Journal of Medicinal Plants Research Vol. 4(2), pp. 104-111, 18 January, 2010
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR09.030
ISSN 1996-0875© 2010 Academic Journals

*Review*

# Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends

**K. Das[1,3]*, R. K. S. Tiwari[2] and D. K. Shrivastava[3]**

[1]International Rice Research Institute, DAPO Box-7777, Philippines.
[2]Department of Plant Pathology, T.C.B. College of Agriculture and Research Station (I. G. K. V.) Bilaspur (Chhattisgarh), India.
[3]Government P.G. Science College, Sarkanda Bilaspur (Chhattisgarh), India

Accepted 8 December, 2009

**Pharmaceutical and scientific communities have recently received the attention of the medicinal plants and various publications have documented the therapeutic worth of natural compounds to validate the claims of their biological activity. Profuse use of commercial antibiotic and synthetic pesticides for human and crop protection is harmful to human health, ecosystem and environment. Attention has also been drawn to the antimicrobial properties of plants and their metabolites due to the growing incidences of drug-resistant pathogens of both clinical and agricultural importance. Medicinal plants have their intrinsic ability to resist pathogenic microorganisms and this has led the researchers to investigate their mechanisms of action and isolation of active compounds. This has enabled exploitation of medicinal plants for the treatment of microbial infections of both plants and humans by developing new antimicrobial agents. This novel search entails extensive research and it is therefore imperative to follow standard methods to authenticate claims of antimicrobial action. This paper reviewed the methods being employed earlier and recently in use related to investigations of the antimicrobial efficacy of medicinal plant extracts. Standard protocols of the various techniques used by different authors are also mentioned.**

**Key words:** Medicinal plants, plant extracts, antimicrobial activity, phytochemicals.

## INTRODUCTION

Finding healing powers in plants is an ancient thought. Plant derived substances have recently become of great interest owing to their versatile applications (Baris et al., 2006). Medicinal plants have been used for centuries as remedies for human diseases and offer a new source of biologically active chemical compound as antimicrobial agent. Medicinal plants are the richest bio-resources of drugs of traditional medicinal systems, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceuticals, intermediate and chemical entitled for synthetic drugs (Hammer et al., 1999). It has been estimated that 14 - 28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on ethno medicinal use of the plants (Ncube et al., 2008). Recently the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led authors to investigate the antimicrobial activity of medicinal plants (Bisignano et al., 1996; Lis-Balchin and Deans 1996; Moaz and Neeman, 1998; Hammer et al., 1999).

Substantial use of chemical pesticides induces problems of health and environmental hazards in agricultural system. So, for human and plants natural products of antimicrobial activity are best biorational alternatives today (Tiwari et al., 2007). Over the last two decades, intensive effort has been made to discover chemically

*Corresponding author. E-mail: kdas_mail@yahoo.com.

useful antibacterial or antifungal drugs of plant origin (Sofowara, 1993; Valsaraj et al., 1997; Perumalsamy et al., 1999). Medicinal plant based antimicrobials represent a vast untapped source of pharmaceuticals and further exploration of plant antimicrobials need to occur for treatment of infectious diseases both in plants and humans while simultaneously for mitigating many of the side effects that are often associated with synthetic antimicrobials. Out of the several hundred thousand medicinal plant species around the globe, only a small portion has been investigated both phytochemically and pharmacologically (Hostettmann, 1999).

In view of the large number of the plant species potentially available for the study, it is essential to have efficient systems of the methods to evaluate efficacy of medicinal plants as antimicrobial agent. The evaluation for antimicrobial agent of plant origin begins with thorough biological evaluation of plant extracts to ensure efficacy and safety followed by identification of active principles, dosage formulations, efficacy and pharmacokinetic profile of the new drug. Many plants have been used because of their antimicrobial traits and antimicrobial properties of plants have been investigated by a number of researchers worldwide. Ethno pharmacologists, botanists, microbiologists and natural product chemists are searching the world for phytochemicals which could be developed for treatment of infectious diseases (Tanaka et al., 2006).

## MAJOR GROUPS OF ANTIMICROBIAL SECONDARY METABOLITES

Traditionally used medicinal plants have recently attracted the attention of the biological scientific communities. This has involved the isolation and identification of secondary metabolites produced by plants and their use as active principles in medicinal preparations (Taylor et al., 2001). Mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics (products of microorganisms or their synthesized derivatives) become ineffective. Another driving factor for the renewed interest in plant antimicrobials in the past 20 years has been the rapid rate of plant species extinction (Lewis et al., 1995) as there is a feeling among natural-products chemists and microbiologists alike that the multitude of potentially useful phytochemical structures which could be synthesized chemically is at risk of being lost irretrievably (Borris, 1996).

Plants have limitless ability to synthesize aromatic secondary metabolites, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). Important subclasses in this group of compounds include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. These groups of compounds show antimicrobial effect and serves as plant defense mechanisms against pathogenic microorganisms. Simple phenols and phenolic acid are bioactive phytochemicals consisting a single substituted phenolic ring. Phenolic toxicity to microorganisms is due to the site(s) and number of hydroxyl groups present in the phenolic compound (Scalbert, 1991; Urs et al., 1975). Quinones are characteristically highly reactive, colored compounds with two ketone substitutions in aromatic ring.

