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Review

Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends

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Medicinal plants have recently received the attention of the pharmaceutical and scientific communities and various publications have documented the therapeutic value of natural compounds in a bid to validate claims of their biological activity. Attention has been drawn to the antimicrobial activity of plants and their metabolites due to the challenge of growing incidences of drug-resistant pathogens. Some plants have shown the ability to overcome resistance in some organisms and this has led to researchers' investigating their mechanisms of action and isolating active compounds. Particular focus is on establishing the effect of the plant(s) extracts in terms of their microstatic and microcidal action and the spectrum of organisms affected. This has enabled exploitation of plants for the treatment of microbial infections and in the development of new antimicrobial agents. This requires rigorous research and it is therefore imperative to follow standard methods to authenticate claims of antimicrobial action. Results comparability is largely dependent on the techniques employed in the investigations and conclusive results can only be obtained if methods are standardized and universal. This paper reviews the current methods used in the investigations of the efficacy of plants as antimicrobial agents and points out some of the differences in techniques employed by different authors.

Key words: Medicinal plants, plant extracts, antimicrobial agents, antimicrobial activity.

INTRODUCTION

Plant-derived substances have recently become of great interest owing to their versatile applications (Baris et al., 2006). Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities 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 ethnomedicinal use of the plants. A number of interesting outcomes have been found with the use of a mixture of natural products to treat diseases, most notably the synergistic effects and polypharmacological application of plant extracts (Gibbons, 2003).

The development of pharmaceuticals begins with identification of active principles, detailed biological assays and dosage formulations, followed by clinical studies to establish safety, efficacy and pharmacokinetic profile of the new drug (Iwu, 1999). The same follows for plant therapeutic agents. Thorough biological evaluation of plant extracts is vital to ensure their efficacy and safety. These factors are of importance if plant extracts are to be accepted as valid medicinal agents. Many plants have been used because of their antimicrobial traits and the antimicrobial properties of plants have been investigated

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by a number of researchers worldwide. Ethnopharmacologists, botanists, microbiologists, and natural-product chemists are searching the earth for phytochemicals which could be developed for the treatment of infectious diseases (Tanaka et al., 2006) especially in light of the emergence of drug-resistant microorganisms and the need to produce more effective antimicrobial agents.

The antimicrobial susceptibility test (AST) is an essential technique in many disciplines of science. It is used in pathology to determine resistance of microbial strains to antimicrobials, and in ethnopharmacology research, it is used to determine the efficacy of novel antimicrobials against microorganisms, essentially those of medical importance. The test is the first step towards new anti-infective drug development. There are various AST methods that are employed by researchers and these could lead to variations in results obtained (Lampinen, 2005).

PLANT PRODUCTS AS THERAPEUTIC AGENTS

Traditionally used medicinal plants have recently attracted the attention of the pharmaceutical and 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). Many of the plant secondary metabolites are constitutive, existing in healthy plants in their biologically active forms, but others occur as inactive precursors and are activated in response to tissue damage or pathogen attack (Osbourne, 1996).

The array of secondary metabolites produced by plants is daunting, with wide ranging chemical, physical and biological activities. These constitute a source of bioactive substances and presently scientific interest has increased due to the search for new drugs of plant origin. A number of plant secondary metabolites (PSM) have been used as anticancer agents. Flavonoid-rich extracts from the mature roots of *Scutellaria baicalensis* have been shown to exhibit anti-proliferative effects on various cancer lines (Scheck et al., 2006). Taxol, a diterpene from the Pacific yew has been widely used as a drug for the treatment of ovarian and breast cancer (Iwu et al., 1999). Limonoids, a group of triterpenes, have been shown to be successful in treatments with *in-vitro* bioassays on human tumor cell lines, with limonin and isofraxinellone being the most active compounds (Ahn, 1994).

Antimicrobial secondary metabolites

Mainstream medicine is increasingly receptive of the use of antimicrobial and other drugs derived from plants, as traditional antibiotics become ineffective and because of the rapid rate of plant species extinction. 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 (Cowan, 1999).

