Austin Journal of Microbiology

Open Access

Austin Publishing Group

Review Article

Recent Trends and Methods in Antimicrobial Drug Discovery from Plant Sources

Bakht Nasir, Humaira Fatima, Madiha Ahmad and Ihsan-ul-Haq*

Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan

***Corresponding author:** Ihsan-ul-Haq, Department of Pharmacy, Quaid-i-Azam University, Islamabad 45320, Pakistan

Received: November 25, 2014; **Accepted:** March 19, 2015; **Published:** March 20, 2015

Abstract

Natural products especially plants represent an inexhaustible reservoir of novel molecules for new drug discovery. Plant derived crude extracts and pure compounds have a long history of management and cure of various ailments. The resurgence of resistant microbial strains and emergence of newer and more lethal infections has called for the need to acquire unique antimicrobials. Standardized tools and techniques are therefore inevitable for identification, isolation, evaluation and authentication of true antimicrobial capabilities of various phytochemicals. This review focuses on classical and advanced extract preparation techniques. The different methods of assessing antimicrobial capabilities of biological extracts along with their advantages and limitations have also been discussed. The variations and modifications reported by different researchers in the existing protocols along with their contributions in bioprospecting of pharmacologically active moieties have also been documented. *In vitro* antimicrobial assessment methodologies are well-established and have quite extensively served the purpose but in order to exploit the true potential of plants as a source of antimicrobial compounds, the need of establishing standardized *in vivo* testing protocols and determining valid correlation with *in vitro* efficacy result is mandatory.

Keywords: Antimicrobial susceptibility testing; Antimicrobials; Natural products; Medicinal plants; Phytochemicals

Abbreviations

AST: Antimicrobial Susceptibility Testing; NAPRALERT: Natural Products Alert; PSM: Plants Secondary Metabolites; SFE: Supercritical Fluid Extraction; MIC: Minimum Inhibitory Concentration; EUCAST: European Committee on Antimicrobial Susceptibility Testing; NCCLS: National Committee for Clinical Laboratory Standards; CLSI: Clinical and Laboratory Standards Institute; BSAC: British Society for Antimicrobial Chemotherapy; PDM: Paper Disc Method; CFU: Colony Forming Unit; TLC: Thin Layer Chromatography; FDA: Food and Drug Administration; ICAAC: Inter Science Conference on Antimicrobial Agents and Chemotherapy

Introduction

The interest in natural product research has revived in recent years due to the failure of alternative drug discovery methods to deliver many lead compounds in key therapeutic areas such as anti-infective, immunosuppression, and metabolic diseases. Natural product research continues to explore a range of lead structures, which may be used as templates for the development of new drugs by the pharmaceutical industry. The importance of natural products in providing a source of new pharmaceutical compounds cannot be denied [1].

Historically, plants have provided a source of inspiration for novel drug compounds, as the contributions to human health and well being made by plant-derived medicine have been outstanding. They may either become the base for the development of a natural blueprint for new drug discovery or a phytomedicine to be used for the treatment of diseases [2]. The World Health Organization reported that 80% of world's population rely chiefly on traditional medicine, and a major part of the traditional therapies involve the use of plant extracts or their active constituents (WHO, 1993). The extensive use of antimicrobial drugs has resulted in the development of resistance by microbes against many antibiotics. So, this has created a serious clinical problem in the treatment of infectious diseases [3]. Apart from this, there are other concerns like serious adverse effects and safety problems associated with antibiotics. Therefore there is a definite need to develop alternative antimicrobial drugs for the treatment of prevailing infectious diseases.

Medicinal plants have been used for centuries as remedy for human ailments and they provide a cheaper and safer source of biologically active chemical compounds as antimicrobial agents. The antimicrobial properties of many plants have been investigated and they are also used extensively in daily clinical practice. Ethnopharmacologists, botanists, microbiologists, and natural-product chemists are searching the world for phytochemicals which could be developed for the treatment of infectious diseases [4]. Only a small number, out of several hundred thousand species of medicinal plants around the globe, have been investigated both phytochemically and pharmacologically [5]. Therefore, the methods required to evaluate the efficacy and potency of such a large number of potentially important medicinal plants and to prove their antimicrobial worth need to be efficient and well validated. The first step towards new anti-infective drug development is the Microbial Susceptibility Test (MST). It is an essential technique used in pathology to determine resistance

Citation: Nasir B, Fatima H, Ahmed M and Haq IU. Recent Trends and Methods in Antimicrobial Drug Discovery from Plant Sources. Austin J Microbiol. 2015;1(1): 1002.

Table 1: Antimicrobial bioactive compounds obtained from medicinal plants.

