ANTI-BACTERIAL PROPERTIES OF THE METHANOL EXTRACT OF *HELICHRYSUM PEDUNCULATUM*

By

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Declaration

I declare that this thesis and the work contained herein is my original work and has not been presented to any other University except the University of Fort Hare for the award of any degree.

Name ……..

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General Abstract

The methanol extract of *Helichrisum pedunculatum* was screened for antimicrobial activity up to a concentration of 5 mg/ml using the agar dilution technique. A number of test bacterial isolates, comprising both Gram negative and Gram positive organisms were susceptible to the crude extract of the plant. The minimum inhibitory concentrations (MICs) of the extract ranged between 1 and 5 mg/ml for the susceptible organisms. The MICs of the selected antibiotics, chloramphenicol and penicillin, ranged between 2 and 4 mg/L, and 2 and 32 mg/L respectively against *Bacillus cereus*, *Proteus vulgaris* and *Staphylococcus aureus* OKOH1. Bactericidal activity was determined by the time kill assay. The methanol extract of the plant was not bactericidal at 1 × MIC for *B. cereus*, *P. vulgaris* and *Staph. aureus* OKOH1. At 2 × MIC the extract was bacteriostatic against *B. cereus* but bactericidal against *P. vulgaris* and *Staph. aureus* OKOH1. Combination studies were done at $^{1}/_{2}$ × MIC, 1 × MIC and 2 × MIC of the plant extract with 1 × MIC of the antibiotics. Combinations of the plant extract and chloramphenicol resulted in mostly indifferent interactions against *P. vulgaris* and *Staph. aureus* OKOH1 but synergistic interactions at higher concentration of the plant extract for *B. cereus*. Penicillin combinations gave synergistic interactions at lower concentrations of the plant for *P.vulgaris* and *Staph. aureus* OKOH1 but was mostly indifferent for *B. cereus*.

CHAPTER 1

INTRODUCTION

Populations throughout Africa, Asia and Latin America use traditional medicine to help meet their primary health care needs, and it is estimated that in developing countries, as much as 80% of the indigenous populations depend on traditional systems of medicine and medicinal plants as their primary source of healthcare. Traditional medicine includes a variety of health practices, approaches, knowledge and beliefs that incorporate plant, animal and/or mineral based constituents, spiritual therapies and exercises used singularly or combined for prevention, diagnosis or treatment of disease (WHO, 2002).

Over the past decade, herbal medicine has become a topic of increasing global importance, with both medical and economic implications. In Australia, Europe and North America complementary and alternative medicine is increasingly used in parallel with allopathic medicine and in European in particular, herbal medicines represent an important share of the pharmaceutical market, with annual sales in the range of US$7 billion (Mukherjee, 2003). In the United States, the sale of herbal products has skyrocketed from $200 million in 1988 to greater than $3.3 billion in 1997 (Parekh *et al*., 2005; Mahady, 2001). There has been an increasing number of countries adopting national policies on traditional medicine and developing regulations with regards to defining the role that traditional medicine plays in their health care delivery systems (WHO, 2002).

Although free health care has become entrenched in South Africa's constitution, many rural people still rely on the cheaper traditional healing methods and it is estimated that between

12 and 15 million South Africans still use traditional remedies from as many as 700 indigenous plant species (Meyer and Afolayan, 1995), thus, the South African government now recognizes the importance of traditional medicine as a key provider of primary health care and is promoting the integration of traditional healing into the official health care system in the Drug Policy of the Government's Reconstruction and Development Programme (Hess, 1998).

Medicinal plants form the principal component of traditional medicine (Eloff, 1998) and have been used for centuries as remedies for human disease because they contain compounds with therapeutic values. Plant derived compounds are of interest because they are believed to be safer (due to their long term use) and more effective substitutes for synthetically produced antimicrobial agents (Maoz and Neeman, 1998), they are culturally acceptable and better compatible with the human body with fewer side effects (Parekh *et al*., 2005).

Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer *et al*., 1999). There has been a global growth in the plant-derived medicinally useful formulations, drugs and healthcare products, its market covering more than 60% of the products of plant origin. 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 plant and that 25% of modern medicine are descended from plants whilst others are synthetic analogues built on prototype compounds isolated from plants (Wakdikar, 2004).

Phytomedicines are defined as therapeutic agents derived from plants or parts of plants, or the preparations made from them. A phytomedicine, ideally, represents the totality of the medicinal plant or one of its parts (such as root, leaf, flower, fruit, etc.), rather than a single

isolated chemical compound (Foster, 1997). 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 *et al*., 1999). The same follows for plant therapeutic agents, and thorough biological evaluation of plant extracts is vital to ensure their efficacy, safety and quality (Eloff, 1998b). These factors are of importance if plant extracts are to be accepted as valid medicinal agents and as a prerequisite for global harmonization of herbal health claims of medicinal plants (Mahady, 2001). With the vast knowledge about plants and their metabolites the challenge is to ascertain their safety and efficacy, especially with regards to treating those diseases which represent the greatest burden, particularly for poorer populations (WHO, 2002). It is therefore important to investigate scientifically those plants which have been used in traditional medicines as potential sources of novel therapeutic compounds. The resurgence of interest in natural therapies and increasing consumer demand for effective, safe, natural products suggests that quantitative data on phytomedicines is needed (Hammer *et al*., 1999).

1.1. The therapeutic value of plant secondary metabolites

Plant secondary metabolites have found application in various types of treatments. They have been used in cancer treatments (Scheck *et al*., 2006; Iwu *et al*., 1999; Ahn, 1994; Matkowski and Wolniak, 2005), as anti-inflammatory agents (Gibbons, 2003; Briskin, 2000), antidiabetic agents (Day, 1990) and as antimicrobial agents with antiprotozoal (Meckes *et al*., 1999), antibacterial (Meyer and Afolayan, 1995), and antifungal (Dwivedi and Singh, 1999) activities, and in that capacity have also been found to mitigate the side effects associated with synthetic antimicrobials (Al-Bakri and Afifi, 2007).

Faced with the emergence of multi drug resistant microorganisms, plant derived compounds have become popular as substitutes for synthetically produced antimicrobial agents and they have been found to be useful as antibiotic resistance-modifying agents (Al-hebshi *et al*., 2006). Antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions while antimicrobials of plant origins treat infections while simultaneously mitigating many of the side effects that are associated with synthetic antimicrobials (Parekh *et al*., 2005).

Moreover, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics (Cowan, 1999). This has resulted in intensive research by ethnopharmacologists, botanists, microbiologists and natural product chemists (Tanaka *et al*., 2006) in the search for newer less expensive, alternative substances (Salie *et al*, 1996) especially with the realization that the effective life span of any antibiotic is limited (Clark, 1996). This has lead to the isolation and identification of a large variety of secondary metabolites produced by plants and subsequently their exploration for use as active principles in medicinal preparations (Taylor *et al*., 2001).

