



Antibacterial and Antifungal Agents of Higher Plants

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Abstract

The extensive search for therapeutic agents derived from higher plants has recently accelerated owing to the antibiotic resistance crisis, which is a serious warning to human healthcare worldwide. It is estimated that antibiotic-resistant infections will lead to nearly ten million yearly deaths at the global level by the year 2050. Thus, it is wise to search for new alternative antibiotics sources derived from higher plants to tackle this alarming antibiotic resistance crisis issue. Amongst the 250,000–500,000 plant species available in the World, only a small percentage (1–10%) of them have been explored by man. This data shows that there is a great opportunity for exploring these higher plants, which are rich in a wide variety of phytochemicals, and could be used as a good source to develop new antibacterial and antifungal agents. Moreover, the World Health Organization (WHO) suspects about four billion humans (about 80% of the world population) currently use herbal medicine as their primary healthcare. Therefore, this chapter is designated to discuss in detail the prospects of higher plants as antimicrobial agents. Also, this chapter attempts to summarize the antibiotic resistance crisis, current status of higher plants as antimicrobial agents, the extraction process of phytochemicals, in vitro and in vivo antimicrobial activity evaluations and the contribution of important phytochemicals, such as polyphenols, as antimicrobial agents. The methods presented in this chapter are illustrated for the bacterial and fungal model.

Keywords

Animal model · Antibiotic-resistant · Antimicrobial · Higher plants · Phytochemical

16.1 Introduction

The higher plants, characterized by vascular tissue and reproducing either by spores, cones, or flowers, dominate the world's flora and vegetation (Akeroyd and Synge 1992). The higher plants are flora comprising various phytochemicals that could be utilized for healing activities or are precursors for the new synthesis of beneficial remedies (Sofowora 2008; Swamy et al. 2016; Arumugam et al. 2016; Swamy et al. 2017). Therefore, since antiquity, Mother Nature has been a treasure of natural remedies for providing relief from various illnesses affecting human beings. In addition, the oldest written records on Indian, Chinese, Egyptian, Greek and Roman folk medicine have recorded numerous medicinal plants with various therapeutic values and prescriptions used in treating numerous illnesses (Pandey and Kumar

2013; Mohanty et al. 2017). Moreover, plant-based remedies have been reported to be inexpensive, safe and without any adverse side effects, particularly when compared with synthetic chemical drugs, and they can serve patients from low income countries. Higher plants also usually yield numerous secondary metabolites, which comprise a significant source of new antimicrobials against pathogenic bacterial and fungal infections. It is projected that only a small percentage (1–10%) of plant species have been explored by man and animals out of the 250,000 to 500,000 in the world. This data shows the great opportunity of exploring the higher plants, which are rich in a wide variety of phytochemicals, as a good source for developing antibacterial and antifungal agents. Moreover, the World Health Organization (WHO) suspects about four billion humans (about 80% of the World population) currently use herbal remedies as their main healthcare (Kumara Swamy et al. 2011; Kumara et al. 2012; Ekor 2013; Mohanty et al. 2017). Thus, exploration for novel drugs with improved therapeutic values and cheaper phytochemicals from higher plants is a natural choice for developing innovative next-generation therapeutics, especially against pathogenic microbial infection. Consequently, the extensive search for therapeutic agents derived from higher plants has recently accelerated owing to the antibiotic resistance crisis, which is a serious warning to human health worldwide (Rudramurthy et al. 2016). Thus, this chapter is intended to deliver insights into the antibiotic resistance crisis, current status of higher plants as antimicrobial agents, important antimicrobial phytochemicals from higher plants, extraction methods of higher plants and in detail *in vitro* and *in vivo* evaluations of higher plants' antimicrobial activities through clinical trials.