Compound	Species	Antimicrobial activity	Reference
Aloe emodin Chrysophanol Aloin	*Aloe ferox*	Antibacterial	[13]
Anolignan B	*Terminalia sericea*	Antibacterial	[14]
3,5-bis-O-caffeoyl quinic acid 4,5-bis-O-caffeoylquinic acid	*Lonicera japonica Thunb*	Antibacterial	[15]
Catechol 2-hydroxybenzyl alcohol	*Salix capensis*	Antibacterial	[16]
Carnosol	*Salvia chamelaeagnea*	antibacterial	[17]
Ceanothic acid Ceanothetric acid	*Caenothus americanus*	Antibacterial	[18]
Diospyrin Isodiospyrin Mamegakinone 7-Methyljuglone Neodiospyrin Shinanolene	*Euclea natalensis*	Antimycobacterial	[19]
Ent-beyer-15-en-19-ol 4-Epimer *ent* beyer-15-en-18-ol 15b,16b-Epoxide-*ent*-beyeran-19-ol Nidoanomalin	*Helichrysum tenax* var *tenax*	Antibacterial and antifungal (yeasts)	[20]
Epicatechin Catechin	*Schotia latifolia*	Antibacterial	[21]
d-Friedoolean-14-en-oic acid (3-acetyl aleuritolic acid)	*Spirostachys africana*	Diarrhoeal pathogens	[22]
Genkwanin 5-Hydroxy-7,4-dimethoxyflavone Rhamnocitrin Rhamnocitrin	*Combretum erythrophyllum*	Antibacterial and antifungal (moulds)	[23]
Helihumulone	*Helichrysum cymosum*	Antibacterial and antifungal (yeasts)	[24]
Lanosol ethyl ether	*Osmundaria serrata*	Antibacterial and antifungal	[25]
Linoleic acid	*Helichrysum pedunculatum*	Antibacterial	[26]
2-Methyl-4-[2,4,6-trihydroxy-3-(2-methylpropanoyl) phenyl]but-2-enyl acetate	*Helichrysum caespititium*	Antibacterial and antifungal (moulds)	[27]
2-Methyl-6-(-3-methyl-2-butenyl)benzo-1,4-quinone Benzopyran,6-hydroxy-8-methyl-2,2-dimethyl-2Hbenzopyran	*Gunnera perpensa*	Antibacterial and antifungal (yeasts)	[28]
Muzigadial	*Warburgia salutaris*	Antibacterial	[29]
Prenyl-butryl phloroglucinol Kaurenoic acid	*Helichrysum kraussii*	Antibacterial	[30]
2,4,6-Trihydroxychalcone (pinocembrin chalcone) 5,7-Dihydroxyflavanone (pinocembrin)	*Helichrysum trilineatum*	Antibacterial	[31]
3,5,7-Trihydroxyflavone (galangin)	*Helichrysum aureonitens*	Antibacterial and antifungal (moulds)	[32]
Vernolide Vernodalol	*Veronia amygdalina*	Antibacterial and antifungal (moulds)	[33]

of microbial strains to antimicrobials, and in ethnopharmacology research it is used to determine the efficacy of novel and medically important antimicrobials. There are various MST methods that are employed by researchers and these could lead to variations in obtained results [6].

Significance of Medicinal Plants as Source of New Drugs/ Antimicrobial Agents

Chemical substances derived from medicinal plants and other natural sources have been used to treat human diseases since the dawn of medicine. Clinical, pharmacological and chemical studies of these chemical substances, which were primarily derived from plants, were the basis of most early medicines such as aspirin, digitoxin, morphine, quinine, and pilocarpine [7]. Biological activity guided isolation has been the most reliable method for the discovery of important novel drugs from medicinal plants. A fair share of new potential drugs especially in areas of anticancer, antihypertensive, anti-infective, immune-suppression and neurological disease management is still provided by natural products despite competition with other methods of drug discovery [8]. Biologically active compounds isolated from plant species used in herbal medicines have demanded great attention in recent times due to the side effects and the serious issues of resistance that pathogenic microorganisms develop against conventional antibiotics [8]. Antimicrobials of plant origin are quite effective in the treatment of infectious diseases while simultaneously alleviating the numerous side effects often associated with synthetic antimicrobials [2].

Terrestrial plants offer a unique and renewable resource for the discovery of new drugs and biological entities due to the biological and structural diversity or heterogeneity of their constituents [9]. They have played an imperative role in the discovery of new chemical entities for drug discovery and development. Plant-derived drugs also serve as lead compounds which can further be optimized by synthetic means [10]. Medicinal plants are undoubtedly among the most perfect "natural laboratories" for the synthesis of various molecules ranging from simple to highly complex chemical structures. Only 5-10 percent

of around 250,000 higher terrestrial plants in existence have been chemically and pharmacologically investigated in systematic fashion. There is an obvious need for improvement on part of the assessment tools and techniques used to explore this natural treasure [11,12]. A summary of few antimicrobial bioactive compounds obtained from medicinal plants is presented in Table 1.

Secondary Metabolites

Role of secondary metabolites as active biological principles

The availability of large number of compounds is an infinite requirement of drug discovery process.

Nature has developed an enormous diversity during several billion years of evolution. It is currently estimated that there are at least 250,000 different plant species, up to 30 million species of insects, 1.5 million species of fungi and similar numbers of algae and prokaryotes in existence. These organisms share an identical biochemistry necessary for a living cell, but in addition, they produce a wide variety of 'secondary metabolites' that are involved in the interactions between organisms. An enormously wide variety of secondary metabolites has evolved which offers an extremely useful source; plant secondary metabolites for example, regarded previously as waste products, are now recognized for their resistant activity against pests and diseases. The exploitation of this resource is now considered vital in the optimization of the lead discovery process [34]. In 1988 the database NAPRALERT already contained greater than 88,000 secondary metabolites, and every year some 4,000 new ones are reported [35]. Thus, the count of secondary metabolites should now be more than 190,000. Extrapolation of the data suggests that from all plant species, at least a million different compounds could be isolated. It is clear that nature provides an enormous potential for the discovery of new bioactive compounds [34].

Cancer, being one of the most deadly diseases requires profound attention and keen interest for its treatment. A number of Plant Secondary Metabolites (PSM) has been used as potent anticancer agents. Extracts rich in flavonoids, from the roots of *Scutellaria baicalensis* have been shown to exhibit anti-proliferative effects on various cancer lines [36]. Taxol, a diterpene from the Pacific yew has been widely used as a drug for the treatment of ovarian and breast cancer [2]. A group of triterpenes, Limonoids have been shown to be successful in inhibition of human tumor cell lines in various *in-vitro* bioassays, the most active compounds being limonin and isofraxinellone [37].