Flavones, flavonoids and flavonols are phenolic structure with one carbonyl group. They are synthesized by plants in response to microbial infection (Dixon et al., 1983) and are often found effective in vitro as antimicrobial substance against a wide array of microorganisms (Bennet and Wallsgrove, 1994).Tannins are polymeric phenolic substances possessing the astringent property. These compounds are soluble in water, alcohol and acetone and give precipitates with proteins (Basri and Fan, 2005). Coumarins are phenolic substances made of fused benzene and α-pyrone rings (O'Kennedy and Thornes, 1997). They have a characteristic odor and several of them have antimicrobial properties.

Fragrance of plant is carried by essential oil fractions which are secondary metabolites and highly enriched in isoprene structure based compounds. They are called terpenes but when the compound contains an additional element as oxygen they are termed as terpenoids. Essential oils also posses strong antimicrobial properties. It was reported early that 60% of the essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria (Chaurasia and Vyas, 1977). Naturally found alkaloids are heterocyclic nitrogenous compounds and are commonly found to have antimicrobial property (Omulokoli et al., 1997). Many of the earliest isolated pure compounds with biological activity were alkaloids and were found in pharmacogenically active basic principles of flowering plants.

Apart from the above which emphasizes major phytochemical groups, it should be mentioned, however that there are reports of antimicrobial properties associated with polyamines (in particular spermidine) (Flayeh and Sulayman, 1987), isothyocyanates (Dornberger et al., 1975; Iwu et al., 1991), thiosulfinates (Tada et al., 1988) and glycosides (Murakami et al., 1993; Rucker et al., 1992).

## METHODS OF PLANT EXTRACT PREPARATION

Extraction methods involve separation of medicinally active fractions of plant tissue from inactive/inert components by using selective solvents and extraction technology. Solvents diffuse into the solid plant tissues and solubilize compounds of similar polarity (Green, 2004). Quality of plant extract depends on plant material, choice

of solvents and the extraction methods.

**Plant material**

Plants are collected either randomly or by following leads supplied by local healers in geographical areas where the plants are found (Martin, 1995). Fresh or dried plant materials can be used as a source for the extraction of secondary plant components. Many authors had reported about plant extract preparation from the fresh plant tissues (Tiwari et al., 2005). The logic behind this came from the ethno medicinal use of fresh plant materials among the traditional and tribal people. But as many plants are used in the dry form (or as an aqueous extract) by traditional healers and due to differences in water content within different plant tissues, plants are usually air dried (Dilika et al., 1996; Baris et al., 2006) to a constant weight before extraction. Other researchers dry the plants in the oven at about 40°C for 72 h (Salie et al., 1996). In most of the reported works, underground parts (roots, tuber, rhizome, bulb etc.) of a plant were used extensively compared with other above ground parts in search for bioactive compounds possessing antimicrobial properties. The following schematic diagram illustrated by Ved et al. (1998) and Uniyal et al. (2006) depicts the percentage of plant parts being preferred in general.

**Choice of solvents**

Successful determination of biologically active compound from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions include low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate (Hughes, 2002) As the end product in extraction will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay (Ncube et al., 2008). The choice will also depend on the targeted compounds to be extracted. Initial screening of plants for possible antimicrobial activities typically begins by using the crude or alcohol extractions and can be followed by various organic solvent extraction methods.

Water is universal solvent, used to extract plant products with antimicrobial activity. Though traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extract (Parekh et al., 2005). Also water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics only important as antioxidant compound (Yamaji et al., 2005; Nang et al., 2007). A study reported that extraction of tannins and other phenolics was better in aqueous acetone than in aqueous methanol. In another study, among the twenty different solvents evaluated, chloroform was found to be the best solvent for the extraction of non-polar biological active compounds (Harmala et al., 1992). Since nearly all of the identified antimicrobial compounds from plants are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction (Serkedjieva and Manolova, 1992). Thus the most commonly used solvents for preliminary investigations of antimicrobial activity in plants are methanol, ethanol and water (Parekh et al., 2006; Bisignino et al., 1996; Lourens et al., 2004; Salie et al., 1996; Rojas et al., 2006).

There is also variation in the solvent used by different workers. The other solvents used by researchers are dicholro-methane (Dilika and Meyer, 1996; Freixa et al., 1996), acetone (Basri and Fan, 2005; Dilika et al., 1996; Lourens et al., 2004; Mathekga, 2001), hexane (Masoko and Eloff 2006). Some authors use a combination of these solvents to obtain the best solvent systems for extraction (Nostro et al., 2000). Recently Eloff (1998a) examined a variety of extractants for their ability to solubilize antimicrobials from plants, rate of extraction, ease of removal, toxicity in bioassay and acetone received the highest overall rating. Though there is a wide diversification in the usage of solvents, it is necessary to focus on a standardized extraction method and solvent system for a wide variety of researchers working in diverse settings to minimize the variability in the antimicrobial efficacy reports.

