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Revealing the Antimicrobial Potential of Plants

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ABSTRACT
Plants being capable of synthesizing a vast array of secondary metabolites with antimicrobial potential are a prospective source of new therapeutic agents. Methods for preparing crude plant extracts, and those for assessing their antimicrobial activity become important in this context, especially when the problem of drug resistance among pathogenic microbes has become prevalent. This review explores the different classes of secondary metabolites, various extraction procedures followed by different assays that can be used to determine their antimicrobial potential. The separation techniques suitable for isolation and identification of the active component(s) are also described.

Key words: Secondary metabolites, Antimicrobial, Extraction.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their uses in traditional medicine. Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines[1]. Medicinal plants have been an important part of the human history, culture and tradition. It is therefore likely that medicinal plants hold the key to new advances of great importance to human health. Plant is a biosynthetic laboratory, comprising a multitude of compounds like glycosides, alkaloids, terpenoids, etc., which are known to exert physiological and therapeutic effect. Out of the estimated 4,22,000 flowering plant species of the world, nearly 35,000 to 70,000 species are used for medicinal purposes all over the globe.

According to one study only 20% of plant flora has been studied and 60% of synthetic medicines owe their origin to plants [2]. Well known examples of plant derived medicines include quinine, morphine, codeine, colchicines, atropine, reserpine, and digoxin as mentioned in the South East Asian regional workshop (2006). Plant derived natural products such as flavonoids, terpenoids, and steroids have received considerable attention in recent years due to their diverse pharmacological properties[3]. The relatively low incidence of adverse reactions to plant preparations as compared to conventional pharmaceuticals, coupled with their reduced cost is encouraging to both, the consuming public and national health care institutions to consider plant medicines as alternative to synthetic drugs [1]. Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate infections.

Pathogen resistance to synthetic drugs and antibiotics already in use makes search for plants with antimicrobial activity more important, as they can substitute for synthetic antibiotics and drugs. Characteristics of plants that inhibit microorganisms have been investigated in laboratories since 1926 [4]. In modern times a large number of research groups are engaged in this area of biology [5-8]. A schematic representation of targeted screening of plant extracts for antimicrobial activity is presented in fig. 1 [9].

This review focuses on the antimicrobial properties of the plant (including but not restricted to medicinal plants) secondary metabolites. This is an attempt to give an introductory overview of the plant antimicrobials to the reader. In depth discussion of each of the points is outside the purview of this work. Those aspects which we have not discussed in detail here are nicely reviewed in references - [10-12].

SECONDARY METABOLITES: A TOOL FOR CLASSICAL DEFENCE

Secondary metabolites are a vast and diverse group of organic compounds which do not participate directly in growth and development but perform other functions. Secondary metabolites (also described as natural products) have often attracted interest because of their biological effect on organisms other than its producer [13]. It is likely that plants produce them *de novo* as part of their phytoalexic response on microbial invasion [10]. External plant tissues are known to be rich in phenolic compounds, alkaloids, diterpenoids, and other compounds which inhibit the development of bacteria and fungi [14]. Secondary metabolites play important role in plant defence by serving as antifeedant, protectants against herbivory and microbial infection, as allelopathic agents, as attractants for pollinators and seed dispersing animals,

and for plant flavour in cases of spices[15]. It should be noted that all of the secondary metabolites do not have antimicrobial property. Those which do have, can be categorized into different groups as described in following paragraphs. Structures of few of them are presented in fig. 2.

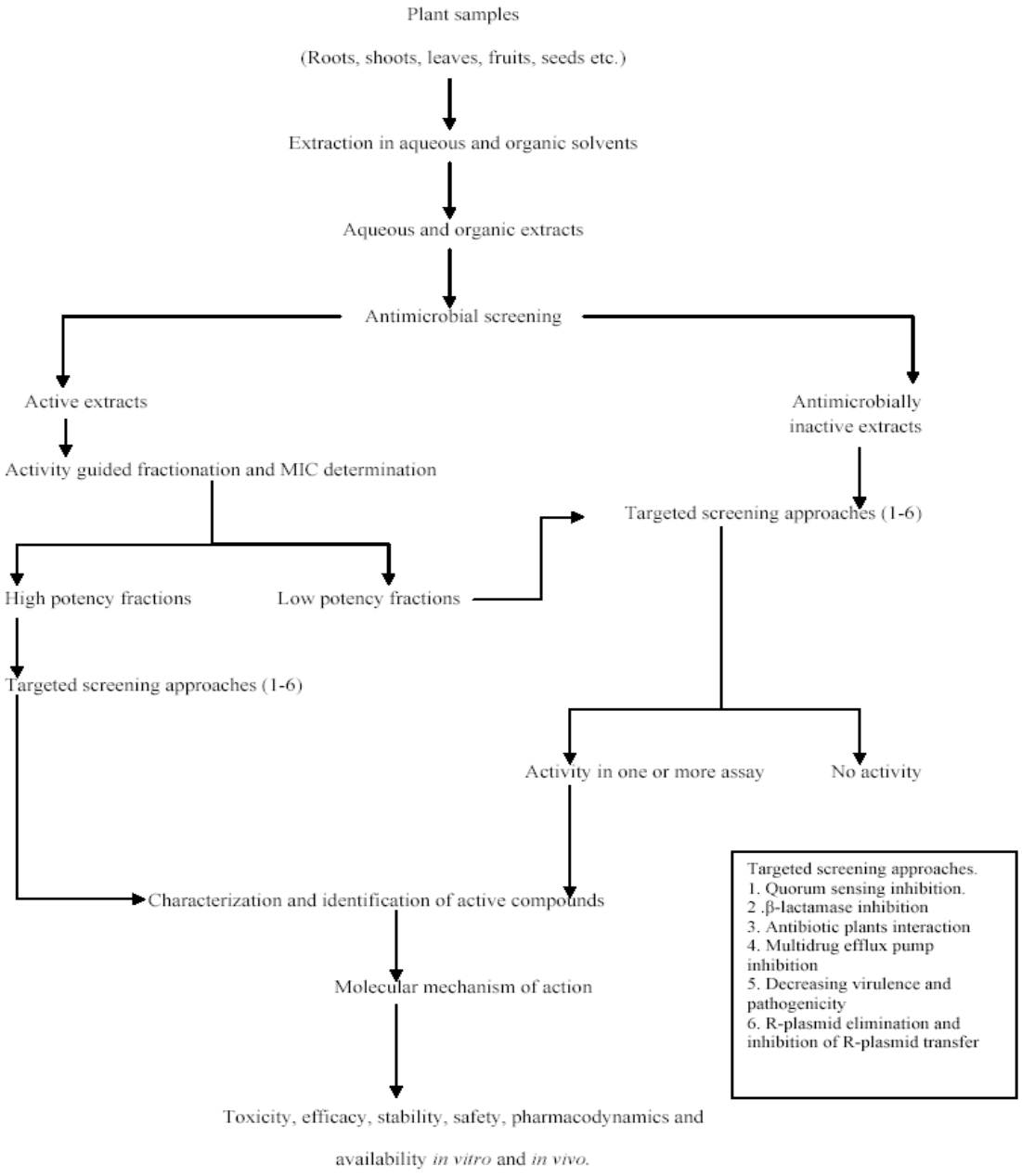

Fig 1. Schematic representation of targeted screening of plant extracts/phytocompounds for antimicrobial activity.

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Phenolics

Plant phenolics include a wide range of aromatic metabolites that posses one or more acidic hydroxyl substituents attached to a phenol ring [16;17]. Phenolic compounds play an important role in plant growth, development, reproduction and defence[3]. Based on their biological functions, phenolic compounds can be classified as flower pigments, fruit pigments, fungicides, phytoalexins, and pesticides. Gallotannine found in *Quercus robur* and flavonols like quercetin-glycosides in *Gossypium* are used as pesticides. Isoflavonols viz. luteon in *Lupinus* and phenolcarboxylic acids in *Allium* are known to be fungicidal. Stilbenes like reservatrol in *Arachis hypogaea* and furocoumarins like psoralen present in *Petroselinum crispum* function as phytoalexins [17].