16.2 Antimicrobial Resistance Crisis

Recently, a rapid rise has been noticed in the cases of multidrug-resistant microorganisms worldwide. This has become a great threat to the use of antibiotics for treating several microbial diseases (Michael et al. 2014; Rudramurthy et al. 2016). Antibiotics are commonly used against many bacterial infections effectively. Since the discovery of antibiotics, the scenario of medicine has significantly transformed towards safeguarding millions of animal lives (Rossolini et al. 2014; Ventola 2015). Because the antibiotics were effective, they became the only option, and were continuously prescribed to patients suffering from microbial diseases. This practice is still in progress owing to various reasons and has resulted in the crisis of antibiotic resistance. In particular, the resistance catastrophe is mainly attributed to the misuse and overuse of antibiotics/drugs. Also, a complete failure in the development of alternative, but clinically effective drugs, led to this crisis. There are a number of microbes that have become resistant to multi-drugs and threaten the medical world. Therefore, there is an urgent need to invent novel and effective antimicrobial agents to overcome this situation and treat patients against multi-drug resistant microbial infections (Michael et al. 2014; Rudramurthy et al. 2016).

Microorganisms, such as bacteria, fungi, viruses, or protozoa, recurrently interact with humans in several ways. The type of interactions may lead to beneficial or

damaging effects in their host organisms. The damaging microbes, also known as pathogens, cause many contagious diseases that can spread from one individual to another. These pathogenic microorganisms are predominantly responsible for human deaths throughout the world. After invading a host body, pathogens multiply quickly by overpowering the immunity or defense lines. The design and function of the microbial genome is responsible for their ability to overcome the defense barriers of the host cell. A wide range of molecular mechanisms, such as enzyme inactivation, target site modification, biofilms formation, evading repair mechanisms and intra-cellular localization, assist in the survival of pathogens in their host cells (Swamy and Rudramurthy 2016). This makes it necessary to use effective antimicrobial agents that can kill these pathogens and control diseases.

Antimicrobial agents are natural, synthetic or semi-synthetic chemical substances with the capability to inhibit or destroy the growth of microbial pathogens. For instance, antibiotics are used against bacteria, while antifungal agents are used against fungi. It is noteworthy that antimicrobial agents cause no harm or very little damage to the host. The era of antimicrobial agents has been in progress since Sir Alexander Fleming discovered the antibiotic penicillin in 1928 (Sengupta et al. 2013; Ventola 2015; Swamy and Rudramurthy 2016). The discovery of antibiotics, such as penicillin, vancomycin, amoxicillin, levofloxacin, tetracycline, rifamycins, etc., in the twentieth century has undoubtedly boosted the state of human health in myriad ways (Laxminarayan et al. 2013; Rudramurthy et al. 2016). Antimicrobial agents destroy pathogenic microbes through various ways, such as the inhibition of DNA replication, inhibiting RNA and protein synthesis, preventing cell wall formation and modifying the other metabolic activities (Swamy and Rudramurthy 2016). Antimicrobial agents are extensively used for treating and preventing many infectious diseases. With the discovery of several new antimicrobial drugs, their application further increased drastically, and this has prompted the development of antibiotic resistance properties in microorganisms. This change can be attributed to the fact that microbes exhibit an unlimited flexibility or adaptableness to different environments because of their incredible genomic manipulability and capability to interchange their genetic material with other species (Rodríguez-Rojas et al. 2013; Ventola 2015). The drug resistance may also occur unexpectedly via mutations (Read and Woods 2014). In addition, incorrect prescription of antimicrobial agents, availability of only a few new antibiotics, extensive agricultural uses and regulatory barriers are some of the other reasons towards the development of microbial resistance. Regardless of warnings on the overuse of antibiotics, even today, they are overprescribed throughout the world leading to serious threats and extensive clinical and economic burden on the healthcare system and patients (Ventola 2015).

Antimicrobial-resistant microbial infections are already prevalent across the globe, and they pose a great challenge to treatment approaches. It is reported that nearly 99,000 deaths occur amongst the two million Americans who develop health associated infections per year, mostly because of antibiotic-resistant pathogens (Ventola 2015). Further, antibiotic-resistant infections are overburdening the nation's healthcare systems. It is estimated that the average medical expense per each patient suffering with a drug-resistant infection ranges between 18,588 and

29,069 US\$ (Golkar et al. 2014; Ventola 2015). Some of the drug-resistant strains of bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), penicillin-resistant *Streptococcus pneumoniae* (PRSP), fluoroquinolone-resistant *Escherichia coli*, carbapenem-resistant *Acinetobacter baumannii*, cephalosporin-resistant Enterobacteriaceae, etc. (Swamy and Rudramurthy 2016).