**The extraction methods**

Variation in extraction methods are usually depend on the length of the extraction period, solvent used, pH of the solvent, temperature, particle size of the plant tissues and the solvent-to-sample ratio. The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction. In the study by Eloff (1998b), 5 min extractions of very fine particles of diameter 10 µm gave higher quantities than values obtained after 24 h in a shaking machine with less finely ground material. Earlier studies reported that solvent to sample ratio of 10:1 (v/w) solvent to dry weight ratio has been used as ideal (Green, 2004).
The extraction method that has been widely used by researchers is plant tissue homogenization in solvent (Meyer and Dilika, 1996; Basri and Fan, 2005; Parekh et al., 2005). Dried or wet, fresh plant parts are grinded in a blender to fine particles, put in a certain quantity of solvent and shaken vigorously for 5 - 10 min or left for 24 h after which the extract is filtered. The filtrate then may be dried under reduced pressure and redissolved in the solvent to determine the concentration. Some researchers however centrifuged (approximately 20,000 x g, for 30 min) the filtrate for clarification of the extract

(Cichewicz and Thorpe, 1996; Taylor et al., 1996).

Another common method is serial exhaustive extraction which involves successive extraction with solvents of increasing polarity from a non polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted (Green, 2004). Other researchers employ soxhlet extraction of dried plant material using organic solvent (Kianbakht and Jahaniani, 2003). This method can not be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (de Paira et al., 2004).

## EXPERIMENTAL APPROACHES

### Efficacy of phytochemicals as antimicrobial agent

Multiple factors affect the outcome of antimicrobial efficacy of plant products by in vitro susceptibility test. Standardization is required for intra and inter-laboratory tests to minimize the significant influence of different methods employed (EUCAST, 2003). Standard criteria for in vitro evaluation of antimicrobial activity of plants differ among authors. Results obtained from antimicrobial efficacy of plant extract is often difficult to compare with published results due to the influence of several factors, that is, environment and climatic conditions during plant growth, choice of plant extracts, choice of extraction methods, antimicrobial tests employed, and on test microorganisms (Nostro et al., 2000; Hammer et al., 1999). The beneficial medicinal effect of plant materials basically results from the secondary products present in the plant and is not usually attributed to a single compound but a combination of the metabolites (Parekh et al., 2005). The phytochemicals may also specifically found in certain taxa of plants and vary in presence among different parts of plant tissues (Gottlieb, 1990).

### Methods for evaluation of efficacy of plant extract

#### *In vitro antimicrobial susceptibility testing*

The antimicrobial susceptibility test (AST) is an essential technique in modern biological science. It is used in pathology to determine resistance of certain microbial strains to different antimicrobials and in pharmacology research it is used to determine the efficacy of novel antimicrobials from biological extract against different microorganisms. The various AST methods employed by researchers worldwide could led to variations in results obtained as AST methods are widely employed now a days to screen the plant extracts for antimicrobial activity and to determine Minimum Inhibitory Concentration (MIC) of the antimicrobial substance.

Although current standard AST methods approved by various organizations like National Committee for Clinical Laboratory Science (NCCCLS), British Society for Antimicrobial Chemotherapy (BBSAC) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) exist, for guidelines of antimicrobial susceptibility testing of convenient drugs, these might not be exactly applicable to plant extracts and modifications have to be made (Hammer et al., 1999). AST standard tests are broadly classified into diffusion and dilution methods for convenience. Diffusion tests include agar well diffusion, agar disk diffusion, poison food technique, and bioautography, while dilution methods include agar dilution, broth microdilution and broth macrodilution technique (Tenover et al., 1995).

#### *Agar disk diffusion assay*

The Agar disk diffusion method of antimicrobial test was developed in 1940 (Heatley, 1944). The procedure which was accepted by NCCLS and widely used now a days, is a modification of that described by Bauer, Kirby, Sherris and Truck (commonly known as Kirby-Bauer test) (Bauer, et al., 1959, 1966). The Agar disk diffusion technique has been widely used to assay plant extract for antimicrobial activity (Freixa et al., 1996; Salie et al., 1996; Ergene et al., 2006). In this method, 6 mm sterilized filter papers disks (Whatmann No. 1) are saturated with filter sterilized (Salie et al., 1996) plant extract of desired concentration. The impregnated discs are then placed onto the surface of a suitable solid agar medium like Mueller Hinton (Mueller and Hinton, 1941), Trypton soy agar (Lourens et al., 2004) or Nutrient agar (Doughari, 2006). The media has been pre-inoculated with test organisms. The standard inoculum size is of 1 x 108 CFU/ml of bacteria for inoculating diffusion plates (Baris et al., 2006) which is equal to McFarland 0.5 turbidity standard. Some researchers impregnate the paper disk with plant extract before putting on the inoculated plates (Lourens et al., 2004; Salie et al., 1996) while others prefer after (Nostro et al., 2000; Baris et al., 2006). The drying time of impregnated paper disk varies among researchers from 2 h to overnight under a laminar flow cabinet (Basri and Fan, 2005). Plates are then incubated for 24 h at 37°C (bacteria) and 48 h at 25°C (fungi) (Salie et al., 1996; Baris et al., 2006). After incubation, zone diameter is measured to the nearest whole millimeter at the point wherein there is a prominent reduction of 80% growth.

