Antimicrobial activity of phytopharmaceuticals for prevention and cure of diseases

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Abstract
Plants have been used for thousands of years to treat various health disorders and to prevent diseases including epidemics. Over the centuries within and among human communities, the knowledge of their healing properties has been transmitted. Attention has been drawn towards the studies on the potential antimicrobial activity of plant-derived substances by pharmaceutical and scientific communities, due to the challenge of growing incidence of drug-resistant pathogens. Recently, focus has been established on the effect of the plant extracts in terms of their microstatic and microcidal action and the spectrum of organism which has enabled exploitation of plants for the treatment of microbial infections in the development of new antimicrobial agents. Antimicrobial action of the plant extract is largely dependent on the techniques employed in the investigations and conclusive results can only be obtained, if methods are standardized and universal. This review focuses on medicinal plants and their active compounds as a useful source of antimicrobial agents and current methods used in the investigation of the efficacy of plants as antimicrobial agents.

Key words: Medicinal plants, phytochemistry, antimicrobial activity, plant extracts, antimicrobial techniques

Introduction
Medicinal plants are used for treating various diseases since prehistory. Around, 250 to 500 thousand plant species are estimated to be present on the planet, out of which 1 to 10% of them are used as food by human beings and other animals (Cowan, 1999). According to World Health Organization (WHO), 80% of the world’s population depends mainly on traditional medicines and use plant extracts in traditional treatments (WHO, 1993). This is commonly found in rural areas where synthetic drugs are not available or too expensive to purchase. Some of the crude drugs used in past are still in use in phytotherapeutics. Cinchona plant is often used in its natural and unrefined form to treat malaria (Trease and Evans, 1985), although some other herbal drugs are refined by isolating their pure active compounds and their active principles, helped in the advancement of scientific medicine. 20th century, the antibiotic era substantially reduced the threat of infectious diseases, but over the years there is a decrease in microbial susceptibility to existing microbial agents, responsible for drug resistance which is exerting global problem today. Infact, the theme of World Health Day 2011 was: no action today, no cure tomorrow. There is an urgent need of new antimicrobial agents to overcome this global problem (Gould, 2008). It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin. Each year, two million people are admitted with bacterial infection in US hospitals, 70% of the cases involve strains that are resistant to atleast one drug (Infectious Diseases Society of America, 2004). Higher plants are a rich source of antibiotics (Trease and Evans, 1972), the antibiotic action of alliline from Allium sativum (garlic), or the antimicrobial action of berberine from...
Brief history

Since the dawn of humanity, war against disease has been a part of everyday life and the use of plant materials to treat the sickness is as old as man (Longer and Robinson, 1985). The Ancient Egyptians in 1500 BC wrote the “Ebers Papyrus” which contain information on around 850 plant medicines, including garlic, juniper, cannabis, castor bean, aloe, and mandrake (Sumner and Judith, 2000). In India, earliest Sanskrit writings such as the Rig Veda, and Atharva Veda are some of the earliest available documents, detailing the medical knowledge that formed the basis of the Ayurveda system (Sumner and Judith, 2000). Ayurveda medicine has used herbs such as turmeric possibly as early as 1900 BC (Aggarwal et al., 2007) and many other herbs and minerals in Ayurveda are later described by ancient Indian herbalists such as Charaka and Sushruta during the 1st millennium BC. Shennong, the mythological Chinese Emperor is said to have written the first Chinese pharmacopoeia, the “Shennong Ben Cao Jing” which contain a list of 365 medicinal plants and their uses - including Ephedra (the shrub that introduced the drug ephedrine to modern medicine), hemp, and chaumoolgra (one of the first effective treatments for leprosy) (Sumner and Judith, 2000). In the late fifth century B.C., Hippocrates wrote “De Materia Medica” in the first century A.D., a medicinal plant catalog which became the prototype for modern pharmacopoeias. During the Early Middle Ages, Benedictine monasteries were the primary source of medical knowledge in Europe and England (Ardall and Anne, 2002). A 12th-century Benedictine nun wrote a medical text called Causae et Curae (Ramos-e-Silva, 1999). The 15th, 16th, and 17th centuries were the great age of herbalists, many of them available for the first time in English and other languages rather than Latin or Greek and the first herbal to be published in English was the anonymous Grete Herball of 1526. In the modern era, pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis, and quinine. Pharmacologists, microbiologists, botanists, and natural-products chemists are combing the Earth for phytochemicals that can be used for the treatment of various diseases. According to the World Health Organisation, approximately 25% of modern drugs used in the United States have been derived from plants. Around 7,000 medical compounds in the modern pharmacopoeia are derived from plants (Interactive European Network for Industrial Crops and their Applications, 2000-2005).

