality and purity of *Cordyceps* products, mainly by looking at the unique nucleosides found only in *Cordyceps* (Holliday et al. 2004; Yu et al. 2006; Li et al. 2006). The large variations in quality found in *Cordyceps* products cultivated under different methods and culture conditions by different companies lead many consumers to believe wild-collected *O. sinensis* is medicinally more potent than the cultivated type. But with continuing advances in our understanding of both the chemistry and the mechanisms of action for *Cordyceps*' medicinal activity, along with the great advances we have seen in biotechnology practices since the beginning of the twenty-first century, companies today are able to cultivate *Cordyceps* under modified culture parameters and by using specially selected substrates, which are chosen specifically for the optimized production of the targeted medicinal compounds sought (Cleaver et al. 2008). This is leading to an ever-increasing quality of commercial *Cordyceps* products available on the market. With habitat destruction and overharvesting, wild *O. sinensis* is becoming more and more scarce and faces the real risk of becoming extinct in the near future, but fortunately, the cultivated variety is becoming ever closer to the potency and wide-ranging effects of the original wild type. As research continues to unfold the secrets of *Cordyceps*, it is allowing cultivators new targets to aim for, and new levels of quality and consistency are evolving in the commercial *Cordyceps* market every day.

One issue that has commercial cultivators looking for answers today is the fact that because *O. sinensis* cannot be readily fruited in culture, the commercial cultivation industry is limited to fermentation technology of the anamorphic stage only – the asexual mycelial growth. This mycelium material is commercially produced and marketed as a substitute for the much more expensive wild-collected variety. This situation is fine in most respects, as the mycelial products can have an almost identical analytical signature to the wild-collected *O. sinensis* fruit body and insect host complex. And because commercial cultivation is typically performed at large scale, the products are far more affordable and available to a much larger market sector than wild-collected types. At least this quality issue is true when the products are cultivated by the larger biotech firms. Unfortunately, the high price and wide demand for these products have led some unscrupulous manufacturers to put

products on the market that sometimes do not contain any *Cordyceps* at all. This tends to be more common in Asian products and low-cost products sold in Africa, rather than in European or American products.

Over the last four decades, there have been 22 different species from 13 different genera identified as the anamorph, or asexual stage, of *O. sinensis* in the scientific literature. In 2001, a group from China led by Yue-Qin Chen published a paper purporting to resolve this question, showing their research group had determined through modern DNA sequencing methods *Hirsutella sinensis* be the true anamorph for *O. sinensis* (Chen et al. 2001). According to decisions made at the Nomenclature Section meeting of the International Botanical Congress in Melbourne, Australia, in July 2011, these new naming regulations allowed each fungus to have only one name (Hawksworth 2011). These regulations took effect on January 1, 2013, and since then the anamorph name *Hirsutella sinensis* was dropped by the *International Code of Nomenclature for algae, fungi, and plants* (formerly called the *International Code of Botanical Nomenclature*) (McNeill and Turland 2011). The name Hirsutella *sinensis* is no longer used, and the currently correct naming for both the anamorph and teleomorph stages of *O. sinensis* are now both the same – *O. sinensis.*

It would seem that with the identification of the true anamorph for *O. sinensis*, it would be relatively simple for cultivators to produce products identical to the wild-type fungus. But this has definitely proven not to be the case. As mentioned earlier, there are many fungi cohabiting and living commensally in the insect host that forms one-half of the *O. sinensis* fungal/insect complex. In fact, when examined closely, the fruiting body and larval remains most closely resemble a colony organism, much the way a coral reef is a colony – made up of many very diverse species. Many to most of these separate species are dependent on some other species for their very survival, and they all unquestionably add their own unique secondary metabolites into the complex. With more than 600 individual species of fungi shown to coinhabit the *Thitarodes* larvae and *O. sinensis* fungal complex, we can assume all of these fungi add something to the complex chemistry found in the wild *O. sinensis*. It is interesting that when *Hirsutella sinensis* is cultivated alone as a single species, the resulting chemistry, the analytical profile, does not look very much like *O. sinensis* at all (Fig. 3.3). With such a dissimilar chemical profile, the cultivated *H. sinensis* does not have the same medicinal properties as the wild fungus. The approach that has been taken by some of the more sophisticated biotech cultivators of *O. sinensis* substitutes today is that rather than strictly matching the DNA of the wild type, they match the *O. sinensis* chemical profile as closely as possible, thereby replicating the entire complex analytical signature and the medicinal properties of wild-collected *O. sinensis*. This often requires the blending of several cultivated anamorph species, usually including both *Hirsutella* spp. and *Tolypocladium* spp., and sometimes adding *Paecilomyces* spp. into the mix. This approach, along with carefully chosen substrates and culture conditions (Cleaver et al. 2008), results in very high-quality commercial *Cordyceps* products, which have the same characteristics, chemical profile, and medicinal activity as the wild-collected *O. sinensis*, at a mere fraction of the price.

Several other species of *Cordyceps* are being cultivated today as alternatives to *O. sinensis*, including large quantities of *Cordyceps militaris*. While *C. militaris* has no historical usage in traditional medicine, it shares many of the chemical constituents of *O. sinensis* and is a species that is much easier and cheaper to cultivate, making it a ready substitute for natural *Cordyceps*. Today, *C. militaris* has become a readily available substitute for *O. sinensis* and has many of the same properties and medicinal qualities (Das et al. 2010). *Cordyceps japonica* (Park et al. 2002) and *Cordyceps cicadae* (Hsu et al. 2015) fall into this same category and are currently being marketed as cultivated substitutes for *O. sinensis*.

## 3.6 Traditional Medicinal Usage of *Cordyceps*

The range of therapeutic uses claimed for *Cordyceps* is quite broad, although many of them have yet to be investigated using the modern gold standard of double-blind, placebo-controlled clinical trials. *O. sinensis* has been used in TCM to treat quite a wide range of medical conditions, including respiration and pulmonary diseases, cardiovascular diseases, renal and liver disease, hypo-sexuality, hyperlipidemia, and many age-related disorders. It is also widely used for boosting weak immune systems and other immune disorders and as an adjunct to the modern cancer treatments of chemotherapy, radiation, and surgery (Bensky et al. 2004). *O. sinensis* is believed by many, particularly those people living in its place of origin, Tibet, to be a remedy for altitude sickness and all manners of general weakness and fatigue. It is often used as a general tonic for increased energy or taken while recovering from any serious illness to give strength, increased vigor, and help speed recovery. *O. sinensis* also has a strong reputation as a treatment for impotence and is often referred to in Asia as "Himalayan Viagra." The treatment of impotence is perhaps the single largest market for wild-collected *O. sinensis* today, with an annual market estimated over a billion US dollars in China alone. There is some basis for this belief, as early clinical trials showed *O. sinensis* to increase sexual function in both men and women (Zhu et al. 1998). However, these trials were poorly designed with only a small patient base and would not stand up to today's more rigorous clinical trial standards. *O. sinensis* is often prescribed in TCM for the elderly to ease general aches and pains. TCM practitioners also recommend the regular use of *O. sinensis* to strengthen the body's immune system and resistance to illness, such as colds and flu, and to generally improve the well-being and health of the patient. *O. sinensis* has historically been used as almost a catchall medication, seemingly prescribed for so many different conditions that it seems like the medical "magic bullet," good for whatever ails the patient. Modern science has proven some of these uses to be valid and is still looking for evidence for some other traditional uses.

## 3.7 The Bioactive Chemistry of *Cordyceps*

*O. sinensis* and other *Cordyceps*, although they are not normally considered as food, contain a broad range of nutrient compounds. *Cordyceps* contains all 18 of the essential amino acids. The content of amino acids tends to be quite high, generally in the range of 5–10%. The highest contents are glutamate, arginine, aspartic acid, tryptophan, and tyrosine (Zhou et al. 2009). Also found are vitamins E and K and the water-soluble B vitamins, including B1, B2, and B12. In addition, *Cordyceps* contains many mono-, di-, and oligosaccharide sugars and contains very unique complex polysaccharides of both the alpha-bound and beta-bound conformation, even some polysaccharides with both beta- and alpha-bonds found in the same molecule, containing both five and six carbon sugars, which make them true heteropolysaccharides, proteins, sterols, many different nucleosides, including some unique nucleosides found nowhere else in nature, and a wide range of minerals, perhaps reflecting the rich mountain soil in which it grows, including K, Na, Ca, Mg, Fe, Cu, Mn, Zn, Pi, Se, Al, Si, Ni, Sr, Ti, Cr, Ga, V, and Zr (Holliday and Cleaver 2008; Mizuno 1999).

### 3.7.1 Polysaccharides

*O. sinensis* contains a large concentration and variety of polysaccharides, which can be in the range of 10–20% of the total weight of wild-collected specimens and typically up to 50% or even higher in *Cordyceps* specially cultivated by solid-state fermentation methods to enhance the production of these unique polysaccharides. The structural cell wall polysaccharides and the exopolysaccharides excreted into the culture medium or into the environment in wild specimens are considered to be the main medicinally active classes of compounds from *Cordyceps*. These polysaccharides are present in the fruiting bodies, the mummified larva, and the mycelium of both solid fermentation and submerged cultures, and the exopolysaccharides are found in the culture broth of liquid cultivated and excreted into the substrate in solid-state fermentation (Zhou et al. 2009). Four different beta-D glucan exopolysaccharide structures exopolysaccharides from *C. militaris* with different molecular masses ranging from 50 to 2260 kDa were found in *C. militaris* cultivated by liquid fermentation (Kim et al. 2003). In the case of *O. sinensis*, most of the heteropolysaccharides contained mannose, galactose, and glucose, with mannose at higher levels and with smaller amounts of the five carbon sugars arabinose, rhamnose, and xylose, respectively. The average molecular mass of *O. sinensis* polysaccharides varies between 7 and 200 kDa, while the *C. militaris* polysaccharides consisted mostly of glucose, galactose, and mannose with only traces of rhamnose and xylose and have an average molecular weight of approximately 60 kDa (Zhong et al. 2009). The polysaccharides of *Cordyceps* are very difficult to analyze and elucidate due to their complex tertiary structure, which is primarily a triple right-hand helix conformation with differing lengths and ratios of pentose and hexose side chains and only recently have analytical methods been developed to accurately purify and assay the various

polysaccharide fractions (Danielson et al. 2010; Megazyme 2008). The attempt to fully elucidate the polysaccharides from *Cordyceps* is an ongoing research area being pursued by many scientists around the world. Much has yet to be learned regarding the tertiary structures of these polysaccharides, their binding to particular receptor sites on the cells, and the subsequent biological actions triggered in the body.

### 3.7.2 Proteins and Other Nitrogenous Compounds

*Cordyceps* contain proteins, peptides, polypeptides, polyamines, all the essential amino acids, and a number of common and uncommon cyclic dipeptides, including cyclo-[Gly-Pro], cyclo-[Leu-Pro], cyclo-[Val- Pro], cyclo-[Ala-Leu], cyclo-[Ala-Val], and cyclo-[Thr- Leu]. *Cordyceps* also contains small amounts of polyamines, such as 1,3-diamino propane, cadaverine, spermidine, spermine, and putrescine (Mizuno 1999).

Many free nucleosides have been found in *Cordyceps*, including uridine, several unique deoxyuridines, adenosine, dideoxyadenosine, hydroxyethyl adenosine, cordycepin [3′deoxyadenosine], cordycepin triphosphate, guanidine, deoxyguanidine, and other altered and deoxygenated nucleosides, many of which are found nowhere else in nature (Fig. 3.6) (Yang et al. 2007).

Many of the altered nucleosides, such as cordycepin, have been shown to have potent antiviral effects, including effects against the HIV virus (Adotey et al. 2011). Cordycepin is found in several of the *Cordyceps* species, in lesser quantities in *O. sinensis*, and larger quantities in *C. militaris*. Cordycepin has a unique way of halting HIV (and other viral) replication: cordycepin is an inhibitor of ribonucleoside triphosphate (rNTP). Macrophages contain high levels of rNTP, which is used in cells in a variety of ways. HIV uses primarily rNTP to replicate inside macrophages but not in CD4+ cells. By inhibiting the rNTP, cordycepin effectively inhibits the replication of the virus, at least in the macrophages (Kennedy et al. 2010). This is just one of the several mechanisms by which cordycepin is thought to exert its antiviral effects. Sugar-binding proteins named lectins were isolated from *C. militaris*. N-terminal amino acid sequence differed greatly from other lectins (Yue et al. 2013). Production of the nonribosomal peptides cicapeptins I and II from *C. heteropoda* was reported. These are compounds unique in their structures that have been found only in one *Cordyceps* species so far, which display antibacterial and antifungal activity (Krasnoff et al. 2005).

The total amount of amino acids in cultivated *Cordyceps* products can be up to about 10% by weight. In *O. sinensis*, the three principal amino acids in both the mycelium-mummified larvae and the fungal fruiting body are glutamic acid, aspartic acid, and arginine, and their contents are in the range of 1.5% up to 2.6% (Hsu et al. 2002). *Cordyceps* is often used to provide mild sedation, and in one trial, a mixture of 18 synthetic amino acids combined to mimic the amino acid composition in natural *O. sinensis* showed the same sedative action as natural *O. sinensis*, confirming this medicinal activity (Zhang et al. 1990).

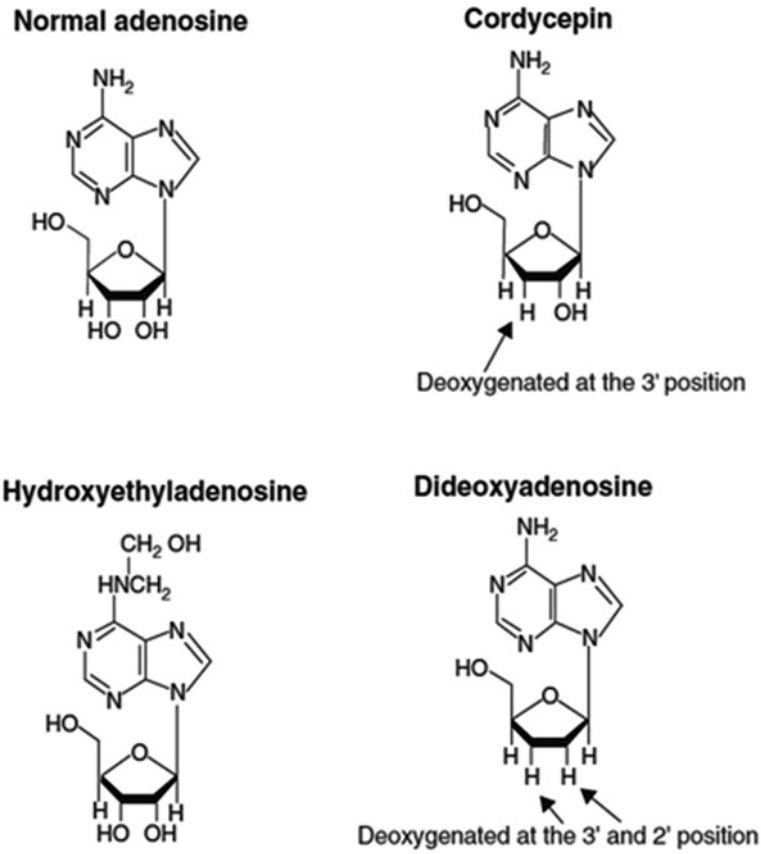


**Fig. 3.6** Some of the unique nucleosides found in *Cordyceps*

A number of unique peptides and cyclodipeptides have been found in *Cordyceps*. One is the cyclodipeptide named cordycedipeptide A, which is a compound isolated from the culture liquid of liquid fermentation-cultivated *O. sinensis*. This compound showed good antitumor effect, showing cytotoxicity when tried against several cancer cell lines, including L-929, A375, and HeLa cells (Jia et al. 2005; Mang et al. 2015).

Another interesting peptide that was isolated from *O. sinensis* was named cordymin. Cordymin was shown in animal trials to have a protective effect in the ischemic rat brain by inhibiting inflammation. If this animal data translates to humans, this compound could prove to become a valuable medication for the prevention or limiting of neurological damage caused by cerebral ischemia-reperfusion injury in stroke patients (Wang et al. 2012b).

A unique and atypical amino acid known as myriocin has been isolated from *O. sinensis* and has been found in several other fungi as well. Myriocin is a potent immunosuppressant agent, up to 100 times more potent than the standard against

which other immunosuppressants are measured, cyclosporine. Myriocin, which also has antibiotic properties and is known as antibiotic ISP-1, is a new type of immune inhibitor. It also very potently inhibits the synthesis of sphingosine, which forms a primary part of sphingolipids. These are a type of cell membrane lipid that includes sphingomyelin, the material covering nerve fibers in humans. Myriocin is used today primarily as a research tool to deplete cells of sphingolipids, although it shows potential for other uses in medicine as well (Zhao et al. 2014; Paterson. 2008).

*O. sinensis* has long been used as an anti-inflammatory medication. The discovery of a group of uniquely altered adenosine-type compounds named cordysinins in *Cordyceps* has proven this usage to be scientifically valid. Five cordysinins, named cordysinins A–E, have been isolated from the mycelia of *O. sinensis.* These compounds have been shown to have potent anti-inflammatory activities (Lo et al. 2013; Shrestha et al. 2014).

### 3.7.3 Sterols

A number of sterol compounds have been found in *Cordyceps*, including the sterol common to all fungi, ergosterol. Ergosterol is important to human health in that it is the precursor of vitamin $D_2$. When ergosterol is exposed to ultraviolet light (as in sunlight), a photochemical reaction is triggered that converts ergosterol into vitamin $D_2$, the biologically active form of vitamin D in the human body. Other sterols have been found in *Cordyceps* as well, including δ-3 ergosterol, ergosterol peroxide, 3-sitosterol, daucosterol, and campesterol (Zhu et al. 1998). Another sterol compound named H1-A was found in *O. sinensis* that has shown to be effective in the treatment of autoimmune disorders, a great challenge to medicine using the current drugs available today, and an area worthy of further research (Yang et al. 1999).

### 3.7.4 Other Constituents

Twenty-eight saturated and unsaturated fatty acids with the function of decreasing blood lipids have been isolated from *O. sinensis*. The unsaturated fatty acid content includes Cl6:1, Cl7:1, Cl8:l, and Cl8:2 (Zhou et al. 2009). Polar compounds of *C. sinensis* extracts include many alcohols and aldehydes (Bensky et al. 2004). Particularly interesting is the range of polycyclic aromatic hydrocarbons produced by some *O. sinensis* strains, called PAH compounds, which were found to react with the polypropylene used in common mushroom culture bags, resulting in the production of by-products toxic *to O. sinensis* and stunting growth as time progresses, limiting the cultivation potential of strains producing these PAHs to glass or metal culture containers (Holliday et al. 2004). Various immune-suppressive compounds have been found in *Cordyceps*, including cyclosporine from *Cordyceps subsessilis*, which is most commonly produced through the cultivation of the *Cordyceps subsessilus* anamorph (*Tolypocladium inflatum*) (Segelken 2002). Other immune-suppressive

compounds have been found in *Isaria sinclairii*, another entomopathic species that is closely related to *Cordyceps* (Halpern 2007).

#### 3.7.4.1 Mannitol

*O. sinensis* contains a high percentage of D-mannitol, up to 10% in wild specimens of *O. sinensis* and up to 14% in cultivated varieties. D-mannitol is the so-called cordycepic acid in Chinese. The name cordycepic acid was thought to be first used by the mycologist G.H. Cunningham around 1951, referring to a slightly acidic compound in *Cordyceps militaris* that he assumed was unique to *Cordyceps*. It was first isolated and purified from *O. sinensis* in 1957 (Lin and Li 2011). Further research into the chemistry of this compound determined it was not unique to *Cordyceps* but was nothing other than the common sugar alcohol D-mannitol. As such, the name cordycepic acid has been considered obsolete since the late 1950s. This compound is not unique to *Cordyceps*, and in fact, D-mannitol is found in much higher concentrations in cranberries than it is in any of the *Cordyceps* species. However, this term is still widely used in the commercial market as a "verification of authenticity" and a "measure of potency," with manufacturers claiming their products to contain this or that percentage of cordycepic acid. This is an unsubstantiated marketing claim used by unscrupulous marketers to confuse the nonscientific public into thinking their products must be better than another company's products because of the "high levels of cordycepic acid" they contain. The term cordycepic acid cannot be found in any modern chemical formularies though, and the correct term D-mannitol should be used in its place. D-mannitol is found in *O. sinensis*, *C. militaris*, and many others of the *Cordyceps* s.lat. group.

## 3.8 Current Medicinal Uses for *Cordyceps* s.lat.

While the long history of and widespread use of *Cordyceps* in TCM has been discussed in the section on Traditional Medicinal Uses above, it is still a favored medicine in TCM today, seemingly gaining popularity year by year as TCM becomes more widely accepted in other areas around the world, far from its birthplace of China. Also, the increased availability through commercial cultivation has allowed its widespread use, such as was never possible in previous times. One of the most significant activities ascribed to almost all the medicinal mushrooms in recent years is their role as immunomodulators, also known as biological response modifiers (BRM) (Zhu et al. 2015; Chen and Seviour 2007; El Enshasy and Hatti-Kaul 2013). Immunomodulation is not the same as immune potentiation or immune suppression, rather it is usually defined as a concurrent bidirectional immune response, with the upregulating of some classes of the immune cell while simultaneously downregulating other cell classes. For example, in clinical trials, it was found in some autoimmune diseases; we find *Cordyceps* downregulates pro-inflammatory cells while upregulating anti-inflammatory cells (Halpern and Miller 2002; Zhu et al. 1998; Lu 2003). This immunomodulation effect is thought to be primarily the result of the polysaccharide component, which are primarily the cell wall structural components,

found in both wild and cultivated varieties of *Cordyceps*. Many other medicinal activities are ascribed to *Cordyceps*, such as anticancer, antitumor, antimetastatic, immunomodulatory, antioxidant, anti-inflammatory, insecticidal, antimicrobial, hypolipidemic, treatments for hypolibido in both males and females, hypoglycemic, antiaging, and neuroprotective and renal protective activities (Holliday and Cleaver 2008).

### 3.8.1 Cancer

One of the major applications of *O. sinensis* and other of the *Cordyceps* s.lat. today are their use in cancer treatments as adjuncts to chemotherapy and radiation therapy and to speed recovery from surgery. Quite a bit of research has been done in the area, including both animal and human trials. In one trial with lung cancer patients, a commercial formulation consisting of several species of medicinal mushrooms named *Immune Assist*™ was used, of which *O. sinensis* is a major constituent. This formula and compared with an anti-side effect medication called *Polyactin A*™. In every side effect parameter measured, such as vomiting, hair loss, appetite, sleep time, etc., the medicinal mushroom formula outperformed the drug (Ruwei et al. 2003). In another 1 yearlong trial with this same *Immune Assist*™ mushroom formula conducted on dogs undergoing chemotherapy for four types of cancer, the mushroom immunomodulator formula showed very good results (Gianotti et al. 2009). The mechanism by which *Cordyceps* inhibits the growth of various cancer cells is thought to occur by several mechanisms of action: by upregulating the body's own immune function; by selectively inhibiting RNA synthesis, thereby affecting protein synthesis; perhaps by restricting the growth of new blood vessels (antiangiogenesis); by inducing tumor cell apoptosis (Tuli et al. 2015; Chen et al. 2014; Li et al. 2015); and by the direct cytotoxic effect of cordycepin and other altered nucleosides being taken up by the tumor cells (Zhou et al. 2009).

Inhibiting growth of cancer cells through enhancing of immune function is usually associated with the fungal polysaccharides, especially beta-D-glucans and the unique heteropolysaccharides in *Cordyceps*. These are specific structures of polysaccharides, which are not found in plants or animals but are found in the cell walls of fungi and some bacteria. Consequently, when orally administered, they are recognized by the intestinal immune system – the gut-associated lymphoid tissue (GALT) – to be pathogen-associated molecular patterns, called PAMPs (Janeway 1992). PAMPs trigger strong immune responses in the gut, similar to what would occur if it was facing a massive fungal infection. This leads to a rapid increase in the number and activity of immune cells, which pass through the intestinal wall and into the bloodstream, where they circulate on a heightened state of alert and are thus available to fight other immune challenges encountered, such as the identification and destruction of aberrant cells. This heightening of nonspecific immune awareness is thought to be one of the main mechanisms by which the fungal polysaccharides work in identifying and downregulating cancer growth.

Studies have shown that there are other ways besides gut immune activation in which beta-D-glucans initiate biological response, such as by directly binding to receptors located on the surface of the immune system effector cells, such as the T cells, B cells, eosinophils, neutrophils, and monocytes, thereby setting up different intercellular activities of the immune system and leading to release of various cytokines, such as tumor necrosis factor (TNF) interleukins and interferons (Battle et al. 1998). Complement receptor type 3 (CR3) receptors, Toll-like receptors (especially TLR-2), and Dectin-1 receptors play an important role in binding and activating responses to fungal beta-D-glucans (Chen and Seviour 2007). It is most likely that other uniquely fungal polysaccharide structures bind to other cell receptor sites, which mechanisms are currently under investigation but have not been fully elucidated yet. The anticancer role of cordycepin and other *Cordyceps*-derived compounds has been extensively investigated in a variety of cancers, including glioma and cancers of oral, breast, lung, hepatocellular, bladder, colorectal, testicular, prostate, melanoma, and blood cell (Tuli et al. 2015; Chen et al. 2008, 2014; Li et al. 2015; Wu et al. 2007; Lee et al. 2009, 2010, 2011, 2012, 2013a, b, 2014a, b; Choi et al. 2011; Kim et al. 2011; Noh et al. 2010; Nakamura et al. 2006; Shi et al. 2008; Lu et al. 2014; Jen et al. 2010; Pan et al. 2011; Jeong et al. 2011, 2012; Yoshikawa et al. 2008, 2009; Thomadaki et al. 2008; Ko et al. 2013).

A search on Google Scholar (https://scholar.google.com) conducted on March 30, 2017, using the keywords *Cordyceps* + Cancer returned almost 11,000 scholarly articles on this topic. This number of published articles indicates the scope and breadth of the research being performed into cancer treatment using *Cordyceps* over the last 30 years. It seems that cancer treatment is one of the traditional uses for *Cordyceps*, which use has been well established as valid today.

### 3.8.2 Antiviral

In a recent murine study on infection and mortality of the H1N1 influenza virus, it was shown that an extract of *C. militaris* had a strong anti-influenza effect (Lee et al. 2014c).

The mechanism of action for the antiviral effect cited in this study was the increase in IL-12 production and an increase in NK cell population. Another 2007 influenza study using an acidic polysaccharide extract from *C. militaris* also showed potent anti-influenza activity and decreased mortality (Lee et al. 2014c). In another recent study using a protein extracted from *Cordyceps sobolifera*, potent HIV reverse transcriptase inhibition was noted, opening the possibility for the development of *Cordyceps*-based anti-HIV medications (Wang et al. 2012b). Two studies using *Cordyceps* and other mushroom polysaccharides in conjunction with the drug lamivudine on chronic hepatitis B patients had shown that when the mushrooms were added to the standard treatment of lamivudine, it increased treatment efficacy and decreased the time to patient's conversion to an HBV seronegative (Li 2004; Ruwei et al. 2004). Many other studies on *Cordyceps*' antiviral activity have shown *Cordyceps* to have potential in treating many viral infections and, as the raw

material source for new antiviral drug development, validating the long-observed antiviral effects by TCM practitioners.

### 3.8.3 Fatigue

Of all the traditional uses that *Cordyceps* is noted for, perhaps the one that is best known is the relief of fatigue. There is an ancient legend told in the Himalayas, relating the way *Cordyceps* was originally found; it is a legend from a long time ago when the tribes' people of Tibet and Nepal took their animals into the high mountain pastures for springtime grazing. There they would see goats and yaks grazing on some sort of a small, brown grasslike mushroom, growing from the head of a caterpillar. After eating this strange-looking creature, the animals would become frisky and start chasing the other goats and yaks around with lustful intent. This added vigor must have looked pretty attractive to those tribes' people, so they started collecting these small mushrooms and eating them as well. They also became frisky, even a bit lustful, or so the story goes (from author's discussions with local nomad people in Tibet).

Today, most of the wild *O. sinensis* collected is considered too valuable to be consumed by the rural people in its natural habitat, so all of it tends to get sold off to buyers for transport to Beijing and on to the rest of the world. It seems that modern conveniences such as motorcycles and TVs have become more valued in those collecting regions than the benefits from consuming the fungus itself. But in previous times, the people living in these harsh climate high mountains would consume *Cordyceps* on a regular basis. It gave them energy and offset the symptoms of altitude sickness. With *Cordyceps* at their disposal, they were able to trek higher into the mountains and stay there for longer periods of time. We now think that one of the reasons for this energy-boosting effect is this increase of cellular ATP (Siu et al. 2004). In addition to increasing cellular ATP, recent studies have shown that oxygen availability is facilitated from taking *Cordyceps* (Hirsch et al. 2017), which would also help to explain how *Cordyceps* assists in these high-altitude jaunts. *Cordyceps* is in regular use today by most high-altitude mountain climbers, and it is doubtful if Mount Everest would get nearly so many visitors if it were not for the remarkable fatigue-reducing effectiveness of *Cordyceps*. *Cordyceps* supplements are also in widespread use by professional athletes in the United States (personal communications between author and several professional NFL football players) and other amateur and professional sports as shown by the popularity of the many *Cordyceps*-based performance-enhancing supplements on the market today, such as the supplements Performax™ by Aloha Medicinals and Optygen™ by First Endurance Company, as well as hundreds of other *Cordyceps*-containing sports products as found through any search on the internet for *Cordyceps* performance enhancement products. One of the simplest and most reliable tests used to determine whether a compound can actually increase energy output or decrease fatigue is the mouse swim test. In this test, two groups of mice are used. One group gets the normal diet and the other group a normal diet with the addition of the test substance, *Cordyceps* in this case.

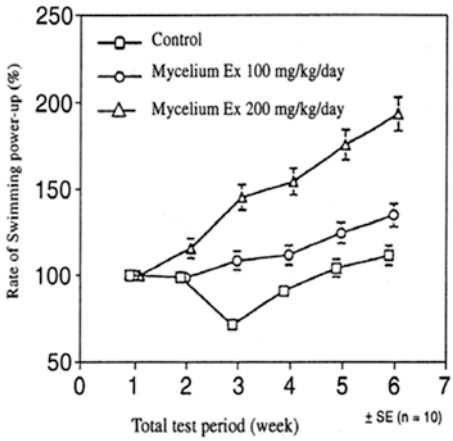


**Fig. 3.7** Mouse swim test with *O. sinensis* extract

After a period of time taking the test substance, the two groups of animals are put into a steep-sided container full of water from which they cannot escape. In that way, they are forced to swim. The time to exhaustion is measured for the two groups and compared. If the group receiving the test compound swims longer than the group on the normal diet, then it has been determined that they had increased energy output and/or decreased fatigue than the other group. Many trials of this nature have been conducted using *Cordyceps*, and invariably they show that the use of *Cordyceps* significantly increases the time to exhaustion in the test animals. An example of this test is detailed in the 1999 review article by T. Mizuno, titled "Medicinal Effects and Utilization of *Cordyceps* (Fr.) Link (Ascomycetes) and *Isaria* Fr. (Mitosporic Fungi) Chinese Caterpillar Fungi" (Mizuno 1999) (Fig. 3.7).

### 3.8.4 Kidney Protection and Repair

Traditional beliefs are that *Cordyceps* mushrooms are useful for strengthening the kidneys. Given its vast array of uses, it is interesting to note that what is being discovered today is that kidney health, perhaps more than that of any other organ, is a virtual cornerstone of the body's health. When the kidneys fail, the effects are normally felt via other organs and systems consequently affected. In this way, taking into consideration only its effect on the kidneys, *Cordyceps* truly was a promoter of overall health and homeostasis. Many other traditional uses for this mushroom can be traced back to proper kidney health. Fatigue, impotence, joint and back pain, and even ringing in the ears are all symptoms of degenerative kidney health. It has been shown that much of *Cordyceps* kidney-enhancing potential comes from its ability to increase 17-hydroxy-corticosteroid and 17-ketosteroid levels (Zhu et al. 1998).

Chronic renal failure is a serious and all too common disease, often affecting the elderly. In a study with 51 patients suffering chronic renal failure, it was found that the administration of 3–5 g per day of *O. sinensis* significantly improved both the kidney function and the overall immune function of the patients receiving the

*Cordyceps*, as compared to the control group who did not receive the *Cordyceps* (Guan et al. 1992). Patients with chronic renal failure or reduced kidney function often suffer from hypertension, proteinuria, and anemia. In a study with such patients, it was found that after 1 month on *Cordyceps*, a 15% reduction in blood pressure was observed. Urinary protein was also significantly reduced validating the long-held TCM belief that *Cordyceps* is valuable for treating diseases and weakness of the kidneys.

In another recent study, the mechanism of action for *Cordyceps*' kidney-protection mechanisms was studied, gaining insight into how and why *Cordyceps* works in preventing and reversing kidney damage (Zhong et al. 2012).

Several studies have shown *Cordyceps* to be especially helpful when used in conjunction with cyclosporine for renal transplant patients, reducing the required dose of cyclosporine and at the same time reducing the cyclosporine-induced kidney damage, toxicity, and side effects (Ding et al. 2010; Wojcikowski et al. 2006).

Listed here are just a few of the many trials that have been conducted on kidney function with *Cordyceps*, both animal and human, showing *Cordyceps* to be of particular value in maintaining kidney health and in restoring function to diseased and damaged kidneys. A search on http://scholar.google.com conducted on April 17, 2017, using "*Cordyceps* AND Kidney" as the keywords, revealed over 5200 scholarly articles, showing the extent to which this use has been researched. From evaluating the results of the many kidney studies, *Cordyceps* appears to be a low-cost, low-toxicity medicine that is well tolerated and has real value for clinical application in this field.

### 3.8.5 Lungs

TCM has characterized *Cordyceps* as a guardian of respiratory health for many hundreds of years. Much of its reputation for protecting the lungs is believed to come from its ability to promote enhanced oxygen utilization efficacy. In environments lacking sufficient oxygen levels, mice treated with *Cordyceps* were able to survive up to three times longer than those left untreated, demonstrating a more efficient utilization of the available oxygen. This is objective confirmation of *Cordyceps* long history of use in preventing and treating altitude sickness (Zhu et al. 1998). Such efficacy alludes to the use of *Cordyceps* as an effective treatment for bronchitis, asthma, and chronic obstructive pulmonary disease (COPD). Extracts of *O. sinensis* have been shown to inhibit tracheal contractions, especially important for asthma patients in that it allows for increased airflow to the lungs (Hsu et al. 2008). In addition, its anti-inflammatory properties bring further relief to asthma patients, whose airways become obstructed due to an allergic reaction resulting in swelling of the bronchial pathways (Rao et al. 2007). In a clinical trial involving 50 asthma patients, efficacy against symptoms among the group treated with *Cordyceps* was 81.3%, within an average of 5 days, while among those treated with conventional antihistamines, the rate was only 61.1% and took an average of 9 days for symptoms to subside (Halpern 1999).

There have been many well-designed trials with *Cordyceps* for improving lung function in humans, using *Cordyceps* to treat all manner of respiratory illnesses including asthma, COPD, and bronchitis, either alone or as an adjunct to other standard therapies. It has proven useful for all of these conditions (Zhu et al. 1998). Other recent studies have elucidated the mechanisms of action whereby *Cordyceps* is able to offset the effects of hypoxia – low oxygen levels (Singh et al. 2013). While the use of Cordyceps for enhanced performance in low-oxygen environments has long been observed, for example its use in high-altitude mountain climbing, this is the first time the actual mechanisms responsible for this activity have been explained. It has been observed for centuries by thousands of TCM practitioners that *Cordyceps* improves respiratory function, and it is now a well-proven and well-accepted scientific fact.

### 3.8.6 Heart

One of the more profound actions of *Cordyceps*, both traditionally and in modern practice, is its ability to stabilize the heart beat and correct heart arrhythmias. *Cordyceps* is one of the first-line medications of choice for this serious condition in China today. While the exact reasons and mechanism of action for *Cordyceps* excellent reputation in controlling arrhythmias are only partly understood, it was proposed in earlier studies that the anti-arrhythmia action is due to the presence of adenosine (Peleg and Porter 1990). A study conducted in 2013 has shown this to be correct (Yan et al. 2013). *Cordyceps* often contains a significant quantity of adenosine, deoxyadenosine, and related adenosine-type nucleotides and nucleosides. It has been shown that these compounds have a widespread effect on coronary and cerebral circulation (Toda et al. 1982; Berne 1980). While no single drug or herb is equally effective in all patients, it is rare that a patient's arrhythmia does not benefit from the addition of *Cordyceps* to the treatment regimen. *Cordyceps* is not known to adversely react with any other arrhythmia medication, and with its low toxicity, it seems to be an excellent choice for this condition.

In studies of patients suffering from chronic heart failure, the long-term administration of *Cordyceps* in combination with conventional treatments, digoxin, hydrochlorothiazide, dopamine, and dobutamine, promoted an increase in the overall quality of life. This included general physical condition, mental health, sexual drive, and cardiac function, compared to the control group (Chen 1995).

### 3.8.7 Uses Against Male and Female Sexual Dysfunction

*Cordyceps* has been used for centuries in traditional Chinese medicine to treat male and female sexual dysfunction, such as hypolibidinism and impotence. Preclinical data on the effects of *O. sinensis* on mice showed sex steroid-like effects. Human clinical trials have demonstrated similarly in the effectiveness of *Cordyceps* in combating decreased sex drive. The results of one such study showed an increase in 24-h

urine 17-ketosteroid, compared to the control group (Zhu et al. 1998). "These results indicated that CS-4 might affect patients' sexual drive and functions, either via sex hormone systems or by directly acting on the sexual organs, in parallel with the effects on the hypothalamic-pituitary-adrenocortical axis" (quote from reference). The presence of amino acids, vitamins, zinc, and other trace elements found in *Cordyceps* is hypothesized to account for increased sperm survival rates, as demonstrated in clinical and preclinical studies (Guo 1986). In three separate studies done in China on a total of 756 patients who were reporting decreased sex drive (hypolibidinism), the patients were given either a placebo or *O. sinensis* at 3 g per day for 40 days. By the completion of the 40-day study, 64.8% of the patients in the *Cordyceps* groups reported improvement in their sex drive, while only 23.8% showed improvement in the placebo group (Zhu et al. 1998). In these three related studies alone, 492 patients with a noted lack of sex drive found relief from this condition by using *Cordyceps.* In another study on both elderly men and women with complaints of decreased libido, impotence, and other sexual malfunctions, *Cordyceps* was given at 3 g per day for 40 days, and several measurements were taken to determine the degree of improvement. Increased sperm survival time, increased sperm count, and decreased number of malformed sperm were noted in the majority of male subjects, as well as more than double the number of patients reporting reversal of their impotence. Improvements in hypoleukorrhagia, menoxenia, and sex drive were noted in the majority of women subjects (Zhu et al. 1998). *Cordyceps* is clearly indicated as a therapeutic agent in treating hypolibidenism and other sexual malfunction in both men and women.

Currently, as of the spring of 2017, the main usage for *O. sinensis* in Asia is for decreasing impotence and improving erectile dysfunction (ED). *O. sinensis* is widely regarded throughout Asia as the "Herbal Viagra" or "Himalayan Viagra." A search of the research literature does not show any reliable studies proving the ED effect claimed for *Cordyceps*, but nonetheless, it is widely believed to be effective for this condition, and ED is thought to be one of the main driving forces of the *O. sinensis* trade today (Winkler 2010).

## 3.9 Safety and Toxicity

*Cordyceps*, both wild-collected and cultivated products, have proven to be a very nontoxic herbal substance for something with the obviously wide-ranging physical effects on the body. While no human toxicity studies have been reported, animal models have found an $LD_{50}$ (median lethal dose) of 27 g/kg when injected IP in mice. Given by mouth to rabbits for months at 80 g/day, no abnormalities were seen from blood tests or in kidney or liver function (Zhu et al. 1998). *Cordyceps* s.lat. is thought to be a very safe substance with a minimal potential of toxicity.

## 3.10 Conclusions

Considering *O. sinensis*' long history of use, it seems quite likely there is some truth behind the observed medicinal efficacy. In our modern age, we strive to test and understand why and how these natural medicines work. With *O. sinensis* and other species of the *Cordyceps* s.lat. group, this challenge has been greater than with many other herbals, primarily due to the enormous cost and scarcity of the wild-collected material. But today, we are in an age of rapidly expanding biotechnological progress. This has provided us with ways to lab produce *Cordyceps* spp. in large enough volume, and at a low enough cost, that research becomes practical for anyone interested in looking into the characteristics and medicinal efficacy of these unique organisms. As we investigate deeper, we shall learn more and more of *Cordyceps* secrets, and we might find that this once rare medicinal herb may hold the key to controlling some of our more difficult medical challenges. More research is needed into this and other species of medicinal mushrooms.

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# Medicinal Mushrooms with Antiallergic Activities

4

Simon Merdivan and Ulrike Lindequist

**Abstract**

Allergies are an increasing problem worldwide, and new strategies for prophylaxis and therapy are urgently needed. Medicinal mushrooms present such an opportunity. Antiallergic activities have been found for *Agaricus subrufescens*, *Armillaria ostoyae*, *Flammulina velutipes*, *Ganoderma lucidum* and *G. tsugae*, *Inonotus obliquus*, *Phellinus linteus*, *Pleurotus ostreatus* and *P. pulmonarius*, *Tricholoma populinum*, and some further mushroom species. Nevertheless, most effects have been detected only in vitro and/or in animal assays, and responsible bioactive compounds have not yet been identified. Besides, only a limited number of mushroom species has been investigated for antiallergic activities until now. The chapter gives an overview about mushrooms with antiallergic activities and describes the challenges for the exploration of the antiallergic potential of mushrooms.

**Keywords**

*Agaricus subrufescens* • Antiallergy • *Armillaria ostoyae* • *Flammulina velutipes* • *Ganoderma lucidum* and *G. tsugae* • Mushrooms • *Inonotus obliquus* • *Phellinus linteus* • *Pleurotus* sp.

## Contents



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## Abbreviations

| | |
|---|---|
| ACE | Angiotensin-converting enzyme |
| cAMP | Cyclic adenosine monophosphate |
| CD | Cluster of differentiation |
| ConA | Concanavalin A |
| DNP | Dinitrophenyl |
| $ED_{50}$ | Median effective dose |
| ERK | Extracellular-signal regulated kinase |
| FIP-*fve* | Fungal immunomodulatory protein *fve* |
| i.p. | Intraperitoneal |
| $IC_{50}$ | Half maximal inhibitory concentration |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| JNK | C-Jun N-terminal kinase |
| MAPK | Mitogen-activated protein kinase |
| OVA | Ovalbumin |
| p.e. | Parenteral |
| p.o. | Per os (oral administration) |
| p38 | Protein 38 |
| PMA | Phorbol-12-myristate-13-acetate |
| RPMC | Rat peritoneal mast cells |
| RRTI | Recurrent respiratory tract infections SLIGRL-$NH_2$ Agonist peptide derived from the *N*-terminus of protease-activated receptor 2 |
| Syk | Spleen tyrosine kinase |
| Th1/2 cells | T helper cell subpopulations |
| TNFα | Tumor necrosis factor α |

## 4.1 Introduction

### 4.1.1 Importance of Medicinal Mushrooms

The medicinal use of so-called "medicinal" mushrooms has a very long tradition, especially in East Asian countries. Since some decades, their use is strongly increasing also in the Western hemisphere, and numerous scientific studies confirm the pharmacological potential of mushrooms. Some species, e.g., *Lentinula edodes* (Berk.) Pegler, *Ganoderma lucidum* (W.Curt.:Fr.) P. Karst, and *Trametes versicolor* (L.:Fr.) Pilát are well-known for their immunostimulating properties and applied, e.g. in the complementary tumor therapy. These activities are mostly attributed to ß-glucans, heteropolysaccharides, or complexes of polysaccharides and proteins (Lindequist et al. 2005; Wasser 2014; Guthmann 2017). In opposite, immunosuppressive and antiallergic activities are less well documented. Nevertheless, the example of fingolimod underlines that mushrooms are able to produce compounds with immunosuppressive activities. Fingolimod is a chemical modification of myriocin from the ascomycete *Isaria sinclairii* (Berk.) Lloyd and was the first registered drug for p.o. treatment of multiple sclerosis, an autoimmune disease.

### 4.1.2 Allergic Reactions

Allergic reactions are hypersensitive and harmful reactions of the human immune system to normally harmless antigens, also called allergens. The situation, which arises as the result of such a reaction, is called allergy (Ferenčík et al. 2006). Allergies can be subdivided into type I–VI. Type I allergy, the most frequent type of a pathogenic immune reaction, is caused by IgE-mediated reactions, which result in the release of vasoactive substances, e.g. histamine from mast cells. The necessary stimulus is the bridging of two allergen-specific IgE molecules on the surface of mast cells or basophils. Allergic rhinitis, allergic bronchial asthma, urticaria, angioedema, and anaphylaxis are all type I allergic reactions. Type II allergic reactions are caused by cytotoxic antibodies against surface molecules of cells. The production of such antibodies can be a consequence of the reaction of a hapten, e.g. a drug to structures on the surface of blood cells, resulting in agranulocytosis or thrombocytopenia. Type III allergic reactions are mainly induced by immune complexes, which activate the complement system or neutrophils and platelets. Type IV allergies are produced by lymphocytes, which react against certain structures. This mechanism is a cause of atopic eczema and drug-induced exanthema. Type V reactions are the reason of granuloma development, e.g. after the injection of xenografts. Type VI reactions can be found in the case of autoimmune diseases such as thyreoiditis or myasthenia gravis, where antibodies exhibit stimulating or inhibiting activity. From the aforementioned classification, type I and IV allergies have the greatest practical importance (Ring 2005).

The pharmacological treatment of allergy consists mainly of administration of immunosuppressants, antiphlogistic, and/or antiallergic drugs. Immunosuppressive agents are effective primarily in the case of type II, III, and IV allergies. Antiphlogistic drugs, e.g. glucocorticoids, can be used in all types of allergy. Antiallergic drugs are degranulation inhibitors (e.g. cromoglicin, nedocromil), anti-IgE antibody omalizumab, and H1-antihistaminic substances like azelastine and loratadine (Freissmuth et al. 2012).

The best option besides avoiding the allergen is a hyposensibilization with specific allergens. It leads to a 50% drop in symptoms and blocks the progression of allergic reactions to other antigens or the switch from allergic rhinitis to allergic bronchial asthma (Ledford 2007).

At this time, some plant preparations for the treatment of allergic rhinitis exist. These are mainly extracts from *Petasites* sp. or *Astragalus membranaceus* (Fisch.) Bunge declared as dietary supplements. For the so-called Ze 339 extract from *Petasites hybridus* (L.) G. Gaertn. et al. inhibition of allergen-induced cell response, airway inflammation, and airway hyperreactivity in mice could be observed (Brattström et al. 2010). In a randomized, double-blind, placebo-controlled crossover study with 18 subjects, it was shown that extract Ze 339 relieves nasal obstruction as a consequence of allergic rhinitis more effectively than desloratadine. The extract consists mainly of sesquiterpene metabolites (Dumitru et al. 2011).

The ISAAC Phase Three studies detected an increase in the prevalence of asthma, allergic rhinoconjunctivitis, and eczema (Asher et al. 2006). As pathogenic immune reactions are on the rise, so will be the cost for the treatment of symptoms. Novel efficient treatment options are highly needed. In this field, mushroom could play at least a supporting role, as some species seem to possess antiallergic activities.

The chapter presents an overview about mushrooms with in vitro and/or in vivo detected antiallergic activities. Immunosuppressive properties which result from cytostatic and other effects which are not directly related to allergies are not in the focus of this review.

## 4.2 Medicinal Mushrooms with Antiallergic Activities

### 4.2.1 *Agaricus subrufescens*

The edible mushroom *Agaricus subrufescens* Peck (Agaricaceae), also known as almond mushroom, is distributed in North and South America and well investigated for its health-promoting properties. Today, it is worldwide cultivated and used as a medicinal food to prevent and to treat many diseases, e.g. cancer and diabetes (Guthmann 2016). It is also named *A. brasiliensis* Wasser et al. or *A. blazei* Murrill. For an explanation of discrepancies regarding its nomenclature, see Wisitrassameewong et al. (2012). The bioactive compounds are mainly polysaccharides (ß-glucans) with immunomodulating activities. Antiallergic effects have been found in vitro and in animal assays.

A chloroform-soluble extract inhibited the degranulation of mouse bone marrow-derived mast cells as shown by the decreased release of ß-hexosaminidase. Besides it reduced the production of IL-6, prostaglandin D(2), and leukotriene C(4) in PMA plus A23187-induced cells (Song et al. 2012).

The influence of a water extract (0.01, 0.1 or 1 g/kg body weight, p.o.) was investigated on mast cell-mediated anaphylaxis-like reactions in mice in comparison to the influence of the reference compound dinatrium cromoglycate. The extract inhibited compound 48/80-induced systemic anaphylaxis-like reaction, ear-swelling response, and passive cutaneous anaphylaxis-like reaction. Moreover, the extract dose dependently reduced compound 48/80-induced or anti-dinitrophenyl IgE-mediated histamine release from rat peritoneal mast cells (Choi et al. 2006a). Asthma-induced mice treatment with a hot water *Agaricus* extract (350 mg/mouse/day, p.o.; day 0 till day 17 after primary immunization with ovalbumin [OVA]) caused significant downregulation of OVA-specific antibody responses of IgG1 and IgE but not of IgG2a and significantly decreased total cell numbers, levels of IL-5, and eosinophil numbers in bronchial alveolar lavage fluids. The results suggest that the extract ameliorates the Th1/Th2 balance from the skewed Th2 conditions. It was assumed that ß-glucans are not the responsible compounds in the extract (Takimoto et al. 2008).

AndoSan™ is a mushroom extract, mainly containing *A. blazei* (82%), but also *Hericium erinaceus* (Bull.:Fr.) Pers. (15%) and *Grifola frondosa* (Dicks.:Fr.) Gray (3%). It was given p.o. either a day before or 19 days after the immunization of mice with OVA (s.c.). The mice were sacrificed on day 26. It was found that the extract both, when given before or after immunization, reduced the levels of anti-OVA IgE (Ellertsen and Hetland 2009). An *Agaricus* extract alone, p.o., had similar effects on the serum IgE levels after OVA sensitization in mice. Besides, IL-4 and IL-5 production in OVA-restimulated splenocytes were significantly decreased (Bouike et al. 2011). The effects were mediated through the activation of macrophages by epithelial cells, the promotion of naïve T cells into Th1 cells (Bouike et al. 2011), and the amelioration of a skewed Th1/Th2 balance (Hetland et al. 2011).

### 4.2.2 *Armillaria ostoyae*

*Armillaria ostoyae* (Romagn.) Herink is a basidiomycete from the family Physalacriaceae (Fig. 4.1a, b). It was first described in 1900 as *A. solidipes* from Peck and later renamed *A. ostoyae* (Peck 1900; Romagnesi 1970). It is an edible mushroom when preprocessed by cooking. *A. ostoyae* is a saprotrophic-parasitic fungus; it infects various trees, in North America especially Douglas firs (*Pseudotsuga menziesii*). The biggest and heaviest organism on earth is an exemplary of *A. ostoyae* in Oregon, USA (Schmitt and Tatum 2008). *A. ostoyae* can produce bioluminescence (Rishbeth 1986). Different metabolites have been isolated from this mushroom, belonging to the class of meroterpenes, in which a sesquiterpene part is connected to a polyketide part. These metabolites are mainly called


**Fig. 4.1** (**a**, **b**) Fruiting bodies of *Armillaria ostoyae*

melleolides and exhibit antibacterial and antifungal effects (Midland et al. 1982; Donnelly et al. 1985; Momose et al. 2000; Bohnert et al. 2011; Bohnert et al. 2014; Chen et al. 2015).

Crude extracts from *A. ostoyae* fruiting bodies and mycelia obtained by soxhlet extraction using dichloromethane inhibited the degranulation of RBL-2H3 cells. The mycelial extract showed higher potency ($IC_{50}$ 21.5 μg/mL), whereas the fruiting body extract had an $IC_{50}$ value of 115.1 μg/mL. Isolated substances melleolide H ($IC_{50}$ value 99.9 μM) and J ($IC_{50}$ value 39.5 μM) also showed this activity (Merdivan 2016; Merdivan et al. 2017a).

### 4.2.3 *Bulgaria inquinans*

*Bulgaria inquinans* (Pers.:Fr.) Fr. (Phacidiaceae), poor man's licorice, is an ascomycete growing on branches and bark of dead *Carpinus*, *Castanea*, *Fagus*, *Quercus*, and other trees. It is distributed worldwide. An ethanol extract, p.o., 300 and 600 mg/kg, dose dependently inhibited histamine release from mast cells induced by histamine and scratching behavior of mice induced by compound 48/80 and serotonin. It did not inhibit scratching behavior induced by histamine (Jiang et al. 2005).

### 4.2.4 *Cordyceps militaris*

*Cordyceps militaris* (L.:Fr.) Link (Cordycipitaceae) grows as a parasite on *Lepidoptera*. Similar to the famous *Ophiocordyceps sinensis*, it is used as a traditional medicine for treating several diseases including allergy in East Asia (Das et al. 2010). An ethyl acetate extract of the fungi, grown on germinated soybean, inhibited antigen-induced degranulation of mast cells (RBL-2H3) with an $IC_{50}$ value of 28.5 μg/mL and the release of IL-4 and TNFα from these cells. In mice, antigen-induced passive cutaneous anaphylaxis response was inhibited with an $ED_{50}$ value of 665 mg/kg. On a molecular level, the inhibition of the phosphorylation of Syk,

ERK, p38, and JNK expression was shown. Isoflavonoids like genistein and daidzein and their glycosides and adenosine should be responsible for the inhibition of degranulation. Cordycepin as a typical compound from *C. militaris* did not exhibit degranulation inhibitory activity (Oh et al. 2011). It can be assumed that the observed activities are at least partly caused by compounds which originate from the soybean substrate.

### 4.2.5 *Flammulina velutipes*

The edible mushroom *Flammulina velutipes* (Curtis) Singer (Physalacriaceae) grows on the stumps of different trees and may be found from September to March in temperate regions. It can be cultivated and is widely used in East Asian cuisine (Japanese name: *Enoki*). An ethanol extract of the mushroom (250 mg/kg body weight/day, p.o.) showed significant antiallergic effects in an oxazolone-induced type IV allergy model in male mice. The extract was given 3 days before challenge with oxazolone (5 days after sensitization). Application of the extract 3 days before the sensitization and hot water extract had no effect (Sano et al. 2002). In a mouse allergy model, antiallergic effects could be detected for the protein FIP-*fve* (114 amino acids, 13 kDa) which has been isolated from the fruit bodies of the mushrooms. BALB7c mice were immunized twice i.p. with OVA in an interval of 2 weeks. Before and during each period of immunization, mice were treated with FIP-*fve* (200 µg/mouse, p.o., total five doses). Mice receiving FIP-*fve* during sensitization had a decreased OVA-specific IgE response with a Th1-predominant cytokine profile. These mice were protected from systemic anaphylaxis-like symptoms induced by subsequent oral challenge with OVA. It could be shown that FIP-*fve* induced a Th1-predominant allergen-specific immune response. Surprisingly the protein retains its activity after p.o. administration (Hsieh et al. 2003).

### 4.2.6 *Ganoderma lucidum* and *Ganoderma tsugae*

Mushrooms of the genus *Ganoderma* (Ganodermataceae, Polyporales) belong to the most important medicinal mushroom species worldwide. The most famous species *G. lucidum* is probably a complex of different species (Zhou et al. 2015) and also known as *Reishi* or *Ling Zhi*. It has been used for medical purposes in China since ancient time. The annual world production of *Ganoderma*-derived products is about 2.5 billion USD (Bishop et al. 2015). *G. lucidum* grows in decaying logs or tree stumps and can effectively be cultivated. *G. tsugae*, the hemlock reishi mushroom, is closely related to *G. lucidum*. Both species are distributed worldwide.

The mushrooms exhibit a broad spectrum of biological activities including antitumor, antiviral, antidiabetic, anti-inflammatory, and antiallergic activities. The beneficial health properties are mainly attributed to polysaccharides and triterpenes, e.g. ganoderic acids (Paterson 2006; Bishop et al. 2015; Guthmann 2016). One investigation found that ganoderic acids C and D (concentration 0.4 and 2 mg/ml),

isolated from *G. lucidum*, inhibited histamine release from rat mast cells that was induced by compound 48/80 and concanavalin A (ConA) (Kohda et al. 1985). The chloroform extract from the culture medium had similar effects. Oleic acid (Tasaka et al. 1988a) and cyclooctasulfur (Tasaka et al. 1988b) have been identified as effective compounds in the medium extract.

A methanol extract of *G. lucidum* (100 and 300 mg/kg p.o., rich in triterpenes) inhibited scratching, an itch-related response, induced by intradermal injections of an extract of salivary gland of the mosquito as pruritogen in mice (Andoh et al. 2010). The extract (10–1000 mg/kg) inhibited dose dependently also scratching induced by 5-hydroxytryptamine, α-methyl-5-hydroxytryptamine, and proteinase-activated receptor-2-activating peptide SLIGRL-$NH_2$ but not those induced by histamine, substance P, and compound 48/80 (Zhang et al. 2010). The results suggest that the extract relieved allergic itch through peripheral action and that mast cells and $H_1$ histamine receptors are not the primary sites of the antipruritic action of the extract (Andoh et al. 2010).

*G. tsugae* supplementation alleviated bronchoalveolar inflammation in an airway sensitization and challenge model with female BALB/c mice. In this allergic model, mice were weekly sensitized by i.p. injection of OVA three times and challenged with aerosolized OVA twice. *G. tsugae*, given p.o. as a supplement to feed (2.0–6.6 g/kg feed) for 5 weeks, significantly decreased infiltration of inflammatory cells and the secretion of inflammatory mediators into the local tissues of lungs and airways (Lin et al. 2006). A triterpene-rich methanol extract (1, 2, or 5 mg/day for 2 weeks p.o.) led to comparable results so that the effects can be attributed to triterpenes (Chen and Lin 2006).

ASHMI® is a traditional Chinese medicine containing *Ganoderma lucidum* as one component (besides *Sophora japonica* and *Glycyrrhiza uralensis*). In a preliminary study with 91 subjects with moderate to severe persistent asthma, the preparation improved lung function and reduced symptom scores (Wen et al. 2005). A phase I study confirmed safety and tolerability of the preparation in patients with asthma (Kelly-Pieper et al. 2009). A review of anti-inflammatory and antiallergic activities of *G. lucidum* is given by Bhardwaj et al. (Bhardwaj et al. 2014).

### 4.2.7 *Hypsizigus marmoreus*

*Hypsizygus marmoreus* (Peck) H.E. Bigelow (Tricholomataceae), beech mushroom, lives as a saprophyte on *Fagus* and other trees. The edible mushrooms are cultivated for culinary purposes especially in Japan and Korea. Besides bioactive polysaccharides and proteins, they contain interesting isoprenoids, the hypsiziprenols (Guthmann 2016). The p.o. application of an ethanol extract of *H. marmoreus* for 3 days at a dose of 250 mg/kg b.w. exhibited antiallergic effects on an oxazolone-induced type IV allergy in male ICR mice, characterized by a severe ear edema, changes in different cytokine levels, and diminished serum antioxidative activity. The mushroom extract prevented the increase of serum IL-12 and the decrease in the serum level of IL-2, spleen natural killer cell activity, and serum

antioxidant activity. The effects result from inhibitory actions on antigen-presenting cells like macrophages, inhibition of production and/or release of IL-12, and suppression of oxidative stress (Sano et al. 2002; Yoshino et al. 2008).

### 4.2.8 *Inonotus obliquus*

*Inonotus obliquus* (Fr.) Pil. (Polyporaceae), the Chaga mushroom, is the sterile stage of a wood-destroying fungus parasitizing on trunks mostly of birch. It has been used in the traditional medicine of Eastern Europe for the treatment of cancer, digestive system diseases, and other illnesses. In Russia, medicinal preparations are commercially available. Triterpenes, polysaccharides, and polyphenolic pigments are the main bioactive compounds (Shashkina et al. 2006).

A hot water extract of Chaga mushrooms inhibited the systemic anaphylactic shock induced by compound 48/80 in mice. The extract was applicated i.p. 30 min before administration of compound 48/80. Whereas 100% of the animals in the control group (only compound 48/80) died, all mice treated with 2.5 mg extract/mouse survived. Besides, the extract given p.e. or p.o. significantly reduced the total IgE levels in the animals sensitized by OVA and slightly affected the production of IgG1. Spleen cells harvested from the OVA-sensitized mice that had received the extract p.o. showed a significant increase in Th1-derived responses, e.g. the production of IFN-γ (Yoon et al. 2013). In another investigation 50, 100, or 200 mg/kg of a hot water extract were given p.o. to OVA-sensitized BALB/c mice. When the extract was administered after the second immunization with OVA, it significantly suppressed the OVA-induced increase in serum IgE and IgG(2a). In ex vivo studies, spleen cells were isolated from mice sensitized with OVA and treated with 100 mg/kg of the extract. Compared to the controls, ConA stimulation resulted in lower IL-4 production and increased IFN-γ production. Moreover, IL-4, IFN-γ, and IL-2 were significantly reduced after ConA stimulation in isolated CD4(+) T cells (Ko et al. 2011). Injection of an ethanol extract (high and low dose) into asthmatic mice (asthma was caused by injection and inhalation of OVA) resulted in a significant alleviation of histopathological damages. It was concluded that the extract inhibited the expression of phosphor-p38 MAPK and corrected the imbalance of IFN-γ/IL-4 and the number of inflammatory cells (Yan et al. 2011).

### 4.2.9 *Phellinus linteus*

*Phellinus linteus* (Berk.:M.A.Curtis) Teng (Hymenochaetaceae) occurs in tropical and subtropical regions and lives as a saprophyte on *Cassia*, *Quercus*, and other trees. The mushrooms are known to have a broad spectrum of biological activities and have been used as traditional medicine in oriental countries for a long time (Silva 2010; Guthmann 2016). Oral application of a water extract from the fruiting bodies inhibited the compound 48/80-induced systemic anaphylaxis reaction and ear-swelling response in mice. Besides, the anti-dinitrophenyl (anti-DNP)

IgE-mediated passive systemic and cutaneous anaphylaxis reaction was inhibited. In vitro, the extract dose dependently reduced histamine release from rat peritoneal mast cells (RPMC) activated by compound 48/80 or anti-DNP IgE, decreased the compound 48/80-induced calcium uptake into RPMCs, increased the level of intracellular cyclic adenosine monophosphate (cAMP), and inhibited the compound 48/80-induced cAMP reduction in RPMC. It was suggested that the extract might serve as an effective therapeutic agent for allergic diseases (Choi et al. 2006b). Besides, extracts prepared from the mycelium of the mushrooms have been tested for inhibiting activities on the IgE-dependent mouse triphasic cutaneous reaction. The triphasic reaction was induced in the ear of BALB/c mice passively sensitized with anti-DNP IgE by painting with DNP 24 h later. Ear swelling appeared triphasically with peak responses at 1 h, 24 h, and 8 days after the challenge. Methanol- and water-soluble fractions given p.o. at a dose of 100 mg/kg inhibited the first and second phase of ear swelling. The most potent fraction was the boiling water-soluble fraction. It inhibited dose dependently (30–300 mg/kg) all phases, inhibited vascular permeability increase caused by passive cutaneous anaphylaxis and histamine, and ear swelling caused by TNFα (Inagaki et al. 2005).

### 4.2.10 *Pleurotus ostreatus*, *Pleurotus pulmonarius*, and *Pleurotus eryngii*

The oyster mushroom – *Pleurotus ostreatus* (Jacq.:Fr.) P. Kumm – is an edible basidiomycete from the Pleurotaceae family. It is a saprophytic and parasitic fungus, mainly thriving on deciduous trees and widely cultivated for culinary purposes. In nature, fruiting bodies occur late in the year, which makes *P. ostreatus* a winter mushroom.

The oyster mushroom was subjected to different clinical trials regarding its antiallergic and antiasthmatic properties. One randomized, placebo-controlled, double-blind clinical trial was investigating the effect of *P. ostreatus* on recurrent respiratory tract infections (RRTI). Atopic persons tend to have a higher risk of such infections in comparison to healthy subjects. The application of a commercially available syrup P4H® p.o. over a period of 6 months resulted in a stable serum IgE titer in the verum group, whereas in the placebo group, the amount of IgE in serum was rising. The blood eosinophil count decreased in the verum group where it remained unchanged under placebo. The effect was stronger in atopic than in nonatopic persons. P4H® comprised of pleuran and vitamin C (concentration 10 mg/mL each), and placebo consisted of 10 mg/mL vitamin C. Pleuran is a branched β-glucan insoluble in alkali (Karácsonyi and Kuniak 1994). One main drawback of this study was the determination of surrogate parameters only. Nevertheless, the results showed a possible response to the administration of *P. ostreatus* in RRTI (Jesenak et al. 2014).

Another study from the same author measured hard clinical endpoints in a multicentric, double-blind, placebo-controlled, and randomized design investigating the effect of pleuran administration on prevention of RRTIs and immunomodulatory activity in children. Verum, placebo, and treatment period were the same as

mentioned in the study above. Treatment with verum led to significantly lower respiratory morbidity, alteration of immunoglobulin composition, and a temporal decrease of $CD8^+$ cytotoxic T cells. This points to a better immune defense against pathogens (Jesenak et al. 2013). Thus, *P. ostreatus* can exhibit an influence on the immune system which increases defense against pathogens and lowers markers for allergy.

*Pleurotus pulmonarius* (Fr.:Fr.) Quél, the Indian Oyster or Lung Oyster (Japanese: *Usuhiratake*) exhibits different beneficial activities. Proteins from the water extract inhibit the angiotensin-converting enzyme (ACE) with an $IC_{50}$ of 12 μg/mL (Ibadallah et al. 2015). A protein/polysaccharide complex of *P. pulmonarius* inhibited development and progression of liver cancer (Xu et al. 2012). Further, β-D-glucans from *P. pulmonarius* have an antinociceptive effect (Smiderle et al. 2008a, b; Baggio et al. 2010; Baggio et al. 2012). The antinociceptive effect could also be beneficial for patients, who struggle with inflammation as a result of an allergic reaction.

Polysaccharides of *P. pulmonarius* attenuated and prevented intestinal inflammation symptoms in a mouse model. TNF-α levels decreased in tissue and increased IL-1β attenuation (Lavi et al. 2010). A β-D-glucan from this mushroom exhibited a dose-dependent anti-inflammatory activity in a mouse model measuring leukocyte migration to inflammatory tissue (Smiderle et al. 2008a).

The powder of fruiting bodies of *P. pulmonarius* caused a significant decrease of sneezing and nasal rubbing in BALB/c mice. The effect was observed when the powder was administered in a dose of 500 mg/kg body weight for 2 weeks or at a dose of 200 mg/kg body weight for 4 weeks. The IgE levels did not decrease. The powder did not reduce histamine-induced nasal rubbing and sneezing via an antagonistic effect. In an in vitro model using RBL-2H3 rat basophils, a decrease of compound 48/80 triggered histamine release could be observed. It seems possible that *P. pulmonarius* reduced the symptoms of allergic rhinitis through inhibition of histamine release from immune cells (Yatsuzuka et al. 2007).

An ethanolic extract (250 mg/kg body weight/day) of *Pleurotus eryngii* (DC.) Quél., the king trumpet mushroom, exhibited antiallergic effects on oxazolone-induced type IV allergy in mice, similar to extracts from *Flammulina velutipes* and *Hypsizygus marmoreus* (see above). The extract was applied p.o. 3 days before the challenge with oxazolone (Sano et al. 2002).

### 4.2.11 *Tricholoma populinum*

*Tricholoma populinum* Lange is a basidiomycete from the family Tricholomataceae. It is a mushroom building mycorrhizas with different poplar species (Grubisha et al. 2012). Expansion of the mycelium is mainly by vegetative growth, not through sexual reproduction by spores (Gryta et al. 2006). As *T. populinum* is a mycorrhiza fungus, fruiting bodies (Fig. 4.2a, b) are only formed in connection to the symbiotic partner and cannot be cultivated but must be collected by wild harvesting. Nevertheless, it is possible to cultivate the mycelium.


**Fig. 4.2** (**a**, **b**) Fruiting bodies of *Tricholoma populinum*

In 1977 mushroom collector Herbert Schäfer published a case report about effects of *T. populinum* against thromboangiitis obliterans, a chronic relapsing inflammatory illness, which affects mainly distal arteries of the extremities. Etiology is mainly unknown but seems to be caused by an allergic-hyperergic effect and based on an autoimmune process. Thromboangiitis obliterans can lead to loss of extremities (Pschyrembel 1998). Mr. Schäfer suffered from severe, tearing pain in the abdominal and pelvic region and felt muscular pain even under light-duty and wound pain, especially in the skin. He was not able to walk longer distances without pain; climbing stairs was nearly impossible. Ca. 1 h after the intake of fruiting bodies of *T. populinum*, he felt a weak, pleasant sensation, which reduced his wound pain and caused subtle tickling. He then investigated the effects of dechallenge and rechallenge. Consuming the fruiting bodies of *T. populinum* for a period of time, a positive effect was observed for a few months after the dechallenge. Thereafter, illness symptoms worsened again. Mr. Schäfer utilized the fruiting bodies of *T. populinum* for two 2-month periods every year. In his report, he has described the positive effect of *T. populinum* consumption on allergic rhinitis by testing on his wife and a complete absence of symptoms was observed (Schäfer 1977).

In the 1980s different studies on the immunosuppressive action of *T. populinum* were conducted, resulting in the isolation of ergosterol peroxide as an active ingredient. Ergosterol peroxide reduced the production of antibodies in the hemolysis-plaque assay and the proliferation of mitogen-stimulated lymphocytes in the lymphocyte transformation assay (Lindequist 1987; Lindequist et al. 1989a, b; Kreisel et al. 1990). The dichloromethane extract from fruiting bodies of *T. populinum* inhibited the degranulation of RBL-2H3 cells and the IL-2 release from Jurkat T cells. The degranulation inhibiting effect could only be observed when fruiting bodies were processed with heat. Extracts from lyophilized fruiting bodies showed no effect ($IC_{50}$ >500 μg/mL), but when lyophilized fruiting bodies were mildly heated for a prolonged period, the extract showed this effect again ($IC_{50}$ 224.2 μg/mL). Extracts from fruiting bodies dried using increased temperature displayed a slightly stronger influence ($IC_{50}$ 161.8 μg/mL) (Merdivan 2016; Merdivan et al. 2017b).

An overview on activities of medicinal mushrooms regarding allergic reactions is presented in Table 4.1.

**Table 4.1** Overview on activities of medicinal mushrooms regarding allergic reactions

| Mushroom | Active principle | Biological effect in respect to allergy | Reference |
|---|---|---|---|
| *Agaricus subrufescens* | Chloroform extract, (hot) water extract, AndoSan™ | Inhibition of IL-5,6, PGD(2), LTE-C(4) release; anaphylaxis-like reaction; IgG1, IgE production | Choi et al. (2006a), Takimoto et al. (2008), Ellertsen and Hetland (2009), Bouike et al. (2011), Hetland et al. (2011), and Song et al. (2012) |
| | | IL-5↓, total cell number and eosinophil count in mice bronchial alveolar fluid↓ | |
| *Armillaria ostoyae* | Sesquiterpene aryl esters | Degranulation of RBL-2H3 cells↓ | Merdivan (2016) and Merdivan et al. (2017) |
| *Bulgaria inquinans* | Ethanol extract | Histamine release from mast cells↓ | Jiang et al. (2005) |
| *Cordyceps militaris* | Ethyl acetate extract, isoflavonoids (?) | Inhibition of degranulation of RBL-2H3 cells, IL-4 and TNF-α release↓ | Das et al. (2010) and Oh et al. (2011) |
| | | Syk, ERK, p38, JNK expression↓ | |
| *Flammulina velutipes* | Ethanol extract, protein FIP-*fve* | Type IV allergy in mice model↓, IgE response in cells↓ | Sano et al. (2002) and Hsieh et al. (2003) |
| *Ganoderma lucidum* | Ganoderic acids (triterpenes), chloroform extract, oleic acid, cyclooctasulfur, medium extract, ASHMI® | Histamine release↓; scratching↓; itch-related response↓ | Kohda et al. (1985), Tasaka et al. (1988a), Tasaka et al. (1988b), Kelly-Pieper et al. (2009), Andoh et al. (2010), and Zhang et al. (2010) |
| *Ganoderma tsugae* | Whole fruiting bodies, triterpene-rich extract | Infiltration of inflammatory cells, secretion of inflammatory mediators into lung and airway tissue in a mouse model↓ | Chen and Lin (2006) |
| *Hypsizigus marmoreus* | Ethanol extract | Type IV allergy in a mouse model↓, blocking of increase in IL-12 and decrease in IL-2 serum levels, spleen natural killer cell activity, and serum antioxidant activity | Sano et al. (2002) and Yoshino et al. (2008) |

(continued)

**Table 4.1** (continued)

| Mushroom | Active principle | Biological effect in respect to allergy | Reference |
|---|---|---|---|
| *Inonotus obliquus* | Hot water, ethanol extract | Inhibition of anaphylactic shock in mouse model | Yan et al. (2011), Ko et al. (2011) and Yoon et al. (2013) |
| | | IgE↓, IFN-γ↑, IL-4↓, IL-2↓, histopathological damages↓ in mouse model | |
| | | Increase in IgE and IgG(2a)↓, IL-4 production↓, IFN-γ production↑ in mouse spleen cells ex *vivo* | |
| | | IL-4, IFN-γ, and IL-2↓ in CD4+ cells | |
| | | Histopathological damages in asthmatic mice↓ (possibly phosphor-p38 MAPK expression↓, imbalance correction of IFN-γ/IL-4, number of inflammatory cells↓) | |
| *Phellinus linteus* | Water extract, fractions | Degranulation of RPMCs↓, intracellular cAMP↑ | Inagaki et al. (2005) and Choi et al. (2006b) |
| | | Systemic anaphylaxis and ear swelling in mice↓, passive systemic and cutaneous anaphylaxis reaction in mice↓ | |
| | | Triphasic cutaneous reaction in mice↓ | |
| *Pleurotus ostreatus* | Pleuran (β-glucan) | IgE in human serum↓, BEC↓, CD8+ cells↓, alteration of immunoglobulin composition | Jesenak et al. (2013, 2014) |
| *Pleurotus pulmonarius* | β-Glucans | Antinociceptive effects, TNF-α↓, IL-1β↓, sneezing↓, nasal rubbing↓ in mice, leukocyte migration to inflammatory tissue in a mouse model↓ | Yatsuzuka et al. (2007), Smiderle et al. (2008b), Lavi et al. (2010) and Baggio et al. (2010, 2012) |
| | | Histamine release from RBL-2H3 cells↓ | |
| *Pleurotus eryngii* | Ethanolic extract | Type IV allergy in mouse model↓ | Sano et al. (2002) |
| *Tricholoma populinum* | Dichloromethane extract, fractions | Degranulation of RBL-2H3 cells↓ | Lindequist (1987, 1989a, b), Kreisel et al. (1990), and Merdivan (2016) |

## 4.3 Conclusions

The analysis of described antiallergic activities of mushrooms shows that only a relatively small number of mushroom species has been investigated for such effects. The available results lead to the conclusion that fungi could possess promising antiallergic properties. To explore this potential, it is necessary to verify the results in further test systems, to elucidate the mode of action, to conduct clinical trials, to identify the responsible bioactive compounds, and to ensure safety and quality of resulting products. In the case of success, mushrooms or mushroom-derived compounds can become a prophylactic and/or therapeutic opportunity in diseases like allergic rhinitis, allergic asthma, allergic itch, food allergy, and urticaria.

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# The Bioactivity of Tiger Milk Mushroom: Malaysia's Prized Medicinal Mushroom

5

Shin-Yee Fung and Chon-Seng Tan

**Abstract**

The tiger milk mushroom has long been extolled for its medicinal properties and has been used for the treatment of asthma, cough, fever, cancer, liver-related illnesses, and joint pains and as a tonic. The history of usage for tiger milk mushroom dated back to almost 400 years ago, but there were no records of scientific studies done due to unavailability of sufficient samples. Even when there were samples collected from the wild, the supply and quality were inconsistent. With the advent of cultivation success of one of the most utilized species of tiger milk mushroom (*Lignosus rhinocerotis*) in 2009, scientific investigation was done to validate its traditional use and to investigate its safety for consumption and biochemical and biopharmacological properties. Among the properties that have been investigated to date are antiproliferative, anti-inflammatory, antioxidative, nutritional, immunomodulatory, and neuritogenesis activities of the *Lignosus rhinocerotis*. The scientific findings have so far verified some of its traditional applications and revealed interesting data which shows potential for it to be further developed into possible nutraceutical. More scientific investigations are much needed to validate the medicinal properties of tiger milk mushroom across its species and to unveil potential biomolecules that may form a valuable foundation in pharmaceutical and industrial applications.

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**Keywords**
Biomedical properties • *Lignosus* • Medicinal mushroom • Tiger milk mushroom • Sclerotia

## Contents



## Abbreviations

| | |
|---|---|
| ABTS | 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) |
| AGE | Advanced glycation end |
| AKT | Protein kinase B |
| CAT | Catalase |
| CBM | Carbohydrate-binding module |
| CWE | Cold water extract |
| DPPH | 1,1-Diphenyl-2-picrylhydrazyl |
| ERK | Extracellular signal-regulated kinases |
| FIP | Fungal immunomodulatory protein |
| FRAP | Ferric reducing ability of plasma |
| G-CSF | Granulocyte colony-stimulating factor |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GST | Glutathione transferase |
| HMW | High molecular weight |
| HWE | Hot water extract |
| IL-6 | Interleukin 6 |

| | |
|---|---|
| iNOS | Inducible nitric oxide synthase |
| NO | Nitric oxide |
| LMW | Low molecular weight |
| MALDI-MS | Matrix-assisted laser desorption/ionization coupled with mass spectrometry |
| LC-MS | Liquid chromatography coupled with mass spectrometry |
| MCHC | Mean corpuscular hemoglobin concentration |
| MCPs | Matricellular proteins |
| MCV | Mean corpuscular volume |
| ME | Methanol extract |
| MMW | Medium molecular weight |
| Mn-SOD | Manganese-superoxide dismutase |
| NF-κB | Nuclear factor-κB |
| MIP-1α | Macrophage inflammatory protein- 1α |
| NOAEL | No-observed-adverse-effect level |
| PCV | Packed cell volume |
| RBC | Red blood cell |
| ROS | Reactive oxygen species |
| SGOT | Serum glutamic oxaloacetic transaminase |
| SGPT | Serum glutamic pyruvic transaminase |
| SOD | Superoxide dismutase |
| TNF-α | Tumor necrosis factor alpha |

## 5.1 Introduction

### 5.1.1 Historical Background

The tiger milk mushroom (also known by a variety of names: *cendawan susu harimau* (in Indonesian/Malay) (Burkill & Haniff 1930, Burkill et al. 1966); *betes kismas* (Haji Taha 2006); *hurulingzhi* (Huang 1999a, b); *hijiritake* (Yokota 2011)) has been documented by Jesuit in 1664 in the "The Diary of John Evelyn" (Evelyn 1664) where it was referred to as *Lac tygridis* (*tigridis*) (tiger's milk) and was recorded to be used by the local communities to treat diseases that "Western druggist and physicians were not able to figure out." Cooke (1879) pioneered the scientific documentation of this fungus and named it as *Polyporus rhinocerus* using a specimen obtained from Penang Island, Malaysia. The subsequent records in Southeast Asia were by H.N. Ridley (Ridley 1890, 1900; Ridley and Curtis 1902) where the mushroom (then referred to as *Polystictus rhinocerotis*) was mentioned to have an important economic value. In "A Dictionary of the Economic Products of the Malay Peninsula," Burkill et al. (1966) listed *Polysticus sacer* as one of the mushrooms that the Malays called *susu rimau*. According to Malaysian folklore, it was believed that the tiger milk mushroom grows where the mother tiger might have disgorged its milk during lactation. There were huge intervals between the historical

mentions, presumably due to the quandary of locating the mushroom for use. The scarcity of the mushroom may be owing to ill-suited weather and growth environment. The decline of this mushroom which is known as an imperative "health guard" to the local communities could also be attributed to its increasing cost due to high demand, overharvesting, deforestation for modern development, pollution (Vikineswary and Chang 2013), and the availability of modern medicine.

## 5.2 The Morphology and Taxonomy of Three Known Tiger Milk Mushroom Species in Malaysia: The *Lignosus rhinocerotis*, *Lignosus tigris*, and *Lignosus cameronensis*

*Lignosus* Lloyd ex Torrend, a genus comprising eight species of polyporales macrofungi, namely, *L. dimiticus* Ryvarden, *L. ekombitii* Douanla-Meli, *L. goetzii* (Henn.) Ryvarden, *L. rhinocerotis* (Cooke) Ryvarden, *L. sacer* (Afzel. ex Fr.) Ryvarden, and *L. hainanensis* B. K. Cui, *L. tigris* C.S. Tan, and *L. cameronensis* C.S. Tan (Ryvarden and Johansen 1980; Douanla-Meli and Langer 2003; Cui et al. 2011; Tan et al. 2013). Species in *Lignosus* genus generally have similar gross morphologies. Variations in size and dimensions of either sclerotia or basidiocarps are not characteristically unique to the species. There is also little variation between the hyphal systems and sclereids on a microscopic level. Hence, the sizes of the pores and basidiospores are the two reliable characters for species identification. In Malaysia, there are three main recognized species, namely, the *Lignosus rhinocerotis* (Cooke 1879; Tan et al. 2010), *Lignosus tigris*, and *Lignosus cameronensis* (Tan et al. 2013; Yap et al. 2014a). The pore sizes of the three species are summarized in Table 5.1 and Fig. 5.1a–d.

The molecular taxonomy of *Lignosus* species is shown in Fig. 5.2 where one can note the intra- and interspecific differences among the species. The phylogenetic tree in Fig. 5.2 shows that *L. rhinocerotis* is distinct compared to other members of the genus. *L. cameronensis*, *L. tigris*, *L. sacer* and *L. ekombitii*, and *L. hainanensis* are within the same clade with *L. ekombitii* appearing to be quite closely related to *L. sacer*, while *L. cameronensis* and *L. tigris* remain distinct species with a genetic distance of up to 7.1 % (Tan et al. 2010, 2013). *L. dimiticus* Ryvarden and *L. goetzii* (Henn.) Ryvarden which are also part of the *Lignosus* genus were not included in the taxonomy study done by Tan et al. (2013) due to unavailability of samples.

**Table 5.1** Pore size of three *Lignosus* species

| Species | Pore size (mm) |
|---|---|
| *Lignosus rhinocerotis* | 5–8 pores per mm |
| *Lignosus tigris* | 1–2 pores per mm |
| *Lignosus cameronensis* | 2–4 pores per mm |

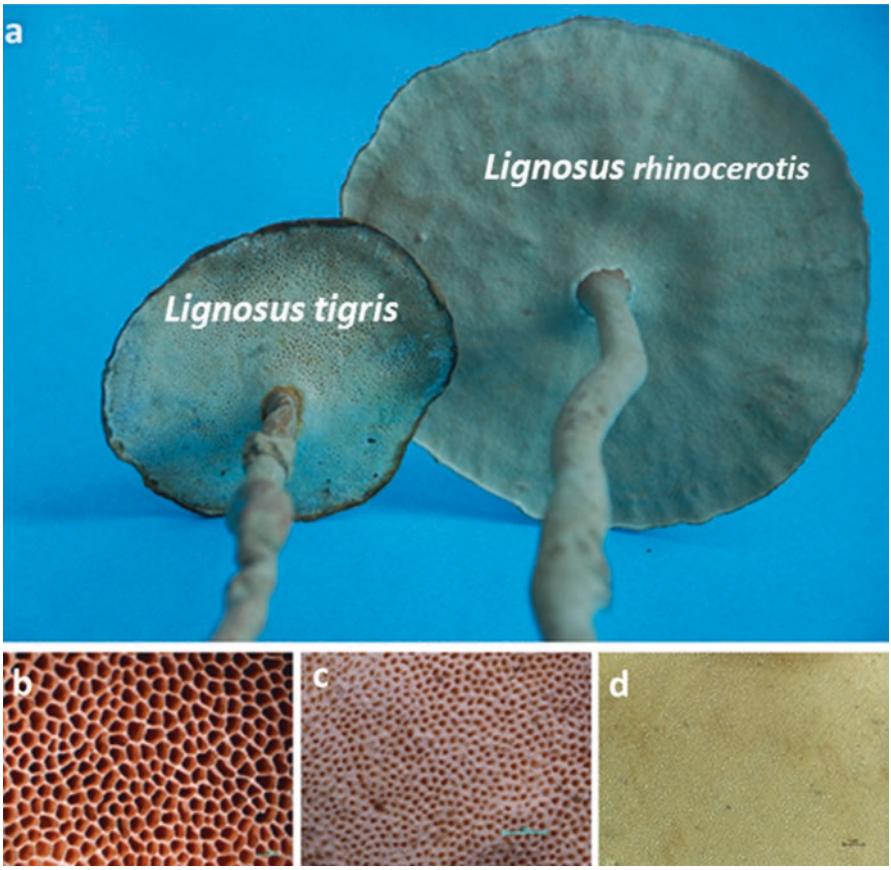


**Fig. 5.1** Pores of *Lignosus* species below the pileus and its differences in sizes. (**a**) The structure of *L. tigris* and *L. rhinocerotis.* (**b**) Pore size of *L. tigris.* (**c**) Pore size of *L. cameronensis.* (**d**) Pore size of *L. rhinocerotis*

## 5.3 Medicinal Usage of Tiger Milk Mushroom: Then and Now

Tiger milk mushroom (particularly referring to the *L. rhinocerotis* species) has been listed as one of the most important medicinal mushrooms used by local communities of Malaysia by a local Malay from Pahang, Tuan Haji Mat Yusop (Corner 1989). Back at the end of the 1890s, Sir H.N. Ridley mentioned that the tiger milk mushroom "has a great reputation for consumption and colds" (Ridley 1897). A technique mimicking cold water extraction has been described by Chan (1953) where the sclerotium is grated on a hard surface such as granite plate along with some water. The resulting mixture is then further diluted with water prior to consumption.

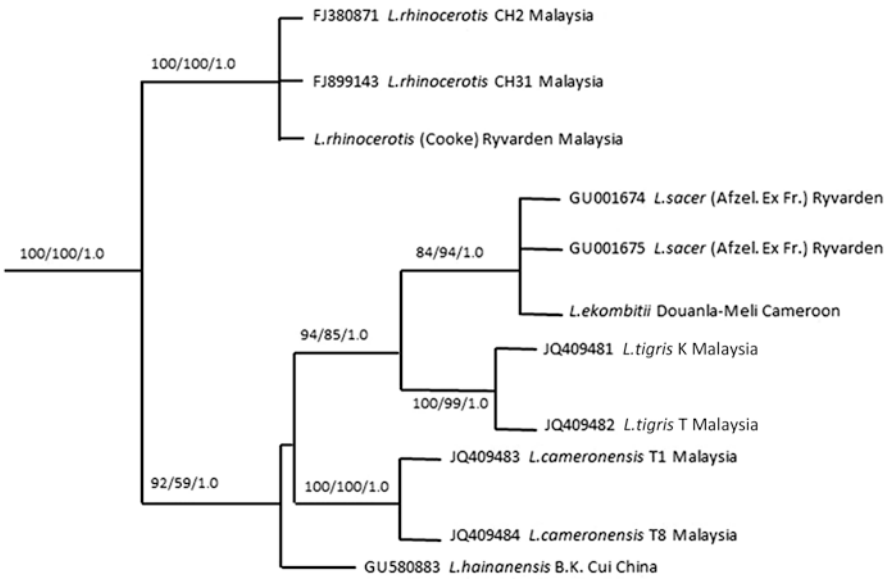


**Fig. 5.2** The molecular taxonomy of *Lignosus* species

The Temuans utilized tiger milk mushroom as medicine to treat coughs and asthma and to strengthen weak constitution by consuming the underground sclerotium (tuber-like; the part with medicinal value) in the form of decoction. The Semai aborigines used *Betes kismas* (a common name for tiger milk mushroom; see Sect. 5.1) for the treatment of asthma, cough, fever, cancer, liver-related illnesses, and joint pains. They are also used by men to revitalize their bodies and as medicines for women after childbirth (Chang and Lee 2006). It was documented that in the state of Kelantan, Malaysia (where the mushroom is often given to mothers after childbirth), the sclerotium is pounded with raw rice, infused, and drunk (Burkill et al. 1966; Burkill and Haniff 1930). The latest survey shows that the local Malay and Chinese communities utilized the sclerotium of tiger milk mushroom to treat food poisoning, wounds, stomach cancer, breast cancer, and swellings (LiGNO Biotech Sdn. Bhd. 2012). In Hong Kong and China, traditional Chinese physicians regarded *L. rhinocerotis* sclerotium as an expensive folk medicine to treat liver cancer, chronic hepatitis, and gastric ulcers (Wong and Cheung 2008). Tun Dr. Mahathir bin Mohamad, the fourth Prime Minister of Malaysia and a medical doctor, has mentioned in his opening speech at the International Convention on Biotechnology 2002 that his chronic intractable cough has been cured by tiger milk mushroom (SMPKE Prime Minister's Office 2017).

The Medicinal Mushroom Research Group (MMRG) from the University of Malaya initiated the safety assessment of the cultivated *L. rhinocerotis* sclerotia powder in 2009 after its successful cultivation (Tan 2009b) to ensure that the cultivar is competent for consumption. Results of the safety assessment are discussed in

Sect. 5.6. Following the results of safety assessment, scientific validation of its nutritive composition and bioactive properties such as antiproliferative and anti-inflammatory was investigated alongside the wild type (see Sects. 5.7 and 5.8).

The method used for the cultivation involves using specially formulated culture medium consisting of rice, water, and food-based materials, subsequently incubated in environmentally controlled culture room for up to 6 months to cultivate the sclerotia before harvesting. The proprietary method used for cultivated could greatly affect the quality of the sclerotia and likely to retain more of its medicinal properties.

A quality of life (QoL) survey was conducted recently among the 100 volunteers who had taken the cultivated *L. rhinocerotis* sclerotia powder for various health concerns. The volunteers were given 500 mg of *L. rhinocerotis* sclerotia powder daily for 1–2 weeks consecutively. The volunteers were interviewed or gave written testimonials at the end of the survey period. The volunteers' testimonials were categorized as shown in Table 5.2.

**Table 5.2** Usage of tiger milk mushroom based on volunteers' testimonials derived from quality of life (QoL) survey conducted among the 100 volunteers

| | Benefit | Summary of comments from volunteers |
|---|---|---|
| 1. | Relief from respiratory-related illnesses | Ease of breathing |
| | | Rid phlegm with ease (especially noted for volunteers who were smokers) |
| 2. | Relief from asthmatic symptoms | Improves breathing |
| | | Reduce the frequency of inhaler usage |
| | | Shorten the recovery period from an asthmatic attack |
| | | Decreases the recurrence of subsequent asthmatic attacks |
| 3. | Relief from a chronic cough and subsequent recovery | Fewer episodes of a cough |
| | | Recover from a cough |
| 4. | Relief from allergy | Relief from respiratory allergy such as nasal and sinus symptoms |
| | | Relief from skin allergy such as eczema |
| | | Relief from an allergy to food or chemicals (rash subsided within a few days of topical application) |
| 5. | Treatment of joint pains | Effective in the treatment of joint pains (i.e., as a result of dengue fever) |
| | | Relieving joint pains in the elderly, rheumatoid arthritis, and osteoarthritis patients |
| 6. | Improved stamina | Improve alertness and stamina |
| | | Prolongs stamina of athletic volunteers |
| 7. | Anticancer | Reduction in the size of the tumor |
| | | Improved the quality of life and more energetic (volunteers who are cancer patients) |

## 5.4 Sclerotia Versus Mycelia of *Lignosus rhinocerotis*

The morphology of *Lignosus rhinocerotis* as a polypore is unique. The fruiting body (also known as the sporophore) has the characteristic consisting of a centrally stipitate pilei that grow from a subterranean sclerotium (plural sclerotia) in a humid environment.

The different developmental stages of *L. rhinocerotis* are shown in Fig. 5.3a–d. The description of various stages of growth: culture mycelial growth, the formation of sclerotia, mature sclerotia, compacted hyphal mass, mushroom with pileus, stipe, root, sclerotium is depicted. Figure 5.3a shows a 2 week culture of mycelial growth from a spore of *L. rhinocerotis*. Expansion of the mycelium is seen as repeated branching of the germ tube (short, initial hypha) which develops into a circular form (known as the "Tiger's Eyes") (Fig. 5.3b). The color of colony ranges from white to yellow upon maturation; appearance is fluff-/velvet-like. The growing mycelia form crosslink structures among the radiating hyphae to enable nutrient uptake and mobilization (Fig. 5.3c). The mycelia had fully colonized the substrate (1–2 months post-inoculation), and sclerotia had begun to form. Sclerotia forms by the initiation of aggregation of hyphae into small knots within the mycelial mass. As the size of the knots increase, central hyphae accumulate nutrients (reserves) from connected mycelia. Cells of the outer layer are seen to shorten and begin to thicken, resembling barrels as the sclerotium increases in size (Fig. 5.3d). Vigorous mycelial growth promotes the development of mature sclerotia (possible harvesting after between 4 and 6 months) under the soil. The reproduction of *L. rhinocerotis* is likely asexual as it is placed under phylum *Basidiomycota* (Abdul Razak 2009).

The sclerotium is the main source of food storage and medicinal material. It is a compact mass of hardened fungal mycelium and represents one of the stages in the fungal life cycle. This structure is a morphologically variable, nutrient-rich, multihyphal aggregate that serves as a food reserve and can remain dormant until favorable growth conditions arise (Willetts and Bullock 1992). They are long-lived compared to mycelia due to the ability to survive environmental extremes. The sclerotium of *L. rhinocerotis* comes in different shapes and sizes from being spherical to oval or irregular with a diameter of 4–5 cm. (Fig. 5.4) The pale to the grayish-brown outer skin (rind) appears rough and wrinkly to keep the internal compacted hyphal mass from drying out (Fig. 5.4).

The rings on the pileus are formed on each rainy (wet) season; active growth of cells is seen and subsequently ends during the dry season. Another ring is formed in the next wet-dry seasonal cycle (Fig. 5.5). The pileus may be eaten by small animal or rot due to humidity. It is interesting to note that under favorable growth conditions, there is root formation after the nutrient of sclerotia is used for initial sprouting into stipe and pileus. For continued survival, rhizobium-like root structure is formed above the "empty" sclerotium, and the sclerotium is filled with mass and grows larger in size.

Apart from the well-established recognition of the superiority of the sclerotium of *L. rhinocerotis* as a part of the mushroom with high medicinal properties, there has also been a growing emphasis on its mycelium as a source of nutraceuticals

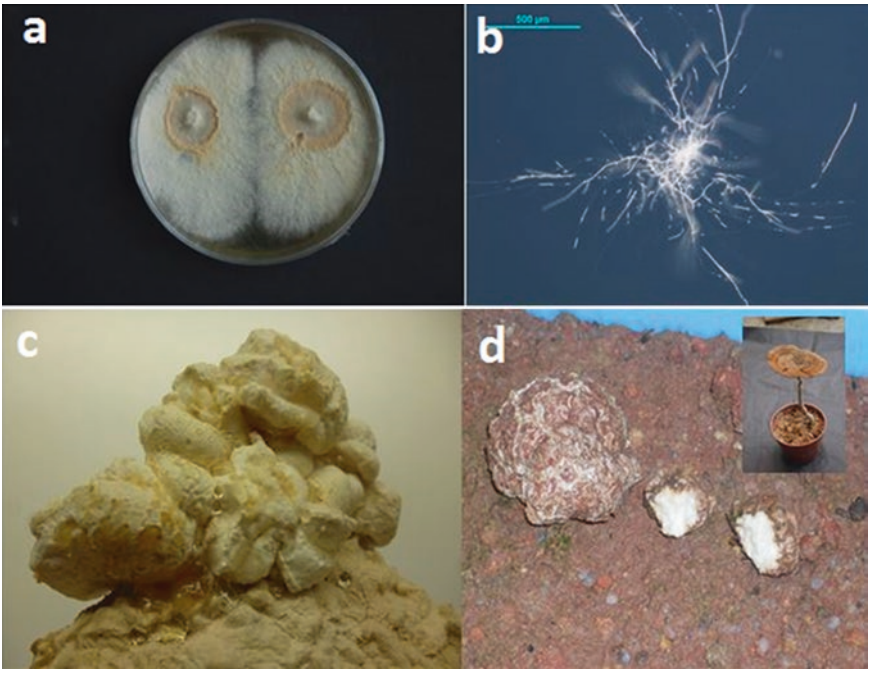


**Fig. 5.3** The different developmental stages of *L. rhinocerotis.* (**a**) Mycelia culture; (**b**) mycelia under microscope; (**c**) sclerotium formation from media; (**d**) hardened sclerotia from soil culture

**Fig. 5.4** The sclerotium of *L. rhinocerotis*

**Fig. 5.5** Rings on the pileus of *L. rhinocerotis*

(John et al. 2013; Lau et al. 2009, 2013, 2014; Phan et al. 2013). Lau et al. (2009, 2013) suggested the use of mycelium as an alternative to sclerotium. They reported the advantages conferred by submerged fermentation as well as the comparable proximate composition and some nutritional attributes of the mycelia. The mycelium was also reported to contain high levels of potassium, phosphorus, magnesium, riboflavin, and niacin and appreciable amounts of essential fatty acids (Lau et al. 2013). They also demonstrated that the mycelium and culture broth of *L. rhinocerotis* exhibited better antioxidant capacity and cytotoxic effect (Lau et al. 2014). However, whether mycelium is a good substitute of sclerotium remains a controversial issue as some researchers are of the opinion that mycelium is not naturally consumed and can only be produced by using artificial chemical-laden liquid culture medium. A simplistic comparison of the bioactivity of mycelium and sclerotia is shown in Fig. 5.6.

## 5.5 Wild-Type Versus Cultivated *Lignosus rhinocerotis*

The wild-type *L. rhinocerotis* can be located in the forest, by chance. This is due to the fact that the sporophore will only sprout from the underground sclerotium when the environment and conditions are optimum. The sclerotium can remain below the ground for months, years, or even decades without sprouting its stipe; hence it is a mammoth task to locate the spot where the sclerotium lies. The irregularity of supply, coupled with the inconsistency quality and nutritional (medicinal) content of the sclerotium (which is highly dependent on the harvesting conditions) along with

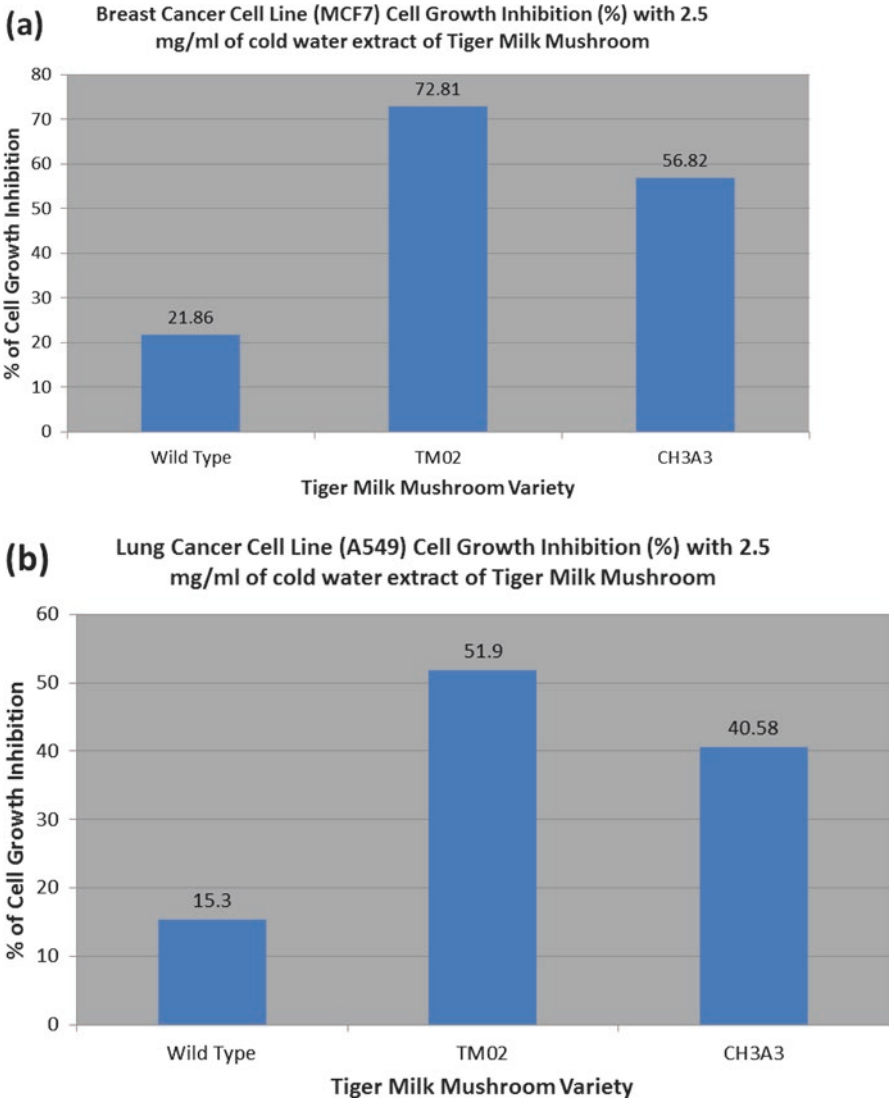


**Fig. 5.6** Cell growth inhibition for two cancer cell lines (**a** MCF7, **b** A549) with the treatment of wild type (wild-type tiger milk mushroom),TM02 (sclerotium of *Lignosus rhinocerotis* cultivar), and CH3A3 (mycelium of *Lignosus rhinocerotis*)

the risk of environmental contamination and adulteration of closely related species, has encouraged methods of cultivation for this prized mushroom.

Successful cultivation technique has been reported by Tan (2009a) who cultured mycelium which is placed in a spawn container containing sawdust and buried in soil for the growth of sclerotia. However, the yield was low. Following the spawning container small-scale cultivation success, LiGNO™ Biotech Sdn. Bhd. in joint efforts with a group of scientists further developed a highly efficient method for tiger milk mushroom sclerotium cultivation in rice-based media using specially formulated culture medium consisting of water and other food-based materials. The cultivation is done using standard, sterile, and hygienic protocol, in a controlled environment and harvested in optimum condition. The latter method is highly successful, and LiGNO™ Biotech Sdn. Bhd. was subsequently recognized as the world's first commercial producer of *L. rhinocerotis*, being able to develop an in-house proprietary method for the quick cultivation and mass production of *L. rhinocerotis* (termed TM02). The prevailing successful cultivated method can produce a consistent supply of the *L. rhinocerotis* sclerotia.

As a result of the initiative of the company, tiger milk mushroom is now listed in traditional medicine active ingredient list under the Malaysian National Pharmaceutical Control Bureau in September 2010 and enabling *L. rhinocerotis* to be commercially available (Ligno LiGNO Biotech Sdn. Bhd. 2012). This cultivar has also been filed in 2011 under the New Plant Variety Protection (NPVP) of Malaysia, recognizing the cultivated variety as a distinct in comparison to that of the wild type.

A simplistic comparison of bioactivity between the wild type and LiGNO's cultivar TM02 was made by comparing the antiproliferative activity (more details in Sect. 5.8.1) (Fig. 5.6). The figure shows that the cultivated *L. rhinocerotis* contains at least three times the bioactivity of the wild type.

## 5.6 Cultivated *Lignosus rhinocerotis* TM02: The Safety Studies (Benefits to Science and Community)

Sclerotia of cultivated *Lignosus rhinocerotis* TM02 are authenticated using a rapid and reliable method. Easily amplified, short, standard genetic markers targeting the internal transcribed spacer (ITS) regions of the ribosomal RNA were developed and used for identification of the cultivated species to ensure that it is identical to the wild type (Tan et al. 2010). Safety studies were conducted (Lee et al. 2011, 2013) to ascertain the innocuous nature of the cultivated species.

### 5.6.1 Subacute Toxicity

Subacute toxicity was carried out in compliance with the guidelines from the Organization for Economic Cooperation and Development (OECD 1995). This was done using repeated doses of 250, 500, and 1000 mg/kg of cultivated *Lignosus*

*rhinocerotis* TM02 sclerotial powder. The highest dose used was 1000 mg/kg; this was chosen based on a preliminary 7-day acute toxicity studies where male and female rats ($n = 5$ each) fed with 2000 mg/kg of the sclerotial powder did not reveal any toxicity. There were no significant differences in the hematological parameters of rats fed with cultivated *Lignosus rhinocerotis* TM02 sclerotia throughout the duration of the 28 days of study and that of the control group. Hematological parameters such as red blood cell (RBC) count, hemoglobin, packed cell volume (PCV), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) of *Sprague Dawley* rats in the same group were also found to be normal. Clinical biochemistry revealed that oral consumption of cultivated *Lignosus rhinocerotis* TM02 did not affect the renal functions (urea and creatinine levels were evaluated), hepatic functions (albumin, total protein, SGOT, and SGPT were evaluated), serum electrolytes (calcium, sodium, potassium were evaluated), as well as glucose and total cholesterol levels. Histological examinations supported the clinical biochemistry results. There were no renal or liver damages. Pathological changes in the heart and spleen of the rats were also absent (Lee et al. 2011).

### 5.6.2 Chronic Toxicity, Genotoxicity, Antifertility, and Teratogenic Effects

The chronic toxicity study was carried out in compliance with the guidelines from the Organization for Economic Cooperation and Development (OECD) (2009). Oral administration of the cultivated *L. rhinocerotis* TM02 sclerotial powder at 250, 500, and 1000 mg/kg did not show any signs of toxicity. There were no significant differences in body weight, urinalysis, hematological examination, and clinical biochemistry, and there was no alteration worthy of note in the microscopic examinations of the organs. In the assessment of fertility and teratogenic effects, the cultivated sclerotial powder did not halt the pregnancy nor alter the number of offspring. It also did not produce any congenital malformation (a birth defect) on any offspring. A biological assay used to assess the mutagenic potential of cultivated *Lignosus rhinocerotis* TM02 sclerotial powder revealed that it did not cause gene mutations (Lee et al. 2013).

The recommended daily consumption of the cultivated *Lignosus rhinocerotis* TM02 as nutraceutical is approximately 0.5 g (on an assumption the average body weight is 50 kg). For cancer patients, ten times the amount of sclerotial powder is recommended (up to 100 mg/kg daily). These dosages were taken into consideration for the safety studies. The outcome of the studies shows that the no-observed-adverse-effect level (NOAEL) dose was more than 1000 mg/kg.

The demonstration of the safety profile of cultivated *Lignosus rhinocerotis* TM02 has open doors to more scientific investigations into the application of this medicinal mushroom as a potential nutraceutical, and possible target molecules can be identified for pharmaceutical use.

## 5.7 Nutritional Value of Wild-Type and Cultivated *Lignosus rhinocerotis* TM02

Wong et al. (2003, 2008) reported that the main components of the dry matter of wild-type *L. rhinocerotis* were mostly made up of carbohydrate (insoluble dietary fiber and non-starch polysaccharides) with low lipid content. In 2013, we reported the comparative nutrition of wild-type and cultivated *Lignosus rhinocerotis* TM02 (Table 5.3) (Yap et al. 2013). The energy value and protein content of cultivated sclerotia are 1.5× and 4.1× higher than the wild type, respectively. The amino acid composition of the protein found in the mushroom sclerotia was also analyzed (Yap et al. 2013). The total essential amino acid content of cultivated sclerotial powder was found to be significantly higher than the wild-type sclerotial powder (6.35 g/100 g dry weight vs. 1.65 g/100 g dry weight).

Carbohydrates are the major constituent of *L. rhinocerotis* sclerotia. Carbohydrate content in cultivated *Lignosus rhinocerotis* TM02 is slightly lower compared to the sclerotia of the wild type. The sclerotial carbohydrate constituent of the wild type is made up of 98.85% insoluble fiber, while the cultivated sclerotia contained 31% insoluble fiber, which is 40.26% of its total carbohydrate content. Further characterization of the sclerotial carbohydrate composition of wild-type and cultivated sclerotial powder using AOAC method shows that there were higher levels of β (1,3/1,6)-glucans in wild-type sclerotial powder, which is 99.78 % of its total glucan content and contains a very low amount of α-glucans. The cultivated sclerotial powder, on the contrary, contains a higher amount of α-glucans (80.44% of its total glucan content) with lower level of β(1,3/1,6)-glucans (14.56% of its total glucan content).

In our most recent study, we investigated the content of β (1,3/1,6)-glucans and α-glucans using Megazyme glucan test kit method. We found that the cultivated sclerotia contain 90.5 % β(1,3/1,6)-glucans and 9.5 % α-glucans. The contradictory results obtained using differing methods of assessment have been discussed by Mccleary and Draga (2016).

**Table 5.3** Comparative nutrition values of wild-type and cultivated *Lignosus rhinocerotis* TM02

| | Wild-type *L. rhinocerotis* | Cultivated *L. rhinocerotis* TM02 |
|---|---|---|
| Energy (kcal/100 g dry weight) | 201.0 | 308.0 |
| Proximate composition (g/100 g dry weight) | | |
| Crude protein | | |
| Fat | 3.4 | 14.1 |
| Carbohydrate | 0.1 | 0.8 |
| Dietary fiber | 93.3 | 77.0 |
| | 93.3 | 32.0 |
| Dietary minerals (mg/100 g dry weight) | | |
| Calcium | 3.7 | 19.3 |
| Magnesium | 75.8 | 147.9 |
| Potassium | 132.2 | 203.2 |
| Sodium | 8.5 | 8.8 |

**Table 5.4** Content of adenosine and its derivatives in cultivated *Lignosus rhinocerotis* TM02

| Component | Amount in mg/g |
|---|---|
| Adenosine | 0.282 |
| Cordycepin (3′-deoxyadenosine) | 0.873 |
| Adenine | 0.024 |
| Hydroxyethyl-adenosine | 0.268 |
| Ethyl-adenosine | 0.399 |

Among the major elements shown in Table 5.3, potassium is the most abundant mineral in the mushrooms sclerotia followed by magnesium. Calcium, magnesium, and potassium contents of cultivated *Lignosus rhinocerotis* TM02 sclerotia are 5.2×, 2.0×, and 1.5× higher than the wild type, respectively. However, the levels of sodium are comparable. We also determined the content of adenosine and its derivatives according to the method of Furuya et al. (1983) as shown in Table 5.4.

The comparative nutritional studies done thus far indicated that the cultivated *Lignosus rhinocerotis* TM02 sclerotia is superior to the wild-type sclerotia in overall nutritional content by having higher-energy value with more proteins and dietary minerals and is more palatable with the umami ratio higher than the mean ratio of 0.22 (Sun et al. 2012) (umami ratio of cultivated *Lignosus rhinocerotis* TM02 sclerotia was determined to be 0.25, while for wild-type sclerotia, the ratio was 0.20).

## 5.8 Bioactivities of Wild-Type and Cultivated *Lignosus rhinocerotis* TM02

### 5.8.1 Antiproliferative Activity

The antiproliferative activity of *Lignosus rhinocerotis* was first reported by Lai et al. (2008). Lai and colleagues reported the growth inhibitory activity of a polysaccharide-protein complex from wild-type *P. rhinocerus* (synonym to *L. rhinocerotis*) sclerotium against a panel of leukemic cell lines mediated by G1 phase cell cycle arrest. We subsequently reported the antiproliferative effect of a sclerotial cold water extract (CWE) from cultivated *L. rhinocerotis* TM02 against breast cancer (MCF7) and lung cancer (A549) cell lines (Fig. 5.6a, b), but not in the two corresponding human non-tumorigenic cell lines. We demonstrated that the antiproliferative activity was due to either the proteins or protein-carbohydrate complex in high-molecular-weight fraction (Lee et al. 2012). In our successive attempt to probe further into the bioactive components responsible for the antiproliferative activity, we characterized the chemical composition of the CWE and found it to contain 77 % carbohydrates and 1.2 % proteins. The extraction at a low temperature of 4 °C likely prevented the excessive degradation of thermolabile constituents including proteins and peptides. Our report was comparable to a cold water extract done reported by Lee et al. (2012) (with 75 % carbohydrates and 1.2 % proteins) and a cold alkaline extract of *P. rhinocerus* sclerotia (with 82 % carbohydrates and 1.3 % proteins) as reported by Lai et al. (2008). Interestingly, Lau et al. (2013) also demonstrated that a cold

aqueous extract preparation from the sclerotium of *L. rhinocerotis* KUM61075 exhibited cytotoxicity against various human cancer cell lines and the cytotoxic component(s) was deduced to be thermolabile, water-soluble protein/peptide(s) (Lau et al. 2013) as cytotoxicity of the cold aqueous extracts diminished when subjected to heat treatment from 60 to 100 °C for 20 min. Further investigations showed that proteins of medium molecular weight could be responsible for the antiproliferative action of *L. rhinocerotis* (unpublished results).

### 5.8.2 Anti-inflammatory Activity

We investigated the in vitro and in vivo anti-inflammatory activity (Lee et al. 2014). In vitro studies with CWE, along with its high- (HMW) and medium-molecular-weight (MMW) fractions, exhibited an inhibitory effect on TNF-alpha production in LPS-induced macrophages. We demonstrated that the cold water extract exhibited anti-acute inflammatory activity by reducing paw edema induced by carrageenan up to 200 mg/kg, in all three phases of edema development. The fashion in which the CWE resulted in anti-inflammatory action was similar to 10 mg/kg of indomethacin (a nonsteroidal anti-inflammatory drug) in all three phases. The CWE (200 mg/kg) showed ~88 % paw edema inhibition (greater than 10 mg/kg indomethacin) during all the phases of edema development. Further investigations showed that the anti-inflammatory activity was mainly contributed by high-molecular-weight fractions (with possible synergistic effect with lower-molecular-weight fractions) of the CWE. 35 mg/kg of high-molecular-weight fraction had comparable activity with 200 mg/kg of CWE. Cotton pellet-induced granuloma test used widely to assess transudative, exudative, and proliferative phase of inflammation (Swingle and Shideman 1972) revealed that 200 mg/kg of CWE did not inhibit the transudative and proliferative phase of chronic inflammation (Lee et al. 2014). In our attempt to elucidate the active principal causing the anti-inflammatory activity, we determined the ratio of protein and carbohydrate content of the most potent fractions (the HMW fraction). The ratio of protein to carbohydrate in the HMW fraction was 1:20. Further isolation of the protein components in the HMW fraction revealed that the isolated proteins contained a large amount of carbohydrate (1 protein to 8 carbohydrates, mostly alpha glucans). The nonprotein components (mainly alpha-glucan and 3.2 % beta-glucan) were devoid of anti-inflammatory activity. There is a possibility that the anti-inflammatory effect could be due to a polysaccharide-protein complex which has yet to be elucidated. Preliminary studies with the CWE on airway relaxation revealed that CWE was able to fully relax both the trachea and bronchus (unpublished results). Its link with anti-inflammatory activity and its possible application to respiratory ailment have yet to be established.

### 5.8.3 Antioxidative Activity and Presence of AGE Inhibitors

The antioxidant capacity of the various *L. rhinocerotis* sclerotial extracts (hot water (HWE), cold water (CWE), and methanolic (ME)) for both the cultivated and wild type was found to be generally comparable and very low reducing activity when compared to the positive controls (Yap et al. 2013). The inhibition of free radicals by the extracts was found to be dose dependent. The $IC_{50}$ values for DPPH• scavenging activity of the extracts were found to be generally comparable or even lower than most hot water extracts of medicinal mushrooms such as *G. lucidum*, *Lentinula edodes*, and *Pleurotus eryngii*, with $IC_{50}$ values, ranging from 5.28 to 19.09 mg/ml (Abdullah et al. 2012). In comparison with other extraction methods, methanolic extracts (ME) were found to exhibit higher activity in FRAP, DPPH•, and ABTS•+ assays in spite of their lower phenolic content in terms of mg GAE/g extract. This suggests that the extracts may contain other types of antioxidant/reducing compounds which might also contribute to their reducing/electron-donating ability (Mau et al. 2004). Similarly, the methanolic extract of *L. rhinocerotis* sclerotia showed remarkably potent O2•− scavenging activity in contrast with their lower activity in FRAP, DPPH•, and ABTS•+ assays which suggest the presence of other non-phenolic compounds that have the ability to scavenge O2•−.

In a separate study done to investigate the antioxidant and anti-glycation properties of the fractions of CWE, the sum of phenolic compounds of the three fractions (18.27 mg GAE/g) was found to be significantly lower compared to CWE (28.23 ± 0.50 mg GAE/g) and also possess weaker antioxidant activities than CWE, with the exception of O2•− scavenging activity. Degradation and loss of the phenolic compounds might have occurred during fractionation due to external factors such as light and air (Khoddami et al. 2013). The highest terpenoid content was found in low-molecular-weight fraction. This corresponded to the whole-genome analysis of cultivated *L. rhinocerotis* which shows a high amount of terpenoid biosynthesis genes (see Sect. 5.9). The MMW was shown to be a promising O2•− scavenger among the three fractions, with activity comparable to CWE and the positive controls suggesting the presence of compound(s) with SOD-like activity in the fraction. This provides support for the idea that complex polysaccharides and secondary metabolites such as phenolics and terpenoids which are abundantly present in HMW and LMW may not play a significant role in the potent O2•− scavenging activity of *L. rhinocerotis* sclerotium. All these results still did not give any conclusion as to what are the responsible agents for the antioxidant activity and warrant further investigations.

There is a positive correlation between glycation inhibitory activity and antioxidative potency as antioxidants have been shown to protect against glycation-derived free radicals and may possess therapeutic potential (Elosta et al. 2012; Ramkissoon et al. 2013). Our studies revealed that these fractions showed overall weaker glycation inhibitory activities than the CWE itself. The ability of the fractions to reduce glucose-induced AGE-derived fluorescence decreased in an order similar to their secondary metabolites content and antioxidant potential: LMW> MMW > HMW (unpublished results).

Even though studies to date has yet to elucidate the responsible agent as antioxidant, these preliminary results indicate that consumption of the whole *L. rhinocerotis* sclerotial powder is beneficial due to the synergistic effect of bioactive compounds and that long-term multi-antioxidant diet may possibly lead to the prevention of AGE-associated diabetic complications development and progression.

### 5.8.4 Immunomodulatory Activity

Liu et al. (2016) reported a novel water-soluble polysaccharide-protein complex (a mannoglucan-type heteroglycan-protein complex) isolated from the sclerotia of *Polyporus rhinocerus* which could significantly activate murine macrophages RAW264.7 in vitro. The signaling pathway involved was reported to be via the activation of ERK and AKT and iNOS (without NF-κB) together with the secretion of NO, IL-6, TNF-α, G-CSF, GM-CSF, MCPs, and MIP-1α. This is a subsequent report of their previous studies (Wong et al. 2009,2011) which documented the involvement of sclerotial polysaccharides (both water and alkaline soluble) to stimulate human innate immune cells. Guo et al. (2011) also reported hot aqueous extract of *L. rhinocerotis* sclerotial which promoted pinocytosis and increased the level of reactive oxygen species (ROS) and nitric oxide (NO), as well as the production of TNF-α. We have also reported a novel fungal immunomodulatory protein (FIP-Lrh) cDNA which was isolated from *L. rhinocerotis*, with the closest protein sequence identity to *G. lucidum* (64.55 %) and very similar predicted 3-D structure to FIP-fve from *Flammulina velutipes*, GenBank: ADB24832.1 (Pushparajah et al. 2016). Like other FIPs, it is a sugar-binding protein, and the more positively charged putative CBM pocket of FIP-Lrh predicted a stronger interaction with N-acetylgalactosamine and N-acetylglucosamine. A functional recombinant 6xHisFIP-Lrh was successfully produced in *E. coli* cells and was shown to be cytotoxic against several tested cells lines, notably the MCF7 cell line.