Coumarins such as xanthotoxins and aflatoxins possess antifungal, allergenic and hepatotoxic activity. Some even fuction as blood anticoagulants. Flavones and flavonols like quercetin and kaempferol function as antioxidants, antimicrobials, while some function as enzyme inhibitors.

Hydroxyquinones viz. primin, hypericin and juglone have allelopathic and allergenic activity. Lignans possess antitumor and antiviral activity. Phenols like urshiol have been reported to be allergenic and antimicrobial. Stilbenoids such as penosylvin are reported for their antifeedant and antimicrobial action [18].

Simple phenols

Among all phenolic compounds, these are the simplest bioactive phytochemicals that consist of a single substituted phenolic ring. Simple phenols are exemplified by catechols, phloroglucinol, salicylic acid, eugenol and hydroquinone [19]. Monosubstituted catechols are active in part against *Pseudomonas* and *Bacillus* species [20]. The mode of action for their antimicrobial activity is enzyme inhibition by the oxidized compounds, through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins.

Phenolic acids

Phenolic acids form a diverse group of secondary metabolites that includes the widely distributed hydroxybenzoic and hydroxycinnamic acids. Anthamic acid in *Matricaria chemomilla* is effective against *Mycobacterium tuberculosis*, *Salmonella typhi*, *Staphylococcus aureus* and helminthes. Also, phenolic acid present in *Humulus lupus* acts as an antimicrobial agent [12].

Flavonoids

These are one of the largest classes of naturally occurring polyphenolic compounds. They were discovered along with vitamin C in 1928 by Albert Szent-Gyogyi who called them as Vitamin P4. Flavonoids have diverse pharmacological properties which include their ability to act as strong antioxidants, to scavenge radicals, chelate metals, interact with enzymes, adenosine receptors and biomembranes [21]. Flavonoids can be further categorized into aurones, flavanones, isoflavonoids, flavones, chalcones, flavonols, xanthones, and biflavones. Methoxy, isoprenyl, and acylated derivatives of these plant metabolites show antimicrobial activity. Aurones from *Cephalocereus senilis* are effective antimicrobial agents. Taxifolin in aspen knots are reported to be effective against *Escherichia coli, S. aureus and Enterococcus faecalis* [22]. Saponarin selectively inhibits a broad spectrum of gram-negative bacteria like *Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, E. coli, Klebsiella pneumoniae* etc. with an MIC range of 4-2048 µg/mL. The MIC values for flavonols; kaempferol-3-O-(3’’,6’’-di-O-Z-p-coumaroyl)-R glucopyranoside and kaempferol-3-O-(3’’-Z-p-coumaroyl)-(6’’-O-E-feruloyl)-R-glucopyranoside against *Bacillus cereus* were 4, 6, and 2 µg/mL, respectively and against coagulase positive *S. aureus* they were 8, 32, and 64 µg/mL [21]. Shetty (2006) [23] has reported another flavonoid, genticin from soybean as an antimicrobial.

Xanthones

These are organic plant phenols found in selected tropical plants. Xanthones are known to possess antiallergic properties, they are useful for treating fungal and viral infections. Xanthones are heat stable and are natural constituents of plants of Bonnetiaceae and Clusiaceae family. They are also found in some species of the family Podostemaceae[23]. Fractionation of stems from *Kielmeyera variabilis*, used in Brazilian folk medicine, yielded a mixture of xanthones containing assiguxanthone-B (1) and 1,3,5,6-tetrahydroxy-2-prenylxanthone (1:1 w/w), which inhibited *S. aureus* and *Bacillus subtilis* [24]. The most plentiful natural source of xanthones is the pericarp of mangosteen [24], which contains over 30 xanthones and is considered to be nature’s largest source of xanthones. Rubraxanthone, isolated from *Garcinia dioica*, shows potent activity against staphylococcal strains (MIC; 0.31-1.25 µg/mL), an activity which was greater than that of the antibiotic vancomycin (MIC; 3.13-6.25 µg/mL) [25]

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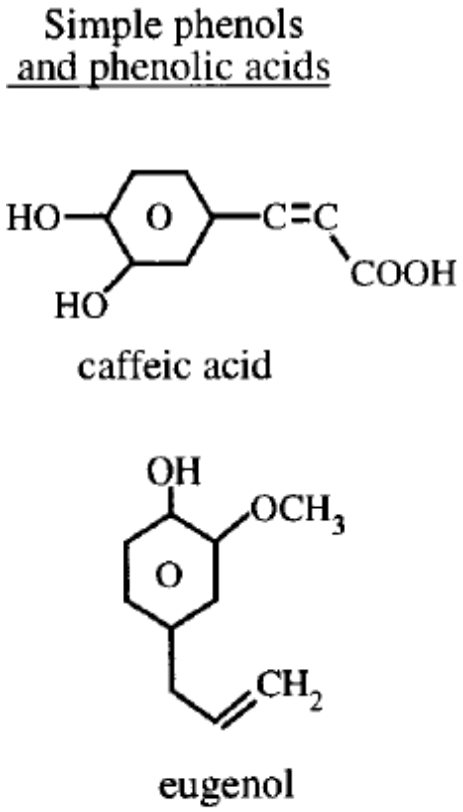
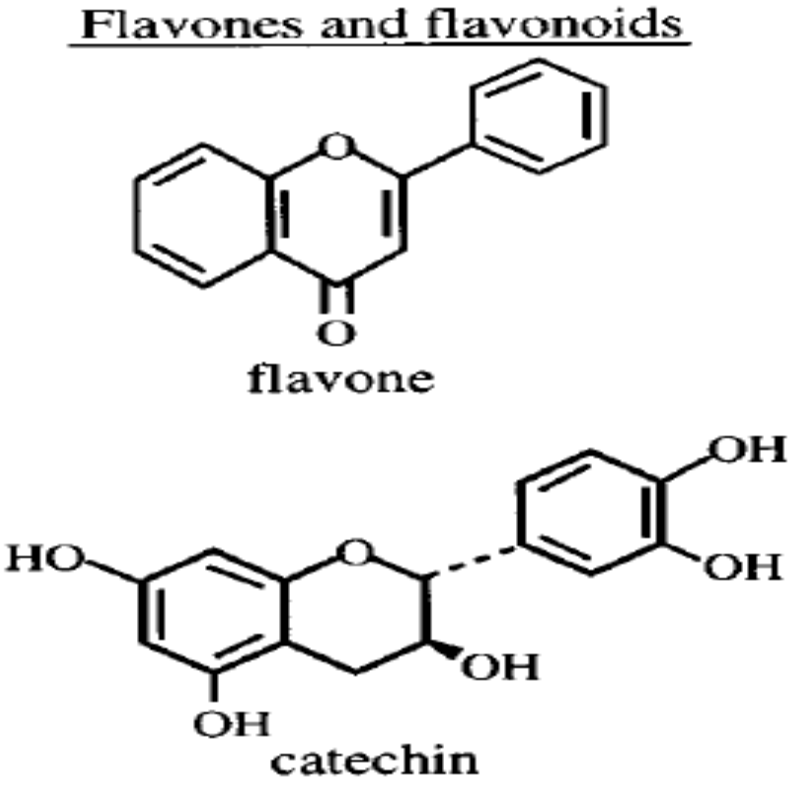


Fig. 2 Structures of few antimicrobial plant chemicals [11]