Antimicrobial properties of medicinal plants

Plant-derived substances have recently gained great interest due to their versatile applications (Baris et al., 2006). Medicinal plants are the richest bioresource of drugs of modern medicines, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer et al., 1999). Baccharis trimera Less “carqueja” showed antimicrobial action on both Gram-positive (Staphylococcus aureus and Streptococcus uberis) and Gram-negative (Salmonella gallinarum and Escherichia coli) bacterial strains and it was found that the former microorganisms are more sensitive to this herb than the latter, which corroborates previous studies (Avancini et al., 2000). Essential oil obtained from stem and leaves of Achillea millefolium showed higher antimicrobial activity than its respective extracts. These oils prevented the growth of Streptococcus pneumoniae, Clostridium perfringes and Candida albicans and inhibited Mycobacterium smegmatis, Acinetobacter Iwoffii and Candida krussei to a lesser extent (Candan et al., 2003). The phenolic compounds present in ethanol extract of chamomile showed antibacterial properties against Staphylococcus aureus (Asolini et al., 2006). Essential oils from Pelargonium graveolens (geranium) showed low values of minimum inhibitory concentration against B. cereus (0.36 mg/ml), B. subtilis (0.72 mg/ml) and S. aureus (0.72 mg/ml), whereas oils from Origanum vulgare (oregano) also showed antimicrobial activity against the same bacteria, in addition to Escherichia coli. However, a concentration of 0.35 mg/ml is required to inhibit B. subtilis whereas 0.70 mg/ml is necessary to inhibit the other bacteria (Rosato et al., 2007) and Centella asiatica L. (Suryakala, 2013). The hydroalcoholic extract of Rosmarinus officinalis Linn. (rosemary) showed antimicrobial activity against Streptococcus mitis, Streptococcus sanguinis, Streptococcus mutans, Streptococcus sobrinus and Lactobacillus casei except S. mitis (Silva et al., 2008). Phytochemicals of plant origin with excellent antimicrobial activity are summarized in Table 1.
Table 1: Plants with antimicrobial activity