Antimicrobial secondary metabolites

Plants produce a huge variety of secondary compounds as natural protection against microbial and insect attack. Some of these compounds are also toxic to animals but others may not be toxic. Indeed, many of these compounds have been used in the form of whole plants or plant extracts for food or medical applications for mankind [38]. Naturally occurring alkaloids are heterocyclic nitrogenous compounds and are commonly found to have antimicrobial properties [39]. Many of the pure compounds isolated earlier with biological activities were alkaloids. Depending on the precursors and the final structure, alkaloids have been divided into different classes including indoles, isoquinolines, piperidines, pyridines, pyrroles and pyrrolidines [40]. True alkaloids, derived from amino acids are basic in nature for example, nicotine. Papaverine, a benzylisoquinoline alkaloid, was shown to have inhibitory effect on several viruses. Activity against gram negative bacteria and yeast was displayed by indoquinoline alkaloids from *Cryptolepsis sanguinolenta* [41]. The alkaloid quinine is popular for its antiprotozoal activity against the malarial parasite [2].

Essential oil fractions carry the peculiar fragrance of plants. These are secondary metabolites highly enriched in isoprene structure based compounds known as terpenes, but when the compound contains an additional element as oxygen, they are termed as terpenoids. Useful effects of essentials oils have been demonstrated against pathogenic microorganisms. Of all the essential oil derivatives studied till 1977, 60% were reported to have inhibitory properties against fungi while 30% inhibited bacteria [42]. Oils from *Cinnamomum osmophloeum* have been shown to possess antibacterial activity against *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* (including methicillin resistant *S. aureus*) *Salmonella* sp. and *Vibrio parahaemolyticus,* with cinnamaldehyde being the main antibacterial component of the particular volatile oil [43]. *Helicobacter pylori* are highly sensitive to spearmint oil [44]. Essential oils are effective against a wide range of oral bacterial population [45], and they are thus used commonly in antiseptic mouthwashes.

Plants have unlimited ability to synthesize aromatic secondary metabolites, most of which are phenols or their oxygen substituted derivatives [46]. Important subclasses in this group of compounds whose antimicrobial potential have been studied include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. These groups of compounds serve as plant defense agents against pathogenic microorganisms. The site and number of hydroxyl groups present in phenolic compounds are responsible for phenolic toxicity to microorganisms [47,48]. Flavones, flavonoids and flavonols synthesized by plants in response to microbial infections [49] are known to be effective *in vitro* as antimicrobial substances against a wide range of microorganisms [40]. Tannins are water soluble polyphenols that are commonly found in higher herbaceous and woody plants [47]. Tannins have been reported to be bacteriostatic or bactericidal against *Staphylococcus aureus* [50]. They also possess astringent properties. Coumarins are phenolic substances made of fused benzene and α-pyrone rings [51]. As a group, coumarins have been found to stimulate macrophages, which could have an indirect negative effect on infections [52].

Biopolymers for example, waxes, fatty esters such as cutin and suberin play a role in protection of external plant surfaces. Cell walls of at least some monocotyledons contain proteins referred to as thionins. These are also known to have antimicrobial properties [53]. In addition to these major phytochemical groups, it should be noted that researchers have also reported antimicrobial properties of polyamines [54], thiosulfinates [55] and glycosides [56,57].

Plant Extract Preparation; Principle/ Methods and Key Factors

Extraction involves the separation of components of plant tissues which are medicinally active from those which are inert by using suitable solvents and appropriate extraction technology. It is

important to extract the desired active chemical components from the plant material for further isolation and characterization; it therefore makes sample preparation a crucial first step in the analysis of plants. The development of efficient sample preparation techniques offering evident advantages over conventional methods for the extraction, isolation and analysis of medicinally important plants is most likely to play a significant role in improving the quality of antimicrobial plant products available to consumers worldwide [58].

The purposes of standardized extraction procedures are to obtain the therapeutically desired portion from crude drugs and to eliminate the inert material by using a solvent system known as menstrum which has the ability to diffuse into the plant tissues and solubilize compounds of similar polarity. The extract obtained may be ready for use in certain ailments or it may be subjected to fractionation to isolate different chemical constituents. Thus the final quality of herbal drugs depends upon standardized extraction procedures to a great extent [59]. The quality of an extract depends upon certain parameters like plant material, choice of solvent and extraction methods employed.

Plant material

The potential active constituents should not be lost, destroyed or distorted during the extraction process from the plant material. If plant selection was done on the basis of traditional uses [12] then the advice of traditional healer should be acted upon in order to exploit the traditional herbal drug as closely as possible. Thus, the traditional use of the plant and the ease of handling of different plant parts are helpful in selecting plant material used in the extraction process.

Plant material can be used both in fresh and dry form. However most scientists working on the chemistry of secondary metabolite components have tended to use dried material for the following reasons; (a) fewer problems linked with the large scale dried plant material extraction as compared to fresh material; (b) isolation of relatively stable secondary metabolite components, especially if these are to be used as antimicrobial agents; (c) difference in water content, caused by time lapse between collection and extraction may adversely affect solubility or subsequent separation by liquid-liquid extraction, thus making it difficult to work with fresh material; (d) use of medicinal plants in the dried form [or as aqueous extract] by traditional healers [60]. Plants are usually air dried [61,62] to a constant weight, while some researchers use oven to dry the plant at about 40°C for 72 hours [63]. In addition to all that is mentioned, climatic condition in which a plant grows affects its constituents. Underground parts (roots, rhizomes, tuber, bulb etc.) of a plant are used mostly compared with the on-surface parts, in search for bioactive compounds possessing antimicrobial properties.