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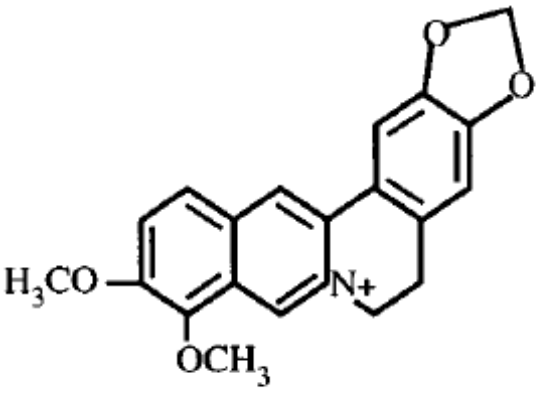
Alkaloids



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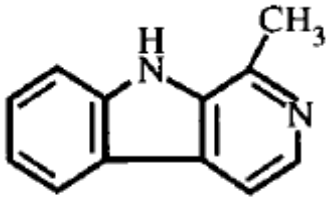

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Condensed tannins

Proanthocyanidins or condensed tannins are water soluble compounds which mainly function as enzyme inhibitors [26]. Condensed tannins found in sainfoin leaves inhibited growth and protease activity of *Butyrivibrio fibrisolvens* A38 and *Streptococcus bovis* 45S1. They show toxicity by changing the morphological features of the microbes by binding to their cell wall polymers [27].

Stilbenes

They are found in bryophytes, pteridophytes, gymnosperms and angiosperms. They serve as both constitutive and inducible defence mechanisms in plants. They display weak antibacterial properties but strong antifungal effect [15]. On exposure to fungi, vine leaves and berries produce resveratrol (trans-3,5,4'-trihyroxystilbene) which shows antifungal activity against a number of fungal pathogens including *Rhizopus stolonifer* , *Plasmopara viticola and Botrytis cinerea* [28]. Stilbene, 2',3',4-trihydroxyl-3,5,4'-trimethoxybibenzyl (combretastatin B5) isolated from the seeds of *Combretum kraussii* showed significant activity against *S. aureus*, and lower activity against *P. aeruginosa*, *E. faecalis* and *E. coli* [29].

Coumarins

Coumarins belong to a group of compounds known as benzopyrones, all of which consist of a benzene ring joined to pyrone. They can be roughly categorized as simple coumarins, furanocoumarins, and pyranocoumarins. Extracts of *Gallium odoratum* showed the presence of coumarin with antiviral activity [12].

Hyroxycinnamic acid

The primary walls of monocots, and the chenopodiaceae family contain significant amount of aromatic hydrocinnamic acid in their non lignified cell walls [15]. reported antiprotozoan activity of 3, 5-diprenly, 4-hydroxycinnamic acid, present in ethanolic extract of Brazilian propolis against trypomastigotes responsible for The primary walls of monocots, and the chenopodiaceae family contain significant amount of aromatic hydrocinnamic acid in their non lignified cell walls [15]. reported antiprotozoan activity of 3, 5-diprenly, 4-hydroxycinnamic acid, present in ethanolic extract of Brazilian propolis against trypomastigotes responsible for Chagas disease.

Terpenes and terpenoids

Terpenes are a large and varied class of hydrocarbons, produced particularly in conifers, in some insects and are main constituents of essential oils of plants and flowers. They are the major components of resin, and of turpentine produced from resin. Triterpenes namely β-sitosterol, 20(29)-lupene-3β-isoferulate isolated from the ethanolic extract of the root bark of *Euclea natalensis* demonstrated antifungal activity against *Aspergillus niger* [30]. The essential oil of basil (*Ocimum basilicum*) containing terpenoids showed antibacterial activity against *Salmonella* and other bacteria [12].

Naphthoquinone

Naphthoquinones are colored substances derived from phenylpropanoid and isoprenoid precursors. Naphtohquinones have been used for medicinal applications and colourants for cosmetics, fabrics and foods [31]. 2-methoxynaphthoquinone from *Swertia calycina*, a small plant found in Rwanda, strongly inhibited the growth of *Cladosporium cucumerinum* [32]. Shinanolone and octahydroeuclein isolated from the ethanolic extract of the root bark of *E. natalensis* demonstrated antifungal activity against *A. niger*, and *Cladosporium cladosporioides* respectively [30]

Alkaloids

Alkaloids are compounds containing nitrogen in a heterocyclic ring, common to about 15 to 20% of all vascular plants. They are synthesized by plants from amino acids. Alkaloids are subclassified on the basis of the chemical type of their nitrogen containing ring. Alkaloids are formed as metabolic byproducts. However, their characteristic bitter taste and accompanying toxicity repels insects and herbivores [17]. Quinine, an alkaloid present in *Cinchona officinalis* is used as antimalarial agent against *Plasmodium falciparum* strains which are resistant to other antimalarial drugs. Another alkaloid sanguinarine from *Eschscholzia californica* also possesses antibacterial properties [15].

Essential oils

These secondary metabolites are highly enriched in compounds based on an isoprene structure and are mainly responsible for the fragrance in plants. Majority of them possess antimicrobial properties [12]. Oils from *Citrus orantifolia* and *Syzygium aromaticum* are known to inhibit gram-positive microorganisms [33]. Antimicrobial activity of plant essential oils against few food-borne pathogens have been investigated [34]. Antibacterial activity of *Ocimum sanctum* L. fixedoil has been reported [35]. The mode of action of essential oils is concentration dependent. Low concentrations inhibit enzymes associated with energy production while higher amounts may precipitate proteins. Essential oils damage the structural and functional properties of membranes, they cause leakage of ions, ATP, nucleic acids and amino acids. Most essential oils impair the respiratory activity of bacteria and yeasts [36].

Table-1 features many of the plants reported for their antimicrobial activity. Unpublished results from author's own laboratory have indicated presence of antibacterial activity in different extracts of –*Syzium cumini, Tamarindus indica, Manilkara zapota*, and *Phoenix sylvestris*– seeds.

EXTRACTION PROCEDURES

Extraction, as the term pharmaceutically used, involves the separation of medicinally active portion from plant or animal tissues using selective solvents through standard extraction procedures. As mentioned in the South East Asian regional workshop (2006), extraction techniques separate out soluble plant metabolites leaving behind insoluble cellular material. The main objective behind standardized extraction procedures is to obtain therapeutically effective portions from the plant and at the same time eliminate the undesired compounds with the help of selective solvents. Properties of common organic solvents used for extraction are presented in table-2.

The basic parameters influencing the quality of an extract [19] are; the plant part used as starting material, the solvent used for extraction, the extraction technology used with the type of equipment employed, and crude-drug:extract ratio. The most important parameters affecting the yield of the extraction procedure are the moisture content of the plant material and temperature.

Traditional extraction processes involve extraction with water or organic solvents. Water is almost universally the solvent used to extract activity. Initial screenings of plants for possible antimicrobial activities typically begin by using crude aqueous or alcohol extractions. Water–soluble antimicrobials are commonly more effective as inhibitors of pathogen adsorption and would not be identified in the screening techniques commonly used. Starches, polypeptides and lectins are better extracted in water. Coumarins and fatty acids are better extracted in ether, whereas methanol is reported to be good for extracting lactones and phenones [12].

Extraction with organic solvents

Percolation

This simple method of extraction is widely used by herbalists all over the world. The construct includes a flask or vessel which is suspended above a cone or tube. The bottom of the tube has a perforated base which holds ground plant material in place. Solvent is

poured into the top of the tube where it soaks through the plant material leaching out the extract and then falling out through the bottom end of the tube into the flask [52]. It is a continuous process in which the saturated solvent is constantly displaced by fresh solvent, but normally the sample is steeped in solvent, unground leaves of artemisia were extracted using continuous hot percolation [53]. Cold percolation was used for extraction of powdered leaves of *Xanthium indicum* [54].