<table>
<thead>
<tr>
<th>S.No</th>
<th>Scientific name</th>
<th>Compound</th>
<th>Class</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Abrus precatorius</em> L.</td>
<td>Hypaphorine</td>
<td>Alkaloids</td>
<td><em>K. pneumonia</em>, <em>S. aureus</em>, <em>Mycobacterium aurum</em></td>
<td>Balungile et al., 2013</td>
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<td></td>
<td></td>
<td>Isohemiphloin</td>
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<td>2.</td>
<td><em>Adesmia aegiceras</em></td>
<td>Quercetin</td>
<td>Polyphenols</td>
<td><em>E. coli</em>, <em>S. aureus</em>, <em>E. coli</em>, <em>S. aureus</em>, <em>S. saprophyticus</em>, <em>P. aeruginosa</em>, <em>P. mirabilis</em></td>
<td>Agnese et al., 2001</td>
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<tr>
<td></td>
<td></td>
<td>Chlorogenic acid</td>
<td></td>
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<td></td>
<td></td>
<td>Isovitexin</td>
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<td>3.</td>
<td><em>Allium cepa</em> L.</td>
<td>Allicin</td>
<td>Sulfoxide</td>
<td><em>Bacteria</em></td>
<td>Vohora et al., 1973</td>
</tr>
<tr>
<td>4.</td>
<td><em>Allium sativum</em> L.</td>
<td>Allicin, Ajoene</td>
<td>Sulfoxide</td>
<td><em>B. subtilis</em>, <em>S. aureus</em>, <em>Mycobacterium phlei</em></td>
<td>Naganawa et al., 1996; San-Blas et al., 1993; San-Blas et al., 1989</td>
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<td>5.</td>
<td><em>Aframomum albiovioleaceum</em> (Ridl.) k schum.</td>
<td>Essential oil</td>
<td>Terpenoid</td>
<td><em>E. coli</em>, <em>E. aerogenes</em>, <em>E. cloacae</em>, <em>K. pneumonia</em>, <em>P. stuartii</em>, <em>P. aeruginosa</em></td>
<td>Doriane et al., 2013</td>
</tr>
<tr>
<td>6.</td>
<td><em>Anthemis xylopoda</em> O. schworz</td>
<td>Essential oils</td>
<td>Terpenoid</td>
<td><em>S. aureus</em>, <em>E. fecalis</em>, <em>B. cereus</em>, <em>B. subtilis</em>, <em>E. coli</em>, <em>K. pneumonia</em>, <em>P. vulgaris</em>, <em>P. aeruginosa</em>, <em>P. fluorescens</em>, <em>S. typhimurium</em></td>
<td>Uzel et al., 2004</td>
</tr>
<tr>
<td>7.</td>
<td><em>Ardrographis paniculata</em> (Burm.f.) Wall ex.Nees</td>
<td>Tannic acid, Gallic acid, Caffeic acid, Vanilli C acid, Ferulic acid, Chlorogenic acid, Cinnamic acid, Salicylic acid</td>
<td>Phenolic acid</td>
<td><em>E. coli</em>, <em>P. aeruginosa</em>, <em>K. pneumonia</em>, <em>S. typhimurium</em>, <em>E. cloacae</em>, <em>S. aureus</em>, <em>B. subtilis</em>, <em>E. fecalis</em>, <em>S. epidermis</em></td>
<td>Somaroy et al., 2010</td>
</tr>
<tr>
<td></td>
<td><strong>Plant</strong></td>
<td><strong>Compounds</strong></td>
<td><strong>Microorganisms</strong></td>
<td><strong>References</strong></td>
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<td></td>
<td>viz., <em>A. scoparia</em>, <em>A. turanica</em> and <em>A. oliveriana</em></td>
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<td>17.</td>
<td><em>Curcuma longa</em> L.</td>
<td>Curcumin Turmeric oil</td>
<td>Terpenoids</td>
<td><em>B. subtilis</em>, <em>M. grisea</em></td>
<td>Apisaryiyakul <em>et al</em>., 1995</td>
</tr>
<tr>
<td>21.</td>
<td><em>Ixora coccinea</em> L.</td>
<td>Lupeol, Quercitin, Gentisic</td>
<td>Ester, Flavonoid, Phenolic acid</td>
<td><em>E. coli</em>, <em>P. aeruginosa</em>, <em>S. typhimurium</em>, <em>S. aureus and B. subtilis</em>.</td>
<td>Annapurna <em>et al.</em>, 2003</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Component</td>
<td>Type</td>
<td>Bacteria</td>
<td>Reference</td>
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<tr>
<td>34.</td>
<td><em>Quercus rubra</em></td>
<td>Quercetin</td>
<td>Flavonoid</td>
<td><em>E. faecalis</em>, <em>B. cereus</em>, <em>S. epidermidis</em>.</td>
<td>Keating and Kennedy, 1997</td>
</tr>
<tr>
<td>36.</td>
<td><em>Rhynchosia beddomei</em></td>
<td>Essential oils</td>
<td>Terpenoids</td>
<td><em>B. subtilis</em>, <em>S. aureus</em>, <em>E. coli</em>, <em>P. aeruginosa</em>.</td>
<td>Bakshu and Venkat Raju, 2001</td>
</tr>
<tr>
<td>37.</td>
<td><em>Rubia peregrina</em> L.</td>
<td>Pentacyclic triterpenes</td>
<td>Terpenes peptide</td>
<td><em>B. subtilis</em>, <em>S. aureus</em>, <em>E. coli</em>.</td>
<td>Ozgena et al., 2003</td>
</tr>
<tr>
<td>40.</td>
<td><em>Satureja montana</em></td>
<td>Carvacrol</td>
<td>Terpenoid</td>
<td><em>S. typhi murium</em>.</td>
<td>Ali-Shtyeh et al., 1997</td>
</tr>
<tr>
<td>41.</td>
<td><em>Satureja khuzestanica</em> L.</td>
<td>Essential oils</td>
<td>Terpenoids</td>
<td><em>S. aureus</em>.</td>
<td>Amanloua et al., 2004</td>
</tr>
<tr>
<td>42.</td>
<td><em>Scutellaria barbala</em></td>
<td>Essential oils</td>
<td>Terpenoids</td>
<td><em>S. aureus</em>, <em>E. coli</em>, <em>P. aeruginosa</em>, <em>S. epidermidis</em>, <em>S. heamolyticus</em>, <em>S. simulans</em>, <em>E. fæcalis</em>, <em>C. freundii</em>, <em>K. pneumoniae</em>, <em>S. flexneri</em>, <em>S. typhi</em>, <em>S. paratyphi-A</em>, <em>S. liquefaviens</em>, <em>S. marcescens</em>, <em>S. maltophilia</em>.</td>
<td>Yu et al., 2004</td>
</tr>
<tr>
<td>43.</td>
<td><em>Salacia oblonga</em> Wall.</td>
<td>Salicic acid di-ethyl bis (tri-methylsilyl) ester</td>
<td>Aliphatic ester</td>
<td><em>B. subtilis</em>, <em>E. aerogene</em>, <em>E. fæcalis</em>, <em>E. coli</em>, <em>K. pneumoniae</em>.</td>
<td>Anjaneyulu et al., 2013</td>
</tr>
</tbody>
</table>
### Antimicrobial compounds and their mode of action on microbes

Plant-derived compounds with therapeutic value are mostly plant secondary metabolites which are traditionally used for medicinal purposes. They exhibit a wide range of activity according to the species, the topography and climatic condition. Plants contain a wide array of active principles (Assob et al., 2011; Arruda et al., 2011), different group of secondary metabolites display varying pharmacological activities.