1.2. The *Helichrysum* species

There are over 600 species of *Helichrysum* occurring worldwide, with 245 found in Southern Africa. The *Helichrysum* species are annuals, herbaceous perennials or shrubs, growing to a height of 60-90 cm. They are usually found in the margins of forest woodlands or in open grasslands. They love the sun and do well in soils that are well drained and water retentive, light sandy or stony. General characteristics of *Helichrysum* species are that they grow as prostrate, semi-erect and erect herbs, are normally many-stemmed, and have woody taproots. The leaves

and stems have a whitish woolly appearance, especially when the plant is young and the flowers are small. The flowers have bracts, which are small modified leaves which grow as cup-like enclosures. The *Helichrysum* species produce many unusual products which are biologically active (Mathekga, 2000) and is known for its aromatic and therapeutic properties. Some members of the genus *Helichrysum* have been well characterized with respect to their secondary metabolites which are used by the plants as a biochemical defense mechanism against bacteria and fungi.

In Southern Africa *Helichrysum* species are used widely for medicinal purposes and in particular the South African species are generally used for infectious diseases and antibiotic activity has been demonstrated for a number of species (Mathkega, 2000). They are traditionally used in the treatment of wounds, infections and respiratory conditions. The extracts as well as the essential oils from *Helichrysum* species have exhibited promising biological activities in many *in vitro* assays which include anti-oxidant, antimicrobial and anti-inflammatory activity (Lourens *et al*., 2004).

1.2.1. *Helichrysum pedunculatum*

The candidate plant in this study is *Helichrysum pedunculatum*, a plant used by the Xhosa people of South Africa to treat traditional circumcision wounds. *Helichrysum pedunculatum* is a perennial herb with a wide distribution, ranging from southern Lesotho to the Eastern Cape Province (Meyer and Dilika, 1996). No specific antimicrobial properties of this plant have been reported although its medicinal values which included its ability to cure stomach ailments and its inflammatory properties have been cited by some authors (Meyer and Dilika, 1996).

The plant is used by the Xhosa and Fengu as a dressing after circumcision and the root is also used for coughs and colds (Hutchings, 1996). Antibacterial assays of *H. pedunculatum* showed that dichloromethane extracts were active against all the Gram positive bacteria tested, as well as two Gram negative bacteria, *Enterobacter cloacae* and *Serratia marcescens* (Meyer and Dilika, 1996). A water extract of *H. pedunculatum* was effective against *Staphylococcus aureus* and *Micrococcus kristinae* and the shaken methanol extract did not show any activity against the microorganisms tested (Meyer and Dilika, 1996). An antibacterial-activity guided fractionation of the dichloromethane extract of the plant resulted in the isolation of linoleic and oleic acids both of which inhibited a number of Gram positive bacteria tested. A strong synergistic effect between the two fatty acids was also observed against *Staph. aureus* and *M. kristinae*. No other constituents have been isolated form this plant except these fatty acids (Dilika *et al*., 2000).

1.3. Risks associated with the traditional circumcision rite

There are various risks associated with the practice of traditional circumcision among the Xhosa. Traditional circumcision is often performed in non-medical settings, under conditions conducive to sepsis. This increases the chances of health problems resulting from these surgical practices by poorly trained or untrained practitioners. In South Africa, every year, young *abakwetha* (Xhosa: male initiates) are hospitalized or die from circumcision wounds undergone during traditional initiation rites (Daily Sun, 2006). Ritual circumcision under some circumstances can put young men at risk of contracting sexually transmitted infections (STIs), HIV/AIDS and other blood-borne infections. Ischaemia (starvation of blood supply) or/and infection from the tight thong bandage wrapped around the wound, leads to penile sepsis and gangrene, with subsequent loss of

penile tissue. Infection can spread throughout the body and ultimately, septicemia is the cause of most deaths from circumcision. Septicemia is an infection in which large amounts of bacteria are present in the blood. It occurs as a result of localized infection in the body after which bacteria spill over the primary infection site into the blood and carried throughout the body.

It is difficult to quantify the morbidity and mortality associated with ritual circumcision in South Africa, as actual numbers of youths partaking in these rites annually is indeterminable. This is largely due to the esoteric nature of the rite and as a consequence, data collection is scant. However deaths, largely due to blood poisoning have been reported in the media (Natal Witness, 2002; Daily Sun, 2006).

1.3.1 Wound infections

All surgical wounds are contaminated by microbes, but a complex interplay between host, microbial, and surgical factors ultimately determines the prevention or establishment of a wound infection. Microbial factors that influence the establishment of a wound infection are the bacterial inoculum, virulence, and the effect of the microenvironment. Most surgical site infections (SSIs) are contaminated by the patient's own endogenous flora, which are present on the skin, mucous membranes, or hollow viscera (Krizek and Robson, 1975). Sources of such pathogens include surgical/hospital personnel and intraoperative circumstances, including surgical instruments, articles brought into the operative field, and the operating room air.

The usual pathogens on skin and mucosal surfaces are Gram-positive cocci (notably staphylococci). However, Gram-negative aerobes and anaerobic bacteria contaminate skin in the groin/perineal areas. Gram-positive organisms, particularly staphylococci and streptococci, account for most exogenous flora involved in SSIs. The most common group of bacteria

responsible for SSIs is *Staph. aureus*. According to a National Nosocomial Infections Surveillance (NNIS) Systems Report (1996), *Staph. aureus* accounts for 20% of the pathogens commonly associated with wound and burn infections and is the most common cause of acute pyogenic (pus-producing) infection in man. The emergence of resistant strains has considerably increased the burden of morbidity and mortality associated with wound infections. *Staphylococcus epidermis* is the name given to a heterogeneous group of coagulase-negative staphylococci which are often isolated from clinical specimens. They are generally of low pathogenicity and can be usually regarded as secondary invaders of little importance but are an increasingly common cause of severe infections such as sepsis associated with intravascular lines (Thomas, 1991).

Most *Bacillus* species are regarded as having little or no pathogenic potential. However, both *Bacillus cereus* and *B. subtilis* have been known to act as primary invaders or secondary infectious agents in a number of cases (Mathekga *et al*., 2000). On a few occasions highly toxigenic strains of *B. cereus* have caused septic conditions in man. Other microorganisms listed in the NNIS Systems Report as important wound infection causative agents include e*nterococci*, *Pseudomonas aeruginosa*, *Enterobacter* species, *Candida albicans* and group D streptococci.

1.4. Aims of the study

The broad aim of the study was to carry out an assessment of the antimicrobial potential of the methanol extract of *H. pedunculatum*. The specific objectives include:

i) to screen the crude methanol extract of *H. pedunculatum* for antibacterial activity against a panel of bacterial isolates;

ii) to determine the Minimum Inhibitory Concentration (MIC) of the crude methanol extract of the plant, against a panel of different microbial species;

iii) to determine the rate of killing of the extract against selected susceptible bacterial strains in order to quantitatively characterize the relationship between bactericidal activity and the concentration of antimicrobial agents;

iv) to investigate possible synergistic interactions of the plant extract with selected antibiotics.

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CHAPTER 2

Assessment of Antimicrobial Properties of Natural Compounds of Plant Origin: Current Methods and Future Trends

Abstract

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 incidence 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 plants/plant 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.

Keywords: medicinal plants, plant extracts, antimicrobial agents, antimicrobial activity,

1.0 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 plant. 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 *et al*., 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 by a number of researchers world wide. 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).

1.1 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 tissues 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 the 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).

1.1.1 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 e.g. 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 *et al*., 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*, *Staph aureus* (including methicillin resistant *Staph. 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 plant secondary metabolites (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 (e.g. tepernoids), those produced by the modification of aromatic amino acids (e.g. 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 e.g. 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).

1.1.2 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 *Ps. aeruginosa*, which is

resistant to different antibiotics, its growth was inhibited by the 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).