#### *Agar well diffusion assay*

The principle of agar well diffusion is similar to that of agar disk diffusion assay. A standardized concentration of inoculum with fixed volume is spread evenly on the surface of gelled agar plate. A hole which ranges from 6 - 8 mm in diameter is punched with a sterile cork borer aseptically in the middle. A fixed volume of plant extract

is then introduced into the bored agar well and incubated at optimum temperature and duration depending upon the test microorganism (Mbata et al., 2006; Norrel and Messely, 1997)

### *Poison food technique*

Generally antifungal activity is determined by poisoned food technique (Grover and Moore, 1962; Mishra and Tiwari, 1992; Nene and Thapliyal, 2000). Five-day old fungal culture is punched aseptically with a sterile cork borer of generally 7mm diameter. The fungal discs are then put on the gelled agar plate. The agar plates have been prepared by impregnating desired concentration of plant extract at a temperature of 45 - 50°C. The plates are then incubated at temperature 26 ± 1°C for fungi. Colony diameter is recorded by measuring the two opposite circumference of the colony growth. Percentage inhibition of mycellial growth is evaluated by comparing the colony diameter of poisoned plate (with plant extract) and non-poisoned plate (with distilled water) and calculated using the formula given below (Verma and Kharwar, 2008);

$$\% \text{ Mycelial inhibition} = \frac{\text{Mycellial growth}_{(control)} - \text{Mycellial growth}_{(treatment)}}{\text{Mycellial growth}_{(control)}} \times 100$$

### *Spore germination assay*

In addition to the above mentioned assays, antifungal activity of plant extracts can be evaluated by spore germination assay using the slide technique (Nair, et al., 1991). Plant extract of desired concentration and volume are added to the surface of dried slides as a film or in a cavity of a cavity slide. Fixed volume and standard concentration of spore suspension of test fungi are spread over the film whereas in controlled treatment, distilled water is added in place of spore suspension. Slides are then placed on a glass rod in Petri dish under moistened conditions and incubated for 24°C. After incubation, slides are fixed in lacto phenol cotton blue and observed microscopically for spore germination. Percentage spore germination is calculated according to the following formula.

$$\% \text{ Spore Germination} = \frac{\text{Germinated spores (No.)}}{\text{Total Spores (No.)}} \times 100$$

### *Broth microdilution*

The microtitre plate or broth microdilution method has provided potentially useful technique for determining Maximum Inhibitory Concentration (MIC) of large numbers of test samples. MIC in microbiology is the lowest concentration of antimicrobials that will inhibit the visible growth of microorganisms after overnight incubation. MIC is important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agent. In the microtitre plate method, a stock solution of the extract is first obtained in solvent (Grierson and Afolayan, 1999) or in DMSO (Salie et al., 1996; Nostro et al., 2000; Baris et al., 2006). Mueller Hinton broth or water is often used as a diluent in the wells of the microtitre plate before transferring an equal volume of stock solution to the plate. EUCAST (2003) recommends certain supplemented Mueller Hinton broth for non-fastidious microorganisms.

Two fold serial dilutions are made from the first well to obtain a concentration range. MIC-5-8 concentration can represent achievable concentrations for the used antimicrobials (Mendoza, 1998). The inoculum size for this procedure is usually 1 x $10^6$ CFU/ml (Lourens et al., 2004, Basri and Fan, 2005). Some researchers have used microbial culture with an optical density of 0.4 at 620 nm or a 12 h broth culture adjusted to a 0.5 McFarland turbidity standard (Baris et al., 2006). An equal volume of microbial culture is added to the wells and incubated at 37°C for 24 h (Lourens et al., 2004). After incubation, plates are examined for changes in turbidity as an indicator of growth. The first well that appears clear is taken to be the MIC of the extract. Some researchers use indicators like tetrazolium salts or resazurin dye (Umesh et al., 2005) or spectophotometry to determine presence of growth (Devienne and Raddi, 2002; Matsumoto et al., 2001). For spectophotometric method the absorbance is usually at 620 nm with negative control as blank (Salie et al., 1996). Concentration with sharp decline in absorbance value (Devienne and Raddi, 2002) or the lowest concentration which gives a zero absorbance reading (Salie et al., 1996) is the MIC of the plant extract or the test phytochemical.

### *Broth macrodilution assay*

The basic principle of this assay is the same as the broth microdilution assay. But the test is performed in a test tube. In macrodilution assay, a set of test tubes with different concentrations of plant extract with the same volume are prepared. Tubes are inoculated with test microorganisms of standard concentrations as discussed above. After incubation, tubes are examined for changes in turbidity as an indicator of growth. MIC of the plant extract or the test phytochemical can be determined using the above discussed methods.