**Phenolics**

Phenolic compounds are one of the most diverse groups of secondary metabolites, found in plants and also found in fruits, vegetables, nuts, seeds, stems and flowers as well as

<table>
<thead>
<tr>
<th>No.</th>
<th>Species/Genus</th>
<th>Compound Type</th>
<th>Active Principle</th>
<th>Antimicrobial Activity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>44.</td>
<td><em>Solanum aculeastrum</em></td>
<td>Essential oils</td>
<td>Terpenoids</td>
<td><em>S. epidermis</em>, <em>S. aureus</em>, <em>B. subtilis</em>, <em>K. pneumoniae</em>, <em>P. aeruginosa</em>, <em>E. coli</em>, <em>E. fecalis</em>.</td>
<td>Wanyonyi et al., 2003</td>
</tr>
<tr>
<td>45.</td>
<td><em>Solidago virgaurea</em> L.</td>
<td>Quercetin, Kaempferol</td>
<td>Polyphenol Flavonoid</td>
<td><em>S. epidermis</em>, <em>S. aureus</em>, <em>B. subtilis</em>, <em>K. pneumoniae</em>, <em>P. aeruginosa</em>, <em>E. coli</em>, <em>E. fecalis</em>.</td>
<td>Thiem and olga, 2002</td>
</tr>
<tr>
<td>46.</td>
<td><em>Strobilanthes callosus</em> (Nees)</td>
<td>Triterpenoids</td>
<td>Terpenoid</td>
<td><em>E. coli</em>, <em>S. aureus</em>, <em>K. pneumoniae</em>, <em>E. coli</em>, <em>E. fecalis</em>.</td>
<td>Singh et al., 2002a</td>
</tr>
<tr>
<td>47.</td>
<td><em>Vaccinium</em> spp.</td>
<td>Fructose</td>
<td>Monosaccharide</td>
<td><em>E. coli</em>.</td>
<td>Ofek et al., 1996</td>
</tr>
<tr>
<td>48.</td>
<td><em>Viola tricolor</em> L.</td>
<td>Vitri peptide A</td>
<td>Cyclic peptide</td>
<td><em>S. epidermis</em>, <em>S. aureus</em>, <em>B. cereus</em>, <em>K. pneumoniae</em>, <em>P. aeruginosa</em>, <em>E. coli</em>, <em>E. fecalis</em>.</td>
<td>Witkowska et al., 2005</td>
</tr>
</tbody>
</table>
Mechanisms of action of organic acids in a bacterial cell. This might be due to the interaction of cinnamaldehyde with the cell membrane, which may cause enough disruption to disperse the proton motive force by leakage of small ions but without leakage of larger cell molecules such as ATP (Raybaudi-Massilia et al., 2009). Organic acids are usually employed in food preservation because of their effects on bacteria. The mode of action of organic acids on bacteria is non-dissociated (non-ionized) and can penetrate the bacterial cell wall and disrupt the normal physiology of certain types of bacteria that is called pH-sensitive, which cannot tolerate a wide internal and external pH gradient (Figure 1).

Figure 1: Mechanisms of action of organic acids in a bacterial cell (Raybaudi-Massilia et al., 2009).

The passive diffusion of organic acids into the bacteria cause dissociation of acids and lower the bacterial internal pH which leads to the situations which impairs or stops the growth of bacteria. On the other hand, the anionic part of the organic acids that cannot escape the bacteria in its dissociated form, gets accumulated within the bacteria and disrupt many metabolic functions, leading to osmotic pressure increase, that is incompatible for bacterial survival. Essential oils, which are concentrated hydrophobic liquid containing volatile aroma compounds from plants, provide a wide research area. Burt (2004) reported that the antibacterial activity of the essential oils is not carried out by one specific mechanism but acts over several specific targets in the cell. Skandamis et al. (1999) have reported the location and mechanism of action of essential oils on bacterial cell such as : degradation of the cell wall, damage to cytoplasmic membrane and membrane proteins (Lambert et al., 2001), leakage of contents out of the cell, coagulation of cytoplasm (Burt, 2004), and depletion of the proton motive force (Figure 2). Raybaudi-Massilia et al. (2009) reported that the mode of action of essential oils depends on the concentration of phytochemicals, indicating that the low concentrations inhibit enzymes associated with energy production, while higher amounts may precipitate proteins.
Flavonoids are phenolic structures, ubiquitous in photosynthesizing cells (Havsteen, 1983) and are commonly found in fruits, vegetables, nuts, seeds, stems, flowers, tea, wine (Middleton et al., 1986), propolis and honey (Grange and Davey, 1990), and represent a common constituent of the human diet (Harborne and Baxter, 1999). The function of flavonoids in flowers is to provide colours, attractive to plant pollinators and in leaves, this compound is believed to promote physiological survival of the plant and protects them from fungal pathogens and UV-B radiation (Harborne and Williams, 2000). Flavonoids are involved in photosensitisation, energy transfer, control of respiration and photosynthesis, morphogenesis and sex determination. (Harborne and Williams, 2000). Flavonoids have been reported to possess many useful properties, viz., anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity (Havsteen, 1983), antiallergic activity, antioxidant activity (Middleton et al., 1986) and cytotoxic antitumour activity (Harborne and Williams, 2000) for which it has become the subject of medical research. For centuries, preparations containing flavonoids are used by physicians in an attempt to treat human diseases (Havsteen, 1983). For example, the plant Tagetes minuta (containing quercetatetgin-7-arabinosyl-galactoside) has been used extensively in Argentine folk medicine to treat infectious disease (Tereschuk et al., 1997). The basic structural feature of flavonoid compounds is the 2-phenyl-benzo pyrane or flavane nucleus, which consists of two benzene rings (A and B), linked through a heterocyclic pyrane ring (C) (Figure 3) (Brown, 1980). Structures of some of the major classes of flavonoids are given in Figure 4.