Maceration

It is the simplest extraction procedure known in which the solvent is poured onto the grounded plant material and after certain time interval the extract is strained and washed with fresh solvent to a prescribed weight [55]. In maceration, the sample is placed in a stoppered container in contact with the solvent. This allows the solvent to penetrate into the cellular structure in order to dissolve the soluble compounds. Maceration technique has been used for extraction of the biologically active constituents from the leaves, flowers and stem of *Chresta exsucca*, *Chresta scapigera*, *Chresta sphaerocephala* [56] and propolis [57].

Extraction with Soxhlet /modified Soxhlet

This technique is named after the German agricultural chemist, Franz Von Soxhlet, who invented it in 1879. For the past 129 years, Soxhlet extraction has been the most respected one among all conventional techniques [52]. The extraction principle is based on continuous extraction of the solid by repeated boiling-condensation cycle of a solvent in such a way that the extraction fluid is continuously refreshed [58]. This technique is based on distillation, in which there is continuous refluxing of the solvent causing the liquid to drip on the sample present in the thimble, which causes leaching process. When the solvent reaches appropriate level, it is driven back to the boiling container by siphoning mechanism [59].

Extraction with water

Infusion

It is a liquid preparation obtained by pouring warm or boiling water over the plant material, the most common method by which tea is made. After cooling the resulting solution can be filtered through cotton wool or gauze and the eluent is then brought upto the prescribed weight by adding hot water. Sometimes small quantities of acid or alkaline substances are added to the solvent to facilitate the extraction of active compounds [60].

Decoction

These preparations are extemporaneously obtained by boiling in water the suitable plant material, from which the active compounds are to be obtained. Boiling time varies depending on the physical characteristics of material to be extracted [60]. Aerial parts of *Tachiadenus longiflorus* have been extracted using decoction procedure [61]. Extraction of the bark of English oak, prepared by decoction method is used for hemorrhoids and other rectal problems [62].

Steam distillation

This procedure is commonly used for extraction of heat sensitive compounds e.g. aromatic compounds. The principle is that when immiscible objects are mixed together, they can lower the boiling point of each other [63]. The plant material is heated, either by placing it in water which is brought to boiling temperature or by passing steam through it. The heat and steam cause the plant cells to burst and break down, thus releasing active compounds like essential oils. After distillation the vapors are condensed as usual, yielding a two-phase system of water and the organic compounds, allowing for simple separation. It is also used for extraction of certain essential oils like eucalyptus oil from its leaves [63] . Steam distillation was used for extraction of leaves of *M. piperita*, and *Hyptis suaveolens* [64] for evaluation of its antimicrobial activity.

Microwave-assisted extraction

Microwave assisted extraction (MAE) was pioneered [65]. Microwaves are electromagnetic waves with frequency between 300 MHz to 300 GHz. The principle of microwave heating is dependent on ionic conductance and dipole rotation. During microwave heating, if moisture is present in the plant material, it will evaporate and cause pressure that will rupture the plant cell wall. So the active constituents from the plant cell leach out in the solvent used. Commercially two types of MAE systems are available; closed vessel and open vessel [66]. The plant material is immersed in a suitable solvent (which can be microwave transparent e.g. hexane or chloroform) and subjected to microwave irradiation by adjusting various parameters like microwave power, exposure time etc. The MAE has been practiced to obtain saponin from chickpea [65], and also for preparing extracts from *Annona squamosa* seeds [67].

Ultrasonic extraction

This method employs ultrasonicator, which produces high frequency sound for extraction from various plant materials. Ultrasonic acoustic cavitations create shear forces causing mechanical breakage of cell wall, which will cause extraction of liquid content from cells. The ultrasound also enhances the diffusion of the solvent into the tissue. Here finely powdered plant material is subjected to sonication in suitable solvent [68].

Menthol was extracted from peppermint plant *(M. piperita*) by subjecting it to ultrasonication for 1 hour at 22° C in 40 KHz ultrasound bath [69]. *Panax ginseng* (leaves and roots), *Flores Crataegi*, *Fructus Crataegi* (flowers and foetus), *Herba hyperici*, *Herba leonuri*, and *Pinus silvestris L.* (needle) were subjected to ultrasonic treatment at the intensity of 1 - 70 w/cm^2 and oscillation frequency of 20-22 kHz for 60-420 s [70] .

Soxwave (microwave assisted Soxhlet) Extraction

This device makes use of Soxhlet apparatus, which is for solid sample extraction and a microwave digester for irradiation of the sample at certain intervals. Fresh solvents from the distillation flask (which forms the condensed vapours), drip on and pass through the solid sample at regular intervals. This causes breaking of analyte-matrix bonds. There is a reduction in extraction time from hours to just 50-60 minutes, which depends on the polarity of the analytes, with the efficiency similar to or higher than the traditional Soxhlet technique [71].

Supercritical fluid extraction

In this method gases like carbon dioxide, nitrogen, methane, ethane, ammonia etc. are compressed into a dense liquid which is pumped through a cylinder that contains the experimental material. This extract laden liquid is then pumped into a chamber where the extract is separated from the gas. The gas can then be reused [52].

The traditional techniques require longer extraction time and thereby there is always a risk of thermal degradation for most of the phyto-constituents. Relative merits and demerits of different extraction procedures are mentioned in table-3. The fact that one single plant can contain up to several thousand secondary metabolites, makes the need for the development of high performance and rapid extraction methods an absolute necessity [66]. Extraction procedures like ultrasonic extraction, MAE, pressurized fluid extraction, sub-critical water extraction and supercritical fluid extraction [72] counter current extraction, spouted bed extraction, slide phase micro extraction etc. have overcome many of the limitations of the traditional extraction procedures [19]. Latest methods for extraction from aromatic plants include head space trapping technique (HSTT), solid phase micro-extraction (SPME), protoplast extraction technique (PET), micro-distillation, thermo-micro-distillation, and molecular distillation techniques.

IN VITRO ASSAY FOR ANTI-MICROBIAL ACTIVITY

Determination of the antimicrobial activity of a compound is essential for its effective use. The activity of the antimicrobial compounds can be determined easily by various antimicrobial susceptibility tests (AST). The assay is categorized into agar diffusion assay and broth dilution assay [77]. AST is performed routinely on bacterial isolates in clinical laboratories. However it was recognized in the beginning itself that many variables affect the results of the AST. Consequently, it was realized that standardization of these techniques was required. This need has led to standardized AST methodologies. The clinical and economic pressures for rapid methods with low labour input led to the development of semi-automated and automated AST methodologies in the 1970s.

A good antimicrobial assay should fulfill two purposes. It should first verify whether the compound actually has the desired antimicrobial activity or not, and second it should indicate the concentration of the antimicrobial that will be needed to inhibit the target organism. To determine the antimicrobial activity of any compound, it is tested under carefully controlled conditions. The effectiveness of a compound is dependent upon a number of factors such as the nature of the target microbes, concentration of the test compound, inoculum density, time in contact with the compound, temperature, pH and degree of aeration [78].

Beijerinck first utilized the agar diffusion method in 1889 for studying the effect of different auxins on bacterial growth. Fleming introduced the ditch plate technique in 1924 for evaluating antimicrobial qualities of antiseptic solutions. The ditch plate technique was further modified by Reddish. Instead of making the ditch, he cut wells into the agar and filled the wells with antiseptic solutions. The cylinder plate synonymous with the ‘Oxford cup’ developed the principle of this technique further. Fleming’s second contribution to modern AST was the development of a broth dilution technique using turbidity as an end point determination. The broth dilution method is generally used for determination of minimum inhibitory concentration (MIC). Fleming later used pH as an indicator for end point determination. Heatley (1940) introduced the use of absorbent paper for carrying antimicrobial solutions. Vincent and Vincent incorporated penicillin in paper discs for assaying the newly discovered compound. Bondi and co-workers (1947), first described the filter paper discs of 6-6.5 mm that are commonly used today. Schmith and Reymann (1940) were the first to describe the use of agar dilution AST method for determining MIC of antimicrobial agent. This method was further modified, which is now commonly referred to as the ‘breakpoint’

technique, a term first used by Ericsson and Sherris in 1971[79]. In 1992, National Committee for Clinical Laboratory Standards (NCCLS) in the United States, proposed a broth dilution method (M-27P) for antifungal susceptibility testing [80].