Choice of solvent

The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted, likewise successful identification and determination of bioactive compounds from plant material predominantly depends on the type of solvent used in the extraction procedure. Therefore the choice of solvent is critical as far as the success of extraction process is concerned. Properties of a good solvent used in plant extraction include low toxicity, relatively low boiling point so as to be easily removed from the compound after extraction, promotion of the rapid physiological absorption of the desired compound in the extract in specific body compartments, preservative action and inability to cause the quenching or dissociation of active principles [64]. The solvent should not interfere with the bioassay as the end product in extraction will contain traces of residual solvent [65].

The biggest problem in drug development from plants is answering a very simple question: what kind of extract should we test? A variety of different solvents have been used to extract plant metabolites. The choice of solvent depends also on the intended use of the extract. If the aim is antimicrobial components screening, then the effect of solvent on subsequent separation procedures is not important, but it should not inhibit the bioassay procedure. If extraction is done for the isolation of chemical compounds without using bioassay, then in this instance, toxicity of the solvent is not important as it can be removed before subsequent isolation procedures [60].

Studies have shown that the extraction yield is dependent on the solvent, time and temperature of extraction as well as the chemical nature of the sample. Under identical conditions of time and temperature, the two most important factors are the solvent used and the chemical property of the sample [66].

In studies where the aim is initial screening of plants for potential antimicrobial activities, the process may begin by using the crude extracts prepared from different organic and aqueous solvents and can be followed by the utilization of various organic solvents for fractionation. A wide range of solvents as well as their combinations including ethanol and ethyl acetate [67], acetone and methanol [68], hexane, ethyl acetate, ethanol, and water [69], petroleum ether, chloroform, ethanol, methanol and water [63] had been used for the extraction of biologically active compounds from plant materials.

Review of different studies show that researchers have used different solvents based on their requirements to extract specific bioactive compounds. Polyphenolic compounds such as flavonols and many other reported biologically active compounds are generally soluble in polar solvents such as methanol [70]. In a study where three kinds of solvents were used to extract phenolic compounds from papaya fruit, 50% methanol and 50% acetone extracts showed the highest total phenolic contents, total flavonoid contents and antioxidant activity [71]. Other studies have also revealed the use of polar solvents. In the recovery of polyphenols from plant matrix, polar solvents like (hot or cold) aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate are frequently employed [72]. If the aim of extraction is recovery of non-polar bioactive compounds then the choice of solvent is somewhat different. For instance, out of twenty different solvents evaluated in a study, chloroform was found to be the best for the extraction of non-polar bioactive compounds from the roots of *Angelica archangelica* [73].

Water is a widely used solvent for extraction of plant material throughout the globe. It is also primarily used by traditional healers but plant extracts from organic solvents have been reported to give more consistent antimicrobial activity compared to water extract [70]. It is also found that water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance [74,75]. Most antimicrobial components identified so far are not water soluble and thus extracts obtained with organic solvents have been found

Table 2: Experimental conditions for various methods of extraction for plant material.

Extraction method	Commonly used solvents	Solvent volume (mL)	Time period	Temperature	References
Soxhlet extraction	Methanol, ethanol or water and alcohol mixture	150-200	3-18 hrs	Depends upon the solvent used	[58,87,88]
Sonication	Methanol, ethanol or water and alcohol mixture	50-100	1 hr	Heat can be used	[58,87]
Maceration	Methanol, ethanol or water and alcohol mixture	Can be varied based on sample size	At least 3 days	Room temperature	[89-92]
Percolation	Methanol, ethanol or water and alcohol mixture	Can be varied based on sample size	Upto 24 hrs	Room temperature	[92]

more potent [76]. Nearly all identified antimicrobial compounds from plants are aromatic or saturated organic compounds and are mostly obtained through initial ethanol or methanol extraction [77]. In a study conducted on *Combretum* species, antifungal activity was investigated and out of different solvents used, acetone and methanol were found to extract more chemical compounds including saponins from the leaves having antimicrobial activities [78]. Water soluble compounds, such as polysaccharides and polypeptides are commonly more effective pathogen absorption inhibitors on the surface of plants and have no significant impact as antimicrobial agents [52]. However, it is still safe to say that the most commonly used solvents for the preliminary investigations of antimicrobial activity in plants are methanol, ethanol and water [63,76,79-81]. Moreover, study of literature reveals that a variety of other solvents are also used by different researchers. Few common examples are dichloromethane [61,82], acetone, although not frequently used [27,61,80,83], as well as hexane [78]. A combination of these is also used by some researchers to get the best extraction results [84].

Previously in a study a variety of solvents were examined for the extraction procedure from *Anthocleista grandiflora* and *Combretum erythrophyllum* , and different parameters were tested like the ability of the solvents to solubilize antimicrobials from plants, ease of handling and removal, rate of extraction and toxicity to the test organisms. Acetone gave better results as compared to the other solvents used (ethanol, methanol, methylenedichoride, methanol/chloroform/water and water) in the study. However it was noted that different results might be obtained with other plant species and acetone cannot be generally preferred as solvent of choice in extraction procedures [60]. A wide diversification in the use of solvents is usually seen and researchers working in different settings need to focus on standardized solvent systems and extraction methods in order to minimize the variations in antimicrobial susceptibility test results.