**Figure 2:** Mechanisms of action of essential oils and their components in a bacterial cell (Burt, 2004).

**Figure 3:** The structure of the flavones (a class of flavonoids) (Harborne and Baxter, 1999).

**Flavonoids as antimicrobial agents**

Flavonoids are being increasingly documented due to their antibacterial activity. Hypericum (Dall’Agnol et al., 2003), Capsella and Chromolaena (El-Abyad et al., 1990) are reported to possess antibacterial activity that are rich in flavonoids. Flavonoids having antibacterial activity have been isolated and identified by many research groups, viz., apigenin, galangin, genkwanin, sophora flavanone G and its derivatives and other flavones such as flavone glycosides, isoflavones, flavanones, isoflavonones, isoflavans, flavonols, flavonol glycosides and chalcones.

**Mechanisms of action of various flavonoids**

In a study, using radioactive precursors showed that DNA synthesis was strongly inhibited by flavonoids such as robinetin, myricetin and (‘)-epigallocatechin in Proteus vulgaris, whilst RNA synthesis was most affected in S. aureus. Protein and lipid synthesis were also affected but to a lesser extent. The inhibitory action on DNA and RNA synthesis depends on the B ring of the flavonoids that play a major role in intercalation or hydrogen bonding with the stacking of nucleic acid bases. Ohemeng et al. (1993) screened 14 flavonoids of varying structure to determine the inhibitory activity against Escherichia coli DNA gyrase, and for antibacterial activity against Staphylococcus epidermidis, E. coli, S. aureus, Stenotrophomonas maltophilia and S. typhimurium. It was found that E. coli DNA gyrase was inhibited to different extents by seven compounds, including quercetin, apigenin and 3,6,7,3X,4X-pentahydroxyflavone. Interestingly, enzyme inhibition was limited to those compounds with B-ring hydroxylation, with the exception (Ohemeng et al., 1993) of 7, 8-dihydroxyflavone. The authors proposed that the antibacterial activity of flavonoids was due to their inhibition of DNA gyrase. However, since the level of antibacterial activity and enzyme inhibition did not correlate always, it was suggested that other mechanisms may also be involved. A research team previously had found sophoraflavanone G to have intensive antibacterial activity against MRSA and Streptococci which made them to elucidate the mechanism of action of this flavanone recently. Using liposomal model membranes, the effect of sophoraflavanone
G on membrane fluidity was studied and compared with the less active flavanone naringenin, which lacks 8-lavandulyl and 2α-hydroxyl groups. Sophorahflavone G was shown to increase fluorescence polarisation of the liposomes significantly at concentrations corresponding to the MIC values which indicated an alteration of membrane fluidity in hydrophilic and hydrophobic regions, suggesting that sophorahflavonone G reduced the fluidity of outer and inner layers of membranes. Naringenin also exhibited a membrane effect but at much higher concentrations. This correlation between antibacterial activity and membrane interference suggest that sophorahflavonone G exhibit antibacterial activity by reducing membrane fluidity of bacterial cells. In addition, galangin which effect the cytoplasmic integrity in *S. aureus*, has been investigated by measuring loss of internal potassium. When high cell densities of *S. aureus* were incubated for 12h in media containing 50 g/ml of the flavonol, a 60-fold decrease in the number of CFUs was noted and cells lost ca. 20% more potassium than untreated control bacteria which strongly suggest that galangin induces cytoplasmic membrane damage and potassium leakage. Flavonoids that interfere with energy metabolism was demonstrated by Merck Research Laboratories which recently reported that the flavanone lonchocarpol A inhibits macromolecular synthesis in *Bacillus megaterium* by using radioactive precursors. It was demonstrated that DNA, RNA, cell wall and protein synthesis were all inhibited at concentrations similar to the MIC value.

**Alkaloids**

Alkaloids are heterocyclic nitrogen compounds which show different antimicrobial activities. The analysis of the leaf extracts of *Gymnema montanum* (Ramkumar et al., 2007) and of ethanol extract of *Tabernaemontana catharinensis* root bark revealed an antimicrobial activity in the first case due to an activity, depending upon the chemical composition of the extracts and membrane permeability of the microbes, and in the second case linked to indole alkaloids responsible for the observed antibacterial and antidermatophytic activity. Diterpenoids show antimicrobial properties which are commonly found in plants of the Ranunculaceae group. Berberine which is a hydrophobic cation and an isoquinoline alkaloid, present in roots and stem-bark of *Berberis* species are widely used in traditional medicine, owing to its activity against bacteria, fungi, protozoa and viruses (Kim et al., 2002). It is an excellent DNA intercalator and accumulates in cells driven by the membrane potential (Iwasa et al., 2001). These compounds are active on several microorganisms and target on RNA polymerase, gyrase and topoisomerase IV and on nucleic acid.