### *Bioautography*

Bioautography is a very convenient way of testing plant

extracts and pure phytochemical compounds for their effect on both human pathogenic and plant pathogenic microorganisms. It can be employed in the target directed isolation of active constituents (Hostettmann, 1999). Bioautography is also employed as a preliminary phytochemical screening technique, by bioassay guided fractionation, to detect active components (Nostro et al., 2000; Schmourlo et al., 2004).

Paper chromatography followed by bioautography was used for the first time in 1946 by Goodal and Levi to estimate the purity of penicillin (Goodall, et al., 1946). In this method, developed paper chromatogram was placed onto the inoculated agar layer enabling the diffusion of antibiotics from paper to agar containing microorganisms. Thin layer chromatography - bioautography was introduced by Fisher and Lautner et al., 1961. Bioautography methods are usually grouped into three categories, agar diffusion or contact bioautography, immersion or agar-overlay bioautography and direct bioautography (Rios et al., 1988).

### *Agar diffusion or contact bioautography*

In contact bioautography, antimicrobials are diffused from a thin layer chromatographic plate (T.L.C.) to an inoculated agar plate. The chromatogram is placed onto the inoculated agar layer and left for some time to enable diffusion. Then the chromatogram is removed and the agar layer is incubated. The inhibition zones are observed on the agar surface in places where the spots of antimicrobials are stuck to the agar. The method resembles a disk assay.

### *Immersion or agar overlay bioautography*

In this method the chromatogram is covered with a molten, seeded agar medium. After solidification, incubation and staining (usually with tetrazolium dye), the inhibition or growth bands are visualized (Harborne, 1973, 1989, 1992). Agar overlay is a hybrid of contact and direct bioautography.

### *Direct bioautography*

A determined amount of plant extract is applied to silica 60 gel plates and developed with an appropriate solvent system to separate the phytochemicals. A suspension of test bacteria is sprayed onto the T.L.C. plate. Some authors reported using an inoculum of 0.84 absorbance at 560 nm. (Meyer and Dilika, 1996) while others reported using a suspension of $10^6$ CFU/ml (Schmourlo et al., 2004). The bioautogram is then incubated at 25°C for 48 h in humid conditions. For visualization of microbial growth, tetrazolium salts (e.g., MTT) are used (Silva et al., 2005), which are converted by the dehydrogenases of living microorganisms to intensely colored formazen. These salts are sprayed onto the bioautogram and are re-incubated at 25°C for 24 h (Meyer and Dilika, 1996) or at 37°C for 3 - 4 h (Dilika et al., 1996; Runyoro et al., 2006). Clear white zones against a purple background on the T.L.C. plate indicate antimicrobial activity of the extract.

## Conclusion

Plants contain thousands of constituents and are valuable sources of new and biologically active molecules possessing antimicrobial property. The ethno-botanical study of plant is important for modern day medicine but its usefulness cannot be overemphasized if methods are not standardized to obtain comparable and reproducible results. At present, scientists are investigating for plant products of antimicrobial properties. It would be advantageous to standardize methods of extraction and in vitro antimicrobial efficacy testing so that the search for new biologically active plant products could be more systematic and interpretation of results would be facilitated. Thousands of phytochemicals which have inhibitory effects on all types of microorganisms in vitro should be subjected *in vivo* testing to evaluate the efficacy in controlling the incidence of disease in crops, plants, and humans. Efficient collaborations with pharmacologists and medical doctors, plant pathologists and microbiologists are crucial to see the complete development of an interesting lead compound into an exploitable product.

## ACKNOWLEDGEMENT

The authors are thankful to Ms. Achu Arboleda of the training centre at the International Rice Research Institute (IRRI), Philippines for the language scrutiny of the manuscript.

## REFERENCES

Baris O, Gulluce M, Sahin F, Ozer H, Kilic H, Ozkan H, Sokmen M, Ozbek T (2006). Biological activities of the essential oil and methanol extract of Achillea Biebersteinii Afan. (Asteraceae). Turk. J. Biol. 30: 65-73.

Basri DF, Fan SH (2005). The potential of aqueous and acetone extracts of galls of Quercus infectoria as antibacterial agents. Indian J. Pharmacol. 37(1): 26-29.

Bauer AW, Perry DM, Kirby WMM (1959). Single disc antibiotic sensitivity testing of Staphylococci. Arch. Int. Med. 104: 208-216.

Bauer AW, Kirby WMM, Sherries JC, Turckp M (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45: 493-496.

Bennett RN, Wallsgrove RM (1994). Secondary metabolites in plant defense mechanisms. Phytother. Res. 127: 617-633.

Bisignano G, Germanó MP, Nostro A Sanogo R ((1996). Drugs used in Africa as dyes: antimicrobial activities. Phytother. Res. 9: 346-350.

Borris RP (1996). Natural products research: perspectives from a major pharmaceutical company. J. Ethnopharmacol. 51: 29-38.