A rapid emergence of drug-resistant microbes is becoming a great threat to the health benefits offered by the use of antibiotics. This global crisis is mainly caused by the overuse or misuse of antimicrobial agents in addition to the failure of pharmaceutical firms to develop novel antimicrobials to address this challenge. In this direction, many efforts are being carried out to mitigate the problem of drug resistance. A variety of natural compounds isolated from plants, microbes, algae, lichens, etc., still remain the major sources of antimicrobials (Swamy et al. 2016). As plants possess many compounds with chemodiversity and high antimicrobial properties, they are highly preferred and widely exploited in the drug discovery research. However, the antimicrobial activity in relation to chemical structure for many phytochemicals is yet to be clearly understood (Swamy et al. 2016; Swamy and Rudramurthy 2016). The class of polyphenolic compounds obtained from plants exhibit a great diversity in their structures; therefore, their antimicrobial effectiveness against pathogens also differs significantly (Gyawali and Ibrahim 2014; Swamy et al. 2016). In addition, various types of nanoparticles can be effectively employed as alternatives against multidrug-resistant pathogenic microbes (Rudramurthy et al. 2016). It is essential to encourage scientists to keep trying towards the discovery of new lead molecules against the drug-resistant pathogens. Moreover, well-coordinated efforts in implementing new policies and refurbishing investigations are imperative to manage the drug-resistance crisis and other clinical challenges.

16.3 Current Status of Higher Plants as Antimicrobial Agents

Infections caused by pathogenic fungi and bacteria affect the lives of millions of people worldwide. Since time immemorial, infectious diseases caused by pathogenic microbes have been a major cause of death throughout the world. Nowadays, clinically significant pathogenic fungi and bacteria are categorized not only by single drug resistance but also by multiple antibiotic resistant microbes caused by the misuse of antibiotics (Levy 2002). Thus, it is necessary to explore newer drugs to overcome multiple antibiotic resistance issues (Sarkar et al. 2003). Nevertheless, past data of the rapid, extensive appearance of resistance to newly introduced antimicrobial agents show that even new families of antibiotics will have a short life expectancy, and new antibiotics are urgently needed (Coates et al. 2002).

Therefore, the use of plant extracts or novel natural compounds in mixture with conventional antibiotics might hold better potential for promptly providing inexpensive treatment choices against multiple antibiotic resistant microbes (Cheesman et al. 2017). Moreover, about 80% of the world population is reported by WHO to rely on traditional medicine, such as medicinal plants, for their primary

healthcare requirements (Bannerman 1983). Furthermore, this view was revised later by WHO and states that the majority of the population of most developing countries now regularly use traditional medicine for their healthcare requirements (WHO 2003). Even in developed countries, complementary or alternative medicine is gaining popularity among the peoples. More importantly, along with the increased incidence of bacterial resistance to antibiotics, there has also been a corresponding decrease in novel antimicrobial discovery. Consequently, there is a great need to search for new classes of antibacterial substances, especially from natural sources such as plant-based medicines in combination with conventional antibiotics. Scientific research shows that plants either contain antimicrobials that can function in synergy with conventional antibiotics or hold compounds that have no inherent antimicrobial activity but are capable of sensitizing the pathogen to a previously unproductive antibiotic (Betoni et al. 2006; Aiyegoro et al. 2009; Aiyegoro et al. 2010). Therefore, a combinational method that allows synergistic interaction between plant extracts and antibiotics is possibly the most effective approach to battle the antimicrobial resistance problem (Inui et al. 2007). Moreover, the drug combination therapy with higher plant extracts can be used to enhance the spectrum of antimicrobial activity, to avoid the development of resistant strains, to reduce toxicity and to achieve synergistic antimicrobial activity (Pankey et al. 2005). Therefore, this line of research of higher plants as antimicrobial agents may eventually prove to be very beneficial.