Agar diffusion assay

This is the most commonly used assay to assess the potency of an antimicrobial agent. The agar diffusion method is most useful in determining the approximate antibiotic susceptibility of a microbe. Using the agar diffusion method it is possible to test a number of different compounds simultaneously on the same agar plate. Agar diffusion assay can be accomplished with the disc, well or ditch loaded with the test compound.

Disc diffusion assay

Disc diffusion assay is the most preferred assay because of its simplicity and low cost [81]. The disc diffusion assay, also known as Kirby-Bauer assay, is a standard procedure used in clinical laboratories to test the susceptibility of bacterial isolates to antimicrobial compounds and antibiotics. The culture inoculum is spread onto the surface of an appropriate medium such as Mueller-Hinton agar and the discs impregnated with antimicrobial compounds are then placed onto the agar. Mueller-Hinton agar is recommended as it is low in thiamine and thymidine content. The plate is then incubated at an appropriate temperature suitable for microbial growth, during incubation the test compound diffuses away from the disc, into the agar creating a concentration gradient that is highest near the disc and decreases as one moves away from the disc. If the agent is inhibitory, organism will be unable to grow near the disc, which is seen as a zone of clearance in the lawn of growth. Farther from the disc, where the concentration of the antimicrobial compound is much lower, growth will be evident. The size of the zone of clearance around the disc is an indication of the potency of the antimicrobial against the test organism. After incubation, diameter of the zones of inhibition are measured and translated into categories such as susceptible, intermediate, or resistant [33].

Agar well diffusion method

For agar well diffusion method, a well is prepared in seeded agar plates with help of a cork-borer, then the test compound is introduced into the well and incubated overnight at appropriate temperature. Effectiveness of the test compound is then determined by measuring the diameter of zone of inhibition [43].

The aqueous and ethanol extracts of *Launaea procumbens* Roxb. (Labiateae), *Vitis vinifera* L.(Vitaceae) and *Cyperus rotundus* L. (Cyperaceae) were evaluated for antimicrobial activity against clinically important bacteria by agar diffusion method [43].

The agar diffusion method is however appropriate only as preliminary screening method due to several limitations. This method is not appropriate for testing the non-polar samples that do not easily diffuse into the agar. It is not recommended for highly lipophilic compounds, such as sesquiterpenes. The antimicrobial activity of different samples may not always be compared, due to differences in physical properties, such as solubility, volatility and diffusion characteristics in agar. Compounds with high diffusion coefficient and low antimicrobial activity may penetrate the agar medium even in small amounts. The reverse might also hold true. Moreover other factors are also responsible for variability in the results such as disc size, amount of compound loaded onto disc, adsorption by the disc, agar type, agar content, pH, volume of agar, and microbial strains used. The size of inhibition zones might be influenced by volatilization of active test material. Furthermore, agar-diffusion methods are difficult to run on high-capacity screening platforms [81]. One major limitation arises when this assay is used to measure antimicrobial potency against anaerobic bacteria. Anaerobic organisms are much more likely to be subjected to lethal doses of oxygen when they are spread onto the surface of agar.

Dilution method

For the limitations of agar diffusion method, cited above, broth dilution is the most preferred method for measuring the MIC and microbicidal concentrations of given test agent [82].

MIC is the lowest concentration of a compound that inhibits microbial growth. MIC can be determined by agar dilution or broth dilution method usually following the guidelines of a reference body such as the Clinical and Laboratory Standard Institute (CLSI), British Society for Antimicrobial Chemotherapy (BSAC) or European Committee of Antimicrobial Susceptibility Testing (EUCAST).

Broth dilution method

The MIC of a given compound for certain bacterial species is determined using a series of test tubes containing medium in which the test organism will normally grow, but where each tube contains progressively higher concentrations of the test compound. Each tube is inoculated with the test organism and after incubation the minimum concentration required to inhibit growth is noted. Since many parameters affect MIC, it is only valid under the specified conditions. If it is desired to compare the effectiveness of different antimicrobials on a certain strain or to examine the resistance of a number of microbes to a single agent, test conditions need to be rigorously standardized [83, 81]. Including a proper negative control is equally important [84].

Microbroth dilution methods have also been developed which utilize microtiter plates. Thus the volume of the extract required in this assay is very low. Here the endpoints are determined spectrophotometrically, either as a measure of turbidity or using a cell viability indicator.

Agar dilution method

In agar dilution method the test compound in varying concentrations is incorporated into the agar and the microbial culture is applied to its surface. Replicate dishes can be set up with a range of concentration of the test substance and by dividing the surface of the agar into wedges or squares, a number of bacterial species can be applied to a single dish. In this way, a large number of bacteria may be incorporated in a single assay. Dishes are then incubated for appropriate period of time and the growth of bacteria is scored either as present/absent in proportion of control. Several limitations of this method include requirement of large volumes of test substance, the difficulty in using essential oils and hydrophobic extracts in aqueous environments etc. [85].

SEPARATION OF ACTIVE COMPONENTS FROM CRUDE EXTRACTS

The choice of technique for separation and purification of plant constituents depends largely on the solubility properties and volatilities of the compounds to be separated [16]. Chromatography is one of the most useful means of separating mixtures of compounds as well as to purify and identify the components. Chromatographic separation of plant saponins has extensively been reviewed by Oleszek [86, 87]. In chromatography, the mixture is separated by differential distribution of the components between a stationary phase and mobile phase [68].

Brief description of various separation techniques follows.

Paper chromatography (PC)

Paper chromatography is based on the mechanism of either partition or adsorption. PC is used for separating dissolved chemical substances by taking advantage of their different rates of migration across sheets of paper. It is an inexpensive but powerful analytical tool which requires small quantity of starting material [88]. The considerable reproducibility of R_f (retention factor) values, determined in PC makes it valuable for use in describing new plant compounds [16].

Thin layer chromatography (TLC)

Thin layer chromatography is the simplest and cheapest method of detecting plant constituents because the method is easy to run, reproducible and requires little equipment [89]. The real breakthrough of TLC as an analytical technique came about 36 years ago as a consequence of the pioneering work of Egon Stahl (1988). Separation is based on distribution of a compound between the solid fixed phase that is applied as a thin layer and a mobile solvent moving over the solid phase. Mobile phase will carry the most soluble compounds furthest up the plate and compounds less soluble in the mobile phase will stay behind. Information on suitable mobile phases for various plant components is given in Table-4.

Column chromatography

Column chromatography is the most preferred technique for the separation of components from crude plant extracts. The adsorbent in a suspension of nonpolar solvent is packed in a vertical glass column and the crude extract to be separated is added to the top of the column. A suitable solvent or a solvent mixture, which should be as nonpolar as practical, is then allowed to flow down through the column, eluting the various components from the bottom of the column. The ratio of mass of adsorbent to mass of extract should be around 40:1 and the ratio of column height of adsorbent to column diameter should be around 15:1[68].

High performance liquid chromatography (HPLC)

HPLC is a chemistry based technique for quantifying and analyzing mixtures of chemical compounds which combines column efficiency with speed of analysis [90]. In HPLC the sample is forced through a column, packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase), by a liquid (mobile phase). HPLC is advantageously used for the quantitative estimation of various flavonoids in plant extracts.

The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. HPLC coupled with different detection methods like UV spectroscopy or mass spectrometry provide a great deal of preliminary information about the content and nature of constituents found in active extracts [89].