Extraction methods

Various kinds of extraction schemes are described in the literature: some highly selective for a certain group of compounds, others very general, using an array of consecutive steps involving non-polar (e.g. petroleum ether) to highly polar (e.g. methanol, water) solvents. The steps included in the basic operation of extraction include; prewashing, drying of plant material and grinding before utilization of solvents. Grinding process helps in getting a homogenous sample and increasing the surface area and contact of the sample surface with the solvent system. Decreasing the size of sample material (dry or wet) also improves the kinetics of analytical extraction and it can shorten the extraction period and increase extraction efficiency. Researchers exploring the antimicrobial activity have widely performed homogenization of plant tissue in a particular solvent [61,70,83]. Plant parts which might either be dried or wet are grinded to reduce the particle size, soaked in certain quantity of solvent and vigorously shaken for 5-10 min or left undisturbed for 24 hrs followed by filtration of the extract. The filtrate may then be dried under reduced pressure. It is also reported by some authors that shaking of unhomogenized dried leaves in solvents for about 5 minutes, followed by filtration and concentration under reduced pressure also resulted in better yield of desired principles [85].

The suitability of the extraction methods must be considered and well examined, as the characteristics of target compounds may vary, they may be polar to non-polar and thermally labile. Similarly, proper precautionary measures must be taken to assure that any of bioactive constituents are not lost, distorted or destroyed during the whole extraction process [86]. Different methods are used by researchers throughout the world. Commonly used methods for the extraction of plant samples are sonication, soxhlet extraction, heating under reflux and a few others. Maceration and percolation are also used since ages to prepare plant extracts using fresh green or dried powdered plant material [86]. Various parameters of these methods are shown in Table 2.

Some modern extraction techniques have evolved recently which present certain advantages like comparatively reduced organic sample consumption and sample degradation, fewer steps, improved extraction efficiency, extraction kinetics and ease of automation. It makes these modern techniques much favorable for the extraction of plant materials. Few examples of these modern extraction techniques are; solid-phase micro-extraction, pressurized-liquid extraction, supercritical-fluid extraction, solid-phase extraction, microwave-assisted extraction and surfactant-mediated extraction [58]. These modern techniques are proving to be more efficient than the conventional methods [93].

Soxhlet extraction is also a widely used technique using organic solvents for the extraction purpose [94]. In this case, fresh solvent is continuously introduced to the dried plant material, which improves the overall extraction efficiency. However, this method cannot be used for thermolabile compounds as prolong heating and exposure of plant material to the boiling solvent may lead to degradation of compounds [95].

Serial exhaustive extraction is another method commonly used. It involves serial extraction with an array of solvents of increasing polarity. A range of solvents from non-polar (hexane) to a more polar (methanol) can be used to ensure that compounds with different polarity range could be extracted successfully [96]. This is the ideal method when the aim is to screen the plant for a variety of compounds. There are some methods which are suitable for the extraction of a particular group of compounds. For instance, steam distillation, volatile solvent extraction or Supercritical Fluid Extraction (SFE) are

the methods of choice when it comes to the extraction of essential oils [97].

Variations exist in extraction methods based on length of the extraction period, solvent used, and particle size, solvent to sample ratio, temperature and pH. In a study, extractions performed using fine particles of diameter 10 μm gave higher extraction efficiency in 5 min as compared to 24 hrs extraction performed in shaking machine using less finely ground plant material [98]. In one experiment, sequential extraction performed at room temperature using various solvents was compared with extraction performed in a water bath with acidified distilled water (pH 2.0 with HCl), which was neutralized with NaOH before extraction with diethyl ether. It was found that the latter method was more efficient due to the easy extraction promoted by the acidified aqueous environment [84]. Similarly, solvent to sample ratio also participate in affecting the quantity and quality of obtained constituents. Studies reported that solvent to sample ratios (v/w) of 10 mL: 1g has been used and found as ideal [96].

Antimicrobial Agents Screening Techniques

A large number of assessment techniques are currently in use for the investigation of plant products as a source of new antimicrobial compounds. Since the principle is not the same for all these methods, results obtained will also be influenced to a great deal by method selected, the test microorganisms and the degree of solubility of each test compound [99,100]. The test systems should ideally be simple, inexpensive, rapid, and reproducible and should maximize high sample throughput in order to deal with a large number of extracts and fractions. The complexity of bioassays must be defined according to the laboratory facilities and skill of available personnel [101,102]. The most common antimicrobial screening techniques employed presently include; bioautographic, diffusion and dilution methods. The first two are the qualitative techniques as these methods only perform a qualitative analysis of the samples and give an idea of the presence or absence of antimicrobial substances. Dilution methods, on the other hand are considered quantitative assays [103].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing is an important technique used in clinical microbiology laboratory. The performance of Antimicrobial Susceptibility Test (AST) is imperative to confirm susceptibility to chosen antimicrobial agents or to detect resistant strains [104]. In ethnopharmacological research, it is used to determine the efficacy of potential antimicrobials from biological extracts against various microorganisms. Plants with prospective antimicrobial compounds are screened using AST methods but their most common uses are; determining the efficacy of an antimicrobial in treating infections by finding its Minimum Inhibitory Concentration (MIC), epidemiological studies of susceptibility and in comparisons of new and existing antimicrobial agents [105].

Keeping in view the recent encroachments in antimicrobial drug discovery from plant sources, the development of more sensitive bioassay techniques has become inevitable. *In vitro* tests for screening of plant extracts should be standardized and in order to get comparable results to currently used antibiotics, further studies should be conducted for MIC determination of natural products [106]. The recent standard AST methods approved by various organizations like National Committee for Clinical Laboratory Science (NCCLS), now known as Clinical and Laboratory Standards Institute (CLSI), European Committee for Antimicrobial Susceptibility Testing (EUCAST) and British Society for Antimicrobial Chemotherapy (BSAC) are available and they serve as guidelines for antimicrobial susceptibility testing of a wide range of drugs. However, these might not be exactly applicable to plant extracts and certain modifications have to be made [107].

For convenience AST methods can be broadly classified into diffusion and dilution methods. Examples of the former include; agar disc diffusion, agar well diffusion and bioautography, while agar dilution, broth microdilution and broth macrodilution techniques are categorized under the dilution methods [108].