Consolidation of present reported results shows that *L. rhinocerotis* inarguably possesses immunomodulation activity for preventive and therapeutic potentials, such as anti-anaphylaxis and antitumor effect. Much remains to be explored in this area for the development and utilization of medicinal proteins and their subsequent connection with polysaccharides in their activity.

### 5.8.5 Neuritogenesis Activity

Studies have been done to investigate the neuritogenesis activity of *L. rhinocerotis* sclerotia for maintenance and regeneration of the neuronal communications network to subsequently be used as a preventative measure and as therapeutic agents for neurodegenerative disorders. Neuritogenesis was seen in PC-12 cells (pheochromocytoma of the rat adrenal medulla which has a mixture of neuroblastic cells and eosinophilic cells) using hot water extract of cultivated *L. rhinocerotis* sclerotia (Eik et al. 2012; Seow et al. 2015). The neuritogenic activity was comparable to nerve

growth factor (NGF) albeit at a much higher concentration (The hot aqueous extract (25 μ g/ml) stimulated neuritogenic activity that was comparable to NGF (50 ng/ml, 500×) and hence could mimic the neuritogenic activity of NGF and induce neuritogenesis in PC-12 cells via the NGF-responsive pathway. It is postulated that the neuritogenesis activity of hot aqueous extract may be mediated through the phosphorylation of TrkA receptor and ERK1/ERK2 signaling pathway in PC-12 cells.

### 5.8.6 Antiviral and Anti-microbial Activities

Wild-type *L. rhinocerotis* sclerotium has been screened for antiviral and antimicrobial activities (Mohanarji et al. 2012; Kavithambigai et al. 2013). It was reported that methanol and aqueous extracts (suggesting that the active components are polar) exhibited significant inhibition against several Gram-positive and Gram-negative bacteria at 30 mg/ml. The aqueous extract (at a concentration of 2.5 mg/ml) also showed moderate inhibitory effect (<40 %) against dengue virus type 2. There have been no other studies done thus far to substantiate the screening results reported.

## 5.9 Future Potentials with the Genome, Transcriptome, and Proteome Data of *Lignosus rhinocerotis*

We reported the genome of *Lignosus rhinocerotis* (Yap et al. 2014b) and revealed that it encodes 10,742 putative genes with 84.30 % of them having detectable sequence similarities to others available in public databases. It has a close phylogenetic relationship with medicinal mushrooms from the polyporoid clade, namely, the *Ganoderma lucidum*, *Dichomitus squalens*, and *Trametes versicolor*. Functional annotation of genes showed that the genome encodes for genes responsible for carbohydrate and glycoconjugate metabolism, cytochrome P450s, putative bioactive proteins (such as lectins, fungal immunomodulatory proteins, and laccases), and secondary metabolite biosynthesis, particularly sesquiterpenoid biosynthesis genes. The revelation of the genome contents has provided valuable insights into the biomolecule discovery and provided the foundation for future research and exploitation of *L. rhinocerotis* in pharmacological and functional food applications. For instance, the putative FIP genes (GME7566_g and GME10641_g) were cloned and expressed and subsequently characterized (structure modeling inclusive of carbohydrate binding site and binding affinity) (as discussed in Sect. 5.8.4). The bioactivity of recombinant FIP-Lrh produced in*E. coli* was also verified. Transcriptomic studies (Yap et al. 2015a) confirm the expression of the large number of transcripts involved in the processing of gene information; genes encoding for carbohydrate metabolism (biosynthesis of glucans), terpene synthases, non-ribosomal peptide synthetases, and polyketide synthases; production of cysteine-rich cerato-platanin, hydrophobins, and sugar-binding lectins; and cytochrome P450 sequences were identified. Systematic profiling and identification of proteins from proteomics studies (Yap

et al. 2015b) revealed only a few identified proteins with public databases using the matrix-assisted laser desorption/ionization coupled with mass spectrometry (MALDI-MS) which confirms that *L. rhinocerotis* proteins are indeed structurally quite different from other known fungal proteins. Using liquid chromatography coupled with mass spectrometry (LC-MS) with *L. rhinocerotis* genome as references database, more proteins were identified. These proteins play roles in nutrient mobilization and defense mechanisms. Putative lectins, immunomodulatory protein, aegerolysin, and antioxidant proteins such as Mn-SOD, CAT, and GST were also identified. These data forms a valuable foundation for future research in the exploitation of the *Lignosus rhinocerotis* in pharmacological and industrial applications.

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# Antioxidant Properties of *Antrodia cinnamomea*: An Extremely Rare and Coveted Medicinal Mushroom Endemic to Taiwan

6

K.J. Senthil Kumar and Sheng-Yang Wang

**Abstract**

*Antrodia cinnamomea* is an extremely rare and endemic fungal species native to forested regions of Taiwan. In modern Taiwanese culture, *A. cinnamomea* is believed to be a valuable gift from the heaven. Thereby, it is claimed as the "National Treasure of Taiwan" and "Ruby" among mushrooms." Traditionally, *A. cinnamomea* was used to prepare Chinese medicine for treating various illness including liver diseases, food and drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and tumorigenic diseases. Recent scientific studies strongly support that the pharmacological activities of *A. cinnamomea* go far beyond the original usage, as *A. cinnamomea* has exhibited various pharmacological properties including anticancer, antioxidant, hepatoprotection, antihypertensive, antihyperlipidemic, immunomodulatory, and anti-inflammatory properties. Till date, more than 400 scientific reports have been published on the therapeutic potential of *A. cinnamomea*, or its closely related species *Antrodia salmonea*, and their compounds. In the present review, the taxonomic description of *A. cinnamomea*, ethnomedical value, chemical constituents, and pharmacological effects particularly antioxidant and Nrf2-mediated cytoprotective effects will be discussed.

**Keywords**

Antioxidant • *Antrodia cinnamomea* • *Antrodia salmonea* • Cytoprotection • Medicinal fungus • Nrf2

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## Contents



## Abbreviations

| | |
|---|---|
| AAPH | 2,2-Azobis(2-amidinopropane)dehydrochloride |
| ALT | Alanine aminotransferase |
| ARE | Antioxidant responsible element |
| AST | Aspartate aminotransferase |
| COX-2 | Cyclooxygenase-2 |
| ERK | Extracellular signal-regulated kinase |
| GSH | Glutathione |
| HO-1 | Heme oxygenase-1 |
| IKK | Iκ-B kinase |
| iNOS | Inducible nitric oxide synthase |
| Iκ-B | Inhibitor of nuclear factor kappa-B |
| JNK | c-JUN N-terminal kinase |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| MDA | Malondialdehyde |
| NF-κB | Nuclear factor kappa-B |
| NO | Nitric oxide |
| Nrf2 | Nuclear factor E2-related factor-2 |
| ROS | Reactive oxygen species |
| SAPK | Stress-activated protein kinase |

## 6.1 Introduction

Free radicals and oxidants are recognized as having both toxic and beneficial components since they can be either harmful or helpful to the body. They are produced either from normal cell metabolism in situ or external sources such as physical

stress, pollution, cigarette smoke, alcohol, radiation, and medication (Pham-Huy et al. 2008). Oxygen is an indispensable element for life forms. Cells utilize oxygen to generate energy, and free radicals are generated as a consequence of adenosine triphosphate (ATP) production by the mitochondria (Pham-Huy et al. 2008). These by-products are reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox processes. However, not all reactive oxygen species are harmful to the body. Some of them are useful in killing and invading pathogens or microbes. The imbalance between the production of ROS and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants triggers oxidative stress (Bhattacharyya et al. 2014). Particularly, intracellular accumulation of relatively high concentrations of ROS induces oxidative damage in DNA and plays a major part in the development of chronic and degenerative ailments such as inflammation, cancer, aging, arthritis, autoimmune disorders, and neurodegenerative and cardiovascular diseases (Pham-Huy et al. 2008). The human body has been architected with several defense mechanisms to counteract oxidative stress by producing antioxidants. Some of the examples are superoxide dismutase (SOD), heme oxygenase-1 (HO-1), glutathione peroxidase (GPx), catalase, and γ-glutamylcysteine ligase (GCL; also known as glutamylcysteine synthetase), whereas nonenzymatic antioxidants are reduced by glutathione (GSH), α-tocopherol, ascorbic acid, ubiquinone, etc., or detoxifying enzymes including NAD(P)H:quinone oxidoreductase-1 (NQO1), glutathione-S-transferase (GST), epoxide hydrolase, and uridine-5-diphosphoglucuronyltransferase (UGT). These are either endogenously produced in situ or externally supplied through antioxidant-rich foods or nutraceutical supplements (Mikhed et al. 2015; Surh 2003; Kinnula et al. 1998). These endogenous and exogenous antioxidants act as "free radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents" by preventing and repairing damages caused by ROS and RNS. Therefore, antioxidants can enhance the immune defense and lower the risk of oxidative stress-related disorders (Bouayed and Bohn 2010; Pham-Huy et al. 2008).

Synthetic and natural antioxidants are currently used in food and pharmaceutical industries, especially those containing oils and fats to protect the substance against oxidation. A number of synthetic phenolic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are widely used in food, cosmetics, and pharmacological industries. In view of increasing risk factors of various deadly diseases to human beings, there has been a global trend toward the use of natural antioxidants derived from dietary vegetables or medicinal plants (Lobo et al. 2010; Sindhi et al. 2013). Also, there have been increasing evidences suggesting that intake of antioxidant-rich food or medicinal plants decreases the incidence of diseases in human. The use of naturally occurring antioxidants in pharmaceutical, nutraceutical, and cosmeceutical industries would be a promising alternative for synthetic antioxidants with respect to low cost, high compatibility with minimal side effects (Lobo et al. 2010). A number of naturally occurring antioxidant compounds from the plant sources have been identified as free radical scavengers or

electron donors. Several attempts have been made to study the antioxidant potential of a wide variety of vegetables like potato, spinach, tomatoes, and legumes and fruits such as olives, citrus, prunes, berries, and cherries. In recent years, the antioxidant properties of green and black teas have been extensively studied and reported to contain up to 30% of the dry weight as phenolic compounds (Lobo et al. 2010). Apart from the dietary sources, medicinal plants used in traditional Chinese medicine and Indian Ayurveda system also provide antioxidants, which protect the body from oxidative injury and boost the immune system (Shukla et al. 2012).

Mushrooms have been valued throughout the world as both food and medicine for thousands of years. Particularly, the Chinese and Egyptians were among the first people to appreciate the value of the mushroom as a specialty in the diet of the royal family. Apart from the diet, many of the world's more than 38,000 species of mushrooms have medicinal uses (Mayell 2001). Among the popular medicinal mushrooms such as *Antrodia cinnamomea*, *Antrodia salmonea*, *Ganoderma lucidum*, *Ophiocordyceps sinensis*, *Phellinus igniarius*, *Trametes versicolor*, *Lentinula edodes*, and *Wolfiporia extensa*, *A. cinnamomea* also known as "niu-chang chih" in Chinese is an extremely rare and endemic species native to forested regions of Taiwan. Taiwanese aborigines utilized this mushroom for treating liver diseases and for protection from food and drug intoxication (Ao et al. 2009; Liu et al. 2012). In modern Taiwanese culture, *A. cinnamomea* is believed to be a valuable gift from the heaven. Thereby, it is claimed as the "National Treasure of Taiwan" and "Ruby among mushrooms" (Geethangili and Tzeng 2011). Traditionally, *A. cinnamomea* was used to prepare Chinese medicine for treating various illness including liver diseases, food and drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and tumorigenic diseases (Ao et al. 2009; Geethangili and Tzeng 2011; Liu et al. 2012). Recent scientific studies have strongly supported that the pharmacological activities of *A. cinnamomea* go far beyond the original usage. As evidenced in several reports, *A. cinnamomea* exhibit various biological activities including anticancer, antioxidant, hepatoprotective, antihypertensive, antihyperlipidemic, immunomodulatory, and anti-inflammatory properties (Ao et al. 2009; Levin et al. 2012; Liu et al. 2012; Geethangili and Tzeng 2011; Lu et al. 2013; Yue et al. 2012, 2013). The first report of pharmacological activities of *A. cinnamomea* was published in 1995. New steroid acids were isolated from the fruiting bodies of *A. cinnamomea* exhibiting cytotoxicity to murine leukemia P-388 cells (Chen et al. 1995). After that, *A. cinnamomea* started attracting scientists' attention due to its richness in bioactive compounds such as polysaccharides, flavonoids, triterpenoids, maleic/succinic acid, benzenoids, and benzoquinone derivatives. The particular pharmacological interest in *A. cinnamomea* and its curative properties originated from the realm of traditional practice. Till date, nearly 400 scientific reports have been published regarding the therapeutic potential of *A. cinnamomea* or its compounds.

## 6.2 Taxonomic Description of *A. cinnamomea* and *A. salmonea*

Both *A. cinnamomea* and *A. salmonea* are parasitic fungi growing in the inner cavity of the endemic tree species *Cinnamomum kanehirae* (Bull camphor tree) Hayata belonging to the family Lauraceae (Geethangili and Tzeng 2011; Lu et al. 2013). Taxonomical description of *A. salmonea* is limited, while *A. cinnamomea* is well studied. The fruiting bodies of *A. cinnamomea* have various forms such as bell-like, hooflike, towerlike, or platelike. They are flat on the surface of wood at the beginning of growth (Fig. 6.1). Then the brim of the front edge rises to roll into plate shaped or stalactites (Geethangili and Tzeng 2011).

*A. cinnamomea* possesses a unique flavor resulting from a mixture of distinctive aromatic components. The odor is mainly because of the host tree *Cinnamomum kanehirae*. However, the pure mushroom does not possess a similar odor. *A. cinnamomea* also possesses a strong bitter taste due to its high triterpenoid content (Lu et al. 2013). The mycelia isolated from the fruiting bodies of *A. cinnamomea* form orange-red and orange-brown to light cinnamon-colored colonies. The hyphae of *A. cinnamomea* possess generative hyphae 2–3.5 $\mu$m. The host species, *C. kanehirae*, possess a high taxonomical importance since the species is endemic to Taiwan (Chang and Chou 1995).

After a long tradition usage, this species was rediscovered by Zang and Su (1990) and placed under genus *Ganoderma* due to the similarity in morphological features with *Ganoderma* species. Five years later, Chang and Chou described the species as *Antrodia cinnamomea*. The specific epithet alludes to the host tree. They properly

**Fig. 6.1** Fruiting bodies of *A. cinnamomea* (*orange colored*) growing in the inner cavity of the endemic tree species

placed their species in *Antrodia* because of its dimitic hyphae system with clamped generative hyphae and brown rot-causing ability (Chang and Chou 1995). However, the types of both *Ganoderma camphoratum* and *Antrodia cinnamomea* were found to be conspecific, while *A. cinnamomea* is reduced to a taxonomic synonym. A new nomenclature *Antrodia camphorata* was proposed by Wu et al. (1997), because the name *Ganoderma camphoratum* originally was based on a polypore with contaminating *Ganoderma* spores and hence *Antrodia cinnamomea* was reduced to a taxonomic synonym of *A. camphorata*. In 2004, a phylogenetic analysis based on sequence data obtained from large subunit (LSU) rDNA indicated that *A. camphorata* is not closely related to the genus *Antrodia* or *Antrodiella*. Therefore, this fungus was moved to the new genus *Taiwanofungus* and named as *Taiwanofungus camphoratus* (Wu et al. 2004). Further polymorphism analysis of international transcribed spacer (ITS) regions of rDNA of 11 *A. cinnamomea* strains revealed that *A. cinnamomea* belongs to the genus *Antrodia* (Chiu 2007). The present taxonomic status of *A. cinnamomea* is as follows *Fungi*, *Basidiomycota*, *Agaricomycetes*, *Polyporales*, *Fomitopsidaceae*, *Antrodia*, and *Antrodia cinnamomea* (Chang and Chou 1995). However, the nomenclature and exact taxonomy (genus and species) of *A. cinnamomea* is still the subject of debate and needs further research. In this article, we have chosen the name as *A. cinnamomea* to describe this unique medicinal mushroom.

## 6.3 Ethnomedical Value

Although *Antrodia cinnamomea* was used by Taiwanese aborigines for several centuries, however most of the anthropological studies did not clearly explain the historical origin of this mushroom as tribal folk medicine in Taiwan or elsewhere. It was originally used by the local tribes to treat food and drug intoxication and abdominal pain and to enhance liver function (Ao et al. 2009). However, its use in urban areas was limited owing to the lack of access and knowledge. In 1773 (38$^{th}$ year of Chien-Lung Years), during the Ching Dynasty, Dr. Wu-Sha was one of the famous physicians in Traditional Chinese medicinal system. Dr. Wu-Sha and his followers moved from Fujian province of China to the Yi-Lan, a northeast province of Taiwan. It was Dr. Wu-Sha who observed that the aborigines were suffering from a headache, hepatitis, and liver cirrhosis due to the frequent alcohol consumption. The locals often chewed the fruiting bodies of *A. cinnamomea* and used to drink its decoction to get relief from the alcoholic hangover. Dr. Wu-Sha adopted this traditional usage and applied to cure a number of illnesses like diarrhea, abdominal pain, hypertension, itchy skin, viral infection, stomatitis, diabetes mellitus, nephritis, proteinuria, hepatitis, liver cirrhosis, hepatoma, influenza, car sickness, calenture, and motion sickness (Levin et al. 2012). After its use for several centuries, the mushroom is now believed to be one of the most potent liver-protecting remedy in Taiwan (Geethangili and Tzeng 2011; Levin et al. 2012). Although primary ethnomedical data describing its liver-protecting ability was recorded in the ancient literature,

however, recently several studies have demonstrated that its pharmacological applications go far beyond the original usage.

## 6.4 Nrf2: A Key Regulator of Cytoprotection

The cellular defense system against oxidative stress can be achieved either by reducing the formation of reactive oxygen species (ROS) or stimulating their detoxification. Many xenobiotic-metabolizing enzymes are involved in both phase I (oxidation and reduction) and phase II biotransformation (conjugation) reactions (Rahman 2007; Khan et al. 2016). In general, by utilizing cytochrome p450 monooxygenases, the oxidative stress activation takes place primarily during phase I metabolism. The phase II reactions eliminate the harmful actions of phase I enzymes by reducing the electrophilicity of ROS through enzymatic conjugation with endogenous ligands such as glutathione and glucuronic acid (Lee and Surh 2005). A wide variety of phase II enzymes including heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO-1), glutathione synthetase, glutathione-S-transferase (GST), γ-glutamate cysteine ligase (γ-GCLC), γ-glutamyltranspeptidase, UDP-glucuronosyltransferase (UGT), aldo-keto reductase, microsomal epoxide hydrolase, leukotriene $B_4$ 12-hydroxydehydrogenase, and aldehyde dehydrogenase were induced by a number of antioxidants (Lee and Surh 2005; Surh 2003). The induction of the phase II enzyme system is an important event of the cellular stress response during which a diverse array of electrophilic and oxidative toxicants can be eliminated or inactivated before they can cause damage to critical cellular macromolecules (Lee and Lee 2011). Both basal and inducible expression of many of these antioxidant enzymes are regulated by the CNC-bZIP (cap'n'collar family of basic leucine zipper) transcription factor Nrf2 through the antioxidant response element (ARE).

Genomic analysis has revealed that the cis-acting ARE [5′-(G/A)TGA(G/C)nnnGC(G/A)-3′], a specific DNA-promoter-binding region, exists in the 5′ flanking region of genes encoding NQO-1, multiple GST and UGT isozymes, and epoxide hydrolase. It can be transcriptionally activated by numerous antioxidants and/or electrophiles (Lee and Surh 2005; Na and Surh 2014). Nioi et al. have reported that the ARE consensus sequences in the mouse NQO-1 promoter are necessary for its function. Moreover, recent gene array analyses have shown that a series of genes in mammalian cells can be regulated by ARE (Nioi et al. 2003).

Under basal conditions, Nrf2 is anchored in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap-1), which in turn mediates proteasomal degradation of Nrf2 by acting as an adaptor protein of the Cul3-based E3 ubiquitin ligase complex (Kang et al. 2004; Padmanabhan et al. 2008). Mild oxidative and electrophilic stresses disrupt the binding of Nrf2 and Keap-1 by modifying several cysteine residues of Keap-1, resulting in the accumulation of Nrf2 within the nucleus and further transactivation of ARE-bearing genes (Kansanen et al. 2013; Cho et al. 2002). Numerous comparative studies of the phenotypes of wild-type and Nrf2-disrupted mice have revealed the pivotal role of Nrf2 in protection against oxidant injuries.

Nrf2-disrupted mice have been much more susceptible to toxicities mediated by environmental chemicals and stresses than the wild-type mice (Cho and Kleeberger 2010; Johnson et al. 2008; Osburn and Kensler 2008).

One important Nrf2-target gene, HO-1, is a ubiquitous and redox-sensitive inducible stress protein that degrades heme to CO, iron, and biliverdin (Kobayashi and Yamamoto 2005). The importance of this protein in physiological and pathological states is underlined by the versatility of HO-1 inducers and the protective effects attributed to heme oxygenase products in conditions that are associated with moderate or severe cellular stress. HO-1 is recognized as a protective gene in the kidney involved in degradation of pro-oxidant heme, resulting in the production of anti-inflammatory, antioxidant, and anti-apoptotic metabolites (Abraham and Kappas 2008). The transcriptional activation of ARE-mediated Nrf2 is induced by various chemical compounds, including curcumin, resveratrol, genistein, capsaicin, caffeic acid, sulforaphane, quercetin, silymarin, lucidone, antroquinonol, etc. (Sung et al. 2011).

## 6.5 Pharmacological Activities of *A. cinnamomea* and *A. salmonea*

In the past two decades, extensive research has been carried on *A. cinnamomea* and *A. salmonea* extracts and its active components in vitro and in vivo. Several review articles have been published on the pharmacological applications and recent research and development on *A. cinnamomea* (Ao et al. 2009; Geethangili and Tzeng 2011; Levin et al. 2012; Liu et al. 2012; Lu et al. 2013; Yue et al. 2012, 2013). The current review presents the antioxidant and cytoprotective effects of the *A. cinnamomea* and its active components in different models of in vitro and in vivo studies. In particular, the Nrf2-mediated cytoprotective effects of *A. cinnamomea* and *A. salmonea* are emphasized.

Several researchers have reported the antioxidant and cytoprotective effects of *A. cinnamomea* and *A. salmonea* in various in vitro and in vivo test models. As summarized in Table 6.1, different extracts and culture conditions of these mushrooms have been found to exhibit antioxidant, hepatoprotective, neuroprotective, and anti-inflammatory activities. The protective effects of *A. cinnamomea* and *A. salmonea* on oxidative stress-induced pathological development of various diseases will be dealt in the following sections. For the preparations of *A. cinnamomea* and *A. salmonea* extracts, we need fruiting bodies and mycelium of *A. cinnamomea* and *A. salmonea*; fermented culture broth (FCBAC); aqueous, ethyl acetate, methanol, ethanol, $CHCl_3$/MeOH, and $CHCl_3$ extracts; submerged cultivation filtrate; and wild and solid-state cultures of *A. cinnamomea* and *A. salmonea.* Due to the lack of evidence of standard designed clinical studies, only some case reports from the medical conference will be described here.

A series of publications have appeared on the structural characterization of the secondary metabolites of *A. cinnamomea* and *A. salmonea*. Most of the investigators have studied the fruiting bodies, though there are a few publications on the

**Table 6.1** A summary of the studies conducted on the antioxidant potential of *A. cinnamomea* and *A. salmonea*

| Sample/s | Dosage and route | Study model | Observation/s | Reference |
|---|---|---|---|---|
| Culture filter extract of C in submerged culture | 0.2 mg/mL | Cell-free | Free radical scavenging | Song and Yen (2002) |
| Aqueous extract from AC | 12.5–50 μL | Erythrocytes, HUVECs, and HL-60 (in vitro) | AC inhibits AAPH-induced erythrocyte hemolysis, lipid peroxidation, and cell damage | Hseu et al. (2002) |
| Aqueous extracts of AC | 250, 750, and 1,250 mg/kg/day, 4 days/week (p.o.) | Male ICR mice (in vivo) | AC exhibits protection against chronic CCl4-induced hepatic injury through the antioxidant and free radical scavenging activities | Hsiao et al. (2003) |
| Dry matter of fermented filtrate from AC (DFAC) | 205 and 500 mg/kg/day (p.o.) | Male Sprague-Dawley rats (in vivo) | DFAC protects rats from CCl4-induced hepatotoxicity through the upregulation of hepatic phase II detoxifying enzymes and free radical scavenging | Song and Yen (2003) |
| Mycelia and fruiting bodies of AC | 500 and 1,000 mg/kg/day (p.o.) | Male Sprague-Dawley rats (in vivo) | AC protects rats from alcohol-induced acute liver injury through the induction of antioxidant enzymes | Dai (2003) |
| Methanolic extracts of mycelia of AC | 0.5–10 mg/mL | Cell-free | Free radical scavenging, reducing power and metal-chelating activities | Mau et al. (2003) |
| Methanolic extracts of mycelia of AC | 0.5–10 mg/mL | Cell-free | Free radical scavenging, reducing power and metal-chelating activities | Mau et al. (2004) |

(continued)

**Table 6.1** (continued)

| Sample/s | Dosage and route | Study model | Observation/s | Reference |
|---|---|---|---|---|
| Filtrate of fermented mycelia from AC (FMAC) | 500 and 1000 mg/kg/day (p.o.) | Male Wister rats (in vivo) | FMAC prevents CCL4-induced liver fibrosis *via* scavenging free radicals | Lin et al. (2006) |
| Fermented filtrate of AC (FFAC) | | Mice (in vivo) | FFAC inhibits CCL4-induced serum GTP levels in mice | Huang et al. (2006) |
| Fermented culture broth of AC and aqueous extract of mycelia of AC | 25–100 μg/mL and 50–100 μg/mL | HUVECs (in vitro) | HUVECs were protected from CuSO4 or AAPH-induced LDL oxidation | Yang et al. (2006) |
| Mycelia extract of AC | 500 and 1000 mg/kg/day (i.g.) | Male Sprague-Dawley rats (in vivo) | AC prevents alcohol-induced elevation of serum ALT, AST, ALP, and bilirubin | Lu et al. (2007) |
| Methanolic extract of AC irradiated with γ-rays | 0.5–10 mg/mL | Cell-free | Free radical scavenging, reducing power and metal-chelating activities | Huang and Mau (2007) |
| Water-soluble polysaccharides from AC in submerged culture | 200 μg/mL | Chang liver cells (in vitro) | Polysaccharides protects Chang liver cells from H2O2-induced oxidative injury through the upregulation of GST activity and free radical scavenging | Tsai et al. (2007) |
| Fermented culture broth of AC and aqueous extract of mycelia from AC | 25–100 μg/mL and 50–200 μg/mL | HUVECs (in vitro) | AC products prevents AAPH-induced apoptosis in HUVECS | Hseu et al. (2008) |
| Ethanolic extract of mycelia of AC (EMAC) | 250, 500, and 1000 mg/kg/day (p.o.) | Male ICR mice (in vivo) | EMAC protects mice from ethanol-induced liver injury through the induction of antioxidant and phase II enzymes vi Nrf2 signaling pathway | Kumar et al. (2011) |

(continued)

**Table 6.1** (continued)

| Sample/s | Dosage and route | Study model | Observation/s | Reference |
|---|---|---|---|---|
| Methanol extract of mycelia of AC (MEMAC) | 25, 50, and 75 μg/mL | RAW264.7 cells (in vitro) | MEMAC protects macrophage cells from immunogen-induced lipid peroxidation | Wen et al. (2011) |
| Methanol extract of mycelia of AC (MEMAC) | 5, 25, and 50 mg/kg (i.p.) | Male ICR mice (in vivo) | MEMAC inhibits λ-carrageenan-induced decrease on CAT, SOD, and GPx in mice | Wen et al. (2011) |
| Ethyl acetate extract of culture broth of AC | | Cell-free | Free radical scavenging, reducing power and metal-chelating activities | Wu et al. (2011) |
| Ethanolic extract of mycelia of AC (EMAC) | 250, 500, and 1000 mg/kg/day (p.o.) | Male ICR mice (in vivo) | Increased serum antioxidant capacity in alcohol-treated mice | Wang et al. (2013) |
| AC extracts | 250, 500, and 1000 mg/kg/day (p.o.) | Male Sprague-Dawley rats (in vivo) | AC alleviates endothelial lipid injury by inhibiting lipid peroxidation of ox-LDL and increase of HDL and SOD levels in high-fat diet rats | Qi et al. (2014) |
| Fermented culture broth of AS | 25, 50, and 100 μg/mL | HUVECs | Protects HUVECS against TNF-α-induced atherogenesis through the upregulation of Nrf2 signaling pathway | Yang et al. (2014) |
| Aqueous extracts of AC | 1–10 mg/mL | Cell-free | Free radical scavenging, reducing power and metal-chelating activities | Hsieh et al. (2015) |
| Crude oil from AC | | Cell-free | Free radical scavenging, reducing power and metal-chelating activities | Zhang et al. (2015) |

(continued)

**Table 6.1** (continued)

| Sample/s | Dosage and route | Study model | Observation/s | Reference |
|---|---|---|---|---|
| Fermented culture broth of AS | 25, 50, and 100 μg/mL | RAW264.7 cells | Inhibits LPS-induced inflammatory response through the upregulation of Nrf2 signaling pathway | Yang et al. (2015) |

constituents of the mycelia of *A. cinnamomea* in submerged cultures. The compounds identified are predominantly polysaccharides, benzenoids, diterpenes, triterpenoids, steroids, and maleic/succinic acid derivatives.

More than 80 compounds have been identified and structurally elucidated. Terpenoids are predominantly found in the fruiting bodies. Nearly 40 compounds have been reported. A few terpenoids have been found in mycelia from solid-state and submerged cultivation. There are about 30 triterpenoids with similar structures. A common feature of these structures is ergostane or lanostane skeleton (Geethangili and Tzeng 2011). Due to the high amount of terpenoids (63%) in the fruiting bodies of *A. cinnamomea* and *A. salmonea*, this group of natural compounds has been the focus of many phytochemical studies. In addition to polysaccharides, several other constituents such as benzenoids, lignans, quinone derivatives, and maleic/succinic acid derivatives have been described from *A. camphorata.* Also, sterols, nucleotides, and fatty acids have been found in these species. Furthermore, unique ubiquinone derivatives such as antroquinonol and 4-acetylantroquinonol B were isolated only from the cultured mycelia of *A. cinnamomea*. These compounds have never been reported from the fruiting bodies of *A. cinnamomea* or *A. salmonea*. The antioxidant potential of compounds isolated from these mushrooms has been summarized in Table 6.2.

### 6.5.1 Hepatoprotective Effects of Crude Extracts of *A. cinnamomea*

A number of scientific studies have demonstrated the potential of *A. cinnamomea* in the treatment of liver diseases, biologically active constituents responsible, and their mode of action (Ao et al. 2009; Geethangili and Tzeng 2011; Levin et al. 2012; Liu et al. 2012; Lu et al. 2013; Yue et al. 2012, 2013). In the present review, we have summarized the hepatoprotective effects of *A. cinnamomea* by modulating Nrf2 signaling pathway. A study by Kumar et al. (2011) evaluated the effect of the ethanolic extract of *A. cinnamomea* (EMAC) in ethanol-induced acute hepatotoxicity in mice. Ethanolic extracts of mycelia of *A. cinnamomea* (250, 500, and 1000 mg/kg BW, once a day for 10 days) were orally administered. At the end of the EMAC treatment, hepatotoxicity was induced by administering 3 doses of ethanol (5 g/kg BW) through oral gavage with 12 h interval. Serum biochemical analysis showed

**Table 6.2** A summary of the studies conducted on the antioxidant potential of phytocompounds derived from *A. cinnamomea*

| Chemical class | Compound name | Dosage and route | Study model | Observation | Reference |
|---|---|---|---|---|---|
| Lanostanes | Lanosta-8,24-diene-3β,15α,21-triol | – | PMA-activated leukocytes | Protects leukocytes through the induction of PCK-mediated antioxidant burst | Shen et al. (2006) |
| Naphthoquinones | 2,3-Dimethoxy-5-(2′,5′-dimethoxy-3′,4′-methylenedioxyphenyl)-7-methyl-[1] [4]-naphthoquinone | – | PMA-activated leukocytes | Protects leukocytes through the induction of PCK-mediated antioxidant burst | Shen et al. (2006) |
| | 2,3-Dimethoxy-6-(2′,5′-dimethoxy-3′,4′-methylenedioxyphenyl)-7-methyl-[1] [4]-naphthoquinone | – | PMA-activated leukocytes | Protects leukocytes through the induction of PCK-mediated antioxidant burst | Shen et al. (2006) |
| Benzenoids | Isobutylphenol | 500 μM | Cell-free | Free radical scavenging | Wu et al. (2007) |
| | Antrocamphin A | 100 μM | fMLP-induced neutrophils | Inhibits fMLP-induced production of superoxide anion | Chen et al. (2007) |
| Benzoquinone | 5-Methyl-benzo[1,3] dioxole-4,7-dione | 500 μM | Cell-free | Free radical scavenging | Wu et al. (2007) |
| | 2,3-Dimethoxy-5-methyl[1,4] benzoquinone | 500 μM | Cell-free | Free radical scavenging | Wu et al. (2007) |
| Phenylmethanoids | 4,7-Dimethoxy-5-methyl-1,3-benzodioxole | 62.5, 125, 250, and 500 μM | RAW264.7 cells | Inhibits LPS-induced inflammation in macrophage cells *via* induction of HO-1 | Shie et al. (2016) |
| | | 100 μM | fMLP-induced neutrophils | Inhibits fMLP-induced production of superoxide anion | Chen et al. (2007) |

(continued)

**Table 6.2** (continued)

| Chemical class | Compound name | Dosage and route | Study model | Observation | Reference |
|---|---|---|---|---|---|
| Polysaccharides | Neutral polysaccharides | 400 and 800 mg/kg/day (p.o.) | Male ICR mice (in vivo) | AC prevents *Propionibacterium acnes* and lipopolysaccharide-induced elevation of ALT and AST in mice | Han et al. (2006) |
| | Antrodan | 40 and 80 mg/kg (o.p.) | Male Sprague-Dawley rats | Antrodan protected against liver damage by suppressing LPS-stimulated serum ALT, AST, and NO through upregulation of CAT, SOD, and GPx | Ker et al. (2014) |
| | Polysaccharides | 4 μL | Cell-free | Free radical scavenging | Wu et al. (2007) |
| Steroids | Antcin C | 5, 10, and 20 μM | HepG2 cells | Protects liver cells from AAPH-induced oxidative stress through upregulation of antioxidant and detoxifying enzymes *via* Nrf2 signaling pathway | Gokila Vani et al. (2013) |
| | | 25, 50, and 100 mg/kg (i.p.) | Male ICR mice | Protects mice liver from AAPH-induced oxidative stress through upregulation of antioxidant and detoxifying enzymes *via* Nrf2 signaling pathway | Gokila Vani et al. (2013) |
| | Antcin A | 100 μM | fMLP-induced neutrophils | Inhibits fMLP-induced production of superoxide anion | Chen et al. (2007) |

| | | | | | |
|---|---|---|---|---|---|
| | Antcin B | 100 μM | fMLP-induced neutrophils | Inhibits fMLP-induced production of superoxide anion | Chen et al. (2007) |
| | Antcin M | | Neutrophils and microglial cells | Inhibits NADPH oxidase activity | Shen et al. (2007) |
| Ergostanes | Methyl antcinate L | – | Neutrophils and microglial cells | Inhibits NADPH oxidase activity | Shen et al. (2007) |
| | Methyl antcinate K | – | Neutrophils and microglial cells | Inhibits NADPH oxidase activity | Shen et al. (2007) |
| Triterpenoids | Eburicoic acid | 1, 5, and 10 mg/kg (i.p.) | Male ICR mice | Inhibits λ-carrageenan-induced decrease on CAT, SOD, and GPx in mice | Deng et al. (2013) |
| | | 5, 10, and 20 mg/kg (i.p.) | Male ICR mice | Inhibits $CCl_4$-induced decrease on CAT, SOD, and GPx and increase GSH level in in mice liver tissues | Huang et al. (2013) |
| | Dehydroeburicoic acid | 1, 5, and 10 mg/kg (i.p.) | Male ICR mice | Inhibits λ-carrageenan-induced decrease on CAT, SOD, and GPx in mice | Deng et al. (2013) |
| | | 5, 10, and 20 mg/kg (i.p.) | Male ICR mice | Inhibits $CCl_4$-induced decrease on CAT, SOD, and GPx and increase GSH level in in mice liver tissues | Huang et al. (2013) |
| Ubiquinone derivatives | Antroquinonol | 5, 10, and 20 μg/mL | HepG2 cells | Protects liver cells from alcohol-induced oxidative stress through upregulation of antioxidant and detoxifying enzymes *via* Nrf2 signaling pathway | Kumar et al. (2011) |

(continued)

**Table 6.2** (continued)

| Chemical class | Compound name | Dosage and route | Study model | Observation | Reference |
|---|---|---|---|---|---|
| | | 250, 500, and 1,000 mg/kg (i.p.) | Male ICR mice | Protects mice liver from alcohol-induced oxidative stress through upregulation of antioxidant and detoxifying enzymes *via* Nrf2 signaling pathway | Kumar et al. (2011) |
| | | 15 mg/kg (p.o.) | B-cell-deficient mice (B6.129S2-Igh-6tm1Cgn/J) | Antroquinonol promoted the Nrf2 antioxidant pathway and inhibited the activation of T cells and NLRP3 inflammasome | Yang et al. (2013) |
| | | 10, 30, and 100 mg/kg (o.p.) | APP transgenic mice | Protects against Aβ-induced oxidative stress and neuroinflammation through the activation of Nrf2 signaling pathway | Chang et al. (2015) |
| | | 50 mg/kg (p.o.) | BALB/c mice | Prevents focal segmental glomerulosclerosis (FSGS) through the inhibition of TGF-β-induced NF-kB activity *via* activation Nrf2 signaling pathway | Tsai et al. (2011) |
| | 4-Acetylantroquinonol B | 5, 10, and 20 μg/mL | HepG2 cells | Inhibits ALT, AST, and MDA levels and enhance GSH level through the upregulation of antioxidant enzyme *via* Nrf2 signaling pathway in alcohol-induced liver cells | Wang et al. (2013) |

that the ethanol-induced elevated levels of serum ALT and AST were significantly reduced by EMAC in a dose-dependent manner. Also, ethanol-induced increased levels of MDA and GSH depletion were prevented by the EMAC treatment. Treatment with EMAC (1,000 mg/kg BW) was comparable to those in the silymarin-positive group (200 mg kg BW). The levels of protein expression of heme oxygenase-1 (HO-1) and Nrf2 were found increased in groups orally administered with EMAC. This study suggested that the hepatoprotective effects of EMAC might be through a mechanism that involves Nrf2 activation and upregulation of the expression of the downstream antioxidant gene.

### 6.5.2 Antioxidant Potential of *A. salmonea*

The fermented culture broth extract of mycelia of *A. salmonea* (FMAS) showed potent antioxidant effects against LPS-induced oxidative stress and inflammation in murine macrophage cells (RAW264.7) *in vitro* (Yang et al. 2015). Incubation of macrophage cells with various doses of FMAS (25, 50, and 100 μg/mL) for 24 h did not show any cytotoxic effect on the macrophage cells. To measure the antioxidative potential of FMAS, the authors measured LPS-induced intracellular ROS accumulation using a $DCFH_2$-DA fluorescence microscopic analysis. Incubation of RAW264.7 cells with LPS (1 μg/mL) caused a significant increase in the intracellular ROS. However co-treatment with AS (25–100 μg/mL) resulted in a significant as well as a dose-dependent reduction in ROS accumulation. This data suggested that FMAS could suppress LPS-induced ROS generation in macrophages. Next, exposure of macrophages to AS (50 μg/mL) found to upregulate antioxidant genes such as HO-1 and NQO-1 in a time-dependent manner. The increase in HO-1 mRNA and protein levels was observed after 3 and 4 h, respectively. However, the increased levels of NQO-1 mRNA and protein levels were found 1 h after the FMAS treatment and then gradually decreased. In addition, total GSH level significantly increased after treatment with FMAS, correlating with the increased protein expression of γ-GCLC. Moreover, the authors found that FMAS treatment significantly increased the total Nrf2 expression in LPS-induced macrophages. Aberrant Nrf2 activation by FMAS was observed within 2 h, whereas the increase in Nrf2 expression gradually decreased when FMAS was applied at later time points. Further studies with immunofluorescence and luciferase reporter assays confirm that treatment with FMAS increased nuclear accumulation of Nrf2, thereby activating ARE-dependent transcription of antioxidant genes including HO-1, NQO-1, and γ-GCLC. These results strongly suggest that FMAS protects macrophages from LPS-induced oxidative stress and inflammation through the induction of Nrf2-mediated antioxidant genes. To further demonstrate the importance of FMAS-induced Nrf2 activation in LPS-induced macrophage cells, Nrf2 activity was determined with a Nrf2 knockdown model using siRNA transfection. The siRNA-induced reduction in Nrf2 was not altered by FMAS treatment even after 18 h. It means that the transfection with siNrf2 abrogated the protective effect of FMAS on LPS-induced production of pro-inflammatory cytokines in RAW264.7 cells

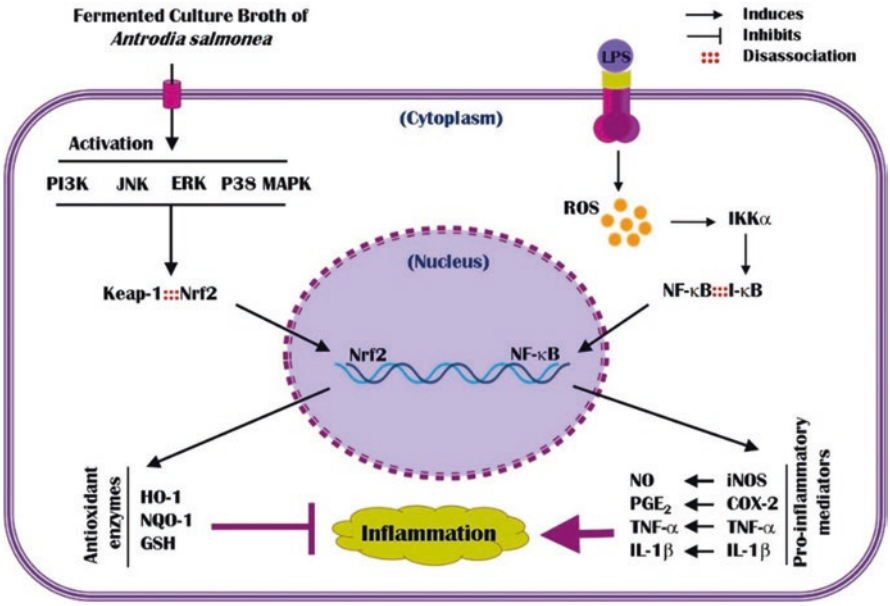


**Fig. 6.2** Fermented broth culture extracts of *A. salmonea* inhibits LPS-induced inflammation in murine macrophage cells *via* induction of Nrf2-mediated antioxidant genes (Yang et al. 2015)

(Fig. 6.2). This finding demonstrates that activation of Nrf2 is directly involved in FMAS-mediated anti-inflammatory effects in macrophages.

The same research group also demonstrated that FMAS protects human endothelial cells (EA.hy926) from TNF-α-induced oxidative stress and inflammation (Yang et al. 2014). The initial study showed that pretreatment with FMAS inhibited TNF-α-induced angiogenic and atherogenic factors such as the protein and enzymatic activity of matrix metalloproteinase-9 (MMP-9) and intercellular adhesion molecule-1 (ICAM-1), which are associated with reduced adhesion of U937 leukocytes to endothelial cells. FMAS treatment suppressed the TNF-α-induced transcriptional activation of nuclear factor κB (NF-κB) through the inhibition of nuclear export. Also, the data revealed that FMAS-mediated inhibition of NF-κB activity is associated with reduced IKKα phosphorylation and increased I-κBα degradation. In addition, the protective effect of FMAS was found to be highly correlated with the increased expression of heme oxygenase-1 (HO-1) and γ-glutamylcysteine synthetase (γ-GCLC), which was induced by the transcriptional activation of Nrf2/ARE. Furthermore, HO-1 knockdown by HO-1-specific shRNA diminished the protective effects of FMAS on TNF-α-stimulated invasion, tube formation, and U937 adhesion in EA.hy 926 cells. These data suggest that FMAS prevents TNF-α-induced NF-κB activation through the induction of antioxidant genes *via* a Nrf2 signaling cascade (Fig. 6.3).

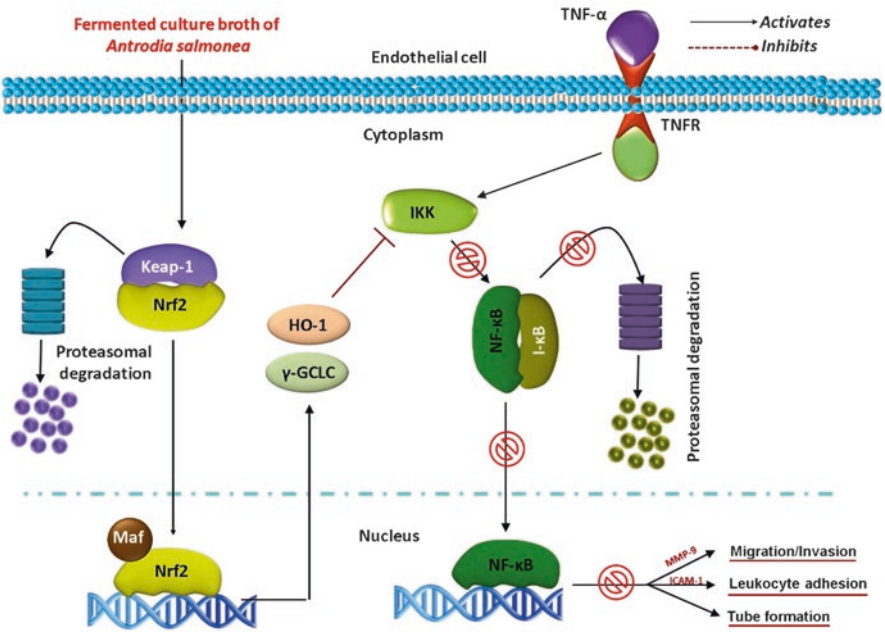


**Fig. 6.3** Fermented broth culture extracts of *A. salmonea* upregulate antioxidant gene expression *via* activation of Nrf2/ARE signaling pathway and suppression TNF-α-induced angiogenesis and atherogenesis in human endothelial cells (Yang et al. 2014)

### 6.5.3 Cytoprotective Effects of Antroquinonol

Antroquinonol (Fig. 6.4) is a ubiquinone derivative isolated from the mycelia of *A. cinnamomea*. Recent studies have indicated that ubiquinone derivatives are potent antioxidant agents and also have shown protection against cancer, male infertility, periodontal diseases, Parkinsonism, and cardiovascular diseases (Lu et al. 2013). Ubiquinone derivatives belong to a larger class of lipophilic benzoquinones. These are structurally correlated with vitamin K and are involved in cellular respiration (Saupe et al. 1994). One of the most extensively studied ubiquinone derivative is coenzyme $Q_{10}$, which is an important component for cell survival because it is a key intermediate in the electron transport system of mitochondria (Jimenez-Santos et al. 2014; Kumar et al. 2016). Recently, antroquinonol has attracted much attention due to its potent antioxidant and hepatoprotective effects.

Kumar and co-workers have reported that antroquinonol exhibits a potent liver protective effect against alcohol-induced oxidative stress *via* induction of phase II detoxifying enzymes and their corresponding regulatory factors which stabilize the hepatotoxic effect of alcohol. The potential liver protective efficacy of antroquinonol was evaluated in both in vitro and in vivo models to understand its mechanism of action (Kumar et al. 2011). In in vitro studies, it was found that pretreatment of human hepatic cells HepG2 with antroquinonol (5, 10, and 20 μM) eliminates

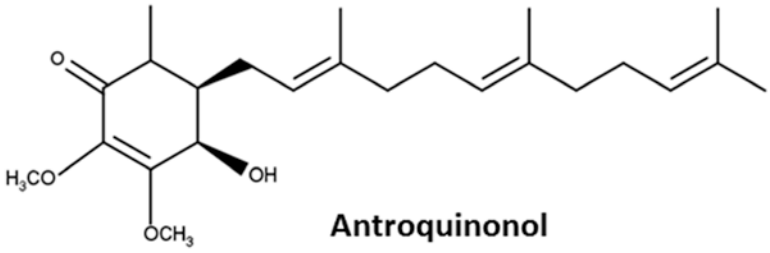


**Fig. 6.4** Chemical structure of antroquinonol

alcohol-induced alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as MDA elevation in a dose-dependent manner. This protective effect was highly comparable with silymarin (100 μM), a known hepatoprotective agent. Also, antroquinonol treatment rendered alcohol-induced ROS and nitric oxide (NO) production in a dose-dependent manner (5, 10, and 20 μM). A significant induction in HO-1 protein and mRNA levels was observed in antroquinonol-treated cells under ethanol treatment condition. It is well known that HO-1 expression was regulated by Nrf2-dependent ARE transcriptional activation (Morse and Choi 2002). Antroquinonol increased the nuclear translocation of Nrf2 as evidenced by accumulation of Nrf2 protein level in the nucleus. This study also revealed that Nrf2 activation was regulated by its upstream kinases including p38 MAPK and JNK/SAPK. Further an in vivo study with male ICR mice strongly supports the *in vitro* data that antroquinonol protects liver cells from alcohol-induced liver damage confirmed by reduced levels of ALT, AST, and MDA as well as increased level of GSH in the bloodstream or hepatic tissues (Fig. 6.5). Tsai et al. (2011) demonstrated that antroquinonol is a potent agent against focal segmental glomerulosclerosis (FSGS). A sequential event of ROS overproduction, inflammation, and fibrosis causes the formation of glomerulosclerosis (Assaily et al. 2011). Antroquinonol, isolated from the solid-state fermented mycelia of *A. cinnamomea*, exhibited a protective effect against FSGS-induced inflammation in experimental mice. Also, the FSGS-induced increase in urine protein and serum creatinine levels was inhibited by antroquinonol. A significant preventive effect against FSGS-mediated deposition of hyaline masses in the glomeruli, podocyte injury, sclerotic lesions, and expansion of cellular matrix was showed in antroquinonol treatment group.

Moreover, antroquinonol treatment blocked FSGS-induced ROS accumulation in the kidneys, glomeruli, and renal tubules. Furthermore, antroquinonol exhibited a significant increase of Nrf2 nuclear export and GPx secretion in mice kidney tissues. These findings suggest that antroquinonol protects kidney through Nrf2 activation thereby inhibiting NF-κB-dependent inflammatory pathway as well as suppressing TGF-β1-mediated fibrosis pathway in FSGS-induced mice.

Another study reported that antroquinonol protects mice kidney from preventing the development of accelerated and progressive IgAN (AcP-IgAN) through the inhibition of inflammasome and activation of Nrf2 (Yang et al. 2013). Excessive ROS generation, systemic T cell activation, and macrophage infiltration in the

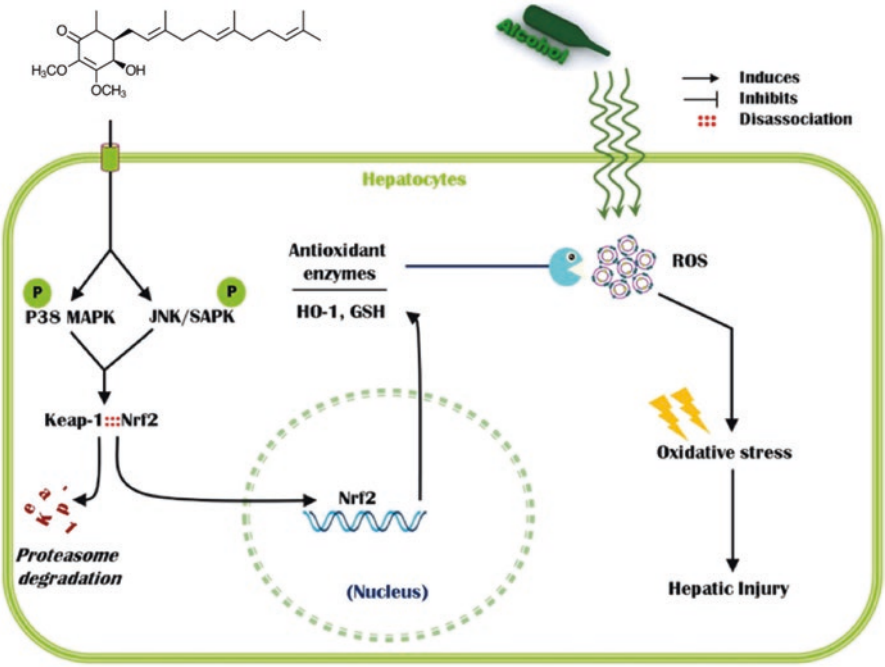


**Fig. 6.5** Schematic representation of antroquinonol-induced upregulation of antioxidant genes *via* Nrf2/ARE signaling pathway, which suppressed alcohol-induced oxidative stress and hepatic injury in human hepatic HepG2 cells

kidney implicated in the AcP-IgAN are the most frequent types of primary glomerulonephritis. AcP-IgAN was induced by daily injection of purified IgA antiphosphorylcholine antibodies and pneumococcal C-polysaccharide antigen (PnC) into B-cell-deficient (B6.129S2-Igh-6tm1Cgn/J) mice. Treatment with antroquinonol (15 mg/kg) reduced urine protein, serum blood urea nitrogen (BUN), and serum creatinine levels in AcP-IgAN-induced mice. Administration of antroquinonol administration substantially impeded the development of severe renal lesions, such as intense glomerular proliferation, crescents, sclerosis, and periglomerular interstitial inflammation, in mice with induced AcP-IgAN. In addition, AcP-IgAN mice showed elevated ROS levels in the serum, urine, and renal tissues, compared to normal controls. However, administration of antroquinonol completely inhibited the increase in serum ROS levels on day 3 and substantially inhibited the increase in ROS levels in the serum, urine, and renal tissues on day 28 compared to disease control mice. Moreover, renal cytoplasmic levels of HO-1 protein and GPx activity were increased by antroquinonol in AcP-IgAN-induced mice. Further mechanistic analysis in AcP-IgAN mice revealed that, during the early developmental stage of the AcP-IgAN model, treatment with antroquinonol augmented the transcriptional activity of Nrf2, thereby suppressing activation of T cells and the activity of NLRP3 inflammasome. Furthermore, antroquinonol treatment improved proteinuria/renal

function, and histopathological analysis supports the potential therapeutic effects of antroquinonol against kidney-related disorders.

Chang et al. (2015) demonstrated that antroquinonol ameliorate the Alzheimer's disease (AD)-like phenotype seen in amyloid precursor protein (APP) transgenic mice. Alzheimer's disease (AD) is the most common form among the chronic neurodegenerative diseases. Accumulation of brain amyloid-β peptides (Aβ), a 40–42 amino acid peptide cleaved from amyloid precursor protein (APP), triggers the pathophysiology of AD. Also, oxidative stress and neuroinflammation induced by Aβ play a critical role in the pathogenesis of AD. In earlier studies, antroquinonol has been reported to reduce oxidative stress and inflammatory cytokines *via* activating the Nrf2 signaling pathway, which was found in lower levels in AD. In this study, the authors found that treatment with antroquinonol improved AD-like pathological and behavioral deficits in the *APP* transgenic mouse. Further analysis showed that oral intake of antroquinonol was able to cross the blood-brain barrier without any adverse side effects. Morris water maze test results showed that consumption of antroquinonol for 2 months improved learning and memory process in mice, reduced hippocampal Aβ levels, and abrogates the degree of astrogliosis. These effects had high correlation with decreased levels of histone deacetylase 2 (HDAC2) and increased transcriptional activation of Nrf2. Together these data strongly suggest that antroquinonol could be a suitable candidate for the prevention of AD-like pathological and behavioral deficits.

### 6.5.4 Hepatoprotective Effect of Antcin C

Drinking alcohol is one of the social behaviors of human since the beginning of civilization. Frequent and high consumption of alcohol results in serious problems in the body including alcohol liver diseases (ALD) (Pari and Karthikesan 2007). ALD is the most common liver disease in Western countries, causing over 20,000 deaths per year in the USA alone. Many cascades were involved in ALD, including oxidative stress and mitochondrial damage (Gilpin et al. 2011). In the human body, ethanol is metabolized to acetaldehyde by a process of enzyme catalysis. The metabolized acetaldehyde is further oxidized into acetate and then converted into carbon dioxide through the citric acid cycle (Das and Vasudevan 2007). Ethanol also affects the immune system *via* modulating cytokine production, in turn decreasing total hepatic glutathione (GSH) and increasing the levels of hepatic triglycerides and lipid peroxidation. GSH is identified as a free radical scavenger and a regenerator of α-tocopherol and plays a significant role in the sustaining of protein sulfhydryl groups (Dey and Cederbaum 2006). Decreased hepatic GSH content results in the increased susceptibility to hepatic injury *via* induction of TNF-α (Fernandez-Checa et al. 2005).

Antcin C (Fig. 6.6), a steroid-like compound isolated from *A. cinnamomea*, protects human hepatic HepG2 cells against 2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress and apoptosis (Gokila Vani et al. 2013).

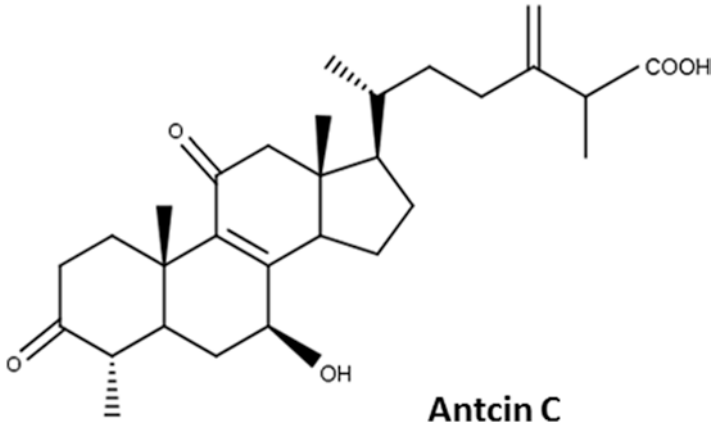


**Fig. 6.6** Chemical structure of antcin C

Exposure of HepG2 cells to 10 mM AAPH markedly increased apoptotic cell death followed by accumulation of intracellular ROS.

However, pretreatment with antcin C (5, 10, and 20 μM) protects hepatic cells from AAPH-induced cell death in a dose-dependent manner. The AAPH-induced accumulation of intracellular ROS was eliminated by antcin C. Also, pretreatment with antcin C prevents AAPH-induced lipid peroxidation, ALT, AST secretion, and GSH depletion in HepG2 cells. The antioxidant potential of antcin C was correlated with induction of antioxidant genes including HO-1, NQO-1, γ-GCLC, and SOD *via* transcriptional activation of Nrf2. The Nrf2 activation by antcin C is mediated by JNK1/JNK2 and PI3K activation, which was confirmed by the fact that pharmacologic inhibition of JNK1/JNK2 and PI3K abolished antcin C-induced Nrf2 activity. In addition, AAPH-induced apoptosis was inhibited by antcin C through the downregulation of pro-apoptotic factors including Bax, cytochrome c, caspase-9, caspase-4, caspase-12, caspase-3, and PARP. An in vivo study showed that 80 mg/kg of AAPH elevated serum ALT, AST, and hepatic lipid peroxidation and depletion of total GSH in ICR mice. Antcin C (25, 50, and 100 mg/kg) treatment protected mice liver from AAPH-induced hepatic injury as evidenced by a reduction in hepatic enzymes including ALT, AST, and MDA levels in circulation. Further, immunocytochemical analyses with mice liver tissues showed that antcin C increased HO-1 and Nrf2 protein expression in a dose-dependent manner. The hepatoprotective effect of antcin C was highly comparable with silymarin (200 mg/kg), a well-known hepatoprotective drug. These results strongly suggest that antcin C could protect liver cells from oxidative stress and cell death *via* Nrf2/ARE activation (Fig. 6.7).

### 6.5.5 Hepatoprotective Effect of 4-Acetylantroquinonol

A previous study by Kumar et al. (2011) reported that ethanol extract of mycelia of *A. cinnamomea* (EMAC) protected alcohol-induced liver injury in mice. Another

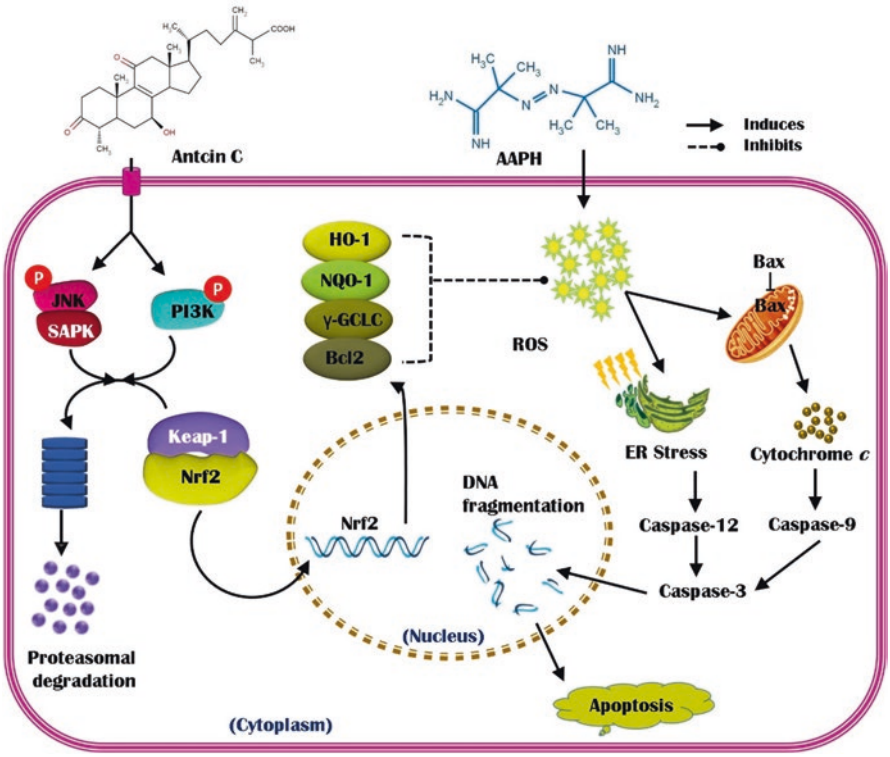


**Fig. 6.7** Schematic representation of antcin C-induced upregulation of antioxidative gene expression *via* the Nrf2/ARE signaling pathway and suppression of AAPH-induced apoptosis in human hepatic HepG2 cells

study by Wang et al. (2013) confirmed that EMAC from the solid-state culture increased serum total antioxidant capacity in LPS-induced mice. The metabolite profiling of EMAC revealed that it consists of nine primary metabolites, cytosine, uracil, cytidine, uridine, adenine, inosine, guanosine, adenosine, and deoxyadenosine, and five representative secondary metabolites, such as dehydroeburicoic acid, dehydrosulfurenic acid, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)-phenyl] furan-2,5-dione, antroquinonol, and 4-acetylantroquinonol B (Fig. 6.8). Quantification of these secondary metabolites showed that antroquinonol was the most abundant compound in the mycelium extract, followed by inosine, 4-acetylantroquinonol B, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione, dehydroeburicoic acid, and dehydrosulfurenic acid. Also, *in vitro* analysis showed that compared to antroquinonol, 4-acetylantroquinonol B exhibited potent anti-inflammatory effects by suppressing NO secretion in LPS-induced murine macrophage cells (RAW264.7).

Also, pretreatment with 4-acetylantroquinonol B downregulated LPS-induced iNOS and COX-2 protein expression in RAW264.7 cells. Next, the authors

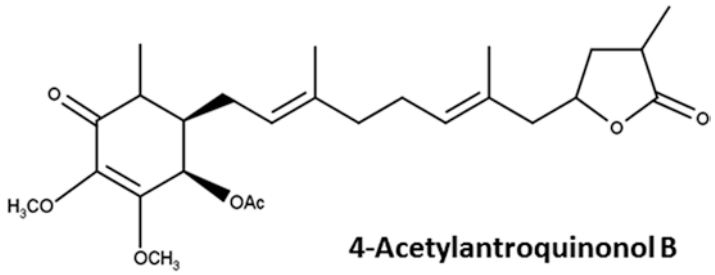


**Fig. 6.8** Chemical structures of 4-acetylantroquinonol B

examined the antioxidant potential of 4-acetylantroquinonol B *in vitro*. The noncytotoxic concentrations of 4-acetylantroquinonol B (5, 10, and 20 μg/mL) showed the protective effect on ALT, AST, and MDA production in EtOH-induced HepG2 cells. Also, the GSH levels were reduced in cultured HepG2 cells treated with ethanol, and pretreatment with 4-acetylantroquinonol B protected against hepatic GSH depletion, as evidenced by the restoration or accumulation of GSH above normal levels. The protection against alcohol-induced oxidative stress by 4-acetylantroquinonol B caused the increase in cellular antioxidant genes such as HO-1. It was found that the HO-1 protein levels significantly increased after incubation with 4-antroquinonol B in ethanol-induced HepG2 cells. Furthermore, it has been proved that HO-1 can be activated by Nrf2, a major transcription factor regulating antioxidant response element (ARE)-driven phase II gene expression. Also, 4-acetylantroquinonol B treatment increased Nrf2 accumulation in the nuclear fraction. These results support that 4-acetylantroquinonol B acts against an ethanol-induced hepatic oxidative stress, at least through activation of Nrf2 and induction of HO-1 expression.

## 6.6 Summary and the Future Perspectives

Recent pharmacological studies on *A. cinnamomea* and *A. salmonea* and their derived components have mostly been performed in vitro and in vivo. *A. cinnamomea* and *A. salmonea* extracts from its fruiting bodies, mycelium, and culture filtrates showed potent antioxidant, anti-inflammatory, and hepatoprotective effects. It took nearly 20 years since the introduction of *A. cinnamomea* to the mainstream research in Taiwan and to shift the research to next levels thus identifying chemical components responsible for its potential biological effects and their targets. However, the new species *A. salmonea* is just the beginning of the exploration. The studies carried out so far clearly demonstrate that compounds isolated from different conditions such as wild harvesting, solid-state cultivation of fruiting bodies, and submerged fermentation culture or parts including fruiting bodies and mycelia showed variation in chemical components. For example, antroquinonol can be found only in mycelia of *A. cinnamomea*, and the compound possesses several

biological activities such as cytotoxicity, anti-inflammation, antioxidant, and hepatoprotective effects. On the other hand, benzenoid and triterpenoids obtained from the fruiting bodies of *A. cinnamomea* showed potent anticancer effects on various cancer types in vitro and in vivo. Particularly, preclinical studies documented a number of antioxidant, and hepatoprotective components from *A. cinnamomea* or *A. salmonea* may provide novel and active medicinal products. Currently, a number of *A. cinnamomea*-based dietary supplements are available. However, the manufacturers cannot claim any specific health benefits, since human clinical trials for studying the effectiveness of *A. cinnamomea* and its active components are still in progress.

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# Part II

# Bioactive Compounds: Medicinal Plants and Fungal Endophytes

# The Fungal Endobiome of Medicinal Plants: A Prospective Source of Bioactive Metabolites

7

Sanjana Kaul, Suruchi Gupta, Supriya Sharma, and Manoj K. Dhar

**Abstract**

Fungal endobiome has over the period of time evolved from being defined just as the microbes living within plants indicating not only their location but also the type of association that they have with the host. They are the organisms that live asymptomatically within the internal tissues of the plant and exhibit a variety of relationships with their host ranging from symbiotic to pathogenic. They have a very intimate and also a co-evolutionary relationship with their host and therefore have the potential to influence the physiology of the plant. Endophytes from medicinal plants especially represent an important and potential source of bioactive compounds. Endophytic fungi under the influence of multiplexed interactions within its niche, viz., host plant, produce a plethora of secondary metabolites which belong to diverse chemical groups including terpenoids, alkaloids, phenylpropanoids, polyketides, peptides, flavonoids, steroids, lignans, etc. Terpenoids and polyketides are most commonly purified from endophytes, whereas flavonoids and lignans are rare. Due to chemical diversity of their secondary metabolites, endophytic fungi have been explored for medicinal, agricultural, and industrial uses. These are proven useful for novel drug discovery and can be used as potential sources of pharmaceutical leads. These metabolites are known to possess a wide variety of biological activities like antimicrobial, antioxidant, immunomodulatory, anticancerous, antidiabetic, acetylcholinesterase, etc. Endophytes are viewed as an outstanding source for bioprospecting new drugs, and their importance lies in the fact that they represent an ecological group which is less explored and develop in special and sequestered environments. Their diversity and specialized habituation make them an exciting field to search for novel bioactive compounds.

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**Keywords**
Fungal endophytes • Endobiome • Bioactive compounds • Medicinal plants • Bioactivity

## Contents



## Abbreviations

| | |
|---|---|
| AgNPs | Silver nanoparticles |
| DPPH | 1, 1-Diphenyl-2-picrylhydrazyl base pairs |
| $IC_{50}$ | Half maximal inhibitory concentration |
| MDR | Multidrug resistance |
| ml | Milliliter |
| MLR | Mixed lymphocyte reaction assay |
| sp. | Species |
| WHO | World Health Organization |
| α | Alpha |
| μg | Microgram |

## 7.1 Introduction

### 7.1.1 Endobiome

The plant endobiome consists of various microorganisms residing inside the endosphere, i.e., internal compartments of the plant. Various studies have significantly advanced the understanding of the composition and structure of plant microbiomes

which indicate that abiotic factors, as well as plant–microbe, microbe–microbe, and plant–plant interactions, contribute to plant endobiome composition and structure. The community structure of the endobiome depends on the combination of ability to colonize and the allocation of plant resources. The drivers for which include soil, host plant, and microbes. Factors such as latitude, elevation, temperature, and precipitation can also interact and influence the endobiome composition. The interactions between the endobiome and plant are highly complex and dynamic and can be beneficial (mutualistic), neutral (commensalism), or detrimental (parasitic). Consequently, the plant endobiome dramatically affects plant health and productivity (Turner et al. 2013; Berg et al. 2014; Schlaeppi and Bulgarelli 2015). The plant microbiome is known to induce or prime plant defenses against a broad range of pathogens and insect herbivores. Studies on the plant–microbe interaction involved in endosphere provide an alternative for the manipulations of different biosynthetic pathways responsible for the production of various bioactive and novel molecules of commercial importance. Additionally, the plant endobiome is a crucial player in global biogeochemical cycles, participating significantly in the biochemical cycling of the products of photosynthesis. Therefore, manipulation of plant endobiome is believed to have the potential to interfere with plant disease development, promote plant secondary metabolite production, and ease chemical inputs, leading to more sustainable agricultural practices and enhanced productivity.

Fungal endobiome can precisely be called as the endophytes and is defined functionally by their occurrence within tissues of plants without causing any immediate overt effects (Bacon and White 2000; Hyde and Soytong 2008). Endophytes are ubiquitous and have been found in all the species of plants studied to date. Host–endophyte interactions fall within a continuum ranging from mutualism to commensalism and ultimately pathogenicity. Mutualistic relationship provides benefit to both the partners. Endophytes get nutrition and shelter from the host, while endophyte contributes to the well-being of the host by providing adequate nutrient supply, improved growth, and resistance from herbivores, pathogens, drought, salinity, etc. Colonization of host plants by endophytic fungi can have a profound effect on the plant ecology, fitness, and plant community health (Gopalakrishnan et al. 2015). Enormous biological diversity coupled with their capability to biosynthesize secondary metabolites has provided the impetus for a number of investigations on endophytes. They are particularly interesting due to their easy biological utilization on the large commercial scale and have proven to be a promising source of novel and biologically active natural products, extracellular enzymes, and plant growth-promoting agents of biotechnological interest.

### 7.1.2 Fungal Endophytes

Over the period of time, the research on endophytes has taken a long leap, but the basic definition given by various researchers remains more or less the same, i.e., “Microbes that exist within the living tissues of plants intercellularly or

intracellularly, at least for a part of their life cycle without causing any harm to their host are known as endophytes" (Nisa et al. 2015). Endophytes in Greek means "within plant" (endo = within, phytes = plants). The term endophyte was first coined by De Bary in 1866 (Jain and Pundir 2015). The endophytic microorganisms which constitute the plant endobiome include bacteria, fungi, and actinomycetes which form a range of relationships with their host plant including symbiotic, mutualistic, commensalism, parasitic, etc. (Stepniewska and Kuzniar 2013; Swarnalatha et al. 2015). The infection caused by endophytes within the invading tissues of the host plant is inconspicuous and symptomless unless endophytes become pathogenic under stressful conditions. Some endophytes assume a quiescent state either for the whole lifetime or for an extended period of time. A harmonious symbiotic association generally exists between plant and endophyte in which both of them are benefitted. Most of the mycologists use the term "endophyte" strictly for those fungi that never cause any visible disease symptoms at any specific moment. Endophytes get nutrition and shelter inside the host and in return provide resistance to the concerned host against biotic and abiotic stresses. They can have a profound effect on the plant ecology, fitness, and plant community health. They reside entirely within host tissues and emerge during host senescence unlike mycorrhizal fungi that colonizes plant roots and grow into the rhizosphere.

The importance of endophytes lies in the fact that they represent the ecological group with less explored fungal species. Each plant, in turn, is host to one or more endophytes. As documented by Hawksworth (2001), only 1 lakh fungal species is presently known out of estimated 1.5 million fungal species. The remaining undiscovered fungi may be in the form of hidden endophytes. Endophytes develop in special and sequestered environments and represent a huge diversity of microbial adaptations. Their diversity and specialized habituation make them an exciting field of study in the search for new medicines or novel drugs. Endophytic fungal diversity is supposed to be high in tropics as compared to temperate regions. It is assumed that fungi within tropical tree leaves may be hyperdiverse. Their diversity even varies from one location to another.

Another interesting point in studying endophytes is because of the hope it brings, by synthesizing diverse and novel secondary metabolites. The new and bioactive compounds can provide assistance and relief in all aspects of human conditions like drug resistance, treatment of new emerging diseases, and safe bioactive compounds (Kaul et al. 2012). A list of all approved agents has been prepared from 1981 to 2006 in which microbes/endophytes were found to be the main source of a significant number of natural products (Pimentel et al. 2011). Synthesis of new active secondary metabolites by the endophytes may be induced by metabolic interaction between the host plant and endophytes.

The discovery of paclitaxel-producing endophytic fungus *Taxomyces andreanae* from *Taxus brevifolia* (yew trees) increased the importance of endophytes and created an impetus among the researchers for a more comprehensive and elaborative examination in this area (Selim et al. 2012). The presence of a microbial source of

the drug could eliminate the need to harvest and extract the slow growing and relatively rare yew trees, and the price for the drug would also be reduced. The drug would be available to cancer patients, since paclitaxel (taxol) could be produced via fermentation in the same way that penicillin is fermented (Strobel 2003). Fermentation of endophytic fungus producing bioactive compounds has several advantages like reproducible and dependable productivity. It can be grown in fermenters to provide an inexhaustible supply of bioactive compound and thus can be exploited commercially. Direct changes in the culture conditions can be explored as a method of optimizing various biosynthetic pathways that lead to the production of derivatives and analogs of novel compounds (Strobel et al. 2004). Later in the years to follow, taxol has been detected in numerous other endophytic fungi, isolated from diverse host plants. In addition to taxol, other medicinally important plant compounds have also been produced by endophytic fungi (Kaul et al. 2012).

There are many assumptions that endophytes uptake plant DNA into their own genome during their long co-evolution with host plants. This adaptation and genetic variation could have led to endophytes with the ability to synthesize phytochemicals originally associated with the host plant. Horizontal gene transfer makes the endophyte capable of producing associated plant compounds. For example, an endophyte *Fusarium solani* could produce precursors of camptothecin. However, in order to synthesize camptothecin, endophyte uses host's strictosidine synthase, an enzyme involved in the synthesis of camptothecin (Kusari et al. 2012). Initially, it was thought and hypothesized that the endophytic fungi do not synthesize paclitaxel independently but derived it from the host and later accumulates it in their cell walls (Heinig et al. 2013). On the contrary, in a recent study, a large set of potential genes involved in paclitaxel synthesis have been reported in *Penicillium aurantiogriseum* NRRL 62421, an endophyte of *Corylus avellana*. This study has however ruled out the possibility of horizontal gene transfer between endophytic fungus and host plant (Yang et al. 2014a).

Host–endophyte association is very important and influences the synthetic ability of endophytes. Under stressful conditions, endophytes affect the host plant in producing defense chemicals against invading pathogens. During this hostile environment, the ability to synthesize diverse and novel bioactive compounds by endophytes also increases. Thus, environment affects host plant which in turn affects endophyte to change its metabolite profile, increasing synthetic ability and overall biological activity of its secondary metabolites (Selim et al. 2012).

## 7.2 Fungal Endophytes from Medicinal Plants

Since time immemorial, medicinal plants have been used as a source of medicine. They produce unique and divergent secondary metabolites and harbor a distinctive microbiome (Qi et al. 2012). The endophytes that live inside the plant have distinct but similar metabolic pathway for the production of secondary metabolites. Inducing

factors from both plants and endophytes affect the accumulation of secondary metabolites. Researchers around the globe have been prompted to explore the medicinal plants for isolation of endophytes. This is because of the importance of secondary metabolites from medicinal plants in pharmaceuticals and their influence on the synthetic ability of endophytes. Endophytes are also known to mimic the bioactive compounds as produced by the plant itself. Therefore, it is significant to bioprospect endophytes from medicinal plants which have been used for centuries as a source of important bioactive compounds. Endophytes isolated from medicinal plants may result in the inexhaustible and cost-effective production of desired compounds and therefore help to conserve the biodiversity. Endophytes from medicinal plants are believed to be the source of unique and novel compounds associated with diverse biological activities (Jain and Pundir 2015). Their capability to biosynthesize bioactive secondary metabolites has provided the impetus on bioprospection of endophytes.

Different workers have investigated and documented the isolation of endophytic fungi and their metabolites from Indian medicinal plants. Eight medicinal plants of Western Ghats of India were sampled for endophyte isolation. Fifteen species were recovered, out of which *Alternaria* sp., *Nigrospora oryzae*, and *Papulospora* sp. showed antimicrobial activity (Raviraja et al. 2006). Similarly, medicinal plants of Jammu and Kashmir, namely, *Digitalis lanata*, *Digitalis purpurea*, *Plantago ovata*, *Dioscorea bulbifera*, and *Crocus sativus*, have been sampled for the isolation of endophytes (Ahmed et al. 2012; Sharma et al. 2015). Furthermore, 30 species of endophytic *Pestalotiopsis* sp. have been isolated from four medicinal plants *Terminalia arjuna*, *T. chebula*, *Azadirachta indica*, and *Holarrhena antidysenterica* (Tejesvi et al. 2007). Likewise, endophytic fungi from *Garcinia atroviridis*, *G. dulcis*, *G.mangostana*, *G. nigrolineata*, and *G. scortechinii* have been documented as the potential source of antimicrobial agents (Phongpaichit et al. 2006). Fungal endophytes isolated from indigenous medicinal plants belonging to North Maharashtra region of India have been investigated for antimicrobial activity. The isolates from roots of *Aloe vera* possess strong antibacterial activity (Jalgaonwala et al. 2010).

Chinese traditional medicinal plants have also been immensely explored for isolation of endophytes. Li et al. (2005) have studied 12 Chinese medicinal plants for fungal endophyte isolation. One hundred thirty endophytic fungi were reported, out of which 9.2% of the isolates exhibited antitumor activity and 30% exhibited antifungal activity. Similarly, Huang et al. (2007) have reported 292 morphologically distinct endophytic fungi from 29 Chinese medicinal plants. The endophytes recovered from *Ginkgo biloba* have shown different bioactivities like antimicrobial, antioxidant, cytotoxic, etc. (Li et al. 2014c; Ye et al. 2013; Yuan et al. 2013). Bioprospecting of fungal endophytes from different medicinal plants for the period 2010–2016 has been tabulated (Table 7.1).

**Table 7.1** Fungal endophytes from medicinal plants showing different bioactivities (Period: 2012–2016)

| S. no. | Medicinal plants | Fungal endophytes | Bioactivity | References |
|---|---|---|---|---|
| 1. | *Hugonia mystax* | *Aspergillus* sp. | Antimicrobial | Abirami and Boominathan (2016) |
| 2. | *Corchorus olitorius* | *Aspergillus terreus* | Extracellular enzymes | Ahmed et al. (2016a) |
| 3. | Marine habitat | *Aspergillus* sp. | L-asparaginase | Ahmed et al. (2016b) |
| 4. | *Sapium ellipticum* | *Chaetomium* sp. | Antimicrobial | Akone et al. (2016) |
| 5. | *Cymbopogon caesius* | *Curvularia lunata* | Antimicrobial | Avinash et al. (2016) |
| 6. | *Glycyrrhiza glabra* | *Phoma* sp. | Antimicrobial | Arora et al. (2016) |
| 7. | *Catharanthus roseus* | *Alternaria alternata* | Acetylcholinesterase inhibitory | Bhagat et al. (2016) |
| 8. | *Cupressus torulosa* | *Penicillium oxalicum* | Antidiabetic and antimicrobial | Bisht et al. (2016) |
| 9. | *Acanthospermum australe* | *Aspergillus calidoustus* | Antimicrobial | Carvahlo et al. (2016) |
| 10. | *Sonneratia ovata* | *Nectria* sp. | Antidiabetic | Cui et al. (2016) |
| 11. | *Nymphaea nouchali* | *Chaetomium globosum* | Antimicrobial | Dissanayake et al. (2016) |
| 12. | *Eichhornia crassipes* | *Aspergillus austroafricanus* | Antimicrobial | Ebrahim et al. (2016) |
| 13. | *Hydrastis canadensis* | *Alternaria* sp., *Colletotrichum fioriniae*, *Diaporthe eres*, *Diaporthe* sp., *Phoma* sp., and *Pyrenochaeta cava* | Antimicrobial | Egan et al. (2016) |
| 14. | *Tamarix nilotica* | *Aspergillus sydowii, Penicillium chrysogenum,* and *Eupenicillium crustaceum* | Not reported | Gashgari et al. (2016) |
| 15. | *Juniperus procera* | *Aspergillus fumigatus* | Antimicrobial | Gherbawy and Elhariry (2016) |
| | | *Hypocrea lutea* | | |
| | | *Penicillium oxalicum* and *Preussia* sp. | | |
| 16. | *Pteris pellucida* | *Emericella quadrilineata* | Antimicrobial | Goutam et al. (2016) |
| 17. | *Silybum marianum* | *Talaromyces minioluteus* | Antimicrobial | Kaur et al. (2016) |

(continued)

**Table 7.1** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Bioactivity | References |
|---|---|---|---|---|
| 18. | *Calotropis procera, Catharanthus roseus, Euphorbia prostrata, Trigonella foenum-graecum,* and *Vernonia amygdalina* | *Alternaria* sp. | Antimicrobial | Khiralla et al. (2016) |
| 19. | *Mentha viridis* | *Fusarium oxysporum* | Antimicrobial | Kumar et al. (2016) |
| 20. | *Nicotiana tabacum* | *Rhizopycnis vagum* | Antimicrobial | Lai et al. (2016) |
| 21. | *Panax notoginseng* | *Chaetomium globosum* | Acetylcholinesterase inhibitory | Lia et al. (2016) |
| 22. | *Mahonia fortunei* | *Fusarium decemcellulare* | Antimicrobial | Li et al. (2016a, b) |
| 23. | *Cephalotaxus hainanensis* | *Diaporthe* sp., *Phomopsis* sp., *Colletotrichum* sp., *Colletotrichum* sp., *Corynespora* sp. | Antimicrobial | Liu et al. (2016) |
| | | *Penicillium* sp. and *Nemania* sp. | | |
| 24. | *Salvia miltiorrhiza* | *Alternaria* sp. | Antimicrobial | Lou et al. (2016) |
| 25. | *Schima wallichii* | *Penicillium simplicissimum and Talaromyces verruculosus* | Antimicrobial | Mishra et al. (2016b) |
| 26. | *Rhizophora annamalayana* | *Trichoderma* sp. | Antimicrobial | Narendran and Kathiresan (2016) |
| 27. | *Pisonia grandis* | *Aspergillus niger, Aspergillus fumigates,* and *Aspergillus japonicus* | Antimicrobial | Nishanthi et al. (2016) |
| 28. | *Garcinia preussii* | *Penicillium* sp. | Antimicrobial | Jouda et al. (2016a) |
| 29. | *Ficus carica* | *Aspergillus tamarii* | Antimicrobial | Ma et al. (2016) |
| 30. | *Cinnamomum iners, Shorea siamensis, Fernandoa adenophylla, Quercus semiserrata* | *Xylaria* sp. | Antimicrobial | Orachaipunlap et al. (2016) |
| 31. | *Cinnamomum malabatrum* | *Colletotrichum gloeosporioides* | Antimicrobial | Packiaraj et al. (2016) |

| | | | | |
|---|---|---|---|---|
| 32. | *Houttuynia cordata* | *Chaetomium globosum* | Antimicrobial | Pan et al. (2016) |
| 33. | *Moringa oleifera* | *Aspergillus flavus* | Antimicrobial | Rajeshwari et al. (2016) |
| 34. | *Cupressus torulosa* | *Alternaria alternata* | Extracellular enzyme | Rajput et al. (2016) |
| 35. | *Hypocrea virens* | *Premna serratifolia* | Antimicrobial | Ratnaweera et al. (2016) |
| 36. | *Rauvolfia serpentina* | *Colletotrichum* sp. | Antimicrobial | Singh et al. (2016) |
| | | *Fusarium* sp. | | |
| | | *Cladosporium* sp. | | |
| 37. | *Datura innoxia* and *Hyoscyamus muticus* | *Aspergillus fumigatus, Aspergillus niger, Aspergillus terreus var. africanus,* | Antimicrobial | El-Said et al. (2016) |
| | | *Cladosporium cucumerinum, Cladosporium oxysporum, Penicillium aurantiogriseum,* and *Penicillium chrysogenum* | | |
| 38. | *Acalypha indica* | *Phoma* sp. | Antimicrobial | Sowparthani (2016) |
| 39. | *Santalum album* | *Fusarium oxysporum, Fusarium solani, Histoplasma* sp., | Antimicrobial | Tapwal et al. (2016) |
| | | *Periconia* sp. and *Pestalotiopsis* sp. | | |
| 40. | *Picea mariana* and *Picea rubens* | *Diaporthe maritime* | Antimicrobial | Tanney et al. (2016) |
| 41. | *Narcissus tazetta* | *Fusarium solani* | Antimicrobial | Wang et al. (2015) |
| 42. | *Huperzia serrata* | *Colletotrichum* sp., *Ascomycota* sp. | Acetylcholinesterase inhibitory | Wang et al. (2016b) |
| 43. | *Eugenia jambolana* | *Aspergillus niger* and *Aspergillus terreus* | Antimicrobial | Yadav et al. (2016) |
| 44. | *Lonicera japonica* | *Fusarium* sp. | Antimicrobial | Zhang et al. (2016b) |
| 45. | *Edgeworthia chrysantha* | *Fusarium oxysporum* | Antimicrobial | Zhang et al. (2016a) |
| 46. | *Sapium ellipticum* | *Penicillium tropicum* | Antimicrobial | Zeng et al. (2016) |

(continued)

**Table 7.1** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Bioactivity | References |
|---|---|---|---|---|
| 47. | *Panax notoginseng* | *Acremonium* sp. | Antimicrobial | Zheng et al. (2016) |
| | | *Alternaria* sp. | | |
| | | *Arthrinium* sp. | | |
| | | *Aspergillus* sp. | | |
| | | *Botryotinia* sp. | | |
| | | *Chaetomium* sp. | | |
| | | *Cladosporium* sp. | | |
| | | *Colletotrichum* sp. | | |
| | | *Dictyosporium* sp. | | |
| | | *Fusarium* sp. | | |
| | | *Humicola* sp. | | |
| | | *Ilyonectria* sp. | | |
| | | *Mucor* sp. | | |
| | | *Myrothecium* sp. | | |
| | | *Penicillium* sp. | | |
| | | *Periconia* sp. | | |
| | | *Pestalotiopsis* sp. | | |
| | | *Phialophora* sp. | | |
| | | *Phoma* sp. | | |
| | | *Phomopsis* sp. | | |
| | | *Plectosphaerella* sp. | | |
| | | *Thielavia* sp. and *Trichoderma* sp. | | |
| 48. | *Centaurea stoebe* | *Trichoderma* sp. | Antifungal, | Abdou and Abdelhady (2015) |
| | | | Cytotoxic | |

| | | | | |
|---|---|---|---|---|
| 49. | *Rhododendron anthopogon* | *Stemphylium* sp. | Antimicrobial | Baral et al. (2015) |
| | | *Alternaria* sp. | | |
| | | *Penicillium* sp. | | |
| | | *Aspergillus* sp. | | |
| | | *Trichoderma* sp. | | |
| | | *Papulaspora* sp. | | |
| | | *Hansfordia* sp. | | |
| | | *Wardomyces* sp. and *Geaotrichum* sp. | | |
| 50. | *Bauhinia forficata* | *Acremonium curvulum* | Antibacterial, Enzymatic | Bezerra et al. (2015) |
| | | *Aspergillus ochraceus* | | |
| | | *Gibberella fujikuroi* | | |
| | | *Myrothecium verrucaria* and *Trichoderma piluliferum* | | |
| 51. | *Curcuma longa* | 44 endophytic fungal isolates | Antioxidant | Bustanussalam et al. (2015) |
| 52. | *Annona crassiflora* | *Rhizoctonia* sp. | Antibacterial | De Mendonca et al. (2015) |
| 53. | *Carapa guianensis* | 35 distinct fungal taxa | Antibacterial | Ferreira et al. (2015) |
| 54. | *Mallotus philippensis* | *Alternaria* sp., | Antimicrobial | Gangwar et al. (2015) |
| | | *Pestalotiopsis* sp. and *Phomopsis* sp. | | |
| 55. | *Curcuma xanthorrhiza* | *Xylaria* sp. | Cytotoxic | Hammerschmidt et al. (2015) |
| 56. | *Kadsura angustifolia* | 42 fungal taxa | Extracellular enzymatic | Huang et al. (2015) |
| 57. | *Azadirachta indica* | *Chaetomium* sp. | Antioxidant | Kumaresan et al. (2015) |
| | | *Colletotrichum* sp. | | |
| | | *Curvularia* sp. and *Trichoderma* sp. | | |
| 58. | *Rauwolfia serpentina* | *Aspergillus awamori* | Antibacterial | Nath et al. (2015) |
| | | *Penicillium* sp. and *Colletotrichum gloeosporioides* | | |

(continued)

**Table 7.1** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Bioactivity | References |
|---|---|---|---|---|
| 59. | *Brucea javanica* | *Trichoderma* sp. | Not reported | Nur and Muh Danial (2015) |
| | | *Fusarium* sp. | | |
| | | *Aspergillus* sp. and *Penicillium* sp. | | |
| 60. | *Solanum xanthocarpum* | *Phomopsis vexans* | Lowering blood cholesterol (lovastatin) | Parthasarathya and Sathiyabama (2015) |
| 61. | *Aegle marmelos* | *Aspergillus flavus* | Antioxidant | Patil et al. (2015b) |
| | | | Antimicrobial | |
| 62. | *Silybum marianum* | 25 fungal taxa | Cytotoxic | Raja et al. (2015) |
| 63. | *Cyperus rotundus* | *Rhizoctonia solani* | Antibacterial | Ratnaweera et al. (2015a) |
| 64. | *Indigofera suffruticosa* | *Nigrospora sphaerica* and *Pestalotiopsis maculans* | Antibacterial | Santos et al. (2015) |
| 65. | *Withania somnifera* | *Fusarium* sp. | Antibacterial | Singh et al. (2015a) |
| 66. | *Limonia acidissima* | *Aspergillus* sp. | Cytotoxic | Siriwardane et al. (2015) |
| 67. | *Huperzia serrata* | *Paecilomyces tenuis* | Anti-Alzheimer's | Su and Yang (2015) |
| 68. | *Bacopa monnieri* | *Aspergillus fumigatus* | Antioxidant and antitubercular | Thakur et al. (2015) |
| 69. | *Alstonia boonei-Ahun, Enantia chlorantha-Awopa,* and *Kigelia africana-Pandoro* | *Aspergillus niger* | Antibacterial | Tolulope et al. (2015) |
| | | *Macrophomina* sp. | | |
| | | *Trichoderma* sp. and four different *Penicillium* sp. | | |
| 70. | *Dracaena draco* | *Botryodiplodia theobromae* | Antibacterial | Zaher et al. (2015a, b) |
| 71. | *Calotropis procera* | *Aspergillus niger* | Antibacterial | Aharwal et al. (2014) |
| | | *Cladosporium herbarum, Aspergillus tamari, Drechslera nodulosa, Fusarium solani* | | |
| | | *Aspergillus japonicas, Alternaria alternata, Alternaria tenuissima, Curvularia pallescens,* and *Curvularia lunata* | | |
| 72. | *Tabebuia argentea* | *Aspergillus niger* | Anticancer | Channabasava and Govindappa (2014) |

| | | | | |
|---|---|---|---|---|
| 73. | *Lantana camara* | Three endophytic strains | Enzymatic, phytochemical screening | Desire et al. (2014) |
| 74. | *Capsicum annuum* | *Alternaria alternata* | Cytotoxic | Devari et al. (2014) |
| 75. | *Gloriosa superba* | *Alternaria solani* and *Penicillium funiculosum* | Antimicrobial | Devi et al. (2014) |
| 76. | *Tabebuia argentea* | *Alternaria alternate* | Cytotoxic | Govindappa et al. (2014) |
| 77. | *Garcinia nobilis* | *Penicillium* sp. | Antibacterial | Jouda et al. (2014) |
| 78. | *Bacopa monnieri* | 26 endophytes | Antimicrobial cytotoxic | Katoch et al. (2014a, b) |
| 79. | *Xanthium sibiricum* | *Eupenicillium* sp. | Antibacterial | Li et al. (2014a) |
| 80. | *Ginkgo biloba* | *Chaetomium globosum* | Cytotoxic | Li et al. (2014c) |
| 81. | *Datura stramonium* | *Aspergillus* sp. | Antimicrobial | Mahdi et al. (2014) |
| | | *Curvularia* sp. | Antioxidant | |
| | *Moringa oleifera* and *Prosopis chilensis* | *Emericella* sp. and *Chaetomium* sp. | | |
| 82. | *Terminalia arjuna* | *Aspergillus flavus,* | Antimicrobial | Patil et al. (2014) |
| | | *Diaporthe arengae* | Antioxidant | |
| | | *Alternaria* sp. and *Lasiodiploidia theobromae* | | |
| 83. | *Polygala elongata* | *Colletotrichum* sp. | Antioxidant | Pawle and Singh (2014) |
| 84. | *Nothapodytes foetida* and *Hypericum mysorense* | *Bionectria ochroleuca* and *Chaetomium globosum* | Antibacterial | Samaga and Rai (2014) |
| | | | Antioxidant | |
| 85. | *Camellia sinensis* | *Colletotrichum* sp. and *Gloeosporioides* sp. | Antifungal | Rabha et al. (2014) |
| 86. | *Cynodon dactylon* and *Dactyloctenium aegyptium* | 26 fungal endophytes 30 fungal endophytes | Antimicrobial | Rekha and Shivanna (2014) |
| | | | Antioxidant | |
| 87. | *Saraca indica* | *Phomopsis* sp. | Antibacterial | Sandhu et al. (2014) |
| | | *Aspergillus terreus* | | |
| | | *Phialophora* sp. | | |
| | | *Alternaria alternata* and *Phyllosticta* sp. | | |

(continued)

**Table 7.1** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Bioactivity | References |
|---|---|---|---|---|
| 88. | *Cinnamomum mollissimum* | *Phoma* sp. | Antibacterial, | Santiago et al. (2014) |
| | | | Antifungal, | |
| | | | Cytotoxic | |
| 89. | *Adiantum capillus-veneris* | *Chaetomium globosum* | Cytotoxic | Selim et al. 2014 |
| | | | Antioxidant | |
| | | | Butyrylcholinesterase inhibitory | |
| 90. | *Allium sativum* | *Trichoderma brevicompactum* | Antifungal | Shentu et al. (2014) |
| 91. | *Curcuma longa* | *Penicillium* sp. | Antibacterial | Singh et al. (2014) |
| 92. | *Phyllanthus amarus* | 30 endophytic fungi | Antifungal, | Taware et al. (2014) |
| | | | Anticancer, | |
| | | | Anti-metastatic | |
| 93. | *Crotalaria pallida* | *Alternaria* sp. | Antioxidant | Umashankar et al. (2014) |
| | | *Penicillium* sp. and *Aspergillus flavus* | Antimicrobial | |
| 94. | *Madhuca indica* | 40 taxa | Antibacterial | Verma et al. (2014) |
| | | Dominant: *Phomopsis* sp. | | |
| | | *Colletotrichum* sp. and *Gloeosporioides* sp. | | |
| 95. | *Baccharis trimera* | *Diaporthe phaseolorum* | Antimicrobial | Vieira et al. (2014) |
| | | *Pestalotiopsis* sp. and *Preussia pseudominima* | | |
| 96. | *Boswellia ovalifoliolata* | 14 fungal species | Not reported | Anitha et al. (2013) |
| | | Dominant: | | |
| | *Pterocarpus Santalinus* | *Colletotrichum falcatum* | | |
| | *Shorea thumbuggaia* and *Syzygium alternifolium* | | | |
| 97. | *Bauhinia guianensis* | *Aspergillus* sp. | Antimicrobial | Pinheiro et al. (2013) |
| 98. | *Pandanus amaryllifolius* | *Colletotrichum* sp. | Antibacterial | Bungihan et al. (2013) |
| 99. | *Camptotheca acuminate* | *Botryosphaeria* sp. and *Fusarium* sp. | Antimicrobial | Ding et al. (2013) |

| | | | | |
|---|---|---|---|---|
| 100. | *Moringa oleifera* | *Alternaria* sp. | Not reported | Dhanalakshmi et al. (2013) |
| | | *Aspergillus* sp. | | |
| | | *Bipolaris* sp. | | |
| | | *Exophiala* sp. | | |
| | | *Nigrospora* sp. and *Penicillium* sp. | | |
| 101. | *Ceratonia siliqua* | *Penicillium citrinum* | Cytotoxic | El-Neketi et al. (2013) |
| 102. | *Tabebuia argentea* | *Aspergillus niger* | Antioxidant | Govindappa et al. (2013) |
| | | *Aspergillus flavus* | | |
| | | *Penicillium* sp. | | |
| | | *Rhizopus* sp. and *Fusarium* sp. | | |
| 103. | *Kigelia africana* | *Cladosporium* sp. | Antibacterial | Idris et al. (2013) |
| | | *Aspergillus flavus* | | |
| | | *Aspergillus* sp. and *Curvularia lunata* | | |
| 104. | *Antidesma madagascariense* | *Aspergillus* sp. *Guignardia* sp. | Not reported | Jeewon et al. (2013) |
| | | *Fusarium* sp. | | |
| | | *Penicillium* sp. | | |
| | | *Pestalotiopsis* sp. and *Trichoderma* sp. | | |
| 105. | *Withania somnifera* | *Chaetomium globosum* | Antifungal | Kumar et al. (2013) |
| 106. | *Jatropha curcas* | *Colletotrichum truncatum* | Antifungal | Kumar and Kaushik (2013) |
| | | *Nigrospora oryzae* | | |
| | | *Fusarium proliferatum* | | |
| | | *Guignardia cammillae* | | |
| | | *Alternaria destruens* and *Chaetomium* sp. | | |
| 107. | *Cannabis sativa* | 30 fungal species | Antifungal | Kusari et al. (2013) |
| | | Dominant: | | |
| | | *Penicillium copticola* | | |
| 108. | *Ocimum tenuiflorum* | *Penicillium citrinum* | Antibacterial | Lai et al. (2013) |

(continued)

**Table 7.1** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Bioactivity | References |
|---|---|---|---|---|
| 109. | *Zingiber zerumbet* | *Fusarium oxysporum* | Antioxidant | Nongalleima et al. (2013) |
| 110. | *Erythrina variegata* | *Alternaria* sp. | Antiangiogenic | Pompeng et al. (2013) |
| 111. | *Garcinia* sp. | *Aspergillus fumigates* and *Fusarium* sp. | Antimicrobial | Ruma et al. (2013) |
| | | | Antioxidant, | |
| | | | Anti-inflammatory | |
| 112. | *Viscum album* | *Aspergillus flavus* | Antioxidant | Sadananda et al. (2013) |
| | | *Fusarium oxysporum* | | |
| | | *Fusarium moniliforme* and *Trichothecium* sp. | | |
| 113. | *Ocimum sanctum* | 147 fungal endophytes | Antioxidant | Sharma and Kumar (2013) |
| 114. | *Elaeis guineensis* | *Trichoderma* sp. | Antifungal | Sundram (2013) |
| 115. | *Rhododendron tomentosum* | *Fusarium tricinctum* | Antibacterial | Tejesvi et al. (2013) |
| 116. | *Glycine max* | *Alternaria alternata* | Not reported | Tenguria and Firodiya (2013) |
| | | *Phoma* sp. | | |
| | | *Penicillium* sp. and *Fusarium* sp. | | |
| 117. | *Sauracia scaberrinae* | *Phoma* sp. | Antibacterial | Wijeratne et al. (2013) |
| 118. | *Panax ginseng* | *Nectria* sp. | Antibacterial | Wu et al. (2013b) |
| | | *Aspergillus* sp. | | |
| | | *Fusarium* sp. | | |
| | | *Verticillium* sp. | | |
| | | *Engyodontium* sp. | | |
| | | *Plectosphaerella* sp. | | |
| | | *Penicillium* sp. and *Cladosporium* sp. | | |
| 119. | *Ginkgo biloba* | *Chaetomium globosum* | Antioxidant | Ye et al. (2013) |
| 120. | *Huperzia serrata* | *Ceriporia lacerate* | Cytotoxic (ceriponols A– K) | Ying et al. (2013) |
| 121. | *Ginkgo biloba* | *Penicillium* sp. | Antioxidant | Yuan et al. (2013) |
| 122. | *Taraxacum mongolicum* | *Phoma* sp. | Antibacterial | Zhang et al. (2013a) |

| | | | | |
|---|---|---|---|---|
| 123. | *Annona muricata* | *Periconia* sp. | Cytotoxic (periconiasins A–C) | Zhang et al. (2013b) |
| 124. | *Terminalia brownii* | *Rhizophus oryzae* | Antimicrobial | Basha et al. (2012) |
| | | *Aspergillus niger* and *Aspergillus flavus* | | |
| 125. | *Ocimum sanctum* and *Sapindus detergens* | 63 endophytic fungal | Antibacterial | Bhagat et al. (2012) |
| | | Isolates | Anticancer | |
| 126. | *Nyctanthes arbor-tristis* | 19 endophytic fungi | Antimicrobial | Gond et al. (2012) |
| | | Dominant: | | |
| | | *Alternaria alternata* and *Cladosporium cladosporioides* | | |
| 127. | *Sapindus saponaria* | *Cochliobolus intermedius* and *Phomopsis* sp. | Antimicrobial | Garcia et al. (2012) |
| 128. | *Cinnamomum camphora* | 20 fungal species | Antimicrobial | Kharwar et al. (2012) |
| 129. | *Dysoxylum binectariferum* | *Fusarium proliferatum* | Anticancer (rohitukine) | Kumara et al. (2012) |
| 130. | *Ophiopogon japonicus* | 30 fungal strains | Antimicrobial | Liang et al. (2012) |
| 131. | *Emblica officinalis* | *Phomopsis* sp. | Antioxidant | Nath et al. (2012) |
| | | | Antimicrobial | |
| 132. | *Piper hispidum* | 21 isolates belonging to 11 genera | Not reported | Orlandelli and Pamphile (2012) |
| 133. | *Trichilia elegans* | *Cordyceps memorabilis* | Antimicrobial | Rhoden et al. (2012) |
| | | *Phomopsis longicolla* and *Dothideomycetes* sp. | | |
| 134. | *Arisaema erubescens* | *Phoma* sp. | Antimicrobial | Wang et al. (2012a) |
| | | | Antitumor | |
| 135. | *Curcuma wenyujin* | *Chaetomium globosum* | Antifungal | Wang et al. (2012b) |
| | | | Cytotoxic | |
| 136. | *Moringa oleifera* | *Nigrospora* sp. | Antifungal | Zhao et al. (2012a, b) |

## 7.3 Fungal Endobiome as a Source of Bioactive Metabolites

Endophytic fungi exist within a niche where it communicates with diverse communities of microorganisms. Variegated cross talks take place among endophytic fungi, endophytes and host, endophytic fungi and endophytic bacteria, etc. Under the influence of such multiplexed interactions and environmental conditions, a plethora of secondary metabolites is synthesized by the fungal endophytes (Kusari et al. 2014). Secondary metabolites are defined as small molecules that are not necessary for normal growth or development. Although it is not possible to reproduce such an array of diverse metabolites by endophytes under in vitro conditions, however, it is interesting that using controlled fermentation condition, by altering the accessible culture and process parameters (media composition, aeration, pH, incubation period, shaking conditions, inoculum size, etc.), the endophyte can be optimized for the production of surplus biologically active secondary metabolites (Kusari et al. 2012).

Secondary metabolites from endophytes have a tremendous impact on the society and proven useful for novel drug discovery and can be used as a potential source of pharmaceutical leads. These belong to diverse chemical groups including terpenoids, alkaloids, phenylpropanoids, aliphatic, polyketides, peptides, flavonoids, steroids, lignans, etc. Terpenoids and polyketides are most commonly purified from endophytes, whereas flavonoids and lignans are rare (Mousa and Raizada 2013). Due to chemical diversity of their secondary metabolites, endophytic fungi have been explored for medicinal, agricultural, and industrial uses. These metabolites are known for a wide variety of biological activities like antimicrobial, antioxidant, immunomodulatory, anticancerous, antidiabetic, antiviral, etc.

Some of the important categories of bioactive secondary metabolites produced by fungal endophytes of medicinal plants are as follows.

### 7.3.1 Anticancer Compounds

Cancer is a killer disease affecting more than six million people every year. It is characterized by unregulated cell proliferation. Due to uncontrollable growth of cells, an abnormal mass of tissue is formed which is generally called as a tumor. Antitumor agents are the compounds that are capable of counteracting the formation of malignant. Plant-based compounds have played an important role in the development of several clinically useful anticancer drugs like taxol, vinblastine, vincristine, topotecan, and etoposide (Nirmala et al. 2011). Despite this, there is a need to explore alternative source with more diversity and novelty. Endophytes with their unique secondary metabolites provide tremendous diversity. Active metabolites isolated from endophytes provide anticancer action with minimum side effects. These compounds could be an alternative approach for discovery of novel anticancer drugs (Kharwar et al. 2011; Kaul et al. 2012; Chen et al. 2014). Various anticancer compounds have been reported from fungal endophytes. For the sake of convenience, some of the anticancer compounds from fungal endophytes have been tabulated (Table 7.2).

**Table 7.2** Anticancer compounds from endophytic fungi of medicinal plants (2012–2016)

| S. no. | Medicinal plants | Fungal endophytes | Anticancer compound | Reference |
|---|---|---|---|---|
| 1. | *Piper hispidum* | *Diaporthe* sp. | (1 → 3,1 → 6)-D-glucans | Orlandelli et al. (2017) |
| 2. | Medicinal plant | *Bipolaris setariae* | Ophiobolin A | Bhatia et al. (2016) |
| 3. | *Acanthospermum australe* | *Aspergillus calidoustus* | Ophiobolin K and 6-epiophiobolin K | Carvalho et al. (2016) |
| 4. | *Uncaria rhynchophylla* | *Colletotrichum gloeosporioides* | Colletotrilactam A–D | Wei et al. (2016) |
| 5. | *Acanthus ilicifolius* | *Aspergillus flavipes* | Meroterpenoids (guignardones) | Bai et al. (2015) |
| 6. | *Hevea brasiliensis* | *Eutypella scoparia* | Cytochalasins | Kongprapan et al. (2015) |
| 7. | *Diphylleia sinensis* | *Aspergillus fumigates* | Fumitremorgin and fumitremorgin D | Liang et al. (2015b) |
| 8. | *Sinopodophyllum emodi* | *Alternaria tenuissima* | Podophyllotoxin | Liang et al. (2015a) |
| 9. | Mangrove plant | *Lasiodiplodia* sp. | Lasiodiplodins | Li et al. (2015b) |
| 10. | *Paris polyphylla var. yunnanensis* | *Aspergillus versicolor* | Versicolols A and B | Zhou et al. 2015 |
| 11. | *Tabebuia argentea* | *Aspergillus niger* | Lapachol | Channabasava and Govindappa (2014) |
| 12. | *Tripterygium wilfordii* | *Penicillium* sp. | Penifupyrone | Chen et al. 2014 |
| 13. | *Ginkgo biloba* | *Chaetomium globosum* | Chaetoglobosins A, G, V, Vb, and C | Li et al. (2014c) |
| 14. | *Ludwigia prostrata* | *Colletotrichum* sp. | Pyrenocines N–O | Yang et al. (2014b) |
| 15. | *Ceratonia siliqua* | *Penicillium citrinum* | Tanzawaic acids G–H, 6-methylcurvulinic acid, 8-methoxy-3, 5-dimethylisoquinolin-6-ol, and 1,2,3,11b-tetrahydroquinolactacide | El-Neketi et al. (2013) |
| 16. | *Ocimum tenuiflorum* | *Penicillium citrinum* | Two new alkaloids | Lai et al. (2013) |
| 17. | *Miquelia dentata* | *Fomitopsis* sp. | Camptothecin | Shweta et al. (2013) |
| 18. | *Tamarix chinensis* | *Penicillium* sp. | Arisugacins I and J | Sun et al. (2013) |
| 19. | *Mentha pulegium* | *Stemphylium globuliferum* | Altersolanol A | Teiten et al. (2013) |

(continued)

**Table 7.2** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Anticancer compound | Reference |
|---|---|---|---|---|
| 20. | *Taxus chinensis* | *Perenniporia tephropora* | Perenniporin A | Wu et al. (2013a) |
| 21. | *Astragalus lentiginosus* | *Emericella* sp. | Secoemestrin D | Xu et al. (2013) |
| 22. | *Avicennia* sp. | *Penicillium* sp. | 4-(methoxymethyl)-7-methoxy-6-methyl-1(3H)-isobenzofuranone | Yang et al. (2013) |
| 23. | *Bruguiera sexangula* | *Pestalotiopsis foedan* | (−)-(4S, 8S)-foedanolide and (+)-(4R, 8R)-foedanolide | Yang and Li (2013) |
| 24. | *Huperzia serrata* | *Ceriporia lacerate* | Ceriponols A–K, | Ying et al. (2013) |
| 25. | *Annona muricata* | *Periconia* sp. | Periconiasins A–C | Zhang et al. (2013) |
| 26. | *Cajanus cajan* | *Hypocrea lixii* | Cajanol | Zhao et al. (2013) |

### 7.3.2 Antioxidant Compounds

Oxidation is an essential process that utilizes oxygen and metabolizes macromolecules for energy production. Paradoxically, this vital mechanism may also lead to cell and tissue damage through production of free radicals and reactive oxygen species. These radicals get stabilized by reacting with cellular components including lipids, proteins, and DNA leading to impairment in their normal structure and function. This ultimately leads to the development of pathologies such as diabetes and cardiovascular and neurodegenerative diseases. An antioxidant is a molecule that slows down the oxidative damage caused by the free radicals and inhibits the deleterious effect caused by oxidation chain reaction. Antioxidant compounds can be obtained from plants, fruits, and vegetables. Since few antioxidants are approved for clinical application due to health safety issues, exploration of novel compounds from endophytes can be considered as an alternative source. Investigation of antioxidant compounds from endophytes gained importance after the discovery of pestacin and isopestacin as antioxidant compounds from endophyte *Pestalotiopsis microspore* residing in *Terminalia morobensis* (Harper et al. 2003). Since then, different studies on isolation of diverse antioxidant compounds have been reported from endophytes.

It is presumed that phenolic and flavonoid compounds are known to possess good antioxidant capacity. Huang et al. (2007) showed a positive correlation between the antioxidant capacity of *Chaetomium* sp., an endophyte of *Nerium oleander*, to phenolic and flavonoid compounds which were the major antioxidant constituents isolated from fungal extract (Huang et al. 2007). Similarly, *Xylaria* sp. isolated from *Ginkgo biloba* has been reported to show antioxidant activity. The activity was due to the presence of phenolic and flavonoid compounds present in the methanolic extract of the fungus (Liu et al. 2007). Similarly, *Chaetomium globosum* (CDW7), an endophyte of *Ginkgo biloba*, has been reported to synthesize antioxidant compound flavipin. The compound has been reported to be used in the therapy for free radical-associated diseases (Ye et al. 2013). Yuan et al. (2013) also isolated *Penicillium* sp. from roots of *Ginkgo biloba*. Six known metabolites have been obtained from this endophyte out of which three compounds, viz., adenosine, adenine, and 2-deoxyadenosine, exhibited potential DPPH scavenging activity.

Some new metabolites possessing antioxidant activity have also been reported from endophytes. In a recent study, phomopsidone A, a novel pentacyclic depsidone, has been reported from mangrove endophytic fungus *Phomopsis*. The compound exhibited antioxidant activity in addition to antifungal and cytotoxic activities (Zhang et al. 2014). Huang et al. (2012) also reported a new isobenzofuranone derivative 4, 6-dihydroxy-5-methoxy-7-methylphthalide from *Cephalosporium* sp. AL031 endophytic in *Sinarundinaria nitida*.

An exopolysaccharide, rhamnogalactan was obtained from endophyte *Fusarium solani* SD5 isolated from *Alstonia scholaris*. The compound showed the significant free radical scavenging effect on DPPH radicals with an $IC_{50}$ value of 578.5 μg/ml (Mahapatra and Banerjee 2014). In another study endophytes for antioxidant compounds glutaminase enzyme possessing free radical scavenging activity were

isolated from the endophytic fungus *Penicillium citrinum*. The $IC_{50}$ value of enzyme for DPPH, reducing power, nitric oxide, and hydroxyl radical scavenging activity were found to be 94.65, 117.73, 87.26, and 105.62, respectively (Sajitha et al. 2014). Antioxidant compounds palmarumycins C2 and C3 from endophyte *Berkleasmium* sp. Dzf12 and terrain from *Aspergillus terreus* have been reported in different studies (Mou et al. 2012; Al-Trabolsy et al. 2014). An antioxidant compound graphislactone A was obtained from endophyte *Cephalosporium* sp. IFB-E00, a resident of *Trachelospermum jasminoides*. The compound was confirmed to have stronger antioxidant activity in vitro as compared to butylated hydroxytoluene and ascorbic acid which were used as positive control (Song et al. 2005). Cajaninstilbene acid, a natural antioxidant, has been reported from *Fusarium*, an endophyte of pigeon pea *Cajanus cajan* (Zhao et al. 2012).

### 7.3.3 Antimicrobial Compounds

Secondary metabolites produced by fungal endophytes having antimicrobial activity are a promising way to overcome the increasing threat of drug-resistant microbes. These can also be used as food preservatives in the control of food spoilage and food-borne diseases (Liu et al. 2007). Antimicrobial metabolites (antibiotics) are low-molecular-weight organic compounds produced by microorganisms that are active at low concentrations against other microorganisms not required for its growth. They are produced as an adaptation for a specific function in nature and are the most frequent bioactive natural products isolated from endophytes.

*Penicillium* sp. has always been the important source of antimicrobial compounds. The literature reviewed revealed several examples where antimicrobial compounds have been reported from diverse species of *Penicillium*. Five new picolinic acid derivatives penicolinates A–E have been isolated from an endophytic fungus *Penicillium* sp. BCC16054. Penicolinates B and C have displayed activity against *Bacillus cereus* and *Candida albicans* (Intaraudom et al. 2013). Chemical investigation of *Penicillium citrinum*, a fungal endophyte of *Ocimum tenuifolium*, has led to the isolation of two new alkaloids along with 14 known polyketides and 4 known alkaloids. Perinadine A, alternariol, and citrinin were found to be moderately active against *Staphylococcus aureus* (Lai et al. 2013). Another *Penicillium* sp. isolated as endophyte of *Curcuma longa* has been reported to exhibit antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* (Singh et al. 2014). *Staphylococcus aureus* was also susceptible to antimicrobial compounds isolated from *Penicillium* sp., an endophyte of *Acrostichum aureum.* The compounds have been identified as cyclo(pro-Thr), cyclo(pro-Tyr), and liquiritigenin (Cui et al. 2008).

Various species of *Aspergillus* isolated as endophytes from different medicinal plants have been described as the promising source of antimicrobial compounds. *Aspergillus* sp. from *Bauhinia guianensis* yielded alkaloidal antimicrobial compounds pseurotin and fumigaclavine C. The latter was found to be active against *Bacillus subtilis* (Pinheiro et al. 2013). Nigerasterols A–B and malformins A–C

have been isolated from culture extract of *Aspergillus niger*, an endophyte of mangrove plant *Avicennia marina*. Malformins A–C displayed weak activity against *S. aureus* (Liu et al. 2013). Another mangrove endophyte *Aspergillus* sp. yielded antimicrobial compound asperterpenoid A. It exhibited strong inhibitory activity against *Mycobacterium tuberculosis* (Huang et al. 2013). *Aspergillus* sp. isolated from *Melia azedarach* afforded seven metabolites. All the isolated compounds have been evaluated against several phytopathogenic fungi and pathogenic bacteria. Compounds asperpyrone A, asperazine, and rubrofusarin B were found to inhibit fungal pathogen *Aspergillus solani*. Asperpyrone A also exhibited antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* with MICs of 25 mm (Xiao et al. 2014b).

A large number of diverse antimicrobial compounds have been reported from *Xylaria* residing in different host plants as endophytes. 7-Amino-4-methylcoumarin was obtained from *Xylaria* sp. YX-28, an endophyte of *Ginkgo biloba*, displayed antibacterial and antifungal activity against many pathogenic organisms (Liu et al. 2007). Three compounds chaetomugilin D, chaetomugilin A, and chaetoglobosin C were isolated from *Chaetomium globosum* endophytic in *Ginkgo biloba*. All of them exhibited significant activity against *Artemia salina* and *Mucor miehei* (Qin et al. 2009). Compounds 2-hexyl-3-methyl butanodioic acid and cytochalasin D possessing antifungal activity were recovered from endophytic *Xylaria* sp. The endophyte was isolated from *Palicourea marcgravii* (Cafeu et al. 2005). Likewise, *Xylaria* F0010, an endophyte of *Abies holophylla*, was found to be a potential producer of antifungal antibiotic agent griseofulvin. The compound has been used for the treatment of human and veterinary mycotic diseases (Park et al. 2005).