The efficiency of HPLC and silica gel thin layer chromatography were compared by applying them for the separation and quantitative determination of the compounds, diterpene glycosides, stevioside and rebaudioside A, in the leaves of *Stevia rebaudiana* Bertoni. It was found that all the basic features such as resolution, accuracy and reproducibility of both the techniques were nearly the same. Thus thin-layer chromatography was found to be more suitable for phytochemical screening of large number of samples [91]. A combination of TLC and HPLC, may be the best approach for separating a particular class of plant compounds [16].

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Table 1: Plants with antimicrobial potential

Sr no	Scientific name	Common name	Plant part/ extract used	Susceptible organisms and MIC*	Reference
1	*Bixa orellana* (Bixaceae)	Achiote	Ethanol extract of leaf	*E. coli (2.1±0.7)* *B. cereus (0.2±0.1)*	[37]
2	*Cecropia peltata* L. (Moraceae)	Trumpet tree	Ethanol extract of leaf	*S .aureus (2.1± 0.1)* *B. cereus (0.2± 0.1)* *E. coli (2.1± 0.7)*	
3	*Gliricidia sepium* (Fabaceae)	Nicaraguan coffee shade	Ethanol extract of leaf	*S. aureus (7.8± 0.5)* *B. cereus (6.8± 0.9)* *E. coli (2.0± 0.0)* *Streptococcus β hemolytic (12.8± 0.0)*	
4	*Spilanthes Americana*	Drury	Ethanol extract of leaf	*S. aureus (4.6± 0.6)* *B. cereus (18.6± 3.2)* *E. coli (15.3± 3.4)* *Streptococcus β hemolytic (15.1± 2.1)*	
5	*Cinnamonum zyelancium* (Lauraceae)	Cinnamon	Bark (cinnamon oil)	*S. aureus* (3.2) *B. subtilis* (> 1.6) *P. aeruginosa* (> 0.8) *E. coli* (> 1.6)	
6	*Citrus limon* (Rutaceae)	Lemon	Lemon oil	Gram-positive organisms *S. aureus* (> 12.8) *B. subtilis* (> 12.8) *P. aeruginosa* (12.8) *E. coli* (> 6.4)	[33]
7	*Pelargonium Graveolens* (Geraniaceae)	Geranium	Geranium oil	Gram-positive organisms *S. aureus* (>12.8) *B. subtilis* (> 6.4) *P. aeruginosa* (> 12.8) *E. coli* (> 6.4)	
8	*Rosmarinus officinalis* (Lamiaceae)	Rosemary	Rosemary oil	Gram-positive organisms *S. aureus* (> 12.8) *B. subtilis* (> 6.4) *P. aeruginosa* (> 6.4) *E. coli* (> 6.4)	
9	*Citrus sinensis* (Rutaceae)	Orange	Orange oil	Gram-positive organisms *S. aureus* (> 12.8) *B. subtilis* (> 12.8) *P. aeruginosa* (> 12.8) *E. coli* (> 12.8)	
10	*Citrus aurantium* (Rutaceae)	Lime	Lime oil	Gram-positive organisms *S. aureus* (12.8) *B. subtilis* (> 6.4) *P. aeruginosa* (> 6.4) *E. coli* (> 6.4)	
11	*Eugenia caryophyllus* (Myrtaceae)	Clove	Clove oil	Gram-positive organisms *S. aureus* (> 6.4) *B. subtilis* (> 3.2) *P. aeruginosa* (> 0.8) *E. coli* (> 1.6)	
12	*Cassia alata* (Fabaceae)	Guajava	Methanol extract of leaves	Dermatophytic fungi	[38]
13	*Allium sativum L.* (Alliaceae)	Garlic	Garlic clove	*Helicobacter pylori* (8-32)	[39]
14	*Bacopa monnieri Linn.* (Scrophulariaceae)	Brahmis	Ethyl acetate and n-butanol fractions of ethanol extract of aerial parts.	*S. aureus, B. subtilis, Bacillus polymexia, Streptococcus feacalis, P. aerugenosa, S. typhi, Vibrio cholerae, Shigella dysenteriae, E. coli, Penicillium notatum, A. niger, Candida albicans* (25-200) for ethyl acetate and (100-600) for n-butanol extract	[40]
15	*Terminallia catappa* (Combretaceae)	Indian almond	Methanol extract of leaves	*B. subtilis (5)* *S. aureus (1.25)*	[41]
16	*Eucalyptus camaldulensis*	Eucalyptus	Methanol extract of leaves	*B. subtilis (5)* *S. aureus (5)*	

	(Myrtaceae)			*C. albicans (< 0.625)*	
17	*Entada Africana* (Fabaceae - Mimosoideae)	Entada	Ethanol and aqueous extracts of bark	10 *E. coli* strains, (1.56-50.00)	[42]
18	*Terminalia avicennoides*	Harad	Ethanol and aqueous extracts of the bark	10 *E. coli* strains, (1.56-50.0)	
19	*Lannae acida*	Mali/volta	Ethanol and aqueous extracts of stem bark	10 *E. coli* strains (1.56-50.0)	
20	*Mitragyana stipulosa* (Rubiaceae)	Nzingu	Ethanol and aqueous extracts of bark	10 *E. coli* strains (1.56-50.0)	
21	*Hibiscus Rosasinensis* (Malvaceae)	Jaasud	Leaf	*B. subtilis*, *Pseudomonas morganii*, *K. pneumoniae*	[1]
22	*Mirabilis jalapa* (Nyctaginaceae)	Gulbas	-	Fungal infection, *P. morganii*	
23	*Sapnidus emarginatus*	Aritha	Leaf	*Pseudomonas testosterone*, *K. pneumoniae* *Micrococcus flavus*	
24	*Aloe barbadensis* (Asphodelaceae)	Aloe vera	Latex	*Corynebacterium*, *Salmonella*, *Streptococcus*, *S. aureus*	[12]
25	*Ocimum basilicum* (Lamiaceae)	Basil (tulsi)	Essential oil	*Salmonella*	
26	*Tanacetum vulgare* (Asteraceae)	Tansy	Essential oil	Helminthes, Bacteria	
27	*Allium cepa* (Alliaceae)	Onion	Onion bulb	Bacteria, *C. albicans*	
28	*Lagenaria vulgaris seringe* (Cucurbitaceae)	Dudhi	Alcoholic and aqueous extract of fruit	*B. cereus*, *K. pneumoniae*	[43]
29	*Coleus aromaticus* Benth (Lamiaceae)	Ajma pan	Alcoholic and aqueous extract of leaf	*B. cereus*, *K. pneumoniae*	
30	*Mangifera indica* (Anacardiaceae)	Mango	Alcoholic and aqueous extracts of leaf	*B. cereus*, *K. pneumoniae*, *S. aureus*, *S. epidermis*, *P. aeruginosa*	
31	*Dalbergia sisoo Roxb* (Fabaceae)	Moto shisham	Alcoholic and aqueous extracts of leaf	*S. aureus*, *S. epidermis* *K. pneumoniae*	
32	*Quisqualis* (Combretaceae)	Madhumalti	Alcoholic and aqueous extract of aerial part	*B. cereus*, *K. pneumonaie*	
33	*Ociumu basilicum* (Lamiaceae)	Takmari	Whole, alcoholic and aqueous extracts	*B. cereus*	
34	*Sesamum indicum* (Pedaliaceae)	Tal	Whole, alcoholic and aqueous extracts	*B. cereus*, *E. fecalis*, *K. pneumoniae*	
35	*Jasminum malabarium* (Oleaceae)	Juhi	Alcoholic and aqueous extract of aerial part	*B. cereus* , *S. epidermis*	
36	*Musa paradisiacal* (Musaceae)	Banana	Alcoholic and aqueous extract of leaf	*S. epidermis*	[43]
37	*Calotropis gigantean* (Apocynaceae)	Ankdo	Alcoholic and aqueous extract of leaf	*B. cereus*, *K. pneumoniae*	
38	*Thymus vulgaris L*	Thyme	Ethanol extracts of dried leaves and flowers	*P. aeruginosa* (70) *E. arogenes* (70)	[44]
39	*Syzygyum joabolanum* (Myrtaceae)	Jambolan	Ethanol extract of leaf	*P. aeruginosa* (50) *E. arogenes* (400) *S. aureus* (300)	
40	*Punica granatum*	Pomegranate	Ethanol extract of pericarp	*P. aeruginosa* (70)	
41	*Psidium guajava*	Guava	Ethanol extract of leaf	*S. aureus* , *C. albicans*	
42	*Coriandrum sativum*	Coriander	Essential oil	*C. albicans;* 0.25 %(v/v) of essential oil *K. pneumoniae ;* 0.5 %(v/v) of essential oil *S. typhimurium;* 1.0 %(v/v) of essential oil.	
43	*Dacus carota*	Carrot seed	Essential oil	*C. albicans* ;2.0 %(v/v) of essential oil.	