Diffusion methods

The earliest methods for detecting the antibacterial effects of extracts involved the use ditches cut into agar plates which were streaked with bacteria. Test solution was poured into the ditch and was allowed to diffuse out and inhibit bacterial growth. As an alternative source of diffusion, some researchers used paper strips impregnated with antibiotics [109]. The agar diffusion principles were further extended to develop the disc diffusion test [110]. Agar wells carrying the antibiotic solution were soon replaced by paper discs impregnated with antibiotics.

Due to its simplicity and ease of performance, diffusion tests were widely adopted by many investigators, but the lack of standardization resulted in unreliable and non-reproducible results. The first article describing a standardized technique called the 'Paper Disc Method' (PDM) for sensitivity testing of bacteria was published by Ericsson in 1954 [111]. Different types of diffusion tests are used nowadays and they are a vital part of current AST methodologies.

Agar disc diffusion assay: Disc diffusion refers to the diffusion of an antimicrobial agent of a specified concentration from discs, tablets or strips, into the solid culture medium that has been seeded with the selected inoculum isolated in a pure culture. The principle of disc diffusion testing was developed in 1940 and has been used in microbiology laboratories for over 70 years [112]. Bauer, Kirby, Sherris, and Turck diligently tested different variables used in the test, such as media, temperature and depth of agar. Their landmark paper was published in 1966, describing the test that is used today [113,114]. The agar disc diffusion susceptibility method (commonly known as Kirby-Bauer test) is simple, practical and has been well standardized [104].

AST of pure substances can be efficiently performed with agar disc diffusion technique but when used for mixtures containing constituents exhibiting different diffusion rates, it may give unreliable results [115]. The test is suitable for lead identification but is not considered much productive for the quantification of antimicrobial response. Furthermore, the bacteriostatic and bactericidal effects cannot be distinguished using this method [84,107,116].

The test is performed by applying a bacterial inoculum of approximately 1–2 $\times 10^8$ CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate [104].The medium of choice is generally Mueller-Hinton although Nutrient agar [117] and Tryptone Soya agar [80] have also been used by various researchers. Once the

agar plate is completely dry, the antibiotic discs are applied either manually or with a dispensing apparatus. In general, no more than 12 discs should be placed on a 150-mm agar plate or 5 discs on a 90-mm plate [118]. Different researchers have reported the drying time of impregnated paper disc and it varies from 2 hrs to overnight under a laminar flow cabinet [83]. The plates are incubated in an inverted position (agar side up) under conditions appropriate for the test microorganism [118]. The incubation time for bacterial strains is 24 h at 37°C and for fungi it is 48 hrs at 25°C. The zones of growth inhibition are then measured to the nearest millimeter around each of the antibiotic discs. The diameter of the zone is related to the susceptibility of the test microorganism and to the diffusion rate of the drug through the agar medium. The results are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI) [119]. Disc diffusion test provides us with "qualitative" results and MIC calculation is not possible. It gives results in the shape of categories of susceptibility and organisms are categorized as susceptible, intermediate or resistant to the tested antimicrobial compound. However, some zone reader systems available commercially claim to give an approximate MIC with certain organisms and antimicrobials by the comparison of zone diameters with the standard curves stored in an algorithm [120.121]. The design of the disc diffusion method for fungal strains is similar to that for bacteria using the same medium (Mueller–Hinton agar) supplemented with glucose and methylene blue dye both of which are easily available [118].

Agar well diffusion: It shares the same principle as the disc diffusion assay. A number of researchers have used this technique to test the antimicrobial action of different compounds extracted from plant sources. The plates inoculated with the test organism (approximately $1–2\times10^8$ CFU/mL) have 6-8 mm wells cut into the surface of the agar using a sterile cork borer. A distance of at least 30 mm is kept between adjacent wells and walls of the petri plate. The wells are filled with a predetermined quantity of antimicrobial samples, the plates are then incubated at 35-37°C for 24-48 hours depending upon the test organism. The diameters of any clear zones around sample containing wells are measured using calipers [122-126]. It has also proven to be an inexpensive, less complicated and reliable method of antifungal drug susceptibility testing for *Candida* sp. and it has given comparable results to the disc diffusion test [127].

Bioautography: Bioautography is a method of separation and detection of plant antimicrobials by performing a suitable chromatographic process followed by a biological detection system. Even though sophisticated online high performance liquid chromatography coupled bioassays are available, bioautography offers process simplicity and rapidity for the chemical and biological evaluation of plant extracts [128]. Bioautography is a very useful and inexpensive method of evaluating antimicrobial potential of plant extracts both in their crude form and as purified phytochemicals. It can also be used effectively in the target directed isolation of active plant secondary metabolites on chromatogram [100]. It has simplified the complex process of isolation of antimicrobial phytoconstituents from crude plant extracts. A very little amount of sample is required to determine the bioactivity moreover it also determines the polarities of active compounds [129]. The three different bioautographic methods are agar diffusion or contact bioautography, direct TLC bioautographic detection and the immersion or agar overlay bioautography [130].

Contact bioautography: In contact bioautography, a TLC or paper chromatogram is developed first from which the antimicrobials diffuse to an inoculated agar plate. The chromatogram is inverted onto the inoculated agar layer for a defined period to allow diffusion, after which the chromatogram is removed and the agar layer is incubated. The zones of inhibition on the agar surface, conforming to the spots in chromatographic plates are the indicators of antimicrobial compounds. Growth incubation time varies between 16–24 hrs [131].