16.4 Important Antimicrobial Phytochemicals from Higher Plants

Higher plants typically contain numerous biologically active, structurally varied phytochemicals that are valuable as medicines, lead structures or fresh materials and are employed mainly for curing various ailments (Kumar and Pandey 2013). Many higher plants have been laboratory tested as antimicrobial agents. Polyphenols or phenolic compounds are a group of secondary metabolites that are major contributors to the observed antimicrobial activity exhibited by the higher plants. Polyphenols are secondary metabolites formed by higher plants that play numerous important functions in plant physiology and have great possible health properties for human beings, mainly as antimicrobial agents against pathogenic bacterial and fungal infections. Polyphenols can be categorized as flavonoids and non-flavonoids based on their chemical structures (Manach et al. 2004). Flavonoids have C6-C3-C6 carbon builds comprising double phenyl rings (A and B) and a heterocyclic ring (C). On the basis of the hydrogenation level of the heterocyclic ring and the bond site of ring B, flavonoids can be further categorized into numerous subclasses, such as flavones, flavonols, flavanones and isoflavonoids (Xiao 2013). Moreover, stilbenes, chalcones, anthraquinones, ellagitannins, ellagic acids and phenolic acids are categorized as non-flavonoids (Xiao and Kai 2012).

Higher floras are rich in a broad diversity of secondary metabolites, namely polyphenols, which hold antimicrobial properties and could act as alternative, efficient, inexpensive and harmless antimicrobials for the cure of microbial infections. Several attractive results have been established using a combination of natural products to cure illnesses; particularly, the synergistic properties and polypharmacological application of higher plant preparations (Gibbons 2003). Furthermore, the antimicrobial activities of polyphenols have attracted great attention among scientists worldwide due to the prospect of dealing with the drug-resistant microbes that are unresponsive to conventional antibiotics (Tangney and Rasmussen 2013). Polyphenols have been recommended for their antimicrobial activities in three modes of action, including direct killing of microbes, synergistic activation of conventional antibiotics and attenuation of microbial pathogenicity (Cushnie and Lamb 2011), in addition to their ability to inactivate efflux pumps, destabilize cytoplasmic membranes and inhibit β -lactamases and topoisomerase to stop the progress of antibiotic resistance in microbes (Daglia 2012). Therefore, polyphenols found in the higher plants can be important phytochemical sources for future and current antimicrobial studies against resistant pathogenic bacterial and fungal infections.

16.5 Extraction Methods of Higher Plants

Plants are highly utilized pharmaceutically due to the rich medicinal values of their phytochemicals, such as phenolics and flavonoids. Various plant extraction methods are widely practiced in galenical development, such as the maceration method, soxhlet extraction method and ultrasound extraction method.

16.5.1 Maceration

Maceration is a well-established plant extraction method which involves soaking coarse powdered plant material in a closed vessel with an appropriate solvent (Jones and Kinghorn 2006; Handa et al. 2008). The selection of solvent mainly depends on the bio-active compounds of interest from the plant material, and thus the solubility of the compounds must be taken into account to choose an appropriate solvent. In addition, chemical characterization of the solvent and extraction yield is also equally imperative to consider while choosing a solvent for plant extraction by the maceration method. Commonly used solvents for the maceration process include hexane, chloroform, ethyl acetate, methanol or ethanol (Yan et al. 2008). The maceration process is usually carried out at room temperature for at least 3 days with occasional agitation to allow the solubilization of phytochemicals from the plant material. The mixture is then filtered, and the final marc is pressed out to completely extract the dissolved bio-active compounds (Pandey and Tripathi 2014).

16.5.2 Soxhlet Extraction

Soxhlet extraction, also known as hot continuous extraction, was developed by van Soxhlet in 1879 (Soxhlet 1879). The finely powdered plant sample is located in a “thimble”, which is usually made from a strong filter paper or cellulose. The sample containing “thimble” is placed in the thimble-holder of the Soxhlet extractor, and the distillation flask at the bottom is filled with the extraction solvent. When the solvent is heated, the vapourized solvent is condensed and consecutively fills the thimble containing the plant material. When the solvent level rises, a siphon tube aspirates it from the thimble-holder and discharges it back into the distillation flask. This procedure is continued until the solvent from the siphon tube does not leave residue when evaporated, and thus it is a continuous–discrete plant extraction method (Luque de Castro and Priego-Capote 2010).

16.5.3 Ultrasound Extraction or Sonication Extraction

This extraction method comprises the utilization of ultrasound with frequencies ranging from 20 kHz to 2000 kHz (Handa et al. 2008). The acoustic result from the ultrasound causes cavitation that in turn upsurges the penetrability of the cell wall promoting the release of phytochemicals from the plant material into the solvent. Although this extraction method is easy and cost-effective, higher ultrasound energy is known to cause undesirable alterations to the bio-active compounds.