				K. pneumoniae; > 2.0 %(v/v) of essential oil. *S. typhimurium;* > 2.0 %(v/v) of essential oil.	
44	*Mentha piperita*	Peppermint	Essential oil	*C. albicans;* 0.5 %(v/v) of essential oil. *K. pneumoniae;* 1.0 %(v/v) of essential oil. *S. typhimurium;* 1.0 %(v/v) of essential oil.	
45	*Santalum album*	Sandalwood	Essential oil	*C. albicans;* 0.06 %(v/v) of essential oil *K. pneumoniae;* > 2.0 %(v/v) of essential oil *S. typhimurium;* > 2.0 %(v/v) of essential oil	[45]
46	*Zingilver Officinale* (Zingiberaceae)	Ginger	Essential oil	*C. albicans;* > 2.0 %(v/v) of essential oil *K. pneumoniae;* > 2.0 %(v/v) of essential oil *S. typhimurium;* > 2.0 %(v/v) of essential oil	
47	*Glycyrrhiza glabra*	Jethimudh	Alcohol,ethyl acetate, acetone, choloroform extract of root were used	*B. cereus,* *Mycobacterium smegmatus,* *S. aureus* *Micrococcus luteus, K. pneumoniae*	
48	*Juniperus oxycedrus*	Juniper	Alcohol,ethyl acetate, acetone, choloroform extracts of seed were used	*B. cereus,* *M.smegmatus* *S. aureus* *M.luteus,* *K. pneumoniae* *B. subtilis*	[4]
49	*Acacia arbica*	-	Aqueous extract of leaf	*Xanthomonas campestris*	[46]
50	*Annona squamosa* (Annonaceae)	Sitafal	Aqueous extract of seed	*B. cereus,* *Clostridium freundii*	
51	*Asparagus racemosus Bak*	Shatavari	Aqueous extract	*B. cereus*	
52	*Embica officinalis*	Amlan	Ethanol extract of leaves	*P. aeruginaosa,* *P. vulgaris,* *Pseudomonas mirabilis ,* *S. epidermis ,* *B. cereus*	[47]
53	*Polyalthia longifolia*	Asopalav	Ethanol extract leaves	*P. mirabilis* *C. freundii* *E. aerogenes,* *S. epidermis*	
54	*Nerium indicum*	Kaner	Ethanolic extract of flower, leaf and stem	*B. subtilis* *Streptococcus pyogenes ,* *Cornybacterium diptheriae* *S.typhi* *Aeromonas hydrophila*	[48]
55	*Pneumatopteris afra*	-	Methanol extract of air dried plant	*E. coli (12.50)* *S. aureus (12.50)* *S. typh*i (25)	[49]
56	*Platycerium bifurcatum*	-	Methanol extract of whole plant	*E. coli (12.50)* *S. aureus (12.50)* *S.typhi (25)*	
57	*Nephrolepsis bisserata*	-	Methanol extract of whole plant	*E. coli (50)* *S. aureus (50)* *S.typhi (100)*	

* MIC (minimum inhibitory concentration); values in italics are in µg/mL and those not in italics are in mg/mL.

[+]Table 2: Properties of common organic solvents used for extraction

Solvent	Formula	M.W. (D)	B.P. (^oC)	M.P. (^oC)	Density (g/mL)	Solubility in water (g/100mL)	Dielectric Constant
Acetic acid	$C_2H_4O_2$	60.05	118	16.6	1.049	Miscible	6.15
Acetone	C_3H_6O	58.08	56.2	-94.3	0.786	Miscible	20.5(25)
Benzene	C_6H_6	78.11	80.1	5.5	0.879	0.18	2.28
1-butanol	$C_4H_{10}O$	74.12	117.6	-89.5	0.81	6.3	17.8
Chloroform	CHC_{l3}	119.38	61.7	-63.7	1.498	0.795	4.81
Chlorobenzene	C_6H_5Cl	112.56	131.7	-45.6	1.1066	0.05	2.71
Diethyl ether	$C_4H_{10}O$	74.12	34.6	-116.3	0.713	7.5	4.34
Ethanol	C_2H_6O	46.07	78.5	-114.1	0.789	Miscible	24.6
Ethyl acetate	$C_4H_8O_2$	88.11	77	-83.6	0.895	8.7	6(25)
Ethylene glycol	$C_2H_6O_2$	62.07	195	-13	1.115	Miscible	37.7
Hexane	C_6H_{14}	86.18	69	-95	0.659	0.014	1.89
Methanol	CH_4O	32.04	64.6	-98	0.791	Miscible	32.6(25)
Toluene	C_7H_8	92.14	110.6	-93	0.867	0.05	2.38(25)

[+] [50,51]

Table 3: Relative merits and demerits of different extraction procedures used for plant materials

Extraction procedure	Advantages	Disadvantages	Reference(s)
Soxhlet extraction	Less expensive, automated and continuous method, with effective extraction yield	Not suitable for thermally labile compounds as they get hydrolyzed, decomposition can occur during the extraction, requires more time, high solvent consumption	[72;59]
Microwave assisted extraction (MAE)	Short operation time, reduced solvent consumption, moderate recovery, less sample manipulation	Due to high pressure there can be risk of explosion in case of closed vessel MAE	
Percolation	Usually depletes the plant material completely of the desired components	Expensive, time-consuming procedure with large solvent requirement	[55]
Maceration	Simple method with easy construct	Low extraction efficiency	
Supercritical fluid extraction	High solute diffusivity, improved mass transfer and reduction of extraction time, high boiling components can be extracted at relatively low temperatures	High pressure is required, expensive	[73; 74; 58]
Steam distillation	Useful for extraction of volatiles, simple to use, gives clear extracts	Useful only for volatiles, time consuming, labor intensive, higher temperature can cause decomposition	[75;76]
Soxwave (microwave assisted Soxhlet) extraction	Less time is required, recycling of the solvent causes less environmental contamination	-	[71]
Ultrasonic extraction	Rapid, reproducible, low cost	Sometimes there is low recovery, lack of automation	[58]

#Table 4: Mobile phase for TLC of various plant components

A. Steroids
1. Benzene: Benzene ethyl acetate (9:1 or 8:2)
2. Chloroform:Ethanol (96:4)
3. Ethyl Acetate:Cyclohexane
4. Benzene: Isopropanol
5. Methanol: Water (95:5)
6. Cyclohexane: Heptane (1:1)
7. Cyclohexane:Ethyl Acetate (9:1)
8. Benzene:Chloroform (9:1)
9. Benzene: Methanol