Direct TLC bioautography: In direct TLC bioautography, the developed TLC plate is sprayed with or dipped into a suitable seeding density of fungal or bacterial inoculum. A suspension of test bacteria or fungi is used for spraying or dipping purpose. The bioautogram is then incubated for 48 hrs at 25°C under humid condition. For visualization of microbial growth, tetrazolium salts are used. These salts are converted by the dehydrogenases of living microorganisms to intensely colored formazen [132]. The bioautogram after been sprayed with the salts are re-incubated at 25°C for 24 hrs [133] or at 37°C for 3-4 hrs [61,129]. Antimicrobial activity is indicated by the appearance of clear white zones against a purple background on the TLC plate.

Immersion/agar overlay bioautography: In Immersion or agar overlay bioautography, the chromatogram is completely covered with an agar medium containing the test microorganism. After solidification, incubation and staining which is usually done with tetrazolium dye, the inhibition zones can be easily visualized to locate the antimicrobial compounds. Agar overlay is a fusion of contact and direct bioautography [115].

Dilution methods

Dilution methods are most commonly used to determine the MIC of antimicrobial agents, including antibiotics and other substances that either kill (bactericidal activity) or inhibit the growth (bacteriostatic activity) of microorganisms [134]. Different types of dilution tests are broth microdilution, broth macrodilution and agar dilution methods [108]. The final volume of the test defines whether the method is termed macrodilution i.e. when using a total volume of 2 mL , or microdilution, if performed in microtiter plates using ≤500 µL per well. In both the agar and broth dilution approaches, the lowest concentration of the antimicrobial agent that prevents visible growth of a microorganism under known conditions is termed as MIC [134]. Dilution methods offer certain advantages over diffusion techniques which include; enhanced sensitivity for smaller extract volumes, quantitative analysis and ability to differentiate bacteriostatic and bactericidal effects of test sample [116]. It is an economical test which can be used for a number of microorganisms and it gives fairly reproducible results. Dilution methods are considered as reference methods for *in vitro* susceptibility testing and are also used to evaluate the performance of other AST techniques.

Broth macrodilution: Broth macrodilution or tube dilution method was one of the earliest AST methods [135]. It was among the first to be developed and still serves as a reference method. The assay is generally performed in test tubes, two fold dilutions of test antibiotic is prepared in a liquid growth medium of volume greater

than 1mL [104]. Test tubes without antimicrobial test sample serving as controls are also included in the test. A standardized bacterial suspension of 1–5×10^5 CFU/mL is used to inoculate the test tube containing the antibiotic. Following overnight incubation, the tubes are examined for changes in turbidity as an indicator of growth. The lowest concentration of antibiotic that prevented growth represents the MIC. The major disadvantage of this method is extensive manual handling of antibiotic solutions, thus increasing the possibility of errors in solution preparation [118].

Broth microdilution: The broth microdilution method, also known as microtiter/microwell plate method is an adaptation of the broth dilution method in which the assays are performed using small volumes of test antimicrobial and allows a number of bacteria to be tested quite rapidly. The mechanization of test by use of microtiter plastic plates and reduction in required test volumes of sample and reagents has made this assay significantly useful and popular. Standard plates having 96 wells are used and it allows approximately 12 antibiotics to be tested in a range of 8 twofold dilutions in a single tray [118].

A stock solution of the extract is obtained in the solvent already been used for extraction [136]. Other investigators have confirmed the use of DMSO as well [62,63,84,103]. Before transferring the predetermined volume of stock solution to the plate, an equal volume of sterile Mueller Hinton broth or water is usually used as diluents in the wells of the microtiter plate [105]. For the susceptibility testing of rapidly growing aerobic or facultative organisms, Mueller-Hinton broth with a pH between 7.2 and 7.4 at room temperature is often recommended as the medium of choice. The media may be supplemented with 2–5% lysed horse blood in case of fastidious organisms [137]. Similarly, EUCAST (2003) recommends certain supplemented Mueller Hinton broth for non-fastidious microorganisms. It should be noted that this assay procedure results the final concentration of the antimicrobial agents in the assay wells being two fold lower than in the aliquots added to the wells [118]. For effective analysis of MIC, 5-8 concentrations representing achievable concentrations for the antimicrobial are usually tested [138].

Inoculation is done with the standard inoculum size of 5×10^5 CFU/mL [104], while some have reported an inoculum size of 1×10^6 CFU/mL [80,83]. It is accomplished by a micropipette or mechanized dispenser that transfers 0.01 to 0.05 mL of standardized bacterial suspension into each well of the microdilution plate [104]. Some investigators have used a microbial culture with an optical density of 0.4 at 620 nm or a 12 hrs broth culture adjusted to a 0.5 McFarland turbidity standard [62]. EUCAST recommended incubation temperature of 35–37°C in air for 16–20 hrs in case of non-fastidious organisms [105].

After incubation, MICs are calculated using either a manual or automated viewing device for the examination of each well for growth. Growth is indicated by change in turbidity and the concentration of first well that appears clear is taken to be the MIC of the extract. The presence of growth is also determined by use of indicators like tetrazolium salts or resazurin dye [139] and also by spectrophotometric analysis [106,140]. The absorbance is usually measured at 620 nm when using spectrophotometric method for growth determination. The lowest sample concentration giving a zero absorbance reading [63] or the concentration with a sharp dip in absorbance value [106] is the MIC of the test compound.

Agar dilution: Agar dilution method is a well-established method for investigating the antimicrobial potential of extracts and is commonly used as a reference method for evaluation of new antimicrobial agents and susceptibility test protocols. It offers various advantages like the simultaneous testing of a number of biological isolates, ability to observe heterogeneous populations or mixed cultures, and the versatility and flexibility in sample selection and concentration range to be tested [118].