2.0 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 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.

2.1 Plant extract preparation

Extraction methods, used pharmaceutically, involve separation 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, solvent concentration and polarity will also affect quantity and secondary metabolite composition of an extract (SEA, 2006).

2.1.1 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°C for 72 hr (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.

2.1.2 Choice of solvent

Successful determination of botanical 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, 1998b). 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 and Raman, 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; Bisignano *et al*., 1999; Lourens *et al*., 2004; Salie *et al*., 1996, Rojas *et al*., 2005). 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*., 1995; Lourens *et al*., 2004, Mathkega *et al*., 2000). 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 (1998b) 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 (1998b) does note that different results may be obtained with other plants and generalization cannot be made on the usefulness of acetone as an extractant.

2.1.3 The extraction method

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), five minute extractions of very fine particles of diameter 10µm gave higher quantities

than values obtained after 24 hr 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 minutes 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 mins 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 minutes or left for 24 hrs after which the extract is filtered and fresh solvent added to the residue for another 24 hrs. Some publishers report shaking unhomogenized dry leaves in solvent for about 5 minutes, 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 (e.g. hexane) to a more polar solvent (e.g. 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 (SFE) (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 cannot 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).

2.2 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), 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.

2.2.1 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) i.e. 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 factors, 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 agar 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 $1x10^8$ cfu/ml of bacteria for inoculating diffusion plates (Baris *et al*., 2006). There has been noted variation on whether the disks are impregnated with antimicrobial agent after or before placing on the inoculated plate. Some impregnate the disks before placing on the agar (Lourens *et al*., 2004; Salie *et al*., 1996) while others place the disk on the plate first before impregnating them (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 hrs 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 hrs for bacteria and 48 hrs for fungi (Salie *et al*., 1996; Baris *et al*., 2006). Some were incubated for 18 hrs at 37 °C for the same species of 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).

2.2.2 Agar-well diffusion method

The principle of the agar well diffusion method is the same as that of the agar-disk diffusion method. A standardized inoculum culture is spread evenly on the surface of gelled agar 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 hrs for bacteria (Mbata *et al*., 2006).

2.2.3 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 gel plates and developed with an appropriate solvent system. A suspension of the test bacteria is sprayed onto the TLC plate. Some authors report 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 hrs in humid conditions. Microbial indicators (usually tetrazolium salts) are used as a growth detectors (Silva *et al*., 2005). These are sprayed onto the plates after which the plates are reincubated at 25

°C for 24 hrs (Dilika and Meyer, 1996) or at 37°C for 3-4 hrs (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).

2.2.4 Broth microdilution

The micro-titre plate/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 dimethyl sulfoxide (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 is 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 hr (Lourens *et al*., 2004) while the recommended temperature by EUCAST (2003) is 35-37°C in air for 16-20 hr for non-fastidious organisms. 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 hour broth culture adjusted to a 0.5 McFarland turbidity standard (Baris *et al*., 2006). EUCAST (2003) recommends that plates be inoculated within 30 minutes 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 (Umoh *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 hours and changes in colour or absence of colour, 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.

2.2.5 Agar dilution assay

The agar dilution test is more versatile than the broth dilution assay and does not present problems encountered with the latter i.e. 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 agar (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 1µl (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 hrs. 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).

2.3 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 British Society for Antimicrobial Chemotherapy (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 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 National Committee on Clinical Laboratory Standards (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.

3.0 Conclusion

The ethnomedicinal study of plants is important for modern day medicine but its usefulness can not be over-emphasized if methods are not standardized to obtain comparable and reproducible results. Other pharmacological studies like time kill studies; potentials for combination antimicrobial chemotherapy and mechanisms 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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References

Acamovic T, Brooker JD (2005). Biochemistry of plant secondary metabolites and their effects in animals. Proceedings of the Nutrition Society 64: 403-412.

Afolayan AJ, Meyer JJM (1997). The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. Journal of Ethnopharmacology 57: 177-181.

Ahn JW (1994). Cytotoxic Limonoids from *Melia azedarach* var. *japonica*. Phytochemistry 36(1): 1493-1496.

Angeh JE (2006). Isolation and characterization of antibacterial compounds present in members of *Combretum* section, *Hypocrateropsis*. PhD Thesis.University of Pretoria.

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). Turkish Journal of Biology 30: 65-73.

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

Bennet RN and Wallsgrove RM (1994). Secondary metabolites in Plant defense mechanisms. New Phytologist 127: 617-633.

Bisignino G, Sanogo R, Marino A, Aquino R, D'angelo V, Germano MP, De Pasquale R, Pizza C (1999). Antimicrobial activities of *Mitracarpus scaber* extract and isolated constituents. Letters in Applied Microbiology 30: 105-108.

Cork SJ and Krochenberger AK (1991). Methods and pitfalls of extracting condensed tannins and other phenolics from plants: Insights from investigations on Eucalyptus leaves. Journal of Chemical Ecology 17:123-134.

Cowan MM (1999). Plant products as antimicrobial agents. Clinical Microbial Review 12(4): 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. An. Acad. Bras. Cienc 76(3): 499-504.

Devienne KF, Raddi MSG (2002). Screening for antimicrobial activity of natural products using a microplate photometer. Brazilian Journal of Microbiology 33(2).

Dilika F, Afolayan AJ, Meyer JJM (1996). Comparative antibacterial activity of two *Helichrysum* species used in male circumcision in South Africa. South African Journal of Botany 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 71:450-452.

Doughari JH (2006). Antimicrobial activity of *Tamarindus indica* Linn. Tropical Journal of Pharmaceutical Research 5(2): 597-603.

Eloff JN (1998a). A sensitive and quick microplate method to determine the minimum inhibitory concentration of plant extracts for bacteria. Planta medica 64: 711-713.

Eloff JN (1998b). Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology 60:1-8.

EUCAST Definitive Document (2000). Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. Clinical Microbiology and Infection 6(9): 503-508.

EUCAST Discussion Document (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clinical Microbiology and Infection 9(8): 1-7.

Freixa B, Vila R, Vargas L, Lozano N, Adzet T, Caniguera S (1996). Screening for antifungal activity of nineteen Latin American plants. Phytotherapy Research 12(6): 427-430.

Giannoccano E, Wang Y, Chen P (2006). Effects of solvent, temperature, time, solvent-to-sample ratio, sample size and defatting on the extraction of soluble sugars in soybean. Journal of Food Science 71(1): C59.

Gibbons S (2003). An overview of plant extracts as potential therapeutics. Expert Opinion on Therapeutic Patents 13(4): 489-497.

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. Journal of Ethnopharmacology 66:103-106.

Hammer KA, Carson CF, Riley TV (1999). Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86(6): 985.

Handa SS (2006). An overview of extraction techniques for medicinal and aromatic plants. South East Asia Regional Workshop on Extraction Technologies for Medicinal and Aromatic plants.

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. Planta Medica 58(2):176-183.

Houghton PJ and Raman A (1998). Laboratory Handbook for the fractionation of Natural extracts.

Hughes I (2002). Science in Africa Magazine. Issue 56.

Iwu MW, Duncan AR, Okunji CO (1999). New antimicrobials of plant origin. In:J.Janick(editor), Perspectives on new crops and new uses. ASHS Press. Alexandria 457-462.