**Terpenes**

Terpenes are compounds also referred to as isoprenoids, and their derivatives, containing additional elements, usually oxygen, are called terpenoids. The antibacterial activity of some monoterpenes (C10), diterpenoids, sesquiterpenes (C15), triterpenoids and their derivatives was recently reviewed (Suryakala and Giri, 2013). The results obtained, illustrate the strong structure-function influence of the antibacterial potential of terpenes. Diterpenoids, and sesquiterpenes, isolated from different plants inhibited the growth of *M. tuberculosis* (Garcia et al., 2012; Kurek et al., 2011) and exhibited bactericidal activity against Gram-positive bacteria. The mechanism of action of terpenoids is not fully understood, but is speculated to involve membrane disruption by the lipophilic compounds.

**Coumarins**

One known coumarin, scopoletin, and two chalcones were isolated as antitubercular constituents of the whole plant, *Fatoua pilosa*. The antibacterial effectiveness of spices and aromatic plants depend on the kind of plant, its composition and concentration of essential oils. The importance of aroma precursors (cysteine sulfoxides) as a potent biologically active agents was revealed in accordance with the popular use of plants belonging to the *Allium* genus in traditional medicine by analyzing the antimicrobial activity of essential oil of *Allium sphaerocephalon* inflorescences (Lazarevic et al., 2011).

**Experimental approaches**

Outcome of susceptibility tests and standardized methods are affected by multiple factors and are more likely to be reproducible than unstandardized methods. When dealing with plant extracts, sometimes it is difficult to compare the results obtained with published results in the literature because several variables influence the results, such as the environmental and climatic conditions under which the plant grew, choice of plant extracts, choice of extraction method, antimicrobial test method and test microorganisms (Nostro et al., 2000; Hammer et al., 1999).

**Plant extract preparation**

Pharmaceutically, extraction methods involve separation of medicinally active components by using selective solvents with appropriate extraction technology. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity (Green, 2004). The quality of an extract is influenced by the basic parameters such as: a) the plant part used as starting material, b) the solvent used for extraction and c) the extraction technology. Effect of plant material depends on the nature of the plant material; its origin; degree of processing; moisture content and particle size, while variations in extraction method include type of extraction; time of extraction and temperature. The concentration, polarity and the nature of solvent affects the quantity and secondary metabolite composition of an extract.
Figure 4: (Tim Cushnie et al., 2005): The skeleton structures of the main classes of flavonoids: aurones (Muziol et al., 2001; Villemin et al., 1998), isoflavones (Havsteen, 1983), chalcones (Harborne and Baxter, 1999; Xu and Lee, 2001), flavanones (Havsteen, 1983; Harborne and Baxter, 1999), flavones (Havsteen, 1983), flavonols (Havsteen, 1983), flavanon-3-ols (Harborne and Baxter, 1999), anthocyanidins (Harborne and Baxter, 1999, Middleton et al., 2000), flavan-3-ols (Havsteen, 1983; Harborne and Baxter 1999), proanthocyanidins (occur as dimers, trimers, tetramers and pentamers; R = 0, 1, 2 or 3 flavan-3-ol structures) (Harborne and Baxter, 1999), flavans (Harborne and Baxter, 1999), flavan-3,4-diols (Harborne and Baxter, 1999) and dihydrochalcones (Harborne and Baxter, 1999).
Figure 5: Chemical structures of some antimicrobial compounds (Cowan, 1999)

**Plant material**

As a source for secondary plant components, fresh or dried plant material can be used. However, dried plant material is used by most of the scientists, working on the chemistry of secondary plant components. Differences in water content may affect solubility of subsequent separation by liquid-liquid extraction and the secondary metabolic components should be relatively stable, especially if it is to be used as an antimicrobial agent. Furthermore, many plants are used in
the dry form or as an aqueous extract by traditional healers. Plants are usually air dried (Dilika et al., 1996; Baris et al., 2006) but some researchers, dry the plants in the oven at about 40°C for 72h (Salie et al., 1996). Plants differ in their constituents, depending on the climatic conditions in which they are growing. The choice of plant material used in the extract preparation is usually guided by the traditional use of the plant and the ease of handling of the different plant parts like the leaves, stems etc.