Endophytic *Phoma* sp. isolated from different medicinal plants has been reported to be a promising source of antimicrobial compounds. Santiago et al. (2012) have reported a polyketide compound 5-hydroxyramulosin from *Phoma* sp., an endophyte of *Cinnamomum mollissimum*. The isolated compound exhibited antifungal activity against *Aspergillus niger*. In another study mycelial extract of *Phoma* sp. NRRL 46751, inhabiting *Sauracia scaberrinae*, afforded three new alkaloids: phomapyrrolidones A–C out of which phomapyrrolidones B and C exhibited weak activity against *Mycobacterium tuberculosis* (Wijeratne et al. 2013). Similarly, a compound phomodione, an usnic acid derivative, was reported to be produced by *Phoma* sp. isolated from *Saurauia scaberrinae*. The compound displayed antibacterial activity against *Staphylococcus aureus* (Hoffman et al. 2008). Bioassay-guided fractionation of culture filtrate of fungal endophyte *Phoma*, isolated from *Taraxacum mongolicum*, led to the isolation of 2-hydroxy-6-methyl benzoic acid. The compound showed antibacterial activity against five bacterial test pathogens: *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Pasteurella multocida* (Zhang et al. 2013a). Four compounds, phomafuranol, phomalacton, (3R)-5-hydroxymellein, and emodin, were isolated from ethyl acetate extract of *Phoma* sp., a marine endophyte isolated from plant *Fucus serratus* (Hussain et al. 2014).

A broad diversity of endophytic fungi exists in the rhizome of *Paris polyphylla var. yunnanensis*, a medicinal plant used in traditional Chinese medicine. *Fusarium*

sp. Ppf4 from this plant yielded two sterols and one fatty acid by bioassay-guided fractionation. The compounds were elucidated as 5alpha, 8alpha-epidioxyergosta-6, 22-dien-3beta-ol and ergosta-8(9), 22-dien-3beta, 5alpha, 6beta, 7alpha-tetraol and displayed antimicrobial activity (Huang et al. 2009). *Fusarium redolens* DzF2 was isolated from Chinese medicinal plant *Dioscorea zingiberensis*. Beauvericin was obtained using bioautographic antibacterial assay. The compound displayed activity against six test bacteria: *Bacillus subtilis*, *Staphylococcus haemolyticus*, *Pseudomonas lachrymans*, *Agrobacterium tumefaciens*, *Escherichia coli*, and *Xanthomonas vesicatoria* (Xu et al. 2010). Taynung et al. (2011) have reported four compounds, 1-tetradecene, 8-octadecanone, 8-pentadecanone, and octylcyclohexane and 10-nonadecanone, from *Fusarium solani* isolated from *Taxus baccata*. All the compounds showed antibacterial as well as antifungal activity. *Fusarium tricinctum* was isolated from *Rhododendron tomentosum*. Transcriptome of this endophyte was sequenced; 12,006 contigs were assembled. On analyzing transcriptomic library, it yielded a peptide resin. The compound was found to be active against *Staphylococcus carnosus*, *Candida albicans*, and *Candida utilis* (Tejesvi et al. 2013).

*Colletotrichum* is one of the important genus frequently isolated as an endophyte from different hosts. The endophyte has been investigated during different studies for the isolation of antimicrobial compounds. Colletotriolide a new macrolide was obtained from *Colletotrichum* sp. residing in *Pandanus amaryllifolius*. The compound showed low activity against *E. coli* (Bungihan et al. 2013). Similarly, Chithra et al. (2014) have reported the ability of *Colletotrichum gloeosporioides* to produce piperine, a compound originally synthesized by the host plant *Piper nigrum*. The compound has antimicrobial activity. One new compound 2-phenylethyl 1H–indol-3-yl-acetate was obtained from endophyte *Colletotrichum gloeosporioides* isolated from *Michelia champaca.* The compound possessed antifungal activity against *Cladosporium cladosporioides* and *C. sphaerospermum* (Chapla et al. 2014). Some of the antimicrobial compounds isolated from fungal endobiome have been discussed, and the rest of the data has been tabulated (Table 7.3).

### 7.3.4 Immunomodulatory Compounds

Immunomodulatory compounds are those compounds that help in modulating the immune system either by stimulating it or suppressing it. Immunosuppressive compounds are required to deal with autoimmune disorders and allograft rejection in transplant patients. Immunomodulatory drugs play a key role in the treatment of cancer. Due to the emergence of new autoimmune disorders and their role in the treatment of cancer, an intensive search is going on for more effective agents that provide aid in this regard. Since fungal endophytes have the capacity to produce novel compounds, these could prove a useful source for potentially active immunomodulatory compounds (Kaul et al. 2012). Several immunomodulatory compounds have been reported from fungal endophytes in the recent past.

**Table 7.3** Antimicrobial compounds from fungal endophytes comprising the endobiome of some medicinal plants

| S. no. | Medicinal plants | Fungal endophytes | Extract/compound isolated | Reference |
|---|---|---|---|---|
| 1. | *Hugonia mystax* | *Aspergillus* sp. | Ethanol | Abirami and Boominath (2016) |
| 2. | *Sapium ellipticum* | *Chaetomium* sp. | Polyketides | Akone et al. (2016) |
| 3. | *Cymbopogon caesius* | *Curvularia lunata* | Ethyl acetate | Avinash et al. (2016) |
| 4. | *Glycyrrhiza glabra* | *Phoma* sp. | Thiodiketopiperazine derivatives | Arora et al. (2016) |
| 5. | *Cupressus torulosa* | *Penicillium oxalicum* | Methanol and chloroform | Bisht et al. (2016) |
| 6. | *Acanthospermum australe* | *Aspergillus calidoustus* | Ophiobolin K and 6-epiophiobolin K | Carvahlo et al. (2016) |
| 7. | Mangrove plants | *Talaromyces amestolkiae* | Isocoumarins and benzofurans | Chen et al. (2016) |
| 8. | *Nymphaea nouchali* | *Chaetomium globosum* | Chaetoglobosin A and C | Dissanayake et al. (2016) |
| 9. | *Hydrastis canadensis* | *Alternaria* sp., *Colletotrichum fioriniae,* | Alternariol, alternariol monomethyl ether, 50 epi-equisetin, equisetin, 10–11 dehydrocurvularin, macrosphelide A, cordipyridone A verticillin A, aurofusarin | Egan et al. (2016) |
| | | *Diaporthe eres, Diaporthe* sp., *Sordariomycetes* sp., *Magnaporthales* sp., *Phoma* sp., and *Pyrenochaeta cava* | | |
| 10. | *Eichhornia crassipes* | *Aspergillus austroafricanus* | Diphenyl ether | Ebrahim et al. (2016) |
| 11. | *Datura innoxia* and *Hyoscyamus muticus* | *Aspergillus fumigatus, A. niger, A. terreus var. africanus, Cladosporium cucumerinum, C. oxysporum, Penicillium aurantiogriseum,* and *P. chrysogenum* | Chloroform | El-Said et al. (2016) |
| 12. | *Juniperus procera* | *Aspergillus fumigatus*, *Hypocrea lutea*, *Penicillium oxalicum*, and *Preussia* sp. | Methanol | Gherbawy and Elhariry (2016) |
| 13. | *Pteris pellucida* | *Emericella quadrilineata* | Benzyl benzoate | Goutam et al. (2016) |
| 14. | *Glycosmis mauritiana* | *Penicillium* sp. | AgNP | Govindappa et al. (2016a) |

(continued)

**Table 7.3** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Extract/compound isolated | Reference |
|---|---|---|---|---|
| 15. | *Curcuma longa* | *Phoma herbarum* | Gentisyl alcohol | Gupta et al. (2016) |
| 16. | *Garcinia preussii* | *Aspergillus japonicus* | Variecolin and neovasifuranone B | Jouda et al. (2016b) |
| 17. | *Silybum marianum* | *Talaromyces minioluteus* | Talarolutins A–D: Meroterpenoids | Kaur et al. (2016) |
| 18. | *Calotropis procera, Catharanthus roseus, Euphorbia prostrata, Trigonella foenum-graecum,* and *Vernonia amygdalina* | *Byssochlamys spectabilis* and *Alternaria* sp. | Ethyl acetate | Khiralla et al. (2016) |
| 19. | *Menthe viridis* | *Fusarium oxysporum* | Broth | Kumar et al. (2016) |
| 20. | *Nicotiana tabacum* | *Rhizopycnis vagum* | Dibenzo-α-pyrone derivatives | Lai et al. (2016) |
| 21. | *Mahonia fortune* | *Fusarium decemcellulare* | Pentapeptides and lipopeptide | Li et al. (2016b) |
| 22. | *Cephalotaxus hainanensis* | *Diaporthe* sp., *Phomopsis* sp., *Colletotrichum* sp., *Corynespora* sp., *Penicillium* sp., and *Nemania* sp. | Ethyl acetate | Liu et al. (2016) |
| 23. | *Salvia miltiorrhiza* | *Alternaria* sp. | Alternariol 9-methyl ether | Lou et al. (2016) |
| 24. | *Ficus carica* | *Aspergillus tamarii* | Cyclic pentapeptide: malformin E | Ma et al. (2016) |
| 25. | *Melastoma malabathricum* | *Diaporthe phaseolorum* | Ethyl acetate | Mishra et al. (2016a) |
| 26. | *Schima wallichii* | *Penicillium simplicissimum* and *Talaromyces verruculosus* | Ethyl acetate | Mishra et al. (2016b) |
| 27. | *Rhizophora annamalayana* | *Trichoderma* sp. | Ethyl acetate | Narendran and Kathiresan (2016) |
| 28. | *Cinnamomum iners, Shorea siamensis, Fernandoa adenophylla,* and *Quercus semiserrata* | *Xylaria* sp. | Ethyl acetate | Orachaipunlap et al. (2016) |

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|---|---|---|---|---|
| 29. | *Cinnamomum malabatrum* | *Colletotrichum gloeosporioides* | Phenol 3, 5- dimethoxy acetate, 4′-isopropylidene-bis-(2-cyclohexyl) phenol, N-didehydrohexacarboxyl-2, 4, 5-trimethylpiperazine and 1, 2, 4-triazolium ylide | Packiaraj et al. (2016) |
| 30. | *Houttuynia cordata* | *Chaetomium globosum* | Ethyl acetate | Pan et al. (2016) |
| 31. | *Moringa oleifera* | *Aspergillus flavus* | Fenaclone | Rajeshwari et al. (2016) |
| 32. | *Hypocrea virens* | *Premna serratifolia* | Epidithiodioxopiperazine, gliotoxin, bisdethiobis(methylthio)gliotoxin | Ratnaweera et al. (2016) |
| 33. | *Rauvolfia serpentina* | *Colletotrichum* sp., *Fusarium* sp., and *Cladosporium* sp. | Methanol | Singh et al. (2016) |
| 34. | *Acalypha indica* | *Phoma* sp. | Terpenoids | Sowparthani (2016) |
| 35. | *Santalum album* | *Fusarium oxysporum, Fusarium solani, Histoplasma* sp., *Periconia* sp., and *Pestalotiopsis* sp. | Distilled water, ethanol | Tapwal et al. (2016) |
| 36. | *Picea mariana* and *Picea rubens* | *Diaporthe maritima* | Dihydropyrones, phomopsolides, alpha-pyrone | Tanney et al. (2016) |
| 37. | *Schinus terebinthifolius* | *Alternaria* sp. | E-2-hexyl-cinnamaldehyde and two compounds of the pyrrolopyrazine alkaloids | Tonial et al. (2016) |
| 38. | *Buxus sinica* | *Colletotrichum* sp. | Colletotrichone A | Wang et al. (2016a, b) |
| 39. | *Eugenia jambolana* | *Aspergillus niger* and *A. terreus* | Ethyl acetate | Yadav et al. (2016) |
| 40. | *Lonicera japonica* | *Fusarium* sp. | Methanol | Zhang et al. (2016b) |
| 41. | *Sapium ellipticum* | *Penicillium tropicum* | Cyclohexapeptide, penitropeptide, and a new polyketide, penitropone | Zeng et al. (2016) |

(continued)

**Table 7.3** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Extract/compound isolated | Reference |
|---|---|---|---|---|
| 42. | *Panax notoginseng* | *Acremonium* sp. | Ethyl acetate | Zheng et al. (2016) |
| | | *Alternaria* sp. | | |
| | | *Arthrinium* sp. | | |
| | | *Aspergillus* sp. | | |
| | | *Botryotinia* sp. | | |
| | | *Chaetomium* sp. | | |
| | | *Cladosporium* sp. | | |
| | | *Colletotrichum* sp. | | |
| | | *Dictyosporium* sp. | | |
| | | *Fusarium* sp. | | |
| | | *Humicola* sp. | | |
| | | *Ilyonectria* sp. | | |
| | | *Mucor* sp. | | |
| | | *Myrothecium* sp. | | |
| | | *Penicillium* sp. | | |
| | | *Periconia* sp. | | |
| | | *Pestalotiopsis* sp. | | |
| | | *Phialophora* sp. | | |
| | | *Phoma* sp. | | |
| | | *Phomopsis* sp. | | |
| | | *Plectosphaerella* sp. | | |
| | | *Thielavia* sp. and *Trichoderma* sp. | | |
| 43. | *Edgeworthia chrysantha* | *Fusarium oxysporum* | Beauvericin | Zhang et al. (2016a) |
| 44. | *Mallotus philippensis* | *Alternaria* sp., *Pestalotiopsis* sp., and *Phomopsis* sp. | Ethyl acetate | Gangwar et al. (2015) |

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|---|---|---|---|---|
| 45. | *Bauhinia forficata* | *Aspergillus ochraceus,* | Ethyl acetate | Bezerra et al. (2015) |
| | | *Gibberella baccata, Penicillium commune,* and *P. glabrum* | | |
| 46. | *Caesalpinia echinata* | *Xylaria* sp. | Ethyl acetate | Campos et al. (2015) |
| | | *Nectria* sp. | | |
| | | *Fusarium* sp., *Epicoccum* sp. | | |
| | | *Talaromyces* sp. and *Aspergillus* sp. | | |
| 47. | *Carapa guianensis* | *Diaporthe mayteni, Endomelanconiopsis, Colletotrichum* sp., *Guignardia mangiferae, Pestalotiopsis* sp., and *Diaporthe melonis* | Ethanol | Ferreira et al. (2015) |
| 48. | *Asclepias sinaica* | *Penicillium chrysogenum* and *Alternaria alternate* | Ethyl acetate | Fouda et al. (2015) |
| 49. | *Dioscorea composita* | *Fusarium* sp. and *Alternaria* sp. | Steroidal saponins | Gupta et al. (2015) |
| 50. | *Opuntia humifusa* | *Biscogniauxia mediterranea* | 5-Methylmellein | Silva-Hughes et al. (2015) |
| 51. | *Senecio kleinia* | *Phoma* sp. | Sclerodione, atrovenetinone | Hussain et al. (2015) |
| 52. | *Avicennia officinalis* | *Acremonium* sp., *Cladosporium* sp., *Curvularia* sp., and *Saccharomyces* sp. | Ethyl acetate | Job et al. (2015) |
| 53. | *Tridax procumbens* | *Alternaria* sp. | Methanol, chloroform, ethyl acetate, and petroleum ether | Kumar et al. (2015) |
| 54. | *Tectona grandis* | *Diaporthe phaseolorum* | Ethyl acetate | Kumala et al. (2015) |
| 55. | *Mahonia fortune* | *Diaporthe* sp. | Tetracyclic Triterpenoid | Li et al. (2015a) |
| 56. | *Taxus chinensis* | *Pestalotiopsis microspora* | α-Pyrone derivative | Li et al. (2015c) |
| 57. | *Tephrosia purpurea* | *Penicillium griseofulvum* and *Aspergillus oryzae* | Broth | Luo et al. (2015) |

(continued)

**Table 7.3** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Extract/compound isolated | Reference |
|---|---|---|---|---|
| 58. | *Avicennia marina* | *Penicillium brocae* | Sulfide diketopiperazines | Meng et al. (2015) |
| 59. | *Rauwolfia serpentina* | *Colletotrichum gloeosporioides, Penicillium* sp., and *Aspergillus awamori* | Ethanol | Nath et al. (2015) |
| 60. | *Panax ginseng* | *Phoma terrestris* | N-amino-3-hydroxy-6-meth oxyphthalimide and 5H–dibenz [B, F] azepine | Park et al. (2015) |
| 61. | *Mikania glomerata* | *Diaporthe citri* | Ethyl acetate | Polonio et al. (2015) |
| 62. | *Crescentia cujete* | *Nigrospora sphaerica, Fusarium oxysporum, Gibberella moniliformis,* and *Beauveria bassiana* | Aspirin and diethyl phthalate | Prabukumar et al. (2015) |
| *63.* | *Artemisia annua* | *Cladosporium* sp. | *Ethyl acetate* | Purwantini et al. (2015) |
| 64. | *Aegle marmelos, Coccinia indica, Moringa oleifera* | *Cladosporium oxysporum* | Taxol | Raj et al. (2015) |
| 65. | *Combretum latifolium* | *Gliomastix polychroma* | Ethyl acetate | Rao et al. (2015b) |
| 66. | *Cryptolepis buchanani* | *Phomopsis liquidambaris* | Ethyl acetate | Rao et al. (2015a) |
| 67. | *Opuntia dillenii* | *Fusarium* sp. | Equisetin | Ratnaweera et al. (2015b) |
| 68. | *Cyperus rotundus* | *Rhizoctonia solani* | Solanioic acid | Ratnaweera et al. (2015a) |
| 69. | *Abies* sp. | *Lophodermium pinastri, L. seditiosum,* and *Phoma herbarum* | Methanol, ethyl acetate, and dichloromethane | Ravnikar et al. (2015) |
| | *Cedrus* sp., *Juniperus* sp., *Larix* sp., *Metasequoia* sp., *Picea* sp., | | | |
| | *Pinus* sp. | | | |
| | *Taxus* sp., *Sambucus* sp., *Calluna* sp., and *Centaurea* sp. | | | |
| 70. | *Calophyllum apetalum* | *Myrothecium* sp. | Methanol | Ruma et al. (2015) |
| | *Garcinia morella* | | | |

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|---|---|---|---|---|
| 71. | *Rhizophora mucronata, Excoecaria agallocha* | *Fusarium proliferatum* | Ethyl acetate | Salini et al. (2015) |
| 72. | *Indigofera suffruticosa* | *Nigrospora sphaerica* and *Pestalotiopsis maculans* | Methanol, ethyl acetate | Santos et al. (2015) |
| 73. | *Cinnamomum camphora* | *Muscodor tigerii* | 4-Octadecylmorpholine, 1-tetradecanamine, N,N-dimethyl ,and 1,2- benzenedicarboxylic acid, mono(2-ethylhexyl) ester. | Saxena et al. (2015) |
| 74. | *Tsuga heterophylla* | *Gloeosporium* sp. | 6-Pentyl-2H-pyran-2-one | Schaible et al. (2015) |
| 75. | *Caesalpinia sappan, Alternanthera sessilis* | *Trichoderma* sp., *Aspergillus* sp., *Fusarium* sp., and *Trichoderma* sp. | Ethyl acetate | Srinivas et al. (2015) |
| | *Sapindus laurifolius* | | | |
| | *Basella alba* and *Acalypha indica* | | | |
| 76. | *Prosopis juliflora* | *Colletotrichum gloeosporioides* and *Paecilomyces lilacinus* | Ethyl acetate | Srivastava and Anandrao (2015) |
| 77. | *Phragmites communis* | *Phoma* sp. | Barceloneic acid C | Xia et al. (2015) |
| 78. | *Cephalotaxus hainanensis* | *Neonectria macroconidia, Xylaria* sp., and *Verticillium bulbillosum* | Ethyl acetate | Yang et al. (2015) |
| 79. | *Swietenia macrophylla* | *Aspergillus terreus* | Di-n-octyl phthalate | Yin et al. (2015) |
| 80. | *Dracaena draco* | *Botryodiplodia theobromae* | Dipeptides (maculosin and L,L-cyclo(leucylprolyl)), alkaloid (norharman), coumarin and isocoumarins (bergapten, meranzin, and monocerin), sesquiterpene (dihydrocumambrin A), aldehyde (formyl indanone), fatty alcohol (halaminol A), and fatty acid amide (palmitoleamide, palmitamide, capsi-amide and oleamide) | Zaher et al. (2015a) |
| 81. | *Ginkgo biloba* | *Aspergillus* sp. | Xanthoascin | Zhang et al. (2015a) |
| 82. | *Acanthus ilicifolius* | *Aspergillus flavipes* | Phenyl derivatives: aromatic butyrolactones, flavipesins A and B | Bai et al. (2014) |

(continued)

**Table 7.3** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Extract/compound isolated | Reference |
|---|---|---|---|---|
| 83. | *Xanthium sibiricum* | *Eupenicillium* sp. | Eupenicinicols A and B, butylitaconic acid, and (2S)-hexylitaconic acid | Li et al. (2014a) |
| 84. | Australian dry rainforests | *Preussia* sp. | Ethyl acetate | Mapperson et al. (2014) |
| 85. | *Hyptis dilatata* | *Pestalotiopsis mangiferae* | Polyhydroxylated macrolide: mangiferaelactone | Ortega et al. (2014) |
| 86. | *Tribulus terrestris* | *Aspergillus fumigatiaffinis* | Neosartorin | Ola et al. (2014) |
| 87. | *Vitex negundo* | *Pestalotiopsis* sp., *Fusarium* sp., *Fusarium* sp., and *Alternaria* sp. | Ethyl acetate | Palanichamy et al. (2014) |
| | *Justicia gendarussa, Ocimum basalicum* | | | |
| | *Costus spicatus* and *Glycosmis pentaphylla* | | | |
| 88. | *Aloe vera* | *Talaromyces wortmannii* | Methanol | Pretsch et al. (2014) |
| 89. | *Anoectochilus setaceus* | *Xylaria* sp. | Helvolic acid | Ratnaweera et al. (2014) |
| 90. | *Plumeria acuminate* and *Plumeria obtusifolia* | *Colletotrichum gloeosporioides* and *Fusarium oxysporum* | Ethyl acetate | Ramesha and Srinivas (2014) |
| 91. | *Nothapodytes foetida* | *Bionectria ochroleuca* | Ethyl acetate | Samaga et al. (2014) |
| 92. | *Cupressus arizonica, C. sempervirens var. cereiformis,* and *Thuja orientalis* | *Alternaria alternata, A. pellucid,* and *A. tangelonis* | Methanol | Soltani and Moghaddam (2014) |
| 93. | *Allium sativum* | *Trichoderma brevicompactum* | Extract | Shentu et al. (2014) |
| 94. | *Madhuca indica* | *Aschersonia* sp. | Ethyl acetate | Verma et al. (2014) |
| 95. | *Pinus wallichiana* | *Tritirachium oryzae, Truncatella spadicea,* and *Fusarium larvarum* | Methanol | Qadri et al. (2014) |
| 96. | *Melia azedarach* | *Botryosphaeria dothidea* | Pycnophorin, stemphyperylenol | Xiao et al. (2014a) |
| 97. | *Brguiera sexangula var. rhynchopetala* | *Stemphylium* sp. | Pyrone derivatives, infectopyrones A and B | Zhou et al. (2014b) |
| 98. | *Bruguiera gymnorrhiza* | *Penicillium* sp. | Penibruguieramine A:Pyrrolizidine alkaloid | Zhou et al. (2014a) |
| 99. | *Rhizophora stylosa* | *Aspergillus nidulans* | Aniquinazolines A–D | An et al. (2013) |

| | | | | |
|---|---|---|---|---|
| 100. | Amazon rainforest biome | *Chaetomium globosum,* | Pyrrocidine C | Casella et al. (2013) |
| | | *Xylaria cubensis* and *Lewia infectoria* | | |
| 101. | Amazon forests | *Xylaria feejeensis* | Xyolide | Baraban et al. (2013) |
| 102. | *Camptotheca acuminata* | *Botryosphaeria dothidea* | 9-Methoxycamptothecin | Ding et al. (2013) |
| 103. | *Vitex negundo* | *Phomopsis* sp. | Ethyl acetate, methanol, hexane | Desale and Bodhankar (2013) |
| 104. | *Ceratonia siliqua* | *Penicillium citrinum* | Alkaloids and polyketides | El-Neketi et al. (2013) |
| 105. | *Trichilia elegans* | *Phomopsis longicolla* | 3-Nitropropionic acid | Flores et al. (2013) |
| 106. | *Artabotrys odoratissimus, Cassia auriculata, Guazuma ulmifolia,* and *Terminalia catappa* | *Phomopsis* sp. | Ethyl acetate | Gopinath et al. (2013) |
| 107. | *Cannabis sativa* | *Aspergillus niger, A. flavus, A. nidulans, Penicillium chrysogenum, P. citrinum, Phoma* sp. | Ethanol | Gautam et al. (2013) |
| | | *Rhizopus* sp., *Colletotrichum* sp., *Cladosporium* sp., and *Curvularia* sp. | | |
| 108. | *Rhizophora stylosa* | *Alternaria tenuissima* | Tricycloalternarene 3, djalonensone | Hong et al. (2013) |
| 109. | *Ocimum tenuiflorum* | *Penicillium citrinum* | Polyketides and alkaloids | Lai et al. (2013) |
| 110. | *Ulmus macrocarpa* | *Microsphaeropsis arundinis* | Arundinols A–C and Arundinones A and B | Luo et al. (2013) |
| 111. | *Bauhinia guianensis* | *Aspergillus* sp. | Alkaloids: fumigaclavine C and pseurotin A | Pinheiro et al. (2013) |
| 112. | Forests of Western Ghats | *Xylaria* sp. | Ethyl acetate | Rajulu et al. (2013) |
| 113. | *Cymodocea serrulata, Halophila ovalis,* and *Thalassia hemprichii* | *Hypocreales* sp., *Trichoderma* sp., and *Penicillium* sp., *Fusarium* sp. and *Stephanonectria* sp. | Ethyl acetate | Supaphon et al. (2013) |

(continued)

**Table 7.3** (continued)

| S. no. | Medicinal plants | Fungal endophytes | Extract/compound isolated | Reference |
|---|---|---|---|---|
| 114. | *Triticum durum* | *Aspergillus* sp., *Alternaria* sp., *Penicillium* sp., *Cladosporium* sp., *Chaetomium* sp., and *Phoma* sp. | Ethyl acetate | Sadrati et al. (2013) |
| 115. | *Dioscorea zingiberensis* | *Berkleasmium* sp. | Palmarumycins C3 and C4 | Mou et al. (2013) |
| 116. | *Cedrus deodara, Pinus roxburghii,* and *Abies pindrow* | *Trichophaea abundans, Diaporthe phaseolorum* and *Fusarium redolens* | Methanol | Qadri et al. (2013) |
| 117. | *Ficus pumila* | *Phomopsis* sp. | Ethyl acetate | Rakshith et al. (2013) |
| 118. | *Theobroma cacao* | *Epicoccum* sp. | Polyketides: Epicoccolides | Talontsi et al. (2013) |
| 119. | *Rhododendron tomentosum* | *Fusarium tricinctum* | Polypeptides | Tejesvi et al. (2013) |
| 120. | *Panax ginseng* | *Fusarium* sp. | Triterpenoid saponin | Wu et al. (2013b) |
| 121. | *Rheum palmatum* | *Fusarium solani* | Rhein | You et al. (2013) |
| 122. | *Taraxacum mongolicum* | *Phoma* sp. | 2-Hydroxy-6-methylbenzoic acid | Zhang et al. (2013a) |
| 123. | *Clidemia hirta* | *Cryptosporiopsis* sp. | 1-(2,6-Dihydroxyphenyl)pentan-1-one (2) and (Z)-1-(2-(2- butyryl-3-hydroxyphenoxy)-6-hydroxyphenyl)-3-hydroxybut-2-en-1-one | Zilla et al. (2013) |

*Pestalotiopsis* sp. isolated from *Taxus brevifolia* (Yew tree) has been reported for immunosuppressive pestalotiopsins A and B (Pulici et al. 1996). Another immunosuppressive compound cytochalasin U has been produced by *Pestalotia* sp. isolated from *Cassia fistula* (Burres et al. 1992).

Subglutinols A and B, two immunosuppressive compounds, have been obtained from *Fusarium subglutinans*, inhabiting *Tripterygium wilfordii*. Both the compounds were nontoxic and very potent in the thymocyte proliferation (TP) assays and mixed lymphocyte reaction (MLR) (Lee et al. 1995). Cyclosporine-A, an immunosuppressive drug isolated from endophyte, was found to be 104 times more potent in the TP assay and roughly as potent in the MLR assay (Bentley et al. 2000). Similarly, *Pestalotiopsis leucothoe* from *Tripterygium wilfordii* has been documented to produce three compounds designated as BS, GS, and YS. All the compounds showed variable effects on T and B cells and monocytes. Hence, these represent a new source of immunomodulatory compounds for the treatment of human immune-mediated diseases. However, the structure of the compounds has not been elucidated yet (Kumar et al. 2005). Another potent immunosuppressive fungal metabolite used for the treatment of autoimmune diseases and organ transplantations has been documented to be produced by fungal endophytes *Penicillium*, *Aspergillus*, *Byssochlamys*, and *Septoria* species. The compound was identified as mycophenolic acid (Larsen et al. 2005).

Ren et al. (2008) have recorded collutelin-A and cyclosporine-A from *Colletotrichum dematium* inhabiting *Pteromischum* sp. growing in the tropical forests of Costa Rica. The compound displayed strong immunosuppressive activity by inhibiting CD4 T-cell activation of interleukin-2 production, whereas cyclosporine-A showed moderate activity in the same experiment.

Recently, an endophyte *Phomopsis longicolla* yielded four tetrahydroxanthone dimers, of which phomoxanthone A showed immunostimulation and pro-apoptotic activity. The compound exhibited immunostimulation by activating T lymphocytes, NK cells, and macrophages (Ronsberg et al. 2013). Similarly, *Botryosphaeria dothidea* isolated from *Kigelia africana* has been evaluated for its immunomodulatory potential. It was found to suppress T-cell proliferation by 50% and also inhibited TNF-α production (Katoch et al. 2014). These examples depict the potentiality of endophytes for exploring rare and uncommon immunomodulatory compounds.

### 7.3.5 Antidiabetic Compounds

Diabetes mellitus is the highest cause of death among other chronic diseases. It cannot be cured but controlled. In 2015, about 415 million people had diabetes worldwide, with type II diabetes accounting for about 90% of the cases (Cui et al. 2016). It can cause complications such as cardiovascular disorders, kidney failure, impotency, blindness, and gangrene. One of the strategies used to cure this is by inhibiting digestion of complex carbohydrates in the small intestine into glucose, resulting in the reduction of intake of glucose into the blood. Alpha-glucosidase and

alpha-amylase inhibitors are known to possess such activity. Medicinal plants for diabetes are a potential source of microbes producing alpha-glucosidase inhibitors.

α-Glucosidase is an important enzyme for breaking down complex carbohydrates for absorption; α-glucosidase inhibitors such as acarbose, miglitol, and voglibose, all originating from natural products, are widely used to treat type II diabetes, indicating that natural products are an important source of antidiabetic drugs.

*Syncephalastrum* sp. isolated from *Adhatoda beddomei* exhibits antidiabetic activity by inhibiting α-amylase (Prabavathy and Valli 2013). The alpha-glucosidase inhibitory activity of endophytic fungi isolated from *Cassia siamea* has also been reported (Munim et al. 2013). α-Amylase inhibitor from endophytic fungi of antidiabetic medicinal plants of the Western Ghats retards the liberation of glucose from dietary complex carbohydrates and delays the absorption of glucose. Antidiabetic activity of ethanolic and acetone extracts of endophytic fungi *Syncephalastrum racemosum* isolated from the seaweed *Gracilaria corticata* by alpha-amylase inhibition has been reported (Ushasri and Anusha 2015). Similarly, endophytic *Alternaria* sp. isolated from *Viscum album* exhibited strong antidiabetic activity on alloxan-induced diabetic rats (Govindappa et al. 2015). The additional examples of antidiabetic activity of fungal endophytes have been tabulated (Table 7.4).

### 7.3.6 Acetylcholinesterase Inhibitory Activity of Fungal Endophytes

Alzheimer's disease is a neurodegenerative disease of the central nervous system. The first clinical manifestation is recent memory dysfunction, which is followed by persistent intellectual impairment, loss of judgment and reasoning abilities, aphasia, and movement dysfunction. A study found that of the 10–15% of elderly people with different degrees of dementia, approximately 60–70% of the cases are due to Alzheimer's disease. However, the pathogenesis of senile dementia is not clear. Cholinergic nerve injury is the most accepted hypothesis of Alzheimer's disease pathogenesis, and if this is true, acetylcholinesterase inhibitors could be developed to effectively improve Alzheimer's disease treatment.

The use of acetylcholinesterase inhibitors is the most effective approach to treating the cognitive symptoms of Alzheimer's disease (Zhang et al. 2011) and has other possible therapeutic applications in the treatment of Parkinson's disease, senile dementia, and ataxia (Zhang et al. 2011; Singh et al. 2012). Acetylcholinesterase inhibitors such as eserine, tacrine, donepezil, rivastigmine, and galantamine are the drugs currently approved for the treatment of Alzheimer's disease (Anand and Singh 2013). The additional examples of acetylcholinesterase inhibitory activities of endophytic fungal isolates have been tabulated (Table 7.5).

**Table 7.4** Antidiabetic activity of endophytic fungi isolated from medicinal plants

| S. no. | Medicinal plants | Fungal endophytes | Bioactivity | Extract/compounds isolated | References |
|---|---|---|---|---|---|
| 1. | *Cupressus torulosa* | *Penicillium oxalicum* | Alpha-amylase inhibitory activity | Chloroform and methanol | Bisht et al. (2016) |
| 2. | *Mangrove plants* | *Talaromyces amestolkiae* | α-Glucosidase inhibitory and antibacterial | Isocoumarins and benzofurans | Chen et al. (2016) |
| 3. | *Sonneratia ovate* | *Nectria* sp. | α-Glucosidase inhibitory activity | Polyketides: Nectriacid B, nectriacid C | Cui et al. (2016) |
| 4. | *Hintonia latiflora* | *Xylaria feejeensis* | α-Glucosidase inhibitors | Pestalotin 4′-O-methyl-β-mannopyranoside and 3S,4R-(+)-4-hydroxymellein | Chavez et al. (2015) |
| 5. | *Viscum album* | *Alternaria* sp. | Antidiabetic | Lectin(N-acetylga lactosamine) | Govindappa et al. (2015) |
| 6. | *Sonneratia apetala* | *Aspergillus* sp. | Antidiabetic | Methanol | Liu et al. (2015b) |
| | | | | Isocoumarin derivatives | |
| | | | | Aspergifuranone | |
| 7. | *Cerbera manghas* | *Penicillium* sp. | α-Glucosidase inhibitory | (±)-penifupyrone and phenolic compounds | Liu et al. (2015a) |
| 8. | *Acacia nilotica* | *Aspergillus awamori* | Antidiabetic | Peptide | Singh and Kaur 2016) |
| 9. | *Tinospora. cordifolia* | *Cladosporium* sp. | α-Glucosidase inhibitors | Phenolic compound | Singh et al. (2015b) |
| 10. | *Gracilaria corticata* | *Syncephalastrum racemosum* | Antidiabetic | Ethanol, acetone | Ushasri and Anusha (2015) |
| 11. | *Momordica charantia* and *Trigonella foenumgraceum* | *Trichoderma atroviride* and *Stemphylium globuliferum* | Antidiabetic | Ethyl acetate | Pavithra et al. (2014) |
| 12. | *Morus alba* | *Alternaria* sp. | Antidiabetic | Ethyl acetate | Zheng et al. (2014) |
| 13. | *Salvadora oleoides* | *Aspergillus* sp. | Antidiabetic | 2, 6-Di-tert-butyl-p-cresol and phenol, 2, 6-bis (1, 1-dimethylethyl)-4-methyl | Dhankar et al. (2013) |
| 14. | *Ficus religiosa* | *Dendrophion nanum* | Antidiabetic | Herbarin (naphthoquinones) | Mishra et al. (2013) |
| 15. | *Catharanthus roseus* | Fungal endophytes | Antidiabetic | Ethyl acetate | Rosaline and Agastian (2013) |
| 16. | *Coscinium fenestratum* | *Fusarium solani* | Antidiabetic | Berberine | Vinodhini and Agastian (2013) |

**Table 7.5** Acetylcholinesterase inhibitory activity of endophytic fungi isolated from medicinal plants

| S. no. | Medicinal plants | Fungal endophytes | Activity | Extract/compounds isolated | References |
|---|---|---|---|---|---|
| 1. | *Catharanthus roseus* | *Alternaria alternata* | Acetylcholinesterase inhibitory | Altenune | Bhagat et al. (2016) |
| 2. | *Panax notoginseng* | *Chaetomium globosum* | Acetylcholinesterase inhibitory | 3-Methoxy epicoccone, epicoccolides B | Li et al. (2016a) |
| 3. | *Huperzia serrata* | *Colletotrichum* sp., | Acetylcholinesterase inhibitory | Ethanol | Wang et al. (2016b) |
| 4. | *Phlegmariurus phlegmaria* | *Ceriporia lacerate* | Acetylcholinesterase inhibitory | Chloroform | Zhang et al. (2015b) |
| 5. | *Huperzia serrata* | *Paecilomyces tenuis YS-13* | Acetylcholinesterase inhibitory | Huperzine A | Su and Yang (2015) |

### 7.3.7 Endophytes as a Source of Silver Nanoparticles

Nanotechnology is the ability to work at atomic, molecular, and supramolecular levels. It involves production, manipulation, and use of material ranging from less than a micron. Nanoparticles have a wide range of applications in diverse fields like catalysis, sensors, medicine, etc., and these depend on the physical and optical properties of the particles. As the field of nanotechnology is progressing, the knowledge of physical and chemical characteristics of nanoparticles has greatly increased. The most well-known nanoparticles are made from silver metal. Silver is used in medical fields as a topical bactericide. Silver nanoparticles possess broad-spectrum multifunctional activities and have the promising therapeutic potential to be used for the treatment of burns and variety of infections. The emphasis is being given to their use in prophylaxis and treatment of different types of cancers and microbial infections. The silver nanoparticles can change the 3D structure of proteins by interfering with S–S bond and block the functional operations of microorganisms (Sunkar and Nachiyar 2013). In the recent years apart from silver, gold nanoparticles have also been the focus of interest because of their emerging applications in the areas such as bioimaging and biosensors (Alappat et al. 2012).

Silver nanoparticles can be synthesized using chemical approaches, but it leads to the presence of traces of toxic chemicals absorbed on the surface which is undesirable in the medical applications (Bharathidasan and Panneerselvam 2012). Moreover, a lot of hazardous by-products are generated using this approach. Considering these facts, an alternative approach for nanomaterial synthesis has to be thought of. An important aspect in the field of nanotechnology is to develop a reliable and eco-friendly process for the synthesis of nanoscale materials.

Green technology is emerging nowadays and involves the use of microorganisms in the synthesis of nanoparticles (Razavi et al. 2015). The synthesis of nanoparticles using biological systems provides new routes to develop nanoparticles with desired

**Table 7.6** Nanoparticle producing endophytic fungi isolated from medicinal plants

| S. no. | Endophyte | Plant | Nanoparticle | Activity | References |
|---|---|---|---|---|---|
| 1. | *Penicillium* sp. | *Calophyllum apetalum* | Ag-Nps | Not reported | Chandrappa et al. (2016) |
| 2. | *Aspergillus versicolor* | *Centella asiatica* | Ag-Nps | Antimicrobial<br>Antioxidant | Netala et al. (2016) |
| 3. | *Fusarium solani* | *Withania somnifera* | Ag-Nps | Antibacterial<br>Cytotoxic | Vijayan et al. (2016) |
| 4. | *Colletotrichum* sp. | *Andrographis paniculata* | Ag-Nps | Antibacterial | Azmath et al. (2016) |
| 5. | *Fusarium* sp. | *Withania somnifera* | Ag-Nps | Antibacterial | Singh et al. (2015a) |
| 6. | *Penicillium* sp. and *Alternaria* sp. | *Gloriosa superba* | Ag-Nps | Antibacterial | Devi et al. (2014) |
| 7. | *Cryptosporiopsis ericae* | *Potentilla fulgens* | Ag-Nps | Antimicrobial | Devi and Joshi (2014) |
| 8. | *Penicillium* sp. | *Curcuma longa* | Ag-Nps | Antimicrobial | Singh et al. (2014) |
| 9. | *Epicoccum nigrum* | *Phellodendron amurense* | Ag-Nps | Antifungal | Qian et al. (2013) |
| 10. | *Penicillium* sp. | *Centella asiatica* | Ag-Nps | Antimicrobial | Devi et al. (2012) |

properties for making their exploitation possible in diverse fields (Pugazhenthiran et al. 2009). The nonpathogenic and eco-friendly behavior of endophytes makes them as good candidates for the synthesis of nanoparticles (Kaul et al. 2014). Fungal endophytes can be exploited for large-scale extracellular synthesis of nanoparticles which makes the downstream processing easier (Verma et al. 2011). Various studies on fungal endobiome described their ability to synthesize nanoparticles particularly silver nanoparticles (Ag-Nps) (Table 7.6).

### 7.3.8 Antitubercular Compounds

Tuberculosis is currently a major public health problem due to the advent of multidrug-resistant (MDR) forms of bacilli as well as human immunodeficiency virus epidemics. The World Health Organization (WHO) estimated that currently 50 million people are infected and 1,500 people die each hour from tuberculosis worldwide. After emergence and spread of *Mycobacterium tuberculosis*-resistant strains to multiple drugs, the search for new antimycobacterial agents is timely. The globe recognized medicinal plants as a repository for fungal endophytes with metabolites containing the novel molecular structure and biologically active compounds against various human pathogenic diseases for potential use in modern medicine. Endophytic fungi are a good source for exploring the possibility of new antimycobacterial drugs. Recently, polyketide such as penialidin C has been isolated from endophytic

*Penicillium* sp. of *Garcinia nobilis* that exhibits significant activity against *Mycobacterium tuberculosis* (Jouda et al. 2016a).

A new isofuranonaphthalenone isolated from endophytic fungus *Nodulisporium* sp. of *Antidesma ghaesembilla* displayed antimycobacterial activity with $IC_{50}$ values of 3.125 μg/mL (Prabpai et al. 2015). Asperlones A and B, dinaphthalenone derivatives, asperterpenoid A, and alterporriol-type dimmers from mangrove endophytic fungus *Aspergillus* sp. and *Alternaria* sp., respectively, exhibited potent inhibitory effects against *Mycobacterium tuberculosis* protein tyrosine phosphatase B (MptpB) with $IC_{50}$ values of $4.24 \pm 0.41$, 8.70, and 2.2 μM, respectively (Xia et al. 2014, 2015; Huang et al. 2013). Peniphenones A−D from endophytic *Penicillium dipodomyicola* of *Acanthus ilicifolius* exhibited strong inhibitory activity against *Mycobacterium tuberculosis* protein tyrosine phosphatase B (MptpB) (Li et al. 2014b).

Numerous secondary metabolites are known from endophytic strains of *Phoma* sp. Phomapyrrolidones A−C, alkaloids from the endophytic fungus *Phoma* sp. of *Saurauia scaberrinae*, possess significant antitubercular activity (Wijeratne et al. 2013). Penicolinates A–E from endophytic *Penicillium* sp. of grasses belonging to Poaceae family were found to possess antitubercular activity (Intaraudom et al. 2013). Colletotriolide, a macrolide isolated from *Colletotrichum* sp. endophytic to *Pandanus amaryllifolius*, exhibited an inhibition of greater than 90% at 128 μg/mL for *M. tuberculosis* (Bungihan et al. 2013).

### 7.3.9 Antihelminthic, Antiplasmodial, and Antileishmanial Compounds

Parasitic diseases such as malaria and leishmaniasis affect millions of people worldwide and pose a major health problem in developing countries. Malaria and leishmaniasis have affected major population with increasing number of new cases each year. Leishmaniasis is caused by protozoan parasites that belong to the genus *Leishmania* and is transmitted by the bite of certain species of sand fly (subfamily *Phlebotominae)*. Most of the current drugs used to treat parasitic diseases are decades old and have many limitations, including the emergence of drug resistance. For leishmaniasis, either the first-line pentavalent antimonials or second-line drugs such as amphotericin B are available, which are costly and have serious side effects and are getting resistant to pathogens after treatment for several weeks, and hence there is a need for new antileishmanial agents with improved efficacy and fewer side effects for both visceral and cutaneous leishmaniasis.

Malaria remains the world's most devastating human parasitic infection, afflicting more than 500 million people and causing about 2.5 million deaths each year. It is an infectious disease caused by the main four protozoan species of the genus *Plasmodium* (*Plasmodium falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*) (Mendis et al. 2009). The increasing resistance to existing antimalarial drugs demands the exploration of novel drugs and treatment efforts to eliminate this deadly disease. Natural products contain a great variety of chemical structures and have been

screened for antiplasmodial activity as potential sources of new antimalarial drugs (De Silva et al. 2013).

The development of anthelmintic resistance in helminths reported in a number of countries gives a clear indication that control programs based exclusively on their use are not sustainable. The development of integrated programs to control helminths is vital, but such control programs require viable alternatives to the use of anthelmintics. The history of herbal medicine is almost as old as human civilization. Medicinal plants have served through ages, as a constant source of medicaments for the exposure of a variety of diseases. The endophytic fungi act as an alternative to provide a rich source of anthelmintics, antibacterials, and insecticides.

Screening of mangrove endophytic fungi for antimalarial natural products displays the most favorable bioactivity profile (Calcul et al. 2013). Two unusual dibenzofurans, preussiafurans A–B, isolated from the fungus *Preussia* sp. occurring in *Enantia chlorantha* possessed antiplasmodial activity against erythrocytic stages of chloroquine-resistant *Plasmodium falciparum* (Talontsi et al. 2014). Reduced perylenequinone derivatives from an endophytic *Alternaria* sp. isolated from *Pinus ponderosa* were found to have antileishmanial and antimalarial activities (Idris et al. 2015). Meroterpenoid, isocoumarin, and phenol derivatives isolated from seagrass endophytic fungi *Pestalotiopsis* sp. exhibited antimalarial activity (Arunpanichlert et al. 2015).

Chemical and biological investigation for the endophytic fungus *Nigrospora sphaerica* led to the isolation of nigrosphaerin A, a new isochromene derivative with moderate antileishmanial activity having IC50 values of 30.2, 26.4, and 36.4 μg/ml, respectively (Metwaly et al. 2013). Enniatins (ENs), a group of antibiotics commonly produced by various strains of *Fusarium*, are six-membered cyclic depsipeptides formed by the union of three molecules of D-α-hydroxyisovaleric acid and three N-methyl-L-amino acids. The endophyte *Fusarium tricinctum* isolated from the fruits of *Hordeum sativum* showed antileishmanial activities (Zaher et al. 2015b).

The genus *Aspergillus* represents a diverse group of fungi, which are among the most abundant fungi in the world. Biologically active metabolites from endophytic fungus *A. terreus* isolated from the roots of *Carthamus lanatus* were found to have antileishmanial activity. Terrenolide S, a new butenolide derivative, together with (22E,24R)-stigmasta-5,7,22-trien-3-β-ol and stigmast-4- ene-3-one exhibited antileishmanial activity toward *Leishmania donovani* with IC50 values of 27.27, 15.32, and 27.27 μM, respectively, and IC90 values of 167.03, 40.56, and 14.68 μM, respectively (Elkhayat et al. 2016).

### 7.3.10 Extracellular Enzymes

Among a large number of microorganisms capable of producing useful enzymes, filamentous fungi are of particular interest due to their easy cultivation and high production of extracellular enzymes. Fungal enzymes are gaining importance in agriculture, industry, and human health as they are often more stable (at high temperature and extreme pH) than the enzymes derived from plants and animals (Maria

et al. 2005). Fungal endophytes known to be the treasure of new compounds represent an interesting alternative to be explored for enzyme production with different potentialities (Bhagobaty and Joshi 2012). Diverse array of extracellular enzymes produced by endophytes include cellulases, chitinases, amylases, lipases and proteases, pectinases, laccase, etc., having wide application in various industrial processes such as baking, brewing, textile, confectionaries, paper, pulp and leather, manufacturing corn syrup, hydrolyzing milk proteins, removing stains, separating racemic mixtures of amino acids, bioremediation, and biosensing (Kaul et al. 2014). The extracellular enzyme production varies among the fungal isolates. By optimizing the conditions, these isolates can prove to be a novel source of industrially relevant enzymes.

Different studies have been carried out on the screening of endophytes for enzyme production. For example, 50 fungal strains isolated from medicinal plants (*Alpinia calcarata, Bixa orellana*, *Calophyllum inophyllum*, and *Catharanthus roseus*) were screened for their ability to produce extracellular enzymes such as amylase, cellulase, laccase, lipase, pectinase, and protease on solid media. Variation in the enzyme production was recorded among the isolates. The array of enzymes produced by different fungal isolates often depends on the host and their ecological factors (Sunitha et al. 2013).

Endophytic fungi isolated from *Opuntia ficus-indica* were analyzed for preliminary screening for enzyme production. Among the 24 isolates which were studied, *Aspergillus japonicus* presented pectinolytic activity, and cellulase activity was exhibited by *Xylaria* (Bezerra et al. 2012).

Similarly, 30 fungal endophytes isolated from indigenous monocotyledonous and dicotyledonous plants have been evaluated for amylase, cellulase, protease, lipase, and laccase activity, and most of them showed positive results (Patel et al. 2013). Endophytes from *Lantana camara* have been screened for amylase, lipase, and laccase production, and three isolates were shown to produce three enzymes (Desire et al. 2014). Fungal endophytes isolated from *Butea monosperma*, a tropical medicinal plant, and *Bacopa monnieri* were found to be potential producers of industrial enzymes such as amylase, cellulase, pectinase, protease, and lipase (Tuppad and Shishupala 2014; Katoch et al. 2014a, b). The extracellular enzymatic activity of endophytic fungi *Cladosporium* sp., *Rhizoctonia* sp., *Aspergillus* sp., *Chaetomium* sp., *Biosporous* sp., *Fuzarium* sp., *Curvularia* sp., *Cladosporium* sp., and *Colletotrichum* sp. isolated from medicinal plants *Azadirachta indica*, *Citrus limon*, *Gossypium hirsutum*, *Magnolia champaca*, *Datura stramonium*, *Piper betle*, and *Phyllanthus emblica* has been reported (Patil et al. 2015a). Endophytic fungi from leaves of *Calophyllum inophyllum* produce extracellular enzymes such as amylase, protease, lipase, and cellulase (Patil et al. 2015a). Endophytic microbial resources producing extracellular enzymes can establish a unique niche for ecological adaptation during symbiosis with the host frankincense tree *Boswellia sacra* (Khan et al. 2016). A few more examples of common enzymes isolated from fungal endobiome of medicinal plants are enlisted below (Table 7.7).

**Table 7.7** Extracellular enzymes produced by fungal endophytes of medicinal plants

| S. no. | Fungal endophytes | Medicinal plants | Enzyme | References |
|---|---|---|---|---|
| 1. | *Corchorus olitorius* | *Aspergillus terreus* | Xylanase | Ahmed et al. (2016a) |
| 2. | Marine habitat | *Aspergillus* sp. | L-asparaginase | Ahmed et al. (2016b) |
| 3. | *Cupressus torulosa* | *Alternaria alternate* | Protease | Rajput et al. (2016) |
| 4. | *Artemisia annua* | *Aspergillus* sp. | Amylolytic | Ogbonna et al. (2015) |
| 5. | *Eurotium* sp. | *Curcuma longa* | Asparaginase | Jalgaonwala and Mahajan (2014) |
| 6. | *Preussia minima* | *Eremophila longifolia* | Amylase | Zaferanloo et al. (2014a) |
| 7. | *Alternaria alternate* and *Phoma herbarum* | *Eremophila longifolia* | Protease | Zaferanloo et al. (2014b) |
| 8. | *Sordaria humana* | *Cedrus deodara* and *Pinus roxburghii* | Cellulase | Syed et al. (2013) |

## 7.4 Conclusions

Fungal endobiome of medicinal plants is considered as an important and viable component of microbial biodiversity that offers a plethora of advantages to its host plant by producing bioactive secondary metabolites. In the continuous search for novel drug sources, endophytic fungi have proven to be a promising, largely untapped reservoir of natural products. A perusal of the literature indicates many ethnomedicinal plant species known to harbor potential endophytes that produce bioactive metabolites. Therefore, it is significant to bioprospect endophytes from medicinal plants for bioactive secondary metabolites. Bioactive metabolites from fungal endobiome could be a resolute solution to the present-day problems like the emergence of new diseases and resistance to existing drugs. The ability of endophytes to produce bioactive metabolites is influenced by its interaction with the host plant and its cross talk with other microbiota associated with the host. So it is very much significant to understand the mechanisms underlying the plant–microbe interaction. Improvization of isolation and purification methods needs to be done for commercial production of bioactive metabolites. Many novel and valuable compounds with antioxidant, anticancer, antimicrobial, immunomodulatory, and antidiabetic activities have been reported from fungal endophytes. This proves that fungal endobiome of medicinal plants certainly holds in them great potential to improve future in medicinal cure along with various industrial applications.

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# Multipotent and Poly-therapeutic Fungal Alkaloids of *Claviceps purpurea*

8

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**Abstract**

*Claviceps* are a group of phytopathogenic ascomycetes which includes around 50 known species. *Claviceps purpurea*, *Claviceps fusiformis*, *Claviceps paspali*, *Claviceps africana*, and *Claviceps lutea* are the most common and well-characterized fungi. Ergot alkaloids and other constituents derived from *Claviceps* are beneficial for various clinical applications in humans and animals. However, they also contain certain chemicals that are extremely addictive, abusive, and lethal. Ergot derivatives exhibit interesting pharmacokinetic and pharmacodynamic effects. Their pharmacodynamic actions are attributed to their agonistic, partial agonistic, and antagonistic effects on different receptors pertaining to the monoaminergic neurotransmitters. Due to their binding (with or without intrinsic effects) ability on the receptors, they induce numerous pharmacological effects which have potential medical values. Methysergide, ergotamine, dihydroergotamine, ergometrine (ergonovine), pergolide, ergoloid mesylates, and bromocriptine are the most popular ergot-based

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drugs used globally for treating numerous diseases. These drugs have been used to treat inflammatory-, infectious-, neurological-, cardiovascular-, gastrointestinal-, endocrinological-, sexual-, and urological-related pathologies. Hence, they are considered as a multipotent and poly-therapeutic fungus.

**Keywords**

*Claviceps purpurea* • Ergots • Medicinal value • Medicines derived from *Claviceps* • Neuroprotection

## Contents



## Abbreviations

| | |
|---|---|
| 5-HT | Serotonin |
| AUC | Area under the curve |
| CNS | Central nervous system |
| D receptor | Dopamine receptor |
| DHE | Dihydroergotamine |
| FFA | Free fatty acid |
| GIT | Gastrointestinal tract |
| GPCRs | G protein-coupled receptors |
| HPPD | Hallucinogen persisting perception disorder |
| LSD | Lysergic acid diethylamide |
| MAO | Monoamine oxidase |
| MDMA | Methylenedioxymethamphetamine |
| PZQ | Praziquantel |
| Src | Proto-oncogene tyrosine-protein kinase |
| Trk | Tropomyosin receptor kinase |

## 8.1 Introduction

The word *Claviceps* is derived from the Latin word "Clava" which means club and "-ceps" referred to as "headed" from the fungal shape (Dennis 1978). The genus *Claviceps* are a group of phytopathogenic ascomycetes of filamentous fungi, known to parasitize over 600 monocotyledonous plants of the families Poaceae, Juncaceae, and Cyperaceae, including forage grasses, corn, wheat, barley, oats, millet, sorghum, rice, and rye. *Claviceps* includes about 50 known species of which significant ones are *Claviceps purpurea* (parasitic on grasses and cereals), *Claviceps fusiformis* (on pearl millet, buffel grass), *Claviceps paspali* (on Dallis grass), *Claviceps africana* (on sorghum), and *Claviceps lutea* (on paspalum) (Bandyopadhyay et al. 1998). *Claviceps purpurea* is an ergot fungus that most commonly affects rye, wheat, and barley. It is the ergot stage of the fungus that harbors various compounds that have been useful as pharmaceutical drugs as well as mycotoxins that can be fatal when consumed. Mycotoxins are secondary metabolites of fungi that can induce pathologies and demise in plants, animals, and humans. Due to their wide pharmacodynamic effects, they have found use as drugs, medicine (antibiotics), and growth inducers and have been implicated as poison and used in chemical warfare agents. The proportion of the compounds produced varies within the species. The ergot family includes many alkaloids which contain the structural elements of the neurotransmitters serotonin, dopamine, and epinephrine. Therefore, ergot alkaloids can be recognized as ligands at a number of neurotransmitter receptors, as agonists or antagonists. They also act directly on the smooth muscles of the uterus causing contractions, thus their early use to induce abortion. The strongest biological effect of the ergots is intoxication, caused by specifically lysergic acid amides, one of which is the recreational (and illegal) drug, lysergic acid diethylamide (LSD). In this chapter, we have reviewed the studies on fungal alkaloids of *Claviceps purpurea having poly-therapeutic applications.*

## 8.2 Composition of *Claviceps* and Chemistry of the Ergots

The ergot sclerotium contains 30–40% of fatty oils and up to 2% of alkaloids (Komarova and Tolkachev 2001). It also contains free amino acids, ergosterin, choline, acetylcholine, ergothioneine, free aromatic and heterocyclic amines (tyramine, histamine), and alkylamines. The outer shell of sclerotium consists of anthraquinolinic acid derivatives (orange-red endocrinin, clavorubin and light yellow ergochromes, ergochrysins). Naturally growing *Claviceps purpurea* species differ both in the qualitative and quantitative composition of alkaloids. Hence these are identified based on a single alkaloid or certain group of alkaloids (ergotamine, ergotoxine, ergocristine) they produce (de Groot et al. 1998). Ergotoxine ethanosulfate was considered a pure substance and used as standard since its isolation. Later ergotoxine was shown to be a mixture of the three alkaloids ergocristine, ergocornine, and ergocryptine (Evans and Trease 1996). Six pairs of alkaloids predominate in the sclerotium and fall into the water-soluble ergometrine group or the water-insoluble

ergotamine and ergotoxine groups (Table 8.1). The ergot alkaloids possess high biological activity and a broad spectrum of pharmacological effects; hence they are of considerable importance to medicine. They affect adrenergic, serotoninergic, and dopaminergic neurotransmission. These compounds are now obtained both by methods of artificial parasitic cultivation on rye and by techniques using in vitro culture (Boichenko et al. 2001).

The naturally occurring ergot alkaloids are all indole-containing heterocycles biosynthesized from the amino acid L-tryptophan and the isoprene dimethylallyl diphosphate. Over 80 different ergot alkaloids have been isolated, mainly from various *Claviceps* species, as well as other fungi and plants. All of the ergots contain a tetracyclic ring system referred to as an ergoline which is a partially reduced indole [4,3-f,g] quinolone (Fig. 8.1). They also contain several centers of chirality, and all ergots have the R configuration at position 5 since this configuration is fixed by the biosynthetic precursor L-tryptophan. Depending on the structure of the ergoline D-ring and the nature of the substituent at C8, the ergots are subclassified as clavines, simple lysergic acid amide derivatives, and peptide ergot alkaloids (ergot peptides). These subclasses are biogenetically controlled, resulting from different biosynthetic pathways in the different organisms that form ergots.

The simple lysergic acid amides and peptide ergot alkaloids are amide derivatives of lysergic acid, with different types of amide functionality at C8; lysergic acid amide derivatives consist of simple secondary or tertiary alkyl amides, while the peptide ergots have a cyclic tripeptide amide (Fig. 8.1). The clavines typically contain a simple alkyl or alcohol group at position 8 instead of an amide and may have other functional groups present at position 9 or the indole ring. Generally, the ergoline ring is responsible for the base pharmacologic activity of the ergot alkaloids (serotonin antagonism, alpha or dopamine agonism, uterine contractility), while the differing ergoline ring substituents in the clavine, lysergic acid, and peptide ergot subclasses impart varying degrees of receptor specificity and agonist/antagonist activity. The only significant clavine derivative in therapeutic use today is the Parkinson drug pergolide. The primary ergot peptides of pharmacologic and

**Table 8.1** Various ergot alkaloids and their formula

| Group | Alkaloid | Formula |
|---|---|---|
| Ergometrine | Ergometrine | $C_{19}H_{22}O_2N_3$ |
| | Ergotmetrinine | $C_{19}H_{22}O_2N_3$ |
| | Ergotamine | $C_{33}H_{35}O_5N_5$ |
| Ergotamine | Ergotaminine | $C_{33}H_{35}O_5N_5$ |
| | Ergosine | $C_{30}H_{37}O_5N_5$ |
| | Ergosinine | $C_{30}H_{37}O_5N_5$ |
| | Ergocristine | $C_{35}H_{39}O_5N_5$ |
| Ergotoxine | Ergocristinine | $C_{35}H_{39}O_5N_5$ |
| | Ergocryptine | $C_{32}H_{41}O_5N_5$ |
| | Ergocryptinine | $C_{32}H_{41}O_5N_5$ |
| | Ergocornine | $C_{31}H_{39}O_5N_5$ |
| | Ergocorninine | $C_{31}H_{39}O_5N_5$ |

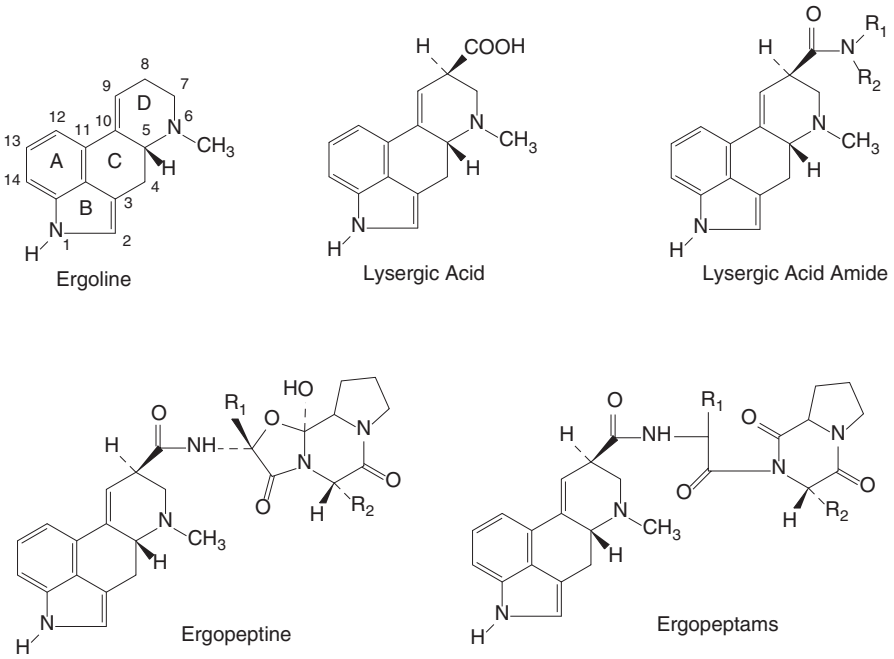


**Fig. 8.1** Structures of the ergot derivatives

therapeutic interest are ergotamine, bromocriptine, and the ergoloid mesylates. LSD, methysergide, and ergonovine represent the principle lysergic acid derivatives of pharmacologic significance.

The ergot peptides traditionally are further subdivided into two classes, the ergopeptines and the ergopeptams (Fig. 8.1). The ergopeptides are the classic cyclic ergot alkaloids with an oxazolopyrrolopyrazine amide functionality derived from cyclization of three amino acids (cyclic tripeptide amides), and these are the most common naturally peptide ergot alkaloids. The ergopeptams, or E-seco-ergot alkaloids, have a diketopiperazine peptide amide substituent, also derived from amino acids. The ergopeptines and ergopeptams can be further subclassified based on their differing R1 and R2 substituents in the peptide fragment. For example, the ergopeptides are classified as valine derivatives when R2 is an isopropyl group (bromocriptine, ergoloid mesylates) or alanines (ergotamine) when R2 is a simple methyl group. The pharmacology of representative members of these series is discussed in the sections that follow. Ergopeptams typically are present in only small amounts and in certain ergot-producing organisms and are not therapeutically significant.

The lysergic acid and ergot peptides have two centers of chirality in their ergoline ring system at C5 and C8 (Fig. 8.1). Pharmacologically active ergots, the so-called left-handed isomers, typically have the 8R and 5R configuration. However, in the presence of a base (and even acid), the lysergic acid and ergot peptides readily undergo epimerization at C8 resulting in the formation of 8S and 5R diastereomers

(right-handed isomers). The epimerization occurs because the proton at C8 of these ergots is relatively acidic since it is on a carbon adjacent to both the amide carbonyl group and the double bond at C9–C10. These two functional groups electronically stabilize the negative charge of the carbanion that forms when the C8 proton is removed in base and thereby facilitate epimerization and loss of the R configuration. The resulting 8S epimers that form the lysergic acid ergots are referred to as isolysergic acid derivatives, and the 8S epimers of the ergopeptines are called ergopeptinines. These epimers typically have only weak or no pharmacological activity. Significant quantities of epimer may form during chemical extraction and processing and even during prolonged, improper storage. Interestingly no 8S isomers have been reported for ergopeptams, presumably because these ergots readily decompose into simpler derivatives in the presence of base or acid. Also, the chiral center at C5 of the lysergic acid and ergot peptides is chemically stable and does not undergo configurational inversion. Finally, the ergot peptides contain an additional four centers of asymmetry. These chiral centers are fixed by the biosynthetic process and do not epimerize. In most ergot alkaloids, the D-ring alkene bond is conjugated with the indole ring and therefore located at positions C9–C10 (Fig. 8.1). Reduction of this bond results in the formation of the corresponding dihydroergot derivative, such as reduction of ergotamine, and yields dihydroergotamine. Reduction of this alkene moiety also introduces a third chiral center into the ergoline nucleus. With respect to chemical reactivity, all of the ergot alkaloids are bases with the most basic atom being the nitrogen at position 6 of the ergoline ring. All other nitrogen atoms are relative nonbasic due to extensive aromatic conjugation as in the indole nitrogen at position 1 or the amide- and peptide-carbonyl conjugation in the case of the lysergic acid amides and ergot peptide side chains. As a result of this basicity, ergot salts can be prepared by reaction with mineral or organic acids. The other significant chemical reaction, the lysergic acid amides, and ergot peptides can undergo hydrolysis of any or all of the amide and peptide linkages. Complete hydrolysis of both lysergic acid amides and ergot peptides results in the formation of lysergic acid and the component amines or amino acids. It should be noted that the ergot peptide ergot alkaloids can also undergo decomposition reactions catalyzed by temperature, light, oxidizers, reducers, bases, and acids. As a result, these ergots can decompose under conditions of improper storage or during isolation and purification and even in the course of chemical analysis. Also, when administered to humans, the ergots are metabolized by several enzyme systems that can oxidize the portions of the ergoline ring or peptide side chain, as discussed in the sections that follow. Since their initial discovery, a large number of ergot derivatives have been synthesized by modifying either the ergoline ring system or amide-peptide portion of the ergot molecule. Most derivatives prepared to date have modified alkyl amides or tripeptide amide moieties or substitutions at positions 2, 5, or 13 of the ergoline ring system. Most noteworthy of these derivatives in terms of therapeutically useful agents have been the substitution of a bromine atom at position 2 of ergocriptine to yield 2-bromocriptine,an agent discussed in more detailed in the sections that follow.

## 8.3 General Pharmacokinetic Aspects Associated with *Claviceps*

With regard to the pharmacokinetic properties, ergot alkaloids are variably absorbed from the gastrointestinal tract (GIT). Bioavailability is of the order of 5% or less by the oral or rectal administration. The oral dose of ergotamine is about ten times larger than intramuscular dose. The rate of absorption and peak blood levels after oral administration can be improved by the administration of caffeine. The amine alkaloids are also absorbed from the rectum, buccal cavity, and aerosol inhaler. Absorption after intramuscular injection is slow but reliable. Bromocriptine is more completely absorbed from the GIT than ergotamine. More than 90% of the absorbed dose undergoes first-pass hepatic metabolism by the cytochrome P450 (CYP3A) system mainly by hydrolysis to lysergic acid and peptides, with the remainder of the dose which is hydrolyzed in the liver to inactive metabolites (Lam 2000; Kvernmo et al. 2008). Hence, patient on these drugs should avoid cytochrome P3A4 (CYP3A4) inhibitors, such as macrolide antibiotics (erythromycin, clarithromycin), antifungal drugs (ketoconazole, itraconazole, fluconazole, and clotrimazole), protease inhibitors (ritonavir, nelfinavir, indinavir, and saquinavir), antidepressants (nefazodone, fluoxetine, and fluvoxamine), cyclosporine, tacrolimus, and grapefruit juice (Wooltorton 2003). After intramuscular or intravenous administration, plasma concentrations decay in a bi-exponential fashion. The elimination half-life is 2–2.5 h, and clearance is about 0.68 L/h/kg. As noted above, metabolism occurs in the liver, and the primary route of excretion is the biliary.

## 8.4 Pharmacology of *Claviceps*

Ergot alkaloids act on several types of receptors (alpha-adrenergic, serotonin, and dopamine). They act as agonists, partial agonist, and antagonists at alpha-adrenergic receptors or serotonin (5-HT) receptors (Table 8.2). However, they only exhibit agonistic effect at the dopamine receptors. Naturally occurring alkaloids present in *Claviceps* exhibit powerful hallucinogenic effects. Lysergic acid diethylamide (LSD25) is a semisynthetic compound demonstrating hallucinogenic action and acts as peripheral (5 $HT_2$) receptor antagonist. Smooth muscles are the supporting tissue of blood vessels, stomach, intestine, and bladder. On vascular smooth muscles, it acts as vasoconstrictors and is due to partial agonistic effects at alpha-adrenergic receptors and 5-HT receptors. Vasoconstriction shows differential vascular sensitivity to ergot alkaloids of which most sensitive are cerebral arteriovenous anastomotic vessels. Anti-migraine specificity is mediated by neuronal or vascular serotonin receptors. Overdosage of ergotamine and related agents cause severe, long-lasting vasospasm which is not reversible by alpha-antagonists and serotonin antagonists. On uterine smooth muscle, ergot alkaloids have a stimulant effect which varies with hormonal status. The stimulant action involves serotonergic, alpha-adrenergic, and other effects. The uterine smooth muscle at term (during childbirth) is more sensitive than early pregnancy and far more so than the

**Table 8.2** Actions of ergot derivatives

| Ergot alkaloids | Alpha-adrenergic receptor | Dopamine receptor | Serotonin receptor (5 $HT_2$) | Uterine smooth muscle stimulation |
|---|---|---|---|---|
| Bromocriptine | Antagonist | Agonist | Antagonist | No effect |
| | Partial agonist | | | |
| Ergonovine | Agonist | Agonist | Partial agonist | Agonist |
| Ergotamine | Partial agonist | No effect | Partial agonist | Agonist |
| LSD | No effect | Agonist | Antagonist | Agonist |
| | | | Agonist (CNS) | |
| Methysergide | None | None | Partial agonist | None |

nonpregnant organ. In small doses, ergot preparations can evoke rhythmic contractions and relaxation of the uterus. At higher doses, ergot preparations can induce a powerful and prolonged contraction. Ergometrine is more selective than other alkaloids and the agent of choice in obstetrics. Gastrointestinal smooth muscles show variable sensitivity causing nausea, vomiting, and diarrhea by activation of gastrointestinal serotonin receptors and central nervous system (CNS) chemoreceptor trigger zone emetic centers. There is no effect on bronchial smooth muscles. In the eye, ergotamine has alpha antagonist effect, producing miosis (constriction). Ergometrine has alpha agonist effect producing mydriasis (dilatation). Psoriasis is a chronic, common, and persistent dermatological pathology which significantly affects the life cycle of cells in the skin leading to increased cell growth on the surface of the skin. These dermal cells form thick, dry, and red patches, and silvery scales are itchy and painful. The various types of psoriasis are plaque psoriasis, nail psoriasis, scalp psoriasis, guttate psoriasis, inverse psoriasis, pustular psoriasis, erythrodermic psoriasis, and psoriatic arthritis. Malfunction of T cells has been an accepted etiology of psoriasis. Infection, obesity, stress, and smoking also can contribute to the etiopathology of psoriasis. The current therapeutic approaches are corticosteroids (topical), retinoids (topical), calcipotriene (Dovonex-vitamin D analog), anthralin, tacrolimus/pimecrolimus (calcineurin inhibitors), immunosuppressants (methotrexate, cyclosporine) salicylic acid, and moisturizers. The other nonpharmacological approaches are light therapy and avoiding alcohol and smoking. Bromocriptine possesses immunosuppressive properties, which may be related to its ability to lower circulating prolactin levels or to its direct suppressive effect on B and T cells (Morkawa et al. 1993; Morikawa et al. 1994). Due to the above effects, several studies reported the efficacy of bromocriptine in the treatment of psoriasis and psoriatic arthritis (Guilhou and Guilhou 1982; Eulry et al. 1995).

## 8.5 Drugs Derived from *Claviceps*

### 8.5.1 Methysergide

It is available as the maleate salt and is an ergot derivative and a congener of lysergic acid diethylamide, possessing nonselective serotonergic blocking activity (Johnson et al. 2003). Metabolites of methysergide also exhibit pharmacological activity. Methylergometrine (one of methysergide's metabolites) is responsible for methysergide's therapeutic effects regarding migraine treatment (Müller-Schweinitzer and Tapparelli 1986). Vascular headaches are a group of headaches that includes migraines. The vascular headaches involve abnormal function of the cerebral blood vessels or vascular system. The most common type of vascular headache is migraine which is characterized by severe pain on one or both sides of the head, nausea and/or vomiting, disturbed vision, and intolerance to light. Other kinds of vascular headaches include cluster headaches and headaches caused by a rise in blood pressure. Migraine is a complex disorder characterized by recurrent episodes of headache, most often unilateral and in some cases associated with visual or sensory symptoms—collectively known as an aura. The current therapy includes analgesics (acetaminophen, aspirin, ibuprofen, naproxen), caffeine, antidepressants, anti-nausea drugs (promethazine, chlorpromazine, prochlorperazine), and triptans (almotriptan, rizatriptan, sumatriptan). The other nonpharmacological approaches are acupuncture, biofeedback and cognitive behavioral therapy, spinal manipulation, diet changes, massage, meditation, neck stretching, and relaxation exercises. Methysergide's prophylactic action against migraine is via its nonselective action on 5-HT2c and 5-HT2b receptors on the vascular endothelium (Silberstein and Goadsby 2002; Johnson et al. 2003), while at the $5\text{-HT}_{1A}$ receptor it serves as a partial agonist. On systemic administration, it reaches maximum vasoconstrictor effect after 25 min and decreases within 50 min (Müller-Schweinitzer and Tapparelli 1986). By methysergide's action on the 5-HT2A receptor subgroup, it inhibits the release of histamine from mast cells (on 5-HT 2A receptor) (Young and Rozen 2005). The FDA-labeled indication is for the prophylaxis of vascular headache, but therapy tends to be limited to those patients who suffer frequent and/or severe and uncontrollable vascular headaches that do not respond to other prophylactic measures. Methysergide is one of the most effective medications for the prevention of migraine, but not for the treatment of an acute attack (Joseph et al. 2003). Methysergide is also used to treat other recurrent throbbing headaches (Alliance 2008) and is also used in carcinoid syndrome to treat severe diarrhea and in the treatment of serotonin syndrome (Sporer 1995). It has a known side effect, retroperitoneal fibrosis which is severe, although uncommon. Other severe but uncommon side effects include pleural fibrosis and subendocardial fibrosis. In addition, there is an increased risk of left-sided cardiac valve dysfunction (Connolly et al. 1997; Joseph et al. 2003). The systemic availability of methysergide after oral administration is only 13%, due to a high degree of first-pass metabolism by N-1 demethylation to methylergometrine. After oral administration, the plasma concentrations of the metabolite are considerably higher than those of the parent drug, and

the area under the plasma concentration curve (AUC) for methylergometrine is more than ten times greater than for methysergide. Methylergometrine is a partial agonist/antagonist on multiple receptors such as serotonergic, dopaminergic, and alpha-adrenergic receptors.

### 8.5.2 Ergotamine

Ergotamine, marketed as the tartrate salt and often in combination with caffeine, is a peptide ergot derivative with nonselective serotonin 5-HT receptor agonistic activity and also has affinity for dopamine and noradrenaline receptors (Tfelt-Hansen et al. 2000; Villalon et al. 2003). The actions of ergot alkaloids at 5-$HT_{1B/1D}$ receptors likely mediate their acute antimigraine effects (Schaerlinger et al. 2003). The reduction product, dihydroergotamine (DHE), is also a α1-adrenoceptor antagonist. Ergotamine (Ergomar®) is available in oral and sublingual tablet formulation and rectal suppositories. With respect to pharmacokinetics, the oral absorption of ergotamine is 60–70 %, and concurrent administration of caffeine improves both the rate and extent of absorption (Bülow et al. 1986). Caffeine is thought to exert its rate-accelerating effect by increasing the water solubility of ergotamine, thereby assisting ergotamine absorption from a lipid phase (GI membrane) into an aqueous phase (blood) (Schmidt and Fanchamps 1974). However, ergotamine has low bioavailability as a result of substantial (greater than 90%) first-pass metabolism by the liver following oral administration, apparently involving CYP3A4. As a result, significant drug interactions can occur when ergotamine is administered with CYP3A4 inhibitors such as the macrolide antibiotics and protease inhibitor drugs. Ergotamine and its metabolites are excreted principally in the feces via biliary elimination.

The use of ergot alkaloids for a migraine should be restricted to patients having frequent, moderate migraines or infrequent, severe migraine attacks (Treves et al. 1998; Boureau et al. 2000). The drugs should be taken as soon as possible after the onset of a headache. The use of ergotamine has declined since the introduction of the triptans. Ergotamine has a more extensive adverse side effect profile (Young 1997; Morren and Galvez-Jimenez 2010) than the triptans, and clinical studies have shown that oral ergotamine plus caffeine is less effective than triptans for an acute migraine. Only the rectal forms of ergotamine are superior to rectal triptans. However, ergotamine may still be helpful for patients with status migrainous or those with frequent recurring headaches.

Side effects and contraindications include nausea and vomiting (occur in 10% of patients taking ergot alkaloids). They are contraindicated in patients with cardiovascular diseases. Due to teratogenic effects (pregnancy category X), it is contraindicated in pregnancy. Category X refers to studies where animals or humans have demonstrated fetal abnormalities and/or there is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience. Risks involved in the use of the drug in pregnant women clearly outweigh potential benefits. It causes uterine contractions, fetal distress, gastrointestinal atresia, and miscarriage. These ergot alkaloids have a Black Box Warning for serious and/or

life-threatening peripheral ischemia which is associated with the coadministration of ergotamine with potent CYP3A4 inhibitors, including protease inhibitors and macrolide antibiotics. As CYP3A4 inhibition elevates the serum levels of ergotamine, the risk for vasospasm leading to cerebral ischemia and/or ischemia of the extremities is increased. Hence, concomitant use of these medications is contraindicated.

### 8.5.3 Dihydroergotamine

Dihydroergotamine (DHE) is synthesized by reducing an unsaturated bond in ergotamine. DHE is the currently used ergot preparation and marketed as the mesylate salt. An orally inhaled formulation of DHE delivered to the systemic circulation will be available in the near future (Aurora et al. 2011). DHE, a semisynthetic product, is currently prepared either by hydrogenation of ergotamine isolated from field ergot/fermentation broth or via synthesis from dihydrolysergic acid and the appropriate synthetic tripeptide. The structural differences between ergotamine and DHE are small but significant. DHE has a hydrogen atom projecting below the plane of the ring at C(10) in the same direction as the proton on N(6), whereas ergotamine has none (Berde and Schild 1978). This modification results in a changed pharmacologic profile with dihydroergotamine exhibiting greater alpha-adrenergic antagonist activity and much less potent arterial vasoconstriction and emetic potential. Both ergotamine and DHE are 5-HT1A, 5-HT1B, 5-HT1D, and 5-HT1F receptor agonists. FDA-labeled indications for the use of dihydroergotamine mesylate are for the acute treatment of the symptoms of a migraine headache (w/wo aura) and for the acute treatment of a cluster headache. Following intranasal administration, the relative bioavailability is 30–40%, with peak plasma concentrations reached in 30–60 min. Dihydroergotamine is administered as a nasal spray form (Migranal®) or by injection. Four metabolites of dihydroergotamine have been identified in human plasma following oral administration. The major metabolite, 8′-β-hydroxy-dihydroergotamine, exhibits affinity equivalent to its parent for adrenergic and 5-HT receptors and demonstrates equivalent potency in several venoconstrictor activity models, in vivo, and in vitro. The other metabolites (i.e., dihydrolysergic acid, dihydrolysergic amide, and a metabolite formed by the oxidative opening of the proline ring) are of minor importance. Following nasal administration, total metabolites represent only 20–30% of plasma AUC. The systemic clearance of dihydroergotamine mesylate following IV and IM administration is 1.5 L/min. Quantitative pharmacokinetic characterization of the four metabolites has not been performed.

### 8.5.4 Ergometrine (Ergonovine)

Ergometrine is an amine ergot alkaloid included in World Health Organization's List of Essential Medicines, the most important medications needed in a basic health system. Ergometrine has effects on uterine and vascular smooth muscle.

Usual therapeutic doses of ergometrine produce intense contractions of the uterus followed by periods of relaxation. The amplitude and frequency of uterine contractions increase which in turn impedes uterine blood flow thereby promoting hemostasis. With larger doses, basal uterine tone is elevated, and these relaxation periods will be decreased. Ergometrine also increases contractions of the cervix. The sensitivity of the uterus to the oxytocic effect is much greater toward the end of the pregnancy. Oxytocin is secreted by the posterior lobe of the pituitary gland. It is a neuropeptide that was primarily known for its role in the birth process and also in nursing. Oxytocin induces uterine contractions during labor and helps shrink the uterus after delivery. Oxytocin also has an antianxiety (anxiolytic) effect and may increase romantic attachment and empathy. It has therapeutic effects in patients with autistic spectrum disorders and appears to play a role in protecting the intestine from damage, with potential for use in the treatment of irritable bowel disease. These oxytocic actions of ergometrine are greater than its vascular effects (Gilman and Gilman 1985). Vasoconstriction is predominant in capacitance vessels thereby increasing central venous pressure and blood pressure. At high doses, ergometrine causes vasoconstriction of coronary arteries (Kimball et al. 1989). Like other ergot alkaloids, ergometrine produces arterial vasoconstriction by stimulation of α-adrenergic and serotonin receptors. The drug has only slight α-adrenergic blocking activity, and its vasoconstrictor effects are less than those of ergotamine (de Groot et al. 1998). In CNS, ergometrine is a partial agonist and partial antagonist at some serotonin and dopamine receptors. Ergometrine also possesses weak dopaminergic antagonist actions in certain blood vessels and partial agonist action at serotonin receptors in umbilical and placental blood vessels (Gilman and Gilman 1985).

Pharmacokinetics: Uterine contractions are usually initiated within 5–15 min following oral administration, within 2–3 min after IM injection, and 1 min following IV injection.
Half-life: 1–5 min (initial phase) and 0.5–2 h (terminal phase).
Duration in uterine effects: 3 h (IM) and 45 min (IV).

With regard to indications, as a prophylactic drug, ergometrine is administered after the delivery of the placenta for the purpose of contracting the uterus in order to prevent postpartum and postabortion hemorrhage due to uterine atony (Goodman et al. 2001). As a treatment, ergometrine is administered after the delivery of the placenta to promote involution of the uterus in order to treat postpartum and postabortion hemorrhage (Goodman et al. 2001); 0.2 mg IM is administered and may be repeated in 2–4 h, not to exceed five doses total. In diagnosing and testing, ergometrine maleate or methylergometrine maleate has been used as a provocation test for the diagnosis of variant angina (Prinzmetal's angina). Ergometrine maleate was used in the past to diagnose esophageal spasm (Schwartz and Kaufmann 1984). The major contraindications are previous hypersensitivity or idiosyncratic reactions to ergometrine, eclampsia or preeclampsia, severe or persistent sepsis, peripheral vascular disease, or heart disease, and in patients with hypertension or a history of hypertension, Raynaud's phenomenon, impaired hepatic, or renal function exists.

Ergometrine is rapidly absorbed after oral or IM administration. Following oral administration, bioavailability is highly variable but averages about 60%. Due to its lipophilic nature, the drug is rapidly distributed to tissues and into breast milk. It is metabolized by the liver by CYP3A4, but the metabolites have not been characterized. As a result, drug interactions are possible when ergometrine is coadministered with drugs that are inhibitors of CYP3A4, increasing the risk for vasospasm, cerebral ischemia, and/or ischemia of the extremities. This drug is excreted principally in feces and bile.

### 8.5.5 Pergolide

Pergolide is a semisynthetic clavine ergot derivative marketed as the mesylate salt and is predominantly D2 receptor agonist, with some weak actions at the Dl and D3 (Perachon et al. 1999) as well as on serotonin receptors. It possesses intrinsic activity at the dopamine D1 receptor which offers an additional advantage compared to other antiparkinson dopamine agonists (McClure et al. 2010). Pergolide also promotes the striatal expression of the dopamine D3 receptor (Perachon et al. 1999) and exerts actions on many subtypes of 5-hydroxytrptamine (5-HT) 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, and 5-HT2C receptors and α-adrenoceptors (Ruffolo, Jr. et al. 1987; Deleu et al. 2002).

Pharmacokinetics: It is well absorbed orally and has plasma half-life in the range of 3–7 h (Gilman and Hardman 1996). The drug undergoes extensive hepatic first-pass metabolism by oxidative N-dealkylation and sulfur oxidation and is approximately 90% bound to plasma proteins. It differs from bromocriptine in that it has a longer half-life, is substantially more potent, and has Dl agonist properties (Gilman and Hardman 1996).

Indications and clinical uses: In Parkinson's disease, levodopa has been considered as the sole drug in the treatment of its symptoms, but because of unwanted adverse effects such as long-term motor fluctuation and dyskinesia, the current guidelines suggest delaying of initiation of levodopa treatment and advocating an early use of dopamine agonists which offer several theoretical advantages over levodopa because of the underlying reasons. First, dopamine agonists act directly on dopamine receptors and do not require metabolic conversion to an active product in order to exert their pharmacologic effect. Hence they act independently of the degenerating dopaminergic neurons, and through this way the increased dopamine metabolism that would result from levodopa administration which may damage the nigrostriatal system through free radical generation is prevented. Nevertheless, dopamine agonists do not undergo oxidative metabolism and do not generate free radicals or induce oxidative stress. In addition, they have the potential to stimulate the presynaptic autoreceptors of dopamine and may, therefore, reduce endogenous dopamine turnover which is enhanced by levodopa and has been proven to be potentially toxic. Second, in contrast to levodopa, circulating plasma amino acids do not compete with dopamine agonists for absorption and transport into the brain. Third, dopamine agonists have a longer half-life than immediate-release and

controlled-release formulations of levodopa, and individual doses, therefore, have the potential to provide more sustained stimulation of striatal dopamine receptors. Finally, there is mounting evidence suggesting that they may have neuroprotective effects (Marsden et al. 1982; Lange 1998; Olanow et al. 2001; Deleu et al. 2002). Other uses of pergolide include treatment of depression in Parkinson's disease (Rektorová et al. 2003) and restless leg syndrome (Earley et al. 1998; Bassetti et al. 2002) and in the symptomatic treatment of Tourette syndrome (Eric et al. 2001; Gilbert et al. 2003) and prolactinoma.

Side effects: The drug is in decreasing use, as it was reported in 2003 to be associated with a form of heart disease called cardiac fibrosis (Breitenstein et al. 2006) (Research; Breitenstein et al. 2006). Pergolide is not currently available in the United States for human use, due to its action at the 5-HT2B serotonin receptors of cardiac myocytes, causing proliferative valve disease by the same mechanism as ergotamine, methysergide, fenfluramine, and other serotonin 5-HT2B agonists (Jähnichen et al. 2007). Pergolide can rarely cause Raynaud's phenomenon. In March 2007, pergolide was withdrawn from the US market for human use, due to serious valvular damage that was shown in two independent studies (Zadikoff et al. 2006; Schade et al. 2007). Pergolide has also been shown to impair associative learning (Markham and Benfield 1997).

### 8.5.6 Ergoloid Mesylates (Hydergine)

Ergoloid mesylates (Hydergine) contain derivatives of three naturally occurring ergot alkaloids – ergocristine (30.0–36.5%), ergocriptine (ergocryptine) (30.0–36.5%), and ergocornine (30.0–36.5%). Ergoloid mesylates have therapeutic efficacy in the treatment of dementia (Alzheimer's disease) and age-related cognitive impairment as well as to aid in recovery after stroke. However, the mechanism of action in dementia is unknown. It stimulates dopaminergic and serotonergic receptors and blocks alpha-adrenoreceptors (Markstein 1985). Modulation of synaptic neurotransmission rather than solely increasing blood flow has been proposed (Rowell and Larson 1999). Also, aging is associated with increase in monoamine oxidase (MAO) levels resulting in decreased availability of catecholamines in the synaptic cleft (Kennedy and Tanenbaum 2000). It possesses antioxidant and vasodilator effects in the vasculature of the brain. Although current lines of evidence suggest that cerebrovascular factors can worsen or accelerate the course of Alzheimer's disease, there is no convincing evidence that it actually increases the effective flow of blood to diseased areas of the human brain or that they have any clinical efficacy in dementia. Hence they are no longer used or recommended drug. It has peripheral alpha-blocking activity and also depresses CNS vasomotor nerve activity resulting in a slight decrease in blood pressure and hypertension.

### 8.5.7 Bromocriptine

Bromocriptine mesylate is a semisynthetic ergot alkaloid with potent dopamine receptor agonist activity. Dopamine receptors are a class of G protein-coupled receptors implicated in many neurological processes, including motivation, pleasure, cognition, memory, learning, and fine motor control, as well as modulation of neuroendocrine signaling. Bromocriptine mesylate inhibits prolactin secretion and lowers blood levels of growth hormone in acromegaly. Quick-release formulation of bromocriptine (Cycloset) is thought to act on circadian neuronal activities within the hypothalamus to reset abnormally elevated hypothalamic drive for increased plasma glucose, triglyceride, and free fatty acid levels in fasting and postprandial states in patients with insulin resistance. With regard to the pharmacokinetic aspect, absorption is 28 % from GI tract with bioavailability of 28% for Parlodel and 65–95% for Cycloset. Bromocriptine binds to albumin (90–96%) with the volume of distribution of 61 L. It is metabolized completely in the liver, with a half-life elimination of 4–4.5 h (initial phase) and 8–20 h (terminal phase). Excretion is mainly by feces (85%) and via biliary elimination.

*Indications of Bromocriptine*: It is used to treat hyperprolactinemia and prolactinomas (Parlodel). Secretion of the anterior pituitary hormone prolactin is inhibited without affecting other pituitary hormones. It is a potent suppressor of lactation. Since dopamine is an inhibitor of prolactin release, drugs increasing dopaminergic neurotransmission such as bromocriptine are useful and widely used in the treatment of these endocrine disorders (McMurray et al. 1995). About 80 % of hyperprolactinemic women treated with bromocriptine resume their normal menstrual cycles, and pregnancy rates may be as high as 70%. It induces menses in amenorrheic women and maintains normal levels of serum prolactin possibly via the release of luteinizing hormone (Cottigham 2004). Bromocriptine is the drug of choice for the treatment of hyperprolactinemia regardless of the etiology. In prolactin-secreting tumors, it reduces prolactin level and causes a reduction in tumor size as long as therapy continues; however tumor size rebounds upon cessation of treatment. The dose of bromocriptine used for hyperprolactinemia, amenorrhea, and male or female infertility is initially 1.25–2.5 mg once per day and then increased to a maintenance dose of 2.5 mg two or three times daily (Delgrange et al. 1998). Bromocriptine is used as a conservative treatment of pituitary micro- or macroprolactinomas, in presurgical reduction of tumor size to facilitate resection, and in postsurgical inhibition of persistent hyperprolactinemia. Bromocriptine is used to suppress or prevent puerperal lactation and prevent lactation following abortion in puerperal breast engorgement and incipient puerperal mastitis (Eftekhari 2004). It is also used in treating benign breast disease and controlling premenstrual symptoms (Delgrange et al. 1998). Acromegaly is caused by excessive secretion of growth hormone after the growth plate cartilage fuses in adulthood. Gigantism refers to abnormally high linear growth due to excessive growth hormone secretion while the epiphyseal growth plates are open during childhood. Somatotroph adenomas (growth hormone-producing adenomas) account for over 80% of the cases of acromegaly for which the treatment of choice is irradiation or surgical removal of the tumor. Drug therapy

is indicated for patients not cured by surgery and those with recurring problems. Growth hormone secretion by adenomas can be suppressed by bromocriptine. The effect of bromocriptine on growth hormone secretion is paradoxical, as it can actually increase growth hormone secretion in normal pituitary. It can reduce serum growth hormone by 50% or more in approximately half of patients treated, although not usually to normal levels. Since the effects of external pituitary radiation may not become maximal for several years, adjunctive therapy with bromocriptine mesylate offers potential benefit before the effects of irradiation are manifested. The dose in acromegalic patients is 1.25 mg two or three times daily, gradually increasing to 10–20 mg daily depending on clinical response and adverse reactions (Cassar et al. 1976).

Most patients with Parkinson's disease are treated with levodopa or with dopamine agonists. Bromocriptine has been used in previously untreated patients with Parkinson's disease to prolong levodopa treatment and delay its complications (Yamashita et al. 1995). While some neurologists use bromocriptine early in the treatment of Parkinsonism in an attempt to delay therapy with levodopa, others reserve it for adjunctive use when levodopa is no longer effective alone or cannot be tolerated. Bromocriptine is sometimes useful in reducing "off" periods with levodopa and in ameliorating other fluctuations of mobility in the later stage of the disease (Parfitt 1999). Bromocriptine mesylate tablets or capsules are indicated for the treatment of the signs and symptoms of idiopathic or postencephalitic Parkinson's disease. As adjunctive treatment to levodopa (alone or with a peripheral decarboxylase inhibitor), bromocriptine mesylate therapy may provide additional therapeutic benefits in those patients who are currently maintained on optimal dosages of levodopa, those who are beginning to deteriorate (develop tolerance) to levodopa therapy, and those who are experiencing end of dose failure on levodopa therapy. Bromocriptine mesylate therapy may permit a reduction of the maintenance dose of levodopa and thus may ameliorate the occurrence and/or severity of adverse reactions associated with long-term levodopa therapy such as abnormal involuntary movements (e.g., dyskinesias) and the marked swings in motor function (on-off phenomenon). Continued efficacy of bromocriptine mesylate therapy during treatment of more than 2 years has not been established. The basic principle of bromocriptine mesylate therapy is to initiate treatment at a low dosage and, on an individual basis, increase the daily dosage slowly until a maximum therapeutic response is achieved. The dosage of levodopa during this introductory period should be maintained, if possible. The initial dose of bromocriptine mesylate is 1.25 mg twice daily with meals. Assessments are advised at 2-week intervals during dosage titration to ensure that the lowest dosage producing an optimal therapeutic response is not exceeded. If necessary, the dosage may be increased every 14–28 days by 2.5 mg/day with meals. The safety of bromocriptine mesylate has not been demonstrated in dosages exceeding 100 mg/day (Willis 2005).

Psoriasis is a complex, chronic, multifactorial, inflammatory disease that involves hyperproliferation of the keratinocytes in the epidermis, with an increase in the epidermal cell turnover rate. Environmental, genetic, and immunologic factors appear to play a role. The disease most commonly manifests on the skin of the

elbows, knees, scalp, lumbosacral areas, intergluteal clefts, and glans penis. In up to 30% of patients, the joints are also affected. Bromocriptine has also been used in the treatment of psoriasis. Increase in the severity and extent of psoriasis correlates with the development of a prolactin-secretory pituitary gland microadenoma. Prolactin may play a role in the pathogenesis of psoriasis because it plays an important part in the immune reactions and exerts a proliferative effect on human keratinocytes. The degree of elevation of prolactin is related to the severity of psoriasis. However, in all patients, administration of bromocriptine as a treatment of prolactinoma was associated with a better therapeutic response in psoriatic cutaneous lesions. Bromocriptine can be useful in the treatment of different autoimmune diseases. It has been used in the treatment of psoriasis vulgaris and psoriatic arthritis with a marked improvement in the lesions (Regaña and Umbert Millet 2000).

Bromocriptine is a sympatholytic D2-dopamine agonist that has been FDA approved for the treatment of type 2 diabetes. Based on animal and human studies, timed bromocriptine administration within 2 h of awakening is believed to augment low hypothalamic dopamine levels and inhibit excessive sympathetic tone within the central nervous system (CNS), resulting in a reduction in postprandial plasma glucose levels due to enhanced suppression of hepatic glucose production. Bromocriptine has not been shown to augment insulin secretion or enhance insulin sensitivity in peripheral tissues. However, the addition of bromocriptine to poorly controlled type 2 diabetic patients treated with diet alone, metformin, sulfonylureas, or thiazolidinediones produces a 0.5–0.7% decrement in $HbA_{1c}$. Bromocriptine also reduces fasting and post-meal plasma free fatty acid (FFA) and triglyceride levels.

Autonomic dysfunction after traumatic brain injury is usually associated with hypertension. The hypertension encountered in traumatic brain injury is described as being part of a hyperadrenergic state because it is associated with elevation of urine and blood catecholamine levels. Bromocriptine can cause hypotension, so it is used in the management of labile hypertension associated with autonomic dysfunction. Treatment is started with a low dose (0.025 mg/kg twice daily) and gradually increased to 0.05 mg/kg t.d.s. as required to treat the autonomic dysfunction. So, bromocriptine is an effective alternative treatment for the episodes of autonomic dysfunction due to severe traumatic brain injury or due to other causes (Russo and O'Flaherty 2000). Bromocriptine is also used to reduce the intensity of psychiatric symptoms associated with cocaine withdrawal (Cottigham 2004). Recently, it has been reported that dopamine receptors are effective targets for nerve regeneration. Höglinger et al. (2004) reported that dopamine $D_2$ receptor agonists, including bromocriptine, stimulate precursor cells to proliferate. Moreover, it increased the number of $BrdU^+$ cells and restored the nigrostriatal pathway in a model of Parkinson's disease (Van Kampen and Eckman 2006). These reports suggest that dopamine agonists increases neurogenesis, neuroprogenitor proliferation, and neuritic outgrowth. However, amine ergot alkaloids, including ergometrine and methylergometrine, did not induce neurite outgrowth. These results indicate that the structure of amino acid ergot alkaloids is important for the effect of bromocriptine. The primary site of action of bromocriptine remains to be identified. It is possible that bromocriptine-induced neurite outgrowth via activation of tropomyosin receptor kinase A (TrkA).

Moreover, K-252a, known as a Trk inhibitor, did not inhibit bromocriptine-induced neurite outgrowth. These results suggest that bromocriptine could not directly activate the signaling pathway mediated by TrkA. Recent reports indicate that G protein-coupled receptors, such as the purine $P2Y_2$receptor, adenosine $A_{2A}$ receptor, and adrenaline $\alpha_2$ receptor, induce neurite outgrowth via the activation of proto-oncogene tyrosine-protein kinase (Src) (Arthur et al. 2006; Karkoulias et al. 2006; Nakata 2007). Thus, amino acid ergot alkaloids, including bromocriptine, might activate Src via G protein-coupled receptors. However, talipexole and pramipexole do not induce neurite outgrowth, and clonidine, adrenaline $\alpha_2$ receptor agonist, also did not. These results suggest that the effect of adrenaline $\alpha_2$ receptor agonist is not sufficient for neurite outgrowth. Bromocriptine stimulates antioxidant mechanisms in the brain and acts as a free radical scavenger in addition to its action at dopamine receptors, thus indicating its strength as a valuable neuroprotectant (Muralikrishnan and Mohanakumar 1998).

Bromocriptine is used in bodybuilding though it is not an FDA-approved indication. It acts by prolactin inhibition, similar to that of progestin-based compounds (trenbolone and nandrolone decanoate). They increase prolactin levels which tend to increase the incidence of prolactin-based gynecomastia. Bodybuilding involves restricted calorie intake, which leads to decrease in leptin levels, and the body responds by holding onto nutrient stores. Bromocriptine helps normalize the slowing metabolism during this time and helps maintain normal leptin stimulation. This phenomenon is connected to its dopamine effects. Another off-label use of bromocriptine is in the treatment of the neuroleptic malignant syndrome. Bromocriptine is marketed as the mesylate salt which has relatively high bioavailability (65–95%) due to its lipophilic nature. The drug is highly bound to plasma albumin (90–95%) and highly distributed (volume of distribution 60 L). Bromocriptine undergoes extensive metabolism primarily by hydrolysis of the amide bond to produce lysergic acid and a peptide fragment, both of which are inactive and nontoxic. Bromocriptine is also metabolized by CYP3A4 and excreted primarily in the feces via biliary secretion (>80%). Because of this clearance profile, patients with a significant hepatic impairment may have elevated plasma levels of the active drug when bromocriptine is administered in standard doses. The half-life of the drug in patients with normal hepatic function is approximately 5–6 h.

### 8.5.8 Lysergic Acid Diethylamide (LSD)

It is a psychedelic drug also known as entactogen (to touch within) which is one of the most potent psychoactive compounds and was first synthesized by Albert Hofmann in Switzerland, in 1938 from ergotamine, while researching the medical effects of ergot-derived synthetic molecules (Wu et al. 2012). However, in 1943, Hofmann unintentionally ingested the substance and experienced stimulatory effects in the CNS, and hence the hallucinogenic properties of LSD were discovered (Sanders-Bush and Hazelwood 2011). Currently, LSD is known for its use as a "club drug," together with 3,4 methylenedioxymethamphetamine (MDMA). LSD

induces intense spiritual experiences, during which users may feel they have come into contact with a greater spiritual or cosmic order. Users sometimes report out of body experiences. LSD interacts with brain 5-HT receptors to produce agonist or partial antagonist effects on serotonin activity (Halberstadt and Geyer 2011). Activation of 5-HT2A also leads to increased cortical glutamate levels probably mediated by thalamic afferents thus producing a dissociation between sensory relay centers and cortical output (Marona-Lewicka et al. 2005). LSD also stimulates dopamine D2 receptors, leading to a biphasic pharmacologic pattern of early serotonin like effects (15–30 min after administration) and late mediated dopamine-like effects (60–90 min after administration). The onset of action of LSD is within 30–60 min, with effects peaking over 1–6 h and dissipating in 8–12 h. LSD is rapidly metabolized in the liver by N-demethylation, N-deethylation, and aromatic hydroxylation, and metabolites are excreted in the urine. The elimination half-life of LSD is 3–5 h. Physical dependence and withdrawal syndromes are absent, and psychological dependence is low. However, users develop a high degree of tachyphylaxis due to downregulation of 5-HT2A receptors. Long-term effects of chronic use can result in persistent psychosis and hallucinogen-persisting perception disorder (HPPD), so-called flashbacks (Abraham 1983). LSD remains one of the most potent mood-altering and perception-altering drugs because of its relatively high effect, and no deaths have been directly attributed to LSD use alone (Mello and Mendelson 2012). LSD is not considered an addictive drug because it does not produce compulsive drug-seeking behavior. However, LSD does produce a physiologic tolerance, requiring subsequently increased doses to achieve the same effect. Acute toxicity includes gastrointestinal upset, chills, hyperglycemia, hypertension, mydriasis, tachycardia, and panic attacks. The chronic effects include flashbacks and exacerbation of latent mental disorders, particularly schizophreniform psychosis and derangements in memory function, problem-solving, and abstract thinking. Overdosage most commonly results in "bad trips" that are characterized by intense anxiety, confusion, and panic. LSD is sold on the illicit market in a variety of forms, including tablets, capsules, and a liquid form. Drops of the solution are added to blotter paper, gelatin wraps, gelatin squares, candies, and sugar cubes. Stamped blotter paper containing cartoon characters is particularly common. When found in a solid form, the substance is a powder or crystal that is incorporated into capsules or tablets. The drug can be detected by radioimmunoassay; levels from 1.5 to 5.5 ng/mL may be found within 24 h after the patient has taken a 300-mcg dose of LSD. However, high-performance liquid chromatography or gas chromatography is required for confirmation. LSD levels in the urine do not correlate with severity of symptoms (Berg et al. 2013). Hope was placed in these substances for new treatments for psychiatric conditions and discoveries that would "unlock the mysteries" of the mind. The research of LSD faded because the clinical promises failed to be realized while the illicit use of the drug grew exponentially. Today, LSD and other hallucinogens are once again being evaluated for specific purposes, such as for treatment of a cluster headache and as tools in therapy for working with those suffering from anxiety provoking end-of-life issues and for posttraumatic stress disorder.

Following oral administration, plasma lysergic acid diethylamide concentrations are detectable for up to 12 h. Maximum concentrations of lysergic acid diethylamide are reached within 1.5 h after administration and then decrease following first-order kinetics with a half-life of 3–4 h up to 12 h and slower elimination thereafter with a terminal half-life of over 9 h. Only a small fraction (1%) of the oral dose of LSD is eliminated in urine as lysergic acid diethylamide, while about 15% is eliminated as 2-oxo-3-hydroxy-lysergic acid diethylamide within 24 h. The acute subjective and sympathomimetic responses to LSD last up to 12 h and closely correlate with plasma concentrations over time.

## 8.6 Future Perspectives

Ergot alkaloids as treatment for schistosomiasis: The parasitic infection schistosomiasis is clinically treated using a single drug, praziquantel (PZQ). However, the molecular basis of action of this clinical agent is poorly understood, hence exploiting the predictive phenology between free-living planarian regenerative screens and parasitic neuromuscular physiology to reveal a broad efficacy of ergot alkaloids in phenocopying the action of PZQ. Ergot alkaloids with efficacy in regenerative assays were also found to modulate the contractility of schistosomules. Overall, these data highlight a possible therapeutic potential of ergot alkaloids as antischistosomals and the action of PZQ as an ergomimetic. Indeed, ergot alkaloids have a well appreciated ability to modulate smooth muscle contraction based on their bioaminergic mimicry, a property that underpins several of their applications in the clinic. This ergomimetic quality to PZQ action provide impetus for considering ergot alkaloids as potential drug leads for manipulating bioaminergic G protein-coupled receptors (GPCRs) to provide next-generation antischistosomals (Ribeiro et al. 2012). In conclusion, the ergot alkaloid scaffold merits further exploration by medicinal chemistry to identify novel chemotherapeutics with efficacy against parasite muscle.

## 8.7 Conclusions

Ergot alkaloids have biotechnological relevance due to their use as medicinal agents and to their role as toxins in agricultural industry. Future research on biotechnological aspects of ergot fungi will therefore most probably focus on the following trend analysis of biosynthetic pathway at enzymatic and genetic level. Alternatively, construction of recombinant peptide synthetases containing the D-lysergic acid-activating module should be a means to produce recombinant ergot drugs. With the availability of the ergot alkaloid biosynthesis gene cluster from *C. purpurea*, it will become possible to determine the regulatory mechanisms directly at the molecular level. This will be of importance for the improved biotechnological production of alkaloids in genetically engineered, alkaloid high-producing strains of *Claviceps*. In summary, detailed biochemical and molecular genetic research covering the various

aspects of ergot alkaloid-based drug development and control of toxins is required in the future. As these new studies move forward, it is hoped that this present paper will be a roadmap for also securing the data missing from our knowledge of the pharmacology of ergot alkaloids in particular *Claviceps purpura*.

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# Recent Advance on Bioactive Compounds from the Edible and Medicinal Fungi in China

9

Yan-Long Yang, Qiao-Qiao Tao, Jun-Jie Han, Li Bao, and Hong-Wei Liu

**Abstract**

Natural products and their derivatives have played a prominent role in the history of drug discovery and remain the most attractive source of potential drugs because of their structural complexity and diversity. Edible and medicinal fungi have been shown to have profound health-promoting benefits and were recognized as an important source of natural products with diverse structures and distinct pharmacological potential. There are about 10,000 fungi species and 473 medicinal fungi species in China. The fruiting bodies of these mushrooms were used in Chinese traditional medicine to treat various diseases. Recently, chemical investigation of edible and medicinal fungi collected in China led to isolation and identification of a huge number of bioactive compounds with various bioactivities including antibacterial, antioxidant, anticancer, antiplasmodial, antiproliferative, antifibrotic, and neurite outgrowth-promoting activities. In this chapter, we review the studies on isolation, structural elucidation and biologically activities of bioactive compounds derived from edible and medicinal fungi conducted by Chinese scientists after 2007. Selected compounds with unique structural features and promising bioactivities have been described herein on the basis of structural types. The main types included are terpenes, meroterpenoids, polyketides, and alkaloids. A table that lists the name of fungi species, bioactive compounds, and medicinal properties is given along with 109 references.

**Keywords**

Biological activities • Edible and medicinal fungi • Meroterpenoids • Natural products • Terpenoids

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## Contents



## Abbreviations

| | |
|---|---|
| Bax | Bcl-2-like protein 4 |
| Bcl-2 | B-cell lymphoma 2 |
| BrdU | 5-Bromo-2-deoxyuridine |
| COX-2 | Cyclooxygenase-2 |
| DPP-4 | Dipeptidyl peptidase-4 |
| DPPH | 2,2-Diphenyl-1-picrylhydrazyl |
| GPP | Geranyl pyrophosphate |
| HMGR | 3-Hydroxy-3-methylglutaryl-CoA reductase |
| $IC_{50}$ | Half maximal inhibitory concentration |
| iNOS | Inducible nitric oxide synthase |
| LPS | Lipopolysaccharide |
| MCP-1 | Monocyte chemotactic protein 1 |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| PTP1B | Protein tyrosine phosphatase 1B |
| ROS | Reactive oxygen species |
| TCM | Traditional Chinese medicine |
| TGF-β1 | Transforming growth factor β1 |

## 9.1 Introduction

Edible and medicinal fungi have been widely consumed as food ingredients for centuries, not only for their good flavor and texture but also for their substantial nutritional value and potential medicinal value (Zjawiony 2004). It is commonly accepted that there are about 10,000 fungi species and 473 medicinal fungi species in China (Dai and Yang 2008). The fruiting bodies of these mushrooms were used in Chinese traditional medicine to treat various diseases, such as the fruiting bodies of *Hericium erinaceus* was used to treat gastricism and hyperglycemia (Mizuno 1999); *Antrodia camphorata* for treating liver diseases, food and drug intoxication, diarrhea, abdominal pain, hypertension, allergies, skin itching, and tumorigenic diseases (Ao et al. 2009); *Ganoderma lucidum* and *Ganoderma sinense* for the treatment of neurasthenia, insomnia, anorexia, dizziness, chronic hepatitis, hypercholesterolemia, coronary heart disease, hypertension, and carcinomas (China Pharmacopoeia Committee 2010); and so on. Recently, edible and medicinal fungi were recognized as a prolific source of natural products with diverse structures and distinct pharmacological potential, such as davallialactone from *Inonotus xaranticus* improving the aging process (Yang et al. 2013a); thelephantin O from *Thelephora aurantiotincta* inhibiting the proliferation of cancer cells (Norikura et al. 2013); 4,7-dimethoxy-5-methyl-1,3-benzodioxole (DMB) from *A. camphorata* exhibiting antiproliferative, antitumor, and anti-inflammatory effects (Chen et al. 2007; Tu et al. 2012); erinacines A-K from *H. erinaceus* possessing stimulatory activity for the biosynthesis of nerve growth factor (Kawagishi et al. 1994, 1996a, b, 2006; Lee et al. 2000); and so on. Herein, we review the studies on isolation, structural elucidation, and biological activities of the bioactive compounds derived from edible and medicinal fungi conducted by Chinese scientists after 2007. The names, fungi sources, and bioactivities, along with the references of these bioactive compounds, have been listed in Table 9.1.

## 9.2 Terpenoids

Terpenoids are the most abundant and structurally diverse higher fungi natural products. Sesquiterpenoids gained attention because of their roles for finding new lead structures for medicinal chemistry. More than 30 skeletons of sesquiterpenoids have been reported in edible and medicinal fungi, including humulane, illudane, tremulane, lactarane, marasmane, and drimane. Diterpenoids have fewer structures than sesquiterpenoids. In this review, we mainly concentrate on cyathane. As for triterpenoids, lanostane is the most classic type.