Joyce LF, Downes J, Stockman K, Andrew JH (1992). Comparison of five methods, including PDM Epsilometer test (E-test), for antimicrobial susceptibility testing of *Pseudomonas aeruginosa*. Journal of Clinical microbiology 30(10): 2709-2713.

Kianbakht S, Jahaniani F (2003). Evaluation of antibacterial activity of *Tribulus terrestris* L. growing in Iran. Iranian Journal of Pharmacology 2(1).

Lampinem J (2005). Continous Antimicrobial susceptibility testing in drug discovery. DrugPlus International.

Langfield RD, Scarano FJ, Heitzman ME, Kondo M, Hammond GB, Neto CC (2004). Use of a modified microplate bioassay method to investigate antibacterial activity in the Peruvian medicinal plant *Peperomia galiodes*. Journal of Ethnopharmacology 94(2-3): 279-281.

Leite SP, Vieira JRC, de Madeiros PL, Leite RMP, Lima VL, Xavier HS, Lima E (2006). Antimicrobial activity of *Indigofera suffruticosa*. Oxford University Press.

Lemberkovics KE, Kakasy A, Szoke E, Simai B (2002). Effect of extraction methods on the composition of essential oils. ISHS Acta Horticulture 597: International Conference on Medicinal and Aromatic plants (Part II).

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. Journal of Ethnopharmacology 9: 253-258.

Masoko P, Eloff JN (2006). Bioautography indicates the multiplicity of antifungal compounds from twenty-four Southern African *Combretum* species (Combretacea). African Journal of Biotechnology 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.

Mathekga ADM, Meyer JJM, Horn MM, Drewes SE (2000). An acylated phloroglucinol with antimicrobial properties from *Helichrysum caesptitium*. Phytochemistry 53: 93-96.

Matsumuto 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. Antimicrobial Agents and Chemotherapy 46(2): 308-314.

Mbata TI, Debiao L, Saikia A (2006). Antibacterial activity of the crude extract of Chinese Green Tea (*Camellia sinensis*) on *Listeria minocytogenes*. The Internet Journal of Microbiology 2(2).

Mendoza MT (1998). What's new in Antimicrobial Susceptibility Testing. Philippines Journal of Microbiological Infectious Diseases 27(3): 113-115.

Meyer JJM, Afolayan AJ (1995). Antibacterial activity of *Helichrysum aureonitens* (Asteraceae). Journal of Ethnopharmacology 47(2):109-111.

Meyer JJM, Dilika F (1996). Antibacterial activity of *Helichrysum pedunctulatum* used in circumcision rites. Journal of Ethnopharmacology 53(1): 51-54.

Mitscher LA, Leu R, Bathala MS, Wu W, Beal JL (1972). Antimicrobial agents from higher plants. Introduction, rational, and methodology. Lloydia 35:157-166.

Nang HLL, May CY, Ngan MA, Hock CC (2007). Extraction and identification of water soluble compounds in Palm Pressed Fiber by SC-CO_2 and GC-MS. American Journal of Environmental Science 3(2): 54-59.

Nascimento GGF, Locatelli J, Freitas PC, Silva GL (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Brazilian Journal of Microbiology 31: 247-256.

Nostro A, Germano MP, D'Angelo V, Marino A, Cannatelli MA, 2000. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Letters in microbiology 30(1): 379-384.

Ogunleye DS, Ibitoye SF (2003). Studies of antimicrobial activity and chemical constituents of *Ximenia americana*. Tropical Journal of Pharmaceutical Research 2(2): 239-241.

Osbourne AE (1996). Preformed antimicrobial compounds and plant defense against fungal attack. The plant cell 8: 1821-1831.

Parekh J, Jadeja D, Chanda S (2005). Efficacy of Aqueous and Methanol Extracts of Some Medicinal Plants for Potential Antibacterial Activity. Turkish Journal of Biology 29:203-210.

Parekh J, Karathia N, Chanda S (2006). Screening of some traditionally used medicinal plants for potential antibacterial activity. Indian Journal of Pharmaceutical Sciences 68(6): 832-834.

Rojas JJ, Ochoa VJ, Ocampo SA, Monoz JF (2005). Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in treatment of non-nosocomial infections. BMC Complementary and Alternative Medicine 6:2.

Runyoro DKB, Matee MIN, Ngassapa OD, Joseph CC, Mbwambo ZH (2006). Screening of Tanzanian medicinal plants for anti-candida activity. BMC Complementary and Alternative Medicine 6(11).

Salie F, Eagles PFK, Lens HMJ (1996). Preliminary antimicrobial screening of four South African Asteraceae species. Journal of Ethnopharmacology 52(1): 27-33.

Scheck AC, Perry K, Hank NC, Clark WD (2006). Anticancer activity of extracts derived from the mature roots of *Scutellaria baicalensis* on human malignant brain tumor cells. Biomed Central Complementary and Alternative medicine 6:27.

Schmourlo G, Mendonca-Filho RR, Alviano CS, Costa SS (2004). Screening of antifungal agents using ethanol precipitation and bioautography of medicinal food plants. Journal of Ethnopharmacology 96(3): 563-568.

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).

Silva O, Duarte A, Cabrita J, Pimentel M, Diniz A, Gomez E (1996). Antimicrobial activity of Guinea-Bissau traditional remedies. Journal of Ethnopharmacology 50: 53-59.

South-East Asian (SEA) Regional Workshop on Extraction Technologies for Medicinal and Aromatic Plants (2006). India.

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, Lewis K (2000). Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. Proceedings of the National Academy of Sciences of the United States of America 97(4): 1433-1437.

Struelens MJ, Nonhoff C, Van Der Auwera P, Mertens R, Serruys E (1995). Evaluation of Rapid ATB Staph for 5-Hour Antimicrobial Susceptibility Testing of *Staphylococcus aureus*. Journal of Clinical Microbiology 33(9): 2395-2399.

Tanaka JCA, da Silva CC, de Oliveira AJB, Nakamura CV, Dias Filho BP (2006). Antibacterial activity of indole alkaloids from *Aspidosperma ramiflorum.* Brazilian Journal of Medical and Biological Research 39(3): 387-391.

Taylor JLS, Rabe T, McGaw LJ, Jäger AK and van Staden J (2001). Towards the scientific validation of traditional medicinal plants. Plant growth regulation 34(1): 324-327.

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*. Journal of Clinical Microbiology 33(6): 1524-1527.

Tepe B, Donmez E, Unlu G, Polissiou M, Sokmen A (2004). Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *Salvia cryptantha* (Montbret et Aucher ex Bench.) and *Salvia multicaulis* (Vahl). Food Chemistry 84(4): 519-525.

Thomas C.G.A (1991). Medical Microbiology, 6th Ed., University Press, Cambridge. Great Britain

Umoh EU, Oluma HOA, Igoli O (2005). Antibacterial screening of four local plants using an indicator-based microdilution technique. African Journal of Traditional Complementary and Alternative Medicines 2(3): 238-243.

Vilegas JHY, de Marchi E, Lancas FM (2002). Extraction of low polarity compounds (with emphasis on coumarin and kaurenoic acid) from *Mikania glomerata* ('guaco') leaves. Phytochemical Analysis 8(5): 266-270.

Wallace JR (2004). Antimicrobial properties of Plant Secondary Metabolites. Proceedings of the Nutrition Society 63: 621-629.