Chaurasia SC, Vyas KK (1977). *In vitro* effect of some volatile oil against *Phytophthora parasitica* var. *piperina*. J. Res. Indian Med. Yoga Homeopath. pp.24-26.
Cichewicz RH, Thorpe PA (1996). The antimicrobial properties of chilli peppers (Capsicum species) and their uses in Mayan medicine. J. Ethnopharmacol. 52: 61-70.
Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12: 564-582.
de Paira SR, Lima LA, Figueiredo MR, Kaplan MAC (2004). Plumbagin quantification in roots of *Plumbago scandens* L. obtained by different extraction techniques. Ann. Acad. Bras. Cienc. 76(3): 499-504.
Devienne KF, Raddi MSG (2002). Screening for antimicrobial activity of natural products using a microplate photometer. Braz. J. Microbiol. 33(2): 97-105.
Dilika F, Afolayan AJ, Meyer JJM (1996). Comparative antibacterial activity of two *Helichrysum* species used in male circumcision in South Africa. S. Afr. J. Bot. 63(3): 158-159.
Dilika F, Bremner PD, Meyer JJM (2000). Antibacterial activity of linoleic and oleic acids isolated from *Helichrysum pedunculatum*: a plant used during circumcision rites. Fitoterapia pp.450-452.
Dixon RA, Dey PM, Lamb CJ (1983). Phytoalexins: enzymology and molecular biology. Adv. Enzymol. 55: 1-69.
Dornberger K, Bockel V, Heyer J, Schonfeld C, Tonew M, Tonew E (1975). Studies on the isothiocyanates erysolin and sulforaphan from *Cardaria draba*. Pharmazie 30: 792-796.
Doughari JH (2006). Antimicrobial activity of Tamarindus indica Linn. Trop. J. Pharm. Res. 5(2): 597-603.
Eloff JN (1998a). A sensitive and quick microplate method to determine the minimum inhibitory concentration of plant extracts for bacteria. Plant. Med. 64: 711-713.
Eloff JN (1998b). Which extractant should be used for the screening and isolation of antimicrobial components from plants? J. Ethnopharmacol. 60: 1-8.
Ergene A, Guler P, Tan S, Mirici S, Hamzaoglu E, Duran (2006). Antimicrobial and antifungal activity of *Heracleum sphondylium* subsp. *artivinense*. Afr. J. Biotechnol. 5(11): 1087-1089.
EUCAST (2003). Discussion document, determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin. Microbiol. Infect. 9(8): 1-7.
Flayeh KA, Sulayman KD (1987). Antimicrobial activity of the amine fraction of cucumber (*Cucumis sativus*) extract. J. Appl. Microbiol. 3: 275-279.
Freixa B, Vila R, Vargas L, Lozano N, Adzet T, Caniguera S (1996). Screening for antifungal activity of nineteen Latin American plants. Phytother. Res. 12(6): 427-430.
Geissman TA (1963). Flavonoid compounds, tannins, lignins and related compounds. In: Florkin M, Stotz EH (eds), Pyrrole pigments, isoprenoid compounds and phenolic plant constituents:, Elsevier, New York, N.Y., 265p.
Goodall RR, Len AAA (1946). Micro-chromatographic method for the detection and approximate determination of the different penicillins in a mixture. Nature 158: 675-676.
Gottlieb OR (1990). Phytochemicals: differentiation and function. Phytochemistry 29: 1715-1724.
Green RJ. (2004). Antioxidant Activity of Peanut Plant Tissues. Masters Thesis. North Carolina State University. USA.
Grierson DS, Afolayan AJ (1999). Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa. J. Ethnopharmacol. 66: 103-106.
Grover RK, Moore JD (1962). Toximetric studies of fungicides against brown rot organisms, *Sclerotia fructicola* and *S. laxa*. Phytopathol. 52: 876-880.
Hammer KA, Carson CF, Riley TV (1999). Antimicrobial activity of essential oils and other plant extracts. J. Appl. Microbiol. 86(6): 985.
Harborne JB (1973). Phytochemical methods. London. Chapman and Hall, Ltd., pp. 49-188.
Harborne JB (1989). Methods in plant biochemistry, In: Plant phenolics: Academic Press, London, UK.
Harborne JB (1992). In: Heftmann E (5th eds) Chromatography: Elsevier, Amsterdam, pp. B363-92.
Harmala P, Vuorela H, Tornquist K, Hiltunen R (1992). Choice of solvent in the extraction of *Angelica archangelica* roots with reference to calcium blocking activity. Plant. Med. 58(2): 176-183.