B. Essential Oils
1. Benzene:Chloroform
2. Petroleum Ether

C. Cardenoloids
1.Chloroform: Pyridine (6 :1)

D. Fatty acids
1. Petroleum ether:Isopropylic ether: Acetic acid (70 : 30 :1)
2. Chloroform:Petroleum ether

E. Vitamins
1. Methanol:Carbon tetrachloride:Xylene:Chloroform (Alumina)
2. Chloroform:Petroleum ether
3. Methanol

F. Alkaloids
1. Chloroform/Ethanol / Hexane:Chloroform (3:7) + 0.051% Diethylamine
2. Benzene: Ethanol (9:1)
3. Chloroform:Acetone:Diethylamine (5 : 4 : 1)

G. Flavonoids and coumarins
1. Petroleum ether: Ethyl acetate (2:1)
2. Methanol: Water (8:2 or 6:4)
3. Toluene:Ethyl formate: Formic acid (5 : 4 : 1)

H. Phenolic Acids
1. HOAc-$CHCl_3$ (1:9)

I. Phenols
1. Benzene-MeOH- HOAc (45:8:4)

#[68,16]

Table 5: Techniques for separation of active components from plant extracts

Separation technique	Advantages	Applicability	Limitations	References
Paper chromatography	High reproducibility, easy to perform	Phenolic compounds, carotenoids	Separation is slow	[16]
Column chromatography	Simple equipment requirement	Almost all compounds	Time consuming, requires constant operator attention	[92]
TLC	Better separation than column chromatography, cheap, simple apparatus	Lipids, steroids, caretenoids, simple quinines, chlorophylls	Only low amounts can be separated	[16; 55]
GC	Method of choice for separation of volatile compounds	Fatty acids, monoterpenes, sesquiterpenes, hydrocarbons, sulphur compounds	Not suitable for heat-labile components	
HPLC	Suitable for separation of thermolabile compounds, automation allows analysis of large number of samples, better reproducibility of results	Less volatile constituents, flavonoids	Equipment cost is high	[93; 16;55]
Electrophoresis	Speedy separation, capillary zone electrophoresis (CZE) can be modified to analyze uncharged molecules too	Polyphenols, amino acids, some alkaloids and organic acids	Limited applicability to uncharged molecules	[92;16]

++Table 6: Absorption maxima of different secondary metabolites

Class of secondary metabolite	Name of the compound	$\lambda_{max\ (nm)}$
Flavonols	Flavones	240-285, 304
	Flavonol	240-285, 352
	Flavanone	270-295, 300-303
	Dihydroflavonol	270-295, 300-320
	Chalcones	220-270, 340-390
	Aurones	370-430
	Anthocynidine	270-280, 465-550
	Yellow flavonols	365-390, 250-270
Coumarins	Coumarins	300
	Unsubstituted coumarins	274, 311
	7-hydroxy coumarins	217, 315-330
	Linear furanocoumarins	205-225, 240-255
Alkaloids	Vasicine	298
	Papaverine	325, 312, 283, 238
	Emetine	235, 285, 360
	Morphine	286, 250, 298
	Quinine	277, 321, 332
	Catharantus	296, 290, 275, 255, 221
	Reserpine	296, 267, 216
	Ergonovine Maleate	311
	Strychine	240, 270, 294
	Nicotine (pyridine)	261
	Atropine(tropane)	246, 251.6, 257, 263.5, 271
	Ephedrine	250, 256, 262
	Piperine	245
	Colchicine	243, 357
	Caffeine	278
Terpenoids	Butadine	217
	Zingieberine	260
	Levopimarua	272
	B-phellandrene	232
	Myrcrene	224
	Citral	236
	Camphor(meoh)	288
	Carvone	235
	Abeitu	237.5, 235, 241.5
Carotenoids		400-500
Phenols	Orcinol	276, 282 (EtOH)
	Resorcinol	276, 283 (EtOH)
	Catechol	279 (EtOH)
	Hydroquinone	295 (EtOH)
Phenolic acids	Gallic	272 (EtOH)
	Salicylic	235, 305 (EtOH)
	Vanillic	260, 290 (EtOH)

++(93; 16)

Electrophoresis

Electrophoresis is applicable to compounds that carry charge, e.g., amino acids, some alkaloids and organic acids. Capillary electrophoresis has proved to be a valuable analytical tool for most classes of secondary metabolites but especially for plant polyphenols [16].

A comparative account of different separation techniques is presented in Table-5.

IDENTIFICATION

After separation of potential secondary metabolites, the biochemist is faced with the task of identifying them. The purity of the separated compound should be confirmed, i.e. it should travel as a single spot on different chromatographic systems. Identification can be achieved with the help of different qualitative tests, by comparing Rf values, melting point and boiling point for solids and liquids respectively, with those mentioned in literature. Equally informative data can be obtained by spectroscopic analysis in UV-Vis and infrared region, followed by nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS) measurements. Absorption maxima of different classes of secondary metabolites are mentioned in table-6. X-ray crystallography can also be used if the compound is available in sufficient amount and in crystalline form [16]. Antibacterial terpenes have been isolated and identified with NMR spectroscopy from *Commiphora molmol* [6]. Identification of vibriocidal compounds through HPLC has been reported [5]. Gas chromatography has been

used for identifying components in leaves and seeds of *Syzium cumini* [94].

FUTURE PROSPECTIVES

Despite the extensive use of plants to cure diseases, many plant species yet remain to be investigated for their medicinal value [19]. The increasing development of drug resistance in human pathogens and because of the adverse effects of certain antimicrobial agents, there is an urgent need to search for new antimicrobial agents [38]. Increasing drug resistance among microorganisms is due to genetic selection because of over exposure to antimicrobials agent [95]. The diversity of bioactive compounds found in plant species makes them promising sources of new antimicrobial agents which will be safer, more dependable and cost effective compared to synthetic drugs [96].

An alternative approach for safe storage of foodstuffs and grains besides the use of synthetic chemical antimicrobial agents is necessary because of the associated risk of toxicity to humans. Fungi and bacteria are significant destroyers of foodstuffs and grains during storage, rendering them unfit for human consumption by reducing their nutritive value and often by producing mycotoxins as in case of fungi. Plant based antimicrobial agents appear to be one of the better alternatives as they are known to have minimal environmental impact and danger to consumers in contrast to the synthetic pesticides and preservatives [46].

If any of the antimicrobial agent(s) isolated from the plants are likely to be toxic upon oral administration to humans, still these agents can be considered for topical application on animate and/or inanimate surfaces [97].

Antimicrobial agents obtained from plants can also be used in veterinary medicine and animal husbandry to eradicate various microbial infections. They can also be used in agriculture to control bacterial and fungal diseases of plants. Other uses of plant antimicrobials are in aquaculture, disinfectants, sanitizers, biocides and wood preservation [95]. Besides antimicrobial potential, active component(s) isolated from plant extracts are known to posses other therapeutic properties such as anticancer activity, antioxidant activity, antihepatotoxic activity [19].

Metabolic engineering of plants for increased biosynthesis of antimicrobial compounds may open a new vista for newer therapeutic agents. Metabolic engineering involves modification of given metabolic pathway or introduction of new pathways so as to increase the production of the compound sought. One of the successful examples of metabolic engineering in plants is the creation of transgenic rice with the antimicrobial peptide defensin from *Brassica*, which act against the fungus *Magnaporthe grisea*, causing rice blast disease [98]. Metabolic engineering was also practiced to enhance the production of enzyme oxidosqualene cyclase that catalyzes biosynthesis of antifungal triterpenoid, saponin, in oat roots [99]. Pathway engineering can be expected to help in development of plants with an improved spectrum of secondary metabolites, including those with antimicrobial activity, which will require expansion of our knowledge of biosynthetic pathways in plants at enzymatic level [15].

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ABBREVIATIONS:

MAE: Microwave assisted extraction;
AST: Antimicrobial susceptibility tests;
MIC: Minimum inhibitory concentration;
TLC: Thin layer chromatography;
HPLC: High pressure liquid chromatography