Yamaji K, Ishimoto H, Usui N, Mori S (2005). Organic acids and water soluble phenolics produced by *Paxillus* species. Mycorrhiza 15(1):17-23.

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant material

The plants used in this study were collected at the University of Fort Hare Research farm between August 2006 and June 2007 and identified by Professor D Grierson, Botany Department as *Helichrysum pedunculatum.* The plants were cleansed with water and the leaves air-dried to constant weight at room temperature (24 °C).

3.2. Preparation of crude extract

Crude extract of the dry leaves of the plant was prepared with methanol, following the method of Basri and Fan (2005). For each extraction, 50 g of plant material was first extracted in 500 ml absolute methanol and left for 24 hours on a shaker at room temperature. Most protocols propose a 1:5 w/v plant material to solvent but because of the nature of the plant material the volume was increased to obtain a 1:10 w/v mixture. After the 24 hr period, the extract was expressed and 500 ml fresh solvent added to the residue and left for another 24 hours after which it was expressed again. The residue was discarded and the extract filtered with Whatman no. 1 filter paper and evaporated to dryness in a rotary evaporator under reduced pressure. The extract was evaporated in a weighed flask to allow for calculation of the yield of the extraction process. The yield of the methanol extraction was 12 % on average. A stock solution of 50 mg/ml of the extract was prepared, from the dry extract, in methanol and stored in the fridge at about 4 °C for future use.

3.3. Test organisms

Bacteria species that have been implicated in wound infections were used in the study and these were selected to represent environmental isolates, reference strains and clinical isolates and they included the following: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 10389, *Streptococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 10702, *Enterobacter cloacae* ATCC 13047, *Klebsiella pneumonae* ATCC 10031, *Proteus vulgaris* ATCC 6830, *Acinetobacter calcoceticus* UP, environmental strains of *Staphylococcus epidermidis* and *Staphylococcus aureus* and clinical staphylococcal isolates (*Staphylococcus aureus* OKOH 1, OKOH 2A, OKOH 3 and *Staphylococcus sciuri* OKOH 2B). These clinical staphylococcal strains have previously been isolated by the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare and their 16S rRNA gene sequences are available in the Gene Bank and their accession numbers are EU244633, EU244634, EU244635 and EU244636.

3.4 Antibacterial Activity of plant extract

The agar dilution method of Afolayan and Meyer (1997) was used for assaying the antibacterial activity of the plant extract. Agar was prepared following the manufacturer's instructions and placed in a water bath at 50 °C. The extract stock solution was filtered through a 0.22 µm filter then incorporated in the molten agar at different volumes to obtain a range of concentrations between 0.1 and 5 mg/ml. The agar-extract was poured into sterile Petri dishes and allowed to cool. Controls were set up, one containing only nutrient agar and the second containing nutrient agar and 10 % methanol (highest concentration of methanol achieved by any dilution). The organisms were radially streaked onto the solidified agar-extract plates. Complete inhibition of bacterial growth was expected for an extract to be declared active and the minimum inhibitory

concentration (MIC) was defined as the lowest concentration of the extract that inhibited growth of the test organism.

3.4.1 Preparation of bacterial inocula

All test bacteria were maintained on nutrient agar and to prepare inoculum for the agar dilution procedure, the test microorganisms were recovered in sterile nutrient broth and incubated overnight at 37 °C. Prior to streaking the inoculum onto the agar extract plates, the overnight culture was diluted 1:100 v/v in fresh sterile nutrient broth.

3.5 Antibacterial Susceptibility Testing of Penicillin and Chloramphenicol

Antibacterial susceptibility testing (AST) of the antibiotics was done following the description of the European Committee for Antimicrobial Susceptibility Testing (EUCAST), (2000). A stock solution of the antibiotic was prepared in accordance with the description of the manufacturer. Different volumes of the stock solution were incorporated in sterile, molten agar to obtain a two fold dilution series ranging from 0.004 mg/L to 512 mg/L. The agar was poured onto sterile Petri dishes and allowed to cool before streaking the test organisms. The plates were inoculated with a wire loop and incubated at 37 °C for 18 hr.

3.5.1 Inoculum preparation

The colony suspension method according to EUCAST (2003) was used for preparation of the inoculum. The test organism was cultured in nutrient agar overnight. Identical colonies from the culture were suspended in sterile saline. The suspension was adjusted with saline to give an

optical density of 0.1 at 600_{nm}. The adjusted inoculum was diluted 1:100 in broth to give an approximate inoculum of 5×10^5 cfu/ml.

3.6. Time kill studies of the methanol extract of *H. pedunculatum*

The broth macrodilution method of Okoli and Iroegbu (2005) was used for the time kill assay. Kill kinetics were done at MIC and 2×MIC (previously determined) of three selected test organisms *B. cereus*, *P. vulgaris* and *Staph. aureus* OKOH1. These concentrations were developed in 10 ml nutrient broth from a 100 mg/ml stock solution of the plant extract. This was inoculated with 0.5 ml of test organism prepared by the colony suspension method described above. The final inoculum density was approximately 10^5 cfu/ml. A negative and a positive control were set up, without the plant extract and the other supplemented with 5 mg/L ciprofloxacin respectively. Incubation was done at 37 °C on an orbital shaker at 120 rpm.

At time intervals ranging from 0 to 24 hr the numbers of surviving microorganisms were counted. About 0.2 ml from each sample was withdrawn and diluted in a ten fold series in sterile normal saline (0.85 % w/v NaCl). About 0.2 ml of each dilution was plated in duplicate in nutrient agar using the pour plate method. Triphenyltetrazolium chloride (TTC) was incorporated in the molten sterile agar before pouring at a ratio of 1:100 v/v, to facilitate easier enumeration of the colonies after the incubation period. The TTC was prepared in sterile water to obtain a 0.5 % solution and filtered in a 0.22 µm filter before incorporating into the agar to maintain its sterility. The plates were incubated at 37 °C for 24 hours after which colonies were counted from the test plates. Colonies were counted from plates containing between 30-300 colonies. The results were expressed as $\log_{10}$ cfu/ml and time kill curves plotted.

3.7. Combination studies

Combination studies were done following the principle of the time kill assay, to measure the total effect of the combined antimicrobial agents compared with the most active agent in the combination. This was done following the guidelines from the methods of Lee *et al.* (2005) and Pankey *et al*. (2005). Combinations were made in 10 ml broth at $^{1}/_{2}$MIC, 1×MIC and 2×MIC of plant extract with 1×MIC of the two antibiotics chloramphenicol and penicillin. Both the extract and antibiotics were tested alone as controls at all concentrations included in the combinations. The test organisms, *B. cereus, P. vulgaris* and *Staph. aureus* OKOH1 were inoculated at a rate of approximately 10^5 cfu/ml and incubated at 37 °C. About 0.2 ml aliquots of each sample were taken at 0 and 24 hr intervals for viable counts. Each aliquot was serially diluted in sterile saline and plated on nutrient agar using the pour plate method. Colonies were counted on plates yielding 30-300 colonies after incubation at 37 °C for 24 hr. Plating was done in duplicate and results expressed as mean $\log_{10}$ cfu/ml. The log change after 24 hr was calculated for each sample by subtracting the $\log_{10}$ at 0 hr from the $\log_{10}$ at 24 hr to determine if there was bactericidal activity. The effect of the combination was calculated by the difference between the survivors in the combination and the more active of the two agents in the combination. Synergy and additivity/indifference were defined, respectively, as a $\geq 2\log_{10}$ cfu/ml decrease and a $< 2\log_{10}$ cfu/ml change in the viable count of test organism in the combination, in comparison with the most active single drug. In an antagonistic combination, the combined effect of the drug is less than their independent effect.