Heatley NG (1944). Method for the assay of penicillin. Biochem. J. 38: 61-65.
Hostettmann K (1999). Strategy for the biological and chemical evaluation of plant extracts. Pure Appl. Chem. 70(11): 1-9.
Hughes I (2002). Isoprenoid compounds and phenolic plant constituents, Elsevier, New York, N.Y. Sci. Afr. Mag. 9(56).
Iwu MM, Unaeze NC, Okunji CO, Corley DG, Sanson DR, Tempesta MS (1991). Antibacterial aromatic isothiocyanates from the essential oil of *Hippocratea welwitschii* roots. Int. J. Pharmacogn. 29: 154-158.
Kianbakht S, Jahaniani F (2003). Evaluation of antibacterial activity of *Tribulus terrestris* L. growing in Iran. Iran. J. Pharmacol. Ther. 2: 22-24.
Lampinen J (2005). Continuous Antimicrobial susceptibility testing in drug discovery. Drug Plus International. pp. 1-3. http://www. thermo-readingroom.com/files/application_pdfs/File_30585.pdf.
Lewis WH, Elvin-Lewis MP (1995). Medicinal plants as sources of new therapeutics. Ann. Mo. Bot. Gard. 82: 16-24.
Lis-Balchin M, Deans SG (1996). Antimicrobial effects of hydrophilic extracts of *Pelargonium* species (Geraniacee). Lett. Appl. Microbiol. 23: 205-207.
Lourens ACU, Reddy D, Baser KHC, Viljoen AM, Van Vuuren SF (2004). *In vitro* biological activity and essential oil composition of four indigenous South African *Helichrysum* species. J. Ethnopharmacol. 9: 253-258.
Maoz M, Neeman I (1998). Antimicrobial effects of aqueous plant extracts on the fungi *Microsporum canis* and *Trichophyton rubrum* and on three bacterial species. Lett. Appl. Microbiol. 26: 61-63.
Martin GJ (1995). Ethnobotany: A methods manual. Chapman & Hall, New York, N.Y.
Masoko P, Eloff JN (2006). Bioautography indicates the multiplicity of antifungal compounds from twenty-four Southern African *Combretum* species (Combretacea). Afr. J. Biotechnol. 5(18): 1625-1647.
Mathekga ADM (2001). Antimicrobial activity of Helichrysum species and the isolation of a new phloroglucinol from *H. caespititium*. PhD Thesis. Botany Department. University of Pretoria. South Africa.
Matsumoto M, Ishida K, Konagai A, Maebashi K, Asaoka T (2001). Strong antifungal activity of SS750, a new Triazole derivative, is based on its selective binding affinity to cytochrome P450 of fungi. Antimicrob. Agents Chemother. 46(2): 308-314.
Mendoza MT (1998). What's new in antimicrobial susceptibility testing? Philipp. J. Microbiol. Infect. Dis. 27(3): 113-115.
Mendoza L, Wilkens M, Urzua A (1997). Antimicrobial study of the resinous exudates and of diterpenoids and flavonoids isolated from some *Chilean pseudognaphalium* (Asteraceae). J. Ethnopharmacol. 58: 85-88.
Meyer JJM, Dilika F (1996). Antibacterial activity *of Helichrysum pedunctulatum* used in circumcision rites. J. Ethnopharmacol. 53(1): 51-54.
Mishra M, Tiwari SN (1992). Toxicity of *Polyalthia longifolia* against fungal pathogens of rice. Indian Phytopath. 45: 56-61.
Mueller, Hinton (1941). A protein-free medium for primary isolation of the gonococcus and meningococcus. Proc. Soc. Exp. Biol. Med. 48: 330.
Murakami A, Ohigashi H, Tanaka S, Tatematsu A, Koshimizu K (1993). Bitter cyanoglucosides from *Lophira alata*. Phytochemistry 32: 1461-1466.
Nair MG, Safir GR, Siqueira JO (1991). Isolation and identification of vesicular arbuscular mycorrhiza stimulatory compounds from clover (*Tnifolium repens*) roots. Appl. Environ. Microbiol. 57: 434-439.
Nang HLL, May CY, Ngan MA, Hock CC (2007). Extraction and identification of water soluble compounds in Palm Pressed Fiber by SC-CO2 and GC-MS. Am. J. Environ. Sci. 3(2): 54-59.
National committee for clinical laboratory standards (1999). Performance standards for antimicrobial susceptibility testing: ninth informational supplement. NCCLS 19: 21.
Ncube N, Afolayan SAJ, Okoh AI (2008). Assessment techniques of antimicrobial properties of natural compounds of plant origin: current