### 9.2.1 Monoterpenoids

Five monoterpenoids (**1**–**5**) were obtained from the solid culture of *Pleurotus cornucopiae*. Compounds **1**–**5** showed moderate inhibition against nitric oxide

**Table 9.1** Bioactive compounds isolated from edible and medical fungi in China

| Fungi species | Compounds | Bioactivities | References |
|---|---|---|---|
| *Pleurotus cornucopiae* | **1** | Inhibition of nitric oxide production | Wang et al. (2013a) |
| | **2** | Inhibition of nitric oxide production | Wang et al. (2013a) |
| | **3** | Inhibition of nitric oxide production | Wang et al. (2013a) |
| | **4** | Inhibition of nitric oxide production | Wang et al. (2013a) |
| | **5** | Inhibition of nitric oxide production | Wang et al. (2013a) |
| | Pleurospiroketal A (**61**) | Inhibition of nitric oxide production | Wang et al. (2013b) |
| | Pleurospiroketal B (**62**) | Inhibition of nitric oxide production | Wang et al. (2013b) |
| | Pleurospiroketal C (**63**) | Inhibition of nitric oxide production | Wang et al. (2013b) |
| | Pleurospiroketal D (**64**) | – | Wang et al. (2013b) |
| | Pleurospiroketal E (**65**) | – | Wang et al. (2013b) |
| | **66** | Cytotoxicity | Wang et al. (2013b) |
| | **67** | Cytotoxicity | Wang et al. (2013b) |
| | **68** | Cytotoxicity | Wang et al. (2013b) |
| *Flammulina velutipes* | 5-(Hydroxymethyl)-2-(prop-1-en-2-yl) cyclohexanol (**6**) 1-en-2-yl) cyclohexanol (**6**) | – | Cai et al. (2013) |
| | Flamvelutpenoid A (**7**) | Antibacterial activity | Wang et al. (2012a) |
| | Flamvelutpenoid B (**8**) | Antibacterial activity | Wang et al. (2012a) |
| | Flamvelutpenoid C (**9**) | Antibacterial activity | Wang et al. (2012a) |
| | Flamvelutpenoid D (**10**) | Antibacterial activity | Wang et al. (2012a) |
| | Flamvelutpenoid E (**11**) | – | Tao et al. (2016a) |
| | Flamvelutpenoid F (**12**) | – | Tao et al. (2016a) |
| | Enokipodin E (**13**) | – | Wang et al. (2012b) |
| | Enokipodin F (**14**) | Antifungal activity | Wang et al. (2012b) |
| | Enokipodin G (**15**) | Antifungal activity | Wang et al. (2012b) |
| | Enokipodin H (**16**) | – | Wang et al. (2012b) |
| | Enokipodin I (**17**) | Antifungal activity | Wang et al. (2012b) |
| | Enokipodin J (**18**) | Cytotoxicity; antioxidant activity | Wang et al. (2012b) |

| | | | |
|---|---|---|---|
| | 2,5-Cuparadiene-1,4-dione (**19**) | Cytotoxicity; antioxidant activity | Wang et al. (2012b) |
| | Enokipodin B (**20**) | Cytotoxicity; antioxidant activity | Wang et al. (2012b) |
| | Enokipodin D (**21**) | Cytotoxicity; antioxidant activity | Wang et al. (2012b) |
| | Flammufuranone A (**22**) | – | Tao et al. (2016a) |
| | Flammufuranone B (**23**) | – | Tao et al. (2016a) |
| | Sterpurol A (**24**) | – | Wang et al. (2012b) |
| | Sterpurol B (**25**) | – | Wang et al. (2012b) |
| | Sterpuric acid (**26**) | – | Wang et al. (2012b) |
| | Flammulinol A (**27**) | – | Wang et al. (2012c) |
| | Flammulinolide A (**28**) | Cytotoxicity | Wang et al. (2012c) |
| | Flammulinolide B (**29**) | Cytotoxicity | Wang et al. (2012c) |
| | Flammulinolide C (**30**) | Cytotoxicity | Wang et al. (2012c) |
| | Flammulinolide D (**31**) | – | Wang et al. (2012c) |
| | Flammulinolide E (**32**) | – | Wang et al. (2012c) |
| | Flammulinolide F (**33**) | Cytotoxicity | Wang et al. (2012c) |
| | Flammulinolide G (**34**) | – | Wang et al. (2012c) |
| | Flammuspirone A (**35**) | HMG-CoA reductase inhibitor | Tao et al. (2016a) |
| | Flammuspirone B (**36**) | – | Tao et al. (2016a) |
| | Flammuspirone C (**37**) | HMG-CoA reductase inhibitor; DPP-4 inhibitory activity | Tao et al. (2016a) |
| | Flammuspirone D (**38**) | DPP-4 inhibitory activity | Tao et al. (2016a) |
| | Flammuspirone E (**39**) | DPP-4 inhibitory activity | Tao et al. (2016a) |
| | Flammuspirone F (**40**) | – | Tao et al. (2016a) |
| | Flammuspirone G (**41**) | – | Tao et al. (2016a) |
| | Flammuspirone H (**42**) | DPP-4 inhibitory activity | Tao et al. (2016a) |
| | Flammuspirone I (**43**) | – | Tao et al. (2016a) |
| | Flammuspirone J (**44**) | DPP-4 inhibitory activity | Tao et al. (2016a) |
| | 7,13,14-Trihydroxy-4-cadinen-15-oic acid methyl ester (**45**) | HMG-CoA reductase inhibitor | Tao et al. (2016a) |
| | | DPP-4 inhibitory activity | |

(continued)

**Table 9.1** (continued)

| Fungi species | Compounds | Bioactivities | References |
|---|---|---|---|
| | 1,2,6,10-Tetrahydroxy-3,9-epoxy-14-nor-5(15)-eudesmane (**46**) | HMG-CoA reductase inhibitor | Tao et al. (2016a) |
| | | DPP-4 inhibitory activity | |
| *Pleurotus cystidiosus* | Pleuroton A (**47**) | Cytotoxicity | Zheng et al. (2015) |
| | Pleuroton B (**48**) | Cytotoxicity | Zheng et al. (2015) |
| | Clitocybulol D (**49**) | Cytotoxicity | Zheng et al. (2015) |
| | Clitocybulol E (**50**) | Cytotoxicity | Zheng et al. (2015) |
| | Clitocybulol F (**51**) | Cytotoxicity | Zheng et al. (2015) |
| | Clitocybulol G (**52**) | PTP1B inhibitory activity | Tao et al. (2016b) |
| | Clitocybulol H (**53**) | – | Tao et al. (2016b) |
| | Clitocybulol I (**54**) | – | Tao et al. (2016b) |
| | Clitocybulol J (**55**) | – | Tao et al. (2016b) |
| | Clitocybulol K (**56**) | – | Tao et al. (2016b) |
| | Clitocybulol L (**57**) | PTP1B inhibitory activity | Tao et al. (2016b) |
| | Clitocybulol M (**58**) | – | Tao et al. (2016b) |
| | Clitocybulol N (**59**) | – | Tao et al. (2016b) |
| | Clitocybulol O (**60**) | – | Tao et al. (2016b) |
| *Pleurotus citrinopileatus* | Pleurospiroketal F (**69**) | – | Tao et al. (2016c) |
| | Pleurotin A (**70**) | PTP1B inhibitory activity | Tao et al. (2016c) |
| | Pleurotin B (**71**) | – | Tao et al. (2016c) |
| | Pleurotin C (**72**) | – | Tao et al. (2016c) |
| | Pleurotin D (**73**) | – | Tao et al. (2016c) |
| | Pleurotin E (**74**) | PTP1B inhibitory activity | Tao et al. (2016c) |
| | Pleurotin F (**75**) | – | Tao et al. (2016c) |
| | 5,7-Dimethoxyisobenzofuran-1(3H)-one (**396**) | Iron-chelating capacity | Li et al. (2013c) |
| | | Antioxidant activity | |
| | 3,5-Dihydroxybenzyl acetate (**397**) | Iron-chelating capacity | Li et al. (2013c) |
| | 2,4-Dihydroxy-6-(hydroxymethyl) benzaldehyde (**398**) | Iron-chelating capacity | Li et al. (2013c) |

| | | | |
|---|---|---|---|
| *Laetiporus sulphureus* | Sulphureuine B (**76**) | Induces apoptosis | He et al. (2015a) |
| | Sulphureuine C (**77**) | – | He et al. (2015a) |
| | Sulphureuine D (**78**) | – | He et al. (2015a) |
| | Sulphureuine E (**79**) | – | He et al. (2015a) |
| | Sulphureuine F (**80**) | – | He et al. (2015a) |
| | Sulphureuine G (**81**) | – | He et al. (2015a) |
| | Sulphureuine H (**82**) | – | He et al. (2015a) |
| | Phellinuin J (**83**) | – | He et al. (2015b) |
| | Sulphureuine A (**84**) | – | He et al. (2015b) |
| | 15α-Hydroxy-3,4-secolanosta-4(28),8,24-triene-3,21-dioic acid (**226**) | – | Yin et al. (2015) |
| | 5α-Hydroxy-3,4-seco-lanosta-4(28),8,24-triene-3,21-dioic acid 3-methyl ester (**227**) | – | Yin et al. (2015) |
| | 15α-Acetoxylhydroxytrametenolic acid (**228**) | – | Yin et al. (2015) |
| | Versisponic acid D (**229**) | – | Yin et al. (2015) |
| | **230** | Cytotoxicity | He et al. (2015a) |
| | **231** | – | He et al. (2015a) |
| | Eburicoic acid (**232**) | Attenuated H+/K+-ATPase activity | Wang et al. (2015b) |
| *Xylaria nigripes* | Nigriterpene A (**85**) | – | Chang et al. (2017) |
| | Nigriterpene B (**86**) | – | Chang et al. (2017) |
| | Nigriterpene C (**87**) | Anti-inflammatory effect | Chang et al. (2017) |
| | Nigriterpene D (**88**) | – | Chang et al. (2017) |
| | Nigriterpene E (**89**) | – | Chang et al. (2017) |
| | Nigriterpene F (**90**) | – | Chang et al. (2017) |

(continued)

**Table 9.1** (continued)

| Fungi species | Compounds | Bioactivities | References |
|---|---|---|---|
| *Boletus edulis* | Boledulin A (**91**) | Cytotoxicity | Feng et al. (2011) |
| | Boledulin B (**92**) | – | Feng et al. (2011) |
| | Boledulin C (**93**) | – | Feng et al. (2011) |
| *Inonotus vaninii* | Inonolane A (**94**) | – | Yang et al. (2013b) |
| *Dictyophora indusiata* | Albaflavenone (**96**) | Camphor-like odor | Huang et al. (2011) |
| *Cyathus africanus* | Cyathin D (**97**) | – | Han et al. (2013) |
| | Cyathin E (**98**) | – | Han et al. (2013) |
| | Cyathin F (**99**) | Inhibition of nitric oxide production | Han et al. (2013) |
| | Cyathin G (**100**) | – | Han et al. (2013) |
| | Cyathin H (**101**) | Inhibition of nitric oxide production | Han et al. (2013) |
| | Neosarcodonin O (**102**) | Inhibition of nitric oxide production; cytotoxicity | Han et al. (2013) |
| | Cyathatriol (**103**) | – | Han et al. (2013) |
| | 11-O-acetylcyathatriol (**104**) | Inhibition of nitric oxide production; cytotoxicity | Han et al. (2013) |
| | Cyathin R (**105**) | Inducing apoptosis | Huang et al. (2015) |
| | Cyathin T (**106**) | Inhibition of nitric oxide production | Han et al. (2015) |
| | Cyathin V (**107**) | – | Han et al. (2015) |
| | Cyathin W (**108**) | Inhibition of nitric oxide production; cytotoxicity | Han et al. (2015) |
| | Cyathin Q (**109**) | Inducing apoptosis | He et al. (2016) |
| *Cyathus hookeri* | Cyathin I (**110**) | Inhibition of nitric oxide production | Xu et al. (2013) |
| | (12R)-11a,14a-epoxy-13a,14b,15-trihydroxycyath-3-ene (**111**) | Inhibition of nitric oxide production | Xu et al. (2013) |
| | Erinacine I (**112**) | Inhibition of nitric oxide production | Xu et al. (2013) |

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| *Cyathus gansuensis* | Cyathin J (**113**) | Inhibition of nitric oxide production | Wang et al. (2014a) |
| | Cyathin K (**114**) | Inhibition of nitric oxide production | Wang et al. (2014a) |
| | Cyathin L (**115**) | – | Wang et al. (2014a) |
| | Cyathin M (**116**) | Inhibition of nitric oxide production | Wang et al. (2014a) |
| | Cyathin N (**117**) | – | Wang et al. (2014a) |
| | Cyathin O (**118**) | – | Wang et al. (2014a) |
| | Cyathin P (**119**) | – | Wang et al. (2014a) |
| | **120** | Inhibition of nitric oxide production | Wang et al. (2014a) |
| | **121** | – | Wang et al. (2014a) |
| *Cyathus striatus* | Striatoid A (**122**) | Enhanced nerve growth factor (NGF)-mediated neurite outgrowth | Bai et al. (2015) |
| | Striatoid B (**123**) | Enhanced nerve growth factor (NGF)-mediated neurite outgrowth | Bai et al. (2015) |
| | Striatoid C (**124**) | Enhanced nerve growth factor (NGF)-mediated neurite outgrowth | Bai et al. (2015) |
| | Striatoid D (**125**) | Enhanced nerve growth factor (NGF)-mediated neurite outgrowth | Bai et al. (2015) |
| | Striatoid E (**126**) | Enhanced nerve growth factor (NGF)-mediated neurite outgrowth | Bai et al. (2015) |
| | Striatoid F (**127**) | Enhanced nerve growth factor (NGF)-mediated neurite outgrowth | Bai et al. (2015) |
| *Hericium erinaceus* | **128** | Cytotoxicity | Zhang et al. (2015a) |
| | Erinacerin C (**351**) | – | Wang et al. (2015c) |
| | Erinacerin D (**352**) | α-Glucosidase inhibitory activity | Wang et al. (2015c) |
| | Erinacerin E (**353**) | α-Glucosidase inhibitory activity | Wang et al. (2015c) |
| | Erinacerin F (**354**) | α-Glucosidase inhibitory activity | Wang et al. (2015c) |
| | Erinacerin G (**355**) | α-Glucosidase inhibitory activity | Wang et al. (2015c) |

(continued)

**Table 9.1** (continued)

| Fungi species | Compounds | Bioactivities | References |
|---|---|---|---|
| | Erinacerin H (**356**) | α-Glucosidase inhibitory activity | Wang et al. (2015c) |
| | Erinacerin I (**357**) | α-Glucosidase inhibitory activity | Wang et al. (2015c) |
| | Erinacerin J (**358**) | α-Glucosidase inhibitory activity | Wang et al. (2015c) |
| | Erinacerin K (**359**) | α-Glucosidase inhibitory activity | Wang et al. (2015c) |
| | Erinacerin L (**360**) | α-Glucosidase inhibitory activity | Wang et al. (2015c) |
| | Erinacerin Q (**361**) | α-Glucosidase inhibitory activity | Wang et al. (2015d) |
| | | PTP1B inhibitory activity | |
| | Erinacerin R (**362**) | α-Glucosidase inhibitory activity | Wang et al. (2015d) |
| | | PTP1B inhibitory activity | |
| | Erinacerin S (**363**) | α-glucosidase inhibitory activity | Wang et al. (2015d) |
| | | PTP1B inhibitory activity | |
| | Erinacerin T (**364**) | α-Glucosidase inhibitory activity | Wang et al. (2015d) |
| | | PTP1B inhibitory activity | |
| | Erinaceolactam A (**365**) | – | Wang et al. (2015c) |
| | Erinaceolactam B (**366**) | – | Wang et al. (2015c) |
| | Erinaceolactam C (**367**) | – | Wang et al. (2015c) |
| | Erinaceolactam D (**368**) | – | Wang et al. (2015c) |
| | Erinaceolactam E (**369**) | – | Wang et al. (2015c) |
| | Erinaceolactone D (**370**) | – | Wang et al. (2016d) |
| | Erinaceolactone E (**371**) | – | Wang et al. (2016d) |
| | Erinaceolactone F (**372**) | – | Wang et al. (2016d) |
| | Hericenone K (**373**) | Stimulate NGF-mediated neurite outgrowth | Zhang et al. (2015b) |
| | Hericenone L (**374**) | Cytotoxicity | Ma et al. (2012) |
| | Erinacerin M (**399**) | Cytotoxicity | Wang et al. (2015d) |
| | Erinacerin N (**400**) | Cytotoxicity | Wang et al. (2015d) |

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| | Erinacerin O (**401**) | Cytotoxicity | Wang et al. (2015d) |
| | Erinacerin P (**402**) | Cytotoxicity | Wang et al. (2015d) |
| *Antrodia camphorata* | Antrocamol LT1 (**375**) | Cytotoxicity | Yen et al. (2015) |
| | Antrocamol LT2 (**376**) | Cytotoxicity | Yen et al. (2015) |
| | Antrocamol LT3 (**377**) | Cytotoxicity | Yen et al. (2015) |
| | Antroquinonol (**378**) | Cytotoxicity | Yen et al. (2015) |
| | Antroquinonol B (**379**) | NO inhibition | Yang et al. (2009) |
| | 4-Acetyl-antroquinonol B (**380**) | NO inhibition | Yang et al. (2009) |
| | Antroquinonol D (**381**) | Inhibiting breast cancer growth and migration potential | Wang et al. (2014b) |
| *Pleurotus eryngii* | Eryngiolide A (**129**) | Cytotoxicity | Wang et al. (2012d) |
| | 2,3,6,23-Tetrahydroxy-urs-12-en-28-oic acid (**233**) | Antiproliferative activity | Xue et al. (2015) |
| | 2,3,23-Trihydroxyurs-12-en-28-oic acid (**234**) | Antiproliferative activity | Xue et al. (2015) |
| | Lupeol (**235**) | Antiproliferative activity | Xue et al. (2015) |
| | 6-Dimethoxyisobenzofuran-1(3H)-one (**395**) | – | Liu et al. (2013) |
| *Ganoderma boninense* | Ganoboninketal A (**130**) | Antimalarial effect | Ma et al. (2014) |
| | Ganoboninketal B (**131**) | Antimalarial effect | Ma et al. (2014) |
| | Ganoboninketal C (**132**) | Antimalarial effect | Ma et al. (2014) |
| | Ganoboninone A (**133**) | Antimalarial effect | Ma et al. (2015) |
| | Ganoboninone B (**134**) | Antimalarial effect | Ma et al. (2015) |
| | Ganoboninone C (**135**) | Antimalarial effect | Ma et al. (2015) |
| | Ganoboninone D (**136**) | Antimalarial effect | Ma et al. (2015) |
| | Ganoboninone E (**137**) | Antimalarial effect | Ma et al. (2015) |
| | Ganoboninone F (**138**) | Antimalarial effect | Ma et al. (2015) |

(continued)

**Table 9.1** (continued)

| Fungi species | Compounds | Bioactivities | References |
|---|---|---|---|
| *Ganoderma leucocontextum* | Ganoleucoin A (**139**) | HMG-CoA reductase inhibitor/cytotoxicity | Wang et al. (2015a) |
| | Ganoleucoin B (**140**) | Cytotoxicity | Wang et al. (2015a) |
| | Ganoleucoin C (**141**) | HMG-CoA reductase inhibitor | Wang et al. (2015a) |
| | Ganoleucoin D (**142**) | – | Wang et al. (2015a) |
| | Ganoleucoin E (**143**) | – | Wang et al. (2015a) |
| | Ganoleucoin F (**144**) | HMG-CoA reductase inhibitor | Wang et al. (2015a) |
| | | Cytotoxicity | |
| | Ganoleucoin G (**145**) | Cytotoxicity | Wang et al. (2015a) |
| | Ganoleucoin H (**146**) | – | Wang et al. (2015a) |
| | Ganoleucoin I (**147**) | – | Wang et al. (2015a) |
| | Ganoleucoin J (**148**) | HMG-CoA reductase inhibitor | Wang et al. (2015a) |
| | | Cytotoxicity | |
| | Ganoleucoin K (**149**) | HMG-CoA reductase inhibitor | Wang et al. (2015a) |
| | Ganoleucoin L (**150**) | HMG-CoA reductase inhibitor | Wang et al. (2015a) |
| | | Cytotoxicity | |
| | Ganoleucoin M (**151**) | HMG-CoA reductase inhibitor | Wang et al. (2015a) |
| | Ganoleucoin N (**152**) | HMG-CoA reductase inhibitors | Wang et al. (2015a) |
| | Ganoleucoin O (**153**) | – | Wang et al. (2015a) |
| | Ganoleucoin P (**154**) | Cytotoxicity | Wang et al. (2015a) |
| | Leucocontextin A (**155**) | – | Zhao et al. (2016a) |
| | Leucocontextin B (**156**) | – | Zhao et al. (2016a) |
| | Leucocontextin C (**157**) | – | Zhao et al. (2016a) |
| | Leucocontextin D (**158**) | – | Zhao et al. (2016a) |

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| | Leucocontextin E (**159**) | – | Zhao et al. (2016a) |
| | Leucocontextin F (**160**) | – | Zhao et al. (2016a) |
| | Leucocontextin G (**161**) | – | Zhao et al. (2016a) |
| | Leucocontextin H (**162**) | – | Zhao et al. (2016a) |
| | Leucocontextin I (**163**) | – | Zhao et al. (2016a) |
| | Leucocontextin J (**164**) | – | Zhao et al. (2016a) |
| | Leucocontextin K (**165**) | – | Zhao et al. (2016a) |
| | Leucocontextin L (**166**) | – | Zhao et al. (2016a) |
| | Leucocontextin M (**167**) | – | Zhao et al. (2016a) |
| | Leucocontextin N (**168**) | – | Zhao et al. (2016a) |
| | Leucocontextin O (**169**) | – | Zhao et al. (2016a) |
| | Leucocontextin P (**170**) | – | Zhao et al. (2016a) |
| | Leucocontextin Q (**171**) | – | Zhao et al. (2016a) |
| | Leucocontextin R (**172**) | Cytotoxicity | Zhao et al. (2016a) |
| | Leucocontextin S (**173**) | – | Zhao et al. (2016b) |
| | Leucocontextin T (**174**) | – | Zhao et al. (2016b) |
| | Leucocontextin U (**175**) | – | Zhao et al. (2016b) |
| | Leucocontextin V (**176**) | – | Zhao et al. (2016b) |
| | Leucocontextin W (**177**) | – | Zhao et al. (2016b) |
| | Leucocontextin X (**178**) | – | Zhao et al. (2016b) |
| | Ganoleucin A (**342**) | α-Glucosidase inhibitory activity | Wang et al. (2016b) |
| | Ganoleucin B (**343**) | – | Wang et al. (2016b) |
| | Ganoleucin C (**344**) | α-Glucosidase inhibitory activity | Wang et al. (2016b) |
| | Ganodilactone (**345**) | Pancreatic lipase inhibitory activities | Chen et al. (2016) |

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**Table 9.1** (continued)

| Fungi species | Compounds | Bioactivities | References |
|---|---|---|---|
| *Ganoderma lucidum* | Ganoderitriol M (**179**) | – | Chen et al. (2009) |
| | Ethyl lucidenate A (**180**) | Cytotoxicity | Li et al. (2013a) |
| | Ethyl 7β-hydroxy-4,4,14α-trimethyl-3,11,15-trioxo-5α-chol-8-en-24-oate (**181**) | NGF-like neuronal survival-promoting activities | Zhang et al. (2011) |
| | 23S-hydroxy-3,7,11,15-tetraoxo-lanost-8,24E-diene-26-oic acid (**182**) | Cytotoxicity | Guan et al. (2008) |
| | 12β-Acetoxy-3β-hydroxy-7,11,15,23-tetraoxo-lanost-8,20E-diene-26-oic acid (**183**) | Cytotoxicity | Guan et al. (2008) |
| | **184** | – | Cheng et al. (2010) |
| | **185** | Cytotoxicity | Cheng et al. (2010) |
| | **186** | – | Cheng et al. (2010) |
| | **187** | Cytotoxicity | Cheng et al. (2010) |
| | **188** | Cytotoxicity | Cheng et al. (2010) |
| | **189** | – | Cheng et al. (2010) |
| | (+)-Lingzhiol (**236**) | Inhibit the phosphorylation of Smad3 | Yan et al. (2013) |
| | (−)-Lingzhiol (**237**) | Inhibit the phosphorylation of Smad3 | Yan et al. (2013) |
| | Chizhine A (**238**) | Inhibit MCP-1 and fibronectin production | Luo et al. (2015a) |
| | Chizhine B (**239**) | Inhibit MCP-1 and fibronectin production | Luo et al. (2015a) |
| | Chizhine C (**240**) | Inhibit MCP-1 and fibronectin production | Luo et al. (2015a) |
| | Chizhine D (**241**) | Inhibit MCP-1 and fibronectin production | Luo et al. (2015a) |
| | Chizhine E (**242**) | Inhibit MCP-1 and fibronectin production | Luo et al. (2015a) |
| | Chizhine F (**243**) | Inhibit MCP-1 and fibronectin production | Luo et al. (2015a) |
| | Lingzhifuran A (**244**) | Inhibit TGF-β1-induced Smad3 phosphorylation | Ding et al. (2016) |
| | Lingzhilactone D (**245**) | Inhibit TGF-β1-induced Smad3 phosphorylation | Ding et al. (2016) |
| | Lingzhilactone E (**246**) | – | Ding et al. (2016) |
| | Lingzhilactone F (**247**) | – | Ding et al. (2016) |

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| | Lucidimine A (**412**) | – | Zhao et al. (2015) |
| | Lucidimine B (**413**) | – | Zhao et al. (2015) |
| | Lucidimine C (**414**) | – | Zhao et al. (2015) |
| | Lucidimine D (**415**) | – | Zhao et al. (2015) |
| *Ganoderma sinense* | Ganosinensine (**95**) | – | Liu et al. (2012) |
| | Ganolactone B (**190**) | – | Qiao et al. (2007) |
| | Ganoderiol A triacetate (**191**) | – | Qiao et al. (2007) |
| | Methyl ganosinensate A (**192**) | – | Wang et al. (2010) |
| | Ganosinensic acid A (**193**) | – | Wang et al. (2010) |
| | Ganosinensic acid B (**194**) | – | Wang et al. (2010) |
| | Ganosineniol A (**195**) | – | Liu et al. (2012) |
| | Ganosinoside A (**196**) | – | Liu et al. (2012) |
| | Ganoderic acid Jc (**197**) | Cytotoxicity | Liu et al. (2012) |
| | Ganoderic acid Jd (**198**) | – | Liu et al. (2012) |
| | Ganodermatetraol (**199**) | Induction ability of hPXR-mediated CYP3A4 expression | Liu et al. (2012) |
| | Ganolucidic acid γa (**200**) | – | Liu et al. (2012) |
| | Ganolucidate F (**201**) | Induction ability of hPXR-mediated CYP3A4 expression | Liu et al. (2012) |
| | Ganoderiol J (**202**) | – | Liu et al. (2012) |
| | Methyl lucidenate Ha (**203**) | – | Liu et al. (2012) |
| | (−)-Sinensilactam A (**261**) | Smad3 phosphorylation inhibitor | Luo et al. (2015b) |
| | (+)-Sinensilactam A (**262**) | Smad3 phosphorylation inhibitor | Luo et al. (2015b) |
| | Zizhine A (**263**) | – | Cao et al. (2016) |
| | Zizhine B (**264**) | – | Cao et al. (2016) |
| | Zizhine C (**265**) | – | Cao et al. (2016) |
| | Zizhine D (**266**) | – | Cao et al. (2016) |

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**Table 9.1** (continued)

| Fungi species | Compounds | Bioactivities | References |
|---|---|---|---|
| | Zizhine E (**267**) | – | Cao et al. (2016) |
| | Zizhine F (**268**) | – | Cao et al. (2016) |
| | Sinensine A (**407**) | Protecting the injury induced by hydrogen peroxide oxidation on HUVEC | Liu et al. (2010) |
| | Sinensine B (**408**) | – | Liu et al. (2011) |
| | Sinensine C (**409**) | – | Liu et al. (2011) |
| | Sinensine D (**410**) | – | Liu et al. (2011) |
| | Sinensine E (**411**) | – | Liu et al. (2011) |
| *Ganoderma cochlear* | Fornicatin G (**204**) | – | Peng et al. (2012) |
| | Fornicatin H (**205**) | – | Peng et al. (2012) |
| | Cochlate A (**206**) | – | Peng et al. (2014a) |
| | Cochlate B (**207**) | – | Peng et al. (2014a) |
| | Fornicatin D (**208**) | Hepatoprotective activity | Peng et al. (2014a) |
| | Fornicatin E (**209**) | – | Peng et al. (2014a) |
| | Fornicatin F (**210**) | Hepatoprotective activity | Peng et al. (2014a) |
| | Ganodercochlearin A (**211**) | – | Peng et al. (2014a) |
| | Ganodercochlearin A (**212**) | – | Peng et al. (2014a) |
| | Ganodercochlearin A (**213**) | – | Peng et al. (2014a) |
| | Ganocochlearic acid A (**214**) | – | Peng et al. (2015a) |
| | Cochlate C (**215**) | – | Peng et al. (2015a) |
| | Cochlearic acid A (**216**) | – | Peng et al. (2015a) |
| | Ganodecochlearin D (**217**) | Cytotoxicity | Peng et al. (2015a) |
| | Ganodercochlearin E (**218**) | – | Peng et al. (2015a) |
| | Cochlearic acid B (**219**) | – | Peng et al. (2015a) |
| | Ganodercochlearin F (**220**) | Cytotoxicity | Peng et al. (2015a) |
| | Ganodercochlearin G (**221**) | Cytotoxicity | Peng et al. (2015a) |

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| | Ganodercochlearin H (**222**) | Cytotoxicity | Peng et al. (2015a) |
| | Ganodercochlearin I (**223**) | – | Peng et al. (2015a) |
| | Ganodercochlearin J (**224**) | Cytotoxicity | Peng et al. (2015a) |
| | Ganodercochlearin K (**240**) | Cytotoxicity | Peng et al. (2015a) |
| | Ganocin A (**269**) | – | Peng et al. (2014b) |
| | Ganocin B (**270**) | – | Peng et al. (2014b) |
| | Ganocin C (**271**) | – | Peng et al. (2014b) |
| | Ganocin D (**272**) | Anti-AChE activity | Peng et al. (2014b) |
| | Cochlearol A (**273**) | – | Dou et al. (2014) |
| | Cochlearol A (**274**) | Inhibitor of p-Smads | Dou et al. (2014) |
| | Cochlearoid A (**275**) | Inhibited Cav3.1 TTCC | Zhou et al. (2015) |
| | Cochlearoid B (**276**) | – | Zhou et al. (2015) |
| | Cochlearoid C (**277**) | – | Zhou et al. (2015) |
| | Cochlearoid D (**278**) | Inhibited Cav3.1 TTCC | Zhou et al. (2015) |
| | Cochlearoid E (**279**) | – | Zhou et al. (2015) |
| | Cochlearine A (**280**) | Inhibited Cav3.1 TTCC | Zhou et al. (2015) |
| | Cochlearine B (**281**) | – | Zhou et al. (2015) |
| | Ganoderin A (**282**) | Antioxidant effect | Peng et al. (2015b) |
| | Ganocochlearin A (**283**) | Antioxidant effect | Peng et al. (2015b) |
| | Ganocochlearin B (**284**) | Antioxidant effect | Peng et al. (2015b) |
| | Ganocochlearin C (**285**) | Antioxidant effect | Peng et al. (2015b) |
| | Ganocochlearin D (**286**) | Antioxidant effect | Peng et al. (2015b) |
| | Fornicin D (**287**) | Antioxidant effect | Peng et al. (2015b) |

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**Table 9.1** (continued)

| Fungi species | Compounds | Bioactivities | References |
|---|---|---|---|
| | Ganomycin C (**288**) | Antioxidant effect | Peng et al. (2015b) |
| | Cochlearoid F (**289**) | Inhibitory activity on fibronectin | Wang et al. (2016a) |
| | Cochlearoid G (**290**) | Inhibitory activity on fibronectin | Wang et al. (2016a) |
| | Cochlearoid H (**291**) | Inhibitory activity on fibronectin | Wang et al. (2016a) |
| | Cochlearoid I (**292**) | Inhibitory activity on fibronectin | Wang et al. (2016a) |
| | Cochlearoid J (**293**) | – | Wang et al. (2016a) |
| | Cochlearoid K (**294**) | Inhibitory activity on fibronectin | Wang et al. (2016a) |
| *Ganoderma applanatum* | Applanatumin A (**295**) | Antifibrotic activity | Luo et al. (2015c) |
| | Applanatumol A (**296**) | ECM inhibitors | Luo et al. (2016a) |
| | Applanatumol B (**297**) | ECM inhibitors | Luo et al. (2016a) |
| | (±)-Ganoapplanin (**298**) | – | Li et al. (2016a) |
| | Spiroapplanatumine A (**299**) | – | Luo et al. (2017) |
| | Spiroapplanatumine B (**300**) | – | Luo et al. (2017) |
| | Spiroapplanatumine C (**301**) | – | Luo et al. (2017) |
| | Spiroapplanatumine D (**302**) | – | Luo et al. (2017) |
| | Spiroapplanatumine E (**303**) | – | Luo et al. (2017) |
| | Spiroapplanatumine F (**304**) | – | Luo et al. (2017) |
| | Spiroapplanatumine G (**305**) | Inhibited JAK3 kinase | Luo et al. (2017) |
| | Spiroapplanatumine H (**306**) | Inhibited JAK3 kinase | Luo et al. (2017) |
| | Spiroapplanatumine I (**307**) | – | Luo et al. (2017) |
| | Spiroapplanatumine J (**308**) | – | Luo et al. (2017) |
| | Spiroapplanatumine K (**309**) | – | Luo et al. (2017) |
| | Spiroapplanatumine L (**310**) | – | Luo et al. (2017) |
| | Spiroapplanatumine M (**311**) | – | Luo et al. (2017) |

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| | Spiroapplanatumine N (**312**) | – | Luo et al. (2017) |
| | Spiroapplanatumine O (**313**) | – | Luo et al. (2017) |
| | Spiroapplanatumine P (**314**) | – | Luo et al. (2017) |
| | Spiroapplanatumine Q (**315**) | – | Luo et al. (2017) |
| | Applanatumol C (**316**) | COX-2 inhibitory effect | Luo et al. (2016b) |
| | Applanatumol D (**317**) | – | Luo et al. (2016b) |
| | Applanatumol E (**318**) | – | Luo et al. (2016b) |
| | Applanatumol F (**319**) | – | Luo et al. (2016b) |
| | Applanatumol G (**320**) | – | Luo et al. (2016b) |
| | Applanatumol H (**321**) | – | Luo et al. (2016b) |
| | Applanatumol I (**322**) | – | Luo et al. (2016b) |
| | Applanatumol J (**323**) | – | Luo et al. (2016b) |
| | Applanatumol K (**324**) | – | Luo et al. (2016b) |
| | Applanatumol L (**325**) | – | Luo et al. (2016b) |
| | Applanatumol M (**326**) | – | Luo et al. (2016b) |
| | Applanatumol N (**327**) | – | Luo et al. (2016b) |
| | Applanatumol O (**328**) | – | Luo et al. (2016b) |
| | Applanatumol P (**329**) | – | Luo et al. (2016b) |
| | Applanatumol Q (**330**) | – | Luo et al. (2016b) |
| | Applanatumol R (**331**) | – | Luo et al. (2016b) |
| | Applanatumol S (**332**) | – | Luo et al. (2016b) |
| | Applanatumol T (**333**) | – | Luo et al. (2016b) |
| | Applanatumol U (**334**) | – | Luo et al. (2016b) |
| | Applanatumol V (**335**) | – | Luo et al. (2016b) |
| | Applanatumol W (**336**) | – | Luo et al. (2016b) |
| | Applanatumol X (**337**) | – | Luo et al. (2016b) |

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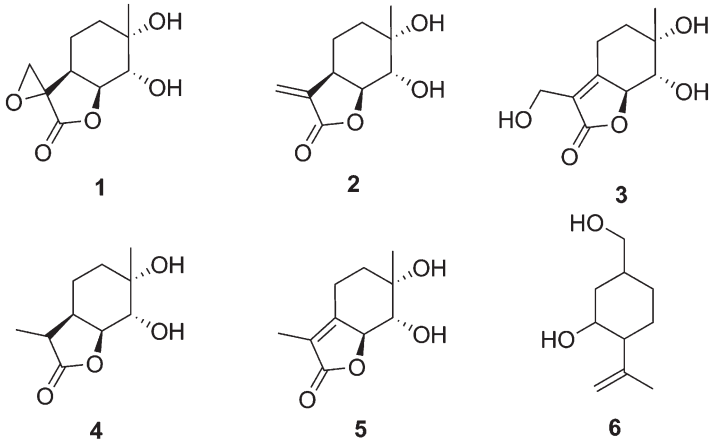
**Table 9.1** (continued)

| Fungi species | Compounds | Bioactivities | References |
|---|---|---|---|
| | Applanatumol Y (**338**) | – | Luo et al. (2016b) |
| | Applanatumol Z (**339**) | – | Luo et al. (2016b) |
| | Applanatumol Z1 (**340**) | – | Luo et al. (2016b) |
| | Applanatumol Z2 (**341**) | – | Luo et al. (2016b) |
| *Ganoderma capense* | Ganocapensin A (**346**) | Antioxidant effects | Peng et al. (2016) |
| | Ganocapensin B (**347**) | Antioxidant effects | Peng et al. (2016) |
| | Ganomycin E (**348**) | Antioxidant effects | Peng et al. (2016) |
| | Ganomycin F (**349**) | Antioxidant effects | Peng et al. (2016) |
| | Fornicin E (**350**) | Antioxidant effects | Peng et al. (2016) |
| *Ganoderma lingzhi* | Spirolingzhine A (**248**) | Affect NSC cell cycle progression | Yan et al. (2015a) |
| | Spirolingzhine B (**249**) | – | Yan et al. (2015a) |
| | Spirolingzhine C (**250**) | – | Yan et al. (2015a) |
| | Spirolingzhine D (**251**) | – | Yan et al. (2015a) |
| | Lingzhine A (**252**) | – | Yan et al. (2015a) |
| | Lingzhine B (**253**) | – | Yan et al. (2015a) |
| | Lingzhine C (**254**) | – | Yan et al. (2015a) |
| | Lingzhine D (**255**) | – | Yan et al. (2015a) |
| | Lingzhine E (**256**) | – | Yan et al. (2015a) |
| | Lingzhine F (**257**) | – | Yan et al. (2015a) |
| | Lingzhilactone A (**258**) | – | Yan et al. (2015b) |
| | Lingzhilactone B (**259**) | Inhibit ROS generation | Yan et al. (2015b) |
| | Lingzhilactone C (**260**) | – | Yan et al. (2015b) |

| | | | |
|---|---|---|---|
| *Armillaria mellea* | 5′-Methoxy-armillasin (**382**) | – | Li et al. (2016b) |
| | 5-Hydroxyl-armillarivin (**383**) | Cytotoxicity | Li et al. (2016b) |
| | Armillaridin (**384**) | Cytotoxicity | Li et al. (2016b) |
| | Armillartin (**385**) | Cytotoxicity | Li et al. (2016b) |
| | Armillarin (**386**) | – | Li et al. (2016b) |
| | Melleolide B (**387**) | – | Li et al. (2016b) |
| | Armillarilin (**388**) | Cytotoxicity | Li et al. (2016b) |
| | Armillasin (**389**) | Cytotoxicity | Li et al. (2016b) |
| | Armillarigin (**390**) | Cytotoxicity | Li et al. (2016b) |
| | Melleolide (**391**) | Cytotoxicity | Li et al. (2016b) |
| *Neolentinus lepideus* | 5-Methoxyisobenzofuran-4,7(1H,3H)-dioneone (**392**) | Inhibition of nitric oxide production | Li et al. (2013b) |
| | 1,3-Dihydroisobenzofuran-4,6-diol (**393**) | Antioxidant activity | Li et al. (2013b) |
| | | Inhibition of nitric oxide production | |
| | **394** | Inhibition of nitric oxide production | Li et al. (2013b) |
| *Lepista sordida* | Lepistamide A (**403**) | – | Chen et al. (2011) |
| | Lepistamide B (**404**) | – | Chen et al. (2011) |
| | Lepistamide C (**405**) | – | Chen et al. (2011) |
| | Diatretol (**406**) | – | Chen et al. (2011) |

"–" means the compound showed no bioactivity or was not studied for biological activity

production in lipopolysaccharide-activated macrophages, with an $IC_{50}$ value of 81.8, 88.8, 80.4, 65.6, and 72.8 μM, respectively (Wang et al. 2013a). A monoterpenoid, 5-(hydroxymethyl)-2-(prop-1-en-2-yl) cyclohexanol (**6**), was isolated from the fruiting body of *Flammulina velutipes* (Cai et al. 2013).

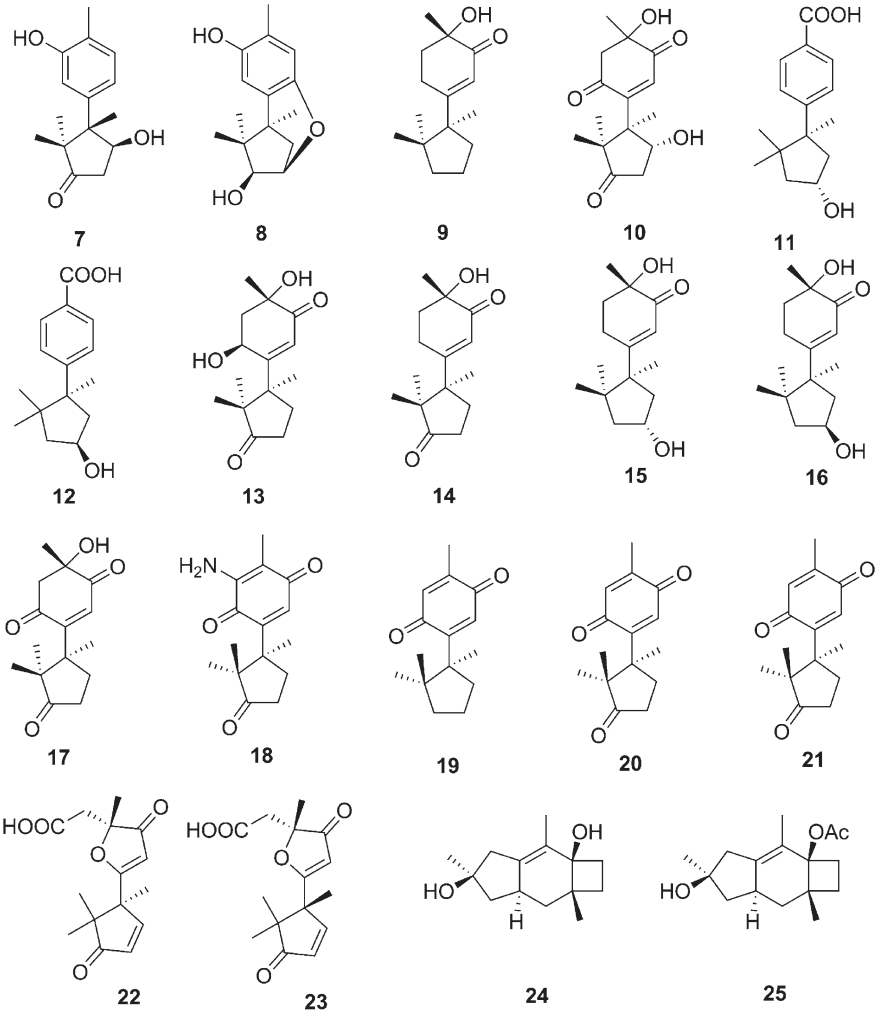



## 9.2.2 Sesquiterpenoids

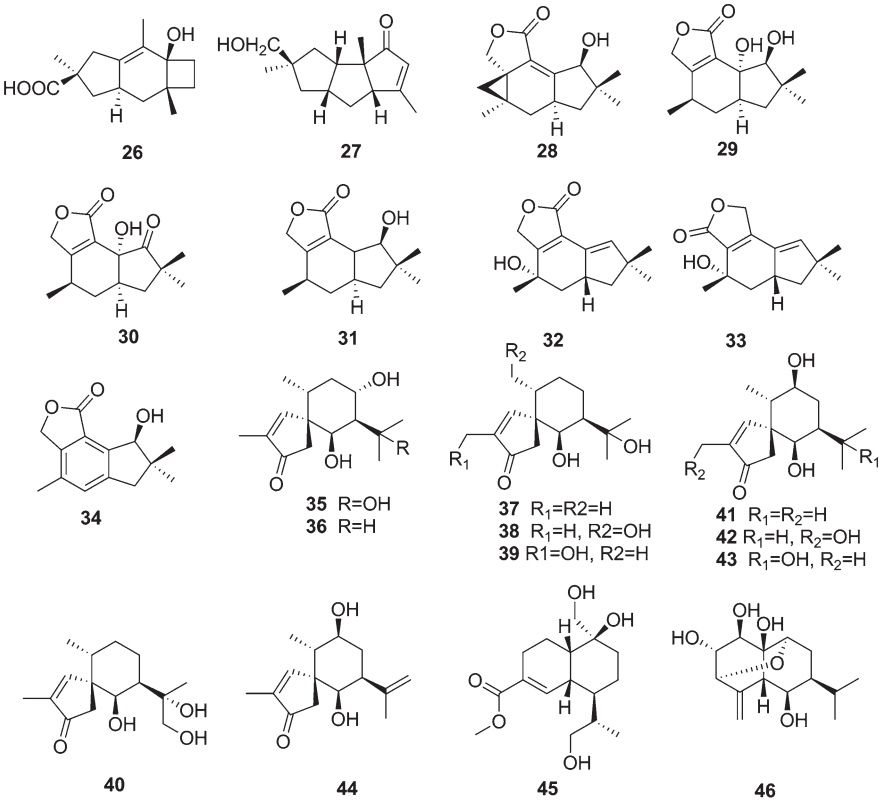
### 9.2.2.1 *Flammulina velutipes*

*F. velutipes* is a rich source of sesquiterpenoids with the various skeleton and interesting medicinal properties. More than 40 sesquiterpenoids have been obtained in recent years. Twelve new cuparene-type sesquiterpenes, flamvelutpenoids A–F (**7–12**) and enokipodins E–J (**13–18**), and three known sesquiterpenoids, 2,5-cuparadiene-1,4-dione (**19**) and enokipodins B (**20**) and D (**21**), were isolated from the solid culture of *F. velutipes* (Wang et al. 2012a, b; Tao et al. 2016a). Flamvelutpenoids A–D (**7–10**) showed weak antibacterial activity against *Escherichia coli*, *Bacillus subtilis*, and methicillin-resistant *Staphylococcus aureus* with MIC values larger than 100 μM (Wang et al. 2012a). Enokipodins F–G (**14–15**) and enokipodin I (**17**) showed weak antifungal activity against *Aspergillus fumigatus*; compounds **18–21** showed both moderate cytotoxicity against the human tumor cell lines (HepG2, MCF-7, SGC7901, and A549) and antioxidant activity in DPPH scavenging assay (Wang et al. 2012b). Flammufuranones A (**22**) and B (**23**) are two *seco*-cuparane sesquiterpenoids which may share a common precursor with cuparene-type sesquiterpene and biosynthesized from 1,6-cyclization (Tao et al. 2016a). Sterpurols A (**24**) and B (**25**), two new sterpurane sesquiterpenes, and sterpuric acid (**26**), a known sterpurane sesquiterpene, were isolated from the solid culture of *F. velutipes* (Wang et al. 2012b). Eight sesquiterpenoids including flammulinol A (**27**) with a new carbon skeleton and flammulinolides A–G (**28–34**), seven isolactarane-related norsesquiterpenes, were obtained from *F. velutipes*

cultivated on cooked rice (Wang et al. 2012c). Flammulinolides A–B (**28–29**) and F (**33**) showed strong cytotoxicity against KB cell line with the $IC_{50}$ of 3.9, 3.6, and 4.7 μM, respectively. Flammulinolide C (**30**) showed strong cytotoxicity against HeLa cell line with the $IC_{50}$ of 3.0 μM. Ten new sesquiterpenes with nor-eudesmane skeletons, flammuspirones A–J (**35–44**), as well as two new cadinene sesquiterpenes, 7,13,14-trihydroxy-4-cadinen-15-oic acid methyl ester (**45**) and 1,2,6,10-tetrahydroxy-3,9-epoxy-14-nor-5(15)-eudesmane (**46**), were obtained from the ethyl



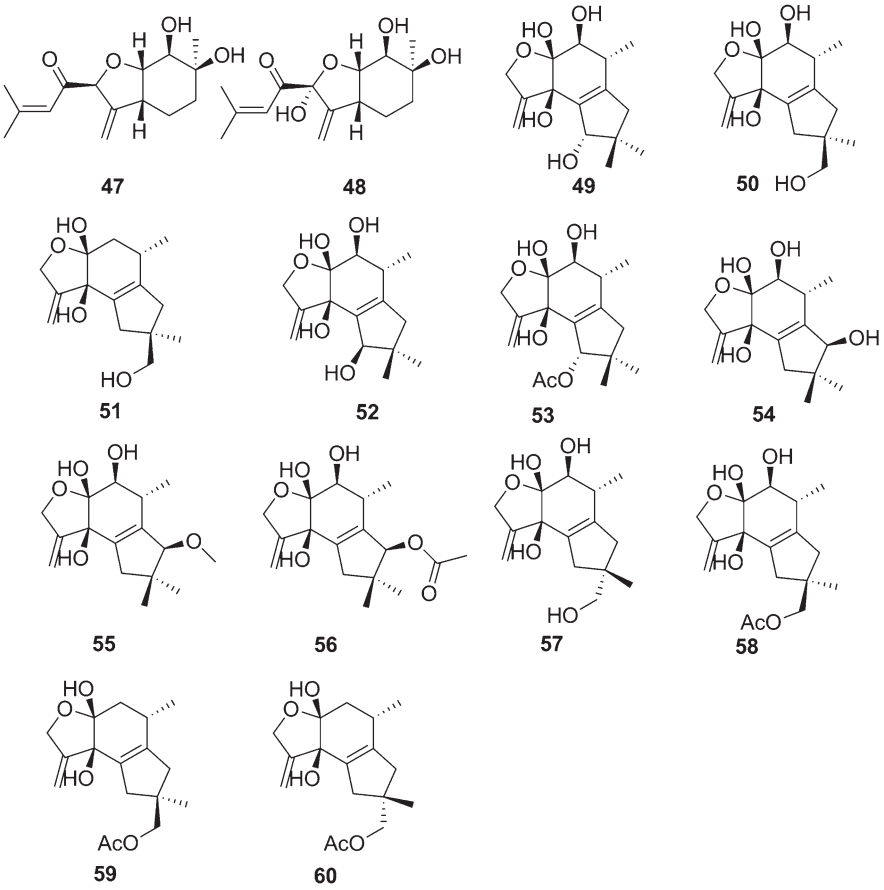
acetate extract of the solid culture of *F. velutipes* which is a wild strain collected in Yunnan province of China (Tao et al. 2016a). Among these compounds, compounds **35**, **37**, and **45**–**46** were found to inhibit the HMG-CoA reductase (HMGR) with $IC_{50}$ of 114.7, 77.6, 55.5, and 87.1 μM, respectively. Compounds **37–39**, **42**, **44** and **46** showed DPP-4 inhibitory activity with $IC_{50}$ of 75.9, 83.7, 70.9, 79.7, 80.5, and 74.8 μM, respectively. Identification of more than 40 sesquiterpenes with diverse skeletons and bioactivity provided evidence for the future application of *F. velutipes* as a functional food.




### 9.2.2.2 *Pleurotus cystidiosus*

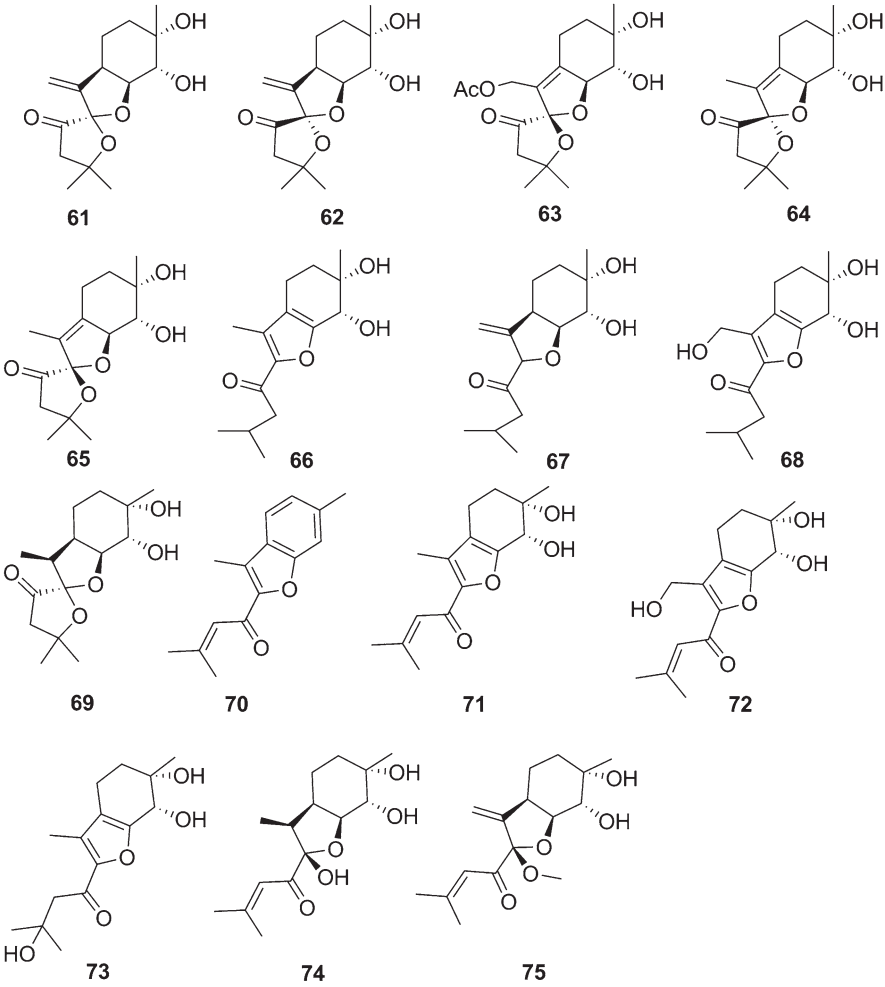
Pleurotons A–B (**47–48**), two bisabolane-type sesquiterpenoids, and clitocybulols D–F (**49–51**), three clitocybulol derivatives, were isolated from the ethyl acetate extract of the solid culture of *P. cystidiosus* (Zheng et al. 2015). Compounds **47–51** showed significant cytotoxicity against two human prostate cancer DU-145 and C42B cells. The $IC_{50}$ of compounds **47–51** was 174, 28, 233, 162, and 179 nM, respectively, against the DU-145 cell and was 104, 52, 163, 120, and 119 nM, respectively, against the C42B cell. A further chemistry investigation of the solid

culture of the *P. cystidiosus* led to the identification of clitocybulols G–O (**52**–**60**) (Tao et al. 2016b). Clitocybulols G (**52**) and L (**57**) exhibited moderate inhibitory activity against protein tyrosine phosphatase 1B (PTP1B) with $IC_{50}$ values of 36.0, 49.5, and 38.1 μM, respectively. All compounds had no significant inhibition against $\alpha$-glucosidase, sucrose, and maltase.




### 9.2.2.3 *Pleurotus cornucopiae*

Pleurospiroketals A–E (**61–65**), five new sesquiterpenes, as well as three related sesquiterpenes (**66–68**) were obtained from the solid culture of *P. cornucopiae* (Wang et al. 2013b). Compound pleurospiroketals A–C (**61–63**) showed inhibitory activity against nitric oxide production in lipopolysaccharide-activated macrophages with $IC_{50}$ values of 6.8, 12.6, and 20.8 μM, respectively. Compounds **66** and **68** exhibited slight growth inhibition against HeLa cells ($IC_{50}$ values of 36.0 and 70.6 μM) and HepG2 ($IC_{50}$ values of 68.6 and 76.8 μM).



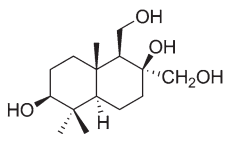

#### 9.2.2.4 *Pleurotus citrinopileatus*

Seven new sesquiterpenes, pleurospiroketal F (**69**) and pleurotins A–F (**70**–**75**), as well as a known sesquiterpene pleuroton B (**48**), were obtained from the solid culture of *P. citrinopileatus* (Tao et al. 2016c). Pleurotins A (**70**) and E (**74**) exhibited inhibitory activity on PTP1B with $IC_{50}$ of 32.1 μM and 30.5 μM, respectively.

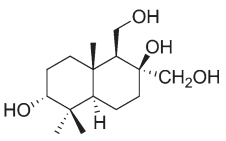
#### 9.2.2.5 *Laetiporus sulphureus*

Seven new drimane-type sesquiterpenoids, sulphureuines B–H (**76–82**), were isolated from cultures of mushroom *Laetiporus sulphureus* (He et al. 2015a). Sulphureuine B (**76**) induces apoptosis in glioma cells through endoplasmic reticulum stress, mitochondrial, and death receptor signaling pathways. Two illudin-type

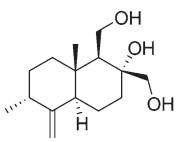
sesquiterpenoids, phellinuin J (**83**) and sulphureuine A (**84**), were obtained from the fermentation extract of *L. sulphureus* (He et al. 2015b). The two compounds were purposely evaluated for their cytotoxicity against HL-60, SMMC-7721, A549, MCF-7, and SW480 cell lines. Unfortunately, no significant inhibitory activity was found.



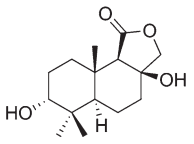
**76**



**77**



**78**



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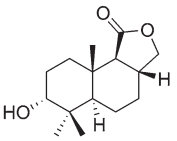
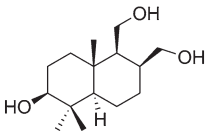


**80**



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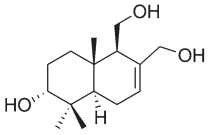
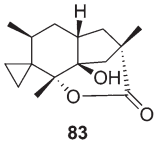
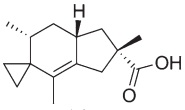


**82**



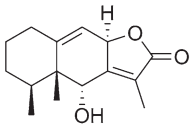
**83**



**84**

### 9.2.2.6 *Xylaria nigripes*

*Xylaria nigripes* has long been used as a traditional Chinese medicine (TCM) for enhancing memory, immunity, and hematopoiesis, treating insomnia and trauma, and as a diuretic, nerve tonic, and antidepressant (Liang et al. 2011; Ko et al. 2011; Zhao et al. 2014). Six new eremophilane-type sesquiterpenes, nigriterpenes A–F (**85–90**), were isolated from the ethyl acetate extracts of the fermented broths of termite nest-derived *X. nigripes* (Chang et al. 2017). These compounds were evaluated against lipopolysaccharide-induced inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) expression, and NO production in murine brain microglial BV-2 cells. Nigriterpene C (**87**) exerted significant inhibitory effects on two induced enzymes and NO production without any significant cellular toxicity. Nigriterpene C (**87**) exhibited concentration-dependent inhibition on NO production and iNOS and COX-2 expression with $IC_{50}$ values of 21.7 ± 4.9, 8.1 ± 2.3, and 16.6 ± 5.5 μM, respectively. The results indicated that the potential anti-inflammatory effects of nigriterpene C (**87**) on murine brain microglial BV-2 cells might provide a rationale for the traditional medical uses of *X. nigripes* for treating insomnia and depression.

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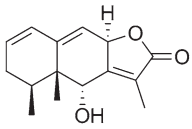


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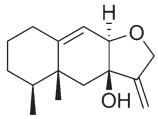


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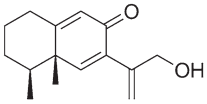


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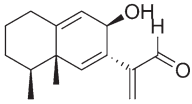


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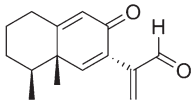
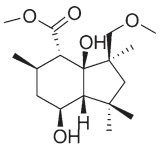



90

### 9.2.2.7 *Boletus edulis*

Three non-isoprenoid botryane sesquiterpenoids, named boledulins A–C (**91–93**), were isolated from the cultures of basidiomycete *B. edulis* (Feng et al. 2011). Boledulin A (**91**) exhibited moderate inhibitory effects on HL-60, SMMC-7721, A-549, MCF-7, and SW480 with $IC_{50}$ values of 2.6, 8.4, 8.3, 3.4, and 3.5 μM, respectively.

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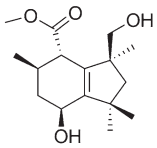


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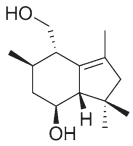


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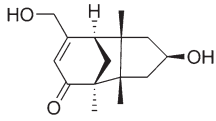


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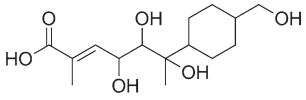


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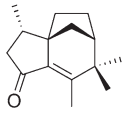



96

### 9.2.2.8 *Inonotus vaninii*

*I. vaninii* has been used in Chinese folk medicine as "sang huang" for the treatment of cancer, diabetes, liver, and heart diseases and stomach ailments in Northeastern China (Dai et al. 2010). A novel sesquiterpene, inonolane A (**94**), was isolated from the EtOAc extract of the medicinal fungus *I. vaninii* (Yang et al. 2013b). Inonolane A (**94**) represents the first bisabolane-type sesquiterpene from the genus *Inonotus*.

### 9.2.2.9 *Ganoderma sinense*

Ganosinensine (**95**), a new sesquiterpene, was isolated from the fruiting bodies of the fungus *G. sinense* (Liu et al. 2012).

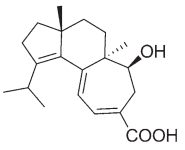
#### 9.2.2.10 *Dictyophora indusiata*

A sesquiterpene antibiotic, albaflavenone (**96**), was isolated from the dried fruiting body of *D. indusiata* by solvent extraction and column chromatography (Huang et al. 2011). The content of albaflavenone (**96**) in the dried fruiting body of *D. indusiata* was quantified by GC through an external standard method to be about 0.0063%. Albaflavenone (**96**) has a camphor-like odor.

### 9.2.3 Diterpenoids

#### 9.2.3.1 *Cyathus* spp.

The species belonging to the genus *Cyathus* (*Nidulariaceae* family) are recognized as prolific producers of bioactive cyathane diterpenoids with a unique [5-6-7] tricyclic ring skeleton. Cyathane diterpenoids represent a group of natural products with great diversity in both structure and bioactivity (Shen et al. 2009). Five novel cyathane diterpenes, cyathins D–H (**97–101**), as well as three known diterpenes, neosarcodonin O (**102**), cyathatriol (**103**), and 11-O-acetylcyathatriol (**104**), were isolated from the solid culture of *Cyathus africanus* (Han et al. 2013). Compounds **99**, **101**, **102**, and **104** showed potent inhibition of nitric oxide production in lipopolysaccharide-activated macrophages with an $IC_{50}$ value of 2.57, 1.45, 12.0, and 10.73 μM, respectively. Neosarcodonin O (**102**) and 11-O-acetylcyathatriol (**104**) showed strong cytotoxicity against HeLa and K562 cell lines with the $IC_{50}$ value less than 10 μM. Cyathin R (**105**), a new cyathane diterpenoid, was isolated from the solid culture of *C. africanus* (Huang et al. 2015). A further chemistry investigation of the solid culture of the *C. africanus* led to the identification of three new cyathane diterpenoids, cyathin T (**106**), cyathin V (**107**), and cyathin W (**108**) (Han et al. 2015). Cyathins T (**106**) and W (**108**) showed moderate inhibition against nitric oxide production in lipopolysaccharide-activated macrophages with an $IC_{50}$ value of 88.87 and 80.07 μM, respectively. In cytotoxicity assay, cyathin W (**108**) showed weak cytotoxicity against K562 cell line with the $IC_{50}$ value of 12.1 μM. Cyathin Q (**109**), a new cyathane-type diterpene, was obtained from the culture of the fungus *C. africanus* by bioactivity-guided separation (He et al. 2016). The bioactivity evaluation shows that cyathin Q (**109**) exhibited anticancer activity via induction of mitochondria- and autophagy-dependent apoptosis in HCT116 cells. Cyathin I (**110**), a new cyathane diterpene, and two related diterpenes, (12R)-11a,14a-epoxy-13a,14b,15-trihydroxycyath-3-ene (**111**) and erinacine I (**112**), were obtained from the fermentation broth of *Cyathus hookeri* (Xu et al. 2013). Compounds **110–112** showed inhibition against nitric oxide production in macrophages with an $IC_{50}$ value of 15.5, 52.3, and 16.8 μM, respectively. Seven new cyathane-type diterpenes, cyathins J–P (**113–119**), and two known diterpenes (**120–121**) were isolated from the solid culture of *Cyathus gansuensis* (Wang et al. 2014a). Bioactivity screening indicated that cyathins J–K (**113–114**), M (**116**), and compound **120** showed moderate inhibitory activity against NO production in lipopolysaccharide-activated macrophages with an $IC_{50}$ value of 42, 78, 80, and 16 μM, respectively. A chemical

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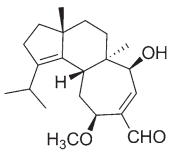


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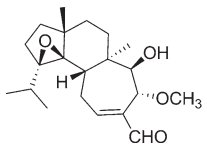


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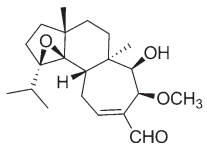


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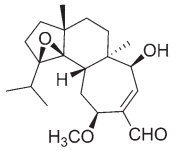


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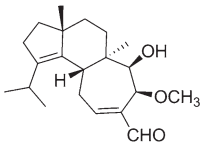


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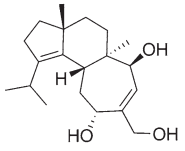


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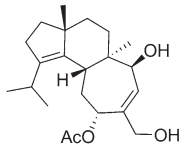


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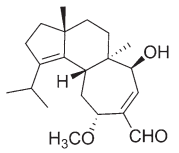


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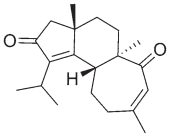


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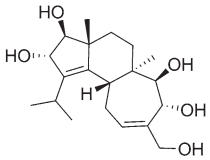


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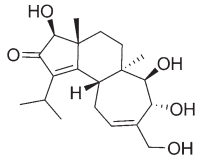


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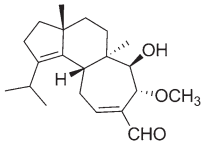


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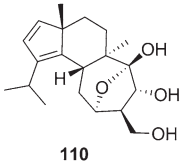


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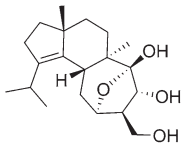


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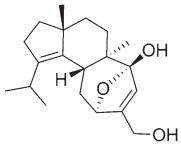


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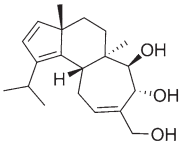


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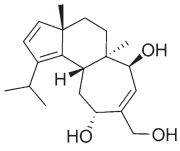


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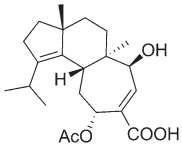


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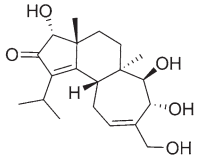


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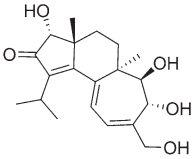


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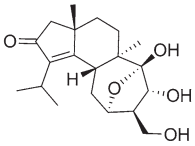


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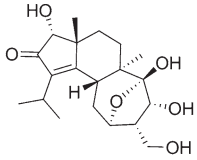


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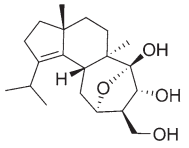


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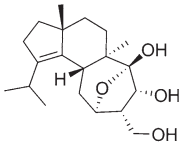


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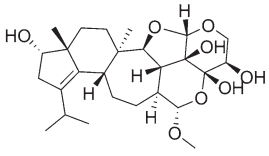


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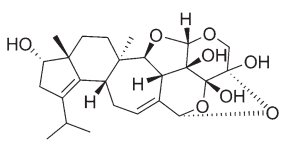
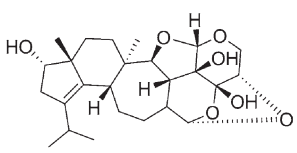



123

investigation of the solid culture of *Cyathus striatus* led to the identification of six new cyathane xylosides, striatoids A–F (**122–127**) (Bai et al. 2015). The bioactivity evaluation shows that striatoids A–F (**122–127**) dose-dependently enhanced nerve growth factor (NGF)-mediated neurite outgrowth.

### 9.2.3.2 *Hericium erinaceus*

The fruiting bodies of this mushroom were used in Chinese folk medicine to treat the tumors of the digestive systems, such as esophageal, stomach, and duodenum cancers and hyperglycemia. The chemical constituents of *H. erinaceum* were widely investigated. Aromatic compounds and diterpenoids with various bioactivities have been isolated from *H. erinaceus*. A new diterpene (**128**) was isolated from the fungal mycelia of *H. erinaceus* by the tracking method of antibacterial activity (Zhang et al. 2015a). Compound **128** showed good cytotoxicity against tumor cell lines (K562 and HEP2) with $IC_{50} < 200$ μM.

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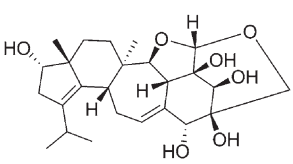


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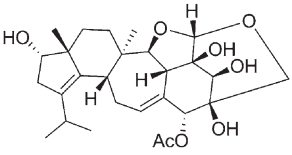


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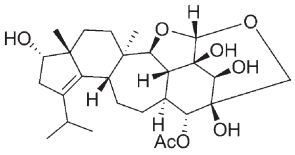


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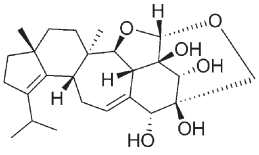


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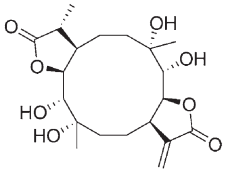
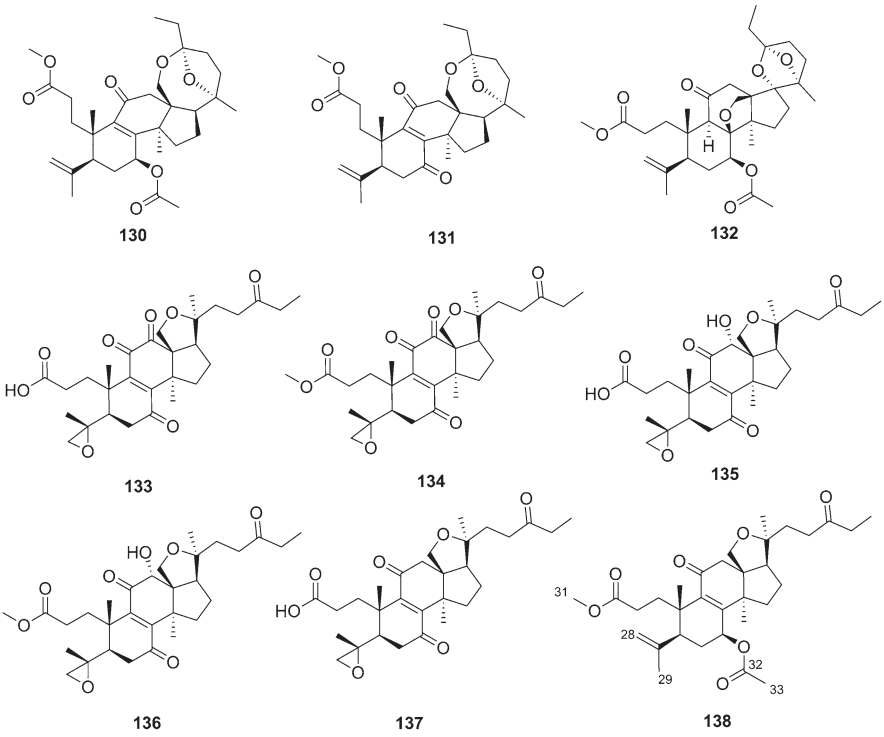



129

### 9.2.3.3 *Pleurotus eryngii*

Eryngiolide A (**129**), a new diterpenoid with unprecedented skeleton, was obtained from the mycelia of edible mushroom *P. eryngii* fermented on rice (Wang et al. 2012d). This compound was tested for their cytotoxic effects against two human cancer cell lines, HeLa and HepG2, using the MTT method. Eryngiolide A (**129**) showed moderate toxicities against two cell lines with $IC_{50}$ values of 20.6 and 28.6 μM, respectively. This macrocyclic diterpene can be formed by a [6+6] cycloaddition from two molecules of geranyl pyrophosphate (GPP).




## 9.2.4 Triterpenoids

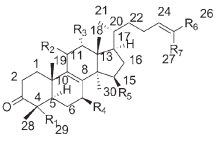
### 9.2.4.1 *Ganoderma boninense*

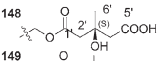
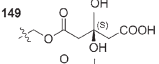
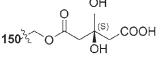
In a searching for new bioactive agents from medicinal mushrooms, the fruiting bodies of *G. boninense* collected from the Hainan province of China (Quinlan Nature Reserve) were chemically investigated. As a result, ganoboninketals A–C (**130–132**), three new nortriterpenes, were obtained from the fruiting bodies of *G. boninense* (Ma et al. 2014). Ganoboninketals A–C (**130–132**) exhibited significant antiplasmodial activity against *Plasmodium falciparum* with $IC_{50}$ values of 4.0, 7.9, and 1.7 μM, respectively. Ganoboninones A–F (**133**–**138**), six new nortriterpenes, were also isolated from the fruiting bodies of the medicinal mushroom *G. boninense* (Ma et al. 2015). Ganoboninones A–B (**133–134**) and F (**138**) showed antimalarial effects with $IC_{50}$ values of 27.36, 15.68, and 2.03 μM, respectively. In a transactivation assay, ganoboninketals A–C (**130–132**) and ganoboninone E (**137**) showed agonistic activity to LXRβ with an $EC_{50}$ value of 8.32, 257.00, 86.70, and 203.00 nM, respectively.

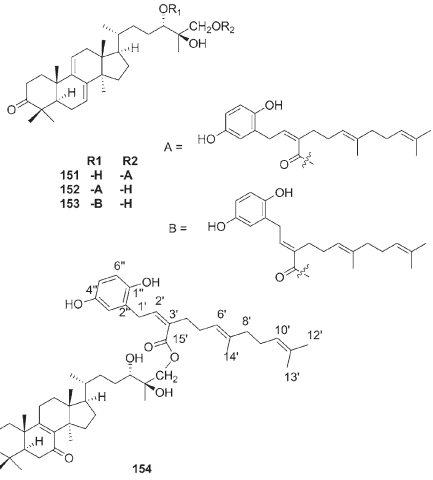
### 9.2.4.2 *Ganoderma leucocontextum*

*G. leucocontextum*, also called "White Lingzhi" due to its white fleshy fruiting bodies, enjoys a strong reputation for being one of the top high-quality Lingzhi in China for its health function from folk usage. Chemical investigation of cultivated and wild *G. leucocontextum* led to the discovery of more than 50 triterpenoids with diverse bioactivity. Ganoleucoins A–P (**139–154**), 16 new lanostane triterpenes, were obtained from the cultivated fruiting bodies of *G. leucocontextum* (Wang et al. 2015a). Compounds **139**, **141**, **144**, and **148–152** exhibited strong inhibitory activity against HMG-CoA reductase. Compounds **139–140**, **144**–**145**, **148**, **150**, and **154** showed cytotoxicity against K562 cells with $IC_{50}$ values in the range 10–20 μM.

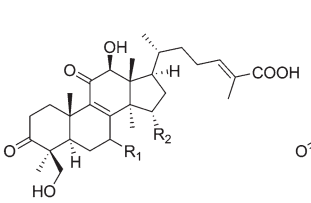
A chemical research on the fruiting bodies of wild *G. leucocontextum* led to the identification of 18 new triterpenoids, leucocontextins A–R (**155–172**), (Zhao et al. 2016a). Leucocontextin R (**172**) presented weak cytotoxicity against K562 and MCF-7 cell lines with the $IC_{50}$ of 20.35 and 28.66 μM, respectively. Leucocontextins S–X (**173–178**), six new triterpenoids, were obtained from the fruiting bodies of wild *G. leucocontextum* (Zhao et al. 2016b). The inhibitory activities against K562, SMMC-7721, and MCF-7 cell lines for leucocontextins S–X (**173–178**) were evaluated. Unfortunately, none of them showed significant activity.



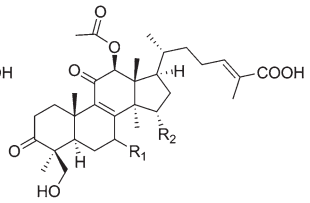
| | R1 | R2 | R3 | R4 | R5 | R6 | R7 |
|---|---|---|---|---|---|---|---|
| 139 | -CH2OH | =O | -H | -OH | -H | -COOH | -CH3 |
| 140 | -CH2OH | =O | -OH | -OH | -H | -COOH | -CH3 |
| 141 | -CH2OH | =O | -OAc | -OH | -H | -COOH | -CH3 |
| 142 | -CH2OH | =O | -OAc | -OH | =O | -COOH | -CH3 |
| 143 | -CH2OH | =O | -OH | -OH | =O | -COOH | -CH3 |
| 144 | -CH2OH | =O | -OAc | =O | =O | -COOH | -CH3 |
| 145 | -CH3 | =O | -H | -OH | -H | -CH2OH | -CH2OH |
| 146 | -CH3 | =O | -H | =O | -H | -CH2OH | -CH2OH |
| 147 | -CH3 | =O | -H | -H | -OH | -CH2OH | -CH2OH |
| 148 |  | =O | -OAc | -OH | =O | -COOH | -CH3 |
| 149 |  | =O | -OH | =O | =O | -COOH | -CH3 |
| 150 |  | =O | -H | =O | =O | -COOH | -CH3 |



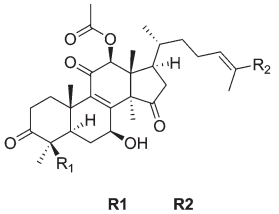
| | R1 | R2 |
|---|---|---|
| 151 | -H | -A |
| 152 | -A | -H |
| 153 | -B | -H |



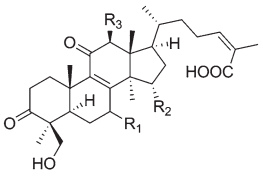
| | R1 | R2 |
|---|---|---|
| 155 | β-OH | =O |
| 156 | β-OH | OH |
| 157 | α-OH | OH |
| 158 | =O | OH |
| 162 | H | OH |



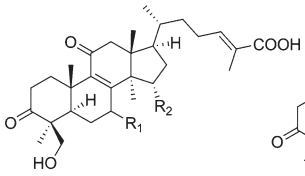
| | R1 | R2 |
|---|---|---|
| 164 | β-OH | =O |
| 165 | α-OH | OH |
| 166 | =O | OH |
| 167 | =O | =O |
| 168 | H | OH |



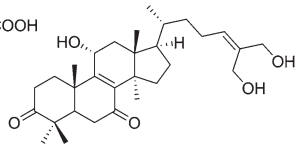
| | R1 | R2 |
|---|---|---|
| 169 | CHO | COOH |
| 170 | CH2OH | COOMe |



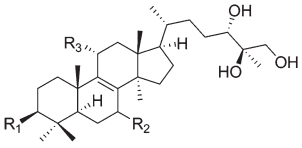
| | R1 | R2 | R3 |
|---|---|---|---|
| 159 | =O | =O | OH |
| 171 | α-OH | OH | OAc |



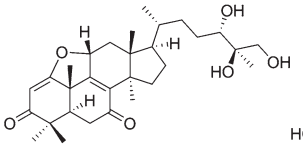
| | R1 | R2 |
|---|---|---|
| 160 | β-OH | OH |
| 161 | =O | =O |
| 163 | H | OH |



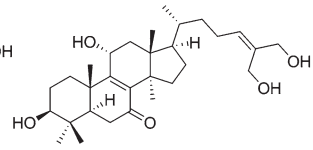
172



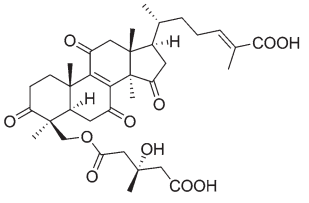
| | R1 | R2 | R3 |
|---|---|---|---|
| 173 | =O | =O | -OH |
| 174 | =O | β-OH | =O |
| 175 | -OH | =O | -OH |



176



177

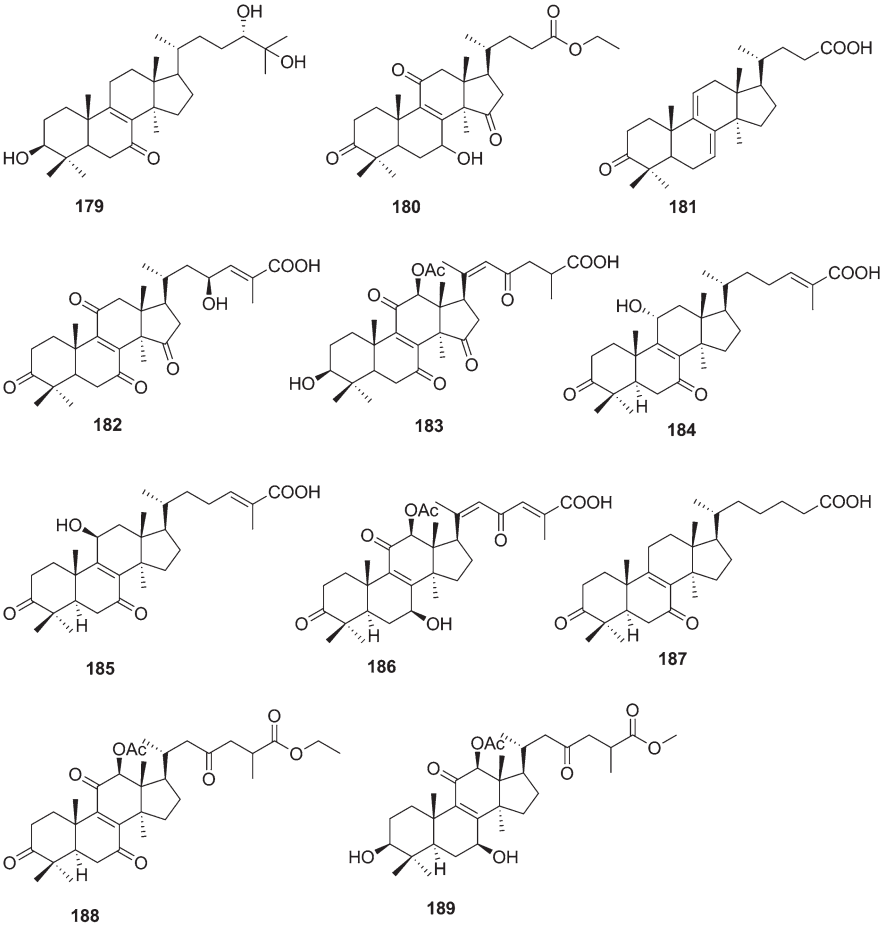


178

## 9.2.4.3 *Ganoderma lucidum*

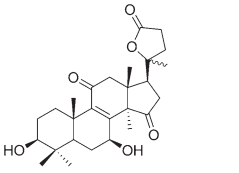
*G. lucidum*, a TCM called Lingzhi, is one of the most highly ranked herbal medicines by Asian people, whose fruiting body, mycelia, and spores were traditionally used as a folk medicine for treatment of debility and weakness, insomnia, hepatitis, cardiovascular diseases, cancer, etc. (Lin 2001; Gao and Zhou 2004; Lin and Zhang 2004). Modern research revealed the bioactivity components of *G. lucidum* to be triterpenes and polysaccharides, which were reported to possess antivirus (Li and

Wang 2006), anti-inflammation (Akihisa et al. 2007), antitumor (Nonaka et al. 2006), immunity-promoting (Zhu et al. 2007), and antidiabetic effects (He et al. 2006). In the latest 10 years, chemical investigation of the metabolites in *G. lucidum* led to identification of 11 new triterpenes, ganoderitriol M (**179**), ethyl lucidenate A (**180**), ethyl 7β-hydroxy-4,4,14α-trimethyl-3,11,15-trioxo-5α-chol-8-en-24-oate (**181**), 23S-hydroxy-3,7,11,15-tetraoxo-lanost-8,24E-diene-26-oic acid (**182**), 12β-acetoxy-3β-hydroxy-7,11,15,23-tetraoxo-lanost-8,20E-diene-26-oic acid (**183**), and compounds **184–189** (Chen et al. 2009; Li et al. 2013a; Zhang et al. 2011; Guan et al. 2008; Cheng et al. 2010). Ethyl lucidenate A (**180**) exhibited cytotoxicity against HL-60 and CA46 cell with $IC_{50}$ values of 25.98 and 20.42 μgmL$^{-1}$, respectively (Li et al. 2013a). Compound **181** had NGF-like neuronal survival-promoting activities (Zhang et al. 2011). Compounds **182–183** exhibited cytotoxicity against four human tumor cell lines, p388, HeLa, BEL-7402, and SGC-7901, with the $IC_{50}$ values in the range of 8–25 μM (Guan et al. 2008).

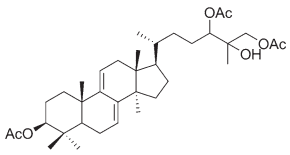


### 9.2.4.4 *Ganoderma sinense*

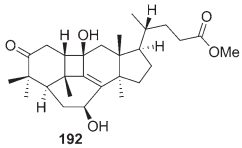
*G. sinense*, a well-known species of *Ganoderma*, is widely distributed in Yunnan province, in the southwest of China. Compared with *G. lucidum*, chemical studies on *G. sinense* have rarely been reported. Ganolactone B (**190**) and ganoderiol A triacetate (**191**), two novel lanostane-type triterpenes, were isolated from the fruiting bodies of *G. sinense* (Qiao et al. 2007). To further discover structurally diverse and biologically significant compounds from the *G. sinense*, three new triterpenoids, methyl ganosinensate A (**192**), ganosinensic acid A (**193**), and ganosinensic acid B (**194**), with an unusual four-membered ring produced by linkage of C-1 with C-11 were isolated from the fruiting body of *G. sinense* (Wang et al. 2010). In 2011, nine new triterpenoids ganosineniol A (**195**), ganosinoside A (**196**), ganoderic acid Jc (**197**), ganoderic acid Jd (**198**), ganodermatetraol (**199**), ganolucidic acid γa (**200**), ganolucidate F (**201**), ganoderiol J (**202**), and methyl lucidenate Ha (**203**) were isolated from the fruiting bodies of the fungus *G. sinense* (Liu et al. 2012). Among these compounds, ganoderic acid Jc (**197**) displayed selective inhibitory activity against HL-60 cells ($IC_{50}$ = 8.30 μM). Ganodermatetraol (**199**) and ganolucidate F (**201**) showed induction ability of hPXR-mediated CYP3A4 expression.



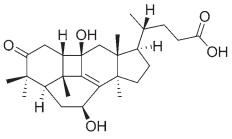
**190**



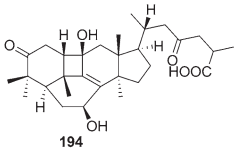
**191**



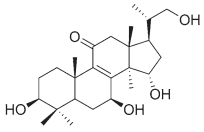
**192**



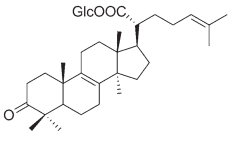
**193**



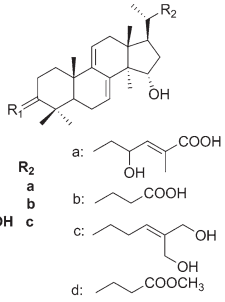
**194**



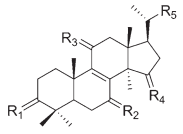
**195**



**196**



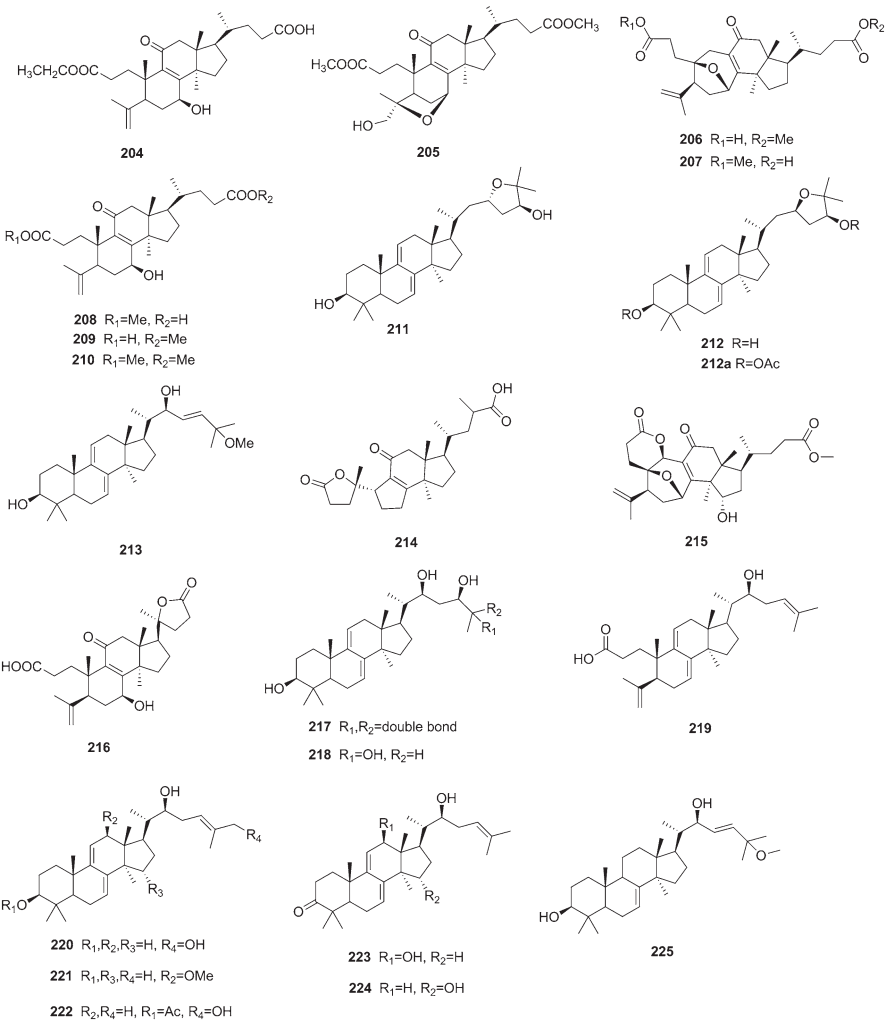
| | $R_1$ | $R_2$ |
|---|---|---|
| 197: | O | a |
| 198: | O | b |
| 199: | α-H,β-OH | c |



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ |
|---|---|---|---|---|---|---|
| 200: | α-H,β-OH | α-H,β-OH | O | α-OH,β-H | a | H |
| 201: | α-H,β-OH | H | O | α-OH,β-H | a | H |
| 202: | O | O | H | H | c | H |
| 203: | α-H,β-OH | α-H,β-OH | O | O | d | OH |

#### 9.2.4.5 *Ganoderma cochlear*

*G. cochlear* has the same morphological characteristics as *G. sinense*, but the fungus stipe of *G. cochlear* lies in the back of the pileus. Initial phytochemical investigation on *G. cochlear* resulted in the identification of two new 3,4-secotrinorlanostane triterpenoids, fornicatin G (**204**) and H (**205**) (Peng et al. 2012). To discover additional biologically functional triterpenoids from *G. cochlear*, the chemical constitutes of *G. cochlear* were studied. As a result, cochlates A (**206**) and B (**207**), two novel trinorlanostanes with a 3,4-seco-9,10-seco-9,19-cyclo skeleton, as well as six new triterpenoids, fornicatins D–F (**208–210**) and ganodercochlearins A–C (**211–213**), were obtained from the fruiting bodies of *G. cochlear* (Peng et al. 2014a). Fornicatins D (**208**) and F (**210**) lowered the ALT and AST levels in HepG2 cells treated with $H_2O_2$, suggesting that both compounds could display in vivo hepatoprotective activities. In 2015, a rearranged hexanorlanostane triterpenoid featuring with a γ-lactone ring and a five-membered carbon ring, ganocochlearic acid A (**214**), and 11 new lanostane triterpenoids cochlate C (**215**), cochlearic acid A (**216**), ganodecochlearin D (**217**), ganodercochlearin E (**218**), cochlearic acid B (**219**), and ganodercochlearins F–K (**220–225**) were isolated from the fruiting bodies of *G. cochlear* (Peng et al. 2015a). Ganodercochlearins F–H (**220–222**) and J–K (**224–225**) showed moderate cytotoxic activities against five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW480) with $IC_{50}$ values ranging from 8 to 30 μM. Ganodecochlearin D (**217**) exhibited relatively potent cytotoxic activity against MCF-7 cells ($IC_{50}$: 9.15 μM), compared to the positive control (cisplatin, $IC_{50}$: 12.7 μM).



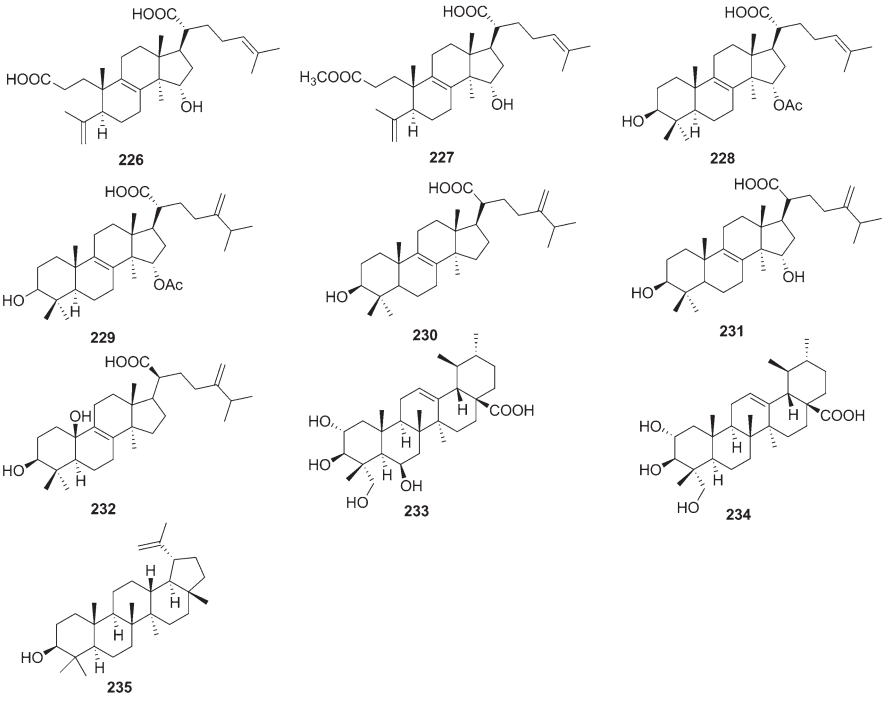

### 9.2.4.6 *Laetiporus sulphureus*

Two 3,4-seco-lanostane-type triterpenes, 15$\alpha$-hydroxy-3,4-secolanosta-4(28),8,24-triene-3,21-dioic acid (**226**) and 5$\alpha$-hydroxy-3,4-seco-lanosta-4(28),8,24-triene-3,21-dioic acid 3-methyl ester (**227**), and one lanostane triterpene 15$\alpha$-acetoxylhyd roxytrametenolic acid (**228**) together with versisponic acid D (**229**) were isolated from the fruiting bodies of *L. sulphureus* (Yin et al. 2015). Compounds **226–229** were evaluated by MTT method for their cytotoxicities against five human cancer cell lines, breast cancer MCF-7, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, colon cancer SW480, and lung cancer A-549. However, none exhibited inhibitory effects. From the EtOAc extracts of the same fungal culture broth, compounds **230** and **231** were obtained (He et al. 2015a). Compound **230**

showed moderate activities against four cells HL-60, SMMC-721, A-549, and SW-480, with $IC_{50}$ values of 37.5, 14.8, 15.6, and 36.1 μM, respectively. Eburicoic acid (**232**) is the main bioactive component in the *L. sulphureus* (Wang et al. 2015b). Eburicoic acid (**232**) protected the gastric mucosa from gastric lesions morphologically and especially attenuated $H^+/K^+$-ATPase activity.

### 9.2.4.7 *Pleurotus eryngii*

2,3,6,23-Tetrahydroxy-urs-12-en-28-oic acid (**233**), 2,3,23-trihydroxyurs-12-en-28-oic acid (**234**), and lupeol (**235**) were identified from the EtOAc-soluble portion of *P. eryngii* extract (Xue et al. 2015). The three isolated compounds were evaluated for the proliferation inhibition activity against the human breast cancer cell line MCF-7 using the MTT assay. All of the compounds significantly inhibited MCF-7 cell proliferation. The $IC_{50}$ values were 15.71, 48.00, and 66.89 μM, respectively. Compound **233** showed greater antitumor activity than compound **234**, indicating that the presence of an additional hydroxyl group at C-6 enhances the cytotoxic effect. Compounds **233–234**, with the carboxylic acid at C-28, showed slightly more potent inhibitory activities in MCF-7 cells than **235**, which lacks a carboxy group at C-28. These results suggest that a free carboxylic group at C-28 may be important to exert antiproliferative activity.



## 9.3 Meroterpenoids

Meroterpenoids are hybrid natural products of both terpenoid and nonterpenoid origin. They have attracted much attention due to their unusual structure features, wide range of bioactivities and interesting biosynthetic mechanisms (Geris and Simpson 2009). Based on the biosynthetic origins, meroterpenoids can be classified into two groups: polyketide-terpenoids and shikimate-terpenoids.

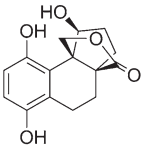
### 9.3.1 Shikimate-Terpenoids

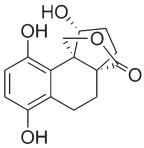
#### 9.3.1.1 *Ganoderma lucidum*

(+)-Lingzhiol (**236**) and (−)-lingzhiol (**237**), a pair of rotary door-shaped meroterpenoidal enantiomers, were isolated from *G. lucidum* (Yan et al. 2013). Lingzhiols (**236–237**) bears an unusual 5/5/6/6 ring system characteristic of sharing a C-3-C-7 axis. The biological evaluation showed that (+)-lingzhiol (**236**) or (−)-lingzhiol (**237**) could selectively inhibit the phosphorylation of Smad3 in TGF-β1-induced rat renal proximal tubular cells and activate Nrf2/Keap1 in mesangial cells under diabetic conditions. Further chemistry investigation on the fruiting body of *G. lucidum* led to the isolation of six new meroterpenoids, chizhines A–F (**238–243**) (Luo et al. 2015a). Chizhines A–F (**238–243**) are isolated as racemic mixtures. Chiral HPLC was utilized to obtain the individual (+)- and (−)-antipodes of these substances. The renoprotective effects of chizhines A–F (**238–243**) were evaluated by using the ELISA technique and high glucose-induced rat mesangial cells. The results show that the individual enantiomers of these substances significantly inhibit monocyte chemotactic protein 1 (MCP-1) and fibronectin production in a dose-dependent manner. Lingzhifuran A (**244**) and lingzhilactones D–F (**245–247**), four new phenolic meroterpenoids, were isolated from the fruiting bodies of *G. lucidum* (Ding et al. 2016). Lingzhifuran A (**244**) and lingzhilactone D (**245**) could selectively inhibit TGF-β1-induced Smad3 phosphorylation in rat renal tubular epithelial cells, representing novel scaffolds of selective Smad3 activation inhibitors.

#### 9.3.1.2 *Ganoderma lingzhi*

*G. lingzhi* is a valuable, edible, and medicinal fungus that has been widely used for the prevention and treatment of a broad range of diseases. Spirolingzhines A–D (**248–251**), four new meroterpenoids with aspiro[benzofuran-2,10-cyclopentane] motif, and lingzhines A–F (**252–257**), six new meroterpenoids with diverse ring systems, were isolated from the fruiting bodies of *G. lingzhi* (Yan et al. 2015a). (−)-Spirolingzhine A (**248**) was shown to affect NSC cell cycle progression using the 5-bromo-2-deoxyuridine (BrdU) incorporation assay. Three new lingzhilactones A–C (**258–260**) containing a fused lactone moiety were isolated from *G. lingzhi* (Yan et al. 2015b). Lingzhilactone B (**259**) could inhibit ROS generation in a dose-dependent manner; inhibit mRNA expression of collagen IV, fibronectin, and IL-6; and increase expression of Nrf2 in rat tubular epithelial cells. Furthermore, we found that compound **259** could reduce urinary albumin levels, abrogate myofibroblastic activation, and inhibit the phosphorylation of Smad3 in Adriamycin-induced mice.


236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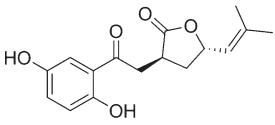
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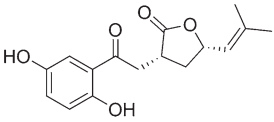
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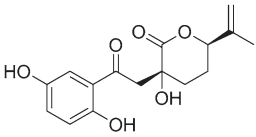
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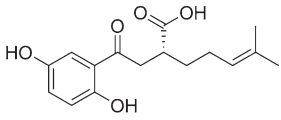
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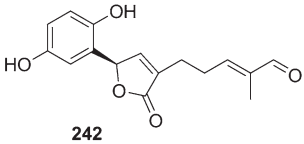
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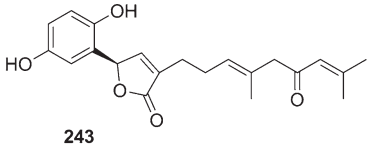
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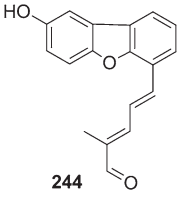
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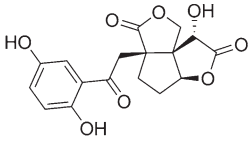
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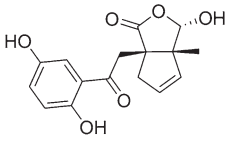
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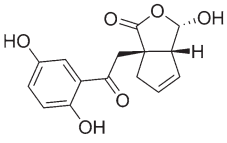
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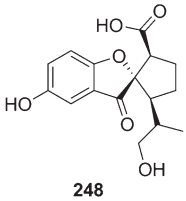
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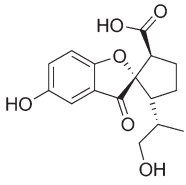
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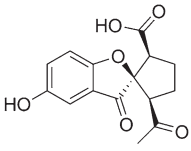
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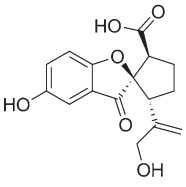
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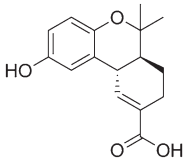
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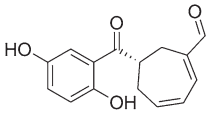
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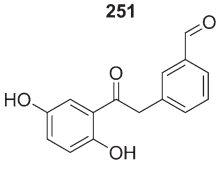
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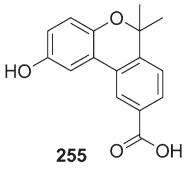
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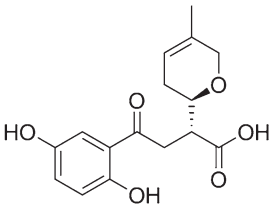


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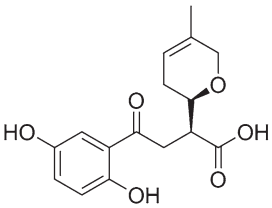


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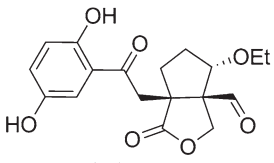


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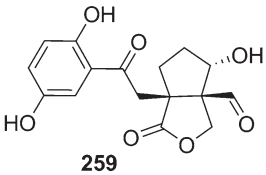


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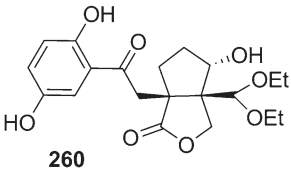
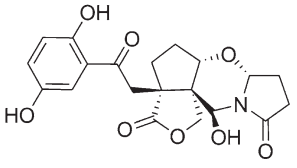



260

### 9.3.1.3 *Ganoderma sinensis*

(−)-Sinensilactam A (**261**) and (+)-sinensilactam A (**262**), novel hybrid metabolites possessing a unique 2Hpyrrolo[2,1-b] [1,3]oxazin-6(7H)-one ring system, were isolated from the fruit bodies of *G. sinensis* (Luo et al. 2015b). (−)-Sinensilactam A (**261**) was found to be a Smad3 phosphorylation inhibitor in TGF-β1-induced human renal proximal tubular cells. Zizhines A–F (**263–268**), six new meroterpenoid, were also isolated from the fruiting bodies of *G. sinensis* (Cao et al. 2016).

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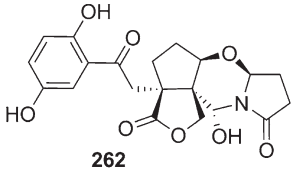


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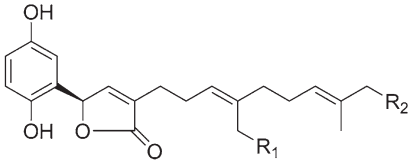
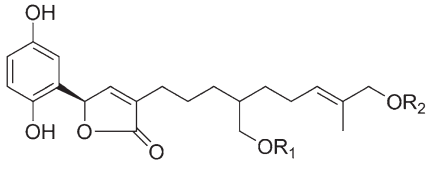
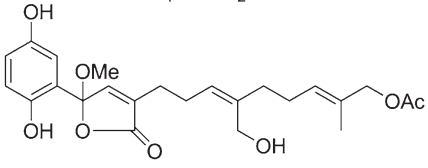



**263** $R_1$=H, $R_2$=OH
**264** $R_1$=OAc,$R_2$=OH
**266** $R_1$=OH,$R_2$=OAc



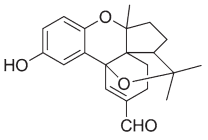

**265** $R_1$=Ac, $R_2$=H
**267** $R_1$=H,$R_2$=Ac




268

#### 9.3.1.4 *Ganoderma cochlear*

Four pairs of new polycyclic-meroterpenoid enantiomers, ganocins A–C (**269–271**) possessing a spiro[4,5]decane ring system, along with ganocin D (**272**) with an eight-membered ring, were isolated from the fruiting bodies of *G. cochlear* (Peng et al. 2014b). Ganocin D (**272**) had weak anti-AChE activity with an inhibition of 32% (50 μM). (+)- and (−)-Cochlearols A (**273**) and B (**274**), two meroterpenoids with novel polycyclic skeletons, were isolated from the fruiting bodies of the fungus *G. cochlear* (Dou et al. 2014). Biological studies showed that (−)-cochlearol B (**274**) is a strong inhibitor of p-Smads, exhibiting renoprotective activities in TGF-β1-induced rat renal proximal tubular cells. Cochlearoids A–E (**275–279**) and cochlearines A (**280**) and B (**281**) were obtained from *G. cochlear* (Zhou et al. 2015). Compounds (+)-**275**, (−)-**278**, and (±)-**280** exhibited significantly inhibited $Ca_v3.1$ TTCC and showed noticeable selectivity against $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$, and $K_v11.1$ (hERG) channels. Five novel meroterpenoids, ganoderin A (**282**) and ganocochlearins A–D (**283–286**), with the polycyclic skeleton and two new prenylated phenols, fornicin D (**287**) and ganomycin C (**288**) with a carbon chain, were isolated from the fruiting bodies of *G. cochlear* (Peng et al. 2015b). All compounds showed an antioxidant effect in radical scavenging assays. Six novel meroterpenoids cochlearoids F–K (**289–294**) were isolated by utilizing phytochemical approaches (Wang et al. 2016a). The biological evaluation shows that compounds **289–292** and **294** exhibit potent inhibitory activity on fibronectin overproduction in TGF-β1-induced HKC-8 cells.



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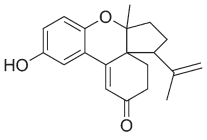
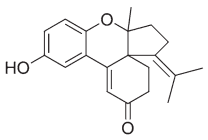


**270**



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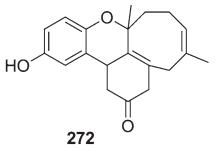

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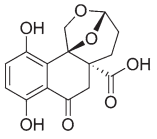

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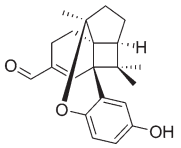

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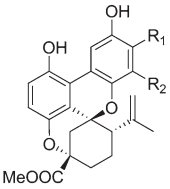
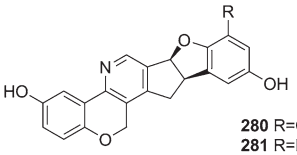
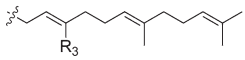


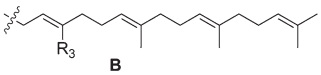
**275** $R_1$=H, $R_2$=**A**, $R_3$=OAc
**276** $R_1$=H, $R_2$=**A**, $R_3$=H
**277** $R_1$=H, $R_2$=**B**,$R_3$=OH
**278** $R_1$=**B**, $R_2$=H,$R_3$=OH
**279** $R_1$=**A**, $R_2$=H,$R_3$=OAc



**280** R=**C**
**281** R=**D**



**A**



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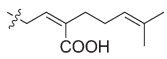
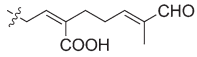
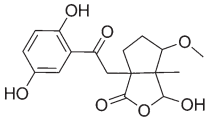


**C**



**D**



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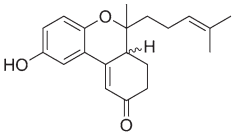

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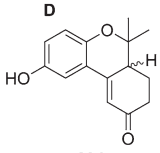

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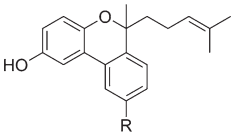
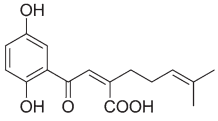
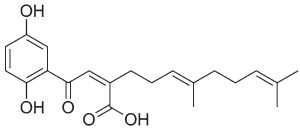


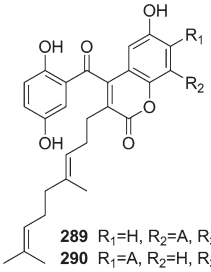
**285** R=CHO
**286** R=COOH



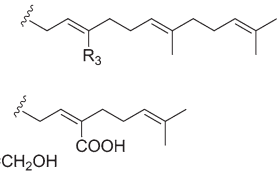
**287**

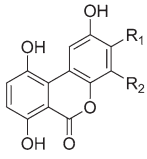


**288**



**289** $R_1$=H, $R_2$=A, $R_3$=$CH_2OH$
**290** $R_1$=A, $R_2$=H, $R_3$=$CH_2OH$

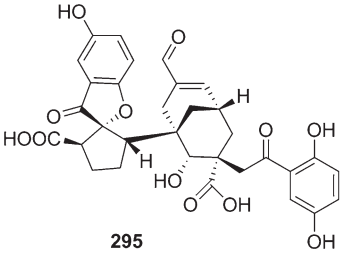




**291** R1=H, R2=A, R3=COOH
**292** R1=H, R2=A, R3=CH2OH
**293** R1=A, R2=H, R3=CH2OH
**294** R1=B, R2=H,

#### 9.3.1.5 *Ganoderma applanatum*

Applanatumin A (**295**), a novel meroterpenoid dimer, was isolated from the fungus *G. applanatum* (Luo et al. 2015c). Applanatumin A exhibits potent antifibrotic activity in TGF-β1-induced human renal proximal tubular cells. Applanatumols A (**296**) and B [(±)-**297**], two unique meroterpenoids, respectively, with a novel spiro[benzofuran-2,2′-bicyclo[3.2.2] nonane] ring system and a naturally unusual dioxacyclopenta[cd]inden motif, were isolated from *G. applanatum* (Luo et al. 2016a). The biological evaluation shows that **296** and (+)-**297** are potent ECM inhibitors in TGF-β1-induced rat proximal tubular epithelial cells, suggesting that these metabolites could be used as novel structure templates for synthesizing more potent agents which are beneficial for CKD. (±)-Ganoapplanin (**298**), a pair of novel meroterpenoid enantiomers featuring an unprecedented dioxaspirocyclic skeleton constructed from a 6/6/6/6 tetracyclic system and an unusual tricyclo-[4.3.3.03′,7′] dodecane motif, were isolated from *G. applanatum* (Li et al. 2016a). Spiroapplanatumines A–Q (**299–315**), 17 new spiro meroterpenoids, respectively, bearing a 6/5/7 or 6/5/5 ring system, were isolated from the fruiting bodies of the fungus *G. applanatum* (Luo et al. 2017). Biological evaluation of spiroapplanatumines A–Q disclosed that spiroapplanatumines G–H (**305–306**) inhibited JAK3 kinase with $IC_{50}$ values of 7.0 ± 3.2 and 34.8 ± 21.1 μM, respectively. Twenty-six new meroterpenoids, applanatumols C–Z (**316–339**), Z1 (**340**), and Z2 (**341**), were isolated from the fruiting bodies of *G. applanatum* (Luo et al. 2016b). Applanatumols C (**316**) was found to have COX-2 inhibitory effect with an $IC_{50}$ value of 25.5 μM.



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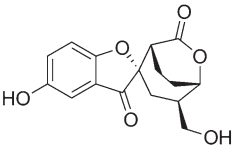

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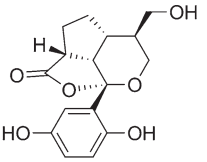

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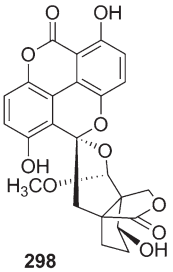
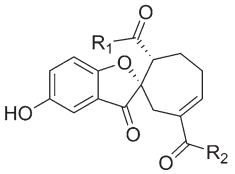
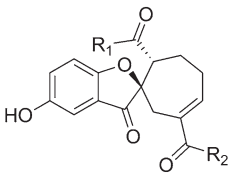


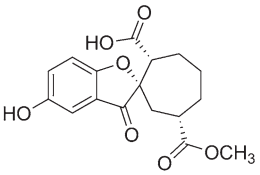
**298**



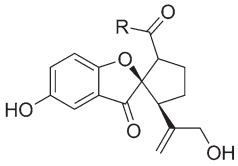


**299** $R_1$=OH $R_2$=OH
**301** $R_1$=$OCH_3$ $R_2$=OH
**303** $R_1$=OH $R_2$=$OCH_3$
**305** $R_1$=OH $R_2$=H
**307** $R_1$=$OCH_3$ $R_2$=H

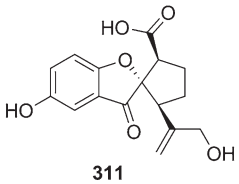
**200** $R_1$=OH $R_2$=OH
**302** $R_1$=$OCH_3$ $R_2$=OH
**304** $R_1$=OH $R_2$=$OCH_3$
**306** $R_1$=OH $R_2$=H



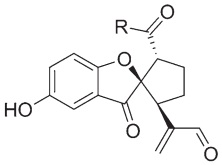
**308**



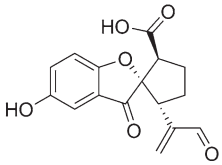
**309** R=OCH3
**310** R=OH



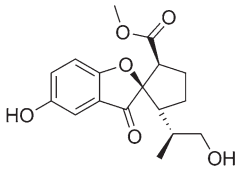
**311**



**312a** R=OH
**313** R=$OCH_3$



**312b**



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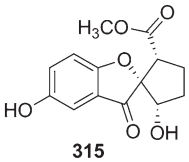

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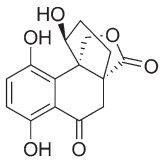

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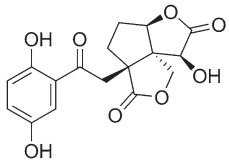

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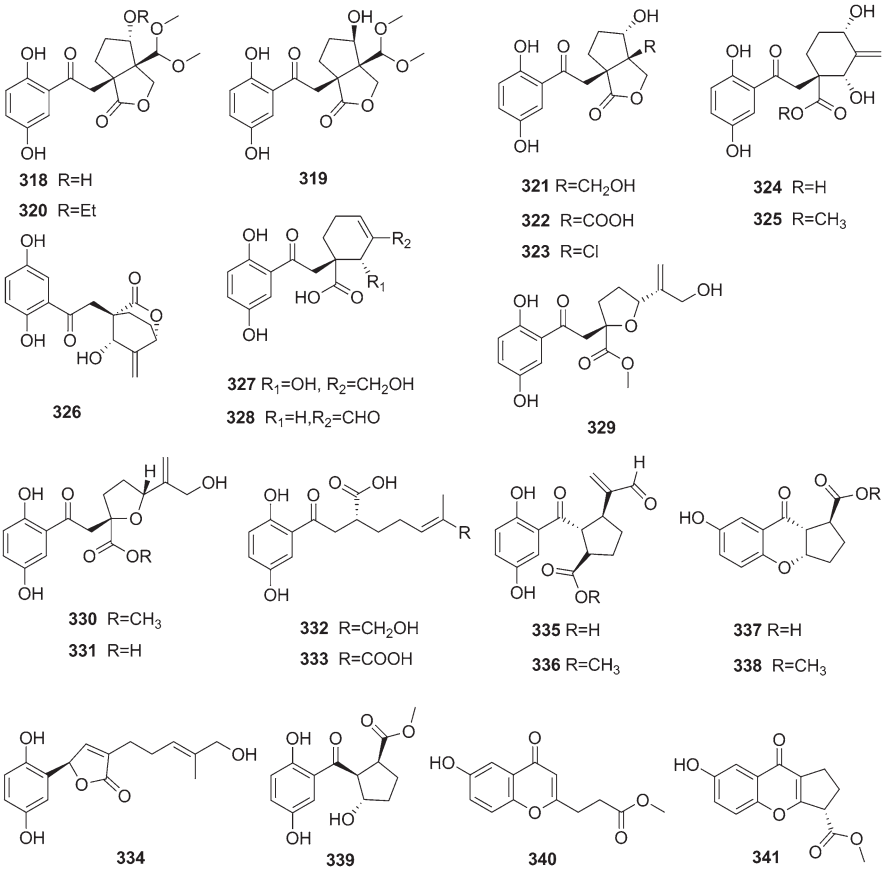
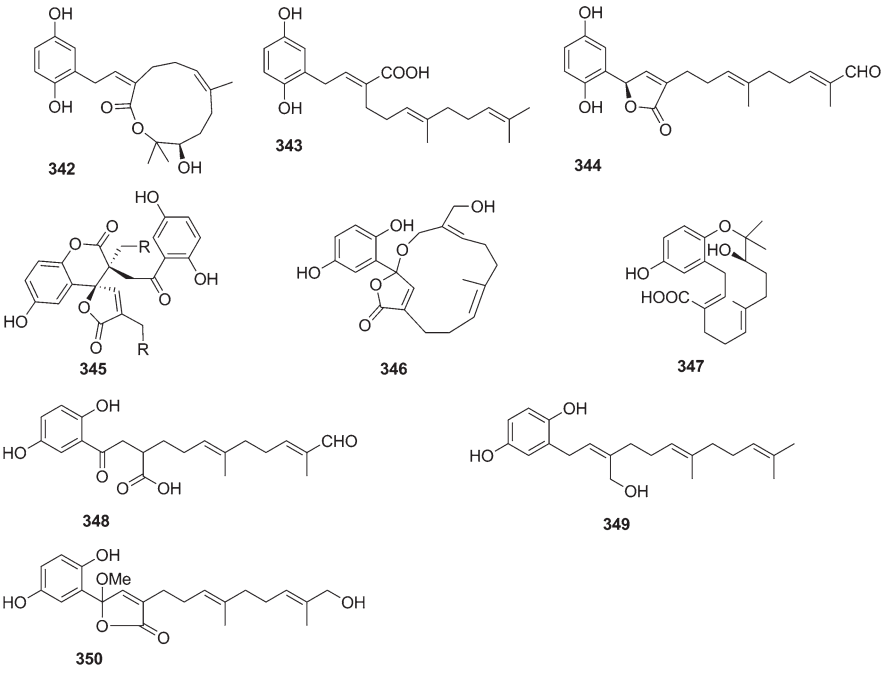



### 9.3.1.6 *Ganoderma leucocontextum*

Three new meroterpenoids, ganoleucins A–C (**342–344**), were isolated from the fruiting bodies of *G. leucocontextum* (Wang et al. 2016b). Ganoleucins A (**342**) and C (**344**) showed noncompetitive inhibitory activity against α-glucosidase. (+)- and (–)-Ganodilactone (**345**), a pair of novel meroterpenoid dimers possessing a unique 5′H-spiro[chroman-4,2′-furan]-2,5′-dione ring system, were discovered from the fruiting bodies of *G. leucocontextum* (Chen et al. 2016). (±)-, (+)-, and (–)-Ganodilactone (**345**) showed pancreatic lipase inhibitory activities and exhibited the $IC_{50}$ values as 27.3, 4.0, and 2.5 μM, respectively.

### 9.3.1.7 *Ganoderma capense*

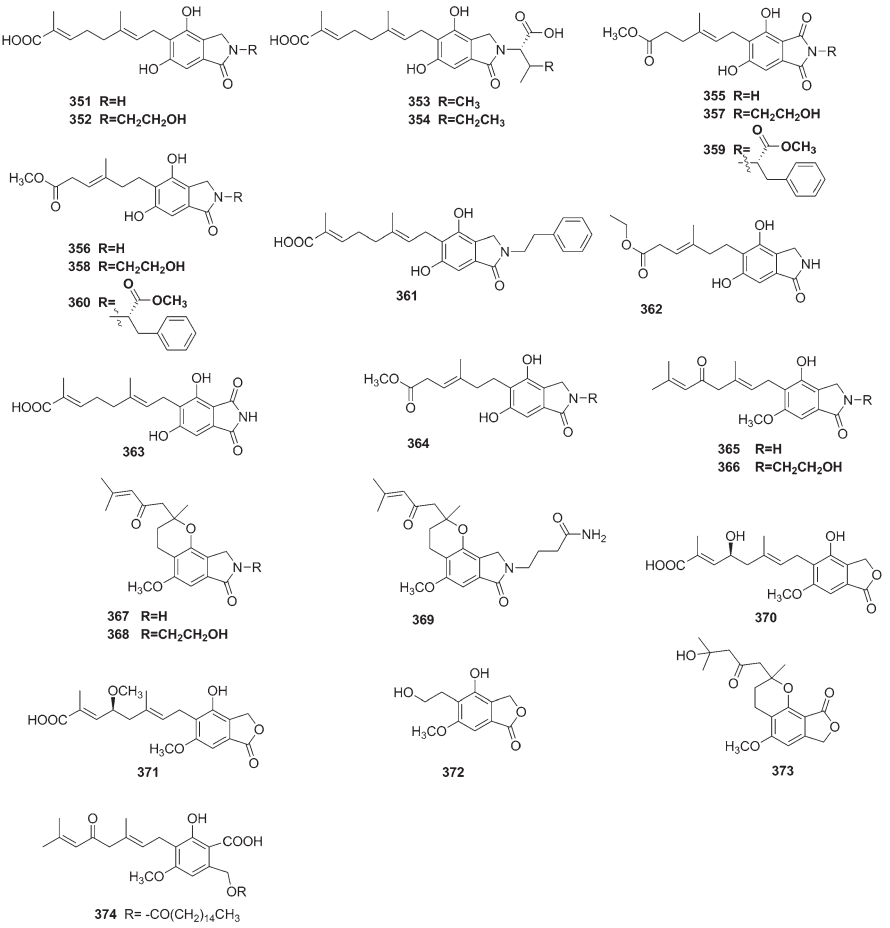
Two new macrocyclic meroterpenoids, ganocapensins A–B (**346–347**), together with three new aromatic meroterpenoids, ganomycins E–F (**348–349**) and fornicin E (**350**), were isolated from the fruiting bodies of *G. capense* (Peng et al. 2016). Compounds **346–350** exhibited antioxidant effects with $IC_{50}$ values ranging from 6.00 ± 0.11 to 8.20 ± 0.30 μg/ml in the DPPH radical scavenging assay.




## 9.3.2 Polyketide-Terpenoids

### 9.3.2.1 *Hericium erinaceus*

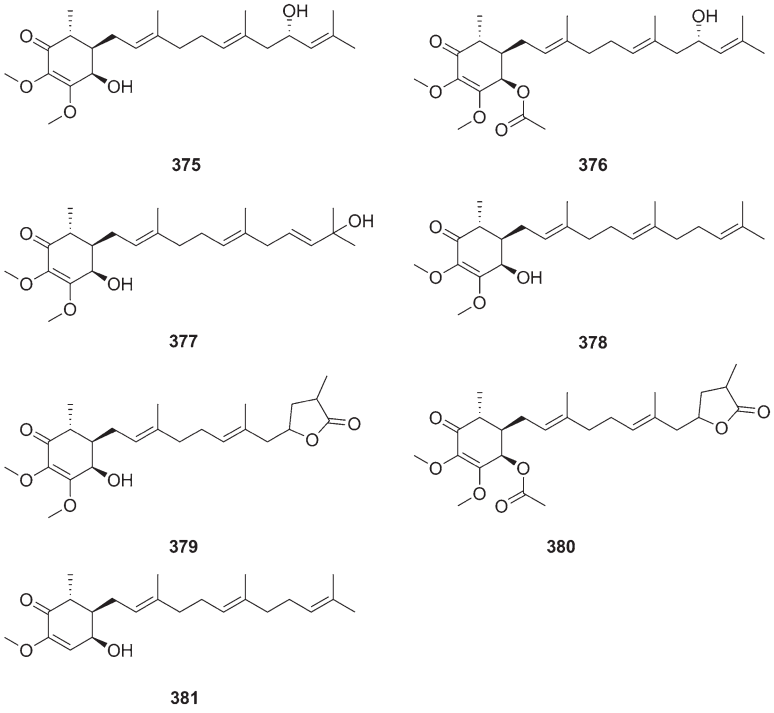
*H. erinaceus* is an important edible and medicinal mushroom. The fruiting bodies and mycelia of this mushroom have been used as an herbal medicine for the treatment of gastricism and hyperglycemia in China. The chemical constituents of *H. erinaceum* were widely investigated. Aromatic compounds and diterpenoids with various bioactivities have been isolated from *H. erinaceus*. Fourteen new meroterpenoids, erinacerins C–L (**351–360**) and erinacerins Q–T (**361–364**), were obtained from the mycelia of *H. erinaceus* fermented on rice (Wang et al. 2015c, d). Compounds **352–364** exhibited inhibitory activity against α-glucosidase with $IC_{50}$ values ranging from 5.3 to 145.1 μM. Erinacerins Q–T (**361–364)** showed inhibitory activities against PTP1B. Erinaceolactams A–E (**365–369**), five new isoindolinones, were isolated from 70% ethanol extract of the fruiting bodies of *H. erinaceus* (Wang et al. 2016c). Five new meroterpenoid, erinaceolactones D–F (**370–372**), hericenone K (**373**), and hericenone L (**374**), were isolated from the fruiting bodies of *H. erinaceus* (Wang et al. 2016d; Zhang et al. 2015b; Ma et al. 2012). Hericenone L (**374**) exhibited cytotoxic activity against EC109 cell line with an $IC_{50}$ of 46 μg·$L^{-1}$. Hericenone K (**373**) exhibited weak neurite outgrowth-promoting activity in NGF-induced PC12 cells.