Many of the earliest isolated pure compounds with biological activity were alkaloids. Naturally occurring alkaloids are nitrogenous compounds that constitute the pharmacogenically active basic principles of flowering plants. Alkaloids have been divided into 3 major classes depending on the precursors and the final structure. The true alkaloids are derived from amino acids, are basic and contain nitrogen in a heterocyclic ring for example, nicotine. Common alkaloid ring structures include the pyridines, pyrroles, indoles, pyrrolidines, isoquinolines, and piperidines (Bennet and Wallsgrove, 1994). A benzylisoquinoline alkaloid, papaverine was shown to have inhibitory effect on several viruses and indoquinoline alkaloids from *Cryptolepsis sanguinolenta* displayed activity against a number of gram negative bacteria and yeast (Silva et al., 1996). Quinine, an alkaloid, is popular for its antiamoebal activity against the malaria parasite (Iwu, 1999). The terpenes are one of the largest and most diverse groups of plant secondary metabolites. They include sterols and triterpenes, complex compounds that are formed by the cyclization of 2,3-oxidosqualene. Sterols and triterpenes can accumulate as glycoside conjugates in substantial quantities in plants. These glycosides, which include steroidal glycoalkaloids, are commonly referred to as saponins. A number of studies have shown saponins to have inhibitory effects on protozoa. Saponins from *Quillaja saponaria* and *Acacia auriculoformis* were found to be antiprotozoal *in-vitro* with butanol as the main active component (Wallace, 2004).

Another important sub-class of compounds under the terpenes are the essential oils of which monoterpenes, diterpenes and sesquiterpenes form the majority of this sub-class. Essential oils possess biological activity including antibacterial, antiviral, antifungal and anti-inflammatory effects. Oils from *Cinnamomum osmophloeum* have been shown to possess antibacterial activity against *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* (including methicillin resistant *S. aureus*) and *Vibrio parahaemolyticus,* with cinnamaldehyde being the main antibacterial component isolated. This compound has also been widely used in antiseptic mouthwashes because of its activity against oral bacteria (Wallace, 2004). Some essential oils are effective against some higher organisms such as nematodes, helminthes and insects. Common active components of the essential oils include thymol, carvacol, camphor and terpinene-4-ol (Acamovic and Brooker, 2005).

The phenolics and polyphenols are another group of PSM that have exhibited antimicrobial activity. Important subclasses in this group of compounds which have been found to have antimicrobial activity include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. This group includes metabolites

derived from the condensation of acetate units (tepernoids), those produced by the modification of aromatic amino acids (phenylpropanoids and coumarins), flavonoids, isoflavonoids and tannins. Flavones, flavonoids and flavonols have been known to be synthesized by plants in response to microbial infection so it is not surprising that they have been found, *in vitro*, to be effective antimicrobial substances against a wide array of microorganisms (Bennet and Wallsgrove, 1994).

Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions have been assigned to tannins. These are soluble in water, alcohol and acetone and gives precipitates with proteins (Basri and Fan, 2005). Tannins have been traditionally used for protection of inflamed surfaces of the mouth and treatment of catarrh, wounds, hemorrhoids and diarrhea (Ogunleye and Ibitoye, 2003). As a group, coumarins have been found to stimulate macrophages, which could have an indirect negative effect on infections (Cowan, 1999).

External plant surfaces are often protected by biopolymers for example, waxes fatty acid esters such as cutin and suberin. In addition, external tissues can be rich in phenolic compounds, alkaloids, diterpenoids, steroid alkaloids and other compounds which inhibit the development of fungi and bacteria. Cell walls of at least some monocotyledons also contain antimicrobial proteins, referred to as thionins (Angeh, 2006).