CHAPTER 4

RESULTS

4.1 Antibacterial activity

The methanol extract of *H. pedunculatum* was screened for antimicrobial activity up to a concentration of 5mg/ml using the agar dilution technique. Eight out of the 14 bacterial isolates tested were found to be susceptible to the effect of the extract at a final concentration of 5 mg/ml (Table 4.1) while the remaining six bacterial isolates were resistant to the extract. The minimum inhibitory concentrations (MICs) of the extract against the susceptible strains were observed to range between 1 and 5 mg/ml. *Acinetobacter calcoceticus* and the environmental *Staphylococcus epidermidis* had MIC of 1 mg/ml, while the other susceptible strains had MIC of 5 mg/ml (Table 4.1).

Table 4.1. Antibacterial activity of the methanol extract of *H. pedunculatum* against selected bacterial strains.

Organism	**Gram reaction**	**Activity**	**MIC (mg/ml)**
Escherichia coli ATCC 8739	-	-	ND
Staphylococcus aureus ATCC 10389	+	-	ND
Streptococcus faecalis ATCC 29212	+	-	ND
Bacillus cereus ATCC 10702	+	+	5
Enterobacter cloacae ATCC 13047	-	-	ND
Klebsiella pneumonae ATCC10031	-	-	ND
Proteus vulgaris ATCC 6830	-	+	5
Acinetobacter calcoceticus §	-	+	1
Staphylococcus epidermidis §	+	+	1
Staphylococcus aureus §	+	+	5
Staphyloccocus aureus OKOH 1♠	+	+	5
Staphylococcus aureus OKOH 2A♠	+	+	5
Staphylococcus sciuri OKOH 2B♠	+	-	ND
Staphylococcus aureus OKOH 3♠	+	+	5

Key: MIC represents minimum inhibitory concentration; § represents environmental strains and ♠ represents clinical isolates. ND – Not investigated beyond 5 mg/ml.

4.2. Time kill assays

Bactericidal activity, measured by assay of viable cell density at different time intervals, was determined by the time kill assay. Cell viability was plotted against time to obtain kill curves to depict the effect of different doses of the extract on test organisms. A 1×MIC concentration of the methanol extract of *H. pedunculatum* did not reduce the initial inoculum of *B. cereus* even after 24 hr of exposure. The bacterial growth was inhibited for the first 4 hr but recovers thereafter and follows a normal growth pattern just slightly lower than the control curve (Fig. 4.1). At 2 × MIC, the initial inoculum does not show any significant reduction for the first 12 hr and is reduced by only about 1.32 Log_{10} units after 24 hr of exposure to the extract. The antibiotic exhibited considerable action on *B. cereus* reducing viable counts to 0 after 24 hr (Fig. 4.1).

Time kill studies for *P. vulgaris* revealed a more bactericidal action of the extract. At 1 × MIC the organism multiplied just slightly slower than in the broth control. At 2 × MIC the bacterial growth remained almost static for the first 4 hr and thereafter reduced to almost zero with a 5 Log_{10} reduction in 8 hr (Fig. 4.2). Ciprofloxacin had a bactericidal effect on the bacteria from the onset of the experiment managing to eliminate the organisms in 4 hr.

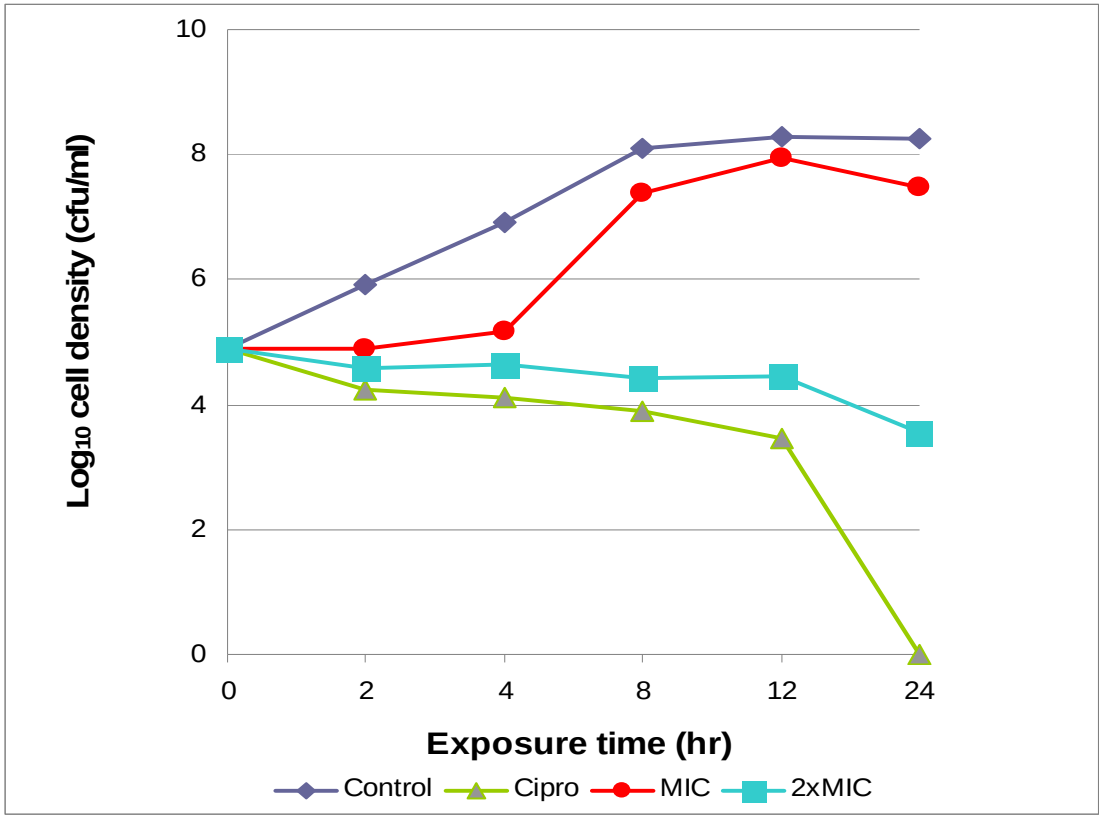

Figure 4.1. Time-kill curve of the methanol extract of *H. pedunculatum* against *B. cereus.*