### 9.3.2.2 *Antrodia camphorata*

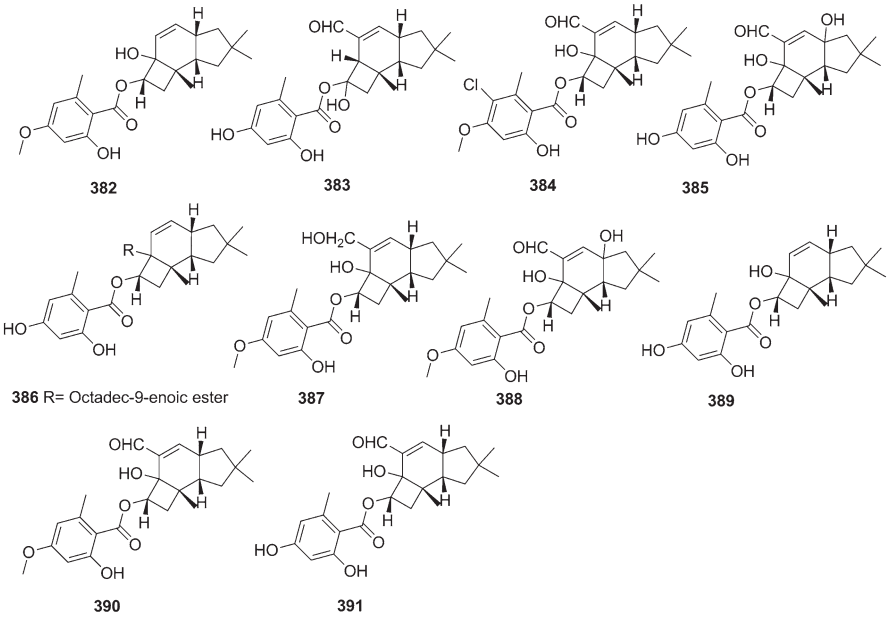
Four ubiquinone derivatives, antrocamol LT1 (**375**), antrocamol LT2 (**376**), antrocamol LT3 (**377**), and antroquinonol (**378**), were isolated from *A. camphorata* mycelium (Yen et al. 2015). Compounds **375**–**378** showed cytotoxicities against CT26, A549, HepG2, PC3, and DU-145 cell lines with $IC_{50}$ values ranging from 0.01 to 1.79 μM. Antroquinonol B (**379**) and 4-acetyl-antroquinonol B (**380**) were obtained from the mycelium of *A. camphorata* (Yang et al. 2009). The two compounds were evaluated for their effects on the inhibition of NO production in LPS-activated murine macrophages. The bioassay displayed that compounds **379** and **380** possessed effects on NO inhibition, with $IC_{50}$ values of $16.2 \pm 0.8$ and $14.7 \pm 2.8$ μg/mL, respectively. Moreover, antroquinonol D (**381**), a ubiquinone derivative, was isolated from the solid-state fermented mycelium of *A. camphorata*

(Wang et al. 2014b). Some research illuminated that antroquinonol D induces DNA demethylation and the recovery of multiple tumor suppressor genes while inhibiting breast cancer growth and migration potential.




### 9.3.2.3 *Armillaria mellea*

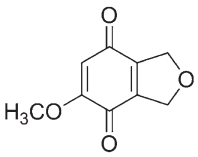
*A. mellea* is an important TCM used in dispelling the wind and removing an obstruction in the meridians and strengthening tendons and bones. Two new protoilludane sesquiterpene aryl esters, 5′-methoxy-armillasin (**382**) and 5-hydroxyl-armillarivin (**383**), as well as eight known protoilludane sesquiterpene aryl esters, armillaridin (**384**), armillartin (**385**), armillarin (**386**), melleolide B (**387**), armillarilin (**388**), armillasin (**389**), armillarigin (**390**), and melleolide (**391**), were isolated from the mycelium of *A. mellea* (Li et al. 2016b). Compounds **383–385** and **388–391** exhibited highly cytotoxic activity against HepG2 cells (4.95–37.65 μg/mL). Among all the ten compounds, melleolide (**391**) showed the best cytotoxic activity for HepG2 cells (4.95 μg/mL) and lower activity for L02 cells (16.05 μg/mL).



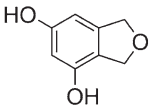

## 9.4 Polyketide

### 9.4.1 *Neolentinus lepideus*

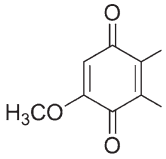
*N. lepideus* is a basidiomycete mushroom of the genus *Neolentinus*, previously well known as *Lentinus lepideus*. It is one of the popular edible mushrooms in China, Japan, and Korea. Three new polyketides, 5-methoxyisobenzofuran-4,7(1H,3H)-dioneone (**392**), 1,3-dihydroisobenzofuran-4,6-diol (**393**), and benzoquinone derivative (**394**), were obtained from the solid culture of *N. lepideus* fermented on cooked rice (Li et al. 2013b). In the DPPH scavenging assay, compound **393** displayed antioxidant activity with $IC_{50}$ of 68.6 μM. Compounds **392–394** showed potent inhibition of nitric oxide production in macrophages with an $IC_{50}$ value of 6.2, 88.8, and 100 μM, respectively.



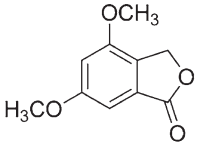

**392**




**393**




**394**

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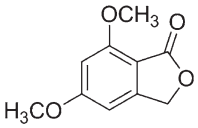
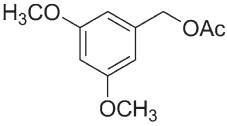



**396**

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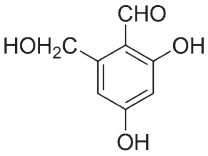



**398**

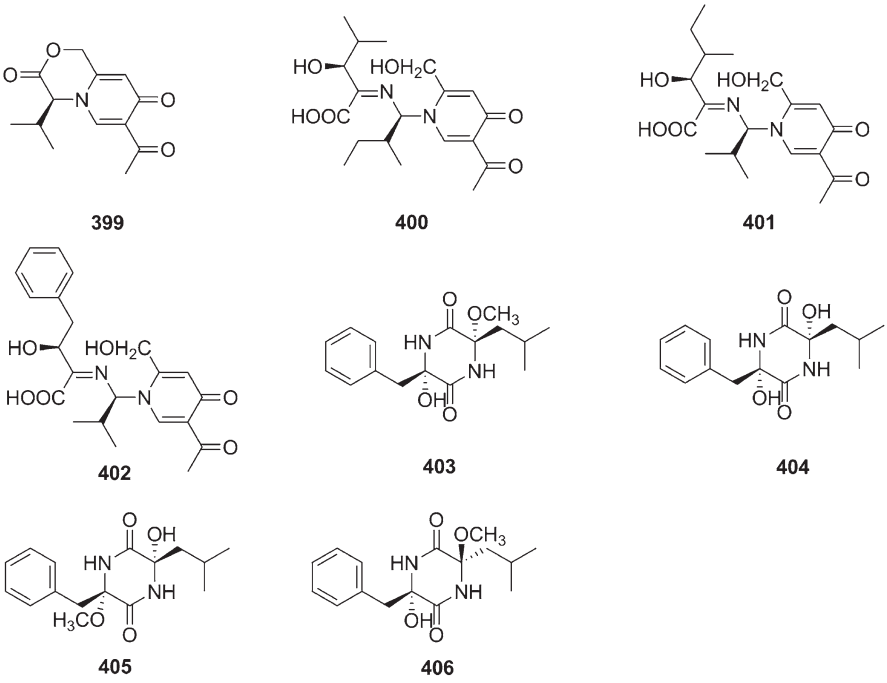
### 9.4.2 *Pleurotus* spp.

6-Dimethoxyisobenzofuran-1(3*H*)-one (**395**), a known polyketide, was isolated from the culture broth of the fungus *P. eryngii* (Liu et al. 2013). Three polyketides, 5, 7-dimethoxyisobenzofuran-1(3H)-one (**396**), 3, 5-dihydroxybenzyl acetate (**397**), and 2,4-dihydroxy-6-(hydroxymethyl) benzaldehyde (**398**), were isolated from the solid culture of *P. citrinopileatus* (Li et al. 2013c). Compounds **396–398** showed moderate chelating capacity with percent chelating value of 28.77%, 29.72%, and 39.47% at a concentration of 200 μmol/L, respectively. Compound **396** showed weak reducing ability with percent reducing the value of (22.22 ± 5.44) % at the concentration of 200 μmol/L.

## 9.5 Alkaloids and Other Nitrogen-Containing Compounds

### 9.5.1 *Hericium erinaceus*

Four new alkaloids, erinacerins M–P (**399–402**), were obtained from the mycelia of *H. erinaceus* fermented on rice (Wang et al. 2015d). Erinacerins M–P (**399–402**) showed moderate cytotoxicity against K562 cells with $IC_{50}$ values of 16.3, 18.2, 15.9, and 11.4 μM, respectively, and also weak cytotoxicity against doxorubicin-resistant K562 cells.

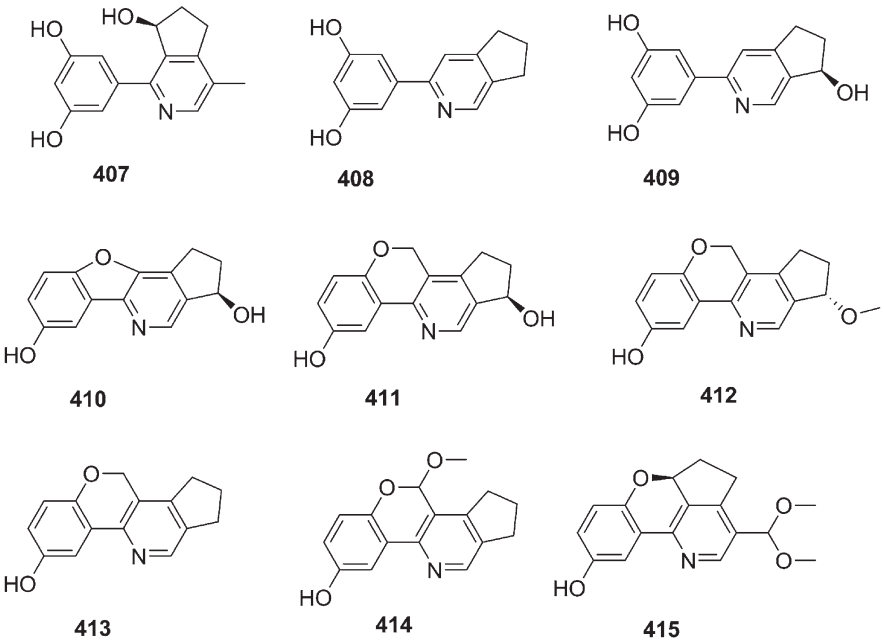



### 9.5.2 *Lepista sordida*

*L. sordida*, a basidiomycetous fungus of the family *Tricholomataceae*, is an edible agaric species. Three new 3,6-dioxygenated diketopiperazines, lepistamides A–C (**403–405**), along with a known compound, diatretol (**406**), were isolated from the mycelial solid cultures of the *L. sordida* (Chen et al. 2011). Compounds **403–406** were all found to be inactive ($IC_{50}$ > 100 μg/ml) in the evaluation of the cytotoxic activity against Astc-a-1 (lung cancer), Bel-7402 (liver cancer), and HeLa (cervical carcinoma) cell lines by means of the MTT assay method.

### 9.5.3 *Ganoderma* spp.

Five new alkaloids, sinensines A–E (**407–411**), were isolated from the fruiting bodies of *G. sinense* (Liu et al. 2010, 2011). Sinensine A (**407**) exhibited activity in protecting the injury induced by hydrogen peroxide oxidation on human umbilical cord endothelial cells (HUVEC), with $EC_{50}$ value of 6.2 mmol/L. Four new polycyclic alkaloids, lucidimines A–D (**412–415**), were isolated from the fruiting bodies of *G. lucidum* (Zhao et al. 2015).




## 9.6 Conclusion

In the past 10 years, substantial progress has been made by Chinese scientists in the field of bioactive metabolites from edible and medicinal fungi. Chemical investigations of the fruiting bodies and culture broth of the edible and medicinal fungi collected in China have resulted in 415 compounds including 90 sesquiterpenoids and 115 meroterpenoids belonging to shikimate-terpenoids. These compounds exhibit various bioactivities including antibacterial, antioxidant, anticancer, antiplasmodial, antiproliferative, antifibrotic, and neurite outgrowth-promoting activities. Edible and medicinal fungi produce enormously diverse metabolites, but only a small number has been explored. It is promising to search for leads of new drugs by continuing the further chemical investigations of edible and medicinal fungi.

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# Endophytes from Malaysian Medicinal Plants as Sources for Discovering Anticancer Agents

10

Ling-Sze Yap, Wai-Leng Lee, and Adeline-Su-Yien Ting

**Abstract**

Malaysia hosts a diverse range of medicinal plants having therapeutic values including anticancer properties. Over the years, a group of microorganisms called endophytes producing bioactive compounds similar to their host plants has been discovered. These endophytes, producing important compounds such as Taxol, L-asparaginase, and sclerotiorin, can be isolated and cultured in large scale to produce valuable compounds. This alternative approach of producing bioactive compounds is more sustainable than plants, as endophytes are renewable sources. In this chapter, endophytes from Malaysian medicinal plants have been discussed, highlighting the diversity of endophytes, the valuable compounds produced, the current methods used in biosourcing of endophytes, and the future prospects of anticancer agents derived from endophytes of Malaysian medicinal plants.

**Keywords**

Anticancer • Bioactive compounds • Endophytic fungi • Malaysian medicinal plants • Natural products

## Contents



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## Abbreviations

| | |
|---|---|
| A549 | Human Caucasian lung carcinoma |
| B16-F10 | Mouse skin melanoma |
| CDK | Cyclin-dependent kinase |
| CEMss | Human T4-lymphoblastoid cell line |
| CPT | Camptothecin |
| D551 | Human normal skin cells |
| DLD-1 | Human colorectal adenocarcinoma |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| HCT116 | Human colon carcinoma |
| HeLa | Human cervix carcinoma |
| HepG2 | Human Caucasian hepatocyte carcinoma |
| HPLC | High-performance liquid chromatography |
| K562 | Human chronic myeloid leukemia |
| Kb | Oral carcinoma cells |
| LLC | Lewis lung carcinoma |
| M059J | Human brain malignant glioma |
| MCF-7 | Human breast adenocarcinoma |
| MCM-B2 | Canine mammary carcinoma |
| MDAMB231 | Human breast adenocarcinoma |
| MG63 | Human bone osteosarcoma |
| MS | Mass spectrometry |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NCI-H1299 | Human lung carcinoma |
| NMR | Magnetic resonance spectroscopy |
| NPAA | Nonprotein amino acids |
| P388 | Murine leukemia cells |
| PBLs | Primary blood lymphocytes |
| PC12 | Rat adrenal pheochromocytoma |
| PC3 | Human prostate adenocarcinoma |
| SkO-3 | Human Caucasian ovary adenocarcinoma |
| T-47D | Human breast ductal carcinoma |
| TCM | Traditional Chinese medicine |
| WEHI-3 | Mouse leukemia cells |

## 10.1 Introduction

Cancer is the world's leading disease affecting as many as 3500 per one million population in the world annually (Pandey and Madhuri 2009). Cancer is characterized by uncontrolled cell division, which results in abnormal cell growth. These abnormal cells spread and metastasize to distant parts of the body, resulting in the formation of malignant tumor cells and destroying normal healthy cells (Pandey and Madhuri 2009; Madhuri and Pandey 2009; Prakash et al. 2013). The risk of cancer occurrence in individuals increases with age, due to higher susceptibility toward DNA mutation (Garinis et al. 2008). Various therapeutic measures have been used to treat cancer including surgery, chemotherapy, and radiation therapy. Nevertheless, these treatments are sometimes invasive and have numerous side effects, with the main concern that the healthy cells are also destroyed. As a result, alternative cancer treatments are explored.

Alternative cancer treatments are primarily focused on the use of medicinal or herbal plants. These plants are rich sources of anticancer agents, as they have long been known to have ethnobotanical properties related to the treatment of various diseases. In the early civilization, herbal knowledge was practiced by referring to Ebers Papyrus, "Shennong Ben Cao Jing," and Atharva Veda and Rig Veda, from Ancient Egypt, China, and India, respectively (Petrovska 2012). As modern medicines were discovered, the role of medicinal plants was relegated to being cheaper alternatives and used only where modern medicines are too costly. Nevertheless, in the last few decades, the limitations of modern medicines, particularly the side effects and drug resistance observed in clinical drugs, have led to the resurgence of the use of traditional medicinal plants as possible alternatives (Lin et al. 2012).

Traditionally, medicinal plants are consumed or used to extract beneficial compounds. For consumption, capsules and tablets containing raw, dried herb or their powdered forms are often used. For extraction, beneficial compounds are extracted using alcohol (tinctures), vinegar (acetic acid extracts), hot water (tisanes or decoctions), or cold water infusion (macerates) (Benzie and Wachtel-Galor 2011). Despite the many benefits and multiuse of these extracts, the levels of bioactive compounds present in the plants are relatively inconsistent (Brusotti et al. 2014). This impacted the wider use of medicinal plants for therapeutic purposes. In addition, the biosafety of medicinal plants was also of concern with reports of heavy metal contamination in medicinal plants (Drew and Myers 1997; Bent 2008). It is evident that despite the many uses of medicinal plants, useful lead compounds are best elucidated through intensive screening, extraction, purification, and quantification processes.

Plants generally produce two types of metabolites; the primary and secondary metabolites. Primary metabolites, also known as central metabolites, are widely distributed and can be found in almost all plants. These metabolites are involved in plant growth and metabolism, usually playing an important role in maintaining the physiological functions of a plant (Balandrin et al. 1985). Examples of primary metabolites are alcohols, amino acids, carbohydrates, and lipids. Secondary metabolites, on the other hand, are the end products or compounds derived from the primary metabolites. These compounds are not found in all plants and are limited to

**Table 10.1** Examples and number of known secondary metabolites of various classes from plants

| Class | Number of known secondary metabolites | Examples |
|---|---|---|
| Alkaloids | 21,000 | Cocaine, morphine, atropine, nicotine |
| Nonprotein amino acids (NPAAs) | 700 | Azatyrosine, canavanine |
| Cyanogenic glycosides | 60 | Amygdalin, prunasin, linamarin |
| Amines | 100 | Hordenine |
| Flavonoids, tannins | 5000 | Tannic acid, luteolin |
| Terpenoids | >22,000 | Artemisinin, tetrahydrocannabinol, azadirachtin |
| Carbohydrates, organic acids | 200 | Stachyose, gentianose |

Adapted from Wink (2010)

the certain taxonomic group. Secondary metabolites do not have a specific function as they are not involved in the plant metabolism, but they do contribute to the host plant defense system. They also contribute as lead compounds with therapeutic functions, produced typically as a response by the medicinal plants to various biotic and abiotic stresses (Bernhoft 2010). Plant secondary metabolites can be classified into two major chemical groups: the nitrogen-containing metabolites such as alkaloids, nonprotein amino acids (NPAAs), cyanogenic glycosides, and amines and the non-nitrogen-containing metabolites such as flavonoids, tannins, terpenes, and carbohydrates (Table 10.1).

Some of these plant secondary metabolites have anticancer properties and have been extensively studied and tested in clinical trials. Primary examples include the discovery and the use of vinca alkaloids and paclitaxel (or Taxol). The vinca alkaloids are derived from the periwinkle plant *Catharanthus roseus*. This plant produces vinca alkaloids such as vincristine, vinblastine, vindesine, and vinorelbine, has antimitotic properties, and thus is highly effective for the treatment of lymphoma, leukemia, testicular cancer, Hodgkin's disease, and lung cancer (Greenwell and Rahman 2015). They induce cell cycle arrest and inhibit angiogenesis by binding onto the β-tubulin. This affects the dynamics of tubulin addition to the end of the mitotic spindle, implicating cell proliferation in cancerous cells as the function of a mitotic microtubule is hampered (Jordan et al. 1991; Umadevi et al. 2013).

In addition to vinca alkaloids, paclitaxel (Taxol) is another well-known anticancer medication used in chemotherapy. Paclitaxel is effective as it targets the tubulin, stabilizing and promoting the polymerization of microtubules. This blocks the progression of cell division and finally triggers the occurrence of apoptosis (Horwitz 1992; Weaver 2014). Paclitaxel was first isolated in 1971 from the inner bark of the Pacific yew tree (*Taxus brevifolia*) and sold under the brand name Taxol. Although paclitaxel is a successful and effective anticancer agent, this anticancer agent is not readily available to most cancer patients as it is extremely expensive. The high-cost of paclitaxel is attributed to the scarcity of the yield of paclitaxel obtained from yew trees. It has been estimated that 10,000 kg of yew tree bark can generate only 1 kg

of paclitaxel upon extraction (Chandra 2012). The low quantities of extracted paclitaxel and the slow growth rate of the yew trees account for the high price of paclitaxel in the market (Joseph and Priya 2011; Kharwar et al. 2011).

Also, it has been discovered that medicinal plants pose several limitations as a feasible source for anticancer agents. Some medicinal plants are rare and slow growing, in which they may require a longer duration to grow and reach maturity and therefore requires several years or decades to be able to harvest the anticancer agents from the plants. For some plants, the harvesting process can only yield a small amount of anticancer agents. The low quantities of anticancer agents and slow growth of medicinal plant will subsequently lead to the need to harvest more plants, resulting in species extinction and environmental degradation. Several attempts have been made to address issues on the low yield of anticancer compounds from plants. For example, scientists have attempted to increase the harvest and extraction of paclitaxel from plants by performing cell, organ, and tissue culture or to increase the yield of paclitaxel through total and semisynthesis (Kharwar et al. 2011; Chandra 2012). The cell and tissue culture approach are, however, both costly and time-consuming.

In 1993, paclitaxel was discovered for the first time from a non-plant origin and endophytic fungus, *Taxomyces andreanae*. This endophyte was isolated from the yew tree, which is the producer of paclitaxel (Gangadevi and Muthumary 2008). With the discovery of *T. andreanae* as producer of paclitaxel, mass production of this anticancer compound is achievable through batch culturing in the bioreactors. This provides an alternative to producing more anticancer compounds in a cheaper manner. Since then, several reports have been published on the production of Taxol by different species of fungal endophytes. The discovery of endophytes producing valuable bioactive compounds such as paclitaxel has opened new chapters in the research of anticancer agents derived from endophytes.

## 10.2 Endophytes from Medicinal Plants

Endophytes can be defined as microorganisms, usually bacteria or fungi, which reside in host plant tissues without any visible symptoms (Petrini 1991; Tan and Zou 2001). Endophytes are ubiquitous and can be found in most of the plant species and throughout almost all plant tissues, including the leaves, stems, barks, roots, and rhizomes (Raviraja 2005). Endophytes form an endosymbiotic relationship with the host plant. The host plants provide protection and nutrients to the endophytes, and in return, the endophytes benefit the host plants by promoting plant growth and tolerance toward biotic and abiotic stresses (Tan and Zou 2001; Ting 2014).

Endophytes produce secondary metabolites that have anticancer, antimicrobial, anti-inflammatory, antioxidant, antidiabetic, and immunosuppressive properties (Joseph and Priya 2011). Some of the compounds produced by the endophytes are similar to compounds produced by the host plants, such as in the case of paclitaxel from *T. andreanae*, which showed similarities to paclitaxel from the host plant (yew tree) (Gangadevi and Muthumary 2008). Chandra (2012) hypothesized that the

**Table 10.2** Anticancer agents derived from fungal endophytes isolated from medicinal plants

| Anticancer drug | Endophytes | Host plant | References |
|---|---|---|---|
| Paclitaxel (Taxol) | *Taxomyces andreanae* | *Taxus brevifolia* | Chandra (2012) |
| | *Pestalotiopsis microspora* | *Taxus wallichiana* | |
| | *Fusarium solani* | *Taxus chinensis* | |
| | *Nigrospora* sp. | *Taxus globosa* | |
| | *Phoma* species | *Aloe vera* | |
| | *Alternaria* sp. | *Ginkgo biloba* | |
| | *Colletotrichum capsici* | *Capsicum annuum* | |
| | *Pestalotiopsis microspora* | *Taxodium distichum* | |
| Camptothecin (CPT) | *Entrophospora infrequens* | *Nothapodytes foetida* *Camptotheca acuminate* | Puri et al. (2005) |
| | *Botryosphaeria parva* | *Nothapodytes nimmoniana* | Chandra (2012) |

production of similar secondary metabolites is due to the long-term coexistence and close metabolic interaction between endophytes and host plants. Endophytes living in tissues of stems and leaves of the host plants may have formed a strong symbiotic association with the host plant over the long period of coexistence. This close relationship may have allowed genetic information to transfer between themselves and the host plants, resulting in the generation of similar secondary metabolites in endophytes as those from the host plant (Strobel 2003). Wang and Dai (2011) suggested that endophytes produce a greater amount of bioactive compounds than their host plants, with the presence of their biotransformation enzymes to change the three-dimensional conformation of bioactive compounds. Hence, endophytes are promising resources of bioactive compounds for anticancer or any other therapeutic applications.

Of the many different compounds produced by endophytes, anticancer compounds are highly sought after as alternatives to synthetic compounds in chemotherapy. Following the discovery of paclitaxel from the first Taxol-producing fungus, *Taxomyces andreanae*, other endophytic species have also been reported to produce Taxol. These endophytes are found naturally colonizing plants of the *Taxus* species (*T. yunnanensis*, *T. wallichiana*, *T. chinensis*, *T. globosa*, *T. cuspidata*) and non-*Taxus* species (*Taxodium distichum*, *Ginkgo biloba*, *Wollemia nobilis*, *Aloe vera*, *Capsicum annuum*) (Table 10.2). Each species of endophyte has a different capacity of producing Taxol. *Colletotrichum capsici* from *C. annuum* and *Pestalotiopsis versicolor* from *T. cuspidata* reportedly have a relatively higher yield of Taxol (687 μg/l and 478 μg/l, respectively). On the contrary, *Tubercularia* sp. isolated from *T. chinensis* var. *mairei* (185.4 μg/l) and *Phoma* sp. from *A. vera* (73.66 μg/l) have a lower yield of Taxol (Chandra 2012). Nevertheless, optimization of media and fermentation conditions are known to increase the production of Taxol (Xu et al. 2006).

Other than Taxol, camptothecin (CPT) is another anticancer compound produced by endophytes. Camptothecin is a pentacyclic quinoline alkaloid and is

typically derived from the bark and stem of the *Camptotheca* plant "happy tree" from China (Kusari et al. 2012). CPT targets tumor cells by binding to the intranuclear enzyme DNA topoisomerase 1 (Topo 1) and the DNA covalent complex, stabilizing and preventing Topo 1 from swiveling and relaxing the DNA during DNA replication and transcription. This deters the religation of DNA, leading to DNA damage and apoptosis (Hsiang et al. 1989). Although CPT has a good anticancer activity, the wide usage of CPT is hampered by poor solubility in aqueous media, high toxicity, and the various side effects. Two of the analogues of CPT from plants, 9-methoxycamptothecin and 10-hydroxycamptothecin, are water soluble, demonstrating potential anticancer activity as the CPT (Kusari et al. 2009). Interestingly, high yield of CPT and its derivatives which are less toxic were found in endophytes isolated from the host plants. Puri et al. (2005) first reported the production of CPT from the fungal endophyte *Entrophospora infrequens*, which was isolated from the Sleumer tree (*Nothapodytes foetida*). CPT has also been found to be produced by endophytes such as *Fusarium solani*, *Nodulisporium* sp., *Neurospora* sp., and *Botryosphaeria parva* from different host plants such as *Camptotheca acuminata*, *N. foetida*, and *Nothapodytes nimmoniana*, respectively (Table 10.2) (Chandra 2012).

Comparatively, bacterial endophytes are less dominant in producing anticancer agents than the fungal endophytes. While the latter has a long list of species and compounds produced (as discussed in the previous section and Table 10.2), the former has only several notable species known to produce anticancer compounds. Most of the bacterial species capable of producing anticancer compounds are of the *Bacillus* species, such as *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus*, and *Lysinibacillus* sp., which produce exopolysaccharide or camptothecin with anticancer potential (Chen et al. 2013; Shweta et al. 2013). These endophytes were isolated from medicinal plants such as *Ophiopogon japonicus* (mondo grass or monkey grass) and the climber plant *Miquelia dentata* Bedd. Taechowisan et al. (2007) also discovered endophytic actinobacteria *Streptomyces aureofaciens* CMUAc130 from the root tissues of *Zingiber officinale*. This isolate produces the bioactive compound 4-arylcoumarins and its derivative 5,7-dimethoxy-4-phenylcoumarin, which exhibited significant anticancer activity toward transplanted Lewis lung carcinoma (LLC). Liu et al. (2012) also documented the antitumor activities of levan-type exopolysaccharide (EPS) produced by *Paenibacillus polymyxa* EJS-3. This bacterium is an endophyte found in the roots of *Stemona japonica* (or "Bai Bu" in Chinese), a Chinese medicinal plant used to treat different types of cough illness. Future research on discovering more bacterial endophytes and their anticancer compounds will benefit the anticancer therapeutic field.

## 10.3 Anticancer Agents Derived from Endophytes from Malaysian Medicinal Plants

The endophytes from Malaysian medicinal plants have been found to produce bioactive compounds with anticancer activities. These endophytes are primarily isolated from medicinal plants, while herbal plants, seaweed, or spices, such as ginger, pandan leaf, hibiscus flower, lemongrass, and bay leaf, are also known to harbor valuable endophytes (Radu and Kqueen 2002; Hazalin et al. 2009). Radu and Kqueen (2002) were among the first few researchers to document the antitumor activities of endophytic fungi from Malaysian medicinal plants. In their study, 13 of the 72 medicinal plants screened were hosts to endophytic fungi with strong antitumor activities (Table 10.3). The antitumor activities from the endophytes were detected using UCK/UCS yeast cell-based assay in which isolates that rescue the growth arrest caused by the hyperactivation of cyclin-dependent kinase (CDK) were considered to possess anticancer properties. In their preliminary study, an accurate comparison of effective isolates was not achieved as crude extracts were used in the bioassay. As such, the low antitumor activity may be due to the lesser amount of bioactive compound obtained and the impurities present in the crude extracts. Therefore it is not an accurate measure of bioactivity of the compounds. The species of fungal endophytes and compounds, which demonstrated anticancer activities, were also not further identified or characterized in their study.

Following the work by Radu and Kqueen (2002), Hazalin et al. (2009) explored native plants from the National Park, Pahang, Malaysia. They isolated 300 endophytic fungi from 43 different plants using the leaf, stem, root, and flower tissues.

**Table 10.3** Summary of fungal endophytes recovered from Malaysian medicinal plants and their antitumor activities using crude extracts

| Endophytic isolates | Local name of medicinal plant (scientific name) | Antitumor activity (mm) | |
|---|---|---|---|
| | | UCS[a] | UCK[b] |
| 5L | Bisa ular (*Barleria lupulina*) | 8 | 8.5 |
| 24L2 | Kesum (*Polygonum* sp.) | 8 | 8 |
| 50ML | Tembaga suasa besar (*Rinum aisiaticum*) | 10 | 10.3 |
| b9L | Cekur (*Kaempferia galanga*) | 10 | 10.3 |
| b14L | Ervalanala (*Aerva lanata*) | 8.2 | 8.2 |
| b20L | Jerangau (*Acorus calamus*) | 7.5 | 7.5 |
| b29L | Lidah mertua (*Sansevieria trifasciata*) | 8.3 | 8.1 |
| b30L | Mengkudu (*Morinda citrifolia*) | 9 | 9 |
| b34L | Pandan (*Pandanus odons*) | 7.2 | 7.1 |
| b49L | Sembong (*Blumea balsamifera*) | 8.7 | 8.5 |
| b53L | Tongkat ali (*Eurycoma longifolia*) | 8 | 8.1 |
| b69L2 | Sambung nyawa (*Gynura procumbens*) | 8.1 | 8 |
| b70L2 | Serai kayu (*Eugenia polyantha*) | 8.6 | 8.3 |

Based on data from Radu and Kqueen (2002)

[a]UCS – Yeast test strain containing plasmid Yep51-SRX5

[b]UCK – Yeast test strain containing plasmid mPR438-CyclinA D 24–62

Crude extracts were prepared and tested for cytotoxic activities against several cancer cell lines. It was discovered that a majority of the extracts were more effective against the murine leukemia P388 cell line compared to the human chronic myeloid leukemia cell line K562. They discovered that extracts from ten endophytic species exhibited higher anticancer activity against P388 than the existing anticancer agents from *Paecilomyces farinosus* (paecilosetin, farinosone) and *Penicillium* sp. GQ-7 (penicillenol). Hazalin et al. (2009) also discovered that the fungal endophyte strain KK29FL1 isolated from the flower of *Costus speciosus* (spiral ginger or "setawar hutan") had the greatest cytotoxic activity against P388 and K562 cell lines. The endophyte was identified as a *Sporothrix* sp., but the extract was not further characterized.

The rain forests offer plants with promising potential to produce anticancer compounds. Ramasamy et al. (2010) isolated 348 endophytes from 24 Malaysian medicinal plants from the Kuala Pilah Rain Forest, Negeri Sembilan, Malaysia. Two selected endophytes, *Aspergillus* sp. HAB10R12 and HAB21F25, were isolated from roots of *Garcinia scortechinii* and fruits of *Smilax myosotiflora*, respectively. The isolates showed potent anticancer activity against MCF-7 (breast cancer) and HCT116 (human colon carcinoma) cell lines. Both isolates were from medicinal plants with ethnobotanical properties. *Garcinia scortechinii* is widely used by Malaysians for the postpartum care and the prevention of peptic ulcer. *Smilax myosotiflora*, also known as "carrion flowers," is a medicinal herb for treatment of syphilis (Lin 2005). Hamiyah et al. (2012) further investigated the antiproliferative effect of extracts from *Aspergillus* sp. (HAB10R12) against four different cancer cell lines using MCF-7 (breast cancer), HCT116 (human colon carcinoma), A549 (human Caucasian lung carcinoma), and HepG2 (human Caucasian hepatocyte carcinoma) using MTT assay. It was reported that the extract was most effective against HepG2, followed by MCF-7, HCT 116, and A549, cancer cell lines for liver cancer, breast cancer, colon cancer, and lung cancer, respectively. The cytotoxic effects of the extracts were linked to the phenolic compounds. However, it needs a further characterization. Hazalin et al. (2012) further explored isolates KK9L2, KK14L1, KK21FL2, and KK30RJ2 from various medicinal plants by Hazalin et al. (2009) and Ramasamy et al. (2010). KK9L2 was isolated from the leaves of *Phyllagathis rotundifolia* "tapak sulaiman" used commonly in the postpartum therapy and as a contraceptive herbal formulation. KK14L1 was isolated from *Ampelocissus cinnamomea* Planch, a climber plant used to treat sinusitis, asthma, and hemorrhoids. KK21FL2 and KK30RH2 were isolated from *Zingiberaceae* sp. (ginger plant), used traditionally for the treatment of diarrhea, sea sickness, migraine, and rheumatism. The extracts of these four endophytes induce apoptosis against the human chronic myelogenous leukemia cancer cell lines HCT116, MCF-7, and K562. Cytotoxic effects have been attributed to the six bioactive compounds identified from the extracts: cytochalasin J, dechlorogriseofulvin, dimethyl-harzianic acid, griseofulvin, harzianic acid, and 2-hexylidene-3-methyl-succinic acid.

Endophytes from another common Malaysian medicinal plant, *Strobilanthes crispus*, known as "pokok pecah kaca" or "pokok pecah beling" also produced effective anticancer compounds (Jinfeng et al. 2017). Traditionally, *Strobilanthes*

*crispus* is used to treat diabetes, hypertension, and cancer (Koay et al. 2013). In the study by Jinfeng et al. (2017), two endophytic isolates, identified as *Sordariomycetes* sp. (PDA)BL3 and (PDA)BL5, were effective toward five different cancer cell lines: the human prostatic, alveolar, colorectal, breast adenocarcinoma, and human hepatocellular carcinoma cells. Gas chromatography-mass spectrometry (GC-MS) indicated that the anticancer activity might be credited to the bioactive compounds pyrrolo[1,2-a]pyrazine-1,4-dione and hexahydro-3-(2-methylpropyl), which are found primarily in the crude extract. These compounds, however, may demonstrate different levels of activities depending on the interaction with other compounds produced by the respective isolates. This phenomenon was observed in a recent report by Jinfeng et al. (2017). It was found that pyrrolo[1,2-a]pyrazine-1,4-dione and hexahydro-3-(2-methylpropyl) in extracts produced by (PDA)BL5 were more efficient in anticancer activity, while the same extract produced by (PDA)BL3 has stronger antimicrobial activity (Jinfeng et al. 2017). Beneficial endophytes have also been discovered from the cinnamon plant *Cinnamomum* sp. (also known as "kayu manis padang"). This plant is sourced mainly as a common spice used in cooking but has also been used traditionally to treat cold and bronchitis and for various antimicrobial, antifungal, and antiseptic uses. Several species of endophytes have been isolated from this plant. Santiago et al. (2012) reported the production of the anticancer compound from the endophyte *Phoma* sp., isolated from the plant *C. mollissimum*. The anticancer compound was identified as 5-hydroxyramulosin, a polyketide produced via the action of pentaketide synthase. The production of the polyketide is from the synthesis of melanin, a metabolic precursor, which increases the potential of virulence in fungal endophytes and to improve its survivability under stressful condition. The result showed that the extracted 5-hydroxyramulosin demonstrated the significant inhibitory effect on the P388 murine leukemia cells. Other endophytic species such as *Colletotrichum gloeosporioides* are also found in *Cinnamomum* trees such as *Cinnamomum malabatrum*. This isolate has shown cytotoxic activity against HeLa (human cervix carcinoma), MCF-7 (human breast adenocarcinoma), and MG63 (human bone osteosarcoma) cancer cell lines. The major metabolites extracted from this endophyte are phenol 3,5-dimethoxy acetate and 4′-isopropylidene-bis-(2-cyclohexyl) phenol.

## 10.4 Malaysian Marine Plants as Source of Endophytic Fungi

Other than terrestrial medicinal plants, studies have also been carried out on endophytic fungi from marine plants, although the medicinal values of marine plants are less understood. Ariffin et al. (2011) isolated 64 endophytic fungi from two marine trees (*Pandanus amaryllifolius* and *Nypa frutican*) and six different types of seaweeds (*Turbinaria conoides*, *Caulerpa lentillifera*, *Caulerpa racemosa*, *Padina australis*, *Caulerpa racemosa* var., *Sargassum polycystum*) from Port Dickson, Negeri Sembilan, Malaysia. The methanolic extracts of the isolates were tested against seven cancer cell lines: M059J (human brain malignant glioma), DLD-1 (human colorectal adenocarcinoma), NCI-H1299 (human lung carcinoma),

MDAMB231 (human breast adenocarcinoma), PC12 (rat adrenal pheochromocytoma), PC3 (human prostate adenocarcinoma), and B16-F10 (mouse skin melanoma). Eight of the most potent extracts with strong cytotoxic activity were reported to be less cytotoxic to the normal cell line D551 as compared to vincristine and 5-flurouracil, the common drugs used in chemotherapy. This suggested that the extracts from the marine endophytic fungi were selective toward cancer cell lines but has less effect on normal cells, which is the main criterion in developing safe and effective anticancer drugs. Ariffin et al. (2014) made similar observations with extracts from endophyte S2 from the marine seaweed *Turbinaria conoides*. In the in vivo test using 12 healthy rats, no mortality or observable adverse effects was shown in the rats suggesting no toxic effects of the extracts. Instead, an increase in the total white blood cell count and hemoglobin levels in the rats was observed. These studies suggested that the marine endophytic fungi are potential sources of bioactive compounds with insignificant toxicity in vivo.

## 10.5 Biosourcing for Endophytes Producing Anticancer Compounds

The process of biosourcing of endophytes and their anticancer compounds begins with the plant selection. Plants that are selected for isolation of endophytes are usually from unique environments, have extraordinary strategies for survival, are endemic and having usual longevity, or have an ethnobotanical history (Strobel et al. 2004). Most of the endophytes from Malaysian medicinal plants mentioned earlier are isolated based on the methods proposed by Strobel et al. (2004), Hazalin et al. (2009, 2012), Ramasamy et al. (2010), Ariffin et al. (2014), and Ting (2014), with minor modifications. A standard protocol for endophyte isolation consists of the following steps: surface sterilization of plant materials using 70% of ethanol, removal of the outer tissues of the plants, excision of the inner tissue, and plating on the agar plates to allow the growth of endophytes. Hallmann et al. (2006) suggested the combinations of sterilizing agents to enhance the effectiveness of surface sterilization process. Pure cultures are subsequently established and mass cultured to obtain bioactive compounds for assays. The effects of the endophyte total crude extract on the viability of different cancer cell lines are evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This assay is a popular method used in preliminary screening of anticancer activities of a large number of samples.

For extracts with bioactivities, further investigation on the mode of action is performed. For example, cancer cell death caused by the apoptosis process can be studied using the “Cell Death Detection” ELISA assay. This assay applies quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies to measure the level of protein markers for apoptosis (Hazalin et al. 2012). The crude extracts showing significant cytotoxicity toward cancer cells are further fractionated, and the aliquots of the extracts are analyzed using high-performance liquid chromatography (HPLC). The aliquots or selected fractions are then tested against

different cancer cell lines, and a dereplication technique is used to examine the peak which exhibits high cytotoxic activity. The dereplication technique of bioactive compound works by comparing the relevance of HPLC-UV data collected from the significant peak from the discovered bioactive compound and the data in the in-house HPLC-UV library database from known fungal and bacterial metabolites. Further identification of compounds will only be carried out if there is no match in the data, thereby indicating that an unknown compound has been found. Identification of compounds, especially unknown compounds, can be carried out by using nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) to elucidate the structure of the bioactive compound. Novel compounds discovered warrants further investigation for its potential to be developed as the anticancer agents.

## 10.6 Malaysian Medicinal Plants: Potential Hosts for Endophytes Producing Anticancer Compounds

It is evident that endophytes from medicinal plants are potential source of anticancer agents. Nevertheless, the number of medicinal plants investigated to date is far from the many that exist in the tropics. There are many more medicinal plants, which have not been studied extensively. Thus there is tremendous potential for further exploration and development of the endophytes from these plants. Almost 10% of the 15,000 species of angiosperms in Malaysia show medicinal properties, reflecting a largely untapped source of endophytes producing valuable bioactive compounds (Batugal et al. 2004). Malaysia is also strategically located as the point of flora diversity, where continental Asiatic flora meets and intermingles in Malay Peninsula that give rise to a greater diversity of plant species (Rao 2010). Typically, the identification of plant species as a medicinal plant is based on the ethnobotanical knowledge of the species. Also, this grouping of medicinal plants is motivated by the knowledge and cultural use by various ethnic groups, ritual practice, location, and availability. Table 10.4 summarizes the common medicinal plants used for cancer treatments in Malaysia. These plants are well known for medicinal properties, but studies on their endophytes have not been fully understood. Therefore, there is a vast potential in developing anticancer agents if endophytes from these plants are isolated. The following section further discusses some of the important medicinal plants in Malaysia, their valuable compounds, and the prospect or potential of isolation of endophytes from these plants.

*Andrographis paniculata*, also known as "King of Bitter" or "hempedu bumi," is a plant of the Acanthaceae family, which has origin in India and Sri Lanka. *Andrographis paniculata* is an annual plant found in moist and shady places and can grow up to 30–110 cm. It is a medicinal plant well known in Asia for its therapeutic benefits such as boosting the immune system, treating infections in upper respiratory tract and gastrointestinal tract, and treating wounds, ulcers, fever, common cold, and hypertension (Wasman et al. 2011). Studies have established that major bioactive compounds of *A. paniculata* include andrographolide, neoandrographolide, and deoxyandrographolide, which are antimicrobial, antioxidant,

**Table 10.4** A summary of Malaysian anticancer plants characterized based on their ethnobotanical use

| Scientific name | Local name | Isolated phytochemicals | Responsive cancer cell lines | References |
|---|---|---|---|---|
| *Andrographis paniculata* | Hempedu bumi | Diterpenoid lactones | HeLa, HepG2, A549 | Kumar et al. (2004) |
| | | | | Zhou et al. (2010) |
| *Eurycoma longifolia* | Tongkat ali | Alkaloids, quassinoids, and diterpenoids | HepG2, MCF-7, A549, Kb | Kuo et al. (2003) |
| | | | | Bhat and Karim (2010) |
| | | | | Tee and Azimahtol (2005) |
| *Goniothalamus umbrosus* | Kenerak | Styryl lactones, acetogenins | Colon, breast, pancreas, kidney | Abdel-Wahab et al. (2009) |
| | | | Breast cancer cells (MCF-7) | Ghazali et al. (2016) |
| *Myrmecodia pendans* | Sarang semut | Terpenoids, phenolics, flavonoids | Ovarian cell (SKO-3) | Hasanuddin et al. (2015) |
| | | | Oral carcinoma KB cells | Yuletnawati et al. (2016) |
| | | | Human cervix cell (HeLa) | Soeksmanto et al. (2010) |
| *Strobilanthes crispus* | Pokok pecah kaca | – | T-47D | Yaacob et al. (2010) |
| | | | MDA-MB-321 | Muslim et al. (2010) |
| | | | MCF-7 | Bakar et al. (2006) |
| *Phyllanthus niruri* | Dukung anak | Terpenes | Liver tumor cell | Mills et al. (1995) |
| | | | Mice skin cancer | Sharma et al. (2009) |
| *Typhonium flagelliforme* | Keladi tikus | Linoleic acid, hexadecanoic acid, pheophorbide compounds | Leukemia WEHI-3 cells, | Mohan et al. (2010) |
| | | | Human T4-lymphoblastoid cell line (CEMss) | Mohan et al. (2011) |
| | | | Lung and breast cancer | Lai et al. (2010) |

"–" Information not found

antidiabetic, anticancer, and immunostimulatory (Rajagopal et al. 2003; Kumar et al. 2004; Chakraborty et al. 2011; Das and Srivastav 2014). Andropholides have strong cytotoxic activities as these are capable of inducing cell cycle arrest at G0/G1 phase through the induction of inhibitory protein p27, thus decreasing the expression of cyclin-dependent kinase (CDK4) (Rajagopal et al. 2003). Andropholides are

also able to initiate cell death signaling by activating the pro-apoptotic Bcl-2 proteins (Bid and Bax) from caspase 8 to mitochondria, resulting in apoptotic cell death (Zhou et al. 2006). Andropholides enhance chemosensitivity of cancer cells to the chemotherapy drug doxorubicin by suppressing the JAK-STAT3 pathway (Zhou et al. 2010). In natural forms, andropholides are diterpenoid lactones, which are the most prominent chemical constituent in *Andrographis paniculata.* It is a colorless crystal with a bitter taste, which was first isolated by Gorter back in 1911. To date, andropholides have demonstrated activity against an array of cancer cells: human cervical cancer cells, human hepatoma cells, human breast cancer cells, human alveolar cell carcinoma, human prostate cancer cells, human non-small cell lung cancer, human colon cancer, and human promyelocytic leukemia cells (Kumar et al. 2004; Zhou et al. 2006; Geethangili et al. 2008; Lee et al. 2010). In recent years, the chemical structures of andropholides have been modified to generate analogues and derivatives such as 14-acetlandrographolide and 3,19-isoproprylideneandrographol ide, which exhibit greater anticancer activity and selectivity against leukemia and colon cancer cells (Jada et al. 2007). As such, the endophytes from *A. paniculata* may have untapped potential to produce compounds that are similar in structure and bioactivity, to the andropholides. Endophytic bacteria found in *Andrographis paniculata* and *Bacillus* sp. were reported to show antimicrobial, plant growth-promoting, enzymatic, biodegrading, and biosurfactant properties, but the anticancer properties of the endophytes are yet to be discovered (Arunachalam and Gayathri 2010).

*Eurycoma longifolia* is also another well-known Malaysian medicinal plant. This plant is fondly known as "tongkat ali" or "Malaysian ginseng" in Malaysia due to the presence of long twisted roots. *Eurycoma longifolia* is a tall tree shrub belonging to the family of Simaroubaceae. The root extracts are primarily used to improve sexual performance and virility and to treat erectile dysfunction (Nasir et al. 2015). Secondary use of extracts from *E. longifolia* includes traditional treatment for fever, dysentery, and diarrhea, to reduce blood pressure and to help in the postpartum recovery. In recent years, *E. longifolia* have been found to have anticancer properties as well, with bioactivity toward several cancer cell lines such as A549 (human lung carcinoma), MCF-7 (human breast adenocarcinoma), Kb (human oral epidermal carcinoma), and Kb-V1 (human cervix carcinoma) (Kardono et al. 1990; Kuo et al. 2003). The isolated compounds are mainly β-carboline alkaloids, quassinoids, canthin-6-one alkaloids, biphenylneolignans, and squalene derivatives (Kardono et al. 1990; Tee and Azimahtol 2005; Bhat and Karim 2010). Tee et al. (2007) reported that the active fraction (F16) derived from *E. longifolia* was able to induce apoptosis in MCF-7 cells by reducing the production of Bcl-2 protein, leading to the specific proteolytic cleavage of polymerase, deterring the formation of DNA thus causing cell death in a cancerous cell. Study on endophytes from this plant is limited to Radu and Kqueen (2002) who discovered endophyte (b53L) with significant antitumor activities in the UCS and UCK bioassay. Wiyakrutta et al. (2004) also reported a number of endophyte isolates found in *E. longifolia* in Thailand, which showed cytotoxicity against KB and BC-1 (breast cancer) cell lines. However, only preliminary screening was carried out in both these studies, needing further validation of the effectiveness of the anticancer compounds produced.

*Goniothalamus umbrosus*, also known as "kenerak" or "sekulai putih," is another important medicinal plant in Malaysia. The plant is a member of the family Annonaceae and is easily recognized by its aromatic bark and fusiform leathery flowers. In the early days, the traditional use of roots of *G. umbrosus* has been primarily to induce abortion and for postpartum healthcare (Tantithanaporn et al. 2011). In recent years, the bioactive compounds (styryl lactones, acetogenins) were discovered to show significant cytotoxic activity toward cancerous cells (Ghazali et al. 2016). Abdel-Wahab et al. (2009) reported that ethyl acetate extracts of *G. umbrosus* induced cell death in MCF-7 breast cancer cells. In these cells, membrane blebs appear as a result of cytoskeleton break up from the plasma membrane, forming apoptotic bodies, and in addition to DNA condensation and fragmentation, resulting in apoptosis. Both styryl lactones and acetogenins were reported to exhibit anticancer, anti-inflammatory, antimalarial, immunosuppressive, and antioxidant properties (Ghazali et al. 2016). Examples of styryl lactones extracted from *Goniothalamus* sp. are goniothalamin, altholactone, and cardiopetalolactone. Goniothalamin activates apoptosis by causing a loss in mitochondrial transmembrane, resulting in the release of mitochondrial cytochrome C, therefore activating the caspases enzymatic cascades (Wiart 2007). Altholactone disrupts the mitochondrial respiratory and causes oxidative stress to the promyelocytic leukemia cells, leading to apoptosis of the leukemia cells (Wiart 2007). Lee et al. (2003) reported the styrylpyrone derivative extract (SPD) from *Goniothalamus* sp. significantly induced apoptosis in MCF-7 by modulating the levels of Bax protein, which is correlated with the level of apoptosis. It will therefore be interesting to isolate endophytes from this plant, which has an array of anticancer compounds. Hsieh et al. (2009) reported the discovery of two different endophytes, *Achromobacter xylosoxidans ss denitrificans* (Gs-01) and *Curtobacterium citreum* (Gr-01), from *Goniothalamus amuyon* in Taiwan which showed significant cytotoxicity toward HepG2 (human liver hepatocellular carcinoma), Hep3B (human hepatoma), A549 (human lung carcinoma), MCF7 (human breast adenocarcinoma), and MDA-MB-231(human breast adenocarcinoma) cell lines. Studies have yet to be carried out on isolating more potential anticancer agent producing endophytes from *G. umbrosus.*

Another interesting Malaysian medicinal plant is *Myrmecodia pendans*, or also known as the "ant plant" or "ant nest plant" or "sarang semut." It is named as such because it has domatia or nesting cavities which are specialized adapted hollow structures to host ant colonies in it (Hertiani et al. 2010; Engida et al. 2013). This plant originates from Papua Island from Eastern Indonesia and is commonly found as an epiphyte on tree branches and trunks of larger trees such as "cajuput" (*Melaleuca*), "cemara gunung" (*Casuarina*), and "kaha" (*Castanopsis*) (Hertiani et al. 2010). *M. pendans* is a myrmecophyte, plant which is associated with ant colonies and is a member of the family Rubiaceae. Among the *Myrmecodia* genus, only *M. pendans* and *M. tuberosa* exhibit medicinal properties (Hasanuddin et al. 2015). Their ethnobotanical uses include treatment for cold, nausea, burn, ascaris, hemorrhoids, and even cancer. Consumption is by boiling the dried cut tubers in water or as mixtures with tea or as a mixture with porridge (Hertiani et al. 2010; Yuletnawati

et al. 2016). *M. pendans* has been reported to be a potential anticancer agent due to its apoptotic and immunomodulatory effects. The presence of terpenoids, phenolics, and flavonoids were reported to be significantly cytotoxic against cancer cells, such as the ovarian cancer cell line SKO-3 in in vitro assays (Hasanuddin et al. 2015). In addition, ethanol extracts of *M. pendans* also showed significant antitumor activity in oral carcinoma KB cells (Yuletnawati et al. 2016). The antitumor activity of *M. pendans* was also evident against HeLa (human cervix epitheloid carcinoma) and MCM-B2 (canine mammary carcinoma) cell lines, despite the use of only water extract (Soeksmanto et al. 2010). Tarman (2015) discovered the antimicrobial properties of endophytic fungi (R53 and RS6B) isolated from *Hydnophytum formicarum*, epiphytic myrmecophytes found in Indonesia. Thus, the potential of endophytes isolated from *M. pendans* to develop anticancer agents is yet to be explored.

*Strobilanthes crispus*, also known as "pokok pecah kaca," "pokok pecah beling," or "jin batu" in Malaysia, is a shrub belonging to the Acanthaceae family. This plant originated in Madagascar then spread to Indonesia. The leaves of *S. crispus* is boiled in water and then consumed for the traditional treatment of cancer, diabetes, and blood pressure (Ghazali et al. 2016). The crude extract of *S. crispus* has significant cytotoxic effect against the human breast cancer cell lines. Further subfractions of the dichloromethane extracts from *S. crispus* showed higher cytotoxicity toward breast and prostate cancer cell lines compared to common chemotherapy drugs such as tamoxifen, paclitaxel, doxorubicin, and docetaxel (Yaacob et al. 2010). Nevertheless, in some cases, the subfractions of *S. crispus* extracts were synergistic with tamoxifen against MCF-7 and MDA-MB-231 human breast cancer cell lines. Apoptosis was induced by tamoxifen and further promoted by the subfraction extracts, leading to the activation of caspase-8 and caspase-9 intrinsic and extrinsic signaling pathways which led to apoptosis (Yaacob et al. 2010). Muslim et al. (2010) reported that the methanolic extracts of *S. crispus* were equally effective toward the human breast ductal carcinoma (T-47D) and breast carcinoma cells (MCF-7). The potency of the extract was somewhat diluted when administered in the form of tea. When consumed as tea, the cytotoxicity was only effective against hormone-dependent breast cancer cell lines (MCF-7) but ineffective against non-hormone dependent breast cancer cell lines (MDA-MB-231) (Bakar et al. 2006). This suggested that the effectiveness of medicinal plants for treatment purposes may be linked to the method of administration. Endophytes isolated from *S. crispus* with potential anticancer activity were mentioned in the earlier section of the chapter. Further studies can focus on discovering more endophytes from *S. crispus* with stronger cytotoxic activity.

*Phyllanthus niruri* also known as "gale of the wind" or "dukung anak" in Malaysia is a tropical herb from the family Phyllanthaceae. This plant has pale green flowers and tiny fruits. It is used traditionally by the Indian community to treat stomach, liver, kidney, and spleen diseases (jaundice). It has also been used in the traditional Chinese medicine (TCM) to treat gallstones and kidney stones. The extracts from *P. niruri* were reported to have antiviral properties (against hepatitis B), anti-inflammatory, antibacterial, antidiabetic, anticancer, and antihepatotoxic

activities (Bagalkotkar et al. 2006). The anticancer properties of *Phyllanthus niruri* are attributed to the terpenes (limonene) found in the plants. Limonene, or monoterpenes d-limonene, with the aid of perillyl alcohol, activates apoptosis in the tumor cells and inhibits the growth of tumor cells (Mills et al. 1995; Nasir et al. 2015). In a study using mice models, Sharma et al. (2009) discovered that the oral administration of the *P. niruri* extract reduced the incidence, yield, and burden of papilloma tumors in mice. Tang et al. (2014) also discovered that the anticancer properties of *Phyllanthus* sp. were credited to the ability of the plant extracts to disrupt various survival signaling pathways, such as MAPKs, P13K/Akt, and NFkB, and to interfere with the regulation of protein involved in the cellular function of tumors such as glycolysis, apoptosis, and metastasis. Kandasamy et al. (2015) reported the antioxidant and antibacterial properties of endophytic fungi isolated from *Phyllanthus amarus*. However, there is still room for further investigations on the anticancer properties of endophytes from *Phyllanthus* sp.

Another medicinal plant valued for its tubers is *Typhonium flagelliforme*, from the family Araceae. This plant is also known as the "rodent tuber" or "keladi tikus" and has long been used for various cancer treatments. Traditionally, the plant extract is obtained by crushing the plants and consuming the extract/juice (Ghazali et al. 2016). In a mice model, Mohan et al. (2010) tested the dichloromethane tuber extracts of *T. flagelliforme* and found that apoptogenic effects were achieved on the leukemia WEHI-3 cells in the in vitro and in vivo assays. Oral administration of dichloromethane tuber extracts significantly reduced the number of immature granulocytes and monocytes in peripheral blood of leukemia mice via the induction of apoptosis. Mohan et al. (2011) also tested the effect of one of the specific fraction (F7) on the human T4-lymphoblastoid cell line (CEMss), with encouraging results demonstrating significant cytotoxicity toward CEMss and selectivity toward noncancerous human primary blood lymphocytes (PBLs). The F7 fraction containing linoleic acid, hexadecanoic acid, and 9-hexadecanoic acid reportedly caused chromatin condensation, cell shrinkage, membrane blebbing, the formation of apoptotic bodies, and cytoplasmic extrusions, leading to cell death. In a separate study, Lai et al. (2010) reported the antiproliferative activity of a specific fraction (D/F19) of *T. flagelliforme* toward lung and breast cancer cells. This fraction has high activity due to the discovery of four pheophorbide-related compounds, which are antiproliferative. On the contrary, Choo et al. (2001) reported that the hexane extract of *T. flagelliforme* demonstrated weak cytotoxicity against murine leukemia cells P388. These observations from the various reports suggest that the solvents used for the extraction of the bioactive compounds may be important factors for consideration. The endophytes from this plant demonstrated antimicrobial and antioxidant properties (Saraswaty et al. 2013; Ling et al. 2014), but the anticancer activity from the endophytes is yet to be explored.

The Malaysian medicinal plants are therefore excellent reservoirs to isolate endophytes that possess anticancer activities. Medicinal plants with anticancer activities are favored for further studies with the aim to obtain endophytes capable of producing anticancer compounds similar to its respective host plants.

## 10.7 Conclusions and Future Prospects

Medicinal plants produce valuable secondary metabolites with potential as anticancer properties, with some of these products developed as clinical drugs. However, there are limitations in relying on plants as a sole source of anticancer agents as discussed earlier in the chapter. Production of anticancer compounds derived from endophytes is an alternative renewable source, more sustainable. Endophytes in medicinal plants have been shown to produce novel compounds or compounds similar to their host plants. These compounds have been studied and documented, although not extensively investigated. Therefore, there is an enormous potential to explore more endophytes from the diverse species of medicinal plants in Malaysia. Concerted research endeavors in this area will lead to the discovery of more anticancer agents.

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# Mushrooms: A Pandora Box of Cardioprotective Phytochemicals

11

Marthandam Asokan Shibu, Dinesh Chandra Agrawal, and Chih-Yang Huang

**Abstract**

Overwhelming consensus among medical authorities and scholarly bodies on the high susceptibility to chronic ailments such as coronary diseases, cancers, and diabetes and the failure to make any leap forward progress in controlling casualties or even to completely understand their pathology is a frightening reality. To comprehend alterations, additions, and management of diet is a preferable approach not only to prevent the occurrence of cardiovascular diseases but also to precise and enhance treatment measures. Proper application of potential drugs is possible only by establishing a systemic correlation and compilation of the knowledge obtained on the possible bioactive drugs. In this perspective gathering knowledge on the health-promoting potential of mushrooms which are considered as one of the promising sources of potential products that provide cardioprotection is indispensable. While there are several mushrooms traditionally utilized around the world for the treatment of cardiovascular diseases (CVD), they are also being cautiously evaluated experimentally for the available evidences of ethnopharmacology. Some therapeutic mushrooms have preclinical studies to demonstrate that uptake of these organic dietary supplements and their

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constituents as a therapeutic alternative or supplement is conceivable, and further evaluations are carried out to help in lessening the prevalence and mortality of CVD by incorporating them either as a population medicine or as a clinical medicine. A few examinations have demonstrated the effect of mushrooms and their bioactive compounds on metabolic markers such as low-density lipoprotein, high-density lipoprotein, total cholesterol, fasting triacylglycerol, and homocysteine levels and on conditions such as hypertension, body hemostasis, oxidative stress, and inflammation which are associated with cardiovascular ailments. The focus of this chapter will primarily be on mushrooms used traditionally for the treatment of CVD.

**Keywords**

Antioxidants•Cardiomyopathies•Cardiovasculardiseases•Hypercholesterolemia • Hypertension • Mushroom

## Contents

## Abbreviations

| | |
|---|---|
| ACE | Angiotensin I-converting enzyme |
| CVD | Cardiovascular diseases |
| *HDL* | High-density lipoprotein |
| LDL | Low-density lipoprotein |
| RAS | Renin-angiotensin system |
| STZ | Streptozotocin |
| VLDL | Low-density lipoprotein |

## 11.1 Introduction

According to the World Health Organization (WHO) report in the year 2010, the mortality rate due to coronary illness, cancers, diabetes, and obesity contributes to about 69% of the total 17.5 million recorded deaths worldwide. Cardiovascular diseases (CVD) are seen as the major contributor to the prevalence of high mortality rate in conditions such as diabetes, hypertension, aging, etc. Therefore, a large part of scientific infrastructure and resources are being utilized on cardiac research. The American Heart Association recommended to various authorities more than a 38.3 billion budget to pursue cardiac research and development during the financial year 2016.

Consumption of naturally available food and food products has long been advocated for healthy well-being. According to the International Life Science Institute, Europe, "A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease" (Roberfroid 2002).

Mushrooms are functional foods with a large number of beneficial bioactive metabolites providing various physiological effects (Abdullah et al. 2012; VanderMolen et al. 2017). Over the years, incorporation of mushrooms, for example, *Lentinus edodes*, *Agaricus bisporus*, and *Pleurotus* spp., in the diet has turned out to be prevalent worldwide (Ferreira et al. 2009; Patel and Goyal 2012; Witkowska et al. 2011). The consumption of mushrooms translates to incorporating a supply of high-fiber, low-fat substance with low unsaturated fats and low amount of sodium along with biologically active phytochemicals such as phenolics; sterols, for example, ergosterol and chitosan; triterpenes; etc. The focus of this chapter will be primarily on those mushrooms used traditionally to treat CVD that have some evidence to warrant clinical evaluations. Mushrooms provide high nutritional value to the diet and delivers proven beneficial effects over obesity, diabetes, and cardiovascular diseases (CVD) (Inoue et al. 2013; Rao et al. 2010; Weng and Yen 2010; Zhou et al. 2009). Therefore, mushrooms are considered as a promising source of naturally occurring therapeutics consequent of which enormous focus is laid on

understanding the pharmaceutical action and value of both edible and wild mushrooms (Wu et al. 2010b).

Mushrooms have long been an integral part of various traditional medicines. Hot water extracts of the fruiting bodies of mushrooms are considered to possess the medicinal properties (Boh et al. 2007; Chen et al. 2016). Cardiac diseases are a leading cause of death across the world. They have a multifactorial etiology arising from various functional disorders like hypertension, metabolic syndromes (diabetes, obesity), drug toxicity, aging, hypercholesterolemia, oxidative stress, etc. (Hobbs 2004). Various mushrooms that correspond to amelioration of such aetiological factors of CVD have been identified and their principle active metabolites explored in detail. Mushrooms such as *Lentinus edodes*, *Auricularia polytricha*, *Flammulina velutipes*, *Pleurotus ostreatus*, *and Agaricus bisporus* are proven hypocholesterolemic agents. Moreover, several mushrooms like *Pleurotus pulmonarius* and *Leucopaxillus tricolor* have also exhibited antihypertensive and blood pressure-lowering effects. Mushrooms with antioxidant and anti-inflammatory properties are appropriate agents that potentially provide protection to heart.

In the same line, mushrooms and their products that beneficially influence renin-angiotensin system, hdl cholesterol, apolipoprotein E, lipoproteins, lipoxidation end products, inflammatory cytokines, and chemokines are also largely considered for their therapeutic value (Ji et al. 1995). Most of these mushroom-based medicines have not been thoroughly investigated, and valuable scientific or clinical data supporting their therapeutic effects are scarce. With this approach of treating CVDs, a few mushrooms are considered as suitable dietary supplements with potential therapeutic value, viz., Chaga (*Inonotus obliquus*), Lion's Mane (*Hericium erinaceus*), Maitake (*Grifola frondosa*), Coriolus (*Trametes versicolor*), Cordyceps (*Cordyceps sinensis*), Agaricus blazei (*Agaricus subrufescens*), Reishi mushroom (*Ganoderma lucidum*), Shiitake (*Lentinula edodes*), and Agarikon (*Laricifomes officinalis*) (Chien et al. 2016; da Costa et al. 2015; Nguyen et al. 2017; Sturm et al. 2016; Wang et al. 2017). Further knowledge on the bioactive constituents are very essential in providing needed resources to fortify mushroom nutrigenomics and pharmacogenomics fields which would help in making the right judgment for the medicinal application of mushrooms.

## 11.2 Pathophysiology of CVD

Cardiomyocytes are highly differentiated matured cells, and they cease to multiply soon after birth of an individual; therefore, event of hyperplasia is not common although recent studies suggest the possibility of cardiomyocyte proliferation in adult heart in exceptional cases. Meanwhile it is well known that cardiomyocytes in the heart undergo adverse hypertrophy and cell death due to changes such as hemodynamic overload (hypertension) or in pathological conditions like chronic inflammation, oxidative stress, hyperglycemia, and hyperlipidemia. The apoptosis of cardiomyocytes and subsequent fibrosis in the heart affect the cardiac contractile function which may lead to heart failure.

Hypertension is a common pathological condition affecting heart and with an epidemic proportion affecting 15–20% of all adults with disorders such as arteriosclerosis, stroke, and myocardial infarction. The renin-angiotensin system (RAS) plays a major role in regulating blood pressure and a critical role in the pathophysiology of cardiovascular diseases including congestive heart failure and hypertension (De Mello and Danser 2000). Renin produces angiotensin-I from angiotensinogen that is cleaved by angiotensin I-converting enzyme (ACE) to form angiotensin II which is a potent vasoconstrictor. Increase in angiotensin II is associated with hypertension and cardiovascular damages. Therefore, inhibition of ACE activity yield major antihypertensive benefits.

## 11.3 Properties of Mushrooms in Defense Against CVDs

### 11.3.1 Antiatherosclerotic Effects

Atherosclerosis is a progressive disease condition associated with increasing accumulation of lipids and fibrous elements in the large blood vessels and is a major risk factor for CVD. In this respect, various edible mushrooms and their mechanisms of actions responsible for the antiatherosclerotic effect have been widely studied. Inflammation and oxidative stress are important critical phenomena causing pathological cardiac events associated with atherosclerosis. Many edible mushrooms are known anti-inflammatory dietary agents, and several active ingredients from mushrooms have been isolated for their anti-inflammatory effects (Barros et al. 2007; Ganeshpurkar et al. 2011). These mushrooms are ideal candidates to treat cardiovascular disorders (Ganeshpurkar et al. 2011). *Grifola frondosa*, *Hypsizygus marmoreus*, and *Pleurotus florida* are some of the potential mushrooms that exert anti-inflammatory effects. Also, *Agrocybe aegerita*, *Boletus edulis*, *Flammulina velutipes*, and *Pleurotus citrinopileatus* are some of the mushrooms having antioxidants with antiatherosclerotic properties.

### 11.3.2 Antioxidative Effects

Dietary intake of antioxidants is an effective strategy to counter the onset of various CVD conditions. Polysaccharides and phenolic compounds of mushrooms possess strong antioxidative properties. They effectively neutralize radical elements by enhancing the activity of oxidative enzymes such as catalase, glutathione peroxidase, and superoxide dismutase and by stabilizing glutathione and malondialdehyde levels (Kozarski et al. 2015; Witkowska et al. 2011). *Ganoderma lucidum*, *G. tsugae*, *Termitomyces heimii*, *T. mummiformis*, *Lentinula edodes*, and *Coriolus versicolor* are mushrooms known for their antioxidant properties. Modification of LDL by oxidation is one of the critical causes of atherogenesis. The antioxidant activity on lipid peroxidation of various commercial mushrooms including *L. edodes*, *F. velutipes*, *P. ostreatus*, *and V. volvacea* have been correlated with the phenolic

contents. In a clinical study, supplementing lyophilized powder of *P. ostreatus* to patients with dyslipidemia resulted in the increased activity of the antioxidant glutathione peroxidase demonstrating the efficient antioxidant effect of dietary intake of mushrooms (Kajaba et al. 2008).

### 11.3.3 Antihypertensive Effects

Hypertension or elevated blood pressure causes great stress and leads to adverse changes in the heart function. As mushrooms contain low concentration of sodium and high levels of potassium (182–395 mg/100 g), they are suitable dietary supplements to prevent hypertension effects. Various investigations reveal the antihypertensive effects of mushrooms such as *Lentinula edodes*, *Ganoderma lucidum*, *Pleurotus narbonensis*, and *Grifola frondosa* (Kabir et al. 1987; Lee et al. 2012). Mushrooms display diverse mechanism in providing antihypertensive effects (Table 11.1); however, ACE inhibition (Table 11.2) is one of the well-known effects of mushroom that contribute to cardioprotection from hypertension.

### 11.3.4 Anti-obesity Effects

Obesity is a widespread problem and it is estimated that more than one third of adults in the United States are obese (Feeney et al. 2014; Poddar et al. 2013). Obesity is often associated with hyperlipidemia and is also commonly related with improper diet and life style. Various mushrooms have shown efficient hypocholesterolemic effects. β-1,3-D-Glucan of mushrooms are known to interact with bile acids and affect micella aggregates that may interfere in the process of cholesterol absorption (Bobek et al. 1996). *Pleurotus ostreatus*, *Lentinus lepideus*, *Panellus serotinus*, and *Lentinus edodes* are some of the well-known mushrooms with anti-obesity effects (Bobek et al. 1996; Handayani et al. 2011; Yoon et al. 2011).

## 11.4 Major Constituents of Mushroom with Cardioprotective Potential

The most commonly cultivated mushroom is *Agaricus bisporus* followed by *Lentinus edodes*, *Pleurotus* spp., and *Flammulina velutipes*. China is one of the leading producers of mushrooms in the world (Aida et al. 2009; Valverde et al. 2015). However, wild mushrooms are attracting increasing interest for their nutritional and pharmacological characteristics (Barros et al. 2008; Kalac 2013).

Mushrooms are rich in carbohydrates and have a high content of proteins and significant levels of vitamins including vitamin B1, B2, B12, C, and D and minerals such as Ca, K, Mg, Na, P, Cu, Fe, Mn, and Se. The moisture content of mushrooms is generally high (81.8–94.8%). The crude proteins content of mushrooms varies from 15.2 g/100 g in *L. edodes* to 80.93 g/100 g dried weight in *A. bisporus.* Total

**Table 11.1** Antihypertensive mode of action and biocomponents from edible mushrooms

| Component | Mode of action | Source |
|---|---|---|
| Tripeptide 3,3,5,5-tetramethyl-4-piperidone (TMP) | Partial ganglionic blocking mediated | *Marasmius androsaceus* |
| | Vasodilation | *Tricholoma giganteum* |
| D-mannitol | Competitive inhibition of ACE | *Pleurotus cornucopiae* |
| Oligo peptides | Competitive inhibition of ACE | *Pleurotus cornucopiae* |
| Potassium | Hyperpolarization of Na + − pump and/or Kir channels | *Lentinula edodes* |
| Lentinan | Vasodilation | *Lentinula edodes* |
| Pentapeptide | Stereoselective and competitive | *Pholiota adiposa* |
| L-pipecolic acid | Inhibition of ACE | *Sarcodona spratus* |
| Hexapeptide | Inhibition of ACE | *Grifola frondosa* |
| Maleic/succinic acid derivatives, triterpenoids, benzenoids, and benzoquinone derivatives | Reduced the aggregation and phosphorylation of PKC in phorbol-12,13-dibutyrate (PDBu)-activated platelets | *Antrodia camphorata* Lu et al. (2014) |
| Oligo peptides, protein extract | Inhibition of ACE | *Agaricus bisporus* Lau et al. (2012) |

Adapted from Yahaya et al. 2014

**Table 11.2** ACE inhibitory activity of extracts of different mushrooms

| Species | ACE inhibitory activity (%) | Reference |
|---|---|---|
| *Leucopaxillus tricolor* | 95.0 | Geng et al. (2015 and Liu et al. 2013) |
| *Grifola frondosa* | 77.2 | Geng et al. (2015) |
| *Boletus bicolor* | 61.3 | Ibadallah et al. (2015) |
| *Tuber micheli* | 56.5 | Geng et al. (2015) |
| *Russula aeruginea* | 53.1 | Geng et al. (2015) |
| *Boletus edulis* | 47.2 | Abdullah et al. (2012) |
| *Morchella vulgaris* | 43.3 | Robati et al. (2014) |
| *Ramaria botrytoides* | 37.8 | Geng et al. (2015) |
| *Oudemansiella radicata* | 30.8 | Geng et al. (2015) |
| *Gloeostereum incarnatum* | 29.2 | Geng et al. (2015) |
| *Tricholoma matsutake* | 95.0 | Geng et al. (2016) |
| *Tricholoma terreum* | 35.3 | Geng et al. (2015) |
| *Tricholoma saponaceum* | 38.2 | Geng et al. (2016) |
| *Tricholoma giganteum* | 29.7 | Geng et al. (2016) |

protein contents (dried weight) account for 0.47 g and 1.29 g per 100 g in *F. velutipes* and *A. bisporus*, respectively.

A plethora of molecules from the mushrooms are known to have bioactive properties and are generally found in the fruiting bodies. Experimental evidences on laboratory and farm-grown mushrooms show high levels of polysaccharides, proteins, fats, minerals, glycosides, alkaloids, volatile oils, terpenoids, tocopherols, phenolics, flavonoids, carotenoids, folates, lectins, enzymes, ascorbic, and organic acids. A large number of bioactive substances, about 150 in *Pleurotus* and 400 in *Ganoderma*, have been studied so far. The bioactive components of mushrooms are potent immune modulators and have systemic effects in the body.

However, polysaccharides especially β-glucans in mushrooms are the most researched bioactive substances and are known for their immune-modulatory effects. With reference to their water solubility, dietary fibers are categorized as insoluble form (chitin, cellulose, lignin) and soluble form (β-glucans and chitosans). Polysaccharides also possess cardioprotective effects against obesity-associated cardiac disorders as they are shown to possess hyperlipidemic effects. The antioxidant properties of polysaccharides (Chen et al. 2015; He et al. 2012) may protect the heart from ischemia/reperfusion injury. Polysaccharides of edible mushrooms are composed of D-mannose, D-galactose and D-glucose, L-Arabinose, L-fucose, L-rhamnose, D-ribose, and D-xylose (He et al. 2012).

Apart from polysaccharides, nucleotides also constitute an important group of bioactive compounds in mushrooms. Adenosine is an important bioactive nucleotide with cardioprotective effects. Nucleotides are known for their vasodilation potential and thus can influence every organ in a systemic way. Also, nucleotides possess antiplatelet properties, enhance circulation, act as a potential muscle relaxant, and reduce stress.

In addition, mushrooms are rich in phenolic compounds particularly phenolic acids with antioxidant properties and act as efficient cardioprotectants. Some of the phenolic acids from mushroom with strong antioxidant activity include caffeic acid, *p*-hydroxybenzoic, gallic acid, protocatechuic acid, p-Coumaric acid, and cinnamic acid which have been proved to be efficient cardioprotective agents both in vitro and in vivo studies (Mattila et al. 2001).

Mushroom proteins are rich in glutamic acid, aspartic acid, and arginine. Uncommon bioactive amino acids such as γ-amino butyric acid (GABA) and ornithine also add up to the biological effects of mushroom proteins. Apart from the protein-associated nitrogen content, mushrooms possess high levels of nonprotein sources of nitrogen (Valverde et al. 2015). Common edible mushrooms contain high levels of proteins in their fruiting bodies (4.5% in *L. edodes*, 31% in *Agaricus blazei) (*Valverde et al. 2015*)*. The total amino acid content and essential amino acid contents were 93.6–230 and 39.7–86.8 g/kg, respectively, in 11 wild species. Methionine was the least abundant, while glutamic acid was the most abundant amino acid. Glutamic acid content was 37.6 g/kg dry weight in *Leucopaxillus giganteus* and 10.9 g/kg dry weight in *Cantharellus tubaeformis*. Total free amino acid content in wild species varied from 1.5 to 72 g/kg of dry weight depending on the mushroom species.

The vitamin and mineral contents in mushrooms are said to be higher than those found in most vegetables (Barros et al. 2008; Mattila et al. 2001). Mushrooms are composed of rich vitamin content such as vitamin D (ergosterol), vitamin B complex (riboflavin, niacin, biotic, folic acid), vitamin B12 (cobalamin) and B5 (pantothenic acid), and vitamin C (L-ascorbic acid), E, H (Biotin), K, and PP (Niacin) and have potent cardioprotective function (Hansen et al. 2015, 2017). The riboflavin contents in edible mushrooms are very high like those present in vegetables, and in some mushrooms like *Agaricus bisporus*, the contents are as high as found in eggs and cheese. Minerals including magnesium, potassium, manganese, sodium, iron, selenium, and zinc have been reported from mushrooms.

Edible mushrooms offer low quantities of dietary fat. Unsaturated fatty acids constitute the major content over the saturated fatty acids particularly palmitic acid, oleic acid, and linoleic acid. Linolenic acid is a precursor for 1-octen-3-ol, commonly identified as mushroom alcohol, a chief aromatic compound present in wide varieties of fungi and responsible for mushroom flavor (Pinho et al. 2008). Unsaturated fatty acids are very critical for cellular metabolism and normal functioning of the human body. The total fatty acid content in the edible fruiting bodies of *Cantharellus cibarius* has been estimated to be 3.6 g/100 g of dry weight. Contents of total lipids are low in wild varieties of mushrooms (between 20 and 30 g/kg dry weight). Some of the common fatty acids in wild mushrooms are linoleic acid, oleic acid, and palmitic acid although in reasonably low quantities (Kalac 2013).

## 11.5 Mushrooms for Reducing the Risk of CVD

### 11.5.1 *Lentinus edodes* (Shiitake)

High cholesterol level is one of the most critical risk factors for cardiovascular diseases. There is a strong correlation between hyperlipidemia and CVD associated mortality. *Lentinus edodes* (Fig. 11.1a), a macrofungus popularly known as shiitake, is used as a traditional medicine in Korea, Japan, and China for its blood cholesterol-lowering effects. According to a study rats fed with a dietary supplement containing 5% *L. edodes* fruiting bodies for 10 weeks showed a significant reduction in their plasma cholesterol levels (Kaneda and Tokuda 1966). In another study, *L. edodes* was found to be effective as an antioxidant at 100 and 400 mg/kg in rats. However, dosage higher than 400 mg/kg caused undesirable changes like reductions in hemoglobin and leukocyte content in the blood (Grotto et al. 2016). Administration of *L. edodes* in rat models caused reduction in the lipidemia-related factors such as cholesterol, HDL cholesterol, and non-HDL cholesterol and serum triglyceride. However, its effect on serum leptin concentrations was noted in females but not in males indicating its potential to modulate estrogen-associated serum leptin level (Shimizu et al. 1997; Tanaka et al. 2001; Yu et al. 2016). Water-soluble components of *L. edodes* containing polysaccharides were capable of inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase, a key enzyme in the endogenous cholesterol

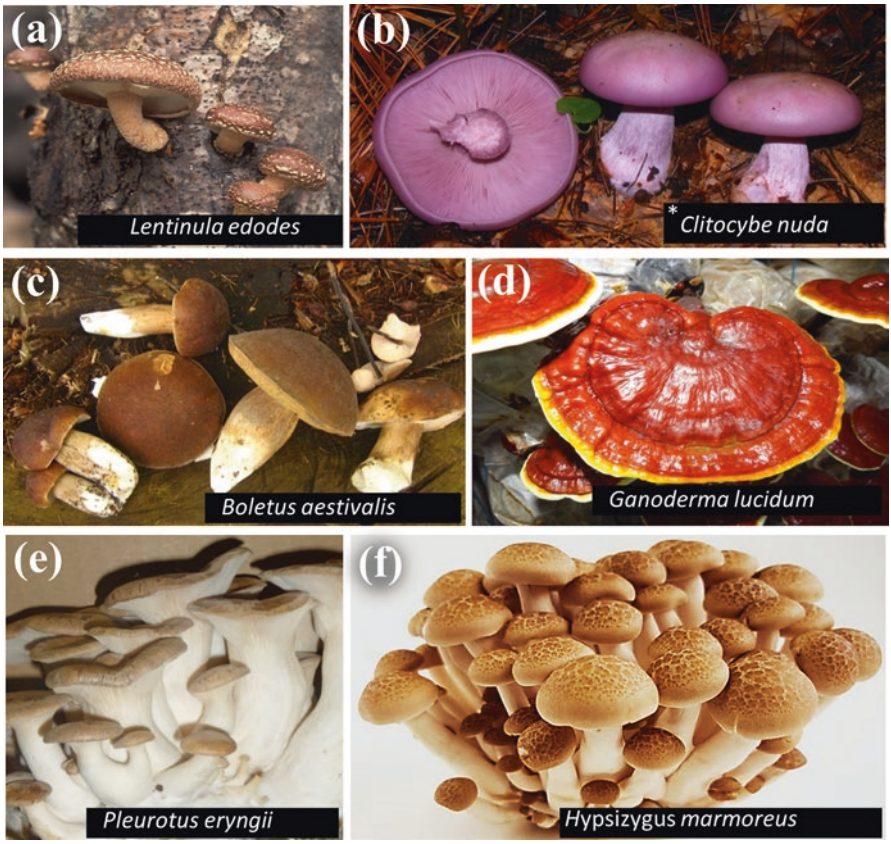


**Fig. 11.1** Fruiting bodies of mushrooms that potentially reduce the risk of CVD. (**a**) *Lintinula edodes*, (**b**) *Clitocybe nuda*, (**c**) *Boletus aestivalis*, (**d**) *Ganoderma lucidum*, (**e**) *Pleurotus eryngii*, (**f**) *Hypsizygus marmoreus* (*Attribution: Dan Molter (shroomydan) (https://commons.wikimedia.org/wiki/File:Clitocybe_nuda_60302.jpg), "*Clitocybe nuda* 60302", https://creativecommons.org/licenses/by-sa/3.0/legalcode

biosynthesis (Gil-Ramirez et al. 2016). In addition to the therapeutic effects in animal models, *L. edodes* also showed effective serum cholesterol-lowering effects in humans. Consumption of fresh *L. edodes* or its dried form or UV-irradiated dried samples reduced serum cholesterol within 7 days in younger as well as in adult women (Bisen et al. 2010).

Eritadenine (2(R), 3(R)-dihydroxy-4-(9-adenyl)-butyric acid) is a hypocholesterolemic factor isolated from *L. edodes* and is considered to be one of the major active components responsible for the cholesterol-lowering properties of this mushroom species (Chibata et al. 1969; Rokujo et al. 1970). The hyperlipidemic effects of eritadenine have been widely explored. It lowers the levels of both high-density and low-density lipoproteins. The cholesterol-reducing effect is not by inhibiting the cholesterol biosynthesis but possibly due to accelerating the cholesterol

excretion and decomposition. Dietary addition of eritadenine (0.005%) in rats resulted in a 25% reduction in total cholesterol within a week. It was found to be more effective in rats on high fat diet than in those on a low-fat diet. A study on humans also indicated a similar effect (Bisen et al. 2010).

### 11.5.2 *Clitocybe nuda*

These edible mushrooms (Fig. 11.1b) are commonly known as wood blewit or blue stalk and are found in Europe, North America, Asia, and Australia (Barros et al. 2008). Due to its distinct fragrance and delicacy, it is preferably cultivated in France, Holland, Britain, and Taiwan. Bioactive extracts of *C. nuda* are known to exhibit antioxidant properties (Murcia et al. 2002). The ethanol extracts of *C. nuda* contain 48.01 μg/mg pyrocatechol, and 8.21 μg/mg quercetin considered as efficient free radical scavengers, terminating the radical chain reactions occurring during the oxidation of triglyceride and account for the antioxidant properties (Mercan et al. 2006; Velioglu et al. 1998).

Drimane sesquiterpenoid such as 3-keto-drimenol, 3β-hydroxydrimenol, and 3β,11,12-trihydroxydrimene from *Clitocybe* has shown to inhibit two isozymes of 11 β-hydroxysteroid dehydrogenases (11 -βHSD1) that are oxidoreductases catalyzing the interconversion of active cortisol and inactive cortisone (Xu et al. 2009). Inhibitors of 11 -βHSD1 are known to have a potential treatment for metabolic syndrome associated with modulation in androgen and estrogen hormones that play a major role in regulating cell survival and protection. Treatment with *C. nuda* extracts has shown promising anti-obesity effects by significantly increasing GLUT4 levels and phospho-AMP-activated protein kinase (AMPK) in the skeletal muscle, adipose, and liver tissues as observed from STZ-induced diabetic mice models (Shih et al. 2014).

### 11.5.3 *Boletus aestivalis*

*Boletus aestivalis* (Fig. 11.1c) is a very popular mushroom species for its elegant aroma particularly in the Europe and used as an ingredient in traditional medicine. Mushrooms of *Boletus* sp. are known for their effects in regulating the blood flow and relieving muscle tension and possessing antioxidant and antitumor potential. Hot water extracts of these mushrooms have been shown to possess potential antihypertensive effects and reduced high heart rate as seen in hypertensive rats. Also, in a study by Midoh et al. (2013), hot water extracts of *B. aestivalis* decreased the levels of blood urea nitrogen, creatinine, and triglyceride and elevated the high-density lipoprotein-cholesterol levels in blood, suggesting that *B. aestivalis* is an excellent natural nutritional source to ameliorate hypertension-associated cardiac effects (Midoh et al. 2013). Acetone and methanol extracts of *B. aestivalis*, respectively, contain about 7 μg and 5 μg of pyrocatechol (equivalent/mg of phenolic compounds), and their corresponding flavonoid contents have been shown to be 3 μg and

2 μg of rutin (equivalent/mg of extract). The presence of these phenolics and flavonoids translates to strong antioxidant potential of the extracts and therefore could be the possible reason behind the cardioprotective function, although a direct correlation has not been established so far. Further, the methanol extracts *B. aestivalis* seems to be less cytotoxic than the acetone extract, a suitable trait for cardioprotective formulations (Kosanic et al. 2012).

### 11.5.4 *Ganoderma lucidum*

*Ganoderma lucidum* (Fig. 11.1d) is a medicinal fungus in the Polyporaceae family and is included extensively as an important constituent in various traditional Chinese medicine formulations. It is mostly cultivated as a medicinal mushroom rather as a dietary mushroom (Jong and Birmingham 1992). *G. lucidum* has been known to contain more than 300 bioactive ingredients including triterpenes, polysaccharides (e.g., β-glucan), ganoderic acids, proteins, peptides, steroids, and sterols to exert different pharmacological properties. Various compounds of the mushroom show promising hypolipidemic, hepatoprotective, antioxidative, antiatherosclerotic, and anti-inflammatory effects (Hajjaj et al. 2005; Pan et al. 2013; Wang et al. 2012). Hydroalcoholic extracts taken from basidiomata of *G. lucidum* are shown to reduce the serum total cholesterol levels in high cholesterol fed obese mice models. Various biologically active oxygenated lanostane-type triterpenoids identified from *G. lucidum* including ganoderal A, ganoderol B, ganoderic acid S, and ganoderic acid K display inhibitory effects on angiotensin-converting enzyme (Morigiwa et al. 1986). *G. lucidum* extracts also significantly reduce hepatic triglycerides and cholesterol and could be a potential hypocholesterolemic agent (Meneses et al. 2016). 26-oxygenosterols from *G. lucidum* inhibits lanosterol 14α-demethylase, which converts 24,25-dihydrolanosterol to cholesterol and thereby lowers blood cholesterol levels (Hajjaj et al. 2005). The hepatic lipid reduction properties of *G. lucidum* extracts are also driven by its ability to reduce key lipogenic genes *Srebp1c*, *Acaca*, and *Fasn*, thereby suppressing hepatic fatty acid synthesis and, hence, hepatic triglyceride accumulation (Meneses et al. 2016).

### 11.5.5 *Pleurotus eryngii*

*Pleurotus eryngii* (Fig. 11.1e) is an edible and therapeutic mushroom in the Pleurotaceae family of the phylum Basidiomycota. The species has shown various biological activities including antioxidant properties and hepatoprotection. Acidic- and alkalic-extractable mycelia zinc polysaccharides extracted from *P. eryngii* have demonstrated prevention of hyperlipidemic effects (Xu et al. 2017). The polysaccharides in the mushroom are known to increase serum HDL cholesterol levels and decrease LDL cholesterol, VLDL cholesterol, total cholesterol, and triglyceride levels and thereby provide positive effects in hyperglycemia and hyperlipidemia conditions (Chen et al. 2016; Xu et al. 2017). *P. eryngii* with its strong effects on

hyperlipidemic conditions has demonstrated a reduction in atherosclerosis formation in apolipoprotein E-deficient mice correlating with the reduced serum total cholesterol (Mori et al. 2008).

### 11.5.6 *Grifola frondosa*

*Grifola frondosa* is a well-known and widely consumed medicinal fungus found in Japan, European countries, and the northeastern states of America. Polysaccharides from the fruiting bodies of *G. frondosa* are known to possess antioxidative activities (Chen et al. 2012; He et al. 2017; Mao et al. 2014). *G. frondosa* also contain active ingredients such as D-(+)-trehalose and crude polysaccharides to counter diabetes and high blood sugar levels due to its inhibitory effects on α-glucosidase (Matsuura et al. 2002). Evidences point out that the antidiabetic effects of an α-glucan from *G. frondosa* are chiefly due to their effects on insulin receptors, which cause enhanced insulin sensitivity (Hong et al. 2007). *G. frondosa* SX-fraction with an average molecular weight of 20,000 is a glycoprotein containing a protein to saccharide ratio in the range of 75:25 to 90:10. The SX-fraction has shown to exhibit hypoglycemic activity in diabetic mice and in type 2 patients under clinical conditions. Also, SX-fraction is shown specifically targeting the insulin receptor and thereby triggering the subsequent signaling events and facilitate glucose uptake (Konno et al. 2013).

### 11.5.7 *Hypsizygus marmoreus*

*Hypsizygus marmoreus* (Fig. 11.1f) is an edible mushroom consumed in Korea, Japan, China, North Europe, and East Asia. Dietary supplement of *H. marmoreus* powder has been found to lower total serum cholesterol and deliver a strong antiatherosclerotic effect in mice (Mori et al. 2008). A purified novel 567.3 Da oligo peptide ACE inhibitor LSMGSASLSP from *H. marmoreus* has demonstrated a clear antihypertensive action in spontaneously hypertensive rat models (Kang et al. 2013).

## 11.6 Cardioprotective Mushrooms and Their Metabolites

### 11.6.1 *Pleurotus ostreatus*

The oyster mushroom *P. ostreatus* (Fig. 11.2a) is one the most popular and effective medicinal mushrooms used for protecting the heart. *P. ostreatus* helps protect from atherosclerosis and reduces cholesterol and diminishes the risk of heart diseases. An extract with 63% polysaccharide content from *P. ostreatus* was found to regulate dyslipidemia in hyperlipidemia rats and has proven antidiabetic effects in type 2 diabetes rat models (Zhang et al. 2016, 2017). *P. ostreatus* has abundance of


**Fig. 11.2** Fruiting bodies of mushrooms effective against CVD: (**a**) *Pleurotus ostreatus*, (**b**) *Auricularia auricular.* *Attribution Thomas Pruß (https://commons.wikimedia.org/wiki/File:Judasohr_(11).jpg), "Judasohr (11)", https://creativecommons.org/licenses/by-sa/3.0/legalcode

phenolic compounds such as protocatechuic acid (81 μg/g dry weight), followed by gallic acid (36 μg/g dry weight), chlorogenic acid (27 μg/g dry weight), formononetin (14 μg/g dry weight), naringenin (10 μg/g dry weight), hesperetin (10 μg/g dry weight), and biochanin A (10 μg/g). Alam et al. have reported phenolic contents of *P. ostreatus* showing antioxidant activity (Alam et al. 2010). Also, these act as anti-hyperlipidemic agents.

### 11.6.2 *Agaricus brasiliensis*

Polysaccharides produced by the edible and pharmacologically important mushroom *A. brasiliensis* (previously name: *A. blazei*) have attracted considerable interest primarily due to its antimutagenic effects (Borchers et al. 2004). Also, recent reports show that *A. brasiliensis* has been known to possess cardioprotective functions and anti-inflammatory activity. The extracts of *A. brasiliensis* are found to contain significant amounts of minerals such as calcium, zinc, iron, magnesium, and phosphorus and are effective against diabetes mellitus, atherosclerosis, and hyperlipidemia which are the major risk factors for CVD. Although consumption of *A. brasiliensis* in rats showed no morphological changes in the heart, it could significantly enhance the antioxidant enzyme super oxide dismutase and reduce the lipid peroxidation-associated oxidative damage marker malondialdehyde (Zhang et al. 2010).

Polysaccharides of *A. brasiliensis* have been identified to be the principle factors that carry out cardioprotective function against ischemia reperfusion. The effective cardioprotective fraction of the *A. brasiliensis* was majorly polysaccharides, typically heteropolysaccharides, and mainly of glucose, arabinose, and mannose in the molar percentages of 78.38%, 10.46%, and 8.51%, respectively (Zhang et al. 2010).

### 11.6.3 *Auricularia auricula*

The fruiting bodies of *A. auricula* (Fig. 11.2b) have long been used both as a food and as traditional medicine in China. The fruiting body of this mushroom is composed of high levels of polysaccharides, containing approximately 630 g $kg^{-1}$ carbohydrates (on dried weight basis). The total polysaccharide content is composed of glucose, 8% mannose, 10% xylose, and 10% fucose (Wu et al. 2010a). Also, fruiting bodies contain higher levels of proteins with Lys and Leu amino acids and minerals such as Ca, P, and Fe. Among the most mushrooms, polysaccharides of *A. auricula* are the most studied and found to be highly effective bioactive ingredients possessing antioxidant, anticoagulant hyperlipidemic, and antidiabetic activities (Chen et al. 2008; Fan et al. 2007; Hu et al. 2017; Luo et al. 2009).

The sulfation of acid *A. auricula* polysaccharides and the sulfation of neutral *A. auricula* polysaccharides derivatives possess considerable antioxidant activity.

Polysaccharides extracted from *A. auricula* evidently regulate serum triglycerides and LDL-C levels and enhance the antioxidant capacity in experimental animals (Chen et al. 2008). Administration of *A. auricula* polysaccharides acts as an effective natural antioxidant that safeguards cardiac function by maintaining the redox levels in the heart. Also, *A. auricula* polysaccharides enhance the ejection fraction (EF) and shot axis fractional shortening (FS) parameters of the left ventricles in aged mice models and therefore are efficient cardioprotective agents that improve heart function and retard the aging process (Wu et al. 2010a).

### 11.6.4 *Inonotus xeranticus*

*Inonotus xeranticus*, a mushroom that lives on deciduous trees such as *Quercus* species, is distributed in Korea, Japan, and China. Davallialactone is an active hispidin analog which is also found in several other mushroom species, e.g., *Davallia mariesii* and *Phellinus igniarius*. Davallialactone is known to possess antiplatelet aggregation activity, antioxidant activity, and free radical scavenging properties (Kim et al. 2008; Lee et al. 2008). Davallialactone has been reported to trigger anti-inflammatory effects by suppressing NfκB via PI3K, Akt, and IKK and independent of MAPKs. Administration of davallialactone strongly affects the LPS-induced phosphorylation and kinase activity of Src, thereby revealing that Src is involved in cardiac hypertrophic conditions (Takeishi et al. 2001). Davallialactone effectively attenuates Adriamycin-associated cardiac damages by maintaining the levels of antioxidant enzymes, protecting mitochondria from ROS effects, suppressing apoptosis effects, and thus restoring cardiac function (Arunachalam et al. 2012).

## 11.7 Active Ingredients and Their Cardioprotective Mechanism

### 11.7.1 Formononetin

As a major isoflavone compound in *P. ostreatus*, formononetin (Fig. 11.3) has exhibited a wide range of pharmacological properties such as anticancer, anti-inflammatory, antioxidant, anti-apoptosis, neuroprotection against ischemia/reperfusion injury, and wound healing (Auyeung et al. 2012; Huh et al. 2011; Jia et al. 2014; Jin et al. 2014; Liang et al. 2014; Ma et al. 2013; Sun et al. 2012). Formononetin protects cardiomyocytes from OGD/reoxygenation injury via inhibiting ROS formation and by promoting GSK-3 β phosphorylation and maintaining mitochondrial membrane permeability (Cheng et al. 2016). As a phytoestrogen, formononetin has been shown to reduce arterial stiffness and regulate blood pressure in overweight men and postmenopausal women (Nestel et al. 2007; Xing et al. 2010).

### 11.7.2 Gallic Acid

Gallic acid (Fig. 11.3b) is a metabolite of propyl gallate and is known to activate diverse pharmacological and biochemical effects including strong anticancer, antioxidant, and anti-inflammatory (Inoue et al. 1995; Kim et al. 2002; Kroes et al. 1992; Shahrzad et al. 2001).

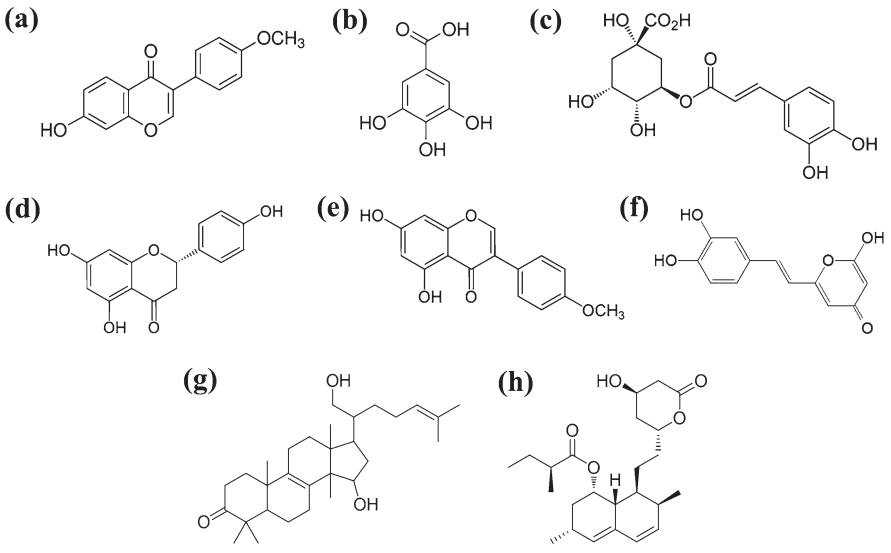


**Fig. 11.3** Chemical structures of compounds with cardioprotective mechanism. (**a**) Formononetin, (**b**) gallic acid, (**c**) chlorogenic acid, (**d**) naringenin, (**e**) biochanin A, (**f**) hispidin, (**g**) fomiroid A, (**h**) lovastatin

Gallic acid has been widely studied for its cardioprotective effects. It effectively lowers blood pressure and attenuates hypertension regardless of the administration route (IP or oral). Gallic acid administration for 3–7 weeks reduced hypertension-associated left-ventricle posterior wall, and septum thickness in chronic L-NAME induced hypertensive mice. Short-term or long-term treatment with gallic acid also attenuates cardiac fibrosis and reduces the levels of histone deacetylase (HDAC) 1 and HDAC 2 in H9c2 cells in rat primary cardiac fibroblasts and as well as in animal models (Jin et al. 2017).

Gallic acid also reduces the DNA-binding ability of phosphorylated Smad3 to Smad binding sites of collagen type I promoter and attenuates cardiac fibroblasts in rats. Further, it also decreases the isoproterenol-induced phosphorylation of c-Jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) protein in mice (Ryu et al. 2016). It was found that gallic acid pretreatment decreased isoproterenol-induced changes in the levels of cardiac marker enzymes such as creatine kinase, aspartate transaminase, alanine transaminase, and lactate dehydrogenase indicating a protective effect against cardiac injury. Also, it elevated the levels of enzymatic and nonenzymatic antioxidants (Priscilla and Prince 2009).

### 11.7.3 Chlorogenic Acid

Chlorogenic acid (Fig. 11.3c) is an ester of caffeic and quinic acids and is considered as one of the most abundant polyphenol compounds in human diet with proven biological effects determined by in vitro and in vivo investigations (Suzuki et al. 2002). Chlorogenic acid exhibits various pharmacological properties such as anticancer, antioxidant, and antihypertensive in humans (Kozuma et al. 2005; Laranjinha et al. 1994; Morishita et al. 1997; Rodriguez de Sotillo and Hadley 2002; Suzuki et al. 2002). Administration of 5-caffeoylquinic acid, a representative of chlorogenic acid, in spontaneously hypertensive rats reduced oxidative stress and improved nitric oxide bioavailability by inhibition of excessive production of reactive oxygen species (ROS) in the vasculature and attenuated endothelial dysfunction, vascular hypertrophy, and hypertension (Suzuki et al. 2006). Administration of chlorogenic acid at a concentration of 40 mg/kg body weight for 19 days effectively ameliorated ISO-induced alterations in cardiac functional parameters and noticeably restored the activities of heart mitochondrial enzymes in ISO-induced rats and reduced the stress in the heart (Akila et al. 2017).

### 11.7.4 Naringenin

Naringenin (Fig. 11.3d) is one of the naturally occurring bioflavonoids with antioxidant, anti-inflammatory, and anticancer properties (Banjerdpongchai et al. 2016). Naringenin regulates the antioxidant enzyme system and controls successive lipid peroxidation to ameliorate doxorubicin induced toxicity and hypoxic stress (Kathiresan et al. 2016). Also, naringenin attenuates the mRNA expression levels of

critical inflammatory markers induced by doxorubicin, alleviates cardiac damage, and improves cardiac functions.

### 11.7.5 Biochanin A

Biochanin A (Fig. 11.3e) possesses a wide range of bioactivities and multiple mechanisms to protect against diabetes, cancer, inflammation. It is a powerful radical scavenger in the presence of a transition metal ion. Biochanin A reverts arsenic challenge caused by oxidative stress, triglyceride, and lipoprotein levels in cardiac tissues. Thereby, it effectively establishes protective effect against arsenic induced cardiotoxicity (Jalaludeen et al. 2015). Biochanin A triggers the relaxation in aortic rings in normal and hypertensive rats by acting on ATP-sensitive potassium channels (Wang et al. 2006).

### 11.7.6 Hispidin

Hispidin (Fig. 11.3f), a phenolic compound isolated from *Phellinus linteus*, has been found to possess effective antioxidant, anticancer, antidiabetic, and antidementia properties. Hispidin is a PKC inhibitor, thereby a potential cardioprotective agent. Hispidin has been shown to protect H9c2 cardiomyoblast cells against $H_2O_2$-induced oxidative stress-associated apoptosis via Akt/GSK-3β and ERK1/2signaling pathways. Hispidin effectively inhibits the intracellular ROS generation by elevating the antioxidant enzymes such as heme oxygenase, catalase, and superoxide dismutase (Kim et al. 2014; Yang et al. 2014).

### 11.7.7 Fomiroid A

Fomiroid A (Fig. 11.3g) has been identified as an active principle of *F. nigra* contributing its hypercholesterolemia property. Fomiroid A, a structural analog of lanosterol (a precursor of cholesterol biosynthesis), directly binds to NPC1L1 which is a key protein in cholesterol absorption. Fomiroid A effectively inhibits NPC1L1-mediated cholesterol uptake via direct interaction with NPC1L1 (Chiba et al. 2014).

### 11.7.8 Lovastatin

Lovastatin (Fig. 11.3h) is a naturally occurring statin drug administered to those with hypercholesterolemia for its cholesterol-lowering effect and thus reduced cardiovascular disease. Lovastatin is found in mushrooms such as *Pleurotus ostreatus* as a phyto-complex, and, therefore, naturally occurring lovastatin does not exhibit the side effects observed in synthetic statins. Mushroom-derived statins contain other ingredients as a complex to mitigate the side effects (Alarcon and Aguila

2006). They act by competitive interaction and inhibition of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, an enzyme of the cholesterol production. Lovastatin effectively prevents angiotensin II-induced cardiac hypertrophy by enhancing p21ras/MAP kinase pathway (Oi et al. 1999). Lipid-lowering effect of lovastatin helped in improving the LV systolic function and decreased myocardial ischemia in patients with coronary artery disease (Kerimkulova et al. 2011).

## 11.8 Conclusions

Drastic changes in the environment and lifestyle-associated effects on healthy life of humans have resulted in the search for various functional foods to acquire desired protective effects. A diverse variety of mushrooms and their constituents have been explored for their distinct flavor and taste for years and have been supplemented in the diet for its therapeutic potential. Mushrooms offer a complete source of nourishment and dietary bioactive elements apart from being rich in protein, dietary fiber, vitamins, and mineral substances. Mushrooms are a Pandora box with a massive number of bioactive components. However, the treasure trove mushrooms offer need to be explored extensively. Identification of bioactive metabolites in mushrooms is a key component to develop its therapeutic values. Mushrooms have shown extraordinary potential to counter or to treat cardiac diseases. However, progresses in development of commercially viable therapeutic drugs are still wanting. Therefore, concerted efforts are needed for further exploration and documentation of potential bioactive ingredients from medicinal mushrooms, its merits and demerits, and finally development of commercially viable drugs/products.

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# Part III

# Production Systems and Biotechnology: Medicinal Plants and Fungi

# Solid-State Fermentation of Plant Residues and Agro-industrial Wastes for the Production of Medicinal Mushrooms

12

Georgios I. Zervakis and Georgios Koutrotsios

**Abstract**

The environmentally acceptable disposal of agricultural wastes and residues constitutes a major scientific challenge, especially when their chemical properties, recalcitrance, and abundance are taken into account. The use of macrofungi, which grow in nature as wood or litter decomposers and excrete nonspecific oxidative enzymes to degrade lignocellulosics, seems to offer solutions that could be widely and readily applied for the biotransformation of such materials. More importantly still, these organisms demonstrate efficient bioconversion of various types of agro-industrial/forestry by-products with low or no economic value to edible biomass. The process involves controlled solid-state fermentation which is optimized for the production of culinary and medicinal mushrooms, thus providing food of high organoleptic and dietetic value. In addition, their content in bioactive compounds related with anticarcinogenic, antidiabetic, antihypertensive, anti-inflammatory, immunostimulating, and other health-beneficial properties has repeatedly been demonstrated. In this chapter, the enzymatic mechanisms behind lignocellulose degradation by fungi are summarized, and several of the most important cultivated mushrooms are presented with respect to their production requirements and medicinal properties.

**Keywords**

Bioactive compound • Bioconversion • Biodegradation • Lignocellulosic • Nutraceutical • White-rot fungi

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## Contents



## Abbreviations

| | |
|---|---|
| BL | Banana leaves |
| BP | Bracts of pineapple crown |
| BS | Broadleaf sawdust |
| BST | Barley straw |
| BWC | Broadleaf wood chip |
| c | Composted |
| CB | Corn powder |
| CC | Corn cobs |
| CH | Coffee husk |
| CP | Coffee pulp |
| CS | Corn stover |
| CSG | Coffee spent ground |
| CSH | Cotton seed hulls |
| CW | Cotton waste |
| EFB | Palm empty fruit bunches |
| GM | Grape marc |
| HH | Hazelnut husks |
| HR | Herbs residue |
| NS | Nut shells |
| OL | Olive leaves |
| P | Paper |
| PN | Pine needles |
| PPF | Palm pressed fiber |

| | |
|---|---|
| PS | Paddy straw |
| RS | Rice straw |
| SB | Sugarcane bagasse |
| SL | Sugarcane leaves |
| SSH | Sunflower seed hulls |
| TPOMW | Two-phase olive mill waste |
| VP | Vineyard pruning |
| WB | Wheat bran |
| WP | Waste paper |
| WPS | Weed plants |
| WS | Wheat straw |

## 12.1 Introduction

Kingdom *Fungi* comprises eukaryotic, heterotrophic microorganisms with absorptive nutrition. They secrete oxidative exoenzymes to degrade their growth substrates, and nutrients are absorbed in soluble form by an extended network of branched hyphae (thread-like tubular structures demonstrating apical growth) which form the mycelium. Fungi demonstrate a large variety of life cycles, modes of sexual/asexual reproduction, and ecological preferences.

At present, ca. 100,000 fungal species are recorded and account for no more than 7% of the estimated total of 1.5 M species (Hawksworth 2001). The phylum *Basidiomycota* comprises about one-third of all described fungi (Stajich et al. 2009); more particularly, *Agaricomycotina* constitutes a diverse clade including 20,000 species (Hibbett 2006), the great majority of which could be described as macrofungi. This term is widely used for fungi forming macroscopically visible reproductive structures (i.e., organs where spores are formed and liberated from) or mushrooms. Although most macrofungi are classified in *Basidiomycota*, some belong to the phylum *Ascomycota*.

The larger part of macrofungi exhibits a saprotrophic lifestyle (as opposed to those demonstrating symbiotic or parasitic ecological preferences), which means that they rely on decomposing animal and plant residues for obtaining nutrients. Therefore, these particular organisms constitute the major agents of degradation and recycling of dead organic matter in nature. The ability of such wood- and litter-decaying microorganisms in converting complex lignocellulosics (the basic structural material of plant cell walls) into simple organic compounds has been widely exploited in numerous biotechnological applications (Harms et al. 2011).

## 12.2 The Enzymatic Arsenal of Macrofungi in the Degradation of Lignocellulosics and Related Organic Compounds

Huge quantities of lignocellulosics are produced every year in nature; it is estimated that the quantity of terrestrial biomass generated annually is $200 \times 10^{12}$ kg (Foust et al. 2008). Lignocellulosics are particularly resistant to microbial degradation because of the complex structure by which polymers of cellulose, hemicellulose, and lignin are bound together. Cellulose forms straight and tightly packed chains of microfibrils composed of β-d-glucose units and linked by H-bonds, whereas hemicelluloses are arranged around the rigid cellulose core, and they consist of various pentoses and hexoses (e.g. xylan, arabinose, glucomannan). In plant cell walls, both cellulose and hemicelluloses are surrounded by a layer of lignin which is particularly recalcitrant due to the existence of three aromatic alcohols known as monolignols (i.e., p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) forming the phenylpropanoid p-hydroxyphenyl, guaiacyl, and syringyl units linked by ether and carbon to carbon bonds (Camarero et al. 2014).

As previously stated, saprotrophic basidiomycetes are among the primary factors of decomposition and recycling of plant debris in nature, and hence they rely heavily on the production of cellulolytic and ligninolytic enzymes for degrading their growth substrates. The former category of enzymes includes endoglucanases and exocellulases (cellobiohydrolases), which decompose cellulose to cellobiose or cello-oligosaccharides that are further processed by β-glucosidases or by cellobiose dehydrogenase. Endo-1,4-β-glucanase (EC 3.2.1.4, endocellulase) is common among *Basidiomycota*, notably in both white and brown rot fungi and in litter-decomposing species (Cohen et al. 2005; Martinez et al. 2009; Steffen et al. 2007). Multiple endoglucanases are generated by fungi; they are monomeric structures, which demonstrate catalytic optima at pH 4.0–5.0, and their activity is mainly oriented toward amorphous regions in the cellulose molecule (Baldrian and Valášková 2008). Cellobiohydrolase (CBH, EC 3.2.1.91; exocellulase) is mainly present in white-rot and litter-decomposing fungi and is typically active on crystalline cellulose, Avicel (Baldrian and Valášková 2008; Rytioja et al. 2014). On the other hand, β-glucosidase (EC 3.2.1.21) is produced by numerous macrofungi and demonstrates high structural variability; it is relatively nonspecific (it cleaves other simple carbohydrates as well), and although it usually attacks cello-oligosaccharides, it is inactive on crystalline cellulose and shows low activity on amorphous cellulose (Baldrian and Valášková 2008). Last, cellobiose dehydrogenase (CDH; EC 1.1.99.18) is a typical oxidoreductase which oxidizes cellobiose and higher cellodextrins by employing a wide range of electron acceptors including quinones, phenoxyradicals, cytochrome c, etc. (Zamocky et al. 2006). CDH is produced mainly by white-rot fungi under cellulolytic conditions together with cellulases and hemicellulases; although it binds specifically to cellulose, it could also degrade hemicelluloses and lignin in the presence of iron and hydrogen peroxide (Henriksson et al. 1997).

The ligninolytic enzymes are mainly produced by wood-rot (notably white-rot) fungi and some related litter-decomposing basidiomycetes which demonstrate

efficient lignin degradation (Hatakka 2001). These enzymes are largely nonspecific and include phenol oxidases (laccase), heme peroxidases (i.e., lignin peroxidase, manganese peroxidase, and versatile peroxidase), as well as several other accessory enzymes (e.g., veratryl alcohol oxidase and glyoxal oxidase), which oxidize the heterogeneous polymeric substrates through multistep electron transfers accompanied by the formation of intermediate cation radicals (Hatakka and Hammel 2011). In particular, lignin peroxidases (LiPs, EC 1.11.1.14) catalyze the oxidation of nonphenolic lignin substructures (in the presence of hydrogen peroxide) to produce aryl cation radicals, which are further degraded/mineralized by nonenzymatic means (Kirk and Farrell 1987). Manganese peroxidase (MnP, EC 1.11.1.13) oxidizes bivalent Mn ($Mn^{2+)}$ to trivalent Mn ($Mn^{3+}$) and the latter catalyzes the oxidation of phenolic lignin moieties leading to their decomposition (Gold et al. 2000). Laccases (Lac, EC 1.10.3.2) are multicopper oxidases catalyzing the conversion of phenolic rings to phenoxy radicals in a reaction without the presence of $H_2O_2$ (Baldrian 2006). In addition, versatile peroxidase (VP, EC 1.11.1.16) can oxidize aromatic compounds and $Mn^{2+}$, while its catalytic mechanism is similar to LiP; VPs have been detected in a few macrofungi, including species of *Bjerkandera* and *Pleurotus* (Ruiz-Dueñas and Martínez 2009). Last, the $H_2O_2$ needed for supporting oxidative reactions during ligninolysis in the presence of LiP and MnP is provided through the action of extracellular oxidases. As such, glyoxal oxidase (GLOX) and aryl alcohol oxidases (AAOs) reduce $O_2$ to $H_2O_2$ by oxidizing the co-substrates (Hammel et al. 1994).

## 12.3 Mushroom Cultivation on Lignocellulosic Residues and Agro-industrial By-Products

A vast amount of plant residues and agro-industrial by-products remains largely unexploited. Only in the European Union, more than 80 million tons of wheat straw and corn cobs are produced annually (data from http://faostat.fao.org, and http://www.indexmundi.com/agriculture), in addition to several other by-products of similar origin, e.g., various grasses, leaves, tree pruning, wood bark and sawdust, cotton gin trash, almond, chestnut and walnut shells, grape marc, olive mill waste, etc. The composition of many such materials, which are rich in lignocellulosics and are already in use or demonstrate a promising potential to be used as growth substrates for macrofungi, is presented in Table 12.1.

Cultivation of mushrooms constitutes a noteworthy case of sustainable exploitation of various lignocellulosics through a controlled solid-state fermentation process which converts them to edible biomass. Such bioconversions are based on well-established methodologies involving discrete phases of substrate preparation (including wetting, mixing, and, often, composting of ingredients), pasteurization, spawning, incubation (spawn run), and mushroom induction, production, and harvest. Optimal environmental conditions for fruit-body primordia formation, minimum crop length, maximum yields, and product quality are under continuous investigation as is the selection of most suitable substrates for each species.