Practical clinical application of plant antimicrobial compounds

Bacteria have evolved numerous defenses against antimicrobial agents, and drug-resistant pathogens are on the rise. This resistance is conferred by multidrug resistance pumps (MDRs), membrane translocases that extrude structurally unrelated toxins from the cell. These protect microbial cells from both synthetic and natural antimicrobials (Stermitz et al., 2000). Secondary metabolites resemble endogenous metabolites, ligands, hormones, signal transduction molecules or neurotransmitters and thus have beneficial medicinal effects on humans due to their recognition in potential target sites (Parekh et al., 2005). The use of plant extracts and phytochemicals can be of great significance in therapeutic treatments and could help curb the problem of these multi-drug resistant organisms. In a study done with *Pseudomonas aeruginosa*, which is resistant to different antibiotics, its growth was inhibited by extracts from clove, jambolan, pomegranate and thyme (Nascimento et al., 2000).

Moreover, the synergistic effects of extracts with antimicrobial activity in association with antibiotics can provide effective therapy against drug resistant bacteria. These synergistic combinations represent a largely untapped source of new pharmaceutical products with novel and multiple mechanisms of action that can overcome microbial resistance. Recent developments in plant biotechnology have created the tools to produce botanical mixtures at a level comparable to that of pure drug compounds (Gibbons, 2003), and through biosynthesis and bioengineering dependence on large amount of plant material is reduced, limiting depletion of biogenetic resources in forests. These compounds, however, should be subjected to animal and human studies to determine their effectiveness in whole-organism systems, including in particular toxicity studies as well as an examination of their effects on beneficial normal microbiota (Iwu et al., 1999). It would be beneficial to standardize methods of extraction and *in vitro* testing so that the search for new antimicrobial drugs from plants could be more systematic and to facilitate proper interpretation of results (Cowan, 1999).

EXPERIMENTAL APPROACHES

There are multiple factors that may affect the outcome of susceptibility tests and standardized methods are more likely to be reproducible than unstandardized methods. Standardization is required for intra- and interlaboratory reproducibility as results may be significantly influenced by the method used (EUCAST, 2003). Standard criteria for evaluation of plant antimicrobial activity are lacking and results greatly differ between authors. Sometimes it is difficult to compare results obtained, when dealing with plant extracts, 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). The beneficial medicinal effects of plant materials typically result from the secondary products present in the plant although it is usually not attributed to a single compound but a combination of the metabolites. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Parekh et al., 2005). They also vary between tissues (higher concentrations occur in bark, heartwood, roots, branch bases and wound tissues), among species from tree to tree and from season to season (Gottlieb, 1990). In their work, Mitscher et al. (1972), found that extracts are generally richest in antibacterial agents after the flowering (sexual) stage of their growth is complete, and that plants taken from stressful environments were particularly active.

Plant extracts preparation

Extraction methods, used pharmaceutically, involve sepa-

ration of medicinally active portions of plant tissues from the inactive/inert components by using selective solvents with appropriate extraction technology. During extraction, solvents diffuse into the solid plant material and solubilise compounds with similar polarity (Green, 2004). The basic parameters influencing the quality of an extract are: 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 nature of solvent as well as solvent concentration and polarity will also affect quantity and secondary metabolite composition of an extract (SEA, 2006).

Plant material

Fresh or dried plant material can be used as a source for secondary plant components. However, most scientists working on the chemistry of secondary plant components have tended to use dried plant material for several reasons. Differences in water content may affect solubility of subsequent separation by liquid-liquid extraction and the secondary metabolic plant 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) to a constant weight but other researchers dry the plants in the oven at about 40^{o}C for 72h (Salie et al., 1996). Also, plants will have different constituents depending on the climatic conditions in which it is 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

Successful determination of biologically active compounds 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). The choice of solvent is influenced by what is intended with the extract. Since the end product will 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 where the optimal conditions for extraction of tannins and other phenolics, aqueous acetone was better at extracting total phenolics than aqueous methanol (Cork and Krochenberger, 1992). In another study where twenty different solvents were evaluated, 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). If the extraction is for general phytochemical analysis or screening then 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 (Eloff, 1998a). Traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extracts (Parekh et al., 2005).

Polyphenolic compounds such as flavonols and most other reported bioactive compounds are generally soluble in polar solvents such as methanol (Houghton et al., 1998). Most antimicrobial active components that have been identified are not water soluble and thus organic solvent extracts have been found to be more potent (Parekh et al., 2006). Water-soluble compounds, such as polysaccharides and polypeptides, including fabatin and various lectins, are commonly more effective as inhibitors of pathogen adsorption and have no real impact as antimicrobial agents (Cowan, 1999). Water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics are only important as antioxidant compounds (Yamaji et al., 2005; Nang et al., 2007).