16.6 In vitro and In vivo Antimicrobial Activities Evaluation

16.6.1 Test Microorganisms and Growth Media

Various Gram-positive and Gram-negative bacteria, yeasts and molds for antimicrobial activity studies can be obtained from the American Type Culture Collection (ATCC). The bacterial strains are cultured in Mueller–Hinton agar (MHA) plates at 37 °C, while the yeasts and fungal are cultured in Sabouraud dextrose agar (SDA) plates and potato dextrose agar (PDA) plates media, respectively, at 28 °C. The stock culture is preserved on agar slants at 4 °C.

16.6.2 Inoculum Preparation for Bacterial and Fungal

One of the most crucial parts in microbiological testing procedures is the preparation of inoculum. This involves a series of steps, including the selection of appropriate colonies, suspension preparation and standardization. There are two approaches for inoculum preparation; direct colony suspension and log phase growth method. For the direct colony method, colonies from cultures not longer than 18–24 h can be used (Cavaliere et al. 2005). Inoculum is standardized immediately after preparation

of suspension unlike the log phase method where inoculum is standardized following inoculation and incubation of bacteria/fungi up to log phase growth.

16.6.2.1 Inoculum Suspension Preparation

To prepare the inoculum, pick 3 to 5 well-isolated colonies from an appropriate bacterial culture (grown at correct temperature on correct media) using a sterile loop or cotton swab. Use morphologically similar colonies to avoid an atypical variant (Schwalbe et al. 2007). Make a suspension by suspending the colonies in 5 mL of sterile saline (0.85% NaCl) or media. Next, homogenize the suspension by using a vortex for about 15 s. If a log phase method is carried out, incubate this suspension up to log phase growth first, prior to the standardization step.

16.6.2.2 Inoculum Suspension Standardization

Set up the spectrophotometer and adjust the wavelength to 600 nm. Add 1 mL of un-inoculated sterile saline or media to a clean cuvette and blank the machine. Transfer the same volume of bacterial suspension into another cuvette and read the optical density. Adjust the cell density of the suspension measured to match that of the standard (0.08–0.13). However, this is subjective as different bacteria give a 10^8 CFU/mL at different absorbance value (Schwalbe et al. 2007). If the turbidity of the suspension is higher than the standard, add more diluent, or if lower than the standard, add more bacteria. Proper adjustment of the inoculum cell density is important to ensure that the resulting growth is confluent or almost confluent (Rao 2011).

16.6.2.3 Principle of Spectrophotometric Method

A spectrophotometer is used to measure the turbidity of bacterial suspension as an indirect measure of cell density (Lumen Learning 2018). This instrument functions by transmitting a light beam through a suspension and measuring the amount of light passing through it. The light intensity is detected and converted to a logarithmic value called absorbance (optical density) (Lumen Learning 2018). The turbidity (optical density) of a sample highly depends on the choice of light wavelength used for measurement. OD600, which refers to “optical density of sample at 600 nm”, is often used to estimate the growth phase of bacteria or other cells in suspension (London BioHackspace 2018). As the number of bacteria in a suspension increases, the turbidity increases, causing less light to reach the detector. The decrease in light intensity is associated with the increase in absorbance measured by the spectrophotometer. The basic idea is to compare a sample of plain media and a sample of media inoculated with bacteria (London BioHackspace 2018).

16.6.2.4 Bacterial Inoculum Preparation

The bacterial inoculum can be standardized according to the CLSI procedures for aerobic bacteria (CLSI 2012). The bacteria is cultured in Mueller Hinton Broth (MHB) for 18–24 h, followed by comparison of the bacterial suspension in the MHB to the turbidity equal to 0.5 McFarland standard solution ($1-2 \times 10^8$ CFU/mL) with the addition of germ-free saline water.

16.6.2.5 Fungal Inoculum Preparation

Sabouraud Dextrose Agar (SDA) or Potato Dextrose Agar (PDA) slants are prepared in order to cultivate the selected fungal strain (Maghsoodi and Yaghmaei 2010.) The fungal species can be cultured in SDA or PDA at 28 °C until the maximum amount of conidia form (3- to 5-day-old). The conidia are recovered with 5 ml of distilled sterile water. Subsequently, the isolates are added to a Vortex mixer for 15 s and are then moved to a sterilized tube. Afterwards, the inoculum is moved to a sterilized syringe fixed to a sterilized filter with a micro-holes diameter of 11 µm. The suspension is filtered and collected in a germ-free tube. This process filters most of the hyphae, generating an inoculum predominantly composed of spores. Finally, the inoculum size is adjusted to 2.0×10^5 spores/ml by microscopic calculations through a cell-counting hemacytometer slide (Neubauer chamber).