**Table 12.1** Content (%, dry weight) in key compounds and elements of raw materials most commonly used in mushroom cultivation substrates

| Material | Hemicellulose | Cellulose | Lignin | C | N | C/N | Ash | References |
|---|---|---|---|---|---|---|---|---|
| Wheat straw | 21.2–35.5 | 29–50 | 5.6–21.0 | 36.4–46.7 | 0.40–1.48 | 48.8–86.0 | 5.6–11.0 | Chandra et al. (2012), Hills and Roberts (1981), Menon and Rao 2012, Saini et al. (2015), nee'Nigam et al. (2009), Philippoussis (2009), Sánchez (2009), and Ward and nee'Nigam (2009) |
| Rice straw | 17.7–28.6 | 22.8–47.0 | 6.4–24.0 | 33.6–41.8 | 0.39–1.15 | 74.2 | 8.37–17.80 | Chandra et al. (2012), Menon and Rao (2012), Obodai et al. (2003), nee'Nigam et al. (2009), Philippoussis (2009), Richard et al. (1996), and Saini et al. (2015) |
| Corn cob | 31.9–43.0 | 28.0–45.6 | 6.1–17.0 | 57.1 | 0.48–1.10 | 64.2–72.0 | 1.36–4.80 | Anwar et al. (2014), Carneiro et al. (2013), Menon and Rao (2012), nee'Nigam et al. (2009), Philippoussis (2009), Sánchez (2009), Richard et al. (1996), and Zych (2008) |
| Cotton stalk | 11.0–14.4 | 31.0–58.5 | 21.5–30.0 | 41.23 | 2.63 | 15.67 | 13.3 | Ioannidou and Zabaniotou (2007), Menon and Rao (2012), and nee'Nigam et al. (2009) |
| Cotton hulls | 5–22 | 49–90 | 4–24 | 47.37 | 0.3–1.4 | 40–59 | 2.6–8.4 | Adenipekun and Dada (2013), Philippoussis (2009), Richard et al. (1996), and Ward and nee'Nigam (2009) |
| Coffee pulp/husks | 15.1–47.5 | 23.0–36.9 | 13.0–26.0 | 50.8 | 0.9–1.9 | 40.02–59.40 | 1.0–6.3 | Dzung et al. (2013), Menon and Rao (2012), Philippoussis (2009), and Sánchez (2009) |
| Sugarcane bagasse | 19.0–37.5 | 25–45 | 10–25 | 65.20 | 0.2–0.8 | 120–190 | 1.5–9 | Anwar et al. (2014), Guan et al. (2013), Menon and Rao (2012), nee'Nigam et al. (2009), Philippoussis (2009), Saini et al. (2015), and Sánchez (2009) |
| Hardwood | 22–40 | 40–55 | 18–26 | 45–50 | 0.1–0.2 | 150–450 | 0.2–0.3 | Anwar et al. (2014) and Philippoussis (2009) |
| Softwood | 10.7–35.0 | 37.5–50.0 | 25–35 | 45–50 | 0.1 | 310–520 | 0.4–0.5 | Anwar et al. (2014) and Philippoussis (2009) |
| Grasses | 13–50 | 25–40 | 6.4–30.0 | 40.8 | 0.81–3.25 | 11.8–42.0 | 4.2–8.5 | Hills and Roberts (1981), Menon and Rao (2012), Philippoussis (2009), and Taylor et al. (1989) |
| Nut shells | 22–30 | 25–30 | 30–40 | 60–62 | 0.60–0.75 | 39.2 | 1.6 | Koutrotsios et al. (2014), Menon and Rao (2012), and Sánchez (2009) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sunflower stalks | 29.66 | 42.1 | 13.44 | 52.9 | 1.38 | 38.3 | 3 | Ioannidou and Zabaniotou (2007) and nee'Nigam et al. (2009) |
| Sunflower seed hulls | 24.0–28.6 | 24.1–49.5 | 19.0–29.5 | 42 | 0.6–0.9 | 60.0–72.4 | 3.0–3.3 | nee'Nigam et al. (2009) and Philippoussis (2009) |
| Two-phase olive mill waste | 6.6 | 14.54 | 8.54 | 49 | 1.31 | 37.7 | 1.4–4.0 | Dermeche et al. (2013)[a] |
| Olive leaves | 5.4 | 8.5 | 39.8 | 47.5 | 1.3 | 35.9 | 14.2 | Garcia-Maraver et al. (2013) |
| Banana leaves | 14.8–21.8 | 13.2–29.5 | 14–15.7 | 44.4–47.8 | 0.94 | 23–43 | 14.2 | Anwar et al. (2014) and Obodai et al. (2003) |
| Vineyard pruning | 17.0–21.0 | 34.0–60.8 | 20.0–22.9 | 47.6 | 1.8 | 26.44 | 3.8 | Ioannidou and Zabaniotou (2007) and Philippoussis (2009) |
| Grape marc | 10.3 | 14.5 | 17.2 | 46.1 | 1.49 | 31.04 | 4.65 | Bayrak (2013) |
| Wheat bran | 35.5–39.2 | 10.5–14.8 | 3.0–12.5 | 60.3 | 2.02–2.74 | 29.8 | 7 | Carneiro et al. (2013), Menon and Rao (2012) and Richard et al. (1996) |
| Rice bran | 11 | 10.7 | 3.3 | 48.3 | 2.08–2.44 | 20 | 8.1–10.0 | Nakagawa et al. (2003) |
| Rice husk | 17.4–29.3 | 28.0–43.0 | 18.3–22.5 | 32.9–44.6 | 0.30–0.76 | 32.8–136.0 | 14.6–21.4 | Chandra et al. (2012), Hills and Roberts (1981) and Nakagawa et al. (2003) |
| Poultry manure | 87.8 | 10.4 | 1.4–3.4 | 26.47 | 4.51–6.87 | 4.4–13.0 | 0.4 | Bernal et al. (2009), Hills and Roberts (1981) and Richard et al. (1996) |

[a]Koutrotsios and Zervakis, unpublished results

Currently, the global mushroom production is estimated at about 27 million tons presenting a 25-fold increase during the last 40 years (Royse 2014); it is estimated that ca. two-thirds of this quantity originates from China. In parallel, per capita consumption of mushrooms has quadrupled from 1997, exceeding nowadays 4 kg per person. Five fungal genera represent 85% of the world's total mushroom production (Royse 2014), i.e., *Agaricus* (mainly *A. bisporus* and substantially lower quantities of *A. brasiliensis*) with almost one-third of totally cultivated mushrooms, *Pleurotus* (principally *P. ostreatus*, but also *P. eryngii*, *P. pulmonarius*, *P. citrinopileatus*, and *P. djamor*) with about 27%, and *Lentinula edodes* at ca. 17% of the world's output. *Auricularia* and *Flammulina* rank fourth and fifth with 6% and 5% of the total volume, respectively. Other cultivated mushroom species with notable medicinal properties are *Ganoderma lucidum* and *Trametes versicolor*, while *Pholiota nameko*, *Hericium erinaceus*, *Grifola frondosa*, *Tremella fuciformis*, *Volvariella volvacea*, and *Cyclocybe cylindracea* are popular edible species in many regions of the world.

In general, mushrooms are a high-protein and low-calorie food which also contains metals, vitamins, and important bioactive compounds. Their water content is high (commonly ranging from 86 to 92%), while their average content in total proteins, total fats, and ash are 250, 30, and 80 g $kg^{-1}$ dry matter, respectively; the rest is mainly composed of various carbohydrates (Kalač 2013). In the protein fraction, the most abundant amino acids are glutamic acid (13–21%), aspartic acid (9–12%), and arginine (4–12%); they also contain tyrosine, leucine, lysine valine, and alanine (usually more than 1 mg $g^{-1}$ fresh weight) (Manzi et al. 1999; Mattila et al. 2002a, b). As concerns their content in lipids, more than ten fatty acids are found in cultivated mushrooms; linoleic, oleic, and palmitic acid are the most representative. Moreover, polyunsaturated fatty acids in cultivated mushrooms exceed 70% of the total (Reis et al. 2012). Mannitol and trehalose are the most common among alcoholic sugars and oligosaccharides, respectively, and β-glucan is the most common and well-known fungal polysaccharide (Kalač 2013; Reis et al. 2012). Potassium is highly accumulated by mushrooms followed by calcium and sodium, while mushrooms also contain significant amounts of zinc and copper when compared to other foods (Kalač 2013; Koutrotsios et al. 2014; Mattila et al. 2001). Trace element content in mushrooms varies greatly among different species, but it also depends on their concentration in the growth substrate. As far as vitamins are concerned, ascorbic acid (vitamin C) content varies from 150 to 300 mg $kg^{-1}$; thiamine ($B_1$), riboflavin ($B_2$), niacin ($B_3$), and pyridoxine ($B_6$) contents are 1.7–6.3, 2.6–9.0, 63.8–83.7, and 1.4–5.6 mg $kg^{-1}$ respectively, and ergosterol (a provitamin to $D_2$) varies from 3 to 7 g $kg^{-1}$ dry matter (Kalač 2013).

## 12.4 Widely Cultivated Species: Mushroom Production and Their Bioactive Compounds and Medicinal Properties

Among the most interesting (in terms of their content in mushrooms and their effect on various functions of the human body) and well-known bioactive compounds produced by cultivated mushrooms are ergothioneine, phenolics, and triterpenoids

(all potent antioxidants), β-glucans with anticancer and prebiotic activities, γ-aminobutyric acid (i.e., the chief inhibitory neurotransmitter in the mammalian central nervous system), and lovastatin with hypocholesterolemic and hypolipidemic properties. An overview of pertinent literature findings is presented in Table 12.2. Moreover, a brief outline of available information for each of the most important cultivated mushroom species is provided below.

### 12.4.1 *Agaricus* spp.

*Agaricus* species include litter-decomposing saprotrophs able to efficiently degrade all components of the lignocellulosic complex. They grow in humus-rich soils and are often found in places used for animal grazing. *A. bisporus* (also known as button mushroom or champignon) in particular has been an important component of human diet for over 200 years, and its worldwide cultivation constitutes a multibillion-dollar industry (Fig. 12.1a). In commercial cultivation, most production substrates are typically composed of wheat straw (in Europe and the USA) or rice straw (in Asia) with chicken or horse manures which are mixed and subjected to a composting process. Moreover, an organic material (casing layer) consisting mainly of peat moss is applied over the colonized by the *Agaricus* mycelium substrate for promoting the initiation and development of fruit bodies (Gülser and Pekşen 2003). Since in many areas horse manure and peat moss are scarce and/or expensive, many efforts have been made to develop alternative inputs for use in substrates or casing layers, e.g., wheat bran, molasses, pigeon manure, coconut fibers, straws from oat and grass, spent mushroom compost, residues from tea cultivation, and olive mill waste (Baysal et al. 2007; Giménez and Pardo-González 2008; Gülser and Pekşen 2003; Yigitbasi et al. 2007).

*Agaricus brasiliensis* (the names *A. subrufescens* or *A. blazei* being also in use for this species) is a mushroom recently discovered (1945) in Brazil, but its popularity is increasing rapidly, especially among Brazilian, Japanese, and Chinese growers. In general, its requirements in infrastructure are similar to those for the cultivation of *A. bisporus*, but formation and maturation of mushrooms are achieved at a higher temperature (22–25 °C vs. 15–18 °C respectively). Therefore, *A. brasiliensis* has the potential to replace the cultivation of *A. bisporus* in the warm summer months when the temperature rises above the optimal range for the latter. Its commercial substrate consists of rice straw or bagasse supplemented with chemical and organic fertilizers (Iwade and Mizuno 1997). However, the suitability of various other materials was also evaluated, e.g., sugarcane, coast cross grass and soybean meal, asparagus straw, and sunflower seed hull-based compost (Matute et al. 2010).

*A. bisporus* mushrooms contain significant amounts of lectins with potent antiproliferative effects on human epithelial cancer cells (Batterbury et al. 2002), antioxidants such as selenium and polyphenols, and vitamins (Koyyalamudi et al. 2009). On the other hand, *A. brasiliensis* contains a higher amount of β-glucans and proteoglycans in comparison to *A. bisporus* (Ohno et al. 2001), while it also produces agaritine and ergosterol which induce apoptosis of leukemic cells, as well as

**Table 12.2** Content in some bioactive compounds present in fruit bodies of widely cultivated medicinal mushrooms

| Mushroom species | Lovastatin (mg/kg) | β-glucan (g/100 g) | Ergothioneine (mg/kg) | γ-aminobutyric acid (mg/kg) | Ergosterol (mg/100 g) | References |
|---|---|---|---|---|---|---|
| *Agaricus bisporus* | 81–565 | 5.7–12.0 | 933–1210 | 125–2400 | 602–794 | Chen et al. (2012), Mattila et al. (2002a), McCleary and Draga (2004), Lee and Kim (2005), Lee et al. (2006, 2009), Phillips et al. (2011), and Sari et al. (2017) |
| *Agaricus brasiliensis (A. blazei)* | 184 | 3.0–13.1 | 39–85 | 360–590 | nd[a] | McCleary and Draga (2004), Lee and Kim (2005), Lin et al. (2013), Lo et al. (2012), Toledo et al. (2013), and Tsai et al. (2008) |
| *Auricularia auricula-judae* | nd | 8.3–42.0 | nd | nd | 70–227 | Banlangsawan and Sanoamuang (2016), Huang et al. (1985), Kim et al. (2012), Lee and Kim (2005), and Sari et al. (2017) |
| *Auricularia polytricha* | 16 | 18.09 | 1.4 | 282 | nd | Kim et al. (2012) and Lo et al. (2012) |
| *Flammulina velutipes* | 40–91 | 18.3–21.0 | 57–455 | 230–339 | 255–417 | Chen et al. (2012), Lee and Kim (2005), Lee et al. (2006), Lin et al. (2013), and Phillips et al. (2011) |
| *Ganoderma lucidum* | 68–254 | 23.5–55.0 | 14.5–80.0 | 17–63 | 403 | Lee et al. (2009), Lin et al. (2013), Lo et al. (2012), McCleary and Draga (2004), and Raina et al. (2014) |
| *Grifola frondosa* | 3–12 | 26.0–32.5 | 143–553 | 18–280 | 692–911 | Chen et al. (2012), Cohen et al. (2014), Dubost et al. (2006), Huang et al. (2011), and Lin et al. (2013), McCleary and Draga (2004), Phillips et al. (2011), and Sari et al. 2017 |
| *Hericium erinaceus* | 14 | 35.3–33.9 | 960 | nd | 240–260 | Banlangsawan and Sanoamuang (2016), Cohen et al. (2014), Koutrotsios et al. (2016), Lee et al. (2009), and McCleary and Draga (2004) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Lentinula edodes* | 3–37 | 20.0–27.4 | 137.7–412.3 | 351–622 | 495–1246 | Bak et al. (2014), Chen et al. (2012), Kim et al. (2010b), Kozarski et al. (2012), Lee and Kim (2005), Lee et al. (2006, 2009), Lin et al. (2013), Lo et al. (2012), Mattila et al. (2002a), McCleary and Draga (2004), Phillips et al. (2011), Sari et al. (2017), and Teichmann et al. (2007) |
| *Pleurotus ostreatus* | 165–980 | 10.1–33.3 | 944–2444 | 6–25 | 427–730 | Chen et al. (2012), Cohen et al. 2014, Mattila et al. (2002a), Lee and Kim (2005), Lee et al. (2006, 2009), Phillips et al. (2011), Synytsya et al. (2008), Teichmann et al. (2007), and Tsai et al. (2009) |
| *Trametes versicolor* | 3 | 22.2–53.0 | 68 | 100 | nd | Lee and Kim (2005), Lin et al. (2013), McCleary and Draga (2004), and Sari et al. (2017) |
| *Volvariella volvacea* | 59 | 36.37 | 537 | 999–1150 | 159 | Eguchi et al. (2015), Lo et al. (2012), and Raina et al. (2014) |

[a]*nd* not determined

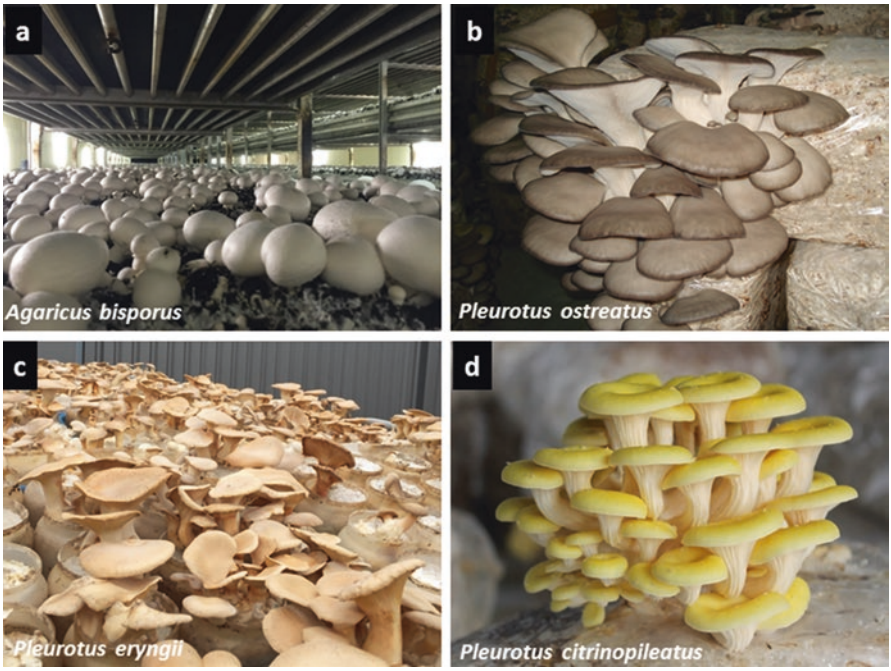


**Fig. 12.1** Mushrooms of (**a**) *Agaricus bisporus* in commercial production and (**b**) *Pleurotus ostreatus*, (**c**) *Pleurotus eryngii*, and (**d**) *Pleurotus citrinopileatus* in commercial production. All photos derived from the authors' personal collections

isoflavonoids with hypoglycemic properties (Oh et al. 2010). Furthermore, compounds from this fungus were demonstrated to possess antitumor properties against fibrosarcoma, myeloma, ovarian and lung cancer in experiments with animals, and gynecological cancer and leukemia (Hetland et al. 2008).

### 12.4.2 *Pleurotus* spp.

*Pleurotus* species (known as "oyster mushrooms") are distributed worldwide and grow on a very large range of substrates (mostly hardwoods). They are potent white-rot fungi with the ability to colonize and form fruit bodies on various lignocellulosic residues; this in combination with the relative ease of cultivation and their culinary/nutritional value are the main reasons for the significant increase in their commercial production during the last 20–30 years. Especially as regards *P. ostreatus* (Fig. 12.1b), the most commonly cultivated species of this genus, a wide variety of available strains are available, with different requirements with respect to climatic conditions and/or mushroom appearance and quality (Koutrotsios et al. 2017), including sporeless strains especially developed to overcome the problems often observed on people working in cultivation rooms due to the large production of

spores. The substrates mostly used are composed of cereal straw supplemented with wheat, rice, or soy bran and/or flours from various leguminous seeds aiming at reducing the cultivation period and/or at increasing mushroom yields. Of interest is that many other plant residues and agro-industrial by-products were exploited for *Pleurotus* cultivation, e.g., sawdust, wood chips, cottonseed hulls, corn cobs, sugarcane bagasse, cotton gin trash, coffee husks, grape marc, vineyard pruning, olive mill wastes, banana straw, soybean stalk, waste paper, and nut shells (Das and Mukherjee 2007; Koutrotsios et al. 2014; Mandeel et al. 2005; Membrillo et al. 2011; Obodai et al. 2003; Pant et al. 2006; Philippoussis et al. 2001; Salmones et al. 2005; Sánchez et al. 2002; Yildiz et al. 2002; Zervakis et al. 1996, 2013). Substrates are usually pasteurized, spawned, and then placed in blocks or bags of 10–20 kg. Biological efficiency (BE, i.e., percentage ratio of fresh mushroom weight over the dry weight of the substrate) differs considerably by ranging from 4 to 74% in various types of sawdust, from 50 to 97% in straw-based substrates, or up to 139% in grape marc, vineyard pruning, and cotton residues (Fig. 12.2a) (Koutrotsios et al. 2014; Mandeel et al. 2005; Pant et al. 2006; Philippoussis et al. 2001; Salmones et al. 2005; Sánchez et al. 2002). Other species, such as *P. eryngii* (Fig. 12.1c), *P. pulmonarius*, *P. citrinopileatus* (Fig. 12.1d), and *P. djamor*, although producing mushrooms of different appearance, taste, and composition, have rather similar requirements in infrastructure, substrate, and growth conditions.

Mushrooms and mycelium of *Pleurotus* spp. contain bioactive compounds such as polysaccharides, lectins, peptides, triterpenoids, etc. with a plethora of medicinal properties including antitumor, anti-inflammatory, and immunomodulatory activities. Among polysaccharides, a β-glucan (pleuran) was identified with antioxidant and anti-inflammatory properties (Bobovčák et al. 2010). Furthermore, trials on humans demonstrated the efficacy of pleuran in the prevention of recurrent respiratory tract infections (Jesenaket al. 2013). Preliminary results also showed that addition of *Pleurotus* mushroom powder in animal diet (at a rate of 4–10%) resulted in lower blood pressure as well as cholesterol levels (Pate et al. 2012). Moreover, when compared to all other cultivated mushrooms, *P. ostreatus* contains the highest concentration of lovastatin and ergothioneine (up to 980 mg/kg and 944–2444 mg/kg respectively; Table 12.2).

### 12.4.3 *Lentinula edodes*

*Lentinula edodes* (commonly known as “shiitake”) grows on dead wood of various deciduous trees in warm and moist regions (Royse 1997; Royse and Sanchez-Vazquez 2001). This is the first mushroom ever cultivated in China 1000 years ago, by placing (in woods) cut logs naturally contaminated by the fungus. Nowadays, it is cultivated in artificial substrates in Asia, Europe, and North America and ranks third in global production because of its flavor, taste, and nutritional and medicinal value (Özçelik and Pekşen 2007). During the last 20 years, the traditional cultivation technique on wood logs has been largely replaced by production on artificial substrates typically consisting of hardwood sawdust supplemented with various

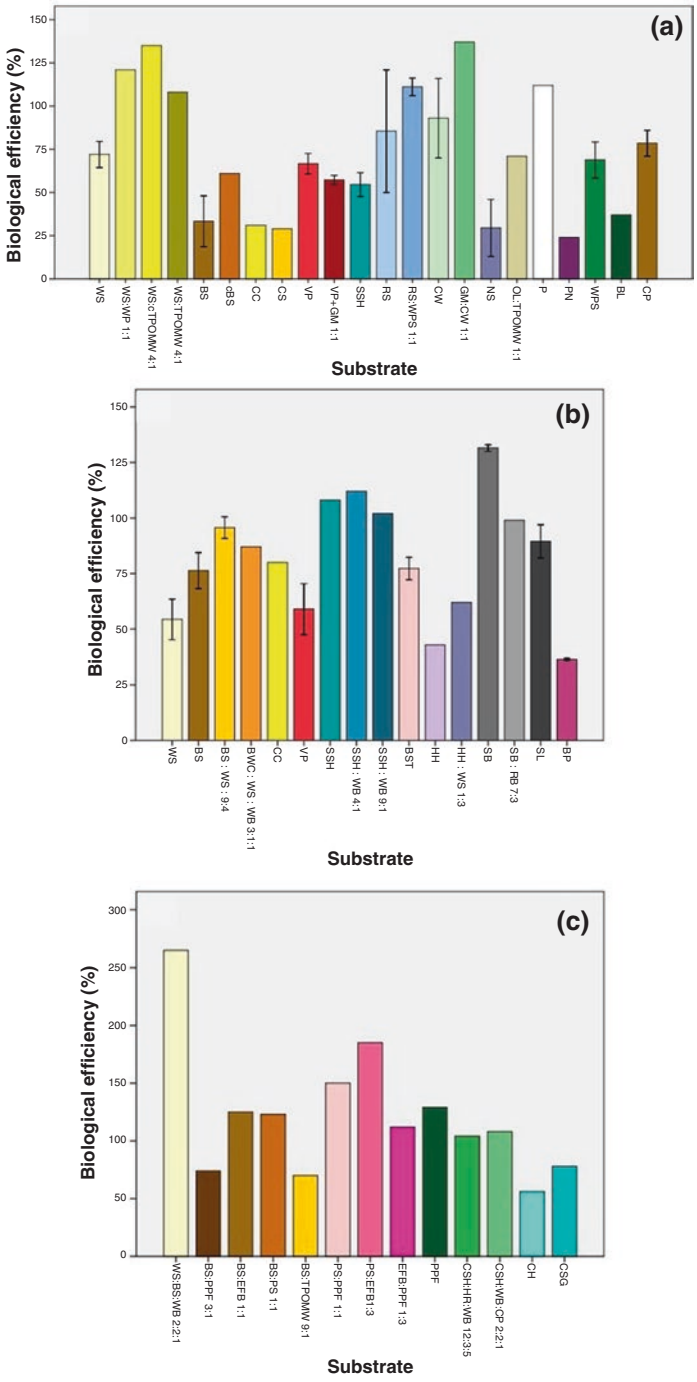


**Fig. 12.2** Biological efficiency (%, fresh mushroom weight/dry weight of the substrate) of (**a**) *Pleurotus ostreatus* (Curvetto et al. 2002b; Das and Mukherjee 2007; Koutrotsios et al. 2014; Mandeel et al. 2005; Obodai et al. 2003; Philippoussis et al. 2001; Salmones et al. 2005; Sánchez et al. 2002; Yildiz et al. 2002; Zervakis et al. 2013; Zhang et al. 2002), (**b**) *Lentinula edodes* (Curvetto et al. 2002a; Gaitán-Hernández et al. 2006; Özçelik and Pekşen 2007; Philippoussis et al. 2007; Rossi et al. 2003; Royse and Sanchez 2007; Salmones et al. 1999), and

nitrogen sources (Özçelik and Pekşen 2007; Philippoussis et al. 2003, 2007; Royse 1997) (Fig. 12.3); these new techniques offer much higher yields in shorter crop periods (Royse 1997). In addition, several plant residues such as wheat straw, corn cobs, sugarcane bagasse, sugarcane leaves, coffee husks, sunflower seed hulls, peanut shells, cotton stalks, hazelnut husks, tea waste, and vineyard pruning were also used for cultivation of shiitake, supplemented with wheat or rice bran, millet, rye, and/or corn in a 10–40% ratio to the main ingredient (Curvetto et al. 2002a, b; Elisashvili et al. 2008; Gaitán-Hernández et al. 2006; Hiromoto 1991; Mata and Savoie 1998; Royse and Sanchez 2007; Salmones et al. 1999). Biological efficiencies ranged from 60 to 65% in hardwood residues and hazelnut husks to 79–89% for cereal straw and sugarcane bagasse and up to 93–108% in vineyard pruning and sunflower seed hulls (Fig. 12.2b) (Curvetto et al. 2002a, b; Gaitán-Hernández et al. 2006; Hiromoto 1991; Özçelik and Pekşen 2007; Salmones et al. 1999). The particular aspect of shiitake cultivation is that the crop requires an extra stage (which extends the length of cultivation) just after the substrate's full colonization by the mycelium; this is known as "browning" since mycelium turns from white to brown and a hard crust of hyphae forms on the surface of the substrate.

Shiitake mushrooms contain several bioactive compounds, i.e., lentinan, lentin, lentinacin or eritadenine, polysaccharide KS-2, and lentinamycin (Enmanet al. 2008; Fujiiet al. 1978; Minato et al. 1999; Shimada et al. 2003), all with interesting medicinal properties. Among them, lentinamycin, lentin, and lentinan (a β-glucan) demonstrate antimicrobial and antiviral activities, lentinan suppresses the proliferation of leukemic cells, ethanol extracts of fruit bodies decrease proliferation of CH72 cells without affecting healthy cells, KS-2 polysaccharides possess properties against Ehrlich and Sarcoma-180 tumors, and eritadenine contributes significantly to the reduction of cholesterol and triglycerides (Casaril et al. 2011; Fujii et al. 1978; Gu and Belury 2005; Handayani et al. 2011; Ngai and Ng 2003). In addition, *L. edodes* mushrooms contain a significant amount of γ-aminobutyric acid (351–622 mg/kg) and ergosterol (495–1246 mg/kg) (Table 12.2).

### 12.4.4 *Auricularia* spp.

*Auricularia auricula-judae* and *A. nigricans* (also known *A. polytricha*) are the two most important representatives of the genus. They are saprotrophs with a worldwide distribution from the temperate regions to the tropics, growing on living and dead deciduous trees, decayed stumps, or logs (Du et al. 2011). Nowadays, *Auricularia* mushrooms are among the top four most important cultivated mushrooms in the

**Fig. 12.2** (continued) (**c**) *Flammulina velutipes* (Chen et al. 2008; Harith et al. 2014; Leifa et al. 2001; Rezaeian and Pourianfar 2016; Rugolo et al. 2016) cultivated on different substrates. The vertical line in columns represents the standard error of the means as derived from calculations made on relevant literature data.


**Fig. 12.3** Mushrooms of (**a**) *Lentinula edodes* in commercial production, (**b**) wild-growing *Auricularia auricula-judae,* (**c**) *Ganoderma lucidum* cultivated on olive mill by-products (Koutrotsios and Zervakis, unpublished data), (**d**) wild-growing *Trametes versicolor*. All photos derived from the authors' personal collections

world with an annual production exceeding 3.5 million tons (Royse 2014). Their form and the unique jelly texture, as well as the distinctive flavor and nutritional characteristics, make them very different from other cultivated mushrooms (Fig. 12.3b). Their cultivation is rather easy, fruit bodies are produced within a relatively short period, and they do not require expensive facilities (Irawati et al. 2012). In addition, they could be obtained from a wide range of plant residues including cotton seed shells, sawdust, sugarcane bagasse, grass and cereal straw, and corn cobs with BEs reaching 70–80% (Liang et al. 2016; Luo 1993; Onyango et al. 2011). *A. nigricans*, in particular, is the best-studied species of the genus as regards cultivation on various lignocellulosic substrates. Hence, sawdust supplemented with different quantities of grass plants provided high BE values (95–148%) (Liang et al. 2016); similarly, sawdust, oil palm fond and spent grain, or sawdust with empty fruit bunch supplemented with spent grains performed very well (BE: 261–290%). In contrast, other substrates such as sawdust, supplemented paddy straw, or palm residues were less productive (BE: 6–114%) (Irawati et al. 2012). Although the addition of a nitrogen source is considered a necessary prerequisite for achieving high yields (Luo 1993), the results of pertinent studies show that the optimum C/N value of substrates is about 100 and that further nitrogen addition leads to a reduction in mushroom yields.

*A. nigricans* and *A. auricula-judae* are two of the most important medicinal fungi in China. Besides their flavor and nutrition value, they also possess significant antitumor, hypoglycemic, anticoagulant, antiviral, and antimicrobial activities (Wasser and Weis 1999).

### 12.4.5 *Flammulina velutipes*

*Flammulina velutipes*, commonly known as enoki ("enokitake") or golden needle mushroom, was first cultivated in China during the eighth century. Nowadays, it ranks fifth among all mushrooms. Recently, its production exceeded 2 million tons, and more than 75% of this quantity originated from China, while Japan, Korea, and Taiwan follow in the leading positions (Royse 2014). Cultivation of *F. velutipes* is commonly performed in polypropylene bottles which are filled with hardwood sawdust and sterilized prior to inoculation. For the production of high-quality mushrooms, low ambient temperature (3–8 °C) is required, and a plastic collar is placed around the bottle neck to promote the formation of fruit bodies with elongated stipes. Apart of sawdust, several other substrates are in use, e.g., sugarcane bagasse, corn cobs, and cottonseed husks (Chang and Miles 1989; Royse 1997). Moreover, additional plant residues have been evaluated either individually or in combination and presented high biological efficiencies (Fig. 12.2c); indicative examples are paddy straw (BE: 90–106%) (Harith et al. 2014); coffee spent ground (78%) (Leifa et al. 2001); maize straw (73%), rubber wood sawdust with paddy straw, palm empty fruit bunches, and palm press fiber in various proportions (100–185%) (Harith et al. 2014); and wheat straw, sawdust, and wheat bran (265%) (Rezaeian and Pourianfar 2016). As regards the effect of nitrogen supplementation, it was reported that *F. velutipes* grows well in media containing soybean powder, beef cream, and yeast powder, whereas growth is slowed when nitrates or amines are used as nitrogen sources (DeChang 2000).

Several bioactive compounds have been isolated from fruit bodies and mycelium of *F. velutipes*, i.e., Fip-FVE protein and enokipodins with proven antimicrobial activity (Ishikawa et al. 2001; Wu et al. 2008), the weakly acidic glycoprotein proflamin with antitumor properties, and the protein flammulin, which is also active against various types of cancer cells (Ikekawa et al. 1985; Gong et al. 1998; Maruyama and Ikekawa 2005). Consumption of enoki mushrooms was demonstrated to reduce the cholesterol level in human body and the allergic immune response in case of food allergies (Hsieh et al. 2003; Ishikawa et al. 2001). In addition, and much like other cultivated mushrooms, it contains antioxidant substances; it is noteworthy that extracts and powder of *F. velutipes* were used as preservatives against meat and fish oxidation (Bao et al. 2008, 2009).

### 12.4.6 *Ganoderma lucidum*

*Ganoderma lucidum*, commonly known as lingzhi or reishi mushroom, is one of the most famous medicinal "herbs" in Asian countries which is extensively used since antiquity (Bishop et al. 2015). Due to the increased demand for its fruit bodies and the qualitative/quantitative differences in the chemical composition of wild *G. lucidum* mushrooms, commercial cultivation has started in the early 1970s, mainly on supplemented hardwood sawdust but also on agricultural residues such as cereal straw, cotton seed husk, corn cobs, and wheat bran (Li et al. 2016; Xia et al. 2003; Zhou et al. 2012) (Fig. 12.3c). However, the use of alternative substrates provided a considerable increase in yields; BEs values of up to 75% was reported when *G. lucidum* was cultivated on maize straw supplemented with wheat and maize bran, sawdust, and lime, or on soy and soybean curd residues (Ji et al. 2001). In addition, such substrates supported the production of mushrooms with higher polysaccharide content and showed enhanced antimicrobial, antioxidant, and cytotoxic activities in comparison to those obtained on conventional substrates.

*G. lucidum* fruit bodies and mycelia contain over 400 different bioactive compounds, mainly triterpenoids and polysaccharides and also steroids, sterols, proteins, peptides, and fatty acids (Paterson 2006). Its medicinal properties have been extensively studied, and several hundreds of relevant papers have been published in the last 30 years. Nowadays, the cultivation of *G. lucidum* on artificial substrates has led to the wide use of reishi mushrooms and its products (80–85% of them derive from fruit bodies) not only as beverages and food additives but also as drugs for the prevention and/or treatment of hepatitis, diabetes, hypertension, nephritis, leukemia, and tumors or as immunoregulating, antiaging, antioxidant, antifatigue, and sleep-regulating agent (Li et al. 2016; Paterson 2006; Zhou et al. 2012).

### 12.4.7 *Trametes versicolor*

*Trametes versicolor*, also known as Turkey-tail fungus, belongs to the family *Polyporaceae*. It is a widely distributed fungus whose mushrooms could be found throughout the year mainly on dead wood of deciduous trees (Fig. 12.3d). Although its fruit bodies are not edible because of its hard-flesh texture, it is among the most potent and best-studied medicinal mushrooms. Cultivation of *T. versicolor* is performed on substrates mainly based on various types of hardwood sawdust, while other tree sawdust (deriving from apple or cherry trees or conifers) is also used (Stamets 2011). Supplementation of such media with sorghum and wheat bran has improved mushrooms yields considerably (Guerrero et al. 2011) and so did alternative substrates such as soybean, camelina seeds, and sunflower seed cakes (Krupodorova and Barshteyn 2015).

*Trametes versicolor* has a long history of use in Japanese and Chinese traditional medicine. The composition of *T. versicolor* mushrooms has been extensively studied, and various ingredients with medicinal properties were detected. In particular, proteoglycans, mainly polysaccharide peptides (PSP) and polysaccharide K (PSK),

are considered as the most representative and were shown to possess antimicrobial, antivirus, antitumor, and immunostimulatory properties. PSK is administered to cancer patients in Japan both during and after chemotherapy (Morimoto et al. 1996). On the other hand, freeze-dried *T. versicolor* fruit-body powder is often prescribed in the USA by homeopathic doctors and oncologists to cancer patients; positive results from such treatment are attributed to stimulation of the immune system (Fisher and Yang 2001). In addition, studies on both animals and humans demonstrated the significant effect of *T. versicolor* water extracts in liver protection and at reducing the level of cholesterol in blood as well as in the treatment of diabetes mellitus (Chiu et al. 1993; Hor et al. 2011).

### 12.4.8 *Grifola frondosa*

*Grifola frondosa* (maitake or hen of the woods) is a relatively rare mushroom occurring in the temperate forests of Asia, Europe, and eastern North America. It grows mainly at the base of oak and chestnut trees, but is also found on dead wood of various other plant species. *G. frondosa* cultivation has spread in the last two decades mainly in Japan and SE Asia, while mushrooms are recently used as dietary supplements too. Mushroom production is mainly performed in polypropylene bottles or in sterilizable bags, and the most common substrate used consists of sawdust supplemented with bran (Fig. 12.4a). However, the BE values reported from cultivation on such media are rather low, i.e., 35% for oak sawdust supplemented with corn bran (Mizuno et al. 1986), or up to 40% for oak sawdust supplemented with rye, millet, and wheat bran (Shen and Royse 2002). In contrast, the use of other substrates such as brewery waste, coffee spent ground, and olive press cake seems to adversely affect yields and quality of the mushrooms produced (Barreto et al. 2008; Gregori et al. 2009; Montoya et al. 2012; Svagelj et al. 2007), probably due to the presence of toxic compound inhibiting mycelium growth and/or fructification. Nevertheless, increase in the demand for *G. frondosa* mushrooms provides sound

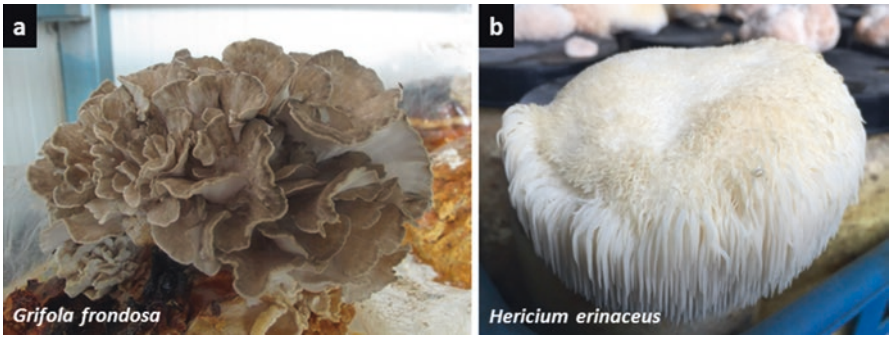


**Fig. 12.4** Mushrooms of (**a**) *Grifola frondosa* in commercial production, (**b**) *Hericium erinaceus* cultivated on olive tree prunings (Koutrotsios et al. 2016). All photos derived from the authors' personal collections

incentives for further research in order to assess the suitability of alternative plant residues as substrates for their cultivation.

The chemical composition and the medicinal properties of *G. frondosa* biomass have been intensely studied in recent years, and as a result, various products were manufactured deriving from mycelium and fruit-body extracts. These were found to contain large amounts of ergothioneine (up to 1840 mg $kg^{-1}$) (Dubost et al. 2006) to which antioxidant and cytoprotective capabilities are attributed (Cheah and Halliwell 2012), as well as lectins, e.g., an N-acetylgalactosamine-specific lectin which could agglutinate erythrocytes and offers cytotoxic properties against HeLa cells (Kawagishi et al. 1990). Furthermore, low molecular mass polysaccharides enhancing phagocytosis of human polymorphonuclear neutrophils and an antivirus protein efficient against HSV-1 were detected (Gu et al. 2006). In addition, *G. frondosa* extracts presented antimicrobial, antioxidant, immunostimulatory, and antitumor properties (Klaus et al. 2015), while they could also be used in cosmetology since they stimulate collagen biosynthetic activity for fibroblasts and show photoprotection of human dermal fibroblasts (Kim et al. 2010a).

### 12.4.9 *Hericium erinaceus*

*Hericium erinaceus* (commonly known as lion's mane or monkey's head due to the fruit-body shape) grows on the wood of deciduous trees and produces fleshy, white mushrooms possessing distinctive elongated spines (Fig. 12.4b). It is traditionally cultivated in Asia, mainly in China, Japan, and Malaysia, by using hardwood sawdust as substrate. However, recent studies have shown that replacing part of the (or the entire) sawdust medium with various agricultural residues, such as sunflower seed hulls (Figlas et al. 2007), sugarcane bagasse, rice hull and/or soybean dregs (Hu et al. 2008), and olive pruning (Koutrotsios et al. 2016), does not only increase productivity but also upgrades mushroom content in bioactive components like antioxidants, phenolics, and α- and β-glucans (Koutrotsios et al. 2016). In general, BE values reported from various *H. erinaceus* cultivation substrates ranged from 31 to 70% (Figlas et al. 2007; Ko et al. 2005; Koutrotsios et al. 2016).

A large number of bioactive substances have been isolated from *H. erinaceus* and were investigated for their medicinal properties in vitro and in animal and human preclinical trials. Among them, 20 aromatic compounds contain a benzene ring, i.e., hericenone A to L, erinacine A to D, and 3-hydroxyhericenones (Ma et al. 2010a, b; Ueda et al. 2008); several of the hericenones were shown to stimulate the synthesis of nerve growth factors and to have an anti-dementia effect in experimental studies on rats and humans. Furthermore, these compounds demonstrated additional medicinal properties, including cytotoxic, antibiotic, and protective against endoplasmic reticulum stress-dependent cell death (Kawagishi et al. 1994a, b; Ma et al. 2010a; Ueda et al. 2008). Other bioactive compounds detected in *H. erinaceus* include diterpenoids (some of which, e.g., cyathane terpenoids, erinacine A to G, promoting biosynthesis of nerve growth factors in cell cultures; Kawagishi et al. 1994a, b) and several lectins with hemagglutinating activity (Gong et al. 2004) or with an effect on adhesive properties of erythrocytes (Kawagishi et al. 1994a, b).

### 12.4.10 *Volvariella volvacea*

*Volvariella volvacea*, commonly known as paddy straw mushroom, is a saprotrophic species fruiting under high temperatures (>30 °C), whose commercial production reaches 180,000 tons per year, making it the sixth largest mushroom crop in the world (Biswas and Layak 2014). It is traditionally cultivated on non-composted and unpasteurized bundles of paddy straw or banana leaves which are laid outdoors to form beds or are placed within wooden frames (Reyes 2000). However, low mushroom yields are often obtained since substrates are rather poor in nutrients and are exposed to contaminations and irregular environmental conditions. In contrast, indoor cultivation provides the prerequisites for higher productivity, as demonstrated by several pertinent studies employing composting and pasteurization of paddy straw supplemented by molasses and NPK fertilizer (Reyes 2000), wheat straw supplemented by wheat or rice bran, and cotton waste or paddy straw (Rajapakse 2011). Moreover, the use of other agricultural by-products, e.g., dried banana leaves and sugarcane bagasse (Oei 2003), oil farm bunch waste (Thiribhuvanamala et al. 2012), and cocoa bean shells (Belewu and Lawal 2003), led to two to three times increase of BE and more stable production yields. In addition, since cellulose/lignin ratio was demonstrated to be positively correlated with *V. volvacea* mycelium growth and mushroom productivity (Philippoussis et al. 2001), the exploitation of additional plant residues is worth examining.

*V. volvacea* mushrooms contain high amounts of essential amino acids as well as vitamins, polypeptides, steroids, terpenoids, lectins, and phenolic compounds (Chang and Buswell 1996; Hung and Nhi 2012). Furthermore, the proteins volvatoxin and flammutoxin, as well as polysaccharides isolated from this fungus, are considered to possess antitumor properties (Cochran 1978). *V. volvacea* contains larger amounts of γ-aminobutyric acid (999–1150 mg/kg) (Table 12.2) than most other cultivated mushrooms; this compound plays a crucial role in reducing neuronal excitability throughout the nervous system and is also directly responsible for the regulation of muscle tone in the human body. In addition, various extracts from paddy straw mushrooms were found to present high antioxidant properties and could, therefore, contribute to the prevention of cardiovascular and neurodegenerative diseases (Cheung et al. 2003; Joseph et al. 1999).

## 12.5 Conclusion

The medicinal use of mushrooms has a very long tradition, especially in Asia, while pertinent interest in Western countries began to develop in the last decades only. Nowadays, research has focused on the investigation of the mechanisms of action of several bioactive compounds isolated from mushrooms and on clinical trials. In particular, those deriving from cultivated species present an enormous – yet largely untapped – potential of applications related to biotechnology, biomedicine, pharmacology, etc. Therefore, mushrooms could significantly contribute not only to the development of such economic activities but most importantly at the generation of

potent medicinal products of natural origin through the valorization of lignocellulosic substrates commonly regarded as waste materials.

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# Making Use of Genomic Information to Explore the Biotechnological Potential of Medicinal Mushrooms

13

Ursula Kües and Susanna M. Badalyan

**Abstract**

Fruiting bodies of fungi are rich in multiple types of bioactive compounds with (potential) pharmaceutical effects. Many kinds of mushrooms are thus highly valued in traditional medicine in different cultures over the world for treatment of diseases and maintenance of good health. Modern science has uncovered functional principles in many medicinal species and assigned beneficial activities (antimicrobial, antiviral, anti-oxidative, immunomodulatory, anti-inflammatory, anti-tumorous, hypotensive, hepatoprotective, antidiabetic/hypoglycemic and hypocholesterolemic, mitogenic/regenerative, etc.) to a wealth of secondary metabolites, peptides, proteins, and sugar-based polymers. Compared to the extensive lists of bioactive compounds and the description of their distinctive effects, the pathways of their biosynthesis and the genes behind are largely understudied. This can now become changed by the many genomes which are provided by large-scale fungal sequencing programs. Among are many assembled genomes for important edible and medicinal mushroom species which can be used in genome mining for genes of interest, both for the synthesis of known products and for the synthesis of novel, so far undetected compounds. Also, genomes of other species offer possibilities to predict genes for the biosynthesis of formerly unnoticed bioactive fungal products of either biochemically already known or novel structure. We present here examples of recent

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identification of genes and gene clusters for bioactive compounds (different terpenoids, phenolics, polyketides, cyclic peptides, aegerolysins, lectins, protease inhibitors, and ribosome-inactivating proteins) in medicinal and edible fungi. Genome comparisons and gene mining identify related genes for similar products in other species. Usually, genes for medicinally interesting products are found in only a restricted range of species, inconsistently distributed over the fungal taxa. Some of the recognized medicinal species probably have genes for a higher variety of bioactive products than species which are estimated purely for their good edible value or species being commonly neglected for exploitation as food and medicine.

**Keywords**

*Agaricomycetes* • *Ascomycota* • Secondary metabolites • Cyclic peptides • Lectins • Aegerolysins • Fungal genomes

## Contents



## Abbreviations

*atf* Gene for acetyltransferase
*cyc* Gene for terpene cyclase

*ggs* Gene for geranylgeranyl pyrophosphate synthase
*p450* Gene for cytochrome P450
*sdr* Gene for short-chain dehydrogenase/reductase

## 13.1 Introduction

The fungal subkingdom *Dikarya* splits into the two phyla *Ascomycota* and *Basidiomycota* that represent the higher fungi, many of which grow in mycelial form and produce compact fruiting bodies with meiotic spores for sexual reproduction (Hibbett et al. 2007). Specific genera of the *Ascomycota*, in particular within the order *Pezizales* (e.g., *Helvella*, *Morchella*, *Terfezia*, *Tirmania*, *Tuber*), and in full extend the class *Agaricomycetes* within the phylum *Basidiomycota* give rise to larger mushrooms (Hibbett 2007; Schoch et al. 2009). Mushrooms are fruiting bodies of macroscopic size, can be seen by the naked eye and be picked by hand, and may grow above (epigeous) or below (hypogeous) ground in soil or a respective growth substrate (Chang and Miles 1992). There is a tremendous variety in mushroom morphologies, such as in their overall shapes, sizes, colors, consistencies, tissue structures, etc. (Schoch et al. 2009; Kües and Navarro-Gonzalés 2015; Sherratt et al. 2005; Halbwachs et al. 2016). Mushrooms attracted humans since ancient times not only by their often beautiful eye-catching looks but for reasons of exploitations such as for food, health care, and use as hallucinogens, among in spiritual practices (Kües and Liu 2000; Boa 2004; Jo Feeney et al. 2014; Sayin 2014; de Mattos-Shipley et al. 2016).

Mushrooms are commonly distinguished into edible, nonedible (e.g., due to no or too bad taste or by too hard structure), medicinal, psychedelic, and poisonous species, with sometimes fuzzy overlaps between the categories (Boa 2004; Guzman 2008; Garibay-Orijel et al. 2009; Gonmori et al. 2011; Grienke et al. 2014; Jo et al. 2014; Santiago et al. 2016). Although controversially discussed, the best known fly agaric *Amanita muscaria*, for example, is reported to be eaten upon decoction in certain locations of the world, possibly in the lack of alternative better and safer food (Rubel and Arora 2008; Viess 2012). The consumption of this unmistakable fungus for the experience of its hallucinogenic effects is also disseminated in certain populations and cultures (Feeney 2010; Viess 2012; Sayin 2014), while (accidental) poisonings with the fly agaric (pantherina-muscaria syndrome based on ibotenic acid as the most potent toxin in the fruiting bodies) are also recorded. However, fatal outcomes are rare (2–5% of cases) by advanced diagnosis and good medical treatment (Michelot and Melendez-Howell 2003; Marciniak et al. 2010; Vendramin and Brvar 2014; Mikaszewska-Sokolewicz et al. 2016).

Global numbers of total fungal species and of different higher fungal taxa are still challenged with contemporary estimates for species between lowest 0.7 and highest 5.1 million, with 1.5–3.0 million being the current working figure (Schmitt and Mueller 2007; Blackwell 2011; Hawksworth 2012; Tedersoo et al. 2014; Dai et al. 2015). In the so far most comprehensive molecular genetic survey of soil

mycobiomes from different continents, latitudes, and ecosystems, about 50% of all detected fungal species (in total 80,486) were *Agaricomycetes* and 1.8% *Pezizomycetes*, with over 40% of the taxa being still unknown (OTUs, operational taxonomic units; Tedersoo et al. 2014). Earlier conservative estimates stated that within a total of 1.5 million fungi, about 140,000 macrofungi exist worldwide of which 10 % were known at the time (Hawksworth 1991; Chang 2001). Of the then known mushroom species, about 7000 and >2000 are considered to be edible and to be entirely safe, respectively (Hawksworth 1991; Chang 2001), while a small percentage (2–3%) of the known species (at least 170) is poisonous to deadly toxic (Gonmori and Yoshioka 2003; Boa 2004). However, the numbers of named fungal taxa (especially genera and species) are rapidly increasing, including that important edible and medicinal fungal species complexes (e.g., within the genera *Morchella*, *Ganoderma*, and *Inonotus*) become better resolved and subdivided into new lineages (Dai et al. 2015).

Regardless of an actual edible status, mushrooms can have many useful medicinal properties, including psychedelic and potentially lethal species which may provide medicinal power also by their Janus-faced toxins (Wasser 2002; Jo et al. 2014; Carhart-Harris et al. 2016; Rahi and Malik 2016). Substantial pharmacological properties were recorded for over 700 species. However, the extensive medicinal potential offered by mushrooms is likely by far not fully perceived and certainly not yet utterly exploited (Wasser and Weiss 1999; Wasser 2002, 2011; Lindequist et al. 2005; Badalyan 2012; De Silva et al. 2013; El Enshasy et al. 2013). Best established in cancer cell line and animal test systems are positive effects of preparations of fungal β-glucans and proteoglycans such as of *Trametes versicolor* (the 100 kDa polysaccharide-K, PSK, krestin), *Schizophyllum commune* (the 45–50 kDa schizophyllan), *Lentinula edodes* (the 45–50 kDa lentinan), *Grifola frondosa* (the 100 kDa maitake polysaccharide D fraction, respectively, purified MD fraction, grifoldan), *Ganoderma lucidum* (the 40 kDa polysaccharide PS-G preparation, ganopoly), and *Phellinus* species (fractions variably sized 8.9–2000 kDa) which are marketed as alternative medicine and used as anticancer therapeutics in Asian countries (Kidd 2000; Lindequist et al. 2005; Lemieszek and Rzeski 2012; El Enshasy and Hatti-Kaul 2013; De Silva et al. 2013; Huang and Nie 2015; Yan et al. 2017). *L. edodes* and *G. frondosa* are widely eaten gourmet mushrooms; *S. commune* fruiting bodies are included in human diets in some tropical countries (e.g., Thailand, Malaysia, and Mexico), whereas the tough sporocarps of *T. versicolor* and the conks of *G. lucidum* are edible but unpalatable and the latter in addition is too bitter (Boa 2004; Chang and Lee 2004; Ruán-Soto et al. 2006). Many versatile medicinal mushrooms, such as from the *Ganoderma* species complex, are therefore applied as food additives (nutriceuticals; Chang and Buswell 2001) in the form of tonics, teas, soups, and alcoholic beverages or as mushroom powders, frequently filled into capsules or pressed into pills, in order to make use of their good ingredients for benefits of human health (De Silva et al. 2012a; Bishop et al. 2015).

Medicinal mushrooms and their multiple bioactive compounds are listed to function in human health care for instance antiviral, antibacterial, antifungal, antioxidative, immunomodulatory and immunosuppressive, anti-inflammatory,

anti-tumorous, hypotensive, hepatoprotective, antidiabetic/hypoglycemic and hypocholesterolemic, anti-obese, mitogenic/regenerative, anti-dementia, and more (Wasser and Weiss 1999; Wasser 2002, 2011; Poucheret et al. 2006; Badalyan 2012; Chang and Wasser 2012; Hassan et al. 2015; Phan et al. 2017). Such claims, however, need a good clinical survey to substantiate these potentials in practice (Chung 2006; Hapuarachchi et al. 2016; Money 2016; Wasser 2017).

Functional compounds of mushrooms, their biochemical nature, their principles of actions, and their production and safe applications are a matter of concern. A wealth of organic bioactive molecules has been identified in worldwide research from mushrooms to have medicinal effects, including different bioactive proteins (e.g., aegerolysins, lectins, ribosome-inactivating proteins, and protein inhibitors), ribosomal and non-ribosomal peptides, polysaccharides, peptidoglycans, alcohols, phenolics, different types of terpenoids (sesquiterpenes, diterpenes, triterpenes, meroterpenes of mixed biosynthetic origin, less often monoterpenes), lipids, steroids, alkaloids, polyketides, and other compounds (Wasser and Weiss 1999; Wasser 2002; Ferreira et al. 2010; Anke and Antelo 2011; Schüffler and Anke 2011; De Silva et al. 2013; Grienke et al. 2014; Duru and Çayan 2015; Sabotič et al. 2016; Dickschat 2017). Functional compounds for applications might be isolated from collected or cultivated mushrooms (Tang et al. 2007; Stadler and Hoffmeister 2015; Bedlovicova et al. 2016; Tang et al. 2016a; Lau and Abdullah 2017) or, when possible, obtained from mycelial fermentation (Zhong and Xiao 2009; De Silva et al. 2012b; Elisashvili 2012; El Enshasy and Hatti-Kaul 2013; Chen et al. 2016a). Not all mushroom species can grow in culture and such therefore fall short for fermentations (Badalyan 2012; Stadler and Hoffmeister 2015). However, polysaccharides (mainly β-glucans) and peptidoglycans as ordinary constituents of fungal cell walls and their external mucilage layers might abundantly be produced in large mycelial fermentation from well-growing species (Fazenda et al. 2008; Wasser 2011; Castillo et al. 2015; Chen et al. 2016a; Money 2016), but also some other bioactive compounds, e.g., phenolic antioxidants and certain terpenoids, can be obtained from cultures (Lorenzen and Anke 1998; Asatiani et al. 2007; Carvajal et al. 2012; Thongbai et al. 2015; Tešanović et al. 2017). Not uncommonly, mycelia produce bioactive compounds which are not seen in the fruiting bodies (Hartley et al. 2009; Thongbai et al. 2015). In other instances, specific proteins and peptides, secondary metabolites, and other potent bioactive molecules might confine purely to the fruiting bodies and even to specific tissues and stages in the development (Enjalbert et al. 1999; Boulianne et al. 2000; Walser et al. 2005; Hu et al. 2012; Li et al. 2014a; Lu et al. 2014; Yilmaz et al. 2015; Zhang et al. 2015a; Sabotič et al. 2016), or specific conditions need to be established for induction of targeted metabolite production in liquid cultures (Xu et al. 2010). Favoring measures can be addition of methyl jasmonate, lipids, or surfactants (Ren et al. 2013a; Xu et al. 2016a,b), addition of suitable precursor molecules (Hu et al. 2014, 2016) or specific known inducers (Liang et al. 2010), addition of effective deregulators of the general metabolism (Ren et al. 2014) and of other severe stressors (You et al. 2013; Cao et al. 2017), application of specific pH control (Wang et al. 2016b) or temperature shifting strategies (Feng et al. 2016), or limitation of oxygen (Zhang and Zhong 2013). Such

environmental and physiological management strategies can become further expanded by genetic engineering of the fungal producers by smart modification of improving expression of genes for the synthesis of precursor molecules and of the compounds of concern and by interrupting gene expression of competitive pathways and unwanted pathway branches (Qin et al. 2015; Xu and Zhong 2015).

Fungal research with the twenty-first century experienced an unprecedented boost by whole genome sequencing programs over the fungal kingdom, many of which are provided to the public in assembled form with gene annotations on the MycoCosm page of the JGI (Joint Genome Institute, Walnut Creek, CA; http://genome.jgi.doe.gov/programs/fungi/index.jsf; Grigoriev et al. 2014) or can be found deposited in the NCBI databases (https://www.ncbi.nlm.nih.gov/). Fungal genome sequencing programs provide extraordinary resources for identifying new genes for the synthesis of bioactive compounds from mushrooms, both of biochemically already known substances and of unforeseen compounds. With respect to edible and medicinal mushrooms, sequencing so far provided from *Ascomycota* in particular the genomes of precious edible truffles such as *Tuber melanosporum* (Martin et al. 2010), *Tuber borchii*, *Terfezia boudieri*, and *Terfezia claveryi*, of the morels *Morchella conica* and *Morchella importuna*, and of the false morel *Gyromitra esculenta* (MycoCosm status from April 2017). Especially many mushroom genomes are published from the *Agaricomycotina* (see MycoCosm page and NCBI databases for full list), among from the edible *Agaricus bisporus* (Morin et al. 2012), *Amanita jacksonii* (Sánchez-Ramírez et al. 2014), *Armillaria mellea* (Collins et al. 2013), *Auricularia subglabra* (Floudas et al. 2012), *Boletus edulis* (MycoCosm page), *Flammulina velutipes* (Park et al. 2014), *Hypholoma sublateritium* (Kohler et al. 2015), *Laetiporus sulphureus* (Nagy et al. 2016), *Lentinula edodes* (Chen et al. 2016b), *Pleurotus eryngii* (Yang et al. 2016b), *Pleurotus ostreatus* (Riley et al. 2014), and *Volvariella volvacea* (Bao et al. 2013), also from the less often eaten *Coprinopsis cinerea* (Stajich et al. 2010) and *S. commune* (Ohm et al. 2010), and as further important medicinal mushrooms from *Amanita bisporigera* and *Amanita phalloides* (Pulman et al. 2016), *Amanita muscaria* (Kohler et al. 2015), *Taiwanofungus camphoratus* (synonym *Antrodia cinnamomea*; Lu et al. 2014), *Fistulina hepatica* (Floudas et al. 2015), *Fomitopsis pinicola* (Floudas et al. 2012), various *Ganoderma* species (Kües et al. 2015; Merciere et al. 2015; Zhu et al. 2015), *Lignosus rhinocerotis* (Yap et al. 2014)), *Omphalotus olearius* (Wawrzyn et al. 2012), *Punctularia strigosozonata* (Floudas et al. 2012), *Serpula lacrymans* (Eastwood et al. 2011), *Stereum hirsutum* (Floudas et al. 2012), *Suillus luteus* (Kohler et al. 2015), *Trametes versicolor*, and *Wolfiporia cocos* (Floudas et al. 2012). More fungal genomes of all categories of mushrooms are likely to come, all of which await exploitation.

## 13.2 The Value of Fungal Genomes

In the past, discoveries of metabolic gene functions were commonly activity driven by encoded enzymes and their products; via appointing mutants, smart selection methods, and function-based genetic screenings; and through elucidation of metabolic synthesis pathways by methods such as of isotope and precursor feedings combined with chemical structure clarifications (Casselton and Zolan 2002; Keller et al. 2005; Bills and Gloer 2016).

The established fungal genomes however offer now in addition invaluable platforms for advanced genome mining, for example, for searching of biosynthesis gene clusters (BGCs) for natural bioactive products generated from multienzyme pathways, applying first in silico sequence searches and suitable bioinformatics methods (Wawryzn et al. 2015; Bills and Gloer 2016; van der Lee and Medema 2016) which subsequently can be combined with experimental activity research on identified genes (Bills and Gloer 2016). The latter might be addressed through heterologous gene expression, e.g., in *Escherichia coli* (e.g., Agger et al. 2009; Quin et al. 2013a; Sun et al. 2016; Yang et al. 2016a; Zhou et al. 2016; Alberti et al. 2017; Braesel et al. 2017; Lin et al. 2017), *Saccharomyces cerevisiae* (Walser et al. 2004; Agger et al. 2009; Ishiuchi et al. 2012; Alberti et al. 2017; Nielsen and Nielsen 2017), *Pichia pastoris* (Xue et al. 2008; Bastiaan-Net et al. 2013; Lin et al. 2013, 2016), or *Aspergillus* species (Yaegashi et al. 2014; Alberti et al. 2017; Braesel et al. 2017). Recently, for the first time, a complete gene cluster from a mushroom has thus been functionally expressed in *Aspergillus oryzae* (Bailey et al. 2016; Alberti et al. 2017). Alternatively, if a transformation system exists for a fungus (in the basidiomycetes still rare), gene functions might be elucidated through homologous expression via in vitro-modified vector constructs providing, e.g., efficient promoters of superior regulatory schemes to cloned genes encoding biosynthesis enzymes (Bailey et al. 2016; Alberti et al. 2017) or gene-specific regulatory proteins (Fox and Howlett 2008; Brakhage and Schroeckh 2011), or respective genes might be knocked out (Bailey et al. 2016; Sun et al. 2016; Yu et al. 2016).

Importantly, genome mining can be used to find the biosynthetic genes for already known products but also for novel products of hitherto unknown existence. Examples of all situations can be found in this article. Different approaches for finding new genes by genome mining are possible. Whole genome comparisons, for example, can identify genes and gene clusters unique to a species as potential candidates for the production of unusual, "exotic," bioactive compounds not found in other species (Wang et al. 2016c). Guesses on gene products, e.g., type of encoded enzyme families, and in the case of gene clusters on the combinations of enzymes provided can direct further research to eventually identify the synthesized metabolic products and their intermediates. Moreover, computer programs can be trained to specifically identify BGCs with relevant (highly conserved) genes but also to identify BGCs with more obscure genes and cluster organization. Motif-independent prediction programs make use of, e.g., transcriptomic data. On the other hand, a known bioactive secondary metabolite might be assigned to genes potentially responsible for their production. Where enzymes for biosynthesis of similar

metabolic products or for potential common precursors and intermediates are known, guesses can be made on the type of genes that should participate in the synthesis of the compound of interest. Genome searches with the respective enzymes of known functions will help by sequence identities and similarities to find candidate genes encoding the desired enzymes for a chemically defined metabolite (Umemura et al. 2013, 2015; Koczyk et al. 2015; Li et al. 2016c; van der Lee and Medema 2016).

Genome search approaches with amino acid sequences can of course also be performed with other, nonenzymatic bioactive proteins and peptides. Sometimes, however, revealing amino acid stretches of identity or similarity between proteins or peptides are very short or key amino acids in motifs are individually spread over larger sequence distances. Various bioinformatics tools are being developed to elegantly address such cases in genome mining. Known structural information from similar proteins and peptides in addition can be included into computer-directed searches and, of course, any possible further knowledge such as related to other genes expected to be positioned in respective BGCs (Nagano et al. 2016; van der Lee and Medema 2016; Hetrick and van der Donk 2017). Nevertheless, busy manual genome mining can be very helpful to (first) identify any informative motifs, and it is sometimes superior and more complete as compared to bioinformatics methods with discrete algorithms designed for finding small motifs (Niculita-Hirzel et al. 2008; Kües et al. 2015; Pulman et al. 2016; van der Velden et al. 2017).

In tendency in fungi, genes for a multienzyme biosynthesis pathway for given secondary metabolites localize together (Brakhage and Schroeckh 2011; Wiemann and Keller 2013; Yaegashi et al. 2014; Li 2016c), although in the so far understudied *Basidiomycota* this might be less true than in the *Ascomycota* (see opposing examples in the text below), or it might depend on the biochemical type of the metabolites of concerns (Schmidt-Dannert 2015). Many novel fungal metabolic gene clusters identified via genome mining appear at first sight to be silent (referred to as cryptic or orphan BGSc), failing to provide any interesting metabolic product(s). However, strategies are presented as for how to activate silent gene clusters through the application of types of stresses, such as co-culturing, e.g., with bacteria, other fungi, or nematodes (Zheng et al. 2011; Plaza et al. 2014; Yao et al. 2016; Tauber et al. 2016). Induced gene expression can then be followed up by transcriptomics, proteomics, and metabolomics (Kuan et al. 2013; Ren et al. 2013a; Plaza et al. 2014; Yap et al. 2015a,b; Yu et al. 2015; Martinez et al. 2016; Yao et al. 2016; Tauber et al. 2016) and coexpression correlations (Umemura et al. 2013, 2015).

In the following, we are reviewing the recent progress made in identification and characterization of genes involved in the production of specific bioactive secondary metabolites, peptides, and proteins with (potential) pharmaceutical value through the now available genomes of many of the interesting mushrooms.

## 13.3 Bioactive Secondary Metabolites and Their Enzymes for Biosynthesis

### 13.3.1 Sesquiterpene Synthases

Sesquiterpenes (C15 terpenoids) are volatile terpenes (isoprenoids) with a skeleton of three isoprene (2-methyl-1,3-butadiene) units. They are produced from the mevalonate pathway-derived five-carbon precursors dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) through the 10-carbon fusion product geranyl pyrophosphate (GPP) and another IPP via the 15-carbon compound farnesyl pyrophosphate (FPP). Sesquiterpene synthases dephosphorylate and cyclize the linear intermediate FPP to a multiplicity of different cyclic sesquiterpene scaffolds (>300) with the formula $C_{15}H_{24}$ (Miller and Allemann 2012; Li and Wang 2016). Sesquiterpenes might be monocyclic, bicyclic, or tricyclic, and they further differ by rearrangements and by side chain modifications through actions of a multitude of cytochrome P450 monooxygenases, oxidoreductases, further oxygenases, and group transferases (Quin et al. 2014). The wealth of different sesquiterpenes described from macrofungi and their medicinal potential is thus stupendous (Lorenzen and Anke 1998; Abraham 2001; Christianson 2006; Ajikumar et al. 2008; Fraga 2011; Li and Wang 2016).

The first sesquiterpene synthase genes from a sequenced mushroom to be cloned were from *C. cinerea* (Agger et al. 2009). This helped to find further sesquiterpene synthase genes in other genomes (>1000 genes in ~100 fungal genomes by year 2014) and, where present, associated gene clusters with genes for precursor biosynthetic enzymes, sesquiterpene-modifying enzymes, transcription factors for putative cluster regulation, and metabolite-related transporters (Wawrzyn et al. 2012; Quin et al. 2014). Table 13.1 gives an overview on so far cloned and characterized sesquiterpene synthases from the *Agaricomycetes* with their specific products as the first entry into the very complex and variable enzymology of sesquiterpene synthesis from FPP in this fungal class. Enzymes differ in their modes of cyclization (by 1,6-, 1,10-, or 1,11-ring closures, respectively). Some of the cloned sesquiterpene synthases share the same products, while others generate sesquiterpene scaffolds of quite different structure. Some of the enzymes are quite specific with only one or mainly one product, and these can be assigned to a specific function, while others are variable promiscuous in their product range. Importantly, however, not all of the detected sesquiterpenes are direct products of the cloned enzymes but result from secondary chemical rearrangements (Table 13.1).

Sesquiterpene synthases are specifically identified by the two characteristic aspartate-rich motifs DDXXD/E and NDE/DTE located at the entrance of the catalytic enzyme center to coordinate a trinuclear $Mg^{2+}$ cluster for binding the pyrophosphate moiety of the FPP substrate, positioning its isoprenyl chain into the pocket, and triggering the pyrophosphate cleavage (Schmidt-Dannert 2015). Enzymatic specificity is then influenced by amino acids in the conserved H-α1 loop which is found C-terminal to the NDE/DTE motif and caps the active site of the enzymes upon FPP binding. Accordingly, amino acid changes in Cop3 and Cop4 of

**Table 13.1** Cloned sesquiterpene synthases from the *Agaricomycetes* and detected products from FPP

| Fungus | Compounds produced by fungus | | Sesquiterpene synthase | | Reference(s) |
|---|---|---|---|---|---|
| | Name | Bioactivities | Gene and enzyme | Enzymatic activities | |
| *Armillaria gallica* | Melleolides, composed of a protoilludane esterified to an orsellinic acid moiety | Antibacterial, antimicrobial, cytotoxic, phytotoxic | *pro1*; protoilludene synthase Pro1 (AGR34199) | Forms exclusively Δ6-protoilludene, involved in biosynthesis of melleoides | Arnore et al. (1986), Obuchi et al. (1990), Bohnert et al. (2011, 2014), Engels et al. (2011), and Kobori et al. (2015) |
| *Boreostereum vibrans* | Cadinane sesquiterpenoids boreovibrins A-G | Boreovibrins E and F are weak inhibitory to 11*β*–hydroxysteroid dehydrogenase activity | *BvCS*; δ-cadinol synthase (KU668561) | Forms mainly δ-cadinol and some germacrene D-4-ol in the biosynthesis pathway of boreovibrins | Ding et al. (2012), Zhao et al. (2013), and Zhou et al. (2016) |
| *Coprinopsis cinerea* | Lagopodin, hydroxylagopodin B, coprinastatins, coprinol, oxazolinone, illudosins, armillols | Antibiotic, anti-tumorous | *cop1*; germacrene A synthase | Forms β-elemene[a], α-muurolene, δ-cadinene, germacrene D | Bottom and Siehr (1975), Bu'Lock and Darbyshire (1976), Johansson et al. (2001); Wihlborg et al. (2008), Agger et al. (2009), Lopez-Gallegro et al. (2010a, b), Pettit et al. (2010a, b), and Lauchli et al. (2014) |
| | | | *cop2*; germacrene A synthase | Forms β-elemene[a], α-muurolene, δ-cadinene, respectively, the alcohols α-cadinol, germacrene D-4-ol | |
| | Volatile sesquiterpenes from culture: pentalenene, α-muurolene, α- cuprenene, δ-cadinene, β-caryophyllene, coprinastatin 1, coprinol, 7,7a-diepicoprinastatin 1, 14-hydroxy-5-desoxy-2S,3S,9R-illudosin, 4,5-dehydro-5-deoxyarmillol | | *cop3*; α-muurolene synthase | Forms α-muurolene, (major product), β-elemene[a], α-muurolene, germacrene D, δ-cadinene | |
| | | | *cop4*; promiscuous δ-cadinene synthase | Forms δ-cadinene (major product), β-cubebene, sativene, β-copaene, cubebol; forms with cis-FPP as substrate α-acoradiene (major product), some β-bisabolene and others | |
| | | | *cop5*; activity unknown | No product detected | |
| | | | *cop6*; specific α-cuprenene synthase | Forms nearly exclusively α-cuprenene in the biosynthesis pathway of lagopodin; forms with cis-FPP as substrate a major unidentified product, α-acoradiene, amorpha-4,11-diene | |

| | | | | | |
|---|---|---|---|---|---|
| *Fomitopsis pinicola* | Δ6-Protoilludene; α-barbatene, β-barbatene, α-muurolene, γ- muurolene, γ- cadinene, δ-cadinene, α-copaene; β-copaene; and several more | | ID84944; specific α-cuprenene synthase | Forms nearly exclusively α-cuprenene | Fäldt et al. (1999), Rösecke et al. (2000), and Wawrzyn et al. (2012) |
| *Omphalotus olearius* | Illudin-S, illudin-M, illudosin, illudol, illudosone, illudiolone, isoomphadione, omphadiol | Antimicrobial, cytotoxic, anti-tumorous | *omp6*/Δ6-protoilludene synthase | Forms in the absence of $Ca^{2+}$ exclusively Δ6-protoilludene in the biosynthetic pathway of illudins; promiscuous in the presence of $Ca^{2+}$: Δ6-protoilludene, β-elemene[a], (*E*)-β-caryophyllene, 4,11-selinadiene, β-selinene, α-selinene | McMorris and Anchel (1965), McMorris et al. (1971, 2000, 2002), Morisaki et al. (1985), Arnone et al. (1991), McCloud et al. (1996), Mayer et al. (1997), Engler et al. (1998), Schobert et al. (2008), Wawrzyn et al. (2012), Quin et al. (2013b, 2015),and Yang et al. (2016a) |
| | Major volatile sesquiterpenes from culture: Δ6-protoilludene, pentalene, african-2-ene, african-3-ene, α-barbatane, δ-cadinene, *trans*-dauca-4(11),8-diene | | *omp7*/Δ6-protoilludene synthase | Forms in the absence of $Ca^{2+}$ mainly Δ6-protoilludene in the biosynthetic pathway of illudins, some pentalenene; promiscuous in the presence of $Ca^{2+}$: Δ6-protoilludene, β-elemene[a], (*E*)-β-caryophyllene, β-selinene, α-selinene | |
| | | | *omp1*/selective α-muurolene synthase | Forms α-muurolene | |
| | | | *omp3*/β-elemene α-muurolene synthase | Forms β-elemene[a], α-muurolene (major product), selina-4,7-diene, δ-cadinene | |
| | | | *omp4*; promiscuous δ-cadinene synthase | Forms primarily δ-cadinene and small amounts of 16 other sesquiterpenes | |
| | | | *omp5a*; δ-cadinol synthase | Forms β-elemene[a], γ- cadinene, *epi*-zonarene (minor product) | |
| | | | *omp5b*; δ-cadinol synthase | Forms β-elemene[a], γ- cadinene | |
| | | | *omp9*; α-barbatene synthase | Forms α-barbatene (major product), β-barbatene | |
| | | | *omp10*; carotene synthase | Forms daucene, trans-dauca-4(11),8-diene | |

(continued)

**Table 13.1** (continued)

| Fungus | Compounds produced by fungus | | Sesquiterpene synthase | | Reference(s) |
|---|---|---|---|---|---|
| | Name | Bioactivities | Gene and enzyme | Enzymatic activities | |
| *Stereum hirsutum* | δ-Cadinol, hirsutenes, hirsutenols, hirsutic acid C, sterins, sterhirtusin C and D, chlorosterone, complicatic acid | Anti-oxidative, nitric oxide inhibitory, antimicrobial, antiviral, immunosuppressive, cytotoxic, apoptosis inducing, autophagy inducing | ID25180; Δ6-protoilludene synthase (1,11-cyclization) | Forms in the absence of $Ca^{2+}$ exclusively Δ6-protoilludene; possibly involved in biosynthesis of sterostreins; promiscuous in the presence of $Ca^{2+}$: Δ6-protoilludene, β-elemene[a], (*E*)-β-caryophyllene, β-selinene, α-selinene | Heatley et al. (1947), Ainsworth et al. (1990), Yun et al. (2002a, b), Yoo et al. (2005, 2006), Li et al. (2006, 2008, 2011a), Opatz et al. (2008), Liermann et al. (2010), Liu et al. (2010), Isaka et al. (2011), Quin et al. (2013a, 2015), Yang et al. (2013), Zheng et al. (2013), Ma et al. (2014), Qi et al. (2014, 2015), andTian et al. (2016) |
| | From *Stereum* sp. strains: stereumins, sterpuranes, sterostreins, cadinanes, sterelactones | Nematicidal, antimicrobial | ID64702 and ID73029; Δ6-protoilludene synthases (1,11- and 1,10-cyclization) | Forms Δ6-protoilludene (major product), β-elemene[a] | |
| | Major volatile sesquiterpenes from culture: 1,11-cyclization products Δ6-protoilludene, α-humulene, hirsutene, pentalene; 1,10-cyclization products β-elemene, δ-cadinene; 1,6-cyclization product sesquisabinene A, (*E*)-β-caryophyllene | | ID159379; β –barbatene synthase (1,6-cyclization) | Forms β-barbatene (major product), α-barbatene, α-cuprene | |
| | | | ID128017; δ-cadinene synthase (1,10- and 1,6-cyclization?) | Forms δ-cadinene (major product; possibly involved in biosynthesis of stereumins), β-coparene, sativene, γ-muurolene, α-muurolene, β-cubebene, germacrene D, sesquisabinene A | |

| | | | | | |
|---|---|---|---|---|---|
| *Taiwanofungus camphoratus* | Antrocin, antroquinonols as sesquiterpene derivatives, aamong various other sesquiterpenes in culture | Cytotoxic, apoptosis inducing, anti-tumorous | M_Fcontig14619; AcTPS1 | Forms δ-cadinol | Geethangili and Tzeng (2011), Rao et al. (2011), Tsai et al. (2011, 2012), Xia et al. (2011), Yeh et al. (2013), Sulake and Chen (2015), Chui et al. (2016), and Lin et al. (2017) |
| | Labdane derivatives | Neuroprotective | M_Fcontig13346; AcTPS2 | Forms acyclic α-farnesol | |
| | | | M_Fcontig26676; AcTPS3 | Forms acyclic α-farnesol | |
| | | | M_Fcontig40411; AcTPS4 | Forms α-cubebene, sibirene, γ-amorphene, γ-cadinene, zonarene, acyclic α-farnesol | |
| | | | M_Fcontig40579; AcTPS5 | Forms α-cubebene, γ-muurolene, γ-amorphene, γ-cadinene, δ-cadinene, T-cadinol, α-cadinol, acyclic α-farnesol | |
| | | | M_Fcontig28068; AcTPS6 | Forms nerolidol | |
| | | | M_Fcontig36944; AcTPS7 | Forms α-cubebene, nerolidol, acyclic α-farnesol | |
| | | | M_Fcontig47706; AcTPS9 | Forms α-cubebene, sibirene, γ-amorphene, cubebol, 1-epi-cubenol, acyclic α-farnesol | |
| | | | M_Fcontig62520; AcTPS10 | Forms nerolidol, α-cadinol, acyclic α-farnesol | |
| | | | M_Fcontig44443; AcTPS11 | Forms acyclic α-farnesol | |

[a]Heat-induced Cope-rearrangement product from germacrene A (Selzer 2008)

*C. cinerea* and replacing the loop of Cop4 by that of Cop6 changed product profiles of the enzymes, whereas mutations in the H-α1 loop of Cop6 and introduction of the Cop4 H-α1 loop into Cop6 had no effects on the product outcomes (Lopez-Gallego et al. 2010a, b). Single amino acid replacements in Cop2 outside the functional center (L59H, S310Y, T65A) improved the selectivity of the enzyme for product germacrene D-4-ol (Lauchli et al. 2014).

Phylogenetic analysis can help to predict specific reactions from sesquiterpene synthases obtained from genome mining. *Basidiomycota* sesquiterpene synthases split by overall sequence conservation into five main clusters which reflect different types of cyclization mechanisms (e.g., 1,6-cyclization; 1,10-cyclization; 1,11-cyclization) and make predictions for their expected types of products possible (Wawrzyn et al. 2012; Quin et al. 2013a; de Sena Filho et al. 2016; Tao et al. 2016).

The analysis on the diverged sesquiterpene biosyntheses in the fungi is however still in its infancy, with only a few cases reported where enzyme activities subsequent to the syntheses of sesquiterpene backbones were identified. The sesquiterpene synthase gene *Pro1* from *Armillaria gallica*, for example, forms Δ6-protoilludene (Engels et al. 2011) to which after oxygenation reactions an orsellinic acid moiety is esterified to give antimicrobial and cytotoxic hybrid terpenoids termed melleolides (Bohnert et al. 2011, 2014; see also Sect. 13.3.4). The monocyclic polyketide orsellinic acid is produced by a seven-domain nonreducing polyketide synthase (NR-PKS) ArmB (Lackner et al. 2013; Sect. 13.3.4). The melleolide-biosynthetic gene cluster with *Pro1* has been deduced from the established genome of *A. mellea* which includes also the characterized gene *ArmB* for the orsellinic acid synthase and 14 other genes for putative cytochrome P450 monooxygenases, $NAD^+$-dependent oxidoreductases, a flavin-dependent oxidoreductase, and an *O*-methyltransferase (Lackner et al. 2013; Wick et al. 2016). However, outside the gene cluster spread over four different genomic locations, there are additional genes for melleolide modification, *ArmH1* to *ArmH5* for FAD-dependent halogenases with a canonical signature sequence (FW[A/V]W[F/L]I). These five halogenases were all able to chlorinate melleolide F as the compound experimentally tested for such a post-PKS-biosynthetic step. In addition, bromonation activity was demonstrated for ArmH4 as one selected enzyme (Misiek et al. 2011; Wick et al. 2016). In the satellite gene cluster for melleolide modification together with *armH1* and *armH2*, a gene *armA* is found as another characterized gene which encodes a tridomain enzyme for adenylation of primarily L-leucine and L-threonine. Although the gene is expressed under melleolide production conditions, any biochemical link to melleolide production is not obvious (Misiek et al. 2011).

Of the six identified sesquiterpene synthase genes in *C. cinerea* (Table 13.1), only the *cop6* gene appears to be part of a biosynthetic gene cluster. *cop6* is flanked by two genes for cytochrome P450 monooxygenases (*cox1*, *cox2* for α-cuprenene oxidases). The P450 enzyme Cox2 oxidizes the α-cuprenene produced by the highly specific enzyme Cop6 to α-cuparene and α-cuparophenol, while Cox1 oxidizes α-cuprenene to unknown hydroxy or ketone derivatives, respectively, and likely also α-cuparophenol into a keto-derivative (Agger et al. 2009). *C. cinerea* produces blue-stained antibiotic lagopodins (Bottom and Siehr 1975; Bu'Lock and Darbyshire

1976) which can principally be generated from α-cuprenene through multiple oxidations to which Cox1 and Cox2 likely contribute (Agger et al. 2009).

*O. olearius* has in total 11 genes for sesquiterpene synthases (Table 13.1). *omp1*, *omp6*, and *omp7* are located in distinct gene clusters. *omp6* is found in the largest cluster of about 25 kb with four P450 genes and 14 other genes for a selection of putative oxidoreductases, transferases, drug transporters, a GTPase1 anthranilate synthase-related enzyme, and a poly-galactonurase. *omp7* clusters with one P450 gene and one gene for a FAD-binding protein, and they originate possibly from a partial duplication of *omp6* cluster genes. Omp6 and Omp7 both produce Δ6-protoilludene. *omp1* for a selective α-muurolene synthase groups with a single P450 gene and three genes for enzymes suggested to act in the modification of α-muurolene. α-Barbatane synthase Omp9 falls into the same clade of enzymes as seven of the total 17 different sesquiterpene synthases of *F. pinicola* (Table 13.1). In this species, only enzyme Fompi1 is encoded in a small gene cluster with two P450 genes (Wawrzyn et al. 2012). α- and β-Barbatane are products of *F. pinicola* (Rösecke et al. 2000). However, Fompi1 is a highly specific α-cuprenene synthase similar to Cop6 of *C. cinerea* (Wawrzyn et al. 2012).

Cluster predictions were also provided for *S. hirsutum*. Genes 159379 for β-barbatene synthase and 128017 for δ-cadinene synthase (Table 13.1) are located in distinct clusters together with genes for a range of oxidoreductases, but there is no P450 gene. δ-Cadinene is the likely precursor for the antibacterial stereumin. P450 monooxygenases required for stereumin synthesis may thus be provided from satellite gene clusters or individual genes still to be defined (Quin et al. 2013a). Notably, the *Agaricomycetes* commonly possess large armies of potential P450 genes (>100, e.g., Doddapaneni et al. 2005; Wawrzyn et al. 2012; Syed et al. 2014; Kües et al. 2015; Qhynya et al. 2015; Mgbeahuruike et al. 2017). Genes 25180, 64702, and 73029 for the three functional Δ6-protoilludene synthases in *S. hirsutum* (Table 13.1) in contrast are all located in larger gene clusters together with genes for a variety of scaffold-modifying enzymes including for several P450 monooxygenases. Some of these are closely related to the P450 enzymes encoded in the *omp6* and *omp7* gene clusters of *O. olearius*, which suggests shared origins of these clusters of the different species. The cluster in *S. hirsutum* with gene 64702 contains also a gene for a transporter. Cytotoxic sterostreins could be products of the three *S. hirsutum* clusters with the functional Δ6-protoilludene synthase genes (Quin et al. 2013a, b). Altogether, there appears to be a trend in the *Agaricomycetes* that genes for Δ6-protoilludene synthases associate with biosynthetic gene clusters (Schmidt-Dannert 2016).

The newest whole genome analysis for sesquiterpene synthase genes is from *T. camphoratus* (Lin et al. 2017; Table 13.1). In this fungus, one or two P450 genes localize in the vicinity of the genes for the enzymes AcTPS1, AcTPS2, AcTPS3, and AcTPS6, the genes for enzymes AcTPS4 and AcTPS9 are linked, and one to a few genes for potential modification have been identified in the closer and wider genomic environments of all sesquiterpene genes. However, there are usually several additional genes for other unrelated or unknown functions, and genes from a shared chromosomal region appear to be not much in parallel regulated in mycelial

growth and during fruiting (Lin et al. 2017), shedding doubt on that many of these genes sit in true sesquiterpene biosynthetic gene clusters. In conclusion, a gene cluster situation for sesquiterpene synthesis is not necessarily the canonical situation in the *Agaricomycetes*.

### 13.3.2 The Pleuromutilin Gene Cluster

Pleuromutilin and its natural and semisynthetic derivatives are antibacterial tricyclic diterpenes (C20 terpenoids) from *Clitopilus* species (Hartley et al. 2009) which block bacterial ribosome function by binding to the peptidyl transferase component of the larger ribosomal subunit (Lolk et al. 2008; Eyal et al. 2016). Some of these compounds are long appointed in veterinary medicine, and some are considered for human use (Paukner and Riedl 2017).

A gene cluster for the synthesis of pleuromutilin has recently been identified from the saprotrophic agaricomycete *Clitopilus passeckerianus* by homologous and heterologous gene expression studies (Bailey et al. 2016). Here, the authors made successfully use of the repeated observation that fungal genes for enzymes of secondary metabolite biosynthesis pathways often come in clusters (Brakhage and Schroeckh 2011; Wiemann and Keller 2013; Yaegashi et al. 2014). They had further the biochemical expectation that a gene for a geranylgeranyl pyrophosphatase (GGPP) synthase (GGS; a diterpene synthase for GGPP production from FPP and IPP as a key step in the synthesis of terpenes) should be implicated in the pleuromutilin biosynthesis pathway. The authors designed degenerate primers to successfully screen a genomic λ phage library for GGS genes and identified by subsequent sequencing and expression studies the complete cluster with seven genes in total (Fig. 13.1). The authors did not provide the actual sequences of the genes and the encoded proteins (Pl-P450-3, cytochrome P450; Pl-ATF, acetyltransferase; Pl-CYC, terpene cyclase; Pl-GGS; Pl-P450-1 and Pl-P450- 2, cytochrome P450s, Pl-SDR, short-chain dehydrogenase/reductase), but a table supplied with the closest known homologues from other organisms (Bailey et al. 2016) allowed us to use these in genome mining of other mushrooms in order to find related gene clusters.

In pBlast searches of the *Agaricomycetes* entries in GenBank at NCBI, all proteins but the *Gibberella fujikuroi* terpene cyclase (*ent*-kaurene synthase; Q9UVY5) gave longer lists of significant hits in various fungal species. However, sequence evidence for potential CYC functions for a two-step cyclation of GGPP was only found in *P. strigosozonata*, *S. lacrymans*, *Gymnopus luxurians*, *Moniliophthora roreri* (partial sequences), and *Moniliophthora perniciosa* (partial sequences; see also Mondego et al. 2008; Fischer et al. 2015). The *cyc* genes in *P. strigosozonata* and *S. lacrymans* cluster with *ggs*, *p450*, and *sdr* genes which resembles the gene composition of the pleuromutilin biosynthesis cluster (Fig. 13.1), unlike the three potential *cyc* genes in *G. luxurians* which locate to fully different gene contexts. A similar analysis of the genomic environment is not yet possible for the two *Moniliophthora* species (synonyms *Crinipellis roreri* and *Crinipellis perniciosa*) by too short available scaffold lengths. However, these fungi synthesize gibberellin-like diterpenoid

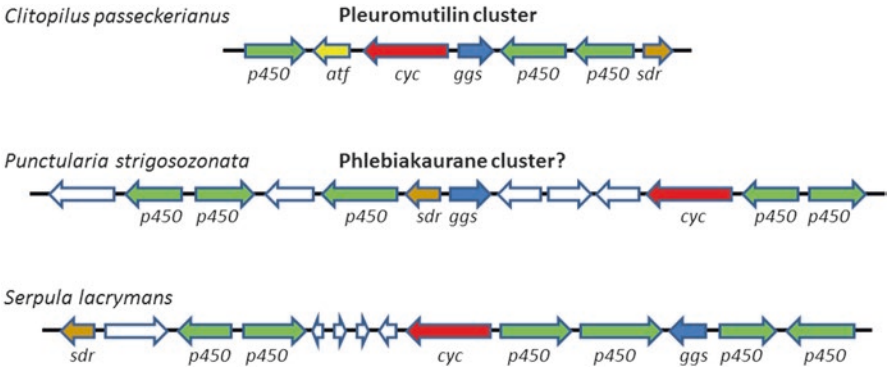


**Fig. 13.1** Gene arrangements in the pleuromutilin cluster of *C. passeckerianus* (Bailey et al. 2016) and putative antibiotic biosynthesis gene clusters of *P. strigosozonata* and *S. lacrymans* S7.9 (as deduced from the MycoCosm page, April 2017). *Note*: A shared *color* indicates same types of gene functions, the *arrows* direction of transcriptions. *ggs* encodes geranylgeranyl pyrophosphate synthase, *cyc* terpene cyclase, *p450* cytochrome P450, *atf* acetyltransferase, *sdr* short-chain dehydrogenase/reductase; *white arrows*: other functions

acids which in analogy of gibberellin biosynthesis is expected to happen via *ent*-kaurene (Mondego et al. 2008). In the past, red and violet terphenyl chinone pigments (phlebiarubrones) and phlebiakauranes with antibacterial and selective antifungal activities have been reported from cultures of *P. strigosozonata* and *Punctularia atropurpurascens* (Lisy et al. 1975; Anke et al. 1984, 1987), and there is also a report on phlebiakaurane production in addition to crinipellins (tetraquinane diterpenoids) by a strain *Crinipellis* sp. 113 (Li and Shen 2010). Synthesis of kaurane diterpenes employs a terpene cyclase and P450 oxygenases and monooxygenases for post-kaurene modifications (Takahashi et al. 2014), and the identified *P. strigosozonata* gene cluster might thus be responsible for phlebiakaurane production. The *S. lacrymans* CYC is reported to give rise to the tricyclic diterpene *ent*-kauranol, differentially from the *C. passeckerianus* enzyme which forms the unique tricyclic pleuromutol core with a C8 ring from GGPP (Proteau et al. 2012). We are however not aware of any kaurane diterpenes described from *S. lacrymans*.

Production of kaurane diterpenes is generally rare in basidiomycetes (Anke et al. 1987; Shen et al. 2009; Yang et al. 2012). An interesting further observation is thus that the GGSs from the putative antibiotic clusters of *P. strigosozonata* and *S. lacrymans* (and also *C. passeckerianus*?) are of the same origin than a larger group of ascomycetous enzymes, unlike the other identified basidiomycetous GGSs coming from *G. luxurians*. An origin from horizontal gene transfer is therefore suggested for the clusters (Fischer et al. 2015). Some *Chytridiomycota*, *Mucoromycota*, and insect GGSs appear also to be closely related (our unpublished observations). A further analysis of the evolutionary origin of the GGSs and the whole gene clusters could thus be very interesting.

### 13.3.3 Ganoderic Acid

*Ganoderma* species are especially rich in secondary metabolites (Peterson 2006; Sanodoya et al. 2009; Batra et al. 2013; Xia et al. 2014; Baby et al. 2015; Duru and Çayan 2015; Richter et al. 2015). From the multitude of bioactive triterpenoids in *Ganoderma* species (>150 different triterpenoids have been isolated at different fungal developmental stages; Chen et al. 2012; Duru and Çayan 2015), ganoderic acids and derivatives are best known as highly oxygenated lanostane-type triterpenoids (C30 terpenoids) with potent anticancer activities (Cheng et al. 2010; Shi et al. 2010; Xu et al. 2010; De Silva et al. 2013; Wu et al. 2013), inhibition effects on cholesterol synthesis (Komoda et al. 1989; Hajjaj et al. 2005), and antioxidant, antidiabetic, hepatoprotective, and other activities (Zhu et al. 1999; Shi et al. 2010; Peng et al. 2013; Liu et al. 2015b; Tang et al. 2016c).

As for other terpenoids, ganoderic acid synthesis takes its origin in the mevalonate pathway, via FPP, squalene, and lanosterol as intermediates (Shiao 2003; Shi et al. 2010; Chen et al. 2012). Thirteen genes for the 11 enzymes (for two enzymes there are two genes in *G. lucidum*) of the mevalonate pathway up to lanosterol cyclization by the 2,3-oxidosqualene-lanosterol cyclase (lanosterol synthase) are known (Chen et al. 2012; Liu et al. 2012a; Kües et al. 2015; Zhu et al. 2015). Genetic engineering to overexpress various cloned genes from the upstream mevalonate pathway successfully leads to improved ganoderic acid synthesis (Shi et al. 2010; Xu et al. 2012, 2015; Ren et al. 2013b; Zhou et al. 2014; Xu and Zhong 2015; Zhang et al. 2017), as did introduction of heterologous chaperones as protectants against accompanied oxidative stress (Li et al. 2016a).

Lanosterol is at a branch point of sterol and terpenoid biosynthesis. The fungal-unique essential membrane organizer ergosterol is produced from lanosterol by actions of the P450 enzyme lanosterol 14α-demethylase (CYP51A; lanosterol synthase) (Lepesheva and Waterman 2008; Shi et al. 2010; Chen et al. 2012; Liu et al. 2012a). A series of oxidations, reductions, and acylation reactions are expected to lead alternatively from lanosterol to the different forms of ganoderic acids (Chen et al. 2012). However, genes for biochemical reactions after lanosterol cyclization are not yet been clearly identified. P450 enzymes and also UDP-glucosyltransferases (with six identified genes for potential transfer of sugar moieties to triterpenoid backbones; Liu et al. 2012a) are discussed to act as candidate enzymes in later steps of ganoderic acid synthesis. Compared to any other fungi (ascomycetes and basidiomycetes), *Ganoderma* species have an exceptional high number of genes for oxidative and hydroxylating P450 enzymes containing heme cofactors (cytochromes P450, CYPs), i.e., nearly 200 and more different functional ones with an additional number of pseudogenes (Chen et al. 2012; Liu et al. 2012a; Syed et al. 2014; Zhu et al. 2015). The P450 proteins cluster into 42 different families. Some have only a single member in *Ganoderma* sp. and others multiple members, some are presented with new subfamilies, and some of the families are novel and unique to *Ganoderma* sp. (Chen et al. 2012; Liu et al. 2012a; Kües et al. 2015). The plenty of *p450* genes makes it difficult to predict their individual enzymatic substrate and product specificities. Expression of *p450* genes is variably differentially regulated, with

subgroups (e.g., 15 *CYP512* and one *CYP5144* gene) which correlate in regulation with the lanosterol synthase gene (Chen et al. 2012). A total of 24 gene clusters with three or more different *CYP* genes (three larger clusters longer than 200 kb might split into two or more different biosynthetic gene clusters) were identified in the genome of *G. lucidum* (Chen et al. 2012; Liu et al. 2012a; Kües et al. 2015). Two of these gene clusters show some parallels to the expression of lanosterol synthase, whereas ten other *p450* genes linked to the lanosterol synthase gene do not (Chen et al. 2012). *G. sinense* distinguishes in the number of gene clusters from *G. lucidum* with 29 in total identified gene clusters. Further, the number of *p450* genes in shared gene clusters can vary between the species. Many of the *p450* genes appear to originate from gene duplications, with options of neofunctionalization, subfunctionalization, and increased gene-dosage advantage (Zhu et al. 2015). Because reliable transformation methods and gene silencing techniques for *G. lucidum* are at hand (Mu et al. 2012; Xu and Zhong 2015), it should be possible with time to address the important *p450* genes in ganoderic acid production. Comparison of the P450omes of different species can possibly give some insight into which candidate genes to select for further study (Lu et al. 2014).

### 13.3.4 Polyketide Synthases

Prenylphenols are hybrid molecules of the monocyclic polyketide orsellinic acid and a prenyl side chain of terpene origin. A stereaceous basidiomycete BY1 produces the novel prenylphenol cloquetin. The fungus has a gene cluster with two functional genes for nonreducing polyketide synthases, *PKS1* and *PKS2*, for the synthesis of orsellinic acid and an unlinked gene *BYBP* for a regiospecific prenyltransferase that attaches a prenyl group to orsellinic acid (Braesel et al. 2017). *S. hirsutum* possesses a highly similar gene (69% protein identity to BYBP; XP_007308836; Floudas et al. 2012) and is as other *Stereum* species also a prenylphenol producer (Omolo et al. 2002; Yun et al. 2002b; Braesel et al. 2017). Other mushrooms (e.g., *A. bisporus*, XP_007334149; *A. muscaria*, KIL64777; *A. subglabra* XP_007349181; *C. cinerea*, XP_001839609, XP_001839607; *G. frondosa*, OBZ66374; *G. luxurians*, KIK57132, KIK57133; *P. strigosozonata*, XP_007385948; *S. commune*, XP_003029237; *S. lacrymans*, XP_007322388, XP_007323929) have genes for less similar, yet uncharacterized prenyltransferases (around 25–40 % identity to BYBP). In general, prenyltransferases catalyze regioselective and stereoselective prenylations of aromatic compounds such as tryptophan, tryptophan-containing peptides, indole derivatives, tyrosine, and also nitrogen-free aromates. Among others, FPP synthases and GGPP synthases producing the precursors for sesquiterpene and triterpene synthesis, respectively (Sects. 13.3.1 and 13.3.2), are prenyltransferases (Winkelblech et al. 2015).

Other than *PKS1* and *PKS2* of strain BY1, only a few other genes for polyketide synthases (PKSs) have been characterized in the *Basidiomycota*, i.e., for the production of orsellinic acid in *T. camphoratus* (Yu et al. 2016; Chou et al. 2017), *A. mellea* (Lackner et al. 2012, 2013; ArmB; see Sect. 13.3.1 above) and *C. cinerea* (Ishiuchi

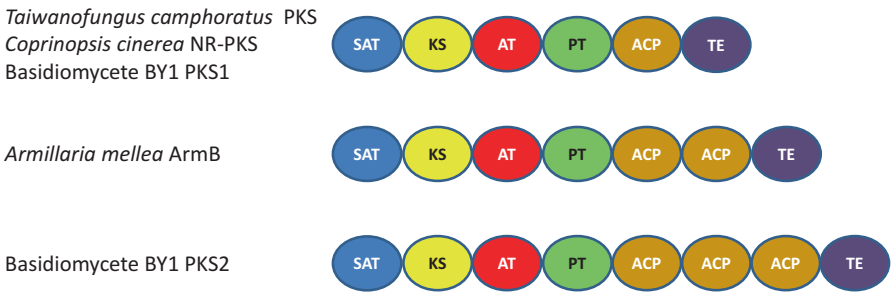


**Fig. 13.2** Domain structures of nonreducing polyketide synthases for the monocyclic polyketide orsellinic acid production from the *Agaricomycetes* (Lackner et al. 2012, 2013; Yu et al. 2016; Braesel et al. 2017) and of the highly reducing polyketide synthases PPS1 and PPS2 for polyene production of the basidiomycete BY1 (Brandt et al. 2017). *Note*: *SAT* starter unit acyl carrier protein transacylase, *KS* β-ketosynthase, *AT* acyl (malonyl-CoA) transferase, *PT* product template domain, *ACP* acyl carrier protein, transacylase, *TE* thioesterase, *DH* dehydratase, *KR* β-ketoreductase, *MT* methyltransferase. Note that orsellinic acid synthases from different fungi differ in numbers of ACP domains

et al. 2012). These enzymes have multi-domain structures as typical for fungal type 1 nonreducing iterative polyketide synthases (NR-PKSs; Fig. 13.2), distinct from modular highly reducing polyketide synthases (HR-PKSs) of clade I (Fig. 13.2) which have additional other domains, from highly reducing polyketide synthases of class II and from hybrid clades I, II, and III polyketide synthases, respectively (Lackner et al. 2012; Du and Lou 2010; Liu et al. 2015a). Basidiomycetous NR-PKSs of class I are monophyletic and divide into two major subclades (Lackner et al. 2013). Genes for similar modular enzymes as PKS1 and PKS2 from strain BY1 are, for example, present in *A. muscaria* (KIL57002), *F. hepatica* (KIY47273), *G. frondosa* (BAO20284), *G. luxurians* (KIK51473, KIK52832), *Hebeloma cylindrosporium* (KIM41076), *P. strigosozonata* (P_007386370), *S. commune* (XP_003038401), *S. lacrymans* (EGN93845), *S. hirsutum* (XP_007307184, XP_007300189; Floudas et al. 2012), *S. luteus* (KIK37237), and *T. versicolor* (XP_008041566). Of these, orsellinic acid metabolites are known from *Stereum* species (Li et al. 2006; Braesel et al. 2017).

Orsellinic acid was further detected in co-culture of *Ganoderma applanatum* and *T. versicolor*. The authors put the production onto *G. applanatum* (Yao et al. 2016), while a respective conserved polyketide synthase gene can also be detected in *T. versicolor* (Koczyk et al. 2015; XP_008041566). The enzyme from gene *pks63787* in *T. camphoratus* is responsible for the synthesis of orsellinic acid from acetyl-CoA and malonyl-CoA. Orsellinic acid is then in parallel routes farnesylated by different CoQ (coenzyme Q)-type synthesizing enzymes (i.e., Coq2, Coq3, and Coq6 which were identified from a pool of total 10 different *coq* genes) to give with additional ring modifications the benzoquinone ring precursors for a variety of antroquinonols with anticancer activities and for the cytotoxic meroterpene 4-acetylantroquinonol B (Yu et al. 2016; Chou et al. 2017).

4-Acetylantroquinonol B production is intimately linked to CoQ synthesis. Production of 4-acetylantroquinonol B can be enhanced by feeding either CoQ or the CoQ precursor 4-hydroxybenzoic acid from the shikimate pathway and by the addition of oleic acids to enhance the mevalonate pathway (Yang et al. 2017).

Most recently, the first modular HR-PKSs have been characterized with the 2736 amino acid long polyene pigment synthases PPS1 and PPS2 of basidiomycete BY1 (Fig. 13.2) from an own clade of fungal PKSs. These enzymes are 99% identical in sequence, likely allelic, and the only HR-PKSs found to be encoded in the host genome. Their genes do not reside in a recognizable gene cluster (Brandt et al. 2017). Genes for enzymes of similar modular structure occur in *M. roreri* and *S. hirsutum* (Brandt et al. 2017), *Fibroporia radiculosa* (XP_012183873, XP_012185398, XP_012183840), *Gloeophyllum trabeum* (XP_007869847), *Jaapia argillacea* (KDQ50123), and *L. sulphureus* (KZT06902, KZT06920). PPS1 was expressed in *Aspergillus niger* and shown to confer production of yellow nematotoxic polyene pigments (18-methyl-19-oxoicosaoctaenoic acid, 20-methyl-21-oxodocosanonaenoic acid) with an unusual shifted conjugated C-C double-bond pattern (Brandt et al. 2017).

Another striking observation from genome mining is that false reports in the literature on functional compounds from mushrooms can be wiped out through genome mining. Together with highlighting misleading flaws in analytical methods used in reports of flavonoid productions of mushrooms, Gil-Ramírez et al. (2016) showed in such manner that mushrooms might have genes for phenylalanine ammonia lyases (e.g., *A. bisporus*, XP_006461241; *P. ostreatus*, KDQ28180) but that they do not have like plants any genes for chalcone synthases and for chalcone isomerases required for flavone synthesis. Distantly related to plant chalcone synthase genes (around 30% amino acid identities between gene products) are exceptional genes (1 or 2 pro genome) for fungal type III polyketide synthases in the mushrooms *Calocera cornea* (KZT59672.1), *Calocera viscosa* (KZO96206), *Dacryopinax primogenitus* (EJT98013), *Exidia glandulosa* (KZW02173), *Phanerochaete carnosa* (XP_007391992, XP_007391993), *Phlebia centrifuga* (OKY58712, OKY58713), and *Sporotrichum laxum* (AMW87979, AMW87980).

Gene knockouts and heterologous expression in *E. coli* proved that the *S. laxum* Pks2 (AMW87980) is an alkylresorcinol synthase which elongates palmitoyl-CoA or palmitoyl-ACP by three malonyl-CoAs and cyclizes the product via a 2,11 intramolecular aldol condensation, with fatty acid acyl-primed triketide and tetraketide pyrenes as by-products (Sun et al. 2016). The alkylresorcinols are precursors of spirolaxine (Sun et al. 2016) which acts as plant growth inhibitor (Arnone et al. 1990), antibacterial (Blaser 1992), cholesterol-lowering (Robinson and Brimble 2007), and inhibitory toward cancer cell lines (Tsukamoto et al. 1998; Gianni et al. 2004). Antibacterial analogs are known from *Phanerochaete velutina* (Dekker et al. 1997). The two *pks* genes in *S. laxum*, *P. carnosa*, and *P. centrifuga* are tail-to-tail arranged and locate in a cluster with a gene for a membrane-bound *O*-acyltransferase (MBOAT) family protein (Sun et al. 2016; XP_007391994 and OKY58711). However, genes for other enzymes expected to participate in the biosynthesis of spirolaxine, and analogs are not present in the cluster (Wang et al. 2013; Sun et al. 2016).

### 13.3.5 The Atromentin Gene Cluster

Atromentin is an NRSP (non-ribosomal peptide synthetase)-like derived terphenylquinone with antibacterial activity and apoptosis-inducing activity in leukemia cell lines (Zeng et al. 2006; Hee and Lee 2009). Atromentin is a central precursor of variegatic acid and various related pigments, for example, for diarylcyclopentenone pigments in *Paxillus involutus* (Braesel et al. 2015) and pulvinic acid-derived pigments in *Suillus* species (Wackler et al. 2012). Variegatic acid and the diarylcyclopentenone pigment involutin have roles in redox cycling during Fenton chemistry in the breakdown of lignocellulosic organic matter (Eastwood et al. 2011; Braesel et al. 2015; Shah et al. 2015).

The synthesis pathway of atromentin has first been elucidated in *Tapinella panuoides*. Atromentin derives from L-tyrosine which is deaminated by the pyridoxal 5′-phosphate-dependent L-tyrosine:2-oxoglutarate aminotransferase AtrD into 4-hydroxyphenylpyruvic acid. Two 4-hydroxyphenylpyruvic acid molecules are then adenylated by the NRSP-like quinone synthetase AtrA (a tridomain enzyme reminiscent to non-ribosomal peptide synthetases, NRPS; see Sect. 13.4.5), ester-bonded to the enzyme, and finally condensed through symmetric C-C bond formation into atromentin. Genes *atrA* and *atrD* are clustered with an *adh* gene (for alcohol dehydrogenase) in between (Schneider et al. 2008). The same genetic arrangement is found in several other basidiomycetes including in *O. olearius*, *Paxillus* species, *S. lacrymans*, and *Suillus* species, and the gene promoters have specific conserved sequence motifs with a potential regulatory function (Wackler et al. 2012; Braesel et al. 2015; Tauber et al. 2016). Confrontation with soil bacteria can elucidate expression of these genes (Tauber et al. 2016).

### 13.3.6 Enzymes for Bioactive Metabolites Evolved from Conventional Fungal Functions

Clavaric acid is a triterpenoid (C30H48) from *H. sublateritium* with an antitumor activity which reversibly inhibits farnesyltransferase activities competitive to Ras (Jayasuriya et al. 1998; Lingham et al. 1998). It is synthesized from squalene alternatively to lanosterol, an essential intermediate in the typical fungal ergosterol pathway. Synthesis occurs in four steps via 2,3-oxidosqualene, squalene, 2,2:22,23-dioxidosqualene, and clavarinone. Steps 1 and probably also 2 are performed by the ERG1 squalene epoxidase, and step 1 is shared with lanosterol production (compare Sect. 13.3.3). Step 3 is performed by the specific oxidosqualene cyclase OCC (Godio and Martin 2009) and step 4 presumably by a clavaric acid synthase. Genes *erg1* and *occ* gene with their encoded products are known. These are unlinked. OCC distinguishes from the lanosterol cyclases by a VSDCVGE motif in its active center (Godio et al. 2007; Godio and Martin 2009). As the only other related enzyme in the NCBI database in April 2017, KIM38979 of *H. cylindrosporum* has the same motif.

### 13.3.7 Others

The FAD-binding monooxygenase and aromatic ring hydrolase VibMO1 of *Boreostereum vibrans* were identified to convert prenyl 4-hydrobenzoate into prenylhydroquinone, likely as one step in the biosynthesis of the unusual fused β-lactone-type metabolites vibralactones and related meroterpenoids (Yang et al. 2016c). Vibralactones can act as lipase inhibitors (Liu et al. 2006) and can have antifungal activities (Schwenk et al. 2016). The gene for the protein closest and related to VibMO1 is from *S. hirsutum* (XP_007305833: 84% identical, 98% similar). Genes for FAD-binding monooxygenases are not uncommon in *Agaricomycetes*. Identity and similarity values for FAD-binding monooxygenases from other mushrooms range around 40% and 60%, respectively. Prenyl hydroquinone is a secondary metabolite also known from *Ganoderma* species (Baby et al. 2015).

A gene *dodA* for a 4,5-DOPA (3,4-dihydroxyphenylalanine) dioxygenase in *A. muscaria* is long known from biosynthesis of the yellow betalain-related pigment muscaflavin with a 7-membered nitrogen heterocycle. DodA opens DOPA either by 4,5-ring cleavage or by an extra 2,3-extradiol cleavage activity to give unstable *seco*-DOPA intermediates which spontaneously recyclize to betalamic acid and muscaflavin, respectively (Hinz et al. 1997; Mueller et al. 1997). DOPA is derived from tyrosine through oxidation by a tyrosinase (a monophenol monooxygenase) restricted to colored mushroom tissues (Mueller et al. 1996). *A. muscaria* has two tyrosinase genes (KIL63081; KIL63082) found in tandem at another chromosomal position than gene *dodA* (our unpublished observation). Betalain pigments are better known from the suborder *Chenopodiniae* of the plant order *Caryophyllales* and are of interest for their antioxidant activities and as food colorants. Muscaflavin is not formed in plants because their enzymes lack significant 2,3-ring cleavage activity (Gandía-Herrero and García-Carmona 2013; Slimen et al. 2017). In the fungi, betalains occur distinctively in species of the three genera *Amanita*, *Hygrocybe*, and *Hygrophorus* (Steglich and Strack 1991; Stintzing and Schliemann 2007; Babos et al. 2011). Many other of the *Agaricomycetes* have also genes for tyrosinases, e.g., DOPA production (Halaouli et al. 2006), and importantly, also genes for potential 4,5-DOPA dioxygenases highly identical in sequence to *A. muscaria* DodA (between 60 and 80% identity and >70 to >90% similarity; e.g., *A. bisporus* XP_007326059: 68% identical, 85% similar). Whether these potential 4,5-DOPA dioxygenases promote 2,3-extradiol cleavage of DOPA or whether they are more stringent in their activities like the plant enzymes remains to be tested.

## 13.4 Peptides: Linear and Cyclic, Ribosomal and Non-ribosomal

### 13.4.1 Ribosomal Cyclic Peptides of the α-Amanitin and Phalloidin Family

Amatoxins and phallotoxins bind to RNA polymerase II and F-actin, respectively (Wieland 1986; Vetter 1998; Göransson et al. 2012). This group of toxins occur in a range of toxic *Amanita*, *Conocybe*, *Galerina*, and *Lepiota* species, with α-amanitin

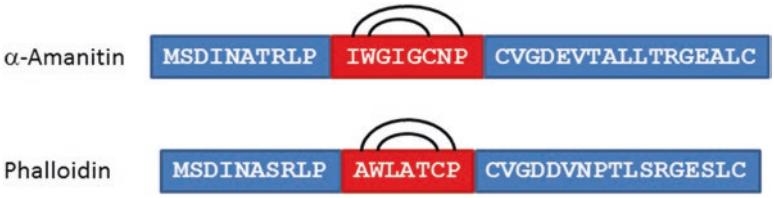


**Fig. 13.3** Precursor proteins for the bicyclic toxins α-amanitin and phalloidin from the MDSIN family of RiPPs (Pulman et al. 2016). *Note*: The conserved leader and follower regions are shown in different shading to the interior core sequences of the toxins. The *bows* mark amino acids involved in macrocyclization and Trp-Cys cross-bridging, respectively

as best known example (Pomilio et al. 2006; Li et al. 2014a, b; Sgambelluri et al. 2014; Pulman et al. 2016; Tang et al. 2016b). They are bicyclic peptides generated from ribosomally produced short precursor peptides (between 33 and 39 amino acids long; Pulman et al. 2016) by the action of a specialized prolyl oligopeptidase B (POPB) of the serine protease family S9a (Luo et al. 2009, 2010, 2014). The precursors are characterized by a conserved 10-aa leader region and a conserved 17-aa follower region with an internal core of different length (between 6 and 10 aa) and sequence which represents the mature cyclopeptide (Fig. 13.3). The conserved sequence of the leader region gave this family of RiPPs (ribosomally synthesized and posttranslationally modified peptides) the name MSDIN family of cyclic peptides (Hallen et al. 2007; Pulman et al. 2016), while the primary sequences outside the core region from *Amanita* species and *Galerina marginata* diverged, and the latter species has only two α-amanitin genes (Luo et al. 2012, 2014; Pulman et al. 2016). Two conserved prolines, one terminal to the conserved leader and one terminal to the core sequence, are required for proteolytic processing (Pulman et al. 2016; Fig. 13.3).

Toxic *Amanita* species have several MSDIN genes (*A. phalloides* and *A. bisporigera* each ~30 with little overlap between the species) mostly for toxins or in some cases also nontoxic cyclopeptides (cycloamanides), but mature products are only found from genes whose predicted precursors have the two conserved prolines (Pulman et al. 2016; Fig. 13.3). The peptide chains are processed and macrocyclized to yield a backbone macrolactam by head-to-tail peptide bonds through peptide bond hydrolysis and transpeptidation by POPB (Luo et al. 2009, 2010; Truman 2016) and further by an unusual internal Trp-Cys cross-bridge via a sulfoxide or a sulfide link for amanitins and phalloidins, respectively (Battista et al. 2000; Göransson et al. 2012). The bicyclic toxins can further diversify by hydroxylations of side chains of their amino acids (Sgambelluri et al. 2014). The *ppob* gene clusters with two *ama* genes for α-amanitin in the *G. marginata* genome and on a genomic cosmid from an *Amanita* species (likely not *A. bisporigera*) with a gene for an MSDIN family cyclic peptide with the sequence GAYPPCPMP (Luo et al. 2010; Pulman et al. 2016).

Another member of the MSDIN family of cyclic peptides is antamanide from *A. phalloides* which is a monocyclic decapeptide (cyclic sequence = cVPPAFFPPFF) with a strong antidote activity against amatoxins and phallotoxins. Moreover, it

shows antitumor and immunosuppressive activities and inhibits the mitochondrial permeability transition pore as an effector of cell death induction through binding of the pore regulator cyclophilin D (Siemion et al. 1992; Azzolin et al. 2011). Immunosuppressive activity has also been reported for *A. phalloides* cycloamanide A (VFFAGP) and B (SFFFPIP) (Wieczorek et al. 1993). Less studied are the monocyclic virotoxins (cVTSPAW) from *A. phalloides* and *Amanita virosa* which bind to actin similar as the phallotoxins (Faulstich et al. 1980; Vetter 1998; Hossain and Park 2016).

### 13.4.2 The Omphalotin Gene Cluster

Omphalotins are cyclic *N*-methylated depsipeptides (WVIVVGVIGVIG) with nematicidal activity from *O. olearius* (Mayer et al. 1997; Sterner et al. 1997). These are members of a new (fourth) class of fungal RiPPs and are produced by a suicidal enzyme OphA. This 399 amino acid-long enzyme has an N-terminal SAM-dependent methyltransferase domain with which it methylates iteratively the amino acids in the depsipeptide motif located at its C-terminus (Fig. 13.3). Methylation occurs intermolecularly. The biochemical process of macrocyclization of the *N*-methylated omphalotins out of their larger protein precursor and the responsible endopeptidase remain to be uncovered. A candidate prolyl oligopeptidase is encoded by gene *ophp* in a gene cluster together with *ophA* and genes *ophB1* and *ophB2* (monooxygenases), *ophC* (NTF2-like), *ophD* (*O*-acyltransferase), and *ophE* (F-box/RNI-like) for putative posttranslational modification functions of the omphalotin core peptide (van der Velden et al. 2017). Posttranslational modifications of the tryptophan residue (to a hexahydropyrrolo[2,3-*b*]indole 2-carboxamide) in the omphalotin core peptide are reported as well as oxidations of other residues (Buchel et al. 1998; Liermann et al. 2009; Ruiz-Sanchez et al. 2011). Importantly, it is possible to exchange amino acids in the depsipeptide motif and its border region without loss of methylation function indicating flexibility in the process (van der Velden et al. 2017).

A related enzyme to OphA with an autocatalytic *N*-methylation function of a similar C-terminal core peptide (Fig. 13.4) has been found in the basidiomycete *Dendrothele bispora* (van der Velden et al. 2017). NCBI pBlast searches with the *D. bispora* protein detect one or more candidate enzymes from a few other agaricomycetes for similar reactions (see selected examples in Fig. 13.3) which could offer potential for further natural cyclic peptides of the new class of RiPPs with the proposed name borosins (van der Velden et al. 2017). Notably, for further attention, a dioxygenase domain with aromatic-ring-cleavage activity is predicted for some of the well-conserved enzymes in between the N-terminal SAM-dependent methyltransferase domain and the C-terminus (around amino acids 315–375). Borosin candidates might also be certain unprecedented highly *N*-methylated cyclopeptides from other agaricomycetes such as the gymnopeptides from *Gymnopus fusipes* and the pteratides from *Pterula* sp. which have antiproliferative activities on human cancer cell lines (Chen et al. 2006; Ványolós et al. 2016).

| | |
|---|---|
| *Omphalotus olearius* (van der Velden et al. 2017) | GFPWVIVVGVIGVIGSVMSTE |
| *Dendrothele bispora* (ID 765759) | GFPWVIVTGIVGVIGSVVSSA |
| *Lentinula edodes* (GAW09067) | GFPWIIVVGVVGVVGSVVSSA |
| *Rhizopogon vinicolor* (OAX31299) | GFPTVLVILPTVIVVLIGRE |
| *Moniliophthora roreri* (XP_007857024.1) | GKPTAFLSAVVIATIIIAL |
| *M. roreri* (KTB34288) | GKPTAFVGLVVIIAVVV |
| *Moniliophthora perniciosa* (EEB90231) | GKPVAFLSAVVIATIIIAL |
| *Fomitiporia mediterranea* (EJD06538) | GTPHPALALLVVIICLI |
| *Hydnomerulius pinastri* (KIJ64101) | NEPAALTTMINIHVTHV |
| *R. vinicolor* (OAX33483) | NGPQGLGTIILVWHTVHGIA |
| *R. vinicolor* (OAX39229) | DGPEGLAVVVIVLVATVALLALLV |

**Fig. 13.4** The C-terminal ends with short sequences for (putative) cyclopeptides (borosins) from a family of some (potentially) autocatalytic SAM-dependent methyltransferases from different agaricomycetes. *Note*: The known sequence of the omphalotins is shaded in *gray*. JGI and NCBI accession numbers are added where known

### 13.4.3 More Potential Cyclic RiPPS

Gene clusters for the synthesis of other fungal cyclic RiPPs (e.g., ustiloxins and phomopsins, antimitotic cyclic mycotoxins which inhibit microtubule assembly and may be of interest for antitumor drug development; Koiso et al. 1994; Cormier et al. 2008) and functions of first genes in these clusters have recently been described in filamentous ascomycetes (Umemura et al. 2014; Tsukui et al. 2015; Ding et al. 2016; Nagano et al. 2016; Ye et al. 2016). Among genes for regulation, transport, and types of posttranslational modifications, these clusters include the genes for protein precursors with repeated peptide motifs and genes for DUF3328 proteins which represent a novel class of oxidases with function in peptide cyclization (Ding et al. 2016; Nagano et al. 2016; Ye et al. 2016). Genes for DUF3328 oxidases (family oxidase ustYa) were found in many ascomycetes and also in a subset of *Agaricomycotina*, often near candidate genes for protein precursors with repeated peptide motifs (Ding et al. 2016; Nagano et al. 2016). Two examples of putative precursors of novel cyclic RiPPs from the *Agaricomycetes* identified via genome mining by DUF3328 sequences are shown in Fig. 13.5. The cyclized products of such precursors were termed dikaritins after the fungal subkingdom of *Dikarya* (Ding et al. 2016).