The most commonly used solvents for investigations of antimicrobial activity in plants are methanol, ethanol, and water (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 and Meyer, 1996; Freixa et al., 1996). Some authors use a combination of these solvents 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 (Basri and Fan, 2005; Dilika et al., 1996; Lourens et al., 2004; Mathkega et al., 2006). In a study by Masoko and Eloff (2006) where they investigated the antifungal activity of *Combretum* species, from the extractants used, which included hexane, dichloromethane, acetone and methanol, they discovered that acetone and methanol extracted more chemical compounds from the leaves than the other solvents. Both acetone and methanol were found to extract saponins which have antimicrobial activity. Eloff (1998a) examined a variety of extractants for their ability to solubilise antimicrobials from plants, rate of extraction, ease of removal, toxicity in bioassay, among other things, and acetone received the highest overall rating. It gave the lowest minimum inhibitory concentration for gram positive organisms tested and the largest number of different components and inhibitors from two plants tested, but Eloff does note that different results may be obtained with other plants and generaliza-

tion can not be made on the usefulness of acetone as an extractant.

The extraction methods

Variations in extraction methods are usually found in the length of the extraction period, solvent used, pH, temperature, particle size and the solvent-to-sample ratio. The longer the contact between solvent and material the more is extracted until all possible materials have been extracted. The extraction period can be shortened by grinding the plant material finer as this will increase the surface area for extraction thereby increasing the rate of extraction. Shaking the plant material-solvent mixture will also increase 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. In one study, sequential extraction with various solvents at room temperature was compared with extraction in a water bath at 37°C for 30 min with distilled water adjusted to pH 2.0 with HCI and then neutralized with NaOH before extraction with diethyl ether. The authors concluded that the latter method had higher activity which was ascribed to the acidified aqueous environment which promoted easy extraction (Nostro et al., 2000). The solvent-to-sample ratio affects the quantity and quality of constituents obtained. In a study to identify the optimal conditions for extracting sugars from non-defatted soybean a solvent ratio of 5:1 at 25°C or 50°C for 15 min was found to give the best yield of sugar (Giannoccano et al., 2006). In some studies solvent to sample ratios of 10 ml : 1 g solvent to dry weight ratio has been used and reported as ideal (Green, 2004).

The method that has widely been used by researchers investigating antimicrobial activity is homogenization in solvent (Meyer and Dilika, 1996; Basri and Fan, 2005; 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. Some authors report shaking unhomogenized dry leaves in solvent for about 5 min, followed by filtering and concentrating under reduced pressure to obtain an epicuticular extract (Mathekga, 2001). This actually gave a higher yield and bioactivity than using the same method but with homogenized (macerated) extract (Dilika et al., 1996; Mathekga, 2001). Of interest are the results obtained by Meyer and Dilika (1996) using these different methods on the same plant. They found that the homogenized dichloromethane extract generally had higher activity than the shaken extract of the same solvent. The trend was the same for the aqueous extract for the same microorganisms tested.

One other 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 compounds could be extracted (Green, 2004). This is ideal when the aim is to screen the plant for a variety of compounds. Some methods are employed when a particular class of compounds is targeted. For example when one is interested in essential oils, then the method of choice would be steam distillation, volatile solvent extraction or supercritical fluid extraction (Lemberkovics et al., 2002). Maceration, maceration with sonication, Soxhlet extraction and SFE with hexane or CO_2 was compared for the extraction of low-polarity compounds from *Mikania glomerata* and SFE-hexane proved to be the most effective. These newer methods, which also include microwave assisted methods, are proving to be more efficient than the conventional methods (Vilegas et al., 2002).