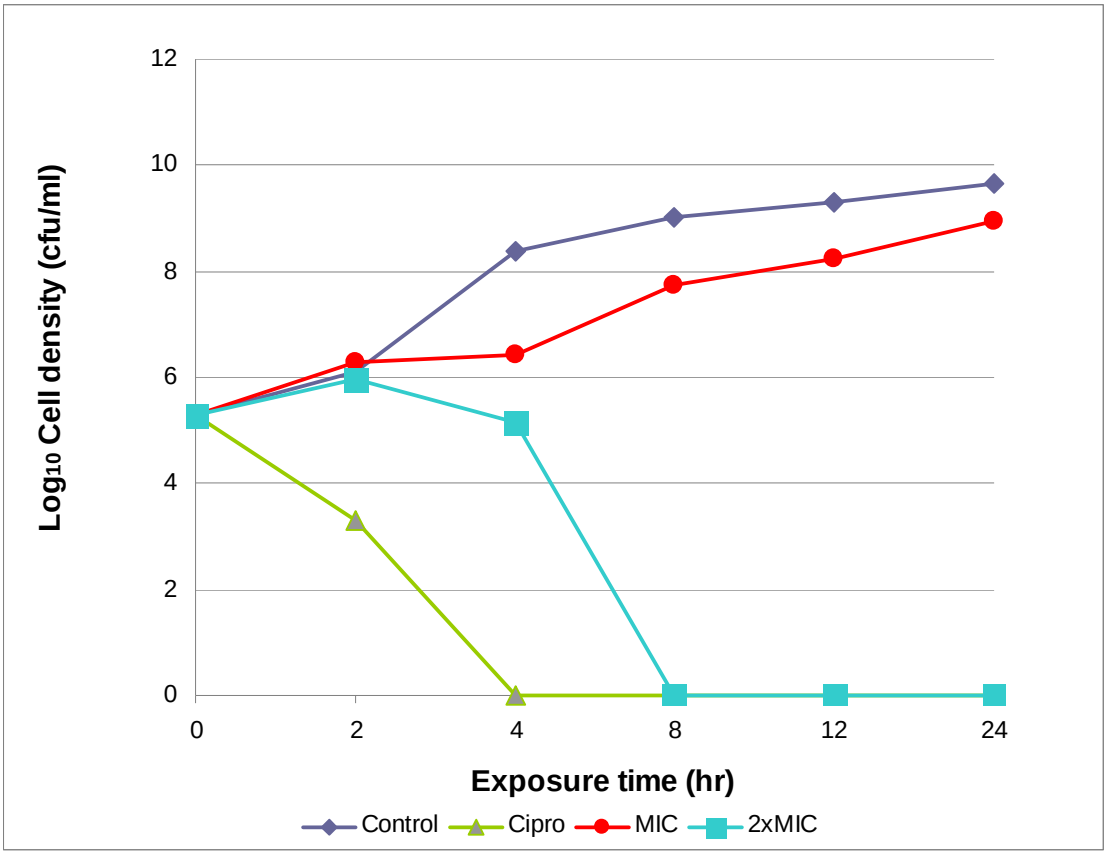

Figure 4.2. Time-kill curve of the methanol extract of *H. pedunculatum* against *P. vulgaris.*

At a concentration of 1 × MIC viable counts of *Staph. aureus* OKOH 1 were initially reduced for the first 4 hr after which the organism multiplied exponentially. At 2 × MIC the microorganisms steadily decreased in growth until completely eliminated in 24 hr. In the ciprofloxacin control, there was an initial slight increase in inoculum after which it steadily decreased by 2 Log_{10} units after 24 hr. The antibiotic did not eliminate all the microorganisms after the 24 hr exposure period (Fig. 4.3).

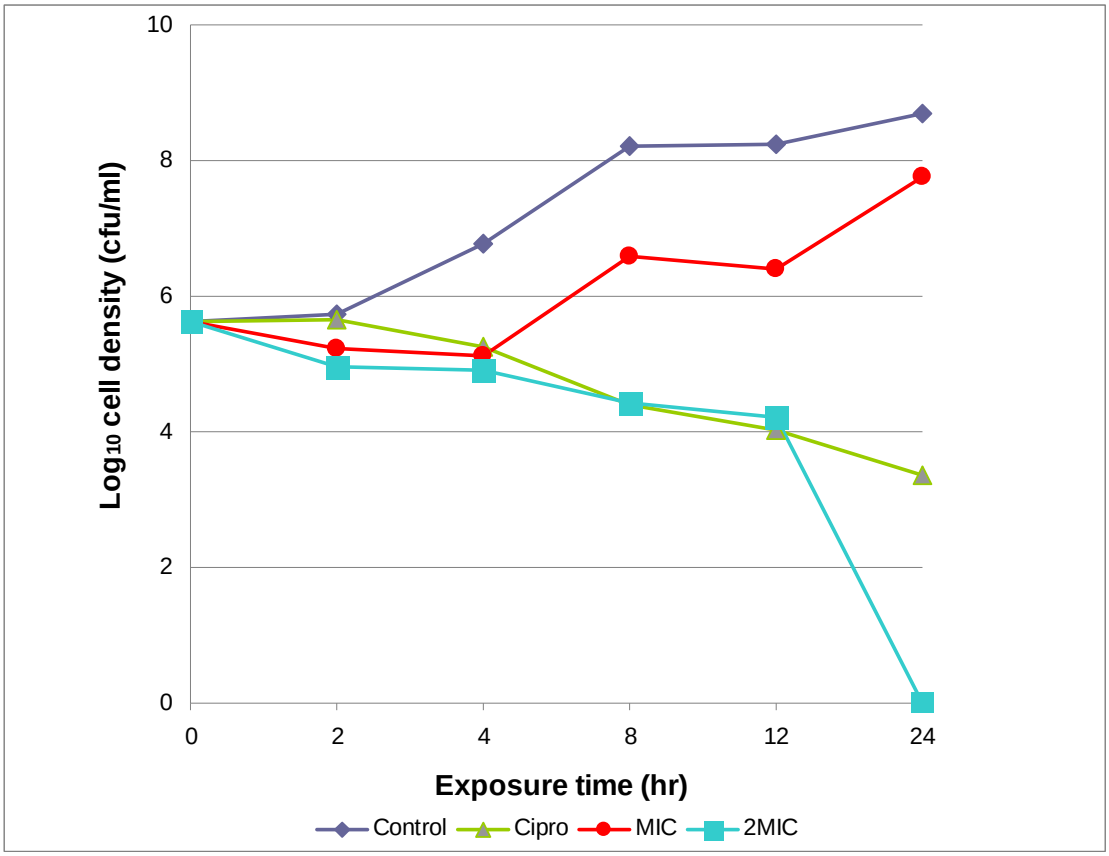

Figure.4.3. Time-kill curve of the methanol extract of *H. pedunculatum* against *Staph. aureus* OKOH1.

4.3. Determination of MIC of Chloramphenicol and Penicillin

Three bacterial strains namely *B. cereus* ATCC 10702, *P. vulgaris* ATCC 6830 and *Staph. aureus* OKOH1 and two antibiotics (chloremphenicol and penicillin) were selected for combination studies. The study demands pre-determination of the minimum inhibitory concentrations of the antibiotics against the test organisms. Chloramphenicol was observed to have MIC of 2 mg/L against *B. cereus* ATCC 10702 and *P. vulgaris* ATCC 6830 while it was slightly higher (4 mg/ml) for the clinical isolate (*Staph. aureus* OKOH1). *B. cereu*s and *P. vulgaris* showed more resistance to penicillin with MICs of 32 mg/L, 16 times higher than that of chloramphenicol. The MIC of penicillin against *Staph. aureus* OKOH1 was 2 mg/L (Table 4.2).

Table 4.2. Minimum Inhibitory Concentrations of Chloramphenicol and Penicillin against the test bacterial strains.