16.6.3 Antimicrobial Disk Diffusion Assay

Antimicrobial activities of the medicinal plant extracts can be determined by the disk diffusion technique (Bauer et al. 1966; Alzoreky and Nakahara 2003). The SDA and PDA plates are seeded with an inoculum size of 10^6 colony-forming units CFU/mL of bacteria or 2×10^5 CFU/mL of yeast cells or fungal spores, respectively, by spreading the inoculum using an L-shaped glass rod. Subsequently, the disks (6.0-mm diameter) are saturated with 25 µL of crude extract at a concentration of 10.0 mg/mL before being placed on the microbe seeded plates. Likewise, each plate also has a blank disk impregnated with solvent alone as a vehicle control at the center of the plate and standard antibiotic disks (6.0-mm diameter) impregnated with 50 mg/mL ampicillin, streptomycin, kanamycin sulfate (for bacteria) and 100 mg/mL nystatin (for fungi) as positive controls. Subsequently, the plates are incubated at 37 °C for 18 h for bacteria and at 28 °C for 48 h for fungi. The inhibition zone around the disk is measured after 18 h of incubation at 37 °C for bacteria and 48 h for fungi at 28 °C, respectively. The antimicrobial activity of the plant crude extracts is measured by determining the diameter of the inhibitory zones (including the diameter of disk) on the agar surface, and values <8 mm are taken as no antimicrobial activity against the tested microbes (Zhu et al. 2005). The antimicrobial activity evaluation should be conducted in triplicate. The qualitative results are reported as the average of three experiments.

16.6.4 Minimum Inhibitory Concentration (MIC) Determination

Normally, the minimum inhibitory concentration (MIC) determinations are employed on plant extracts that show positive results against tested microorganisms by the disk diffusion assay. The maximum dilution of a plant extract that still inhibits the growth of a microorganism is known as the MIC (Misra and Dixit 1978). The comprehensive procedure of the MIC determination is found in the M7-T2 publication of the National Committee for Clinical Laboratory Standards (NCCLS 2002). In short,

plant crude extract preparations are serially diluted by using sterile nutrient broth or Sabouraud dextrose broth medium as diluents to produce final plant crude extract concentrations between 1.275 and 200.000 mg/mL. Subsequently, the tubes with various concentrations of plant extract are inoculated with 20 μ L/mL of bacterial or yeast suspension, homogenized and incubated at 37 °C for 24 h. The tube with the lowest dilution of the plant extract that showed an inhibitory effect against the tested microbe, resulting in no visible growth of microorganism or absence of turbidity, is verified as the MIC value for the plant extract. Usually, the microorganism growth is shown by the turbidity exhibited in the test tube. The experiment can be performed in triplicate and repeated twice to verify the result. A control experiment can be conducted in parallel to evaluate the influence of the solvent alone (without the crude plant extract) on growth of the microorganisms. The solvent (5% DMSO) is diluted by the same approach with sterile nutrient or Sabouraud dextrose broth, as mentioned above, and inoculation by microbial suspensions accompanied by incubation is done similarly to evaluate the influence of the solvent.

16.6.5 In Vivo Antimicrobial Activity in Animal Model

16.6.5.1 Animals Used in the Experiment

The animal experiments should be conducted following the guidelines of the Animal Care Council of the particular country. Protocol of animal experiments should also be specifically approved by the institutional ethics committee on animal experimentation to perform the animal studies. Pathogen-free adult female laboratory-mice *Mus musculus* (White albino) weighing 30–35 g, 10–12 weeks in age can be used for this study. The mice are familiarized with the room temperature (23 ± 2 °C) and a standard 12 h light/dark cycles in cages (1 mice/cage) for 7 days before the beginning of the research. Briefly, mice are immunosuppressed via intraperitoneal injection with cyclophosphamid as demonstrated by Shah et al. (2008) to facilitate the infection. At the third day of immunosuppression, the mice are fasted overnight.