Other researchers employ soxhlet extraction of dried plant material using organic solvents (Kianbakht and Jahaniani, 2003). In soxhlet extraction, the sample is continually exposed to fresh solvent, which improves the efficiency of the method. The method works well for compounds that can withstand the temperature of the boiling solvent, but can not be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (de Paira et al., 2004). Other common extraction methods include maceration (for fluid extract) where whole or coarsely powdered plant-drug is kept in contact with the solvent in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved; percolation; and infusion which is prepared by immersing the plant material for some time in cold or hot water (Handa, 2006).

Antimicrobial susceptibility testing

In ethnopharmacology research the antimicrobial susceptibility test (AST) is used to determine the efficacy of potential antimicrobials from biological extracts against a number of different microbial species. AST methods are used to screen plant extracts for antimicrobial activity but are largely used to determine the usefulness of an antimicrobial in combating infections by determining its minimum inhibitory concentration (MIC). In clinical research *in vitro* susceptibility tests are particularly important if an organism is suspected to belong to a species that has shown resistance to frequently used antimicrobial agents. They are also important in epidemiological studies of susceptibility and in comparisons of new and existing microbial agents (EUCAST, 2003).

Successful discovery of novel natural antimicrobials has necessitated the development of new bioassay techniques which are sensitive enough to detect small amounts of biologically active chemicals (Lampinen, 2005). Standardized *in vitro* tests are essential for screening plant extracts or compounds and more studies

should be conducted for MIC determination of natural products in order to get results that are comparable to those of currently used antibiotics (Devienne and Raddi, 2002). Evaluation of the performance of a susceptibility test should include criteria such as ease of use, reproducibility, i.e. the ability to yield the same result on repeat testing, test sensitivity and specificity (Struelens et al., 1995). Although current standard methods, approved by various bodies like the National Committee for Clinical Laboratory Science (NCCLS) [now known as Institute of Clinical Laboratory Standards (ICLS)], British Society for Antimicrobial Chemotherapy (BSAC) and the European Committee for Antimicrobial susceptibility testing (EUCAST), exist for guidelines of antimicrobial susceptibility testing of conventional drugs, these might not be exactly applicable to plant extracts and modifications have to be made (Hammer et al., 1999).

AST standard tests can be conveniently divided into diffusion and dilution methods. Common diffusion tests include agar well diffusion, agar disk diffusion and bioautography, while dilution methods include agar dilution and broth micro/macrodilution. The broth and agar based methods are the conventional reference methods for AST (Tenover et al., 1995). There are other commercial custom-prepared methods like the agar screen plate, Epsilometer test and the Vitek system which could be used in place of the standard reference methods but these are not common in routine AST (Joyce et al., 1992) and are not common for testing activity of plant extracts. The major problem in the diffusion and dilution based AST is one of availability of the active principles which is a function of the solubility of the test compound.

Agar disk diffusion assay

Agar diffusion techniques have been widely used to assay plant extracts for antimicrobial activity (Freixa et al., 1996; Salie et al., 1996), although there are limitations with the technique. Disk diffusion is suitable for identification of leads but not effective for quantification of bioactivity (Hammer et al., 1999; Nostro et al., 2000; Langfield et al., 2004). These diffusion techniques generally do not distinguish bactericidal and bacteriostatic effects. The MIC can not be determined and these are usually used for preliminary screening (Parekh et al., 2006; Tepe et al., 2004) that is, as qualitative tests, since the amount of extract that adheres to the disk is not quantitatively determined. Some researchers however have reported MIC values obtained by the agar diffusion method (Dilika et al., 2000; Leite et al., 2006) although high activity in the disk diffusion assay does not necessarily correlate to low MIC values in the microtitre plate method (Lourens et al., 2004). The agar disk diffusion technique can only be used for AST of pure substances because when it is applied to mixtures containing constituents, which exhibit different diffusion rates, results may be unreliable (Silva et al., 2005).