### 13.4.4 Linear Peptides

Linear oligopeptides with cytotoxic activities against cell lines [pterulamides I–VI, highly *N*-methylated pentapeptides consisting of modified valine, isoleucine, and alanine residues (sequences: AIVVL; VVVVI) from *Pterula* sp. mushrooms; Lang et al. 2006] were reported, and several inhibitors with variable short sequences (between 3 and 17 amino acids) of specific enzymatic activities (angiotensin I-converting enzyme) with antihypertensive effects and anti-oxidative actions from

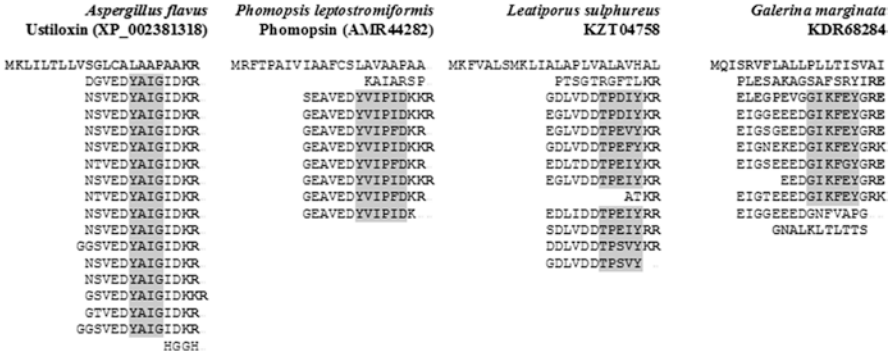


**Fig. 13.5** Precursor sequences for cyclic mycotoxins (dikaritins) from the *Ascomycota* and newly reported precursor molecules for putative ribosomally synthesized cyclic peptides from two mushrooms. *Note*: The (predicted) core sequence for peptide cyclization are shaded in *gray* (Tsukui et al. 2015; Ding et al. 2016) and dipeptides for potential proteolytic processing by KEX proteases are given in *bold*

different mushrooms were described (Kang et al. 2013; Geng et al. 2016). Their origins (ribosomal or non-ribosomal) are currently unclear.

### 13.4.5 Non-ribosomal Peptides

Non-ribosomal peptide synthetases (NRPSs) are multifunctional enzymes with multiple core domains (Fig. 13.6) which resemble polyketide synthases by a modular multi-domain structure (Du and Lou 2010; Wang et al. 2015; compare Fig. 13.2). NRPSs assemble their products (non-ribosomal peptides, NRPs) progressively while moving the growing peptide chain directionally along the protein template. A typical single NRPS elongation module therefore includes all reactive domains required for incorporation of an element to the chain (at minimum adenylation domain A with a non-ribosomal specific code for selection and activation of a monomeric substrate, thiolation domain T to hold the activated substrate, and condensation domain C to catalyze amide bond formation between adjacent T-domain bonded substrates). An NRPS can have a single A-T-C module (monomodular) or several (multimodular). An initiation module used to initiate the peptide synthesis misses the condensation domain. However, NRPS can be very diverse in a number of domains and by the inclusion of various other types of domains for product modifications (Finking and Marahiel 2004; Du and Lou 2010; Kalb et al. 2013; Singh et al. 2017; (Fig. 13.6).

A first NRPS gene cloned and also the first gene cluster detected in a basidiomycete were *fso1* and the ferrichrome A siderophore gene cluster of *O. olearius* with the additional genes *ato1* and *omo1*, respectively (Welzel et al. 2005). Ferrichrome A is made up of a glycine, two serines, and three *trans*-(α-methyl)-glutaconic acid-acylated $N^5$-hydroxyornithine amino acid residues (Zalkin et al. 1966). The synthesis occurs in at least three steps: hydroxylation of L-ornithine by an

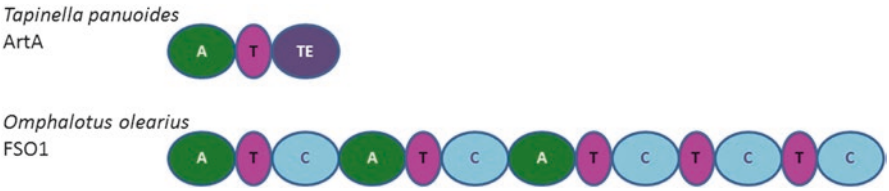


**Fig. 13.6** Domain structures of the multimodular non-ribosomal peptide synthetase (NRPS) Fso1 for hydroxamate siderophore production in *Omphalotus olearius* (Welzel et al. 2005). *Note*: Fso1 is compared to the monomodular L-tyrosine:oxoglutarate aminotransferase AtrD from *Tapinella panuoides* (Schneider et al. 2008; Sect. 13.3.3). *A* adenylation, *T* thiolation, *TE* thioesterase/cyclase, *C* condensation

L-ornithine-$N^5$-monooxygenase, acylation of $N^5$-hydroxy-L-ornithine by an acyl-CoA:$N^5$-hydroxy-L-ornithine *N*-acyl-transferase, and incorporation of other amino acids by a modular NRPS. *omo1* of *O. olearius* encodes an L-ornithine-$N^5$-monooxygenase and *ato1* an acetylase. The NRPS Fso1 is 4548 amino acids long with a weight of about 500 kDa. It consists of repeated A-T-C and T-C modules (Welzel et al. 2005; Fig. 13.6). Adenylation (A) domains are the gatekeepers for NRPSs and select, activate, and load the substrate onto thiolation domains (the molecular carrier) for subsequent condensation reactions. A domains have signature sequences for the selection of cognate substrates. Attempts are made to define non-ribosomal codes for amino acid selection of different A domains (Schwecke et al. 2006; Kalb et al. 2013; Lee et al. 2015). A selective sequence of DIITITATLR has been deduced for the third A domain (A3) in Fso1 for $N^5$-hydroxy-$N^5$-methylglutaconyl-L-ornithine (MGHO) binding (Kalb et al. 2013). Basidiomycete mushroom genomes, in general, do not contain as many genes for modular NRPSs, NRPS-like enzymes, and also polyketide synthases as genomes of ascomycetes. In average, there are about four genes for such modular enzymes in a respective genome, with 0 up to 9 different genes for NRPSs spread into different gene clusters (Lackner et al. 2012; Liu et al. 2012a; Wawrzyn et al. 2012; Riley et al. 2014; Sasso et al. 2014).

## 13.5 Proteins

### 13.5.1 Aegerolysins and Other Pore-Forming Proteins

Aegerolysins represent an aerolysin superfamily (Pfam 06355) of short microbial proteins (ca 140 amino acids, molecular weights of 15–20 kDa) with two conserved cysteines and potential hemolytic activities. They are named after the primordia-specific protein Aa-Pri1 from *Agrocybe aegerita* (Fernandez Espinar and Labarère 1997). Well-known other members of this family are erylysin A from *P. eryngii* (Shibata et al. 2010) and pleurotolysin A (ostreolysin) of *P. ostreatus* (Berne et al. 2002) which come together with the larger 59 kDa MACPF (membrane attack

complex/perforin; Reboul et al. 2016) proteins erylysin B and pleurotolysin B, respectively (Shibata et al. 2010; Ota et al. 2013). Typically in mushrooms, these types of proteins are expressed in stages of fruiting body development and in specific cells of the fruiting bodies (Fernandez Espinar and Labarère 1997; Berne et al. 2002; Schlumberger et al. 2014) or in multicellular sclerotia such as in *Lignosus rhinocerotis* (Yap et al. 2015b).

*A. aegerita* aegerolysin and the *Pleurotus* proteins are reported to be hemolytic acting against mammalian erythrocytes (Berne et al. 2002). However, erylysin A and pleurotolysin A exert such activities only in bicomponent complexes with the MACPF perforins erylysin B and pleurotolysin B, respectively (Ota et al. 2013; Schlumberger et al. 2014). Erylysin A and pleurotolysin A are pore-forming proteins which interact with lipid rafts in cholesterol- and sphingomyelin-rich cellular membranes and insert into cellular membranes (Berne et al. 2009; Hullin-Matsuda et al. 2016; Yamaji-Hasegawa et al. 2016). Erylysin A and pleurotolysin A need to bind to the membranes to then recruit the perforins erylysin B and pleurotolysin B, respectively, and to further assemble as heteromeric complexes with β-barrel protein shapes the transmembrane pores in the cellular membranes (Tomita et al. 2004; Ota et al. 2013; Schlumberger et al. 2014; Lukoyanova et al. 2015; Skočaj et al. 2016). The assembled pore complexes influence transport processes of the cells such as increasing $Ca^{2+}$ influx, thereby disturbing $Ca^{2+}$ homeostasis of the cells (Vrecl et al. 2015a, b).

Aegerolysins occur in about 20% of the *Agaricomycetes*, inconsistently distributed over the species (Lakkireddy et al. 2011; Nayak et al. 2013; Novak et al. 2015), among in *G. marginata* (KDR71454, KDR68296, KDR71457), *H. sublateritium* (KJA14257, KJA14258), and *T. versicolor* (XP_008043601). Of these species, *T. versicolor* (XP_008043602) also has an MACPF perforin-type protein. Apart of a link to fruiting body development, the biological functions of the proteins for the mushrooms are unclear (Berne et al. 2009; Lakkireddy et al. 2011; Schlumberger et al. 2014).

Other types of membrane-integrating pore-forming mushroom toxins are hemolytic chimerolectins (Lakkireddy et al. 2011; Sabotič et al. 2016). *L. sulphureus* has a family of chimerolectins of which the hemolytic LSL has been studied in detail (Mancheno et al. 2010). The 315 amino acid-long LSL consists of an N-terminal β-trefoil-type lectin domain and a C-terminal aerolysin-like pore-forming domain. LSL binds to β-galactosides such as lactose and LacNAc. The crystal structure of LSL resembles that of bacterial aerolysins. LSL is therefore believed upon insertion into membranes to adopt an oligomeric β-barrel protein structure like the aegerolysins (Mancheno et al. 2004, 2005, 2010; Angulo et al. 2011). Genes for LSL-like proteins occur singly or as families inconsistently distributed over the *Agaricomycetes* (Lakkireddy et al. 2011). Highly similar, full-length proteins, for example, exist in *G. marginata* (KDR66599), *G. frondosa* (OBZ73067), *F. pinicola* (EPT04861), and *Sphaerobolus stellatus* (KIJ34088). Protein CC1G_11805 of a family of LSL homologues of *C. cinerea* (Lakkireddy et al. 2011) has been shown to be nematotoxic (Plaza et al. 2014).

A further novel family of mushroom lectins (referred to as FB_lectin superfamily) with structural similarities to hemolytic actinoporins is presented by the

insecticidal XCL of *Xerocomus chrysenteron* which binds to *N*-acetyl-galactosamine (Bleuler-Martinez et al. 2011; Yan et al. 2012). XCL forms tetramers in solution. Upon internalization into insect and human cells by a clathrin-dependent pathway, lectin XCL is delivered to endosome compartments, and it induces changes to the actin cytoskeleton (Francis et al. 2003; Birck et al. 2004). Other characterized lectins of this family are ABL from *A. bisporus*, SRL from *Athelia rolfsii*, and BEL from *B. edulis* (Lakkireddy et al. 2011; Sabotič et al. 2016). FB_lectin genes are more common in species of the *Agaricomycetes*, while the presence of such genes (singly or in families) is also irregularly distributed over the mushrooms (Lakkireddy et al. 2011).

### 13.5.2 Fungal Immunomodulatory Proteins (FIPs)

A first fungal immunomodulatory protein (FIP, Fve superfamily) identified from mushrooms is the well-studied 111-aa-long asparagine-rich protein LZ-8 (FIP-glu) from *G. lucidum* (Kino et al. 1989) with anti-allergy, wound-healing, and manifold antitumor activities with partially understood mechanisms (Li et al. 2011b; Wang et al. 2012; Lin et al. 2014; Wu et al. 2015). Similar immunomodulatory 13 kDa proteins (110–114 amino acids) are available from other *Ganoderma* species (Lin et al. 1997, 2010; Zhou et al. 2009; Wang et al. 2012; Yu et al. 2015), *Chroogomphus rutilus* (Lin et al. 2016), *Dichomitus squalens* (Li et al. 2017), *F. velutipes* (Ko et al. 1995), *L. rhinocerotis* (Pushparajeh et al. 2016), *Postia placenta* (Li et al. 2015), *T. versicolor* (Li et al. 2012), and *V. volvaceae* (Hsu et al. 1997; Wang et al. 2016c). There are also genes for FIPs in *A. muscaria* (KIL61952, KIL58061), *A. subglabra* (XP_007340731, an immunomodulatory 13.4 kDa protein has been isolated from *Auricularia polytricha*, Sheu et al. 2004), *Botryobasidium botryosum* (KDQ10166), *G. subvermispora* (EMD41188, EMD41189, EMD41190), *H. sublateritium* (C-terminal linked to a protein kinase domain, KJA26560), *Obba rivulosa* (C-terminal linked to a protein kinase domain, OCH90019), *P. carnosa* (XP_007392586), *Schizopora paradoxa* (C-terminal linked to a protein kinase domain, KLO20748), *Trametes pubescens* (OJT03424, OJT03425), and more (Lakkireddy et al. 2011), while they are extremely rare in the *Ascomycota* with only two host species detected so far (Bastiaan-Net et al. 2013; Li et al. 2016b). FIPs of closer-related species might be up to 100% identical and sequences from more distantly related fungi between 56 and 75%. Depending on the species, FIPs are variably asparagine- and valine-rich (Li et al. 2011b, 2017; Lin et al. 2016; Pushparajeh et al. 2016).

Importantly, the sequences of the FIPs show similarities to sequences of immunoglobulins (Igs; Tanaka et al. 1989; Ko et al. 1997) although they usually lack cysteine, methionine, and histidine residues which are regularly present in Igs (Paaventhan et al. 2003; Huang et al. 2008). FIPs adopt a Ig-like β-sandwich fold from the seven β-sheets in their C-terminal fibronectin III-type domain like Igs, and they form homodimers or homotetramers (FIP-gmi from *Ganoderma microsporum*) via dimerization through an N-terminal α-helix stabilized by hydrophobic amino acid interactions and assisted by a β-sheet formed by the two dimerizing proteins

from the β-strand following directly after the N-terminal α-helix (Lin et al. 1997; Paaventhan et al. 2003; Wu et al. 2007; Huang et al. 2008; Pushparajah et al. 2016). Variably strong in their cytotoxic, mitogenic, and anticancer activities (Li et al. 2011b; Wang et al. 2012; Ou et al. 2015), FIPs are capable to perform Ig-like reactions and stimulate, e.g., proliferation of lymphocytes and macrophages and modify the production of cytokines by T cells (e.g., Ko et al. 1997; Hsieh et al. 2003, 2007; Hsu et al. 2008; Ou et al. 2009; Lin et al. 2010; Lee et al. 2013; Li et al. 2015; Wu et al. 2015), and they can be inhibitory to virus infections (Chang et al. 2014).

FIPs' activities partially depend on their 3D structure and homodimerization (Lin et al. 1997; Huang et al. 2008, 2014b). The difference in activities between FIPs probably base on the variable amino acid sequences found in loopDE and loopFG of the Ig-like β-sandwich structure (Huang et al. 2008; Wang et al. 2016c) and possibly on strength and structural differences of the α-helices mediating the N-terminal dimerization (Lin et al. 1997; Wang et al. 2016c). The proteins provide a carbohydrate-binding pocket (CBM, family CBM34-like; key residues in *F. velutipes* FIP-fve: W24, T28, D34, R90, I91, W111) by the β-sandwich structure, and the folded proteins can bind to complex cell-surface sugars such as dextrin, cyclodextrin, and *N*-acetyl neuraminic acid (Kino et al. 1989; Ko et al. 1995; Liu et al. 2012b; Pushparajeh et al. 2016). FIPs therefore have a lectin character and can cause blood cell aggregation, while individual FIPs differ in specificity to agglutinate different animal (rat, mouse, sheep) or human red blood cells (Li et al. 2012; Liu et al. 2012b; Lin et al. 2016; Pushparajah et al. 2016).

Interesting for future studies could be functional analyses on the potentially bifunctional proteins of *H. sublateritium*, *O. rivulosa*, and *S. paradoxa* with a protein kinase domain linked to one or two FIP units, because FIP-fve has been shown to activate protein kinase C-α in human peripheral blood mononuclear cells (Ou et al. 2009).

### 13.5.3 Other Lectins

The catalog of different lectins, i.e., carbohydrate-binding proteins which are also known as hemagglutinins, is large in mushrooms as are the lists of types of sugars they interact with and the potential medicinal functions attributed to them including antitumor, mitogenic/antimitogenic, immunomodulatory, antiviral, and other activities (Lakkireddy et al. 2011; Erjavec et al. 2012; Hassan et al. 2015; Singh et al. 2015; Sabotič et al. 2016). Moreover, 3D structures have been established for several distinct fungal lectins, and different structural families of fungal lectins have been defined (Varrot et al. 2013; Hassan et al. 2015; Sabotič et al. 2016). Related genes for the diverse types of lectins are often found in only selected species scattered over the *Agaricomycetes*, commonly not much linked to any taxa relationships. Sometimes, the distribution of related genes within different mushrooms is very restricted to only a low number of species (Lakkireddy et al. 2011; Sabotič et al. 2016).

By the wealth of different lectins which occur in mushrooms and which are moreover possibly even characterized, we can present here only a few selected examples of different groups of lectins and document the quite irregular dissemination of respective lectin genes over the species ranges. The examples discussed should emphasize the value of looking into more (types of) lectins of more mushroom species. The chimeric lectins and the FIPs discussed in Sects. 13.5.1 and 13.5.2 belong to other families of lectins, and further lectin-like proteins with protease inhibitory functions and protease activities are presented in Sect. 13.5.4.

Genes for galectins binding β-galactosides are, for example, present in *Agrocybe cylindracea* (ACG; Yagi et al. 1997, 2001; Ban et al. 2005), *A. aegerita* (the fruiting body-specific AAG; Yang et al. 2009; Luan et al. 2010), *C. cinerea* (the fruiting body-specific Cgl1 and Cgl2; the related Cgl3 binds LacdiNac and chitobiose; Cooper et al. 1997; Boulianne et al. 2000; Walser et al. 2004; Wälti et al. 2008; Plaza et al. 2014), *Heterobasidion irregulare*, *Laccaria amethystina*, *L. bicolor* (Lakkireddy et al. 2011; Lyimo et al. 2011), *G. marginata* (KDR68111), *Pisolithus microcarpus* (KIK30469), and a few others (Sabotič et al. 2016). The fungal galectins adopt a typical β-sandwich structure composed of two antiparallel, six-stranded β-sheets. CGL2 and CGL3 of *C. cinerea* form homotetramers; ACG and AAG from *Agrocybe* species form homodimers (Walser et al. 2004; Ban et al. 2005; Wälti et al. 2008; Yang et al. 2009). CGL1 and CGL2 show toxic activity against nematodes, mosquitos, and amoebae, by binding to *N*-glycans of glycoproteins of attacked organisms (Butschi et al. 2010; Bleuler-Martinez et al. 2011). ACG recognizes specifically *N*-sialoglycans specific to human leukemic cells in a comparably well-understood binding mode (Hizukuri et al. 2005; Parasuraman et al. 2015). Antitumor activities and binding to tumor-related glycan antigens have been demonstrated for AAL (Zhang et al. 2015b; Jin et al. 2016; Liu et al. 2017).

The β-trefoil family of fungal lectins (from the ricin-B superfamily) is much larger with the majority of *Agaricomycetes* having one or more members. However, overall sequence identities and similarities between individual members of this family can be very low with values of 7–26% and 25–40%, respectively. The lectins of the β-trefoil family also vary in their carbohydrate-binding specificity and are often fruiting body- or sclerotia-specific (Lakkireddy et al. 2011; Žurga et al. 2014; Sabotič et al. 2016). Well-studied examples for the β-trefoil family of lectins are the fruiting body-specific CCL1 and CCL2 of *C. cinerea* which have nematicidal activities like many other members of this family, while others are acting entomotoxic (Schubert et al. 2012; Plaza et al. 2014; Sabotič et al. 2016). CCLl and CCL2 are 55% identical and 73% similar in sequence. Most similar proteins, e.g., to CCL2 from other mushrooms, are from *S. lacrymans* (XP_007323761: 43% identical, 60% similar), *Coniophora puteana* (XP_007765699: 43% identical, 59% similar), *Rhizopogon vinicolor* (OAX40927: 45% identical, 56% similar), *S. stellatus* (KIJ40569: 43% identical, 55% similar), *P. ostreatus* (KDQ26460: 45% identical, 59% similar), and *L. amethystina* (KIJ95877: 41% identical, 54% similar). CCL2 binds to a GlcNAc-β1,4-(Fuc-α1,3)GlcNAc (anti-HRP, anti-horseradish peroxidase) epitope *N*-glycans of nematodes (Schubert et al. 2012; Stutz et al. 2015). Another well-studied lectin from the β-trefoil family in the *Agaricomycetes*, the

entomotoxic agglutinin RSA from *Rhizoctonia solani* (Candy et al. 2001; Hamshou et al. 2013), interacts in contrast with Gal-/GalNAc-containing insect glycans (Hamshou et al. 2012; Walski et al. 2014). RSA is shown to also bind to proteins α-2-macroglobulin and IgA from human serum (Van Leuven et al. 1993). The nematotoxic ricin B-like lectin CNL from *Clitocybe nebularis* (26% identical, 40% similar to RSA; Sabotič et al. 2016) immunostimulates dendritic cells via the toll-like receptor 4 pathway (Svajger et al. 2011; Pohleven et al. 2012). A lectin in other species closest to CNL is from *Leucoagaricus* sp. (KXN92490: 32% identical, 50% similar), while other related lectins from *P. placenta* and *C. cinerea* are only 26% identical and 46–44% similar to CNL (Lakkireddy et al. 2011).

Another group of interesting lectins is represented by the 42 kDa PVL from *Lacrymaria* (*Psathyrella*) *lacrymabunda*. This is an integrin-like protein which adopts a six-bladed β-propeller fold with six carbohydrate-binding sites for terminal GlcNAc and sialic acid residues (Ueda et al. 2002; Cioci et al. 2006; Audfray et al. 2015). This lectin binds truncated *N*-glycans on human cancer cells (Audfray et al. 2015). A highly similar lectin PAL from *Lacrymaria* (*Psathyrella*) *asperospora* of high sequence identity (87%) and similarity (92%) and with a similar 3D structure also binds terminal GlcNAc and sialic acid. PAL has a strong cytotoxic effect on human colon cancer cells and on monkey kidney cells (Rouf et al. 2014; Ribeiro et al. 2017). PVL- and PAL-related lectins are however also not as widely distributed in other mushroom species (Lakkireddy et al. 2011). *A. aegerita*, *C. cinerea*, and *L. bicolor* each have one gene (Lakkireddy et al. 2011; Rouf et al. 2014; Ren et al. 2015), and there are related genes in *G. marginata* (KDR83047, KDR74277), *H. cylindrosporium* (KIM35067, KIM47177, KIM35070), *H. sublateritium* (KJA19519), and *L. amethystina* (KIK03462, KIJ92371, KIJ96935, KIJ93857).

Tectonins are lectins of an interesting structure by having tandem β-propeller repeats which form blades by a four-stranded antiparallel β-sheet (Low et al. 2009). Tectonin Le-Tec2 of *L. bicolor* agglutinates *E. coli* and recognizes *O*-methylated mannose and fucose residues, typically present in the outer bacterial lipopolysaccharide layers (Wohlschlager et al. 2014). Genes for tectonins appear not to be much distributed over all the various mushroom species, but they are repeatedly found in species with an ectomycorrhizal lifestyle (Sabotič et al. 2016) such as in *H. cylindrosporum* (KKIM38814, KIM38815, KIM38816, KIM38829, KIM38830, KIM38831, KIM35453, KIM35454, KIM35464, KIM35465), *L. amethystina* (KIJ90107, KIJ94531, KIJ94903), *L. bicolor* (XP_001876432, XP_001877906), *Paxillus rubicundulus* (KIK93906), while the saprotrophic *G. marginata* (KDR71733, KDR71773) has also gene copies.

### 13.5.4 Proteinase Inhibitors and Lectins with Protease Activities

Another type of small proteins found often specifically expressed in stages of fruiting body development and in specific mushroom tissues concerns protein inhibitors. While many mushroom crude extracts have been shown to confer proteinase-inhibiting activities, few inhibitors have been isolated and characterized. Among

these are mycospins (e.g., cospin of *C. cinerea* and cnispin from *C. nebularis*) as serine proteinase inhibitors and mycopins (clitopypins from *C. nebularis* and macrocypins from *Macrolepiota procera*) as cysteine protease inhibitors (Renko et al. 2012; Dunaevsky et al. 2013; Sabotič et al. 2016). Cospin as an example of the mycospin family is a 150 amino acid-long antibiotic protein (Sabotič et al. 2012), cnispin is 146 amino acid-long (Avanzo Caglič et al. 2014), and LeSP1 from *L. edodes* 152 amino acid-long (Odani et al. 1999). These proteins belong by sequence similarities all to the ricin B-like superfamily and adopt a β-trefoil structure with 12 β-strands and 11 loops, and they show high activity against trypsin (Odani et al. 1999; Avanzo Caglič et al. 2014). Cnispin inhibits proteases by its β11-β12 loop with Lys-127 as the P1 amino acid which binds to the active site in trypsin (Avanzo Caglič et al. 2014). In contrast, loop β2-β3 with Arg-27 is responsible for the inhibitory activity of cospin (Sabotič et al. 2012). The mycopins macrocypin 1 and clitocypin are also members of the large fungal ricin B-like family. However, they block proteases through interaction with their β5-β6 loops. Mycopins are strong inhibitors of papain-like proteases (Sabotič et al. 2007, 2009; Renko et al. al. 2010). Genes for the superfamily of ricin-like proteins are widely distributed in the *Agaricomycetes* and often occur in the species in larger diverse families (Lakkireddy et al. 2011; Sabotič et al. 2016). Fungal β-trefoil proteases and fungal ricin B-like lectins of β-trefoil structure interact with each other which is considered as a regulatory mechanism of respective protein activities (Žurga et al. 2015).

The second group of β-trefoil chimerolections encloses MOA from *M. oreades*. This lectin consists of an N-terminal lectin domain and a C-terminal cysteine protease domain. MAO binds to terminal Gal-α1,3-Gal/GalNAc-β epiotopes (Grahn et al. 2007, 2009; Wohlschlager et al. 2011). The homologous lectins PSL1a from *P. squamosus* and SCA from *S. commune* in contrast recognize terminal Neu5Acc-α2,6-Gal-β epitopes (Kadirvelraj et al. 2011; Wohlschlager et al. 2011). MAO forms dimers of a dumbbell shape with the dimerized cysteine protease domain connecting the two β-trefoil lectin domains. Protease activity requires binding of divalent ions such as $Ca^{2+}$ for induction of conformational changes (Cordara et al. 2011, 2016; Wohlschlager et al. 2011). When taken up into mammalian cells, MOA inhibits protein and DNA biosynthesis and induces degradation of β1-integrin, disruption of integrin-dependent cell adhesion signaling, rearrangements of the cytoskeleton, and cell death (Cordara et al. 2014; Juillot et al. 2016). Toxic activities toward mammalian cells depend on the protease domain (Manna et al. 2017). Also the presence of genes for MAO-like genes is inconsistently spread over the *Agaricomycetes*. Related genes (protein similarity ca 35 to >40%) were further found in *Peniophora* sp. (KZV64829, KZV65628), *Plicaturospis crispa* (KII87094), *Polyporus umbellatus* (ANC28063), *Pycnoporus coccineus* (OSC96843, OSD05564), *S. lacrymans* (EGN95167), *S. stellatus* (KIJ35645), and *Trametes cinnabarina* (CDO76070).

### 13.5.5 Ribosome-Inactivating Proteins (RIPs)

Ribosome-inactivating proteins (RIPs) are *N*-glycosidases which depurinate an adenine residue in the conserved α-sarcin/ricin loop of eukaryotic 28S rRNA, thereby blocking the binding of elongation factor 2 (EF-2) and in consequence protein biosynthesis. Type I RIPs (chimero-RIPs) are enzymes with a single RNA *N*-glycosidase activity domain and molecular weights from ca 20 up to 40 kDa. Type 2 RIPs consist of two different chains of distinct function, an *N*-glycosidase A-chain (similar to type 1 RIPs) and a lectin-like B-chain (for cell binding and transport into cells), which are interlinked by a disulfide bridge. Type 3 RIPs are atypical RNA *N*-glycosidases with two domains, an N-terminal type-1 RIP-like domain and a C-terminal domain of unknown function which must be removed to activate the enzymatic function of the N-terminus (Girbes et al. 2004; Akkouh et al. 2015; Wang et al. 2016a).RIPs are best known from plants (Akkouh et al. 2015; Wang et al. 2016a) while ribosome-inactivating effects have also been attributed to several proteins isolated from various mushrooms (Table 13.2). These are however all not fully characterized. Enzymatic depurination activities on 28S rRNA are deduced for marmorin of *H. marmoreus* and volvatin of *V. volvaceae* from gels showing a 28S rRNA fragmentation band of about 0.4 kb which was similar in size to the specific "Endo's" bands generated from 28S rRNA through depurination by plant RIPs (Endo et al. 1988; Yao et al. 1998; Wong et al. 2008). Indication for a member of the classical RIP superfamily in fungi could come indirectly from a comparison of full-length protein sequences (Lapadula et al. 2013). Significantly, the sequence of a RIP from maize (2PQI_A) detected genes for RIP-like proteins only in exceptional cases in very few fungal plant and insect pathogens from the *Ascomycota* and in *S. stellatus* (KIJ38555; KIJ48721) as the only basidiomycete (Lapadula et al. 2013; this report).

Usually, the N-terminal sequences of reported fungal ribosome-inactivating proteins and the protein sizes as determined by migration in gel electrophoresis were published (Table 13.2), whereas the actual nature of the proteins always remained open by the lack of complete sequences. Now the published N-terminal sequences can be used in tBlastn or pBlast searches in the databases against the genomic sequence or against the proteome deduced from a fungal genome, respectively. We have done this here for a range of fungal ribosome-inactivating proteins (Table 13.2). Even with complete genomes available for *H. marmoreus* (GCA_001605315) and two distinct strains of *F. velutipes* (Park et al. 2014; BDAN01000000; both only little annotated), we could not detect 100% perfect sequence matches with any of the experimentally established N-terminal peptide sequences. However, the *H. marmoreus* marmorin and *F. velutipes* velin peptides hit the N-termini of matured secreted proteins of expected sizes which have suggested lectin functions. Marmorin belongs thus to a novel family of fungal lectins together with a crystallized α-galactosyl-binding lectin of *Lyophyllum decastes* and some proteins from a few ascomycetes and basidiomycetes (Goldstein et al. 2007; van Eerde et al. 2015; this study). It is striking that the *L. decastes* lectin shares galabiose-binding activity with the Shiga toxin from the RIP superfamily (Goldstein et al. 2007). Another interesting finding concerns flammin. It appears to be related to or be in fact the formerly characterized *F. velutipes* endo-β-1,3-galactanase FcEn3GAL (Kotake et al. 2011).

**Table 13.2** Fungal ribosome-inactivating proteins and their possible genes and functions

| Fungus | Protein name/ best hit in database[a] | N-terminal sequence/ related sequence deduced from DNA sequences[b] | MW in kDa | | Reference/ proposed function |
|---|---|---|---|---|---|
| | | | In gel | Calculated[c] | |
| *Calvatia caelata* | Calcaelin<br>No revealing hit | ANPIYNIDAFRV | | | Ng et al. (2003a) |
| *Hypsizygus marmoreus* | Hypsin<br>KYQ37785 | **ITFQG**DLD**ARQ**Q**VITNA**D**TR**RKR**DVRAA**<br>**ITFQG**CSP**ARQ**T**VITNA**I**TR**ARA**DVRAA** | 20 | <br>36.19 (38.24) /18.25 (19.16)[d] | Lam and Ng (2001a)<br>Peptidyl-Lys metalloendopeptidase |
| | Marmorin<br>KYQ33543<br>KYQ33548<br>KYQ33492<br>KYQ33491 | **A**EGTLLGSRA**TCES**G**NSMY**<br>A**A**TCWKTSKCSP**CES**A**NSMY**<br>**A**TCWKTSKCSP**CES**A**NSMY**<br>**A**QCFSQHGCGN**CES**RDT**MY**<br>**A**TCWERWGCV**TCES**KDP**MY** | 10 | <br>10.7 (12.67)<br>10.61 (12.71)<br>10.95 (12.86)<br>11.1 (13.17) | Wong et al. (2008)<br>Family of α-galactosyl binding lectins |
| *Flammulina velutipes* | Flammin<br>F7J1C8 | SP**VIPAN**T**F**VA**F**RL**Y**EVG**F**U**P**A<br>AT**VIPAN**S**F**SS**F**ST**Y**WNN**F**Y**P**W | 30<br>30 | <br>24.5 (26.69) | Ng and Wang (2004a)<br>Endo-β-1,3-galactanase Kotake et al. (2011) |
| | Flammulin<br>BDAN01000649:<br>184794-184631<br>181700-181537<br>152142-152305<br>148068-147905<br>*Gymnopus luxurians* KIK67684[e] | **APS**H**F**S**HPGVL**A**DRAQ**I**DF**I**X**GKVN**E**GAEPW**X**SAYN**<br><br>**APS**V**F**T**HPGVL**I**DRAQ**L**DF**I**KD**KVN**S**GAEPW**A**SAYN**<br>**APS**T**F**T**HPGVL**I**DRAQ**L**DF**I**K**GKVN**S**GAEPW**T**SAYN**<br>**APS**T**F**T**HPGVL**I**DRAQ**L**DF**I**K**GKVN**SS**A**Q**PWT**SAYN**<br>**APS**T**F**A**HPGVL**I**DRAQ**L**DF**I**K**GKVN**S**GA**Q**PWT**SAYN**<br>**AP**AT**F**K**HPGI**L**I**DR**Q**Q**L**DF**I**KG**KVN**S**GAEPW**T**K**AYN** | 40 | <br><br><br><br><br><br>44.45 (46.59) | Wang and Ng (2000a)<br><br><br><br><br><br>Alginate lyase |

| | | | | | |
|---|---|---|---|---|---|
| | Velin<br>BDAN01003789:<br>122030- 121947<br>*Moniliophthora roreri*<br>XP_007856901[e] | **SGSPLTQAQAEALLKPQG**L-**AYSSGG**N**T**<br><br>**SGSPLTQAQAEALLKPQG**I**TAYSSGG**C**T**<br>**SGTPLTQAQAEALL**I**PQG**I**TA**S**S**T**GG**C**T** | 19<br><br><br><br>16.97 (18.94) | | Ng and Wang (2004a)<br><br><br><br>Peptidoglycan-binding domain 1 protein |
| | Velutin<br>BDAN01002178:<br>202395- 202327<br>*Fomitiporia mediterranea*<br>XP_007269873 | X**HPDLF**X**X**R**P**D**NT**A**SPKFE**D**P**R**LNP<br><br>MS**HPDLF..R**K**G**N**T**S**SPKFE**S**V**R**KGV<br>MS**HPDL**Y**..R**S**G**N**TTSP**R**F**D**NV**R**EGT** | 13.8<br><br><br><br>27.78 | | Wang and Ng (2001a)<br><br><br><br>Unknown |
| *Lentinus tuber-regium* | Pleuturegin<br>No meaningful hit | ARTQPGNIAPVGDFTLYPNAPRQGHIVA | 40 | | Wang and Ng (2001b) |
| *Lyophyllum shimeji* | Lyophyllin<br>Serine metalloprotease<br>KYQ37785<br>(*Hypsizygus marmoreus*) | **ITFQGASPARQTVITNAITRARADVRAA**<br>**IRFQ**S**ASP**<br>**ITFQG**C**SPARQTVITNAITRARADVRAA** | 20<br>21<br><br>36.19 (38.24)<br>/18.25 (19.16)[d] | | Lam and Ng (2001b)<br>Moon et al. (2014)<br>Peptidyl-Lys metalloendopeptidase |
| *Polyporus adusta* | Adustin<br>No revealing hit | ADVVED | 16.5 | | Ng and Wang (2004b) |

[a]Best hits are either from the Basidiomycota protein databank at NCBI (pBLAST searches) or DNA sequence positions from whole genome sequences (tBLASTn searches)
[b]Amino acids shared between an experimentally determined N-terminal sequence and a hit in the databases are marked in bold
[c]For proteins which have secretion signals, two distinct values are given for the precursor protein (number in brackets) and the mature protein
[d]Values are given for a complete protein sequence as deduced from the whole open reading frame and for sequences of a truncated proteins from the 3´ half of the putative gene
[e]Best hit from another species

Notably, the gene for En3GAL was only found in one of the two released *F. velutipes* genomes (BDAN01000000; Table 13.2).

Further interesting cases are *H. marmoreus* hypsin and *Lyophyllum shimeji* lyophyllin. The experimentally determined N-termini of hypsin and lyophyllin (Lam and Ng 2001a, b) recognize in pBlast searches internal sequences of a larger *H. marmoreus* protein (KYQ37785) with an undefined N-terminal half and an M35-like $Zn^{2+}$-metallopeptidase/deuterolysin domain in the C-terminal half (Table 13.2). Moreover, the N-terminal sequences match that of a 21 kDa serine metalloproteinase characterized from *L. shimeyi* (Moon et al. 2014). The smaller size suggests either a posttranslational splitting of the two domains to give the experimentally determined N-terminus (the calculated protein size is 18.25 kDa), or the enzyme is produced from an ATG start codon in close vicinity to the coding sequence of the established N-terminus (the calculated size is 19.16 kDa). The N-terminal sequence analysis data as presented in Table 13.2 in effect suggest that none of the fungal ribosome-inactivating proteins are classical RIPs.

Most fungal ribosome-inactivating proteins have been classified as such by proof of inhibitory activities in cell-free translation assays, while RNA *N*-glycosidase activity is often claimed but not shown in the publications (Lam and Ng 2001a, b; Wang and Ng 2000a, 2001a, b; Ng et al. 2003; Ng and Wang 2004a, b). Looking at the shown or proposed biological functions of the diverse candidate proteins listed in Table 13.2, it is difficult to imagine that all of them should be true bioactive RIPs with specific 28S RNA cleavage function (see also the discussion by Lapadula et al. 2013). The proteins listed in Table 13.2 have variably been assigned further activities to, for example, being antiproliferative against cancer cell lines (hypsin, lyophyllin, marmorin), anti-mitogenic (calcaelin, hypsin, lyophyllin), antifungal (hypsin, lyophyllin), RNase active (calcaelin), HIV reverse transcriptase inhibiting (hypsin, lyophyllin, marmorin), and β-glucosidase and β-glucuronidase inhibiting (velutin), (Yao et al. 1998; Wang and Ng 2000a, 2001a, b; Lam and Ng 2001a, b; Ng et al. 2003; Ng and Wang 2004a; Wong et al. 2008). Such variability in multiple extra functions is shared with many other proteins isolated over the years from mushrooms and published under diverse headings such as antifungal, antibacterial, antiviral, and antitumor proteins (Akkouh et al. 2015; Ng et al. 2016). Strategies of their isolation and characterization are also commonly shared with the fungal ribosome-inactivating proteins, including that the protein identity is regularly defined via determination of N-terminal sequences. It is thus possible that also behind many of these yet to clearly identify proteins are such diverse kinds of proteins as suggested for the denoted fungal ribosome-inactivating proteins from the here presented peptide pBlast and tBlastn searches (Table 13.2). For further interest, tables with published N-terminal sequences of mushroom bioactive proteins of pharmaceutical interest are compiled, e.g., in Ng (2004) and Ng et al. (2016).

Other short mushroom proteins with reported antitumor-, deoxyribonuclease-, and ribosome-interfering activities are, e.g., the 8.5 kDa protein CULP from the puffball *Calvatia cealata* (Wang et al. 2003), the 9 kDa protein CCULP from *Cantharellus cibarius* (Wang et al. 2003), the 12.5 kDa glycosylated protein PULP

from *P. ostreatus* (Wang and Ng 2000b), and the 18.5 kDa RBUP from *Ramaria botrytis* (Zhou et al. 2017) from a longer catalog of ubiquitin-like proteins compiled from work of similar experimental systematics on many fungal species. No full-length protein sequence of any of the isolated and analyzed ubiquitin-like proteins seem to have ever been published, but obtained N-terminal sequences and MS/MS-covered internal peptides (69% in case of the *R. botrytis* protein) always match the ubiquitin of *Coprinellus congregatus* which as highly conserved protein is 99–100% identical in sequence to ubiquitins of other basidiomycetes and of ascomycetes.

### 13.5.6 Others

TFP of *Tremella fuciformis* fruiting bodies is a new 135 amino acid-long non-glycosylated homodimeric macrophage-activating protein. TFP cannot agglutinate blood cells and has likely no lectin activity. Its closest relatives are small expressed proteins from *S. commune* and *L. bicolor* classified as nonenzymatic proteins from the expansin superfamily which are plant-cell-wall binding and loosening proteins (Huang et al. 2014a).

Another novel immunomodulatory macrophage-activating protein is the glycosylated and cysteine-rich secreted ACA from mycelium of *T. camphoratus*. This protein belongs to the fungal phytotoxic cerato-platanin protein family and also shows no lectin agglutination properties. The ACA precursor is 136 amino acids long (Sheu et al. 2009). Members of the cerato-platanin family have been well studied in the *Ascomycota* (Gaderer et al. 2014; Pazzagli et al. 2014), while information in the *Basidiomycota* is scarce. Many of the *Agaricomycetes* do have variable genes for cerato-platanin-like proteins, among them are *Trametes* species (four genes in *T. cinnabarina*, five genes in *T. pubescens*, five genes in *T. versicolor*). *T. versicolor* protein YZP (alleles from different strains: AGH06133 and XP_008037058; 56% identical and 74% similar to ACA of *T. camphoratus*) stimulates B lymphocytes and induces production of interleukins IL-6 and IL-10 (Kuan et al. 2013). Notably, YZP is present in the Krestin polysaccharide-peptide extracts of *T. versicolor* and could be part of the effective principle of Krestin, similarly to lectin LZ-8 in *G. lucidum* polysaccharide PS-G (Yeh et al. 2010; Kuan et al. 2013).

A highly toxic protein for human cell lines via promotion of apoptosis is the 143 amino acid-long toxophallin from stems of *A. phalloides* mushrooms. It is structurally related to a functional amino oxidase of *L. bicolor* but has no mono- and diamine activity and acts as L-amino acid oxidase (L-AAO). Enzymatic activity generates aggressive $H_2O_2$ which is suggested to lead to activate apoptosis by a caspase-independent pathway (Stasyk et al. 2010). Similar enzymatic activities are known from snake venoms (Doley and Kini 2009). Other mushrooms including *L. bicolor* contain related genes for enzymes with 30–60% identity and 40–74% similarity to toxophallin.

## 13.6 Conclusions

In medicinal and other kinds of mushrooms, there is a fortune of unexploited secondary metabolites which have potential therapeutic values (Wasser and Weiss 1999; Wasser 2002; Anke and Antelo 2011; Schüffler and Anke 2011). Various unique types of proteins, both enzymes and non-enzymatic proteins, have also been reported to be linked to pharmaceutical effects of mushrooms (Lakkireddi et al. 2011; Xu et al. 2011; Erjavec et al. 2012; Sabotič et al. 2016). Modes of actions are being elucidated from many of fungal bioactive compounds, and the spectrum of activities is as broad as the biochemical nature of mushroom bioactive compounds.

Naturally by the high species number of the *Agaricomycetes* in nature as compared to the less studied ascomycetous mushrooms, most of the molecules discussed in this chapter originate from the *Agaricomycetes*. Although overlaps in the outfits of bioactive molecules are recorded between the *Agaricomycetes* from the *Basidiomycota* and the *Ascomycota*, there seems to be a tendency that different types of metabolites have preferentially been adopted in the two phyla of *Dikarya*. This has also been noted before. Comparably, much more polyketides and non-ribosomal peptides are found with corresponding genes in the ascomycetes and much more terpenoids and terpene synthase genes in contrast in the basidiomycetes (Bushley and Turgeon 2010; Chen et al. 2012; Liu et al. 2012a; Schmidt-Dannert 2015, 2016) and possibly (noncore) genes for lectins and lectin-like proteins (Sabotič et al. 2016). Production of secondary metabolites and specific proteins are often but not always connected to mushroom development. However, the vegetative mycelia may also produce the same or other bioactive compounds and proteins. Biological functions of mushroom compounds for the producer often remain elusive. Defense reactions against competing and adverse organisms are trendy postulates, while in some instances experimental evidence has been provided for such (Schwenk et al. 2014; Sabotič et al. 2016). Whatever the actual biological functions for the mushrooms are, their mode of actions might be exploited in tasks of pharmacology.

Research on bioactive metabolites and proteins classically starts by a screening of mushrooms for effective principles, possibly supported by ethnobotanical knowledge on traditional medicinal species. Now with the many fungal genomes at hand, there is a paradigm shift in approaches. Earlier in 2011, we performed a genome mining study with studied fungal lectins and pore-forming proteins with available genome sequences. Although complete at the time, the study was only on a small scale and finished quickly due to the restricted number of available sequences (Lakkireddy et al. 2011). Until recently, reports with sequences for metabolic genes were very rare and "anecdotal" (Schneider et al. 2008). When we agreed last year to write this chapter, we expected that this would not have changed as much. As documented in the chapter, genome-driven publications however now pop up like mushrooms in the forests. The recent flood of excellent papers in the field describing genes and their products indicates that a change has happened through the availability of the genomes. It is now much easier to target interesting genes and functions of pharmaceutical interest, both from medicinal species where such are

expected but also from genome mining in other mushrooms not considered before for potential medicinal applications. However, this makes classical experimental research on genes and compounds not obsolete.

We blasted with selected protein sequences (mostly by pBlast against defined protein sequences) the mushroom genomes deposited at NCBI (including all those mentioned in the introduction). A reoccurring observation is that usually only a few of all tested species contain specific genes of concern. Usually, genes of the same kind are distributed independently of clear species connections. Some of the species (e.g., *G. marginata* or *T. versicolor*) appeared more often in the searches than others mentioned in the text, and there are other species which were never hit. It is possible that recognized medicinal species are equipped with many more genes for pharmaceutically interesting secondary metabolites and unique type of proteins than other fungal species. This notion should be better elucidated in the future.

Detection of gene clusters in fungal genomes can be an entry point into the biosynthesis of known and of novel metabolites. However, on an average there seems to be fewer gene clusters to be present per species in basidiomycetes as compared to ascomycetes (Schmidt-Dannert 2015; Wawrzyn et al. 2015). Examples are accumulating from the *Agaricomycetes* that genes for a distinct biosynthetic pathway are not necessarily completely kept together in a single cluster (Wick et al. 2016; Braesel et al. 2017; this chapter). Moreover, there are also numerous examples of single genes in genomes (e.g., genes for sesquiterpene synthases; Sect. 13.3.1) suggesting that gene clustering for metabolic pathways in basidiomycetes might not be as strict as compared to ascomycetes. Possibly, this makes the systems more versatile and allows producing a much larger diversity of compounds. Also, this idea will need to become substantiated in the future.

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# 14 Biotechnology of Medicinal Plants and Fungi in Taiwan: Production of Bioactive Secondary Metabolites in In Vitro Culture Systems

Dinesh Chandra Agrawal, Emily Chin-Fun Chen, Hung-Chi Chang, Chia-Chen Chen, Chao-Lin Kuo, and Hsin-Sheng Tsay

**Abstract**

Plants and fungi provide a vast range of natural products including pharmaceuticals with diverse chemical structures and a broad array of biological activities. In the past few decades, there has been a worldwide resurgence of interest in the study and use of medicinal plants and fungi in the health-care system. Several medicinal plants and fungi are involved in the industrial processing of profitable products used in human medicines. However, due to severe constraints on the availability of desired plant and fungus materials, different strategies, including tissue culture, have been extensively studied. The present chapter focuses on the application of various in vitro culture systems in the production of bioactive secondary metabolites in medicinal herbs and fungi in Taiwan. All the species described in this article are sources of traditional Chinese medicines. The review

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article includes the research work carried out in our laboratory on the production of plant secondary metabolites in callus cultures of *Saussurea involucrata*, *Solanum melongena*, and *Salvia miltiorrhiza* and in cell suspension cultures of *Taxus mairei*, *Gnetiana davidii* var. formosana, and *Angelica dahurica* var. formosana. Also, plant metabolites were obtained from in vitro shoots of *Glossogyne tenuifolia*, *Saussurea involucrata*, and *Polygonum multiflorum*, aerial parts of *Scrophularia yoshimurae*, tubers of *Corydalis yanhusuo*, tissue culture plants, and hairy roots of *Gentiana scabra*. Also, we could achieve the production of terpenoids in *Antrodia cinnamomea*, a medicinal fungus, and tanshinones in *Salvia miltiorrhiza* using T-DNA activation-tagging technique (*Agrobacterium tumefaciens*-mediated transformation).

**Keywords**

Activation tagging • Bioactive secondary metabolites • Callus cultures • Cell suspension • Hairy roots • Medicinal plants and fungi • Tissue culture

## Contents

## Abbreviations

| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| ABA | Abscisic acid |
| ACE | Angiotensin-converting enzyme |
| ATM | Activation-tagging mutagenesis |
| ATMT | *Agrobacterium tumefaciens*-mediated transformation |
| BA | 6-Benzyladenine |
| CaMV | Cauliflower mosaic virus |
| DNA | Deoxyribonucleic acid |
| $GA_3$ | Gibberellic acid |
| GMM | Genetically modified mycelia |
| GMT | Genetically modified transgenic () |
| GUS | β-Glucuronidase |
| HPLC | High-performance liquid chromatography |
| HPT | Hygromycin phosphotransferase |
| MS | Murashige and Skoog |
| NAA | α-Naphthaleneacetic acid |
| PCR | Polymerase chain reaction |
| PGR | Plant growth regulator |
| T-DNA | Transfer DNA |
| WPM | Woody plant medium |

## 14.1 Introduction

Currently, many natural products are obtained solely from massive quantities of wild populations of several species of medicinal plants and fungi. Demand of the herbal products is increasing each passing day. However, supply from wild sources is scarce and unable to cope up the requirements. Therefore, efforts are being made all over the world to develop alternative approaches for the production of valuable pharmaceutical compounds in the controlled conditions of a laboratory. Plant tissue culture system is an alternative for the production of plant secondary metabolites. The most significant advantage of in vitro culture system is its independence of geographical, seasonal variations, and environmental factors. Also, specific genetic transformation can be performed for the production of bioactive secondary metabolites in medicinal plants and fungi.

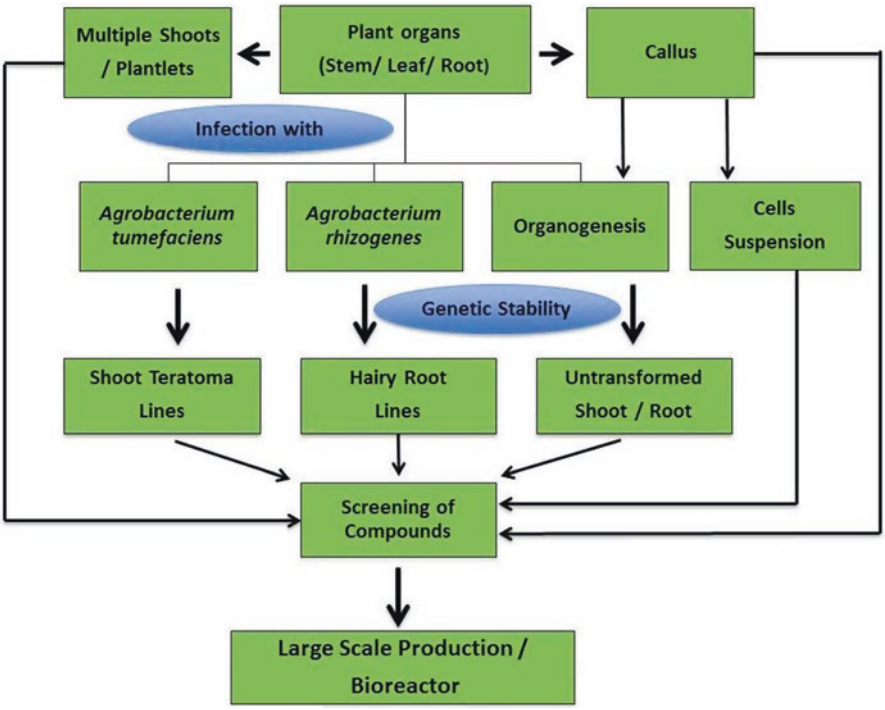


**Fig. 14.1** Schematic representation of secondary metabolite production in different in vitro culture systems

In our laboratory, several bioactive secondary metabolites have been produced in in vitro culture system and in some cases have been compared with commercially available market crude drug samples. The present article reviews the work carried out in our laboratory for the production of secondary metabolites from different culture systems such as callus (*Saussurea involucrata*, *Solanum melongena*, *Salvia miltiorrhiza* Bunge), cell suspension (*Taxus mairei*, *Gnetiana davidii* var. formosana (Hayata) T.N.Ho, *Angelica dahurica* var. formosana), in vitro shoots (*Glossogyne tenuifolia, Saussurea involucrata, Polygonum multiflorum*), aerial parts (*Scrophularia yoshimurae* Yamazaki), tubers (*Corydalis yanhusuo*) of tissue culture plants, and hairy roots (*Gentiana scabra*). Also, the production of bioactive secondary metabolites in *Antrodia cinnamomea*, a medicinal fungus, and *Salvia miltiorrhiza* Bunge using T-DNA activation-tagging technique (*Agrobacterium tumefaciens*-mediated transformation) has been described.

A schematic representation of plant secondary metabolite production in different in vitro culture systems is shown in Fig. 14.1, and studies on different medicinal plants carried out in our laboratory have been listed in Table 14.1.

**Table 14.1** Production of bioactive secondary metabolites in medicinal plants in Taiwan using different tissue culture systems

| Tissue culture material | Plant species | Bioactive secondary metabolites | References |
|---|---|---|---|
| Callus | *Saussurea involucrata* Kar. et Kir. | Syringin; rutin | Kuo et al. (2015) |
| | *Salvia miltiorrhiza* Bunge | Cryptotanshinone | Wu et al. (2003) |
| | *Solanum melongena* L. | Nasunin; *cis*-coumaroyl isomer of nasunin | Hua et al. (2001) |
| Cell suspensions | *Gentiana davidii* var. *formosana* (Hayata) T. N. Ho | Gentiopicroside; Swertiamarin | Chueh et al. (2001) |
| | *Taxus mairei* | Taxol | Lee et al. (1995) |
| | *Angelica dahurica* Benth. et Hook. f. var. *formosana* Yen | Imperatorin | Tsay et al. (1994) and Tsay (1999) |
| | *Dioscorea doryophora* Hance | Diosgenin | Yeh et al. (1994) |
| In vitro shoots | *Glossogyne tenuifolia* Cassini | Oleanolic acid; luteolin | Chen et al. (2014) |
| | *Saussurea involucrata* Kar. et Kir., | Syringin; rutin | Kuo et al. (2015) |
| | *Polygonum multiflorum* Thunb. | Emodin; physcion | Lin et al. (2003) |
| In vitro plants (aerial parts) | *Glossogyne tenuifolia* Cassini | Oleanolic acid; Luteolin | Chen et al. (2014) |
| | *Peucedunum japonicum* Thunb. | Chlorogenic acid; rutin | Unpublished |
| | *Dendrobium tosaense* Makino; *D. moniliforme* Sw. | Alkyl ferulates | Lo et al. (2004a) and Lo et al. (2004b) |
| | *Scrophularia yoshimurae* Yamazaki | Harpagoside | Sagare et al. (2001) |
| | *Gentiana davidii* Franch. var. *formosana* (Hayata) T. N. Ho | Gentiopicroside; swertiamarin | Chueh et al. (2001) |
| Tubers of tissue culture plants | *Corydalis yanhusuo* W. T. Wang | Tetrahydropalmatine; Corydaline | Lee et al. (2001) |
| Hairy roots | *Gentiana davidii* var. *formosana* (Hayata) T. N. Ho | Gentiopicroside, swertiamarin, loganic acid | Huang et al. (2014) |

## 14.2 Production of Bioactive Secondary Metabolites in Callus Cultures

A callus represents an undifferentiated mass of cells derived from plant tissues and has several applications in biotechnology. Plant parts (explants) are surface sterilized and then cultured onto a defined nutrient medium to induce callus for secondary metabolite production. In the following section, secondary metabolite production from callus cultures of *Saussurea involucrata*, *Salvia miltiorrhiza*, and *Solanum melongena* has been briefly described.

### 14.2.1 Production of Syringin and Rutin in Callus Cultures of *Saussurea involucrata* (Kar. et Kir.)

Genus *Saussurea* belonging to family Asteraceae consists of 300 species, mostly growing in the highest diversity in alpine habitats in the Himalaya and Central Asia. *Saussurea* species are perennial herbaceous plants and are commonly known as "Snow Lotus" or "Xuě liánhuā." In China, it grows only in a very specific habitat in the alpine regions of the Tianshan and Kunlun mountain ranges in the Xinjiang Province. The species has been overly harvested from the natural habitats for commercial exploitation and difficult to cultivate. Therefore, in China, the species is listed as a second-grade national protected wild plant (Fu 1992). *S. involucrata* is an important herb in traditional Chinese system of medicine used for treating rheumatoid arthritis, gynopathy, and high-altitude diseases. Two flavonoids, syringin and rutin, have been reported to possess antirheumatic, anti-inflammatory properties and can prevent cardiovascular diseases, enhance immunity, and act as anti-aging, anticancer, and anti-fatigue agents (Jia et al. 2005; Yi et al. 2010; Zhang et al. 2007). In vitro propagation system is a powerful tool which can help in the mass multiplication and germplasm conservation of endangered plant species. In view of ever increasing demand and scarcity of plant materials, tissue culture studies were carried out in our laboratory (Kuo et al. 2015). The HPLC analysis of callus lines showed much higher syringin content as compared to the commercially available market crude drug (Table 14.2). Thus, two bioactive secondary metabolites, syringin and rutin (Fig. 14.2a, b), could be produced in callus cultures without sacrificing the endangered *Saussurea involucrata* plants (Kuo et al. 2015).

### 14.2.2 Production of Cryptotanshinone from Callus Cultures of *Salvia miltiorrhiza* Bunge

*Salvia miltiorrhiza* Bunge belonging to family Lamiaceae is a well-known oriental medicinal herb. The roots of *S. miltiorrhiza* known as "Dānshēn" in the traditional Chinese medicinal system have been used in many therapeutic remedies. The main active compounds of *S. miltiorrhiza*, the tanshinones, are a group of quinoid diterpenes (tanshinone I, tanshinone IIA, and cryptotanshinone). These bioactive

**Table 14.2** HPLC analysis of wild plants, market crude drug, and tissue culture materials for estimation of secondary metabolites in a few medicinal plant species

| Plant species | Bioactive secondary metabolites | Quantities of compounds (mg/g dw) | | | Plant material | References |
|---|---|---|---|---|---|---|
| | | Wild plants | Market crude drug | Tissue culture plants | | |
| *Glossogyne tenuifolia* Cassini (Hsiang-Ju) | Oleanolic acid | 13.78[a] | 6.51 | 16.89[b] | [a]Aboveground parts | Chen et al. (2014) |
| | Luteolin | 0.82 | 0.13 | 0.84 | [b]Tissue culture plants (3 months old) | |
| *Saussurea involucrata* Kar.et Kir | Syringin | – | 0.95 | 13.52 | Callus | Kuo et al. (2015) |
| | Rutin | – | 0.24 | 0.34 | | |
| *Peucedunum japonicum* Thunb. | Chlorogenic acid | – | 0.55[c] | 10.5[d] | [c]Leaf powder from Japan | Chen et al. (2016a) |
| | Rutin | – | 0.33[c] | 0.0 | [d]Tissue culture plants (4 months old) | |
| *Gentiana davidii* var. *formosana* (Hayata) T. N. Ho | Gentiopicroside | 78.35 | – | 52.40[e] | [e]Aerial parts of tissue culture plants (6 months old) | Chueh et al. (2001) |
| | Swertiamarin | 0.79 | – | 0.29[e] | | |

- Not analyzed

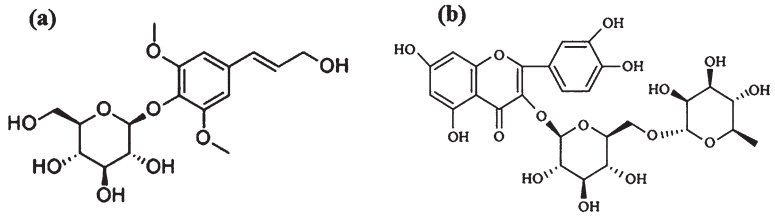


**Fig. 14.2** Chemical structures of (**a**) syringin and (**b**) rutin

secondary metabolites exhibit several pharmaceutical properties, such as antibacterial, antioxidant (Yagi et al. 1989), anti-inflammatory (Kim et al. 2002), cytotoxic (Sung et al. 1999), and antiplatelet aggregation activities (Wang et al. 1989), and have been used in the treatment of certain cardiac disorders (Bruneton 1995). Therefore, *S. miltiorrhiza* has attracted continued interest in the development of biotechnology-based approaches to the production of these bioactive secondary metabolites (Miyasaka et al. 1989; Shimomura et al. 1991; Hu and Alfermann 1993; Bruneton 1995).

In our laboratory, callus induction in leaf explants of *Salvia miltiorrhiza* was obtained on Murashige and Skoog's basal medium (Murashige and Skoog 1962) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0 mg/L) and incubation in the dark.

Further proliferation of callus could be achieved on MS basal medium containing 2,4-D (1.0 mg/L) + 6-benzyladenine (BA) (0.5 mg/L). HPLC analysis of callus showed the presence of small quantities of cryptotanshinone (0.26 mg/g dw). However, on the omission of 2,4-D from the culture medium, callus acquired a deep red color and resulted in a dramatic increase in cryptotanshinone content. It was observed that the cryptotanshinone content in callus cultured on MS basal medium supplemented with 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L BA was significantly higher compared to marketed crude drug (processed from underground parts of *S. miltiorrhiza*). The maximum yield of cryptotanshinone (4.59 mg/g dw) was obtained in the callus cultured on MS basal medium supplemented with 0.2 mg/L BA for 60 days (Wu et al. 2003).

### 14.2.3 Production of Anthocyanins in Callus Cultures of *Solanum melongena*

*Solanum melongena* commonly known as "eggplant" is an important and a highly popular vegetable crop. Eggplant fruits play an inevitable role in many diets worldwide due to its high fiber and low soluble carbohydrate content. The juice obtained from eggplant fruits is used to treat certain liver ailments, and the fluid from the macerated roots is used to treat asthma and syphilis (Jain and Defillips 1991). Recently, a positive influence of certain eggplant types on hyperglycemia risk factors and biomarker of hypertension (ACE) has been demonstrated (Kwon et al. 2008). Nasunin, an important component of anthocyanin pigment of eggplant peels, is known to inhibit peroxidation induced by a linoleic acid–lipoxygenase system (Igarashi et al. 1993). A potent superoxide anion radical scavenging activity and iron-chelating activity of nasunin have been demonstrated (Noda et al. 2000). The ability of eggplant to regenerate in tissue culture has allowed the application of biotechnology.

In our laboratory, an efficient method for callus induction in *S. melongena* was established by culturing pericarp tissues on Murashige and Skoog's basal medium supplemented with α-naphthaleneacetic acid (5.4 μM) and kinetin (2.3 μM) (Hua et al. 2001). HPLC analysis results of pericarp-derived callus showed the presence of two major anthocyanins, nasunin and a *cis*-coumaroyl isomer of nasunin.

## 14.3 Production of Bioactive Secondary Metabolites in Cell Suspension Cultures

Cell cultures are ideal systems for large-scale production processes because the rapid cell growth cycles permit the generation of large quantities of cells. This section briefly describes the work carried out in our laboratory concerning the production of plant secondary metabolites in cell suspension cultures of *Taxus mairei*, *Gentiana davidii*, and *Angelica dahurica.*

### 14.3.1 Production of Gentiopicroside and Swertiamarin in Cell Suspension Cultures of *Gentiana davidii* var. formosana

Recent studies have shown *Gentiana* species possessing anti-inflammatory, analgesic, antirheumatic, antipyretic, diuretic, and hypoglycemic properties (Chen et al. 2008; Sezik et al. 2005; Wani et al. 2011). There are a total of 400 species of genus *Gentiana* distributed in alpine habitats in temperate regions of Asia, Europe, and America (Shimada et al. 2009). Taiwan has 11 *Gentiana* species (Chen and Wang 1999). Among these, *Gentiana davidii* var. *formosana* is the most widespread. Taiwan government has enacted the law to protect the species and collection of wild plants is completely prohibited. This necessitated development of an alternative approach of propagation of *G. davidii*. Cell suspension cultures present an alternative approach to the production of bioactive compounds in *G. davidii.*

In our laboratory, a protocol was optimized for the establishment of cell suspension cultures of *G. davidii* for the production of gentiopicroside and swertiamarin, two pharmacologically important compounds. The maximum gentiopicroside and swertiamarin contents were recorded in 24-day- and 12-day-old cell cultures, respectively (Chueh et al. 2001).

### 14.3.2 Production of Taxol in Cell Cultures of *Taxus mairei*

Paclitaxel (Taxol™), a diterpenoid alkaloid originally isolated from the bark of the Pacific yew (*Taxus brevifolia*), is a valuable pharmaceutical compound. Although the compound is well known primarily for the treatment of cancer, it has also been found to reduce major adverse cardiac events when coated onto coronary stents and is under investigations for the treatment of Alzheimer's disease and other neurodegenerative disorders (Zhang et al. 2005). Since biosynthesis of paclitaxel is a highly complex process, attempts have been made to produce this compound by biotechnological tools (Jaziri et al. 1996; Zhong 2002; Wilson and Roberts 2012). Since the first report of cell cultures of *T. brevifolia* (Gibson et al. 1993), great advances in cell cultures of *Taxus* spp. for the production of Taxol (generic name: paclitaxel) and other taxanes (taxoids) have been achieved in the past decade (Wilson and Roberts 2012).

In our laboratory, *T. mairei* calli were induced from needle and stem explants on B5 medium (Gamborg et al. 1968) supplemented with 2,4-D or NAA at 2 mg/L. Several cell lines were established using these callus cultures. One of the cell lines, after precursor feeding and a 6-week incubation, produced 200 mg/L of taxol (Lee et al. 1995).

### 14.3.3 Production of Imperatorin in Cell Cultures of *Angelica dahurica* var. formosana

*Angelica dahurica* var. *formosana*, commonly known as "Bǎi-Zhì" in Chinese, is a perennial and indigenous plant in Taiwan (Chen et al. 1994). In China, the herb has been used to treat headache and psoriasis (Zhou 1980). The constituent imperatorin

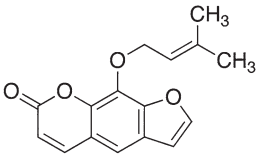


**Fig. 14.3** Chemical structure of imperatorin

(Fig. 14.3) is the major active ingredient in curing the skin disease (Zhou et al. 1988). In recently carried out studies, Deng and coworkers have detected several new coumarins in the roots of *A. dahurica* (Deng et al. 2015). Also, they found that these coumarins have effects on inhibition of NO production in LPS-activated RAW264.7 cells.

In our laboratory, a protocol was optimized for cell suspension cultures of *Angelica dahurica* var. *formosana* using half strength MS basal medium supplemented with 2,4-D (1.0 mg/L) + kinetin (0.1 mg/L) and 3% sucrose. Cultures were routinely subcultured at an interval of 14 days. It was observed that the maximal imperatorin content in the cells ensued between 10 and 14 days of culture, and medium devoid of auxins showed higher imperatorin production. The addition of BA (0.5–1.0 mg/L) or ammonium nitrate/nitrate ratio (2:1) and increased phosphate concentration (from 1 to 2 mM) in the medium led to increased imperatorin production. Glucose was found to be a better carbon source than either sucrose or fructose regarding imperatorin production. A study on the stimulatory effects of elicitors on imperatorin content in cells was also carried out. Vanadyl sulfate (30 mg/L) added at tenth day of cell suspension cultures led to significant increase in imperatorin content, while the addition of Amberlite XAD-7 (20 g/L) on the tenth day of cell suspension cultures resulted in the 140-fold increase of imperatorin compared to control cells (Tsay et al. 1994; Tsay 1999).

## 14.4 Production of Corydaline and Tetrahydropalmatine in the Tubers of Somatic Embryo-Derived Plants of *Corydalis yanhusuo*

*Corydalis yanhusuo* W. T. Wang belonging to the family Fumariaceae or Papaveraceae is a perennial herb cultivated in China as an annual crop by using tubers. Of the 320 species of genus *Corydalis* distributed in the northern hemisphere, about 70 species are used in traditional herbal remedies in China, Japan, and Korea (Kamigauchi and Iwasa 1994). Dried and pulverized tubers of *Corydalis* are utilized in the treatment of gastric and duodenal ulcer, cardiac arrhythmia disease, and several other ailments (Kamigauchi and Iwasa 1994). *Corydalis* tubers contain many pharmacologically active alkaloids such as D-corydaline, DL-tetrahydropalmatine, and corydalis H, I, J, K, and L (Huang 1993). Fungal diseases, mainly downy mildew, often infect *C. yanhusuo* tubers (Gao et al. 1991). Infected tubers if planted in soil resulted not only in 30–50% loss in yield (Gao et al. 1991) but also affected the quality of the crude drug. Thus, it became essential to develop pathogen-free high-quality planting material to boost the production of tubers.

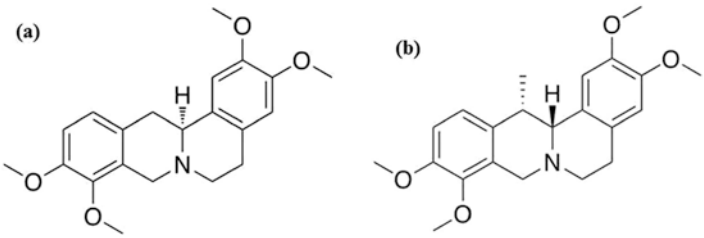


**Fig. 14.4** Chemical structure of (**a**) tetrahydropalmatine, (**b**) corydaline

A method was developed in our laboratory for regeneration of complete plants via somatic embryogenesis in *C. yanhusuo* using tuber-derived callus (Sagare et al. 2000). Primary callus was induced in the culture of tuber slices on MS basal medium supplemented with 6-benzyladenine (BA) (2.0 mg/L) + α-naphthaleneacetic acid (NAA) (0.5 mg/L) and incubation in the dark. On the transfer of this primary callus to MS basal medium supplemented with different concentrations of cytokinins (BA, kinetin, and zeatin), and incubation of cultures in light, induction of somatic embryos was observed within 2 weeks. Well-developed somatic embryos were transferred to a medium supplemented with abscisic acid (ABA), paclobutrazol, or ancymidol, (0.5–10.0 mg/L), GA3 (0.5–5.0 mg/L), polyethylene glycol (PEG-4000) (15–100 mg/L), and sucrose (6%) for their further development into plantlets and in vitro tuberization.

HPLC analysis was carried out to evaluate the alkaloid content in tubers of 1- and 6-month-old plantlets of *C. yanhusuo.* It was observed that tubers of 6-month-old plantlets grown on medium with 0.1 mg/L $GA_3$ had the highest contents of both tetrahydropalmatine (Fig. 14.4a) and corydaline (Fig. 14.4b) (Lee et al. 2001).

## 14.5 Production of Bioactive Secondary Metabolites in In Vitro Shoots Cultures

There are several reports from our laboratory where secondary metabolite production has been achieved in in vitro shoot cultures. This section includes a few examples.

### 14.5.1 Production of Emodin and Physcion in Shoot Cultures of *Polygonum multiflorum*

*Polygonum multiflorum* Thunb. (Polygonaceae) is a perennial herb officially listed in the Chinese Pharmacopeia as one of the most popular Chinese traditional medicines. The tubers known as "Hé shǒuwǔ" in China and East Asia and "Fo-Ti" in North America are used as a tonic and in many Chinese medicines (Chen and Li 1993). Mounting pharmacological studies have stressed out its key benefits for the treatment of various diseases and medical conditions such as liver injury, cancer,

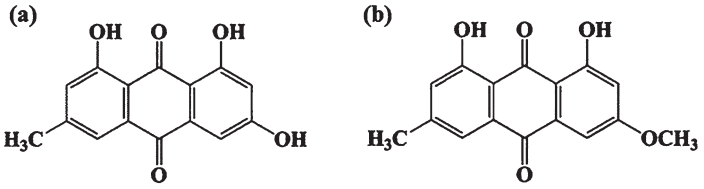


**Fig. 14.5** Chemical structure of (**a**) emodin and (**b**) physcion

diabetes, alopecia, atherosclerosis, and neurodegenerative diseases as well. Most recently, literature on pharmacokinetics-pharmacodynamics analysis, sleep disorders, dyslipidemia treatment, and neurodegenerative diseases and other clinical studies has been reviewed (Bounda and Feng 2015).

An efficient plant regeneration protocol of *P. multiflorum* has been developed in our laboratory (Lin et al. 2003). HPLC analysis of in vitro shoots and tissue culture plants grown in the greenhouse was carried out to evaluate the presence of emodin and physcion. Results showed that emodin and physcion (Fig. 14.5a, b) contents in 6-week-old in vitro shoots and 3-month-old tissue culture were higher compared to the marketed crude drug samples of *P. multiflorum* (Lin et al. 2003).

### 14.5.2 Production of Syringin and Rutin in In Vitro Shoots of *Saussurea involucrata*

Recently, in our laboratory, a complete plant regeneration protocol for *S. involucrata* was established (Kuo et al. 2015). Stages of induction and proliferation of shoots, in vitro rooting, hardening, transfer to soil, and survival in the greenhouse were optimized. It was observed that besides growth regulators and chemical constituents in the culture media, the type of container closures had an influence on the production of syringin and rutin in in vitro shoots and the callus. Also, the HPLC analysis results demonstrated much higher amounts of syringin in in vitro shoots as compared to the commercially available market crude drug (Table 14.2). The tissue culture method optimized in our laboratory has potential application in the production of these two important flavonoids and also conservation of this threatened plant species in wild.

### 14.5.3 Production of Oleanolic Acid and Luteolin in In Vitro Shoots of *Glossogyne tenuifolia*

*Glossogyne tenuifolia*, a perennial herb belonging to Asteraceae family, is native to Penghu Islands, Taiwan (Li 1978). The herb has been used as herbal tea on the island for a long time. In the traditional Chinese system of medicine, it has been used for its antipyretic and hepatoprotective properties (Anonymous 1999). Several recent studies have demonstrated *G. Tenuifolia* possessing several medicinal

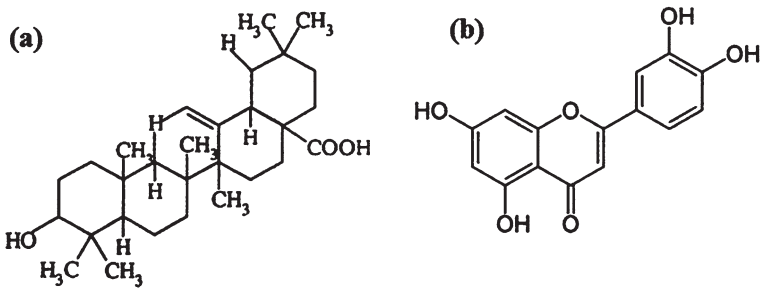


**Fig. 14.6** Chemical structure of (**a**) oleanolic acid (**b**) luteolin

properties such as antioxidant (Yang et al. 2006), anti-inflammatory (Hsu et al. 2005a), immunomodulatory (Ha et al. 2006), and cytotoxicity on several human cancer cell lines (Hsu et al. 2005a). However, supply of natural plant material of *G. Tenuifolia* is seasonal and restricted to only a few months. Given this severe constraint of short supply of plant materials, we carried out in vitro propagation studies in our laboratory. Also, we wanted to explore if we could obtain oleanolic acid and luteolin in shoot cultures of *G. Tenuifolia*. Luteolin, luteolin-7-glucoside, and oleanolic acid are the main bioactive secondary metabolites of *G. tenuifolia.*

HPLC analysis revealed the varying quantities of oleanolic acid and luteolin (Fig. 14.6a, b) in in vitro shoots, tissue culture plants in the greenhouse, and wild-type and commercial crude drug materials (Table 14.2). The oleanolic acid and luteolin contents were found to be significantly higher (16.89 mg/g and 0.84 mg/g, respectively) in 3-month-old tissue culture-raised plants in greenhouse compared to the commercially available crude drug (6.51 mg/g, 0.13 mg/g, respectively). In vitro shoots also showed the presence of active compounds. However, amounts were lower in comparison to the tissue culture-raised greenhouse plants (Chen et al. 2014).

## 14.6 Production of Bioactive Secondary Metabolites in Aerial Parts of Tissue Culture Plants

### 14.6.1 Production of Harpagoside in Aerial Parts of *Scrophularia yoshimurae* Yamazaki In Vitro Plants

*Scrophularia yoshimurae* Yamazaki is a perennial herb native to Taiwan with populations distributed in the central mountain ranges. The species belonging to family Scrophulariaceae is adapted to a narrow set of environmental conditions (Liu 1998). In traditional Chinese medicines, it is called "Xuán shēn" and is a substitute for *Scrophularia ningpoensis* (Chiu and Chang 1998). Roots of *S. yoshimurae* have been reported to be cardiotonic (Reid 1996), possess anti-inflammatory property, and have been used for the treatment of laryngitis, tonsillitis, abscesses of carbuncles, and constipation (Qian et al. 1992).

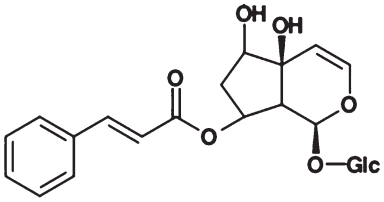


**Fig. 14.7** Chemical structure of harpagoside

Due to short supply of plant materials in Taiwan, roots are imported from China. Therefore, in our laboratory, in vitro plant regeneration method of *S. yoshimurae* was standardized (Lin et al. 1998; Sagare et al. 2001). HPLC analysis results showed that harpagoside (Fig. 14.7) content in the aerial and underground parts of *S. yoshimurae* was significantly higher than the marketed crude drug. The cultures could be maintained for over 2 years without losing their morphogenetic potential. In another study, in our laboratory, gentiopicroside and swertiamarin contents in aerial parts of tissue culture and wild plants of *G. davidii* were recorded (Table 14.2) (Chueh et al. 2001).

### 14.6.2 Production of Chlorogenic Acid and Rutin in Aerial Parts of *Peucedanum japonicum* Thunb. Tissue Culture Plants

*Peucedanum japonicum* Thunb., also known as "the longevity herb," belongs to the family Umbelliferae. The species has distribution in Japan, the Philippines, China, Taiwan, and Korea. In Japan, leaves of *P. japonicum* are consumed for the treatment of cough, while in Taiwan, roots of *P. japonicum* have been used as a folk medicine for the treatment of cold and neuralgic diseases (Chen et al. 1996). Also, in several studies, coumarins from roots and whole plants of *P. japonicum* have shown properties such as antiplatelet aggregation (Chen et al. 1996), antioxidant (Hisamoto et al. 2003), anti-inflammatory, antibacterial (Yang et al. 2009), antidiabetic (Nukitrangsan et al. 2012), and anti-obesity (Nugara et al. 2014; Nukitrangsan et al. 2012; Okabe et al. 2011). Chlorogenic acid and rutin are two important flavonoids responsible for several therapeutic effects of *P. japonicum*. Hsu et al. (2006) have demonstrated that chlorogenic acid inhibited preadipocyte population growth, which may provide a proposed mechanism for reducing obesity. Rutin, a common natural flavonoid, has potential antitumor efficacy and anti-inflammatory effects (Deschner et al. 1991).

In our laboratory, we carried out research on *P. japonicum* with the aim to develop micropropagation system through somatic embryogenesis (SE) and also to carry out LC–MS analysis of syringin and rutin in tissue culture plants derived from somatic embryos and a few commercial products of *P. japonicum* marketed in Japan and Taiwan (Chen et al. 2016). It was observed that SE-derived greenhouse plants (4 months old) had a significantly higher level (10.5 mg/g dw) of chlorogenic acid (Fig. 14.8) compared to commercial product sold in the Japanese market (0.55 mg/g dw). However, rutin was absent in these tissue culture plants in contrast to commercial sample (0.33 mg/g dw) (Table 14.2) (Chen et al. 2016).

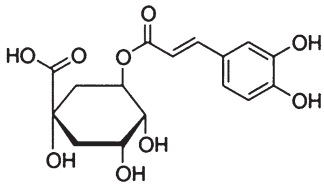


**Fig. 14.8** Chemical structure of chlorogenic acid

## 14.7 Production of Gentiopicroside, Swertiamarin, and Loganic Acid in Hairy Root Cultures of *Gentiana scabra*

Soil bacterium *Agrobacterium rhizogenes* containing root inducing plasmids (Ri plasmids) is responsible for the induction of hairy roots in certain plants by genetic transformation events. The neoplastic roots produced by *A. rhizogenes* infection have a high growth rate as compared to non-transformed roots and possess a high genetic and biochemical stability. Once established, hairy roots are an ideal system for the production of secondary compounds. These roots can easily grow indefinitely on culture medium devoid of plant growth regulators (PGRs) and usually have tremendous growth potential due to profuse lateral rooting. In a recent research development, Ri plasmids can be engineered to contain T-DNA used for genetic transformation of plant cells. The resulting genetically transformed root cultures can produce high levels of secondary metabolites, comparable or even greater than those of intact plants (Georgiev et al. 2007). A large number of studies on hairy roots in different plant species carried out in various parts of the world have been summarized in several reports (Shanks and Morgan 1999; Guillon et al. 2006; Georgiev et al. 2007, 2010).

In our laboratory, an efficient hairy root culture system of *G. scabra* was established (Huang et al. 2014). Leaf explants infected with *Agrobacterium rhizogenes* (strain ATCC15834) induced hairy roots (Fig. 14.9a) 21% explants after 4 weeks of infection. These hairy roots could be easily multiplied on Woody Plant Medium (WPM) (Lloyd and McCown 1981) (Fig. 14.9b, c). Further investigations were carried out to study the influence of different plant growth regulators (PGRs) on the growth rates of hairy roots and on the production of gentiopicroside, swertiamarin, and loganic acid (Fig. 14.10a–c) in the developed hairy roots. Among the various solid and liquid media tested, it was found that B5 (Gamborg et al. 1968) liquid medium resulted in the maximum root biomass in a 4-week culture (Fig. 14.9d). The highest production of loganic acid, swertiamarin, and gentiopicroside in the hairy was obtained with the use of the zeatin in the medium. The quantitative analysis showed that contents of loganic acid and gentiopicroside accumulation were 6.6-fold and 1.8-fold higher in the presence of zeatin (1 mg/l) and α-naphthaleneacetic acid (NAA, 1 mg/l), respectively, as compared to the roots of plants grown in the greenhouse. Also, it was noted that iridoid and secoiridoid contents in hairy roots were significantly affected by the age and growth environment of the *G. scabra* plants. The transformed hairy root lines were confirmed by PCR using rolB and

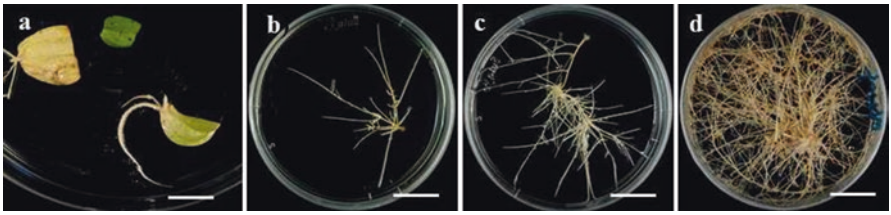


**Fig. 14.9** Hairy roots of *Gentiana scabra* induced by *Agrobacterium rhizogenes*, (**a**) hairy roots induced at cut end of a leaf, (**b**) hairy roots growth on WPM medium (2 weeks), (**c**) hairy roots growth on WPM medium (4 weeks), (**d**) hairy roots growth on WPM medium (8 weeks). Bar = 2 cm

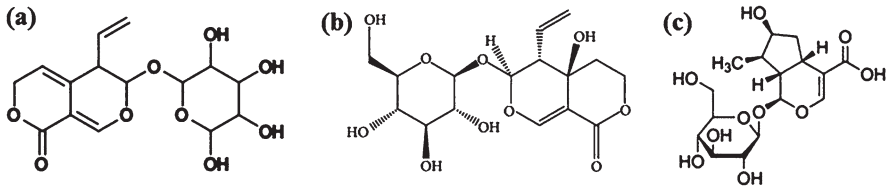


**Fig. 14.10** Chemical structures of (**a**) gentiopicroside, (**b**) swertiamarin, (**c**) loganic acid

rolC gene-specific primers. The method developed has application in the production of gentiopicroside, swertiamarin, and loganic acid in hairy roots of *G. scabra* and could be an alternative to the use of wild plants in the natural habitats.

## 14.8 Secondary Metabolite Production Through T-DNA Activation Tagging

### 14.8.1 T-DNA Activation Tagging in *Antrodia cinnamomea*: An Extremely Rare Medicinal Fungus

*Antrodia cinnamomea* belongs to kingdom Fungi, division Basidiomycota, Class Agaricomycetes, order Polyporales, and family Fomitopsidaceae (Chang and Chou 1995). *A. cinnamomea* is an extremely rare and important medicinal fungus native to Taiwan. The orange-colored fruiting bodies grow slowly on the inner cavity of tree *Cinnamomum kanehirai* (Lauraceae), an endemic and endangered species in Taiwan. In traditional medicines, the fungus locally known as "Niu-Chang-Chih" has been widely used in the treatment of diarrhea, abdominal pain, hypertension, itchy skin, poisoning, hepatic diseases, and tumorigenic diseases, as well as in food, alcohol, or drug detoxification (Tsai and Liaw 1982). In several studies carried out during last decade, bioactive compounds isolated from *A. cinnamomea* have shown properties such as antitumor (Nakamura et al. 2004; Hsu et al. 2005b), anti-hepatitis B virus (Lee et al. 2002; Shen et al. 2005), vasorelaxation (Wang et al. 2003), and neuroprotective (Chen et al. 2006). Also, fruiting bodies have immune-modulating

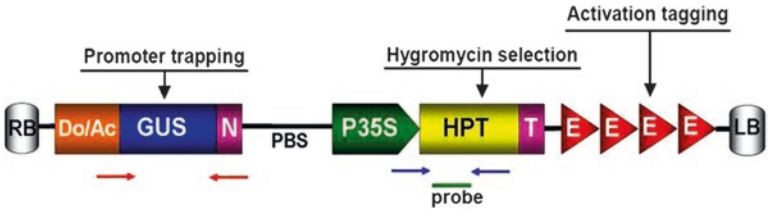


**Fig. 14.11** Schematic representation of the T-DNA of tagging vector pTAG-8. *LB* left border, *RB* right border, *GUS* β-glucuronidase, *N* nopaline synthase terminator, *P35S* cauliflower mosaic virus (CaMV) 35S promoter, *HPT* hygromycin phosphotransferase, *T* CaMV 35S terminator, *E* CaMV 35S enhancer

effects on human leukocytes (Shen et al. 2004), as well as anti-inflammatory effects (Wu et al. 2007; Chen et al. 2007). Mycelia of *A. cinnamomea* mainly consist of triterpenoids (Cherng and Chiang 1995), polysaccharides (Wu et al. 2007), and dicarboxylic acids (Nakamura et al. 2004). Currently, *A. cinnamomea* is used as a remedy for cancer, hypertension, and hangover in Taiwan (Liu et al. 2012). Fruiting bodies of *A. cinnamomea* carry an exorbitantly high price due to the short supply. There is an annual market of over US$100 million in the island.

Fruiting bodies of *A. cinnamomea* have been reported to contain certain triterpenoids, responsible for the medicinal properties of fungus. A total of 78 compounds, including terpenoids, benzenoids, lignans, and benzoquinone derivatives, have been identified. Among terpenoids group, the triterpenoids are considered as the most biologically active components (Geethangili and Tzeng 2011). These triterpenoids are normally absent in mycelia. However, compared to mycelia, fruiting bodies are difficult to grow in the artificial culture medium. Given this, extensive research on isolation of bioactive compounds and their medicinal properties has been done on mycelia of *A. cinnamomea*, and results are quite encouraging.

In our laboratory, an activation-tagging method was developed to obtain mycelial transgenic lines of *A. cinnamomea*, which could produce higher amounts of total triterpenoids (Chen et al. 2009, 2016). For successful *Agrobacterium tumefaciens*-mediated transformation (ATMT), different factors were investigated. *Agrobacterium tumefaciens* strain EHA105, carrying a binary vector pTag8 (Fig. 14.11), was used for the transformation. The binary vector contained a selectable marker coding for the hygromycin phosphotransferase (hphII) gene under the control of the CaMV35S promoter (Fig. 14.11).

Activation tagging is a method to generate dominant mutations by random insertion of enhancer sequences into the genome. Such insertion can produce gain-of-function or loss-of-function mutants. T-DNA activation tagging using cauliflower mosaic virus (CaMV) 35S enhancers is the preferred method due to its efficiency and a high percentage of single T-DNA insertion. The activation-tagging approach has been used successfully in several plant species, such as *Arabidopsis* (Kardailsky et al. 1999), *Catharanthus roseus* (Van der Fits et al. 2001), tomato (Mathews et al. 2003), poplar (Busov et al. 2003), and rice (Hsing et al. 2007).

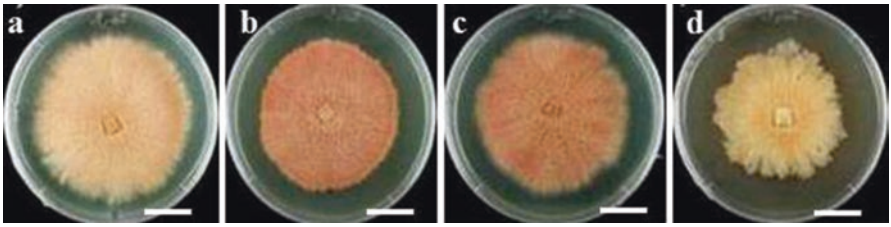


**Fig. 14.12** Mycelia culture of wild type and three genetically modified transgenic (GMT) lines of *Antrodia cinnamomea.* (**a**) Wild-type (control) mycelia of *A. cinnamomea*, (**b**) GMT line H (**c**) GMT line J-1 (**d**) GMT line J-2. Culture medium: MEA+2% agar. Incubation – in dark for 30 days. Bar = 2 cm

It was observed that *Agrobacterium* concentration of $5 \times 10^8$ cfu $ml^{-1}$, 1 mM acetosyringone, 25-day-old mycelia at 0.2 $g/ml^{-1}$, and co-culture period of 6 days were the optimal conditions for enhancing the transformation efficiency. Forty-seven activation-tagging mutants were developed using this protocol. Results showed that 88% of the mutants contained a single T-DNA insertion. Two of the mutants were observed with different triterpenoid profiles compared with the non-transformed line. It was reported that the medicinal properties of this fungus are mainly due to the presence of certain triterpenoids in fruiting bodies which are absent in mycelia. More recently, in our laboratory, it was demonstrated that in contrast to mycelia of wild type of *A. cinnamomea* (Fig. 14.12a), three genetically modified mycelia (GMM) lines of (Fig. 14.12b–d) showed production of both mycelia and fruiting body-specific triterpenoids (dehydrosulphurenic acid, dehydroeburicoic acid, antcin A, antcin B, and antcin C) (Fig. 14.13a–e) (Chen et al. 2016b). These results suggest a new functional genomics approach to tag the triterpenoid biosynthesis genes in *A. cinnamomea*. Since mycelia are becoming increasingly popular as an important functional food for the treatment of several critical illnesses, these findings have significance and demonstrate that it is feasible to produce unique mycelia containing fruiting body-specific triterpenoids.

### 14.8.2 T-DNA Activation Tagging Yields Higher Tanshinone Contents *in Salvia miltiorrhiza*

Background information and medicinal properties of *Salvia miltiorrhiza* Bunge have been briefly described in Sect. 2.3 of this chapter. Further, in our laboratory, an activation-tagging mutagenesis (ATM) population of *S. miltiorrhiza* Bunge was established by *Agrobacterium*-mediated transformation (Lee et al. 2008). *Agrobacterium tumefaciens* strain EHA105 containing pCAMBIA 1302 was used to optimize the conditions for the *Agrobacterium*-mediated transformation of *S. miltiorrhiza*. A binary vector (pTAG-8) was used for promoter trapping and gene activation tagging. The factors affecting the efficiency of *Agrobacterium* transformation such as the conditions of cell growth, *Agrobacterium* concentration,

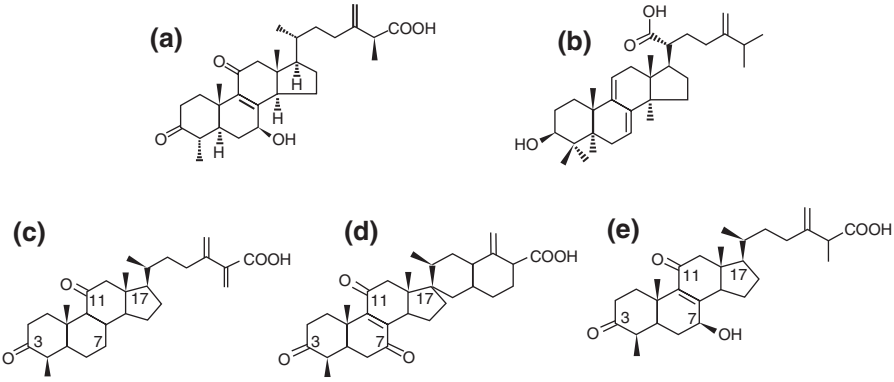


**Fig. 14.13** Chemical structures of (**a**) dehydrosulphurenic acid, (**b**) dehydroeburicoic acid, (**c**) antcin A, (**d**) antcin B, (**e**) antcin C

acetosyringone concentration, and the cocultivation period were optimized. In functional genomic studies, it is necessary to establish a large ATM population of the plant cells. Also, the ATM population should focus on a specific purpose with a specific screening model. In the case of our study on *S. miltiorrhiza*, the optimum conditions for *Agrobacterium* transformation were determined by the expression of the green fluorescent protein. Under these optimized conditions, 1435 ATM cell lines were isolated by antibiotic selection. Of these 700 cell lines tested for the β-glucuronidase (GUS) assay, 35 showed GUS activity. Of these six lines (T1–T6), showed a red color on a selective medium containing 4.5 μM 2,4-D. This red color is used as a phenotypic model system to identify the accumulation of tanshinones. Southern blotting analysis revealed that the T1–T7 ATM cell lines have a single copy of the T-DNA insertion.

A comparative HPLC analysis of ATM-transformed and non-transformed calli showed varying quantities of three tanshinones (Fig. 14.14a–c). There were negligible tanshinones in non-transformed white calli induced with 2,4-D. ATM lines T1–T6 showed a significant increase in tanshinone I (up to 43-fold), tanshinone IIA (up to 26-fold), and cryptotanshinone (up to 104-fold) contents compared with those of the non-transgenic lines on the medium with 2,4-D. Interestingly, the yield of cryptotanshinone from line T4 on 2,4-D medium was two times higher than that of the non-transgenic lines on a medium with *trans*-zeatin riboside. Furthermore, a color-based phenotypic model system was devised to screen the ATM population for transgenic lines with elevated yields of tanshinones. Ours was the first report of a quantitative and qualitative improvement in quinoid diterpene production achieved in a medicinally important plant species by the "T-DNA activation-tagging" technique.

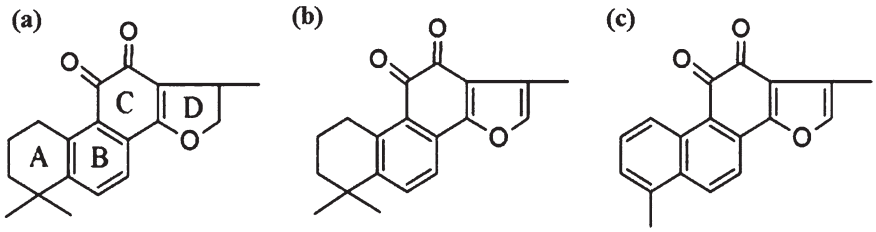


**Fig. 14.14** Chemical structure of (**a**) cryptotanshinone, (**b**) tanshinone IIA, (**c**) tanshinone I

## 14.9 Conclusions

Taiwan is a home to a large number of medicinal plants and fungi used in traditional Chinese medicines. The limited supply of wild plant materials is a severe constraint which could be overcome by adopting modern alternative cultivation technologies. Studies on different medicinal plants carried out in our laboratories during the last two decades demonstrate that it is possible to achieve production of planting materials of medicinal plants. Also, studies presented in the article validate that it is feasible to produce specific and sometimes new bioactive compounds by employing a combination of in vitro culture systems including tools of genetic transformation.

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# Part IV

# Resources/Techniques: Medicinal Plants and Fungi

# Memory-Enhancing and Memory-Related Beneficial Effects of Selected Medicinal Plants from the Nigerian Flora

15

Taiwo O. Elufioye

**Abstract**

The cholinesterase inhibitory activity and the memory-enhancing effects of 22 Nigerian medicinal plants belonging to 16 different families were investigated. The acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory potentials of extracts, fractions, and isolated compounds were evaluated by Ellman colorimetric and thin-layer chromatography (TLC) bioautographic assay techniques. Bioactivity-directed phytochemistry, as well as spectroscopic analysis, was carried out. Morris water maze test was used to assess the cognitive enhancing potential of some of the most active plants. Some plants such as *Morinda lucida, Spondias mombin, Pycnanthus angolensis*, *and Peltophorum pterocarpum* showed inhibitory activity on both enzymes, while others exhibited some remarkable selectivity in their actions. *Alchornea laxiflora* stem bark and root bark, *Calophyllum inophyllum* root bark, and *Crinum jagus* leaves were selectively active against AChE, while *Antiaris africana*, *Bombax bromoposenze*, *Combretum molle*, and *Garcinia kola* were selectively active against BuChE. Activity-directed phytochemistry led to the isolation of bioactive compounds which may lead to drug development. The in vivo effect of four most active plants, *S. mombin, P. angolensis, P. pterocarpum*, *and M. lucida*, on scopolamine-induced memory loss was also confirmed.

**Keywords**

Alzheimer's disease • Anticholinesterase • Medicinal plants • Memory-enhancing effects • Morris water maze

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## Contents



## Abbreviations

| | |
|---|---|
| AChE | Acetylcholinesterase |
| AD | Alzheimer's disease |
| APT | Attached proton test |
| ATCHI | Acetylthiocholine iodide |
| BUCHCL | Butyrylcholine chloride |
| BuChE | Butyrylcholinesterase |
| COSY | Correlation spectroscopy |
| DEPT | Distortionless enhancement by polarization transfer |
| DMSO | Dimethyl sulfoxide |
| DTNB | 5,5′-Dithiobis-(2-nitrobenzoic acid) |
| H2SO4 | Sulfuric acid |
| HMBC | Heteronuclear multiple-bond correlation spectroscopy |
| HMQC | Heteronuclear single quantum correlation spectroscopy |
| IMRAT | Institute of Advanced Medical Research and Training |
| NMR | Nuclear magnetic resonance |
| NOESY | Nuclear Overhauser effect spectroscopy |
| OAU | Obafemi Awolowo University |
| PTLC | Preparative thin-layer chromatography |
| TLC | Thin-layer chromatography |
| VLC | Vacuum liquid chromatography |

## 15.1 Introduction

Apart from pathological memory loss of neurodegenerative diseases, memory impairment and dementia are on the increase due to an increase in aging population. Medicinal plants have significant therapeutic value in the treatment of the above disorders (Li and Vederas 2009; Silverman and Holladay 2014; Link et al. 2015), and memory-related diseases have been managed with plant remedies for centuries (Perry et al. 2000). Alzheimer's disease (AD) is a neurodegenerative disease characterized by cholinergic neurodegeneration in the brain leading to cognitive deficit and memory impairment (Murraya et al. 2013)**.** Cholinesterase inhibitory activity of plants, used traditionally for managing memory loss, has been reported by many researchers (Ingkaninan et al. 2003; Oh et al. 2004; Elufioye et al. 2010). Scopolamine-induced amnesic animal model has been widely used for screening compounds for anti-dementia effects (Lee et al. 2009; Rubaj et al. 2003). Morris water maze test is widely accepted for the assessment of spatial memory in an experimental animal model (Lee et al. 2009; Moris 1984; Kim et al. 2003). Our findings on cholinesterase inhibitory and memory-enhancing potentials of selected species of the Nigerian flora are presented in this chapter.

## 15.2 Plant Materials

The plant parts used were collected from various locations (Table 15.1) and were properly identified.

## 15.3 Extraction

The powdered parts of the different plants were macerated separately with 80% methanol for 72 h and extracts concentrated in vacuo at 40 °C.

## 15.4 Anticholinesterase Assay Procedures

### 15.4.1 Spectrophotometric Analysis

The inhibitions of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) were determined spectrophotometrically using acetylthiocholine iodide (ATCHI) and butyrylcholine chloride (BUCHCL) as substrates, respectively (Ellman et al. 1961). Used as a positive control was physostigmine (eserine). In the assay, 2.0 ml of 100 mM of sodium phosphate buffer (pH 8.0), 100 µl of enzyme preparation ($2.55 \times 10^{-3}$ units/µl), and 100 µl of test samples (10 mg/ml) dissolved in methanol were mixed and incubated for 30 min. One hundred microliter of DTNB was then added to the mixture, and the reaction started with the addition of 100 µl of appropriate substrate dissolved in buffer. The hydrolysis of acetylthiocholine and

**Table 15.1** Medicinal plants selected for the screening of anticholinesterase activity

| Plant species | Family | Collection site |
|---|---|---|
| *Tetrapleura tetraptera* | Leguminosae | Medicinal farm, OAU |
| *Markhamia tomentosa* | Bignoniaceae | Ede road, Ile Ife |
| *Jatropha curcas* | Euphorbiaceae | Medicinal farm, OAU |
| *Spondias mombin* | Anacardiaceae | Medicinal farm, OAU |
| *Alchornea laxiflora* | Euphorbiaceae | Medicinal farm, OAU |
| *Morinda lucida* | Rubiaceae | Medicinal farm, OAU |
| *Peltophorum pterocarpum* | Leguminosae | Road 7, OAU campus |
| *Dioscorea dumetorum* | Dioscoreaceae | Medicinal farm, OAU |
| *Capsicum frutescens* | Solanaceae | Medicinal farm, OAU |
| *Ceiba pentandra* | Bombacaceae | Medicinal farm, OAU |
| *Combretum molle* | Combretaceae | Road 1, OAU campus |
| *Holarrhena floribunda* | Apocynaceae | Medicinal farm, OAU |
| *Pycnanthus angolensis* | Myristicaceae | Road 7, OAU campus |
| *Bombax bromoposenze* | Bombacaceae | Medicinal farm, OAU |
| *Garcinia kola* | Guttiferaceae | Medicinal farm, OAU |
| *Antiaris africana* | Moraceae | Medicinal farm, OAU |
| *Calophyllum inophyllum* | Guttiferaceae | Road 7, OAU campus |
| *Crinum jagus* | Amaryllidaceae | Medicinal farm, OAU |
| *Jatropha tanjorensis* | Euphorbiaceae | Medicinal farm, OAU |
| *Cissampelos owariensis* | Menispermaceae | Road 1, OAU campus |
| *Croton zambesicus* | Euphorbiaceae | Ede road, Ile Ife |
| *Ipomea involucrata* | Convolvulaceae | Road 1, OAU campus |

*OAU* Obafemi Awolowo University

butyrylthiocholine was determined spectrophotometrically at 412 nm by the formation of yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine catalyzed by enzymes. Negative control was methanol, and all assays were carried out in triplicates. Percentage enzyme inhibition was calculated as

$$= \frac{a-b}{a} \times 100$$

where $a$ = ΔA/min of control, $b$ = ΔA/min of test sample, and ΔA = change in absorbance.

For the $IC_{50}$ study, 2 ml of phosphate buffer (100 mM, pH = 8.0) and varying concentration of extracts were added together, followed by the addition of 100 µl of the enzyme. The resulting mixture was vortexed and incubated for 30 min at 37 °C. After 30 min, 100 µl of DTNB was added, and the reaction was initiated by the addition of 100 µl of the substrate. The change in absorbance was monitored spectrophotometrically for 4 min at 412 nm. The data recording from the spectrophotometer was subjected to a linear regression analysis using the SigmaPlot Graphical Software, Version 1.02, to obtain the change in absorbance per minute (ΔA/min) which was used to calculate the percentage inhibitions.

The anticholinesterase activity of extracts of several plant species against acetylcholine esterase (AChE) and butyrylcholine esterase (BuChE) has been reported earlier (Elufioye et al. 2010).

### 15.4.2 Thin-Layer Chromatography (TLC) Bioautographic Assay

The TLC bioautographic assay was performed according to Rhee et al. (2001a). Crude extracts, fractions, and subfractions were spotted on the TLC plates followed by the development of appropriate solvent systems. The developed plates were air-dried and sprayed first with $2.55 \times 10^{-3}$ units/ml of AChE until saturated and then incubated at 37 °C for at least 20 min before spraying with 0.5 mM of the substrate (ATCHI), and then DTNB. Eserine (physostigmine) was co-chromatographed as standard AChE inhibitors.

A duplicate plate was run to detect false-positive effects due to the interaction between the components of the extract chromogenic reagents, according to the method cited by Rhee et al. (2001b). Developed TLC plates were sprayed with DTNB/ATCHI reagent (ImM DTNB and ImM ATCHI in phosphate buffer) until the plates were saturated. The plates were allowed to air-dry for about 5 min before they were sprayed with the enzyme solution. A yellow background appeared with white spots caused by inhibiting compounds. Thus, it could be established whether the inhibition was in the enzymatic reaction or in the chemical reaction between thiocholine and DTNB.

### 15.4.3 Fractionation of Methanolic Extracts

The different parts of eight active plants selected for fractionation were partitioned into n-hexane, ethyl acetate, and water. The various fractions were concentrated in vacuo at 40 °C and assayed for AChE and BuChE inhibitory action (Table 15.2).

### 15.4.4 Ethyl Acetate Extraction and Precipitation Studies

The leaves of four most active plants (i.e., *Pycnanthus angolensis, Morinda lucida, Spondias mombin,* and *Peltophorum pterocarpum*) were selected for further investigation. They were bulk extracted separately with 100%, ethyl acetate and extracts were concentrated in vacuo. Lipid constituent of the ethyl acetate extracts was precipitated out by gradual addition of methanol.

**Table 15.2** Anticholinesterase activity of fractions of selected plants against acetylcholine esterase (AChE) and butyrylcholine esterase (BuChE)

| Samples | | % Inhibition (AChE) | | | % Inhibition (BuChE) | | |
|---|---|---|---|---|---|---|---|
| Plant species | Plant part | Hexane | Ethyl acetate | Aqueous | Hexane | Ethyl acetate | Aqueous |
| Eserine | | 92.63 | | | 89.30 | | |
| *Crinum jagus* | Bulb | 45.31 | | | 44.71 | | |
| *Croton zambesicus* | Leaves | 21.29 | 64.81 | 16.79 | 12.66 | 58.34 | 20.11 |
| *Pycnanthus angolensis* | Root | 24.92 | 68.48 | 23.46 | 13.19 | 49.66 | 30.21 |
| *Pycnanthus angolensis* | Stem | 21.74 | 66.70 | 34.46 | 13.28 | 40.02 | 6.98 |
| *Pycnanthus angolensis* | Fruits | 20.02 | 55.59 | 33.16 | 17.39 | 43.14 | 19.40 |
| *Pycnanthus angolensis* | Leaves | 23.94 | 65.66 | 48.80 | 11.49 | 49.38 | 42.17 |
| *Spondias mombin* | Stem bark | 17.24 | 67.80 | 30.08 | 23.00 | 68.51 | 19.93 |
| *Spondias mombin* | Root bark | 14.60 | 88.13 | 57.10 | 21.08 | 76.38 | 49.66 |
| *Spondias mombin* | Leaves | 36.16 | 58.10 | 23.00 | 24.86 | 52.66 | 18.84 |
| *Calophyllum inophyllum* | Stem bark | 27.18 | 47.81 | 11.75 | 14.06 | 21.11 | 8.67 |
| *Calophyllum inophyllum* | Leaves | 10.46 | 46.04 | 18.89 | 4.88 | 23.12 | 13.28 |
| *Calophyllum inophyllum* | Flowers | 10.69 | 38.15 | 10.53 | 9.32 | 24.67 | 18.28 |
| *Calophyllum inophyllum* | Root bark | 16.19 | 44.92 | 19.77 | 14.63 | 43.43 | 18.11 |
| *Calophyllum inophyllum* | Fruits | 10.69 | 28.15 | 13.53 | 10.10 | 29.68 | 5.04 |
| *Tetrapleura tetraptera* | Fruits | 10.19 | 44.32 | 15.77 | 17.38 | 48.13 | 19.37 |
| *Tetrapleura tetraptera* | Leaves | 10.15 | 39.85 | 19.37 | 7.94 | 43.20 | 11.49 |
| *Tetrapleura tetraptera* | Root bark | 7.94 | 46.48 | 10.17 | 5.80 | 42.00 | 12.50 |
| *Tetrapleura tetraptera* | Stem bark | 14.08 | 64.30 | 19.40 | 13.23 | 49.11 | 13.28 |
| *Ipomea involucrata* | Aerial part | 10.55 | 38.00 | 10.13 | 9.08 | 43.19 | 11.69 |
| *Alchornea laxiflora* | Stem bark | 12.31 | 28.10 | 10.69 | 4.02 | 16.60 | 13.33 |
| *Alchornea laxiflora* | Root bark | 13.10 | 25.04 | 12.55 | 18.46 | 15.68 | 13.88 |
| *Alchornea laxiflora* | Leaves | 10.69 | 34.20 | 17.38 | 7.73 | 18.15 | 4.88 |
| *Peltophorum pterocarpum* | Leaves | 28.62 | 66.10 | 18.00 | 22.01 | 46.32 | 19.23 |

(continued)

**Table 15.2** (continued)

| Samples | | % Inhibition (AChE) | | | % Inhibition (BuChE) | | |
|---|---|---|---|---|---|---|---|
| Plant species | Plant part | Hexane | Ethyl acetate | Aqueous | Hexane | Ethyl acetate | Aqueous |
| *Peltophorum pterocarpum* | Stem | 26.66 | 70.10 | 29.14 | 14.44 | 63.84 | 21.74 |
| *Peltophorum pterocarpum* | Fruits | 10.90 | 40.58 | 31.07 | 12.63 | 22.69 | 38.08 |
| *Peltophorum pterocarpum* | Root | 34.02 | 69.91 | 13.28 | 20.63 | 70.13 | 18.15 |

### 15.4.5 Phytochemical and TLC Cholinesterase Analysis of the Selected Plants

The TLC of both the precipitates and the supernatant of the selected most active plants were carried out using chloroform-hexane 7:3 as the solvent system. The developed plates were sprayed with different phytochemical reagents such as vanillin/sulfuric acid, antimony trichloride, Dragendorff's reagent, and anisaldehyde spray reagents. Some of the developed plates were also subjected to TLC autobiographic enzyme assay. After spraying with vanillin/$H_2SO_4$, it was observed that supernatant of most of the plants gave better color reaction to the spraying reagent. Harborne (1973) showed that concentrated sulfuric acid is useful in the general detection of organic compounds such as steroids, terpenes, and lipids. Vanillin/$H_2SO_4$ is also used in the detection of essential oils with positive detection indicated by several different colors (Pothier 2000).

Spraying with Dragendorff's reagent indicated the presence of alkaloid in some of the plants. Alkaloids have been implicated as cholinesterase inhibitor by several researchers (Houghton et al. 2004). Both eserine from *Physostigma venenosum* and galanthamine from *Crinum* are alkaloids which have been reported as AChE inhibitors. Alkaloidal spots were observed as orange-brown zones against a yellow background (Pothier 2000).

Antimony trichloride is used for detecting cardiac glycosides and saponins (Pothier 2000). Precipitates of *S. mombin, M. lucida,* and *P. Pterocarpum* and the supernatant of *C. zambesicus* and *S. mombin* showed positive results with antimony trichloride.

Spraying with anisaldehyde is useful for the detection of terpenoids (usually purple, blue or red) and some other compounds such as ligands, sugar, and flavonoids (Pothier 2000). A number of terpenoid spots were observed in the tested extracts.

Both the precipitates and the supernatants were also subjected to quantitative and qualitative AChE inhibitory activities. The activity was higher in the supernatant when compared with the precipitate (Table 15.3).

**Table 15.3** Cholinesterase inhibitory activity of precipitate and supernatant of the four most active plants

| Name of plants | Plant part | Methanolic extracts (AChE) | Methanolic extracts (BuChE) | Ethyl acetate extracts | Weight (g) | % AChE inhibition |
|---|---|---|---|---|---|---|
| *Morinda lucida* | Leaves | 40.15 ± 2.57 | 34.09 ± 1.93 | Precipitate | 28.95 | 53.20 |
| | | | | Supernatant | 38.66 | 82.35 |
| *Spondias mombin* | Leaves | 48.58 ± 4.56 | 47.34 ± 2.55 | Precipitate | 18.09 | 71.52 |
| | | | | Supernatant | 19.20 | 87.33 |
| *Peltophorum pterocarpum* | Leaves | 47.5 ± 2.41 | 48.9 ± 0.71 | Precipitate | 36.71 | 42.65 |
| | | | | Supernatant | 28.45 | 86.25 |
| *Pycnanthus angolensis* | Leaves | 43.96 ± 3.04 | 43.59 ± 1.77 | Precipitate | 23.31 | 72.60 |
| | | | | Supernatant | 40.78 | 77.44 |
| *Crinum jagus* | Internal standard | | | Extract | 38.93 | 42.83 |

### 15.4.6 *Peltophorum pterocarpum*

*Peltophorum pterocarpum* is a deciduous tree from the family Leguminosae and subfamily Caesalpiniaceae. Several pharmacological activities have been reported for the plant including hepatoprotective (Kaushik et al. 2010) and antioxidant effects (Sridharamurthy et al. 2012). Several bioactive compounds have also been isolated. One new derivative of peltogynoid ophioglonin and a new 2-phenoxychromone with its 3′-O-β-D-glucoside derivative have been reported in the dichloromethane leaf extract (Polasek et al. 2013). Terrestribisamide (Karunai et al. 2012) and sitosterol-β-D-glucopyranoside tetraacetate (Pathipati et al. 2014) have also been isolated from the plant.

#### 15.4.6.1 Isolation of Bioactive Components

The powdered sample (1 kg) was extracted with 80% methanol and concentrated in vacuo. The methanol extract (44.34 g) was successively partitioned into n-hexane, ethyl acetate, and water. Vacuum liquid chromatography (VLC) of the ethyl acetate fraction on silica gel with gradient elution from n-hexane through ethyl acetate to methanol yielded five subfractions. These were tested, and the most active fraction was further purified by column chromatography on silica gel 60 with gradient elution from n-hexane in ethyl acetate through 100% ethyl acetate to 100% methanol. This yielded 112 subfractions bulked into 25 based on their TLC patterns. One compound was isolated following repeated crystallizations in methanol of subfractions d and e pulled together (Elufioye et al. 2016).

#### 15.4.6.2 Spectroscopy Analysis

$^{1}H$ and $^{13}C$ NMR (in both methanol and acetone), COSY, NOESY, and HMBC were recorded on a 600 MHz instrument.

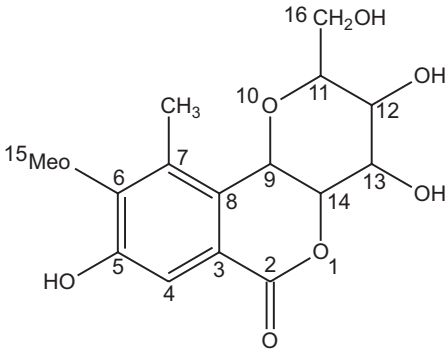


**Fig. 15.1** Chemical structure of bergenin

#### 15.4.6.3 Spectra Data

$^{13}$C NMR: δ 165 (C-2), 119 (C-3), 111 (C-4), 152 (C-5), 142 (C-6), 149 (C-7) 117 (C-8), 74 (C-9), 83 (C-11), 71 (C-12), 75 (C-13), 81 (C-14), 60 (C-15), and 62 (C-16). $^{1}$H NMR: δ 7.08 (s), 4.95 (d), 4.90 (dd), 4.06 (dd), 3.94 (s), 3.80 (dd) 3.70 (m), and 3.49 (dd).

Following data analysis and comparison with literature (Nunomura et al. 2009), the compound was identified as bergenin (Fig. 15.1) with an $IC_{50}$ of 13.17 μM toward AChE and 14.60 μM toward BuChE.

### 15.4.7 *Pycnanthus angolensis*

*Pycnanthus angolensis* (African nutmeg) is an evergreen tree from Myristicaceae family. Flavonoids with cytotoxic effect have been isolated from the plant (Mansoor et al. 2011). Analgesic and anti-inflammatory fatty acids have also been reported (Brill et al. 2004). Other reported activities include antioxidant (Oladimeji and Akpan 2015), antimalarial (Ancolio et al. 2002), antihelminthic (Onocha and Otunla 2010), cholesterol lowering (Leonard 2004), and antinociceptive/antiulcer effects (Sofidiya and Awolesi 2015).

#### 15.4.7.1 Isolation of Bioactive Components

The supernatant (120.36 g) was subjected to vacuum liquid chromatography (VLC) on silica gel using hexane, dichloromethane, and methanol mixtures as the solvent system. A total of 53 fractions were collected and bulked into 6 based on their TLC profile. The bulked fractions were subjected to TLC autobiographic assay, and fractions showing activity were further purified by repeated VLC and PTLC leading to the isolation of the compounds.

#### 15.4.7.2 Spectroscopic Analysis

Both 1D and 2D NMR spectroscopic analyses were carried out. Structure elucidation was done based on $^{1}$H and $^{13}$C NMR, COSY, HMQC, and HMBC spectra data.

#### 15.4.7.2.1 Spectra Data for Compound 1

Compound **1** was brownish yellow in color and oily. The $^{1}H$ NMR spectrum, ($CDCl_3$, 300 Hz) showed the following signals – δ6.4(s), δ5.4 (t), δ4.1(d), δ2.0(d), δ1.4(m), δ0.85(m), δ0.87(m), δ0.9(m), δ 1.70(s), and δ1.60(s) – while the $^{13}C$ NMR ($CDCl_3$, 300 Hz) data are δ 59.63 (C-1), 123.30 (C-2), 140.50 (C-3), 40.08 (C-4), 26.93 (C-5), 37.51 (C-6), 33.90 (C-7), 37.64 (C-8), 25.35 (C-9), 39.95 (C-10), 33.01 (C-11), 39.58 (C-12), 25.00 (C-13), 37.50 (C-14), 28.19 (C-15), 29.91 (C-16), 36.88 (C-17), 135.50 (C-18), 123.48 (C-19), 24.68 (C-20), 16,23 (C-21), 16.38 (C-22), 19.96 (C-23), 22.83 (C-24), 22.92 (C-25), and 19.93 (C-26).

The signal at 5.4 (t) is an olefinic proton assigned to the protons on C-2 and C-19. The signal at δ4.1 (d) represents an alcohol proton and is assigned to the proton residing on C-1. There is a multiplet at δ1.40 to δ1.35 which represents the methylene protons on C-7, C-11, and C-15, while multiplets at δ 1.30 to δ 1.00 were assigned to the protons on C-6, C-8, C-9, C-10, C-12, C-13, C-14, C-16, and C-17. The signal at δ 1.60 (s) was assigned to the methyl protons on C-22 and C-26, while the signal at δ 1.70 was assigned to the OH group. Other assignments include the signals at δ 0.85 (m), δ 0.87 (m), and δ 0.9 (m) which were assigned to the methyl protons on C-21, C-23, C-24, and C-25.