16.6.5.2 In vivo Assay Using Mice

An example of using an in vivo antimicrobial assay to validate the in vivo antimicrobial activity of medicinal plants on the example of candidiasis is instigated by *Candida albicans* infection, which is an induced yeast infection model discussed in the following sections. Candidiasis infection is triggered by intravenous (i.v.) inoculation of 0.1 mL of a 10^6 UFC/mL inoculum in germ-free saline water from a fresh 48 h *Candida albicans* culture to mice. Twenty-four hours after infection, 3 mice are euthanized to verify the success of the infection by evaluating the fungal burden in the blood and kidneys samples. Infected mice are divided into 3 groups of 10 animals each, are housed in cages and have access to food and water ad libitum. Extract at the dosage determined from the toxicity study can be administered to treat the infected animals. As for treatment, the intraperitoneal injection (i.p.) technique

is used over 3 consecutive days, starting 24 h after infection. Two experimental control groups can be designed; an untreated negative control group is administered with distilled water alone and a positive control group is administered with standard antifungal drug nystatin at 10 mg/kg of bw. On day four, the mice are killed by cervical dislocation; blood and kidney are collected from each mouse. Kidney tissues are homogenized in 5 mL of germ-free saline water and then serially diluted. Blood samples are also serially diluted; 0.1 mL of each dilution is plated onto SDA and incubated for 24 h at 37 °C. The colonies are calculated and the colony forming units (CFU) are enumerated per gram of organs (CFU/g) and per milliliter of the blood sample (CFU/ml) (Sasidharan et al. 2008). The kidney samples for histopathology evaluation are stained using the Periodic Acid Schiff (PAS) reagent and also haematoxylin to identify the *C. albicans* infection in kidney cells. Differences in mean CFU in kidneys and blood samples compared to the negative and positive control can be analysed by using a one-way analysis of variance (ANOVA) with a post hoc Tukey test. A P value of <0.05 is considered statistically significant for all comparisons.

16.7 Clinical Trials

Medicinal plants have become an essential and crucial part of public healthcare around the globe. Numerous reports have emphasized the usage of therapeutic plants in traditional and alternative remedies (Eisenberg et al. 1993). Many laboratory level scientific research studies have also proven the efficacy of the medicinal plant to treat various diseases, including issues related to pathogenic microbial infection. Nevertheless, in order to further extend their acceptance among the patient, clinical trials of these medicinal plant products should be fortified. Even though traditional medicine practitioners do not necessitate clinical trials, for its authorization and existence at the global pharmaceutical industry together with modern drugs, it has become a crucial need of the time (Mills 2003). Therefore, to prove the effectiveness of medicinal plant-based products in clinical trials, it is advised to use pharmacological formulations of these products (US Food and Drug Administration 2004). A clinical trial of medicinal plant-based products poses several challenges that need to be addressed, including issues such as those connected to the monetary, moral, product standardization through quality control, the strategy of the study and the law requirements before filing an investigational novel medicine for assessing large phase III trials (Parveen et al. 2015). However, in 2005, the World Health Organization (WHO) distributed working guidelines concerning relevant law requirements needed to support clinical trials of herbal remedies (WHO 2005); hence, clinical trials on medicinal plant-based products based on these WHO working procedures should be encouraged.

16.8 Conclusions and Future Prospects

The potential use of brand new plant-based bio-active products, such as crude extract, active fraction and isolated compound(s), from higher plant origins, is still a very fruitful activity for the production of novel therapeutic agents to advance healthcare, especially against pathogenic microbial infections. It is important to highlight that widespread *in vitro*, *in vivo* and clinical trials need to be carried out frequently to identify the active and nontoxic antimicrobial phytochemicals such as polyphenol from higher plants as plant-derived antimicrobial agents. It is beneficial to standardize the procedures of extraction, *in vitro* and *in vivo* testing so that the exploration to developed antimicrobial agents from higher plants is more orderly and explanation of results would be eased. This chapter also sheds light on the future prospects of combination therapy comprising plant-derived agents, which is certainly very promising. Future research might offer other new or innovative ways of attaining combinational therapy between plant-derived agents with known antibiotics. Therefore, there is great necessity to continue the exploration for antimicrobial agents from higher plants to combat the antibiotic resistance crisis.

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