In the 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. Muller Hinton is usually the medium of choice although Tryptone soy agar (Lourens et al., 2004) or Nutrient agar (Doughari, 2006) have sometimes been used by other researchers. The media is preinoculated with the test organism and authors have reported inoculum sizes of 1×10^8 cfu/ml of bacteria for inoculating diffusion plates (Baris et al., 2006). There have been some variations noted on whether the disks are impregnated with antimicrobial substances after or before placing on the inoculated plate. Some impregnate 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 while Basri and Fan (2005) left the disks to dry under a laminar flow cabinet overnight. 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). Some incubate for 18 h at 37°C for the same bacteria (Nostro et al., 2000; Lourens et al., 2004). Zones of inhibition are then measured from the circumference of the disks to the circumference of the inhibition zone or recorded as the difference in diameter between the disks and the growth free zones around the disks (Salie et al., 1996).

Agar well diffusion

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

Bioautography

This is a variation of the agar diffusion method where the analyte is adsorbed onto a Thin Layer Chromatography (TLC) plate. 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). Bioautography overcomes the challenge of isolating antimicrobial compounds from crude extracts with complex chemical components by simplifying the process of their

isolation and identification. It relatively uses very little amount of sample which is ideal for plant extracts and also allows the determination of the polarity of the active compounds (Runyoro et al., 2006). In their study, Silva et al. (2005) compared different methods of AST, and they concluded that bioautography is a practical, reproducible test which is easy to perform.

In the bioautography agar overlay procedure, a determined amount of the extract is applied to silica 60 gel plates and developed with an appropriate solvent system. A suspension of the test bacteria is sprayed onto the TLC plate. Some authors reported using an inoculum of 0.84 absorbance at 560 nm (Meyer and Dilika, 1996), while others report using a suspension of 10^6 CFU/ml (Schmourlo et al., 2004). The bioautograms are then 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). These are sprayed onto the plates after which the plates are reincubated at 25°C for 24 h (Dilika and Meyer, 1996) or at 37°C for 3 – 4 h (Dilika et al., 1996; Runyaro et al., 2006). Clear (white) zones on the TLC plate indicate antimicrobial activity of the extracts. Some authors' state that direct bioassay on TLC plates is not an ideal method for the quantification of bioactivity of plant extracts. They suggest that TLC causes disruption of synergism between active constituents in an extract thereby reducing its activity (Schmourlo et al., 2004).

Broth microdilution

The micro-titre plate or broth microdilution method has provided a potentially useful technique for determining MICs of large numbers of test samples. Its advantages over diffusion techniques include increased sensitivity for small quantities of extract which is important if the antimicrobial is scarce as is the case for many natural products; ability to distinguish between bacteriostatic and bactericidal effects; and quantitative determination of the MIC (Langfield et al., 2004). This method can also be used for a wide variety of microorganisms, it is not expensive and it presents reproducible results. In the micro-titre plate method, a stock solution of the extract is first obtained in solvent, usually the solvent used for extraction (Grierson and Afolayan, 1999) or in DMSO (Salie et al., 1996; Nostro et al., 2000; Baris et al., 2006). Methanol and acetone are sometimes chosen as solvents because, in addition to dissolving the extracts completely they show no inhibition of the microorganisms even at 2% final concentration (Meyer and Afolayan, 1995; Afolayan and Meyer, 1997; Mathekga et al., 2000). Most authors report on filter sterilizing with a 0.22 or 0.45 µm membrane filter before the procedure (Meyer and Afolayan, 1995; Kianbakht and Jahaniani, 2003). The EUCAST (2003) document states that when membrane filtration is used the samples, before and after sterilization, should be compared by assay to ensure that adsorption has not occurred. Mueller Hinton Broth or water are often used as diluents in the wells of the microtitre plate before transferring an equal volume of stock solution to the plate. EUCAST (2003) recommends cation-supplemented Muller-Hinton broth for non-fastidious microorganisms. 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.

Two fold serial dilutions are then made from the first well to obtain a concentration range. For full range MIC 5 - 8 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); the EUCAST recommended temperature is 35–37°C in air for 16–20 h for non-fastidious organisms (EUCAST, 2003). The inoculum size for the microtitre plate procedure is usually 1×10^6 cfu/ml (Lourens et al., 2004; Basri and Fan, 2005). Others have used a microbial culture with an optical density of 0.4 (log-phase) at 620 nm or a 12 h broth culture adjusted to a 0.5 McFarland turbidity standard (Baris et al., 2006). EUCAST (2003) recommends that plates be inoculated within 30 min of standardization of inoculum, to avoid changes in inoculum density.