Compound **1** appears to be a C-26 carbon compound since the $^{13}C$ spectrum showed that there were $6CH_3$, $13CH_2$, 5CH, and 2C. Characteristic are the oxygenated terminal methylene carbon resonating at δ 59.39 (C-1), the methine carbons resonating at $\delta_c$ 123.39 and $\delta_c$ 123.48 (C-2 and C-19), respectively, and the quaternary carbons C-3 and C-19 resonating at $\delta_c$ 140.50, and $\delta_c$ 135.50, respectively. The tertiary methyl groups (C-22 and C-26) on C-3 and C-18 resonated at $\delta_c$ 16.38 and $\delta_c$ 19.93, respectively; the secondary methyl groups (C-23, C-24, and C-25) resonated at $\delta_c$ 19.96, $\delta_c$ 22.83, and $\delta_c$ 22.92; while the terminal methyl group (C-21) resonated at $\delta_c$16.23.

On critical examination of the spectra, compound 1 appears to be an extension of phytol by additional double bound and methyl groups. Phytol is a C-20 compound, while compound **1** is a C-26 compound with additional $CH_3$ at C-22; $CH_2$ at C-16, C- 17, and C-20; CH at C-19; and Cq at C-18. This compound with IUPAC name (2*E*, 18*E*)-3,7,11,15,18-pentamethylhenicosa-2,18-dien-1-ol, and named eluptol (Fig. 15.2), appears new, and it is also being reported for cholinesterase inhibitory activity for the first time with an $IC_{50}$ of 22.26 μg/ml (AChE) and 34.61 μg/ml (BuChE).

#### 15.4.7.2.2 Spectra Data for Compound 2

1H NMR ($CDCl_3$, 300 Hz): δ6.6(s), δ6.4(s), δ6.0(t), δ5.1(m), δ4.2(t), δ3.1(d), δ2.6(dd), δ2.2(m), δ2.0(m), δ1.6(m), and δ1.2(m). $^{13}C$ NMR ($CDCl_3$, 300 Hz): δ 132.30 (C-1),124.73 (C-2), 132.42(C-3), 134.77 (C-4), 139.94 (C-5), 123.74 (C-6), 145.69 (C-7), 118.32 (C-8), 173.52 (C-9), 68.33 (C-10), 28.11 (C-11), 26.56 (C-12), 28.38 (C-13), 29.29 (C-14), 34.76 (C-15), 29.91 (C-16), 29.58 (C-17), 27.76 (C-18), 39.26 (C-19), 25.85 (C-20), 39.79 (C-21), 146.10 (C-22), 133.33 (C-23), 130.91 (C-24), 148.68 (C-25), 188.18 (C-26), 16.11 (C-27), 17.88 (C-28), 16.15 (C-29), and 16.28 (C-30).

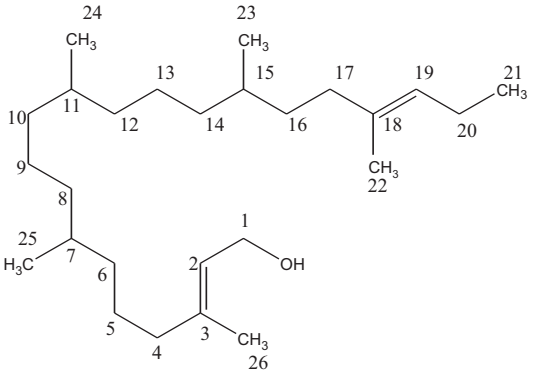


**Fig. 15.2** Chemical structure of eluptol [(2*E*, 18*E*)-3,7,11,15,18-pentamethylhenicosa-2,18-dien-1-ol]

The $^{13}C$ spectrum showed $4CH_3$, $11CH_2$, 7CH, and 8C. Thus, the compound is a C-30 compound. Diagnostic are the carbonyl carbons C-9 and C-26 resonating at $\delta_c$ 173.52 and $\delta_c$ 188.18, respectively. Also important is the oxygenated methylene carbon at C-10 that acts as a bridge between the two aromatic ring systems and resonated at $\delta_c$ 68.33. Also, the methine carbons C-7 and C-8 resonated at $\delta_c$ 145.69 and $\delta_c$ 118.32, respectively, which are in HMBC correlating with the carbonyl at C-9. The hydroxyl group on C-25 ($\delta_c$ 148.68) which made it absorb at a higher value differentiated it from that at C-24 ($\delta_c$ 130.91) even though both are quaternary carbons. The secondary methyl carbon C-28 resonated at $\delta_c$ 17.88, while tertiary methyl groups C-27, C-29, and C-30 resonated at $\delta_c$ 16.11, $\delta_c$ 16.15, and $\delta_c$ 16.28.

The proton signal at δ 6.6 (s) represents the methylene proton on C-23 resonating at δ 133.33 in the HMQC, while that at δ 6.4 (s) resides on C-3 at δ 132.42. The triplet at δ 6.0 was shown to reside on the carbon signal at δ 145.69 assigned as C-7.

In the HMQC spectra, the multiplet at δ 5.1 showed correlation with the carbons at δ 124.73 (C-2), δ 123.74 (C-6), and δ 118.32 (C-8), while the signal at δ 4.2 (t) showed correlation with the diagnostic $OCH_2$ carbon at δ 68.33 and is thus assigned to C-10. The signal at δ3.1 (d) correlated with the carbon at δ 27.76 assigned to C-18, and the multiplet at δ 2,2 to δ 2.0 correlated with carbon signals at δ 26.56, δ 34.76, δ 29.58, and δ 39.79 and was assigned to carbons C-12, C-15, C-17, and C-21. The multiplet at δ 1,6 to δ 1,2 were assigned to the methyl groups at C-27, C-28, C-29, and C-30.

In the HMBC, the $CH_2$ at C-10 showed correlation with the $CH_2$ signal at δ 28.11 which was assigned to C-11 is diagnostic. Also, the $CH_2$ at δ 39.79 (C-21) couples to the quaternary carbon at δ 146.10 (C-22), while the carbonyl carbon at δ 188.18 (C-26) is coupled to the carbon resonating at δ 133.33 (C-23). The HMBC spectra also showed that the CH at δ 145.69 (C-7) coupled with the quaternary carbon at δ 173.52 (C-9) (Fig. 15.3).

Upon comparison with literature, (Renmin et al. 2004; Venkateswara et al. 2011), compound **2** appears to be a cinnamic acid derivative with differences at C-4 and C-5 of the isolated compound and cinnamic acid because of the 4,5-dimethyl substitution on compound 2 which made the carbons absorb at a higher δ values (δ 134.77 and δ 139.94), respectively. Most common cinnamic acid derivative in

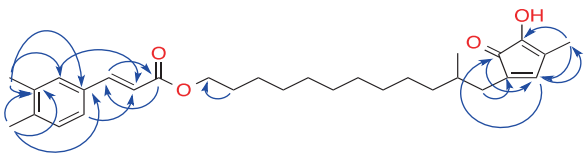


**Fig. 15.3** HMBC correlations of compound 2

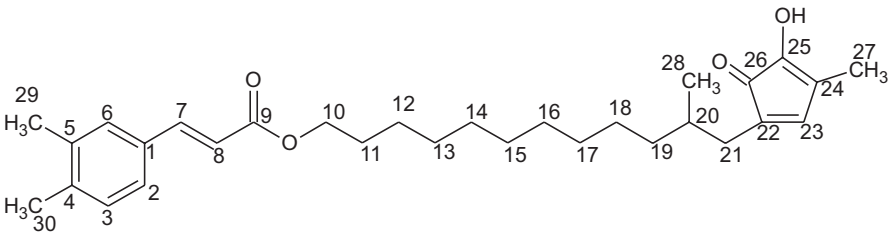


**Fig. 15.4** Chemical structure of omifoate A

literature are 2,3-dimethoxy or 2,3-dihydroxy, unlike the isolated compound which is a 2,3-dimethyl derivative. Also, the attached group to the cinnamic acid through the ester linkage appears new. Thus compound 2,[12-(4-hydroxy-3-methyl-oxo-cyclopenta-1,3-dien-1yl)-11-methyl-dodecyl] (*E*)-3-(3,4-dimethylphenyl)prop-2-enoate, named omifoate A (Fig. 15.4), appears to be new, and it is being reported as cholinesterase inhibitor for the first time with an $IC_{50}$ of 6.51 μg/ml (AChE), 9.07 μg/ml (BuChE).

### 15.4.8 *Spondias mombin* L.

*Spondias mombin* is a medium-sized, occasionally large deciduous tree of the family Anacardiaceae. Biological activities reported on the plant include antiviral (Corthout et al. 1991, 1992, 1994), antifertility (Uchendu and Isek 2008), molluscicidal (Corthout et al. 1994; Abo et al. 1999), β-lactamase inhibitory (Coates et al. (1994), anti-inflammatory (Abad et al. 1996), hematinic (Asuquo et al. 2013), anticonvulsant, antipsychotic, and sedative properties (Ayoka et al. 2005a, b) and abortifacient (Offiah and Anyanwu (1989), oxytocic (Nworu et al. 2007), antimicrobial (Amadi et al. 2007), antigonadotropic (Asuquo et al. 2012), antioxidant (Maduka et al. 2014), and antidiabetic actions (Moke et al. 2015). Isolated compounds include caryophyllene, myrcene, hexanal, 3-hexenol, and (ε) –2-hexenal (Ceva-Antunes et al. 2003), cinnamic acid, 4-hydroxycinnamic acid, 3-methoxy-4-hydrocinnamic acid, 3-methoxy-4-hydroxycinnamic acid, benzaldehyde, linalool, hexanoic acid, alpha-terpineol, palmitic acid and octanoic acid (Adedeji et al. 1991) anacardic acid (Coates et al. 1994), phytosterols mombintane I and II (Olugbuyiro et al. 2013), coumarin, and new flavonoids mombinrin, mombincone, mombinoate, and mombinol, respectively (Olugbuyiro and Moody 2013).

#### 15.4.8.1 Isolation of Bioactive Components

Vacuum liquid chromatography (VLC) of *Spondias mombin* supernatant (19.20 g) was carried out on silica gel 60 with n-hexane, dichloromethane, and methanol. Monitoring of fractions was by thin-layer chromatography (TLC) on pre-coated silica gel 60 F254 (0.25 mm) plates and spraying with vanillin/sulfuric acid reagent. The subfractions collected (103) were bulked into six based on their TLC pattern. The bulked samples were tested for AChE inhibitory activity using TLC bioautographic assay method. Active subfractions further purified using VLC and three bioactive compounds were isolated by preparative thin layer chromatography (PTLC).

#### 15.4.8.2 Spectroscopic Analysis

The isolated compounds were analyzed spectroscopically ($^{1}H$, $^{13}C$, DEPT, COSY, APT, HMQC, HMBC). TLC analysis in different solvent systems, solubility in water, and determination of $IC_{50}$ was also carried out.

#### 15.4.8.3 Spectra Data

Compound (1) 35 mg was a white powder with $R_f$ of 0.46 in hexane: chloroform 3:7 and $R_f$ of 0.35 in 100% chloroform. It gave purple color to both vanillin and $H_2SO_4$ and anisaldehyde spray reagent indicating the steroidal nature of the compound (Osman et al. 2015).

The $^{1}H$ NMR spectrum ($CDCl_3$, 300 Hz) gave signals at δ7.8(m), δ7.75(m), δ5.45(t), δ4.6(s), and δ4.5(d), and the $^{13}C$ NMR spectrum ($CDCl_3$, 300 Hz) gave signals at 38.71 (C-1), 20.90 (C-2), 78.83 (C-3), 35.57 (C-4), 55.24 (C-5), 18.30 (C-6), 34.06 (C-7), 39.35 (C-8), 54.96 (C-9), 37.34(C-10), 27.22 (C-11), 24.92 (C-12), 37.83(C-13), 39.99 (C-14), 27.19 (C-15), 29.48 (C-16), 47.08 (C-17), 50.22 (C-18), 48.97 (C-19), 150.8 (C-20), 29.66 (C-21), 36.65 (C-22), 27.92 (C-23), 15.96 (C-24), 15.46 (C-25), 16.64 (C-26), 14.33 (C-27), 59.41 (C-28), 109.40 (C-29), and 19.70 (C-30). The DEPT experiment showed that there were $6CH_3$, $11CH_2$, 6CH, and 7C. Thus, compound 1 appeared as a C-30 carbon compound.

In the proton NMR, there was a proton at δ4.5 (d) geminal to the hydroxyl group, with a corresponding carbon chemical shift at δ59.41. It also had an olefinic proton at δ4.6 which resided on the carbon at δ109.40. This proton is a terminal $CH_2$ and was assigned to C-22. In comparison with literature data (Tolstikov et al. 2005; Sharma et al. 2010; Uddin et al. 2011), compound 1 was identified as betulin (Fig. 15.5). Betulin has been reported previously in many plant species for various biological activities (Tolstikov et al. 2005). However, its cholinesterase inhibitory activity is reported here for the first time with an $IC_{50}$ of 0.88 μg/ml against AChE and 4.67 μg/ml against BuChE. However, previous researchers (Kim et al. 2006) have reported the activity of some oleanane triterpene saponin compounds in the treatment of dementia and mild cognitive impairment.

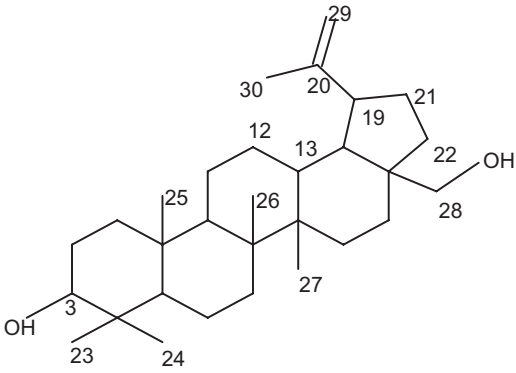


**Fig. 15.5** Chemical structure of betulin

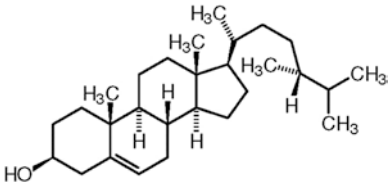


**Fig. 15.6** Chemical structure of campesterol

### 15.4.8.4 Spectra Data of Compound 2

Compound 2 20 mg gave purple color to both vanillin and $H_2SO_4$ and anisaldehyde spray reagent and had $R_f$ values of 0.2 and 0.27 in hexane: chloroform 2:8 and 100% chloroform, respectively.

The $^{13}C$ NMR data are 36.92 (C-1), 34.35 (C-2), 72.22 (C-3), 42.73 (C-4), 141.17 (C-5), 122.14 (C-6), 28.67 (C-7), 32.80 (C-8), 50.53 (C-9), 32.33 (C-10), 21.50 (C-11), 37.66 (C-12), 40.18 (C-13), 57.17 (C-14), 23.42 (C-15), 26.45 (C-16), 56.45 (C-17), 12.26 (C-18), 19.82 (C-19), 36.56 (C-20), 19.44 (C-21), 32.31 (C-22), 24.72 (C-23), 46.23 (C-24), 29.54 (C-25), 20.25 (C-26), 19.20 (C-27), and 12.40 (C-28).

$^{13}C$ NMR spectral data of compound 2 suggested that it is a C-28 compound with the APT experiment revealing three quaternary (3 C), ten methylene (10 $CH_2$), six methyl (6 $CH_3$), and nine methine (9 CH) carbons. The proton NMR showed an olefinic proton at δ5.40 with a corresponding carbon chemical shift of δ121.14 in the HMQC spectrum as well as an oxygenated methylene proton at δ3.5. In the HMBC data, the diagnostic olefinic proton and the proton geminal to the OH had connectivity with the quaternary carbon resonating at 141.17. From the summary of $^{1}H$, $^{13}C$ NMR, APT, HMQC, and HMBC data as well as comparison with literature (Jaju et al. 2010; Jain and Bari 2010), compound 2 was identified as campesterol (Fig. 15.6) with an $IC_{50}$ of 1.89 μg/ml (AChE), 4.08 μg/ml (BuChE),

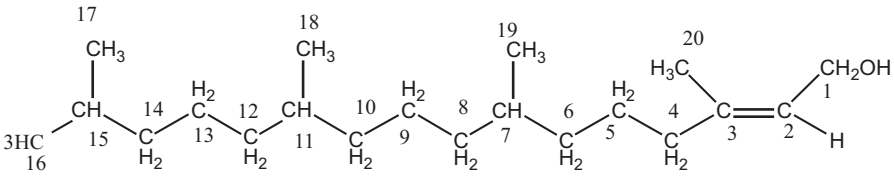

**Fig. 15.7** Chemical structure of phytol (3,7.11.15-tetramethyl-2-hexadecen-1-ol)

Campesterol was earlier reported in many plant species such as soybean oil (*Glycine max*) (Shi et al. 2010), rapeseed oil (*Brassica napa*), (Amar et al. 2008), and wheat germ oil (*Triticum* spp.) (Ruibal-Mendieta et al. 2004), but it is being reported for cholinesterase inhibitory activity for the first time with an $IC_{50}$ of 1.89 µg/ml (AChE) and 4.08 µg/ml (BuChE).

#### 15.4.8.5 Spectra Data of Compound 3

Compound 3 19 mg was isolated as a yellowish liquid and had Rf of 0.64 in hexane: chloroform 1:1 and 0.51 in chloroform 100%. It gave pink color to anisaldehyde spray reagent and purple color with vanillin/$H_2SO_4$.

$^{13}C$ NMR spectral had signals at 59.85 (C-1), 123.48 (C-2), 130.92 (C-3), 40.29 (C-4), 25.55 (C-5) 33.21 (C-6) 30.13 (C-7), 37.78 (C-8), 24.89 (C-9), 37.08 (C-10), 33.11 (C-11), 37.70 (C-12), 25.22 (C-13), 39.79 (C-14), 28.40 (C-15), 23.15 (C-16), 23.05 (C-17), 20.17 (C-18), 20.14 (C-19), and 16.86 (C-20) and revealed $5CH_3$, $10CH_2$, 3CH, and 1C=C suggesting a C-20 compound. The $^1H$ NMR had a signal at δ 5.4(t) which is an olefinic proton assigned to C-2. The alcoholic proton at δ 4.1(d) was assigned to the proton residing on C-1while, the triplet at δ1.98 was assigned to the proton on C-4. The multiplets at δ 1.44 and δ1.35 were the methine protons on C-7 and C-11. Also, the multiplets at δ1.30 to δ1.03 were assigned to protons residing on C-6, C-8, C-9, C-10, C-12, and C-13, while the signal at δ1.65 (s) is the methyl proton on C-20. The signal at δ1.66 represents the OH group. Analysis of the spectral data and comparison with literature (Arigoni et al. 1999) showed compound 3 as phytol (Fig. 15.7). Phytol has been previously reported for its cholinesterase inhibitory activity (Elufioye et al. 2015).

### 15.4.9 *Morinda lucida*

*Morinda lucida* from the family Rubiaceae is a medium-sized tree that grows in tropical West Africa rainforest. Activities reported for the plant include hepatotoxicity and nephrotoxicity (Oduola et al. 2010) and antimalarial (Makinde and Obih

1984) and molluscicide properties (Adewumi and Adesogan 1983). Adesogan (1973) reported the isolation of 18 anthraquinones and its derivatives: lucidin, soranjidiol, damnacanthal, nordamnacanthal, morindin, munjistin, and purpuroxanthin from the wood and bark of *Morinda lucida.* In addition, tannins, flavonoids, and saponosides have been isolated. Adewumi and Adesogan (1983) reported the isolation of anthraquinones and oruwacin from the roots of *Morinda lucida*. Two known triterpenic acids (ursolic and oleanolic acids) were isolated from the leaves (Cimanga et al. 2006). Koumaglo et al. (1992) reported the isolation of three compounds (digitolutein, rubiadin 1-methyl ether, and damnacanthal) from the stem bark of *Morinda lucida*.

#### 15.4.9.1 Isolation of Bioactive Constituents

*Morinda lucida* supernatant (36.66 g) was subjected to repeated vacuum liquid chromatography (VLC) on silica gel with n-hexane, dichloromethane, and methanol as the solvent system. One hundred thirteen subfractions collected were bulked into 7 based on their chromatographic pattern. The bulked fractions were assayed for AChE inhibitory activity using TLC bioautographic method. Fraction $M_1$ showing highest activity was further purified by VLC. The active subfraction ($M1_b$) was subjected to PTLC and active compound isolated (Elufioye et al. 2015).

#### 15.4.9.2 Spectroscopic Analysis

The isolated compound was analyzed spectroscopically ($^1$H NMR, $^{13}$C NMR).

##### 15.4.9.2.1 Spectral Data

$^{13}$C NMR: 59.65 (C-1), 123.30 (C-2), 140.55 (C-3), 40.10 (C-4), 25.36 (C-5), 36.89 (C-6), 32.92 (C-7), 37.66 (C-8), 24.70 (C-9), 37.51 (C-10), 33.01 (C-11), 37.59 (C-12), 25.02 (C-13), 39.59 (C-14), 28.20 (C-15), 22.94 (C-16), 22.85 (C-17), 19.97 (C-18), 19.94 (C-19), and 16.41 (C-20).

The $^{13}$C NMR data of ML-2 showed $5CH_3$, $10CH_2$, 3CH, and 1C=C making compound ML-2 a C-20 carbon compound. The $^1$H NMR had signals at δ 5.4(t), δ 4.1(d), δ1.98 (d), δ1.65 (s), δ1.52. δ 1.44 (m), δ1.35 (m), and δ1.30(m) to δ1.03 (m). Analysis of the spectra showed that compound ML-2 is phytol when compared with literature data (Arigoni et al. 1999) and has been previously reported (Elufioye et al. 2015).

### 15.4.10 Cognitive Enhancement Study

#### 15.4.10.1 Animals

Sixty-five albino mice purchased from the Institute of Advanced Medical Research and Training (IMRAT), College of Medicine, University of Ibadan, were used for this study.

#### 15.4.10.2 Administration of Doses for the Various Groups

The mice were labeled, weighed and grouped into 13 groups of five animals each. All animals were preinjected with 3 mg/kg scopolamine intraperitoneally. Groups 1–3, 4–6, 7–9, and 10–12 were given 0.2 ml equivalent doses of 4 mg/kg, 6 mg/kg, and 8 mg/kg of the extracts of *Morinda lucida*, *Peltophorum pterocarpum, Pycnanthus angolensis*, and *Spondias mombin*, respectively, while the control group 13 was given 0.2 ml of distilled water for 3 consecutive days.

#### 15.4.10.3 Morris Water Maze Test Procedure

Morris water maze is a test usually done to assess spatial memory function. In this study, it was carried out according to the method of Morris (1984) as described by Kim et al. (2003) and Lee et al. (2009). The water maze is made up of a circular pool (90 cm in diameter and 45 cm in height) filled with a mixture of water and evaporated milk to a height of 30 cm. The pool was usually divided into four quadrants with a platform submerged at 1 cm below the water level in one of the quadrants. On day 1 of the assays, the animals were trained to swim for 60 s without the platform. Thereafter, the animals were given two swimming trial sessions per day for 4 consecutive days with the platform in place. Average escape latencies were calculated for each trial session by measuring the locations of each animal from starting position to the platform. After locating it, each mouse was allowed to stay on the platform for 10 s. However, any animal which failed to locate the platform after 120 s was also placed on the platform for 10 s before taking away from the pool. A 30 min interval was observed between daily trials. The point of entry into the pool for the animals and the location of the platform were changed on a daily basis but remained unchanged between daily trials. Changes in the escape latency from day to day represent long-term or reference memory, while changes from trial 1 to trial 2 on the same day represent working or short-term memory. Amnesia was induced in all animals by intraperitoneal injection of 3 mg/kg scopolamine dissolved in water/DMSO. To establish amnesia, all the animals were assessed for spatial memory 24 h after the administration of scopolamine. Treatment with different doses commenced after establishing amnesia in the animals. The control group was given 0.2 mL distilled water instead of extracts.

#### 15.4.10.4 Histopathology

At the end of the experiments, all animals were sacrificed by cervical dislocation. The brains were removed and preserved in phosphate formalin. Slides of the forebrain and hippocampus were prepared and observed under a light microscope with photomicrographs taken and the number of cells in the CA1 region of the hippocampus estimated.

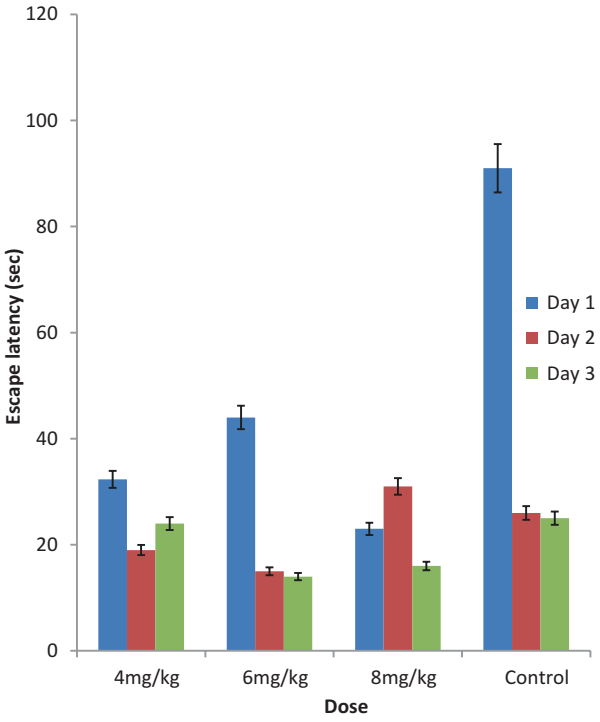


**Fig. 15.8** Escape latency time of *Morinda lucida* extract

### 15.4.10.5 Observations

Impairment of memory and learning is the most characteristic manifestation of cognitive dysfunction, and it can be induced chemically in people (Broks et al. 1988) as well as in experimental animals by scopolamine, a cholinergic antagonist known to interfere with acetylcholine transmission in the central nervous system (Misane and Ogren 2003). The effect of *Morinda lucida* (Fig. 15.8), *Peltophorum pterocarpum* (Fig. 15.9), *Spondias mombin* (Fig. 15.10), and *Pycnanthus angolensis* (Fig. 15.11) showed that the escape latency time of animals induced by scopolamine was significantly reduced by ethyl acetate extracts of the plants when compared with the control group that received distilled water.

The extracts showed dose-dependent cognitive enhancing activity. The histopathology study revealed no significant change in the histology of the brain. However, a reduction in density of cells in the hippocampus of the control mice pretreated with scopolamine only and an increase in the number and density of cells in the animals treated with extracts were observed.

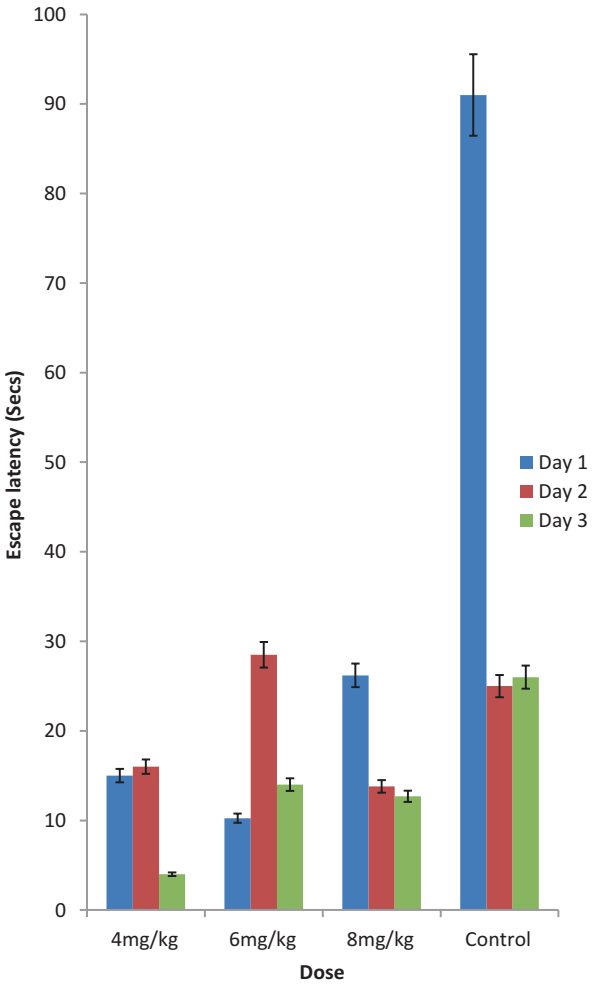


**Fig. 15.9** Escape latency time of *Peltophorum pterocarpum* extract

## 15.5 Conclusion

Several plants have been used in many traditional medical systems all over the world for the management of memory-related problems. This study showed the potential of *S. mombin, P. angolensis, P. pterocarpum*, and *M. lucida* as cholinesterase inhibitors as well as memory enhancers. Thus, the inclusion of these plants in remedies used for managing memory dysfunctions in Nigerian ethnomedicine is justified.

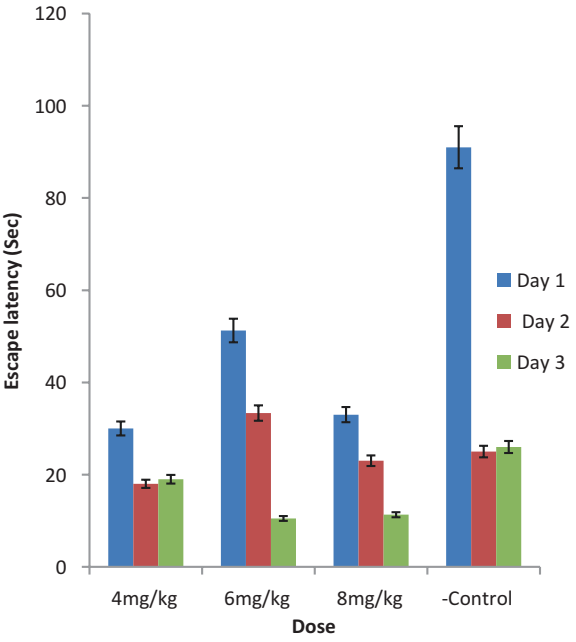


**Fig. 15.10** Escape latency time of *Spondias mombin* extract

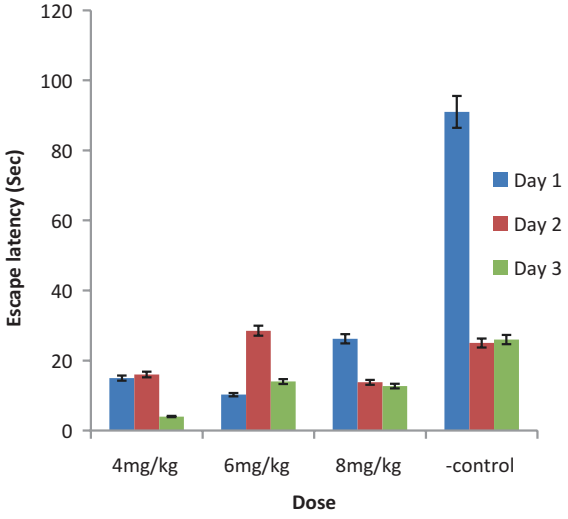


**Fig. 15.11** Escape latency time of *Pycnanthus angolensis* extract

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# Identification Strategies for Bioactive Secondary Metabolites of Fungal Origin

16

İsmail Hakkı Akgün and Fazilet Vardar-Sukan

**Abstract**

In the present review article, we have described several fungi as a promising and evolving source of new and bioactive secondary metabolites for drug discovery processes. Also, the review has outlined isolation and identification strategies of known and novel fungal metabolites from endophytic fungi, fungi isolated from marine sources and extreme environments, etc. The chapter highlights how recent advances in biochemistry, genetics, analytical technologies and bioinformatics have significantly influenced the process of discovery of novel bioactive compounds in fungi. Combination of microfractionation, high-resolution mass spectroscopy and liquid chromatography with emerging technologies such as microfluidics has potential to augment the efficiency of identification procedures for novel bioactive molecules and become effective tools for bioengineers to adapt these microorganisms for industrial applications.

**Keywords**

Bioactive • Biotechnology • Chromatography • Fungi • Natural products • Screening

## Contents



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## Abbreviations

| | |
|---|---|
| CIEIA | Competitive inhibition enzyme immunoassay |
| DAD | Diode array detector |
| ECD | Electrochemical detector |
| ELS | Evaporative light scattering |
| FDA | Food and drug administration |
| FT-IR | Fourier transform infrared spectroscopy |
| HPLC | High-performance liquid chromatography |
| HRESIMS | High-resolution electrospray ionisation mass spectroscopy |
| HTS | High-throughput screening |
| IR | Infrared |
| LC-MS | Liquid chromatography-mass spectroscopy |
| MIC | Minimum inhibitory concentration |
| MS | Mass spectroscopy |
| NMR | Nuclear magnetic resonance spectroscopy |
| PCR | Polymerase chain reaction |
| PDA | Photodiode array |
| QTOF | Quadrupole time of flight |
| SPE | Solid-phase extraction |
| TLC | Thin-layer chromatography |
| UHPLC | Ultrahigh-performance liquid chromatography |
| UV | Ultraviolet |

## 16.1 Introduction

Natural products in the form of secondary metabolites are the most often exploited molecules in drug discovery processes. Although drug companies have decreased their screening activities for natural products in the recent years (Lam 2007), the efforts for identifying new secondary metabolites and their derivatives and defining their biological activities are still very much in demand in academia. In total, 23 new drugs based on natural products or their derivatives were introduced between 2001 and 2005. Two of these have been approved as immunosuppressant molecules despite the reduced interest of drug companies for this class of molecules (Butler 2005).

Although discovering new bioactive entities from natural product extract is a time-consuming, laborious and an expensive process, there remains a vast pool of resources still untapped. Complex chemical compositions, low concentrations of the bioactive compounds, structural similarities of secondary metabolites in the extracts and sometimes limited amount of the raw material or extracts are some of the obstacles to the discovery of new drug molecules (Siddiqui et al. 2014). A crude microbial extract normally is composed of primary and secondary metabolites with fermentation media components (Ito et al. 2011). Heterogeneous chemical characteristics of extracts make it nearly impossible to isolate bioactive compounds in a single-step process. Many different approaches with the help of developing technologies have been followed to overcome these problems (Duarte et al. 2012). For example, development of smaller particle sizes with different chemical processes and compatible high-performance liquid chromatography (HPLC) devices enabled researchers to run separations to get chromatographic fingerprints of microbial extracts with a higher resolution. Combination of chromatographic fingerprints of microbial extracts and their subfractions with bioassays reduced the ratio of false positives and hastened the process of identification of active molecules. Also, tandem usage of these devices with other analytical tools like mass spectroscopy (MS) and nuclear magnetic resonance (NMR) by hyphenated interfaces increased the chances of identification of new entities in a shorter time (Wolfender et al. 2006). In addition, recent findings reveal that silent genes in fungal genome offer vast opportunities for the identification of new bioactive compounds (Chiang et al. 2009).

Low-yield production of these molecules is a more serious problem than their complex structures. With the help of microorganisms obtained from new-strain screening, optimisation of fermentation conditions, genetic modifications, etc., it has been possible to produce high-demand drugs like β-lactam antibiotics penicillins and cephalosporins. In 2009, penicillins had a total revenue of 7.9 billion USD with 19% market share of antibiotics sales, while the same year, 11.9 billion USD were spent on cephalosporins (28% of global antibiotics market). In addition to these, cholesterol-lowering agents, statins (lovastatin, mevastatin, etc.), immunosuppressant cyclosporin (Alberti et al. 2017) and anticancer molecule paclitaxel are other important examples. The above-mentioned molecules are not only secondary metabolites produced in large scale, but these are also produced by fungi.

Fungi are a very large family of microorganisms with nearly 75,000 known species out of an estimated number of 1.5 million. It is possible to encounter fungi both in moderate and very harsh climatic conditions in extreme parts of the world. They survive in soils, at seas, on the surface of rocks, etc., either as a single colony or as parasites (Hawksworth 2001). The ability of adaptation to different environments enables fungi to produce many secondary metabolites with various structures.

According to Brakhage and Schroeckh (2011), nearly 97,000 fungal secondary metabolites have been isolated from fungal sources. Some of these secondary metabolites have demonstrated biological activities such as antimicrobial, antifungal, antiviral, anticancer, immunosuppressant, antiprotozoal, etc.

The aim of this chapter is to emphasise the importance of various types of fungi as a promising and evolving source of new and bioactive secondary metabolites for

drug discovery processes. At the same time, we would like to outline isolation and identification strategies of known and novel fungal metabolites from different fungus types like endophytic fungi and fungi isolated from marine sources and extreme environments.

## 16.2 Identification Methods for Novel Bioactive Molecules

Purification or downstream processing is intended to increase the purity or activity of the molecules of interest. For this purpose, we need to increase the purity of the molecules in every step of the purification process. Many different analytical methods can be used to determine the purity of the molecules or success of the purification step. Analytical methods that measure the qualities and quantities of the substances can be applied to both bioactive and non-bioactive molecules. Contrary to this, bioassays can only give information about the purity of the bioactive molecule. Different methods from simple to complex such as thin-layer chromatography (TLC), HPLC and LC-MS to NMR methods are frequently used in the determination of purity of a molecule.

Thin-layer chromatography is a simple, cheap, fast and flexible analytical method which separates molecules based on their movement on a thin solid surface such as silica gel by using solvent mixtures. However, this method does not give direct information about molecules that are separated. When the solid surface is treated with spraying agents, ultraviolet (UV) radiation or heat, it may be possible to obtain clues about their class. Scott and colleagues determined the presence of nearly 20 mycotoxins using thin-layer chromatography method by using ultraviolet light (Scott et al. 1970). In other cases, retention factors ($R_f$) of the molecules in TLC and reactivity of mycotoxins p-anisaldehyde were taken into account for the identification process (Scott et al. 1970). Based on their experimental results, it was reported that aflatoxin B1 has 0.31 $R_f$ value on toluene, ethylacetate and formic acid mixture and exhibits blue colour on long wavelength UV and faint blue in short wavelength UV. They identified this molecule in the extracts of *Aspergillus flavus* and *Aspergillus sulphureus* successfully. Despite its simplicity, TLC cannot separate molecules with high resolution. Also, some molecules especially at low concentrations cannot be detected.

High-performance liquid chromatography (HPLC) is probably the most commonly used instrument for the determination of molecules among the analytical devices listed above. HPLC is a very flexible and robust device because it can be combined with many different detectors and precise pumps. Woldender reviewed the use of ten different detectors (UV, DAD, ECD, ELS, MS, NMR, etc.) which could be combined with HPLC in the analysis of phytochemicals (Wolfender 2009). A similar approach can be applied to microbial extracts as well.

HPLC analysis of microbial extracts can be divided into two groups. The first is the untargeted profiling of whole extract. In this technique, extracts are separated with a HPLC method which allows the identification of as many molecules as possible. This makes it possible to observe nearly all secondary metabolites produced

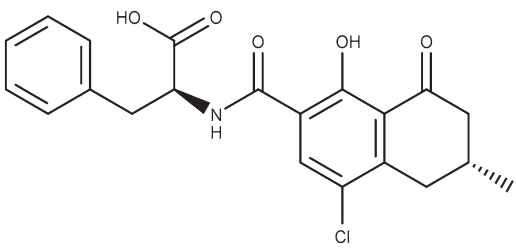



**Fig. 16.1** Chemical structure of Ochratoxin A

by a microorganism. It is also a very useful method which provides information about the complexity of the extract. The high-resolution chromatograms which mostly are called 'chemical fingerprints' (chemotaxonomy) can be used to compare microorganisms and their secondary metabolite profiles. For example, Hansen and colleagues developed an automated and unbiased/unsupervised classification method using HPLC-UV chemical fingerprints of *Penicillium* and *Alternaria* sp. (Hansen et al. 2005). In another report, Andersen and colleagues classified 53 *Alternaria* strains using HPLC fingerprints with the help of multivariate statistical models (Andersen et al. 2008). Besides this method they also produced group-specific HPLC-DAD profiles.

HPLC method also provides a good tool to check the presence of a molecule of interest in a crude extract. This approach is called targeted analysis. Screening of fungal metabolites with HPLC also enables bioactivity-guided isolation or selection of most promising or valuable fungal extracts. For example, Bragulat and his colleagues used HPLC in the detection of ochratoxin A (Fig. 16.1) producing fungi (Bragulat et al. 2001). They screened the quantity of the ochratoxin A produced by the fermentation of 13 *Aspergillus* sp., using two different media for 7, 14 and 21 days. According to their results, ochratoxin levels of *A. foetidus* in CYA medium were relatively higher than other 12 species. However, *A. niger* var. *niger* was the best producer of ochratoxin A in YES medium (Bragulat et al. 2001).

According to Duarte and colleagues, two approaches can be followed to identify bioactive molecules in microbial extracts (Duarte et al. 2012). The first is bioassay-guided fractionation and the second is pure-compound screening (Duarte et al. 2012). In bioassay-guided isolation, the fractions obtained in each purification step are tested for activity, and only active fractions are further purified. This technique is known by different names like bioassay-guided fractionation, activity-guided fractionation, bioassay-directed fractionation and bioactivity-guided fractionation (Weller 2012). There are some recent reports on application of bioassay-guided isolation approach to various fungal extracts. Ali and colleagues isolated α-glucosidase and urease inhibitor from endophytic fungus *Boswellia sacra* (Ali et al. 2017). In another study, Crespo and colleagues reported three long chain alkenyl sulphate molecules, linoleyl sulphate (Fig. 16.2a), linolenyl sulphate (Fig. 16.2b) and oleyl sulphate (Fig. 16.2c) with antifungal activities isolated by bioassay-guided fractionation from *Chaetopsina* sp. These molecules were extracted from 1 L fermentation broth using SP207ss resin and were eluted with water and acetone mixtures. Fractions were tested on an antifungal bioassay, and combined active fractions

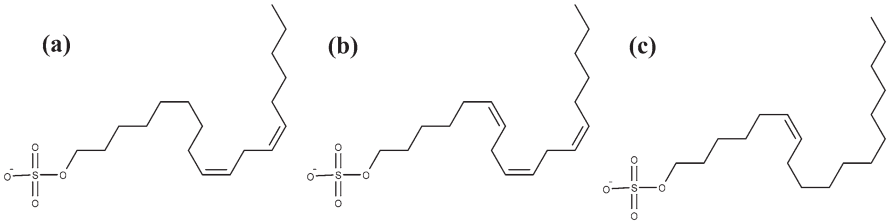
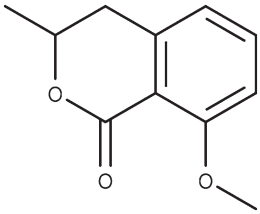



**Fig. 16.2** Chemical structures: (**a**) linoleyl sulphate, (**b**) linolenyl sulphate, (**c**) oleyl sulphate

**Fig. 16.3** Chemical structure of 3-O-methylmellein



were separated on semi-preparative HPLC equipped with an UV detector. Among these three molecules, linoleyl sulphate and oleyl sulphate were determined as active antifungal molecules against a group of fungal strains (Crespo et al. 2016).

Another bioactive secondary metabolite 3-O-methylmellein (Fig. 16.3) was isolated using bioassay-guided fractionation by Rakshith and colleagues (2016). This molecule was isolated from the ethyl acetate extract following an 8-week fermentation process in 1 L fermenters. Crude ethyl acetate extract was subjected to a silica gel column and eluted with petroleum and ethyl acetate mixtures. Molecular and bioactivity profiles of each fraction obtained from silica gel column were monitored on TLC plate by using TLC bioautography method. Bioactive fractions were further purified, and structures were determined using FT-IR, HPLC, LC-PDA-MS and NMR techniques. Minimum inhibitory concentration of 3-O-methylmellein (Fig. 16.3) was determined against various fungi and bacteria. The highest activity of this molecule was observed against *Candida albicans* (MTCC 183) with 0.78 μg/mL (Rakshith et al. 2016).

While bioactivity-guided fractionation studies have many advantages, there are also some difficulties or limitations (Duarte et al. 2012). For example, isolation work should be delayed until a bioassay response is obtained; otherwise, time and resources can be wasted for the isolation of inactive molecules. Generally, isolation studies are shorter than bioassay studies. The differences in timescales make bioactivity studies difficult. Also, the quantities of secondary metabolites in the extracts are often different from each other. Isolation and bioactivity studies of abundant molecules are easier to perform than the scarce ones, preventing exploitation of the minor metabolites. Furthermore, the effect of the selected bioactivity type on the process should not be overlooked. For example, bioassay for the screening of anticancer molecules can lead to overlooking the molecules with antibacterial potential.

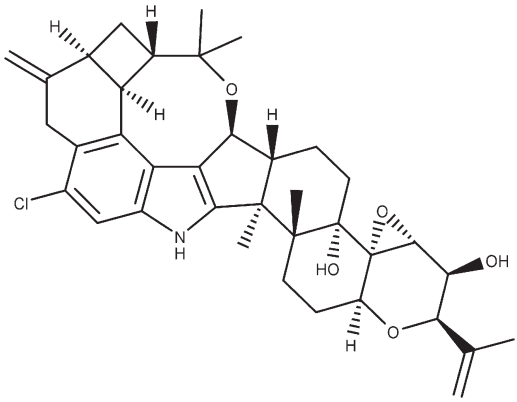



**Fig. 16.4** Chemical structure of penitrem F

It is necessary to test as many fractions as possible in one batch to obtain the highest yield from the bioassays and reduce the waiting time. The developments on the particle technology used in columns and on analytical devices have enabled researchers to separate extracts with higher resolution (Yang et al. 2014a). An effective separation of molecules shortens the time for the identification of the active ones. Since these molecules can be obtained in small volumes with microfractionation techniques, they can be stored for further testing in bioassays. The microfractionation process is carried out by using a fraction collector attached to a HPLC equipped with various detectors like DAD and MS (Ito and Masubuchi 2014). Spectroscopic data of the molecules are collected by the DAD detector, and information about the molecular mass is collected by the MS detector, as it is separated in the HPLC column. Thus, microfractionation and gathering information about structures of the molecules can be synchronised. The combination of mass and DAD detectors allows fingerprinting of molecules. Obtained fingerprints or profiles can be compared with molecular libraries to determine the structure of active molecules. Nielsen and Smedsgaard reported a HPLC-UV-MS data library of 474 mycotoxin or fungal metabolites (Nielsen and Smedsgaard 2003). With the help of this library, two undescribed derivatives of penitrem F (Fig. 16.4) named as boromopenitrem F and dehydropenitrem F were dereplicated in *Penicillium ochrochloron*, which were not indexed in SciFinder software before. Klitgaard and colleagues extended this number to 3000 with the analytical methods they optimised using UHPLC-DAD-QTOF device (Klitgaard et al. 2014). With the help of this new method, they identified nitrogen-containing molecules that may be used as biomarkers for *Aspergillus carbonarius* and *Penicillium melanoconidium* (Klitgaard et al. 2014). Furthermore, the authors improved the ability to detect new molecules using spectroscopic data of fractions, demonstrating the power of microfractionation with HPLC.

Microfractionation method is not only used in academia but also the in industry. Wagenaar's article in 2008 summarises how Wyeth used this method to build natural product libraries to identify new active pharmaceutical candidates (Wagenaar 2008). Fractions obtained from raw extracts were evaluated by bioassays such as

kinase inhibition or ion channel. Statistical evaluations showed 2750 hits representing 1882 crude extracts in 9 bioassays. Only 80% of the 1882 extracts were active in one fraction, and nearly 15% of extracts were active in two fractions. Interestingly, almost 10% of the extracts, from which these active fractions were obtained, had activities. This indicates that activity in minor molecules can be detected with detailed analysis using this method.

Microfractions can be enriched with methods like solid-phase extraction (SPE) to detect bioactive minor molecules. In the liquid-liquid extraction methods, a relatively high amount of organic solvent is used. High costs and environmental drawbacks of organic solvents have led to the development of solvent-free extraction methods such as solid-phase extraction (SPE). In SPE methods, molecules in liquid phase (in our case in fermentation broth) or in HPLC eluent are adsorbed on the solid phase based on molecular structures and physicochemical properties (Poole 2003). SPE methods traditionally have four steps (Hennion 1999). The first step is the conditioning of sorbent with appropriate solvents in order to interact with molecules in liquid phase better. The second step is the application of the sample, where the molecules compatible with sorbent are held on the surface, provided that the rate of elution is slow enough to achieve sufficient interaction. The third step is the cleaning of the sample by rinsing with appropriate solvents. In this step, molecules which do not adsorb or are weakly adsorbed onto the sorbent are eluted. Elution solvent used in this step should not desorb the analyte or the molecule of interest. The fourth and the last step is desorption of the analyte molecules with a strong solvent from the surface of sorbent. SPE is usually used during adsorption of molecules that are eluted from HPLC as a post-column adsorbent, as well as to clean the extracts before injecting them to the HPLC column (Bucar et al. 2013). The molecules separated from the column are diluted with water and automatically directed to a SPE cartridge to be adsorbed (Wolfender et al. 2006). In order to increase the success rate in the microfractionation of the minor molecules, adsorption to the SPE cartridge must be carried out more than once or concentrated sample must be injected to HPLC column (Wolfender et al. 2006). Thus, the minor molecules are deposited on the SPE cartridge. After the deposition process, the molecules can be transferred directly into the liquid phase using a suitable solvent. After evaporation of the solvent, bioassays can be carried out with sufficient amount of metabolite. Several researchers who applied SPE to natural products have used the combination of LC-SPE-NMR (Wolfender et al. 2006; Jaroszewski 2005a, b). Wubshet and colleagues have used LC-HR-MS-SPE-NMR technique to isolate antioxidant molecules griseophenone C (Fig. 16.5a) and peniprequinolone (Fig. 16.5b) and a novel molecule dechlorodehydrogriseofulvin (Fig. 16.5c) from ethyl acetate extract of endophytic fungus *Penicillium namyslowskii* (Wubshet et al. 2013). They diluted HPLC elute with water using a secondary pump and directed the mixture to a SPE unit. The SPE cartridge was previously conditioned with 500 µL of acetonitrile followed by 500 µL of acetonitrile water, and minor peaks of the extract were trapped by six successive injections. Trapped molecules were eluted with 30 µL deuterated acetonitrile. Three-dimensional structures of isolated molecules were determined using 1D and 2D NMR experiments.

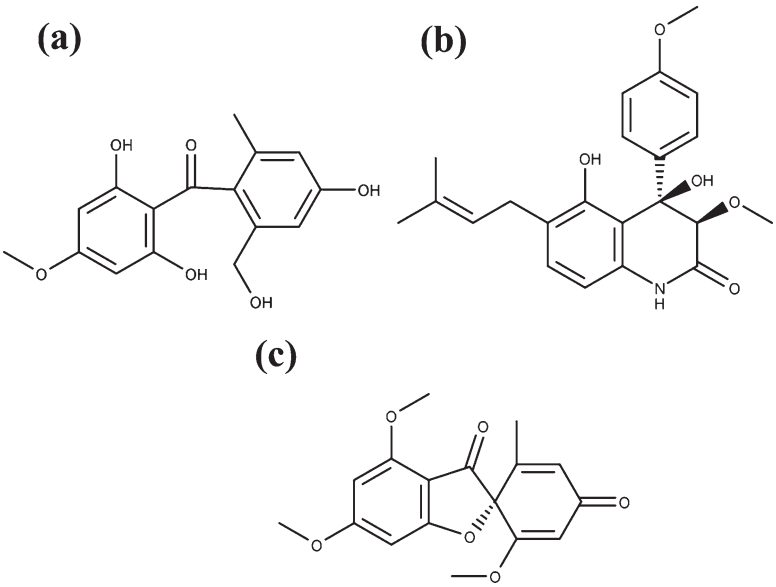



**Fig. 16.5** Chemical structures of (**a**) griseophenone C, (**b**) peniprequinolone, (**c**) dechlorodehydrogriseofulvin

In a pure-compound screening methodology, bioassays are applied to isolate molecules with elucidated structures (Duarte et al. 2012). However, this method is more time-consuming and labour intensive than the bioassay-guided work. Also, since biological activity is checked in the last step, it is possible to waste resources for a non-bioactive molecule.

However, the spectroscopic data of the pure molecules and their isolation methods are invaluable sources for future studies. Watts and colleagues have used pure-compound screening methodology to discover a lead compound that inhibited the growth of the causative parasite of neglected disease *Human African trypanosomiasis (HAT)* and *Trypanosoma brucei* (Watts et al. 2010). They tested inhibition levels of 25 natural products and semi-derivatives isolated from two deep sea-derived fungi *Aspergillus fumigatus* and *Nectria inventa*. Among the screened molecules, dehydrobis(methylthio)gliotoxin (Fig. 16.6a), 6-acetylbis(methylthio)gliotoxin (Fig. 16.6b) and 12,13-dihydroxyfumitremorgin (Fig. 16.6c) were highlighted as potent, together with selective inhibitors with $IC_{50}$ values of 8.5, 6.5, 6.4 μM, respectively (Watts et al. 2010).

It is possible to increase the number of methods and approaches related to identification and isolation of natural products. HTS studies in which a large number of molecules or extraneous matter are screened rapidly and intensively, or hyphenated techniques that help to reduce the time of analysis and isolation, could be given as examples. However, regardless of the method used, at least one of the extraction, separation, identification, structure elucidation and bioactivity studies is involved for each method. In any isolation or dereplication process of fungal secondary

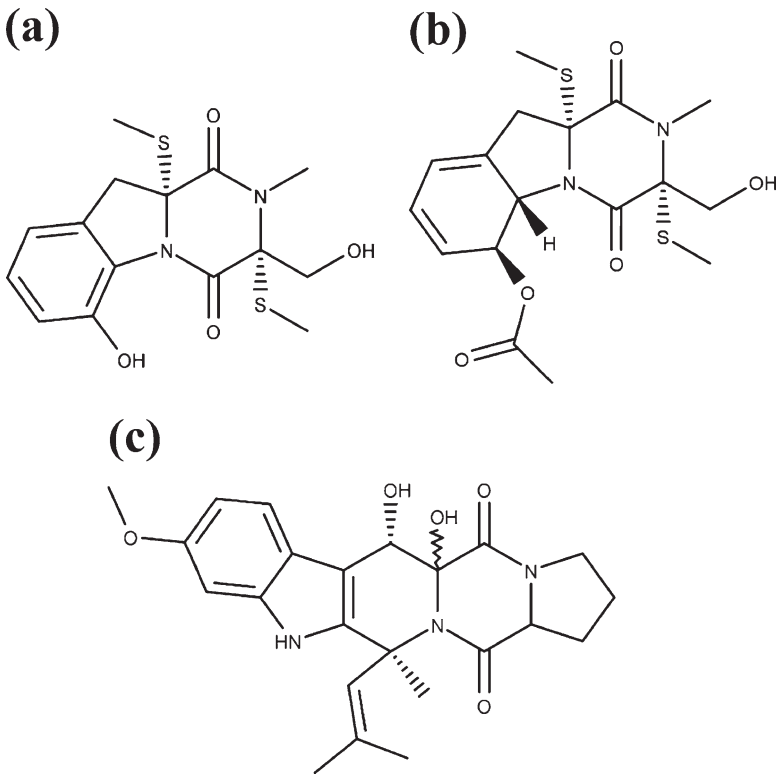



**Fig. 16.6** Chemical structures of (**a**) dehydrobis(methylthio)gliotoxin, (**b**) 6-acetylbis(methylthio) gliotoxin, (**c**) 12,13-dihydroxyfumitremorgin

metabolites, the most suitable technique should be chosen after a careful study of extracts. Good planning and successful practices will allow the process to be completed in the shortest possible time without spending too many raw materials.

## 16.3 Promising Sources for Novel Bioactive Secondary Metabolites

### 16.3.1 Endophytic Fungi

Endophytic fungi are promising and very abundant sources for bioactive compounds or their derivatives. These most likely produce bioactive molecules of their host plants as well as other novel bioactive molecules. Screening of endophytic fungi for bioactive compounds has resulted in mainly novel molecules with antimicrobial, insecticidal and cytotoxic activities (Zhao et al. 2011). Endophytic fungi have been extensively investigated for their potencies for the production of known bioactive compounds such as paclitaxel (anticancer activity), podophyllotoxin (antitumor

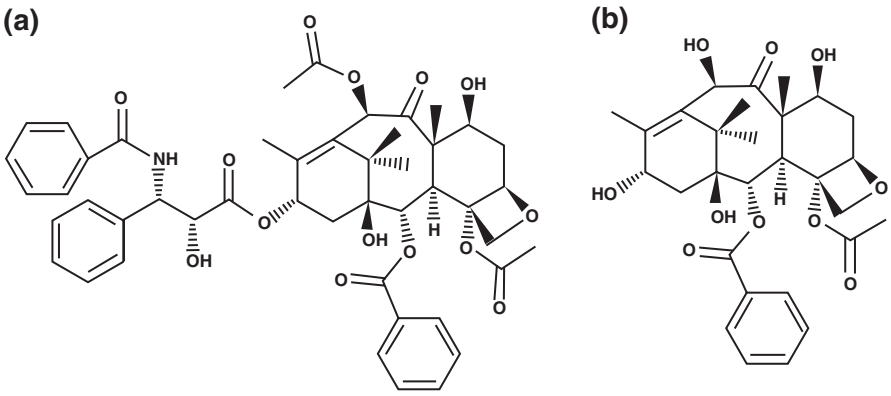



**Fig. 16.7** Chemical structures of (**a**) paclitaxel, (**b**) 10-deacetylbaccatin III

agent), camptothecin (DNA topoisomerase I (Topo I) inhibitors) or vinca alkaloids vinblastine and vincristine (anticancer activity).

Paclitaxel (Fig. 16.7a) is a chemotherapy agent used for the treatment of breast, lung and pancreatic cancer. An ever increasing demand of this compound is probably the most important reason for the interest in endophytic fungi. Paclitaxel was isolated by Wani and colleagues from the bark of a slow-growing tree, Pacific yew (*Taxus brevifolia*) (Wani et al. 1971). Low yields of paclitaxel in Pacific yew led researchers to search for better sources and alternatives. The needles of European yew (*Taxus baccata*) were identified as feasible alternative to produce paclitaxel with semi-synthesis reactions from 10-deacetylbaccatin III (Fig. 16.7b) (Denis et al. 1988; Holton 1993).

Soon thereafter, Stierle and colleagues identified an endophytic fungus *Taxomyces andreanae* which could produce paclitaxel and related taxanes (Stierle et al. 1993). Following these results, concerted efforts have been made to find new paclitaxel-producing fungi especially endophytes (Flores-Bustamante et al. 2010). Over 160 reports and patents have been published on microorganisms producing paclitaxel or taxane derivatives with inconsistent yields. For instance, Zhang and colleagues reported 800 ng/mL paclitaxel yield by an endophytic fungus *Cladosporium cladosporioides* MD2, which was isolated from *Taxus media* (Zhang et al. 2009). The identification of paclitaxel was carried out by thin-layer chromatography with silica gel plate using $CHCl_3$ and MeOH mixture (7:1 v/v) as eluent. They used similar conditions to purify paclitaxel from fermentation broth by sculpting silica followed by MeOH extraction. Quantification of paclitaxel was carried out with UV spectrometer by measuring the absorbance of solution obtained from silica sculpt at 273 nm. Furthermore, HPLC-UV, MS and NMR analyses were carried out as qualitative measurements. In HPLC-UV analysis, presence of paclitaxel was confirmed by comparing retention times of standard paclitaxel solution and a purified sample. The purified sample was analysed by MS by-loop injection method instead of LC-MS technique (Zhang et al. 2009).

In another report, Chi and colleagues analysed the quantity of the paclitaxel produced by endophytic fungus *Nodulisporium sylviforme* isolated from *Taxus cuspidata*, using HPLC-PDA by monitoring peak area at 227 nm (Chi et al. 2008). According to their quantitation, *Nodulisporium sylviforme* produced nearly 500 ng/mL paclitaxel (Chi et al. 2008). Kumaran and Hur followed nearly the same route as Zhang et al. (2009), during the investigation of potency of *Phomopsis* sp. isolated from *Taxus cuspidata*. IR spectra of both standard and purified paclitaxel were recorded (Kumaran and Hur 2009). A positive signal of taxadiene synthase gene in PCR experiments, cytotoxic activity and spectral conformity of the purified extract enabled researchers to identify *Phomopsis* sp. as a potential genetic-engineered species for production of paclitaxel. In spite of relatively high production capabilities, there are some endophytic fungi which do not produce enough paclitaxel, such as *Pestalotiopsis microspora*, isolated from *Taxus wallichiana* by Shrestha and colleagues (Shrestha et al. 2001). The quantity of the paclitaxel was measured by indirect competitive inhibition enzyme immunoassay (CIEIA) as 0.026 ng/mL which is very selective to paclitaxel and can detect very low amounts, while the presence of paclitaxel was also confirmed with HPLC-ESI-MS/MS (Shrestha et al. 2001). Indirect competitive inhibition enzyme immunoassay method was extensively used by Caruso and colleagues (2000) for screening of taxane-producing fungi. They screened a total of 150 endophytic fungus strains including *Aspergillus* sp., *Fusarium* sp., *Alternaria* sp. and *Phomopsis* sp., isolated from *Taxus baccata* and *Taxus brevifolia* and identified 15 of them as taxane producers with very low yields barring a few with 0.05–0.1 ng/mL titres. Despite these successful examples of paclitaxel-producing endophytic fungi, inconsistencies of production capabilities were questioned by Heinig et al. (2013). Their investigation included cultivation of endophytic fungi, taxoid extraction, CIEIA, LC-MS/MS analysis, genomic DNA analysis, diterpene synthase expression and functionality tests. Only 2 of 34 cultivated endophytic fungal extracts had positive results in CIEIA test with taxane concentrations of 7.8 and 2.5 ng/L in culture medium in contrast to $173.3 \times 10^3$ ng/g yield obtained from a *Taxus baccata* plant (Heinig et al. 2013).

Podophyllotoxin (Fig. 16.8) is a potent anticancer compound exhibiting its activity by blocking division of cancer cells and affecting assembly of microtubules (Bruschia et al. 2010). Podophyllotoxin is also a raw material for well-known anticancer drug ingredients, etoposide (Fig. 16.9a) and teniposide (Fig. 16.9b), which are still used for the treatment of brain cancer, lymphoma, ovarian cancer, etc. (Bruschia et al. 2010). *Sinopodophyllum* plant is a major source of podophyllotoxin and is now considered as an endangered species because of over-exploitation (Zhao et al. 2011). In order to satisfy the demand for podophyllotoxin and its derivatives, new sources like endophytic fungi have been screened. Most of the isolated endophytic fungi are not identified as the producers of podophyllotoxin, but only a small portion of them are able to produce the active ingredient. Eyberger and colleagues (2006) screened 215 fungal isolates from explants of *Podophyllum peltatum* and determined 18 unique endophytic fungi based on morphology. Fermentation broths of these endophytes were screened using LC-MS to identify the presence of podophyllotoxin by examining signals of m/z 415.2 $[M+H]^+$, 437.2 $[M+Na]^+$, 453.1

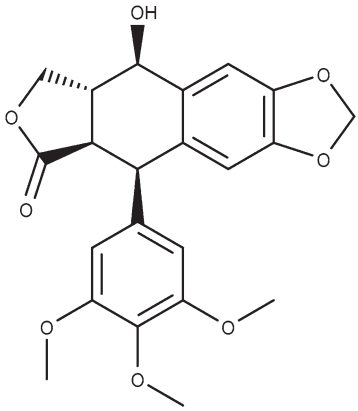



**Fig. 16.8** Chemical structure of podophyllotoxin

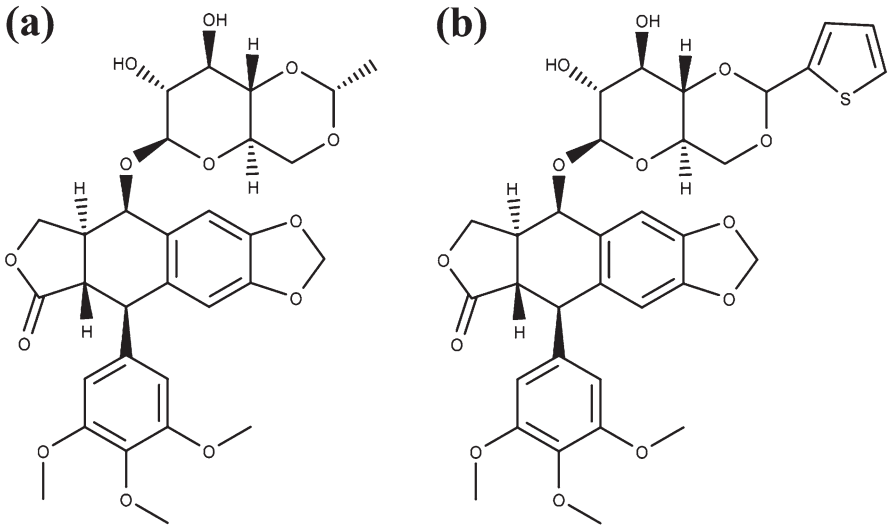



**Fig. 16.9** Chemical structures of (**a**) etoposide, (**b**) tenoposide

$[M+K]^+$, 397.2 $[M\text{-}OH]^+$, etc. Two isolates were determined as producers of podophyllotoxin with yields ranging from 0.5 to 189 μg/L in a 4-week culture. These two podophyllotoxin-producer fungi were identified as *Phialocephala fortinii* based on genetic analysis. Also, there are some other endophytic fungi like *Alternia neesex*, *Aspergillus fumigatus*, *Fusarium oxysporum* and *Trametes hirsuta* with lower yields of podophyllotoxin (Zhao et al. 2011). Kusari et al. (2009) screened the extract of endophytic fungus *Aspergillus fumigatus* Fresenius, isolated from the twigs of *Juniperus communis* with LTQ-Orbitrap ® LC-MS/MS system in single-ion monitoring (SIM) and multiple-reaction monitoring (MRM) modes to qualitatively analyse and quantify podophyllotoxin and deoxypodophyllotoxin (3 μg/L). In spite of very low yields, their LC-MS/MS screen protocol is a great example for identification of minor bioactive molecules (Kusari et al. 2009).

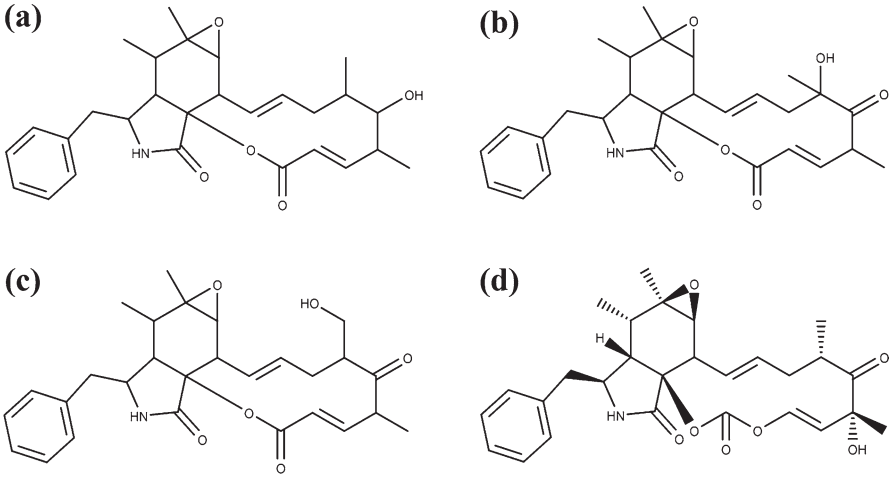



**Fig. 16.10** Chemical structures of cytochalasin derivatives (**a**–**d**) cytochalasin E

Kaul and colleagues published an extensive review in 2012 which covered the subject of secondary metabolites isolated from endophytic fungi from medicinal plants (Kaul et al. 2012). They screened secondary metabolites which were reported until 2012 and presented structures of 43 secondary metabolites with anticancer activities. Four of the metabolites were identified as novel structures within a known molecular family as cytochalasins. Three of these novel molecules, cytochalasin derivatives (Fig. 16.10a–c) and cytochalasin E (Fig. 16.10d) were reported by Wagenaar et al. (2000). These three molecules were isolated from the fermentation broth of endophytic fungus *Rhinocladiella* sp. In spite of their novel structures, these molecules displayed lower cytotoxic activities than the known molecule cytochalasin E. In contrast to the previous example, presence and quantities of these novel molecules were not checked by either HPLC or LC-MS devices. Instead, these were isolated from filtered liquid fermentation broth of endophytic fungus *Rhinocladiella* sp. Fermentation broth was extracted twice with ethyl acetate (EtOAc) and once with dichloromethane. Combined organic extract was evaporated until dry and then subjected to C18 column and eluted by water and acetonitrile mixtures to obtain subfractions. Some of the combined subfractions were fed into a C18 HPLC column with smaller particle size (5 um) for better separations. Structures of isolated molecules were determined by $^{1}H$ and $^{13}C$-NMR spectroscopy, and HRCIMS data confirmed identified structures (Wagenaar et al. 2000). Another recent bioactive molecule, chaetoglobosin U, was isolated from endophytic fungus *Chaetomium globosum* IFB-E019. Unlike the *Rhinocladiella* sp., *Chaetomium globosum* IFB-E019 was fermented by using solid-state fermentation method (Ding et al. 2006). Harvested solid culture of *Chaetomium globosum* IFB-E019 was dried and extracted with MeOH:$CHCl_3$ (1:1) mixture five times and then evaporated until brown oil was obtained. This brown concentrated extract was then subfractioned by liquid-liquid extraction method to simplify complexity of secondary metabolites.

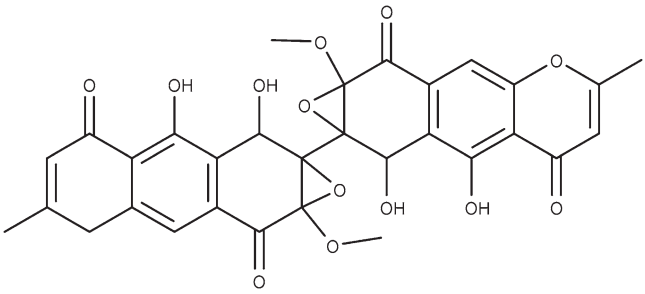



**Fig. 16.11** Chemical structure of Diaporin

Again unlike the isolation process of cytochalasins, subfraction was obtained from liquid-liquid extraction and was tested for cytotoxic activities. Cytotoxic ethylacetate fraction was subjected to silica gel column, eluted with mixtures of $CHCl_3$ and MeOH to afford seven subfractions. Cytotoxicities of seven subfractions were also tested focusing on bioactive subfraction two (Fr 2). Successive separations of Fr 2 on silica gel and Sephadex LH-20 columns resulted in a novel molecule chaetoglobosin U. Similarly, structure of chaetoglobosin U was identified with $^{1}H$ and $^{13}C$-NMR spectroscopy, and HRESIMS data confirmed structure of the molecule. These two instances are good examples of untargeted isolation and bioassay-guided isolation strategies. In addition to Kaul's review, Kharwal and colleagues published an extensive review on anticancer molecules from endophytic fungi (Kharwar et al. 2011).

In the past few years, new entities from endophytic fungi have been reported. Biological activities and the mode of actions of the fungal secondary metabolite named diaporin (Fig. 16.11), symmetrical polyketide isolated from endophytic fungus *Diaporthe* sp. IFB-3lp-10 (Wu et al. 2014), obtained from the leaves of *Rhizophora stylosa* were investigated both in non-small cell lung cancer (Zhao et al. 2011) and breast cell cancer (Feng et al. 2016). Diaporin was stated as a cell cycle inhibitor; thus, it can be used to downregulate proliferation of cancer cells (Song et al. 2014). Investigations on the mechanism of action have shown that diaporin initiated apoptosis by inducing production of reactive oxygen species (ROS) in breast cancer cells (Wu et al. 2014).

Another novel anticancer molecule discovered recently is peniproline A (Fig. 16.12) which was isolated together with 11 known compounds from fermentation broth of *Penicillium decumbens* CP-4 obtained as endophytic fungi from *Cephalotaxus mannii* Hook. f. Despite its novel structure, peniproline A displayed a weak cytotoxic activity against Bel-7402 (8.1 μM) and Hela cell lines (15.5 μM). However, it was noted as the most potent among the 12 molecules (Wang et al. 2017). There is another recent report on secondary metabolites rhytidenone G (Fig. 16.13a) and rhytidenone H (Fig. 16.13b). These two secondary metabolites are produced by endophytic fungus *Rhytidhysteron rufulum* AS21B under acidic fermentation conditions (pH~5) after the optimisation of fermentation conditions (Siridechakorn et al. 2017). In this study, a total of 17 secondary metabolites and

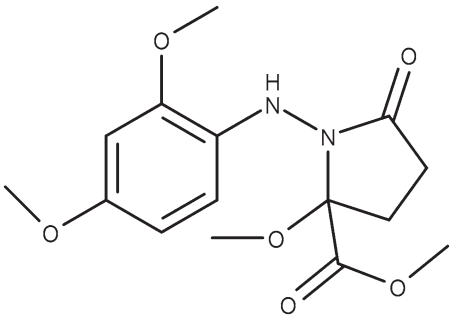


**Fig. 16.12** Chemical structure of peniproline A

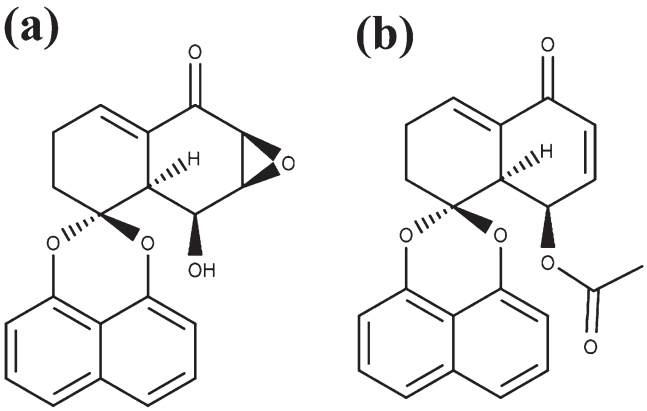



**Fig. 16.13** Chemical structures of (**a**) rhytidenone G, (**b**) rhytidenone H

their cytotoxic activities on cancer cell lines Ramos and H1975 were investigated. One of the novel molecules among these secondary metabolites, rhytidenone H, was determined as very active against Ramos cell with 0.018 μM $IC_{50}$ value. In fact, in the place of high biological activity of rhytidenone Hs, a change in fermentation media conditions leading to production of new and active molecules is an indication that many more molecules can be obtained from the endophytic fungus. Also, with the help of bioengineering applications, richer extracts could be obtained with more efficient production.

### 16.3.2 Marine Sources

Seas have always been generous in feeding the humanity. The seas or in a wider sense aquatic environments provide a very rich unexplored source for new species of microorganisms. Marine environments covering approximately 75% of the surface of the earth host thousands of living organisms including a large number of fungal species. By the end of 1999, 444 marine fungi were identified out of which 235 were totally new (Hawksworth 2001). This number is lower than that of

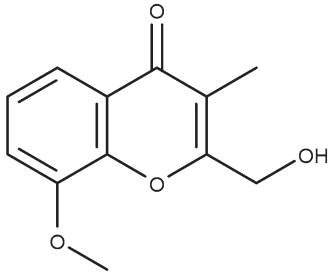



**Fig. 16.14** Chemical structure of chromanone A

terrestrial fungi indicating that there may be many more marine fungi to explore (Raghukumar 2006). A relatively high probability of obtaining new molecules particularly bioactive ones along with new species has led to an increased interest in marine fungi. According to Duarte et al. (2012), about 100 fungal metabolites were isolated from marine fungi between 2000 and 2005, and this number increased to 690 between 2006 and 2010. Similar to terrestrial fungal secondary metabolites, molecules isolated from marine fungi have also been extensively studied for anticancer, antibacterial, anti-inflammatory and antiviral activities (Hasan et al. 2015).

Kjer et al. (2010) summarised the procedures required to obtain secondary metabolites from marine endophytic fungi including estimates of the length of the process, the production in small and large scales and the steps required for downstream processes. In small-scale submerged culture production, it is suggested to homogenise cells without separating them from the medium and then mixing the final solution with ethyl acetate. After filtration, the dense extract obtained from the volatile organic solvent is demonstrated to be rich in new and bioactive molecules. In contrast to small-scale production, for large-scale submerged production, they recommended separation of micelles and media at the initial stage of the downstreaming process. Extraction of micelles using MeOH in a homogeniser was advised. They estimated total production time of at least 7 weeks including the fermentation and the preliminary downstream processes (Kjer et al. 2010).

Chromanone A (Fig. 16.14) is a benzopyrone derivative isolated from *Penicillium* sp.-type fungus obtained from seaweed. Chromanone A was tested for its inhibitory effects on carcinogen-metabolising enzyme. Studies have shown that chromanone A reduces stimulated CYP1A activity by 60% in murine hepatoma cells 'Hepa 1c1c7', at a concentration of 4 μg/mL. Also, it has been found that chromanone A exhibits selective effect against the hydroxyl radicals, thus inhibiting induced DNA damage (Gamal-Eldeen et al. 2009). *Penicillium* sp. fungus was cultivated at room temperature for 2 months with the artificial seawater medium in 2 L fermenters. Crude fermentation broth containing the fungus was homogenised after dilution by one tenth with water. The obtained suspension was extracted three consecutive times with ethyl acetate. Combined organic fractions were evaporated to yield 0.9 g of crude extract. This crude extract was fractionated on silica gel column by eluting molecules with petroleum ether, EtOAc and MeOH mixtures. Some of the fractions obtained from silica gel were refractionated on reverse-phase C-18 material using

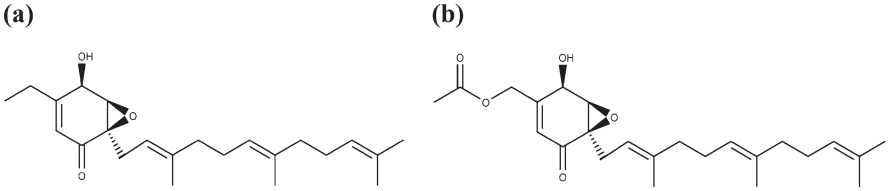



**Fig. 16.15** Chemical structures of (**a**) 7-deacetoxyyanuthone, (**b**) yanuthone A

vacuum liquid chromatography method. The final purification step was carried out on HPLC again with reverse-phase material using water and MeOH mixtures (Gamal-Eldeen et al. 2009).

Studies on the anticancer effects of secondary metabolites from marine fungi and the discovery of effective molecules against methicillin-resistant microorganisms have been carried out. In a study conducted by Li et al. (2003), 7-deacetoxyyanuthone (Fig. 16.15a) and yanuthone A (Fig. 16.15b) were isolated from marine-derived *Penicillium* sp. While these molecules showed moderate activity in cancer cell lines, 7-deacetoxyyanuthone exhibited mild activity (MIC, 50 μg/mL) against methicillin-resistant and multidrug-resistant *S. Aureus*. *Penicillium* sp. was cultivated at 29 °C in SWS medium for 30 days. Filtration of the mycelia-containing broth was carried out as an initial step of the downstream process. Freeze-dried mycelia were extracted using a dichloromethane and MeOH mixture. Evaporated organic phase was subjected to reverse-phase column eluted with water and MeOH mixtures. Consequently, fraction 3 was purified on a silica gel column with hexane and ethyl acetate followed by reverse-phase HPLC method (Li et al. 2003).

Among the secondary metabolites isolated from the marine fungi, plinabulin (Fig. 16.16a) which is a synthetically produced bioactive molecule derived from halimide occupies a different place among bioactive molecules. Plinabulin (NPI-2358) (Fenial et al. 2000; Kanoh et al. 1997) is a drug candidate which is now at phase 2 trials. Plinabulin binds to a region between α and β tubulin and inhibits tubulin polymerisation (Nicholson et al. 2006). Halimide was extracted from marine fungus *Aspergillus* sp. CNC139 isolated from the sample of green alga called *Halimeda copiosa* and incubated for 5 days using a medium containing seawater with yeast extract, peptone and glucose. The 5-day culture was transferred into 1 L fresh medium of the same composition and was incubated for an additional 21 days at 27 °C. Fermentation broth of *Aspergillus* sp. CNC139 was extracted with EtOAc, and combined organic extracts were subjected to silica gel column. Subsequently, the molecules were eluted with hexane and EtOAc mixtures, and presence of halimide was checked by NMR spectroscopy. Halimide-rich fractions were repurified by using silica gel HPLC column with EtOAc and were crystallised (Fenial et al. 2000). Halimide was also isolated from *Aspergillus ustus* by Kanoh et al. (1997) and was named (−)-phenylahistin (Fig. 16.16b). Unlike the marine fungus *Aspergillus* sp. CNC139, *Aspergillus ustus* was inoculated at 28 °C for 8 days on agar medium (Nicholson et al. 2006).

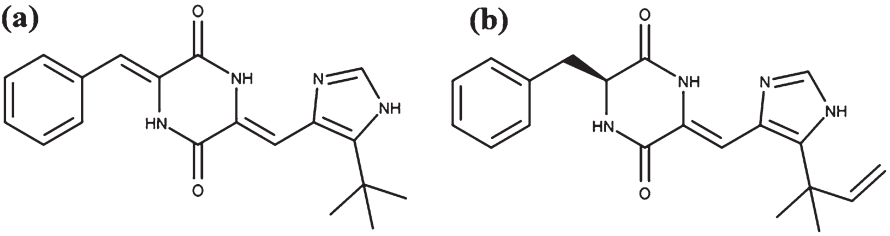



**Fig. 16.16** Chemical structures of (**a**) plinabulin, (**b**) (-)-phenylahistin

Recently, Guo et al. (2015) working with marine fungi described antimicrobial activities of penicyclone A–E (Fig. 16.17a–e) isolated from a deep sea-derived fungus *Penicillium* sp. F23-2. They detected unusual HPLC-UV peaks in the extract of *Penicillium* sp. F23-2, cultured on a rice-based solid medium and isolated five penicyclone derivatives, penicyclone A–D. These five molecules were isolated from the MeOH extract of the fermentation broth of *Penicillium* sp. F23-2 using reverse-phase or size exclusion chromatography column, followed by HPLC separations with a reverse-phase and chiral columns. Three-dimensional structures were determined using 1D and 2D NMR spectroscopy techniques. Among these five penicyclones, penicyclone A was determined as the most active one with its 0.3 μg/mL MIC value against *Staphylococcus aureus* (Guo et al. 2015).

In a study carried out by Kong et al. (2010), 71% of the 32,000 marine natural products in the databases have a novel scaffold. Also, 53% of these new scaffolds were observed only once in the marine natural products. Furthermore, it was demonstrated that marine natural products are highly hydrophobic (Kong et al. 2010). Also, it was noted that the increasing number of oxygen and nitrogen atoms in the molecules made the structures more hydrophilic. Relatively low oxygen levels in the marine environments could be the reason for the hydrophobic character of these molecules. This also suggests that marine microorganisms cultivated in oxygen-rich fermentation environment may produce novel and specifically hydrophilic molecules. When preliminary cytotoxic activity screening results of the National Cancer Institute were examined, it was found that the possibility of discovering active molecules from marine natural products is ten times higher than the terrestrial sources. As a final note, three derivative molecules, cytarabine (Fig. 16.18a), vidarabine (Fig. 16.18b) and trabectedin (Fig. 16.18c), from marine natural products have been accepted by the FDA as drug, while phase studies of 13 are still in the process (Mayer et al. 2010).

### 16.3.3 Extreme Environments

Polar regions, deserts, deep seas, rainforests, highly salty or acidic lakes possess special stress factors for living organisms compared to temperate zones. Microorganisms that can survive in such extreme environments are called

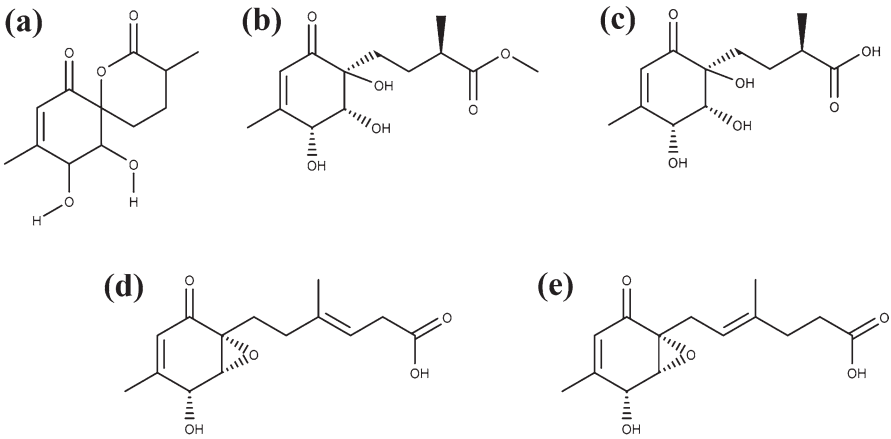



**Fig. 16.17** Chemical structures of (**a**) penicyclones A, (**b**) penicyclones B, (**c**) penicyclones C, (**d**) penicyclones D, (**e**) penicyclones E

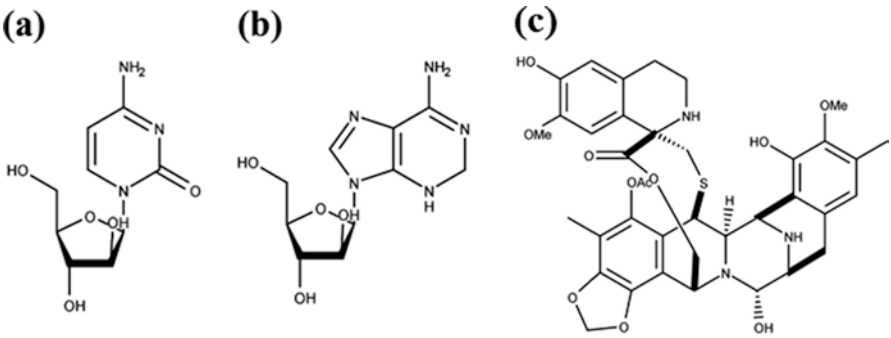



**Fig. 16.18** Chemical structures of (**a**) cytarabine, (**b**) vidarabine, (**c**) trabectedin

extremophiles. There are many species and isolates of extremophiles reported from different environments. Magan (2007) published a very comprehensive book chapter on extremophilic fungi summarising their characteristics and cultivation conditions. Extremophiles are classified according to the conditions in which they can sustain their existence. For example, microorganisms that can grow at high temperatures are called thermophiles, while those that can grow at low temperatures are called psychrophiles. In addition to these, there are acidophiles which can survive under acidic conditions and alkaliphiles that can survive under basic conditions. The microorganisms that may exist under high-pressure and low-temperature conditions in deep seas are called barophiles or barotolerants (Raghukumar 2008). There are also hydrothermal vents exhibiting both high pressure and temperature in the deep seas. It should be kept in mind that metal and sulphur ratios are high near the hydrothermal vents. However, there are microorganisms that can survive under

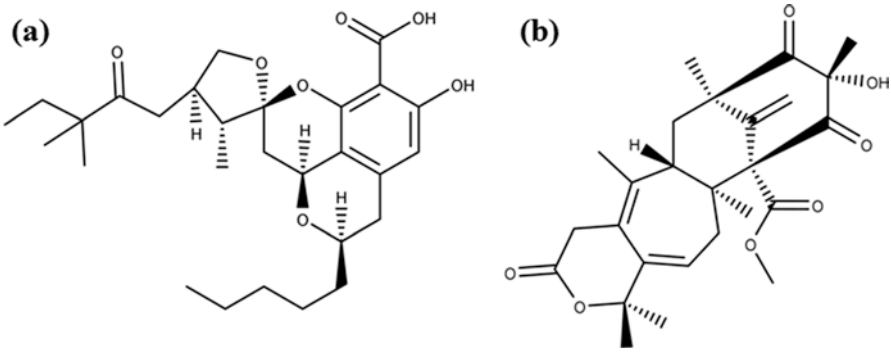



**Fig. 16.19** Chemical structures of (**a**) berkelic acid, (**b**) berkleydione

such difficult conditions (Amend 2014). Isolation and growth of the extremophiles require special cultivation conditions (Raghukumar 2008). For example, *Aspergillus terreus*, which is a deep sea isolate, was successfully cultivated at 200 bar at 5 °C and 200 bar at 30 °C. These organisms have differentiated metabolism compared to ordinary microorganisms and possess structures that can withstand extreme conditions. These organisms and their secondary metabolites have been relatively less investigated making extremophiles important targets for both academia and drug companies. Although some new molecules have already been isolated from extremophiles, however, their promising activity levels are relatively low.

Nevertheless, there are promising molecules like berkelic acid (Fig. 16.19a), isolated from *Chlorella mutabilis* associated with *Penicillium* sp. from extreme acidic environments (pH 2.5–2.7) of Berkeley Pit Lake (Stierle and Stierle 2014). Berkelic acid is a potent enzyme inhibitor of MMP-3 and Caspase-I with 1.87 μM and 98.0 μM $IC_{50}$ values. It is also a selective cytostatic agent against ovarian carcinoma cell line (OVCAR-3) with a $GI_{50}$ value of 90 nM (Stierle et al. 2006). Another extremophile fungus called *Penicillium rubrum* is an acidophile isolated from a depth of 270 m from Berkeley Pit Lake (Stierle et al. 2004). A secondary metabolite isolated from the extract of this acidophile is called berkleydione (Fig. 16.19b) and was identified as a selective cytostatic agent against non-small cell lung cancer NCI-H460 with a GI50 value of 398 nM (Stierle et al. 2004). These two molecules (berkelic acid and berkleydione) were extracted from liquid fermentation media containing potato dextrose broth with $CHCl_3$. Berkelic acid was isolated from the fraction obtained by the elution of silica gel column with hexane, isopropyl alcohol and MeOH mixtures. Enzyme activities of the obtained fractions were determined, and those with the highest activity were chosen for further purification with HPLC. In the second step of purification procedure, fractions were subjected to preparative Sigel HPLC column and eluted with hexane/IPA gradient. In many purification processes, bioactive molecules are isolated with the help of preparative reverse-phase HPLC columns. However, in their study Stierle et al. (2006) used a normal-phase HPLC column.

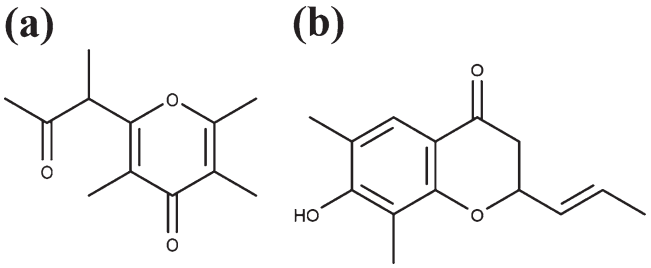



**Fig. 16.20** Two novel molecules isolated by Tian et al. (2007)

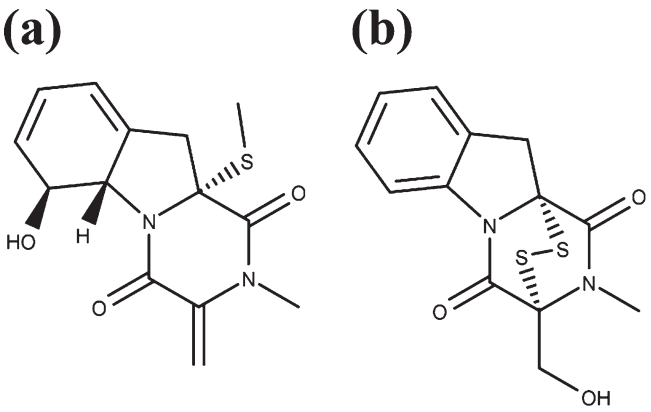



**Fig. 16.21** Two novel molecules isolated from a deep sea extremophile fungus *Penicillium* sp. JMF034

Tian et al. (2007) reported two novel molecules together (Fig. 16.20a, b) with six known secondary metabolites with relatively high cytotoxic activity on P388 cell line (murine leukaemia cells). One of these two novel molecules exhibited a relatively high cytotoxicity with $IC_{50}$ value of 0.14 μM. Fermentation broth (50 L) was extracted three consecutive times with EtOAc, and the evaporated organic layers yielded 38.0 g of crude extract. In the first step of the purification process, crude extract was subjected to silica gel column and eluted with gradient mixtures of petroleum ether-$CHCl_3$ and $CHCl_3$ and MeOH mixtures. The active fractions of the first step purification were subjected to size exclusion column Sephadex LH-20 and eluted with $CHCl_3$ and MeOH mixture. Further purification of active fraction was carried out by using reverse-phase HPLC column with 70% MeOH and 30% water mixture to give 10 mg of (0.026% yield from crude extract) compound one and 12 mg (0.027% yield from crude extract) compound two.

Another good example of bioactive secondary metabolite isolation from a deep sea extremophile fungus is *Penicillium* sp. JMF034 obtained from deep sea sediments of Suruga Bay, Japan. Two novel (Fig. 16.21a, b) and seven gliotoxin analogues were isolated from *Penicillium* sp. JMF034 with potent cytotoxic activities against a P388 cell line. Some of these secondary metabolites, especially ones with

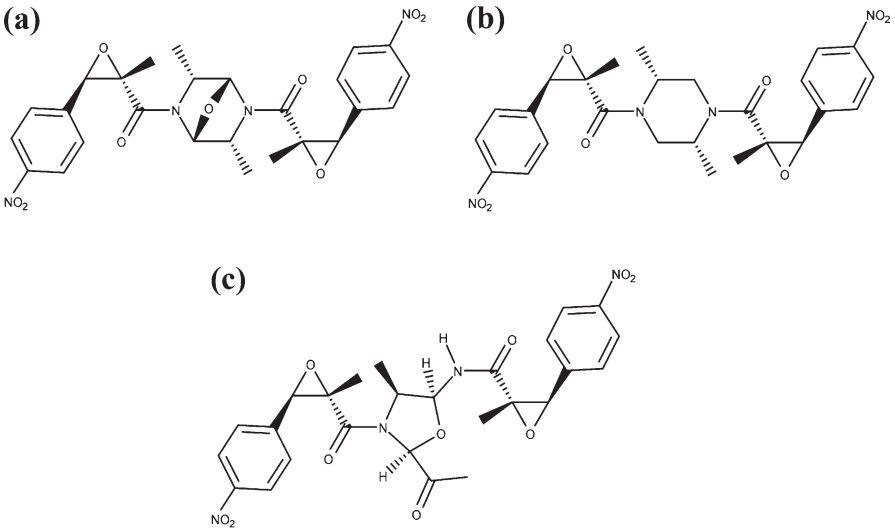



**Fig. 16.22** Chemical structures of (**a**) chrysamide A, (**b**) chrysamide B, (**c**) chrysamide C

disulphide bonds, also exhibited inhibitory activity on histone methyltransferase G9a enzyme (HMT G9a) (molecule 2; HMT G9a inhibition $IC_{50}$ = 55 μM, cytotoxic $IC_{50}$ = 0.058 μM, molecule 3; HMT G9a inhibition $IC_{50}$ = 2.6 μM, cytotoxic IC50 = 0.056 μM, molecule 3; HMT G9a inhibition IC50 = 6.4 μM, cytotoxic IC50 = 0.024 μM). Unlike the isolation procedures mentioned so far, the first fractionation was carried out on a reversed phase column by elution with mixtures of water and MeOH as a stepwise gradient. The fraction obtained by elution of 60% MeOH was further purified with Sephadex LH-20 column using a $CHCl_3$ and MeOH mixture followed by purification on reverse-phase preparative HPLC columns (Sun et al. 2011).

In another report published recently, Chen et al. (2016) isolated three new, rare dimeric nitrophenyl trans-epoxyamides (chrysamides A, B, C) (Fig. 16.22a–c) from deep sea-derived fungi *Penicillium chrysogenum* SCSIO41001. They reported that chrysamide A is the first example of dimeric nitrophenyl trans-epoxyamide in nature. However, despite their very rare skeletons and novel structures, none of these isolates showed cytotoxic activity against K562, A549 and HUH7 cancer cell lines. Also, they did not exhibit any antibacterial activities against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp. Only chrysamide C moderately suppressed the production of pro-inflammatory cytokine interleukin-17 (40.06% inhibition rate at 1 μM concentration) (Chen et al. 2016).

### 16.3.4 Genetic Modification Techniques

Developments in genome sequencing have enabled researchers to constitute genetic maps of many living organisms. Recent genetic studies on fungi have revealed that genes responsible for the production of secondary metabolites are more than

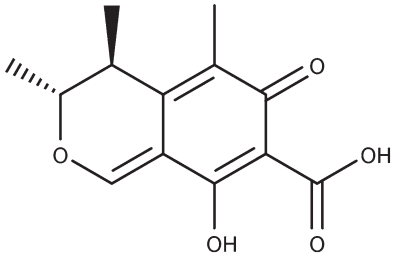



**Fig. 16.23** Chemical structure of Citrinin

expected (Schneider et al. 2008). Therefore, the number of secondary metabolites already isolated from fungi is just a small portion of its potential. There are also reports of re-isolation of known molecules (Hong et al. 2009).

The silent gene clusters of the fungal genome may further initiate the discovery of both novel and bioactive molecules (Brakhage and Schroeckh 2011). There are two types of multifunctional enzyme classes in fungi called as polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) which control the production of secondary metabolites. Although these two enzyme classes have different names, their catalytic domains are similar to each other. The combined effect of these multifunctional enzymes is the main reason that fungi are able to produce different secondary metabolites, and single-point mutations on these enzymes yield differences in mode of action (Brakhage et al. 2008; Williams 2013). The discovery of silent genes which can produce these enzymes led researchers to focus on activating them. There are a number of methods inducing the activation of these silent genes. Heterologous expression, homologous expression with promoter exchange, overexpression of transcription factors (Brakhage et al. 2008), epigenetic modifiers (Yang et al. 2014b; Shwab et al. 2007) and coculture fermentation (Akone et al. 2016) are some examples of this approach.

#### 16.3.4.1 Heterologous Expression of Genes

Heterologous expression of genes related to secondary metabolites has some challenges such as the large sizes of the whole gene clusters of PKSs and NRPSs, replacement of native promotors, etc. (Chiang et al. 2011). Sakai and colleagues transferred gene clusters of *Monascus purpureus* (which are responsible for production of citrinin) (Fig. 16.23) to *Aspergillus oryzae*. Compared to the native fungus, they could increase the yield of citrinin by 400-fold. The concentration of citrinin was determined online, directly from fermentation media without solvent extraction, using HPLC coupled to a fluoresce detector. This is the first report which describes functional fungal secondary metabolite production in *Aspergillus oryzae* by the heterologous gene expression (Sakai et al. 2008).

#### 16.3.4.2 Promotor Exchange

Promotor exchange is a cumbersome process, and sometimes the expression of gene does not yield a desired product (Bergmann et al. 2007; Chen et al. 2013). However, more recently, Yeh et al. (2016) have succeeded in exchanging the promoter of *inpE* gene cluster which encodes proteasome subunit in *Aspergillus*

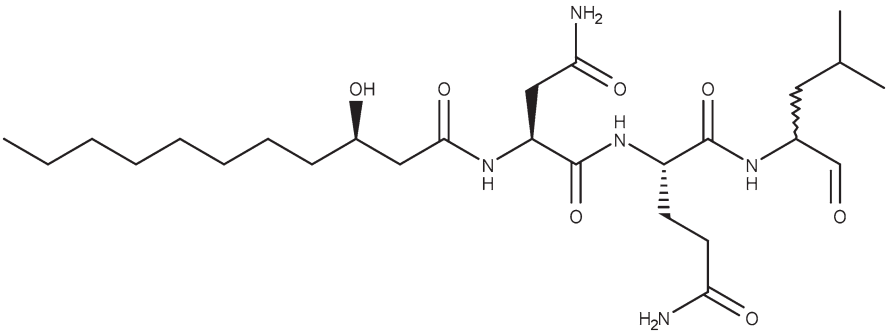



**Fig. 16.24** Chemical structure of fellutamide B

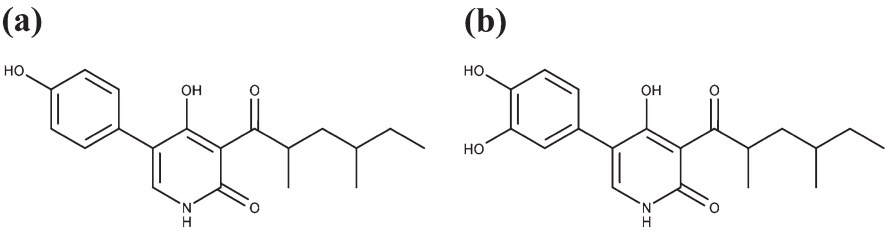



**Fig. 16.25** Chemical structures of (**a**) aspyridone A, (**b**) aspyridone B

*nidulans*. Their efforts resulted in the production of proteasome inhibitor fellutamide B (Fig. 16.24). Strains produced fellutamide B (above 20 mg/L). Optimisation of fermentation parameters and design of suitable growth media may increase the production yield of this inhibitor molecule making it suitable for industrial application (Yeh et al. 2016).

Bergmann and colleagues have successfully transferred the regulatory gene, *apdR*, to *Aspergillus nidulans* to generate mutant clones. Some of the induced clones have shown different secondary metabolite profiles than their native forms. Large-scale fermentation of the best producer mutant *A. nidulans* SB4.1 has resulted in sufficient amount of extracts to enable the isolation of two novel secondary metabolites later named as aspyridone A (Fig. 16.25a) and aspyridone B (Fig. 16.25b). These metabolites were isolated using a three-step purification process. In the first step, the extract was fractionated on a silica gel column followed by Sephadex LH-20 size exclusion column as the second step. The final purification was carried out on preparative HPLC (Bergmann et al. 2007).

### 16.3.4.3 Epigenetic Modifiers

Probably the easiest way to activate silent genes related to secondary metabolite production is to treat fungi with epigenetic modifiers added into the fermentation media. Deletion of gene *hdaA* which encodes histone deacetylase (HDAC) in *Aspergillus nidulans* has resulted in the increased production of secondary metabolites (mostly toxins and antibiotics) (Shwab et al. 2007). Histone deacetylase

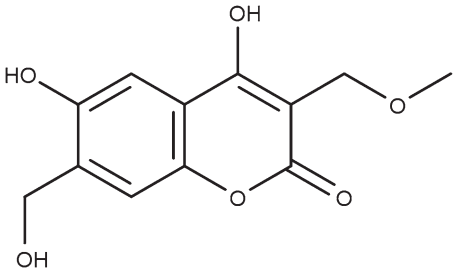



**Fig. 16.26** Novel coumarine-type molecule isolated by Yang and colleagues

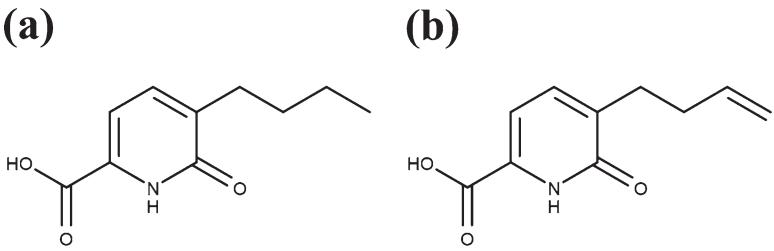



**Fig. 16.27** Two novel fusaric acid derivatives isolated by Chen et al. (2013)

(HDAC) is an enzyme which removes acetyl groups of lysine on histone and is also an epigenetic modifier. It was shown HDAC resulted in overproduction of some secondary metabolites under fermentation conditions (Shwab et al. 2007).

In a report by Yang et al. (2014b), cultivation of endophytic fungus *Pestalotiopsis crassiuscula* using DNA methyltransferase inhibitor 5-azacytidine has significantly changed the secondary metabolite profile of this fungus. As a result of this differentiation, one novel coumarine-type secondary metabolite (Fig. 16.26) along with six known molecules was isolated from the extract. All isolated molecules showed significant antifungal activity (Yang et al. 2014b). These molecules were isolated directly on a preparative HPLC following EtOAc extraction (Yang et al. 2014b).

Chen et al. (2013) reported another case of effect of epigenetic modifier on the secondary metabolite profile of fungi. They could produce two novel fusaric acid derivatives (Fig. 16.27a, b) by using different type of epigenetic modifier, a histone deacetylase (HDAC) inhibitor and suberoylanilide hydroxamic acid. These molecules were isolated on a preparative HPLC column after extraction with EtOAc without prior purification (Chen et al. 2013).

#### 16.3.4.4 Coculture Techniques

Another way of activating silent fungal genes is the cocultivation with other microorganisms like bacteria. In a recent study, Akone et al. (2016) isolated five novel secondary metabolites (Fig. 16.28a–e) along with seven known metabolites from the coculture of endophytic fungi *Chaetomium* sp. and *Bacillus subtilis*. The microorganisms were grown on solid rice media for 5 weeks at 23 °C. At the end of this period, EtOAc was added to media, and cultures were shaken at 140 rpm for 9 h to

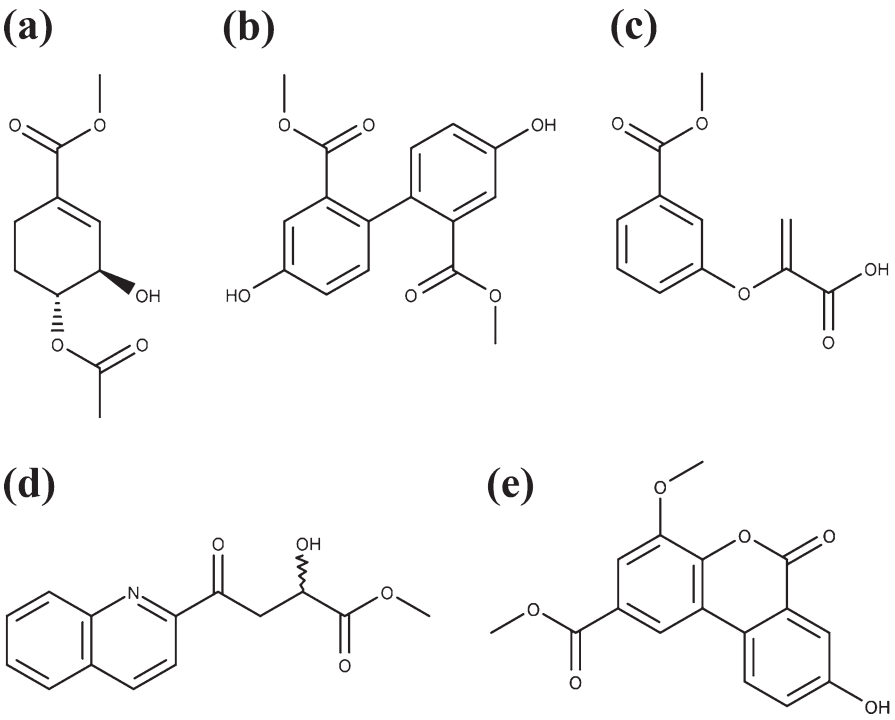



**Fig. 16.28** Five novel secondary metabolites isolated by Akone et al. (2016)

extract the secondary metabolites. The filtered EtOAc phase was evaporated to dryness and then dissolved in MeOH for further purifications. Nearly the same procedures were followed to investigate the effects of epigenetic modifiers. Preparative isolation of secondary metabolites was carried out in three steps according to the report by Bergmann et al. (2007).

## 16.4 New Trends in Separation and Purification of Bioactive Molecules

Phenotypic assays aiming to detect bioactive molecules have been the basis of drug development studies until recently. Although such screening is useful in identifying precursor-lead molecules, however, it is often slow and sometimes it is difficult to determine the pathways or targets the molecules (Kotz 2012). Developments in molecular biology and genetics in the last 30 years have directed researchers to focus on disease-related targets. In phenotypic assays, large molecule libraries need to be tested to determine the active molecule. Conversely, a limited number of molecules may be sufficient for target-based studies. If the structures of enzymes or receptors targeted for diseases are known, then testing of selected molecules is possible. A review published by Swinney in 2013 showed that the number of drug

molecules identified in the past using phenotypic assays was higher. However, surveys indicate that half of newly developed drug molecules were identified by target-based studies (Swinney 2013). Increased emphasis on targeted drug discovery efforts indicates the importance of target-based bioassay studies. The understanding of target-based screening had a positive effect on determination and isolation methods for bioactive molecules. The methods developed enable the activity to be detected in a shorter time, even when the differentiation is made. Ultrafiltration, ligand fishing, online activity detection and biochromatography or bioaffinity chromatography are some of the most convenient methods to identify target-specific molecules from extracts (Cieśla and Moaddel 2016).

### 16.4.1 Ultrafiltration

Ultrafiltration or pulse ultrafiltration technique was developed by van Breemen et al. (1997) to detect enzyme inhibitors from combinatorial libraries in a shorter time and generally involves four steps. The first step is the incubation of free enzyme or receptor and ligand to bind each other. During incubation, molecules with different $K_d$ values are bound in different levels, mostly by competing with other. In some cases ligands do not bind to the active site, and false-positive results are obtained. Incubation with a known strong inhibitor solves this problem. The second step of the method is separation or concentration of enzyme or receptor from incubation media by using ultrafiltration membrane. In the third step, elution of ligand is carried out by using organic solvent or buffers. In this way, the ligand is transferred to the liquid phase passes. The fourth and last step of the method is the analysis of molecules in the solution using HPLC, LC-MS, etc. (van Breemen et al. 1997). By using this method, several novel enzyme inhibitors have been determined from plant extracts (van Breeman et al. 2011; Johnson et al. 2002; Ingkaninan et al. 2000). These reports clearly show that new bioactive molecules from fungal extracts can be obtained by screening via ultrafiltration method.

### 16.4.2 Online, Real-Time Bioassays

Another important target-based method is online, real-time bioassays. In online HPLC-bioassays, flow from the column is separated in two unequal flows. The first smaller-volume flow is directed through MS detector to record mass spectroscopic data of the separated molecule, and the rest of the flow is directed to another line, which contains the target enzyme. The resulting solution is directed into the reaction coil. As long as the mixture travels through this coil, binding takes places. The resulting mixture from the reaction is added to the substrate of the enzyme and transferred to the second reaction coil. The substrate does not react or bind to target; if there is an inhibitor, it elutes from the HPLC column. It will result in an increase in substrate signals. If the intensity of the signal is high, it is possible that a molecule binds well to the target (Cieśla 2016).

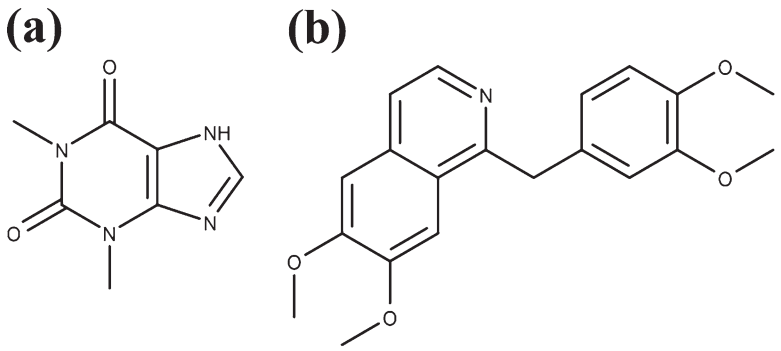



**Fig. 16.29** Chemical structures of (**a**) theophylline, (**b**) papaverine

One of the first examples of this method was reported by Ingkaninan et al. (2000). They used known acetylcholinesterase (AchE) inhibitors to develop and understand drawbacks of online bioassay methods. Yet in another report Falck et al. (2010) generated and optimised an online bioassay method to detect binders of p38α, mitogen-activated protein kinase, in an artificially created mixture. It was demonstrated that different binding modes of ligand could be identified on an online bioassay (Falck et al. 2010). By using online bioassays, enzyme inhibitors have also been identified along with ligand molecules. For example, Schenk and colleagues have identified phosphodiesterase inhibitors from plant extract by using online bioassay methodology (Schenk et al. 2003). When spectroscopic data showing the peaks of the molecules causing the inhibition were investigated, two possible matches, theophylline (Fig. 16.29a) and papaverine (Fig. 16.29b), were identified. In recent years, the online bioassay approach has begun to evolve into microfluidic droplet assays. This method, in which the results are obtained using a very small amount of substance, has shown promising results. For example, Ochoa and colleagues have identified possible inhibitors of *Clostridium perfringens* neuraminidase inhibitors from the extract of *Pelargonium sidoides* plant using droplet-based microfluidics device (Ochoa et al. 2017). Two important reviews by Potterat and Hamburger (2013) and Price and Paegel (2016) on this subject provide a comprehensive approach on the issue.

Another recent development in target-based approach is magnetic beads. Molecules such as proteins, antibodies, enzymes and ligands can be covalently attached to magnetic beads. These magnetic beads then could be separated from media using a relatively small magnetic field (Zhuo et al. 2016). Accuracy, reproducibility and efficiency of this method enable researchers to use it in the studies of protein detection, cancer treatment and drug-protein binding (Zhuo et al. 2016). This technique was also used to search for possible enzyme inhibitors or ligands in the extracts. Yasuda and colleagues have successfully identified quercetin (Fig. 16.30a) and vitexin (Fig. 16.30b) as inhibitors of SIRT6 histone deacetylase (Yasuda et al. 2011). Yet in another report, Jonker et al. (2009) successfully identified norethisterone (Fig. 16.31) as a ligand of His-tagged human oestrogen receptor by coupling magnetic beads with LC-MS device.

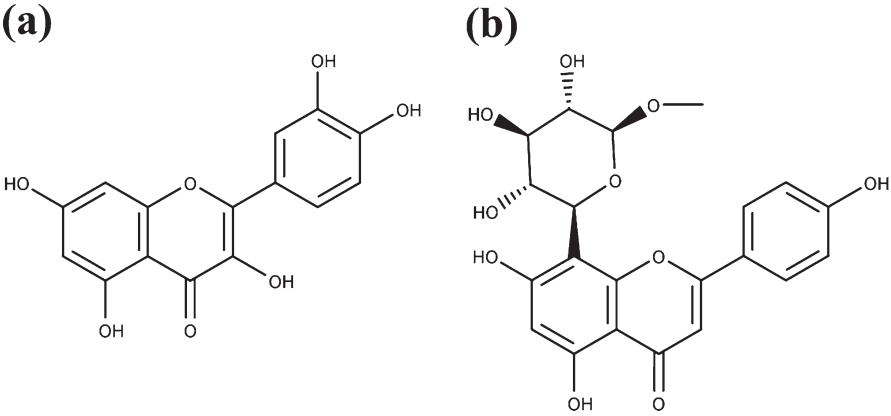
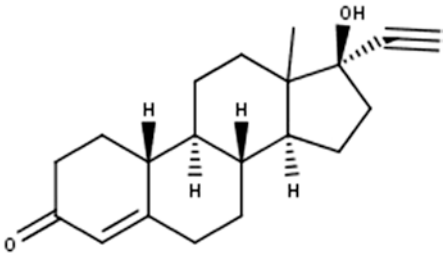



**Fig. 16.30** Chemical structures of (**a**) quercetin, (**b**) vitexin

**Fig. 16.31** Chemical structure of norethisterone




In addition to the methods mentioned above, modified chromatographic columns such as biochromatography and two-dimensional chromatographic separations are developing techniques with great potential for determination of bioactive molecules.

In the cellular membrane affinity chromatography, cell membranes are attached to the surface of solid material to study ligand receptor interactions (Zhuo et al. 2016; Moaddel and Wainer 2009). Extensive information on this method and the work undertaken has been reviewed by de Moraes et al. (2014, 2016). Luo et al. (2003) aimed to identify binder ligand molecules to a polyclone antibody from the extract of *Phyllanthus urinaria L.* According to the results obtained from frontal immunoaffinity chromatography, brevifolin (Fig. 16.32a), brevifolin carboxylic acid (Fig. 16.32b), corilagin (Fig. 16.32c), ellagic acid (Fig. 16.32d) and phyllanthusiin U were identified as possible ligands of HCV NS3 protease (Luo et al. 2003).

Finally, the applications of two-dimensional chromatography and possible developments in this methodology are worth mentioning. Despite improvements in particle technologies, column geometries and devices used in liquid chromatography, high-resolution separation of molecules from complex mixtures such as natural product extracts is still a very challenging task. Sometimes molecules that are very similar to each other make it difficult to achieve high-resolution separation (Stoll and Carr 2016). According to simulations performed by Davis and Giddings (1983),

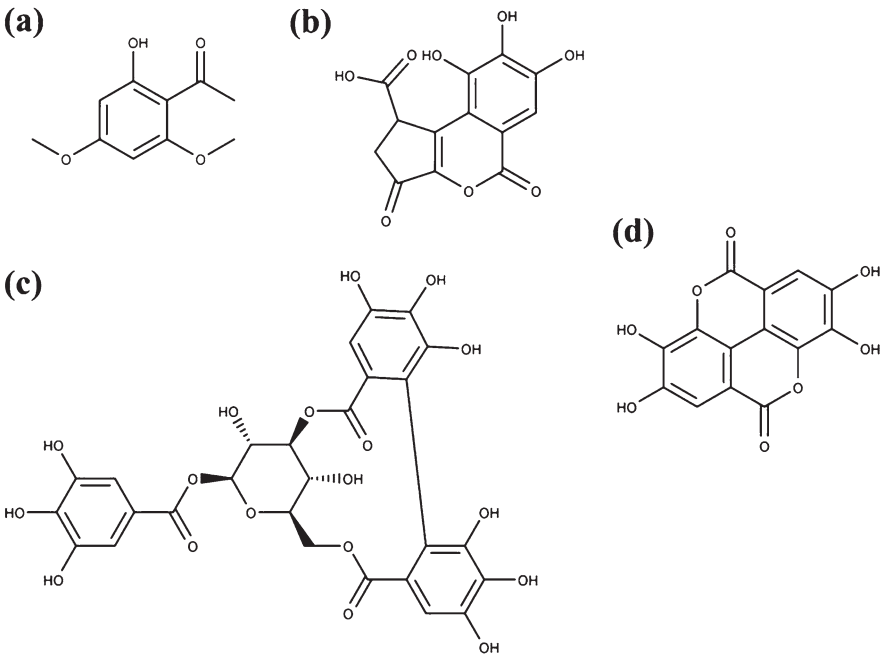



**Fig. 16.32** Chemical structures of (**a**) brevifolin, (**b**) brevifolin carboxylic acid, (**c**) corilagin, (**d**) ellagic acid

it was demonstrated that one-dimensional chromatography only has the ability to separate 37% of peaks and only 18% of them are single molecules. These simulations showed that almost 60% of complex samples such as fungal extracts cannot be analysed in a single dimension (Davis and Giddings 1983). The ability to carry out selective analysis by mass spectroscopy removes the problem in qualitative analysis of co-eluting peaks. However, this is still a problem on preparative scale. Separating co-eluting peaks in a second dimension column with orthogonal character right after they leave the first column may be a solution (Stoll et al. 2007). This can be achieved by the utilisation of the second column synchronously with the first dimension analysis, enabling the identification of many more secondary metabolites in the same analysis (Sarrut et al. 2015). Two-dimensional separation techniques have been analytically applied to many extracts of natural product. However, preparative applications of this separation method need further improvement. Success in this field will enable natural product chemists to separate and isolate many more molecules even with minor ones efficiently.

## 16.5 Conclusions

We have described several examples of the recent technologies and research involving bioactive secondary metabolites of fungal origin from different sources including marine and extreme environments. Recent advances in biochemistry, genetics,

analytical technologies and bioinformatics have crucial roles in the discovery of novel bioactive compounds from fungi. Combination of microfractionation, high-resolution mass spectroscopy and liquid chromatography with emerging technologies such as microfluidics is expected to augment the efficiency of identification procedures for novel bioactive molecules. Also, scale-down techniques with improved efficiency will prove to be effective tools for bioengineers to adapt these microorganisms for industrial applications.

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