methods and future trends. Afr. J. Biotechnol. 7(12): 1797-1806.
Nene Y, Thapilyal L (2002). Poisoned food technique of fungicides in plant disease control (3rd eds). Oxford and IBH Publishing Company, New Delhi.
Norrel SA, Messley KE (1997). Microbiology Laboratory Manual Principles and Applications: Prentice Hall. Upper Saddle River. New Jersey.
Nostro A, Germano MP, D'Angelo V, Marino A, Cannatelli MA (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett. Microbiol. 30(1): 379-384.
O'Kennedy R, Thornes RD (1997). Coumarins: biology, applications and mode of action. (eds), John Wiley & Sons, Inc., New York, N.Y.
Omulokoli E, Khan B, Chhabra SC (1997). Antiplasmodial activity of four Kenyan medicinal plants. J. Ethnopharmacol. 56: 133-137.
Parekh J, Jadeja D, Chanda S (2005). Efficacy of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity. Turk. J. Biol. 29: 203-210.
Parekh J, Karathia N, Chanda S (2006). Screening of some traditionally used medicinal plants for potential antibacterial activity. Indian J. Pharm. Sci. 68(6): 832-834.
Perumalsamy R, Ignacimuthu S, Raja DP (1999). Preliminary screening of ethnomedicinal plants from India. J. Ethnopharmacol. 66: 235-240.
Fisher R, Lautner H (1961). Arch. Pharm., (Weinheim), 294: 1.
Rios JL, Recio MC, Villar A (1998). Screening methods for natural products with antimicrobial activity: A review of the literature. J. Ethnopharmacol. 23: 127-149.
Rojas JJ, Ochoa VJ, Ocampo SA, Monoz JF (2006). Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in treatment of non-nosocomial infections. BMC Complement. Alternat. Med. 6: 2.
Rucker G, Kehrbaum S, Sakulas H, Lawong B, Goeltenboth F (1992). Acetylenic glucosides from *Microglossa pyrifolia*. Plant. Med. 58: 266-269.
Runyoro DKB, Matee MIN, Ngassapa OD, Joseph CC, Mbwambo ZH (2006). Screening of Tanzanian medicinal plants for anti-candida activity. BMC Complement. Altern. Med. 6(11): doi: 10.1186/1472-6882-6-11.
Salie F, Eagles PFK, Lens HMJ (1996). Preliminary antimicrobial screening of four South African *Asteraceae* species. J. Ethnopharmacol. 52(1): 27-33.
Scalbert A (1991). Antimicrobial properties of tannins. Phytochemistry 30: 3875-3883.
Schmourlo G, Mendonca-Filho RR, Alviano CS, Costa SS (2004). Screening of antifungal agents using ethanol precipitation and bioautography of medicinal food plants. J. Ethnopharmacol. 96(3): 563-568.
Serkedjieva J, Manolova N (1992). Anti-influenza virus effect of some propolis constituents and their analogues (esters of substituted cinnamic acids). J. Nat. Prod. 55: 294-297.
Silva MTG, Simas SM, Batista TGFM, Cardarelli P, Tomassini TCB (2005). Studies on antimicrobial activity, in-vitro, of *Physalis angulata* L. (Solanaceae) fraction and physalin B bringing out the importance of assay determination. Memorias do Instituto Oswaldo Cruz. 100(7): 779-782.
Sofowara A (1993). Medicinal plants and traditional medicine in Africa. Spectrum Books Ltd., Ibadan, Nigeria, 289p.
Tada M, Hiroe Y, Kiyohara S, Suzuki S (1998). Nematicidal and antimicrobial constituents from *Allium grayi* Regel and *Allium fistulosum* L. var. *caespitosum*. Agric. Biol. Chem. 52: 2383-2385.
Tanaka JCA, da Silva CC, de Oliveira AJB, Nakamura CV (2006). Dias Filho BP Antibacterial activity of indole alkaloids from *Aspidosperma ramiflorum*. Braz. J. Med. Biol. Res. 39(3): 387-391.
Taylor JLS, Rabe T, McGaw LJ, Jäger AK, van Staden J (2001). Towards the scientific validation of traditional medicinal plants. Plant Growth Regul. 34(1): 23-37.
Taylor RSL, Edel F, Manandhar NP, Towers GHN (1996). Antimicrobial activities of southern Nepalese medicinal plants. J. Ethnopharmacol. 50: 97-102.
Tenover FC, Swenson JM, O'Hara CM, Stocker SA (1995). Ability of commercial and reference antimicrobial susceptibility testing methods to detect vancomycin resistance in Enterococci. J. Clin. Microbiol. 33(6): 1524-1527.
Tiwari RKS, Chandravanshi SS, Ojha BM (2005). Efficacy of extracts of medicinal plant species on growth of *Sclerotium rolfsii* root rot in tomato. J. Mycol. Plant Pathol. 34(2): 461-464.
Tiwari RKS, Singh A, Das K, Sinha A (2007). Efficacy of extracts of medicinal plants against *Rhizoctonia solani*. Ann. Plant Prot. Sci. 15(2): 460-539.
Umeh EU, Oluma HOA, Igoli O (2005). Antibacterial screening of four local plants using an indicator-based microdilution technique. Afr. J. Tradit. Complement. Altern. Med. 2(3): 238-243.
Uniyal SK, Singh KN, Jamwal P, Lal B (2006). Traditional use of medicinal plants among the tribal communities of Chhota Bhangal, Western Himalaya. J. Ethnobio. and Ethnomed. 2(14): 1-8.
Urs NVRR, Dunleavy JM (1975). Enhancement of the bactericidal activity of a peroxidase system by phenolic compounds (*Xanthomonas phaseoli* var. *sojensis*, soybeans). Phytopathol. 65: 686-690.
Valsaraj R, Pushpangadan P, Smitt UW, Adersen A, Nyman U (1997). Antimicrobial screening of selected medicinal plants from India. J. Ethnopharmacol. 58: 75-83.
Ved DK, Anjana M, Shankar D (1998). Regulating export of medicinal plant species: need for scientific rigour. Curr. Sci. 75: 341-344.
Yamaji K, Ishimoto H, Usui N, Mori S (2005). Organic acids and water soluble phenolics produced by *Paxillus* species. Mycorrhiza 15(1): 17-23.