**Choice of solvent**

The choice of solvent is influenced by what is intended with the extract. Since the end product contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay. The choice will also depend on targeted compounds. In a study, aqueous acetone was better at extracting total phenolics than aqueous methanol. In another study, out of twenty different solvents, chloroform was found to be the best solvent for the extraction of non-polar, biologically active compounds from the roots of Angelica archangelica (Harmala et al., 1992). For general phytochemical analysis, the larger the variety of compounds, the extractant will extract the better, because there is a better chance that biologically active compounds will be present in the extract (Eloff, 1998a). Compared to water extracts, plant extracts from organic solvents have been found to give more consistent antimicrobial activity (Parekh et al., 2005). Methanol, ethanol, and water are the most commonly used solvents for investigations of antimicrobial activity in plants (Parekh et al., 2005; Bisignino et al., 1999; Lourens et al., 2004; Salie et al., 1996; Rojas et al., 2006). Dichloromethane has also been used by a number of researchers (Dilika et al., 1996; Freixa et al., 1996). Some authors use different solvents in a combination to obtain the best solvent systems for extraction (Nostro et al., 2000). Acetone, although not a very commonly used solvent, has been used by a number of authors (Dilika et al., 1996; Lourens et al., 2004; Mathkega, 2001). In a study, Masokoa et al. (2007) investigated the antifungal activity of Combretum species in which, the extractants used include hexane, dichloromethane, acetone and methanol. They discovered that more chemical compounds from the leaves are extracted by acetone and methanol than other solvents. Saponins are extracted by both acetone and methanol which have antimicrobial activity. Eloff (1998a) studied a variety of extractants for their ability to solubilise antimicrobials from plants. Rate of extraction, ease of removal, toxicity in bioassay, acetone received the highest among all other solvents and gave the lowest minimum inhibitory concentration for Gram-positive organisms tested and the largest number of different components and inhibitors from two plants tested. However, different results may be obtained with other plants and generalization can not be made on the usefulness of acetone as an extractant (Eloff, 1998a).

**The extraction methods**

Variations in extraction methods are due to the extraction period, solvent used, pH, temperature, particle size and the solvent-to-sample ratio. Homogenization in solvent is the widely used method by researchers investigating antimicrobial activity (Meyer and Dilika, 1996; Parekh et al., 2005). Dried plant material is ground in a blender, put in solvent and shaken vigorously for 5 min or left for 24 h after which, the extract is filtered and fresh solvent added to the residue for another 24 h. To obtain an epicuticular extract, some authors suggested the shaking of unhomogenized dry leaves in solvent for about 5 min, followed by filtering and concentrating under reduced pressure (Mathkega, 2001) which actually gave a higher yield and bioactivity than using homogenized (macerated) extract (Dilika et al., 1996; Mathkega, 2001). Of interests are the results, obtained in a study by Meyer and Dilika et al. (1996), using these methods on the same plant found that the homogenized dichloromethane extract generally had higher activity than the shaken extract of the same solvent.

**Antibacterial susceptibility tests**

Antibacterial susceptibility testing are performed by either dilution or diffusion methods. The choice of methodology are based on many factors such as relative ease of performance, flexibility, use of automated or semi-automated devices for both identification and susceptibility testing. AST standard tests are conveniently divided into diffusion and dilution methods. Diffusion tests usually include agar well diffusion, agar disk diffusion and bioautography, while dilution methods include agar dilution and broth microdilution. Conventional reference methods for AST are broth and agar based methods (Tenover et al., 1995).

**Agar disk diffusion assay**

Agar diffusion techniques have been widely used to evaluate antimicrobial activity of plant extracts (Freixa et al., 1996; Salie et al., 1996), although there are limitations with the technique. For identification of leads, disk diffusion techniques are suitable but not effective for quantification of bioactivity (Hammer et al., 1999; Nostro et al., 2000; Langfield et al., 2004), which generally do not distinguish bactericidal and bacteriostatic effects. Since MIC can not be determined by these techniques, it is usually used for preliminary screening (Parekh et al., 2005; Tepe et al., 2004). Some researchers have reported that by the agar diffusion method, MIC values can be obtained (Leite et al., 2006), although high activity in the disk diffusion assay does not necessarily correlate to low MIC values in the microtiterplate method (Lourens et al., 2004). The agar disk diffusion technique can only be applied to determine the AST of pure substances because the results may be unreliable when it is applied to mixtures, containing different constituents, which exhibit different diffusion rates (Silva et al., 2005). In this method,
6 mm paper disks saturated with filter sterilized (Salie et al., 1996) plant extract at the desired concentration, are placed onto the surface of a suitable solid agar medium. Usually Muller Hinton is the choice of medium by most of the researchers, although, tryptone soy agar (Lourens et al., 2004) or nutrient agar (Doughari, 2006) have sometimes been used by other researchers. Some authors have reported the inoculum sizes of $1 \times 10^5$ cfu/ml of bacteria to pre-inoculate the media for inoculating diffusion plates (Baris et al., 2006).