Plates are then 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 (Umeh et al., 2005) or spectrophotometry to determine presence of growth in microtitre plates (Devienne and Raddi, 2002, Matsumoto et al., 2001). Indicators (usually tetrazolium salts or resazurin dye) are added after the incubation period and left for about 6 h and changes in color or absence of color, depending on the indicator, is used to detect the MIC breakpoint. The use of calorimetric indicators eliminates the need for a spectrophotometric plate reader and avoids the ambiguity associated with visual comparison or measurement of growth inhibition rings on agar plates. When the spectrophotometric method is used the absorbance, usually at 620 nm with the negative control as a blank, is used to detect the breakpoint (Salie et al., 1996). The concentration at which there is a sharp decline in the absorbance value (Devienne and Raddi, 2002), or the lowest concentration which gives a zero absorbance reading (Salie et al., 1996) is deemed to be the MIC.

The minimum bactericidal concentration (MBC) is determined by subculturing the preparations that would have shown no evidence of growth in the MIC determination assay. These subcultures are made either in broth or in agar plates. In broth, the MBC is regarded as the lowest concentration of extract which does not produce an absorbance reading at 620 nm relative to the negative control (Salie et al., 1996). On agar the lowest concentration showing lack of growth represents the MBC.

Agar dilution assay

The agar dilution test is more versatile than the broth dilution assay and does not present problems encountered with the latter that is, sample solution, contamination and determination of MIC breakpoints (Silva et al., 2005). In this method a stock solution of the extract is prepared in its extracting solvent, filter-sterilized (0.22 µm) and then incorporated in molten agar, cooled to 50°C in a water bath, to obtain different concentrations of the extract in the agar. Usually Muller-Hinton (EUCAST, 2003) is used although some authors have used nutrient agar (Grierson and Afolayan, 1999; Meyer and Afolayan, 1995). Inoculum preparation also differs between authors and others have used overnight culture dilutions of 1:100 (Meyer and Afolayan, 1995) or 1:10 (Meyer and Dilika, 1996) in broth. EUCAST (2003) recommends an inoculum density of about 10^7 cfu/ml and using replicator pins, micropipette or standard loop to transfer about 1 µl (10^4 CFU/ml) of the inoculum.

Some publications have reported leaving the plates overnight, before streaking, to allow the solvent to evaporate (Grierson and Afolayan, 1999). The organisms are streaked in radial patterns on the agar plates and incubated at 37°C for 24 to 48 h. The MIC is defined as the lowest concentration of the extract inhibiting the visible growth of each microorganism on the agar plate (Nostro et al., 2000; Hammer et al., 1999).

Guidelines from standardization bodies

Given the diversity of the methods used in the study of the antimicrobial potential of plant compounds, the comparison of results by different laboratories is very difficult. Breakpoints for MIC and MBC are defined differently by researchers and standardization bodies. The BSAC defines MIC as the lowest concentration of antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation and MBC as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on antimicrobial free media. The EUCAST (2000) defines MIC as the lowest concentration, expressed in mg/L, that under defined *in vitro* conditions, prevents the growth of bacteria within a defined period of time and the MBC as the lowest concentration of an antimicrobial agent, expressed in mg/L, that under defined *in vitro* conditions reduces by 99.9% (3 logarithms) the number of organisms in a medium containing defined inoculum, within a defined period of time (EUCAST, 2000). The NCCLS has guidelines for AST which include guidelines for inoculum preparation, medium choice; incubation conditions and minor changes in these protocols may have a significant impact on susceptibility test results.

CONCLUSION

The ethnomedicinal study of plants is important for modern day medicine but its usefulness cannot be overemphasized if methods are not standardized to obtain comparable and reproducible results. Other pharmacological studies like time kill assays; potentials for combination antimicrobial chemotherapy and mechanicsms of action are important ingredients as additional tests to fully assess antimicrobial activity of plant extracts, and these are subjects of intensive investigation in our laboratory.

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