In agar dilution method, the antimicrobial agent after been filter sterilized (0.22μm) is incorporated in the molten agar medium with each plate containing a different concentration of the agent. Muller-Hinton agar [105] is used most commonly although the media used for testing is determined on the basis of the organisms and in some cases the antibiotic to be tested [105]. Some researchers have used nutrient agar [136,141]. Supplemented Muller-Hinton agar is used for non-fastidious bacteria [105,118].The pH of the agar must be between 7.2 and 7.4 at room temperature. The inoculum can be applied rapidly and simultaneously to the agar surfaces, EUCAST (2003) recommends an inoculum density of about 10^7 CFU/mL and using micropipette or replicator pins to transfer about 1μL (10^4 CFU/mL) of the inoculum. Most available replicators transfer 32–36 inoculums to each plate [137]. Streaking is done in radial patterns and the plates are then incubated at 37°C for 24-48 hrs. The MIC is read as the lowest antibiotic concentration that inhibits the growth of the organism completely [84,107]. A faint haze caused by the inoculum or a single colony should not be read as growth. The disadvantages of this method include the labor required to prepare the agar dilution plates and their relatively short shelf life. In addition, the manual evaluation and interpretation of susceptibility results can be time consuming [118].

Etest

It is an innovative commercial antimicrobial susceptibility test developed in Sweden, a gradient technique that combined the principles of both disc diffusion and agar dilution methods. It was first presented at the Inter-science Conference on Antimicrobial Agents and Chemotherapy (ICAAC) meeting in 1988. Etest was approved by U.S. FDA in 1991.

Plastic strips are impregnated with a gradient of antimicrobial concentrations in dry form. The upper surface of the Etest strip has a scale; it is calibrated across a corresponding MIC range. When placed on the surface of the agar plate that has been inoculated with the test organism, the antimicrobial on the test strip is immediately transferred to the plate in a continuous gradient manner. After optimum incubation period, the antimicrobial activity is seen as an ellipse of inhibited growth around the strip. MIC value is obtained from the point where the ellipse intersects the scale (Figure 1).

The gradient remains stable for up to 18-20 hrs so the critical times of a wide range of pathogenic bacteria and fungi is covered. The stability of the gradient also provides inoculum tolerance and the MIC results are minimally effected by variation in CFU/mL so the variability experienced in routine susceptibility testing is significantly avoided. In addition, it also gives highly reproducible results in most

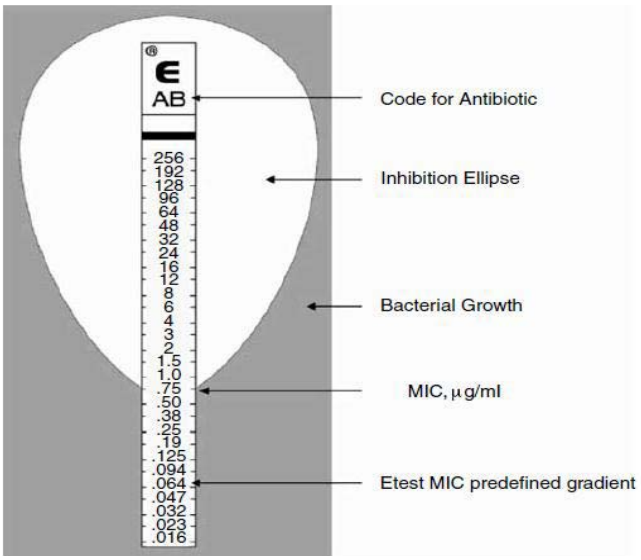

Figure 1: Etest inhibition ellipse showing the MIC at the intersection of the growth of the organism and the calibrated strip [118].

laboratory settings. Numerous researchers have validated the method and their investigations have documented Etest performance to be equivalent to standard MIC methods [118].

Future Prospects

Despite the success of antimicrobial drug discovery in past, infectious diseases still remain the second leading cause of death with approximately 17 million deaths globally, particularly in the children and elderly. The increase in the drug resistance to mainstream antimicrobials has been of particular concern. The pace of drug resistance is by far greater than the discovery of new antimicrobials and there is a definite need for new antimicrobials with novel mechanisms of action.

The current AST methods provide reliable results when the tests are performed according to the directions provided by CLSI. However, there is room for improvement in the area of accurate and rapid recognition of microbial resistance to antibiotics. The development of new automated instruments is highly necessary for the provision of faster and reliable results; it will also be economical by virtue of lower reagent costs and comparatively reduced labor requirements. The detection of microbial growth and the efficiency of susceptibility testing need further standardization which makes it quite obvious to explore new and advanced testing techniques. Already established *in vitro* testing techniques need further fortification by development of validated *in vivo* susceptibility testing techniques.

Conclusion

Despite the extensive development in synthetic medicine, extracts obtained from flora still hold an important stature. Even at present, the medicinal worth of several plant species remains unexplored. Another constraint that restricts the use of plants for screening of antimicrobial compounds is the limitations in the current screening techniques. Though *in vitro* AST methodologies have developed quite extensively and they have served the purpose, but in order to exploit the true potential of plants as a source of antimicrobic compounds, there is a dire need of establishing standardized *in vivo* testing protocols and determining valid correlation with *in vitro* efficacy results. The confluence of these assessment technologies with advancements in instrument automation will offer remarkable possibilities to exploit the chemical diversity of plant's bioactive molecules in the quest for new antimicrobic drugs.

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Austin J Microbiol - Volume 1 Issue 1 - 2015
ISSN: 2471-0296 | www.austinpublishinggroup.com

Citation: Nasir B, Fatima H, Ahmed M and Haq IU. Recent Trends and Methods in Antimicrobial Drug Discovery from Plant Sources. Austin J Microbiol. 2015;1(1): 1002.