Some impregnate the disk before placing on the agar (Lourens et al., 2004; Salie et al., 1996), while others place the disk on the plate first before impregnating (Nostro et al., 2000; Baris et al., 2006). In a report by Mbata et al (2006), the paper disks were soaked in the leaf extract for about 2 h. Basri and Fan (2005) left the disks to dry under a laminar flow cabinet overnight while other authors refrigerate the plates for an hour or two at 4°C to allow pre-diffusion of the extracts from the disk into the seeded agar layer before incubation (Lourens et al., 2004; Tepe et al., 2004; Schmourlo et al., 2004). The plates are then incubated at 37°C for 24 h for bacteria and 48 h for fungi (Salie et al., 1996; Baris et al., 2006). Zones of inhibition are then measured from the circumference of the disks to the circumference of the inhibition zone (Salie et al., 1996).

**Agar well diffusion**

The agar well diffusion is similar to that of the agar disk diffusion method. A standardized inoculum culture is spread evenly on the surface of gelled agar plates. Using a sterile cork borer wells of between 6 and 8 mm are aseptically punched on the agar, allowing at least 30 mm between adjacent wells and the petridish. Fixed volumes of the plant extract are then introduced into the wells and incubated at 37°C for 24 h for bacteria (Mbata et al., 2006).

**Bioautography**

In this method, the analyte is adsorbed onto a Thin Layer Chromatography (TLC) plate which is different from that of agar diffusion method. Bioautography is a preliminary phytochemical screening technique used to detect active components (Nostro et al., 2000; Schmourlo et al., 2004). It also overcomes the challenge of isolating antimicrobial compounds from crude extracts with complex chemical components. This method relatively uses very little amount of sample and also determine the polarity of the active compounds (Runyoro et al., 2006). In a study by Silva et al. (2005), it was concluded that bioautography is a practical, reproducible test and easy to perform when they compared different methods of AST. In the bioautography agar overlay method, the extract of determined amount is applied to silica 60 gel plates and developed with an appropriate solvent system and a suspension of the test bacteria is sprayed onto the TLC plate and incubated at 25°C for 48 h in humid conditions. Microbial indicators (usually tetrazolium salts) are used as a growth detector (Silva et al., 2005) and are sprayed onto the plates after which the plates are reincubated at 25°C for 24 h (Dilika et al., 1996) and the clear (white) zones on the TLC plate indicate antimicrobial activity of the extracts. Some authors state that this method is not an ideal method for the quantification of bioactivity of plant extracts because TLC causes disruption of synergism between active constituents in an extract, thereby reducing its activity (Schmourlo et al., 2004).

**Broth microdilution**

For determining MICs of large numbers of test samples, the microtitre plate or broth microdilution method have been proved to be a potentially useful technique. This technique has advantages over diffusion techniques because of its increased sensitivity for small quantities of extract, ability to distinguish between bacteriostatic and bactericidal effects and quantitative determination of the MIC (Langfield et al., 2004). This method is inexpensive and presents reproducible results and can also be used for a wide variety of microorganisms. In the microtitre plate method, a stock solution of the extract is obtained in solvent usually used for extraction (Grierson and Afolayan, 1999) or in DMSO (Salie et al., 1996; Nostro et al., 2000; Baris et al., 2006). Most authors prefer filter sterilizing with a 0.22 or 0.45 μm membrane filter before the procedure (Meyer and Afolayan, 1995; Kianbakht and Jahaniani, 2003). As diluents in the wells of the microtitre plate, Mueller Hinton Broth or water are often used before transferring an equal volume of stock solution to the plate. Cation-supplemented Muller-Hinton Broth is recommended by Eucast (2003) for non-fastidious microorganisms. In a study, Kianbakht and Jahaniani (2003) discovered that the MIC values for Tribulus terrestris L. did not depend on the type of media used, when comparing the performance of Brain Heart Infusion Broth and Muller-Hinton Broth. Twofold serial dilutions are then made from the first well to obtain a concentration range. For full range MICs to 1 concentrations representing achievable concentrations for the antimicrobial are usually tested (Mendoza, 1998), although some authors have reported from even 3 concentrations. An equal volume of a fixed bacterial culture is added to the wells and incubated at 37°C for 24 h (Lourens et al., 2004). Eucast (2003) recommended a temperature of 35-37°C for 16-20 h for nonfastidious organisms (Eucast, 2003). Usually $1 \times 10^6$ cfu/ml of inoculum size for the microtitre plate procedure is recommended (Lourens et al., 2004). Eucast (2003) also recommended that the plates should be inoculated within 30 min of standardization of inoculum to avoid changes in inoculum density. As an indicator of growth, plates are examined for changes in turbidity. The well that appears clear, is taken as the MIC of the extract. To determine the presence of growth in microtitre plates some researchers use indicators
sources of secondary metabolites such as, phenols, flavonoids, tannins, alkaloids and terpenoids which have been found to have antimicrobial properties. The study of plants for their medicinal properties is important for modern day medicine, but its usefulness cannot be over emphasized if methods are not standardized to obtain comparable and reproducible results. Phytopharmaceuticals as antimicrobial agents has led to the discovery of bioactive compounds for which ethnopharmacologists, botanists, microbiologists and natural-product chemists are standardizing universal methods to produce effective and safe natural products to treat various diseases.

Conflict of interest
We declare that we have no conflict of interest.

References