Organism	**MIC (mg/L)**	
	Chloramphenicol	**Penicillin**
B. cereus ATCC 10702	2	32
P. vulgaris ATCC 6830	2	32
Staph. aureus OKOH 1	4	2

4.4. Combination Studies

Combination studies were done following the principle of the time kill assay. Reduction in bacterial counts (in Log_{10} units) was calculated by subtracting viable counts of the most active antimicrobial alone from the counts obtained in the combination. The antibiotics were combined at 1× MIC while the concentration of the plant extract was varied. The results of the combination studies are as shown in Table 4.3.

A combination of ½× MIC plant extract with 1× MIC of chloramphenicol gave an indifferent interaction against *B. cereus* (a reduction of 0.17892 Log units). Increasing the concentration of the extract, while the antibiotic was kept constant, improved the activity of the two antimicrobials. At 1× MIC and 2× MIC of plant extract there was a total of 3.43933 and 3.04139 log reductions respectively. The same trend was observed with *P. vulgaris*, as increasing the concentration of the extract appeared to improve the activity of the combination with chloramphenicol. At $^{1}/_{2}$× MIC of the extract the combination was antagonistic with the combination resulting in an increase of 1.308329 log units in viable counts compared to the more active of the two antimicrobial agents. At 1× MIC and 2× MIC the activity was improved to achieve indifferent interactions. All combinations with chloramphenicol gave indifferent interactions against *Staph. aureus* OKOH 1, but the figures, however, suggest that further increasing the concentration of the plant extract might have resulted in antagonistic interactions. Combining $^{1}/_{2}$× MIC of the plant extract with 1× MIC of penicillin did not improve the activity of the antimicrobials against *B. cereus* but resulted in a 0.192422 log increase in viable counts compared with the more active of the antimicrobials alone. The activity was however enhanced by increasing the concentration of the plant extract achieving a 0.44494 and 0.54921 log reductions with 1× MIC and 2× MIC of the extract respectively. In contrast, increasing the

concentration of the extract reduced the activity of the antimicrobials against *P. vulgaris*. At $^{1}/_{2}\times$ MIC the interaction was synergistic achieving a 5.43246 log reduction of the bacteria. The interactions are however indifferent at 1× and 2× MIC. The phenomenon is the same for *Staph. aureus* OKOH1 with a bacterial reduction of 3.62492 log at $^{1}/_{2}\times$ MIC of plant extract but indifferent interactions at higher concentrations of the methanol extract (Table 4.3).

Table 4.3. *In vitro* activity *H. pedunculatum* of extract-antibiotic combinations against 3 selected bacterial strains.

Organism	**Reduction in bacterial counts (log_{10} cfu/ml) by combination compared with the most active antimicrobial alone**					
	$^{1}/_{2}$ MIC E/1MIC Ch	1MIC E/1MIC Ch	2MIC E/1MIC Ch	$^{1}/_{2}$ MIC E/1MIC Pn	1MIC E/1MIC Pn	2MIC E/1MIC Pn
B. cereus	-0.17892 (A/I)	-3.43933 (S)	-3.04139 (S)	0.192422 (AN)	-0.44494 (A/I)	-0.54921 (A/I)
P. vulgaris	1.308329 (AN)	0 (A/I)	0 (A/I)	-5.43246 (S)	0 (A/I)	0 (A/I)
S. aureus OKOH1	-1.77477 (A/I)	0 (A/I)	0 (A/I)	-3.62492 (S)	0 (A/I)	0 (A/I)

Ch - chloramphenicol, Pn - penicillin. S - synergistic, I - Indifferent/additive, AN – Antagonistic.

CHAPTER 5

DISCUSSION AND CONCLUSION

The results obtained from these investigations show that eight out of fourteen bacterial isolates tested against the methanol extract of *H. pedunculatum* were susceptible at a final concentration of 5 mg/ml. These are mostly Gram positive bacteria but, however, one can not pick out a trend to compare susceptibilities of the Gram negative and Gram positive bacteria due to the small range of concentrations that were used in the investigation. However, from previous studies, the plant has been shown to inhibit mostly Gram positive bacteria. These were also shown to be inhibited at relatively lower concentrations than Gram negative bacteria (Dilika *et al*., 2000; Meyer and Dilika, 1996). This is consistent with findings from other authors that Gram negative bacteria are usually more resistant to antimicrobial agents than Gram positive bacterial species (Basri and Fan, 2005; Meyer and Afolayan, 1995). This is unexpected considering that major plant pathogens belong to the Gram-negative bacteria, but could be due to the fact that Gram-negative bacteria have a permeability barrier (Mathkega, 2001), comprised of the outer membrane, which restricts the penetration of amphipathic compounds. For a biologically active compound to have activity in a cell it must first diffuse from its site of application, usually the exterior of the cell, to its site of action, often within the cell and partition itself onto the active site (Hansch and Lien, 1971). The rate of these events will depend on the lipophilicity of the compound and the presence of multidrug resistance pumps (MDRs), which extrude toxins across this barrier. Gram negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic

solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da (Nostro *et al*., 2000).

Meyer and Dilika (1996) reported on the antimicrobial activity of a shaken methanol extract of *H. pedunculatum*. They reported that the extract was not effective even up to a concentration of 100 mg/ml against a number of Gram positive bacteria used in this investigation. This is discordant with the results obtained in this study unless one considers the difference in the extraction methods which is supported by the findings by the same authors that the homogenized dichloromethane extract had higher activity (lower MIC) than the shaken extract for the same microorganisms. While shaking was employed in the previous study, in this particular study the ground plant material was homogenized in solvent for almost 48 hr. Sometimes reports are not directly comparable due to methodological differences such as choice of plant extract, extractant, test microorganisms and antimicrobial test method (Hammer *et al*., 1999). Different extraction solvents yield different resultant active fractions. For example studies have shown that while methanol would yield mainly anthocyanins, terpenoids, saponins, tannins, xanthoxyllins, lactones, flavones and polyphenols, acetone would yield mainly flavonols (Cowan, 1999). In a study where the quantity and diversity of compounds extracted, rate of extraction, toxicity in a bioassay, ease of removal of solvent and biological hazard were evaluated for several solvents and solvent mixtures, acetone gave the best results (Eloff, 1998b). The larger the variety of compounds that are extracted by the extractant, the better the chance that biologically active components will also be extracted if a specific class of chemical component is not targeted. Fresh or dried plant material can be used as a source for secondary plant components. Most scientists working on the chemistry of secondary plant components have tended to use dried material because 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. The composition of plant extracts is known to vary according to local climate and environmental conditions. Differences in microbial growth, exposure of microorganisms to extract, solubility of extract may also affect results of susceptibility tests (Hammer *et al*., 1999).

Time kill studies allow for a more rational exploitation of medicinal plants both in traditional medicine and in the empirical development of new antimicrobials (Ngemenya *et al*., 2006). The effectiveness of an antimicrobial agent is measured by its ability to inhibit and kill bacteria. Time kill curves show the bactericidal activity of a fixed concentration of an antimicrobial agent expressed and are one of the most reliable methods for determining tolerance of microorganisms and also show a clear relationship between the extent of inhibition and the concentration of the extract (Nostro *et al*; 2000). Figure 4.1. shows that a dose of 1×MIC of the extract was neither bacteriostatic nor bactericidal against *B. cereus* because the microorganism's growth is not inhibited at this concentration. However, at 2×MIC the extract shows some activity which is largely bacteriostatic for the first 12 hr. The total effect of the extract is a reduction in the initial inoculum, of *B. cereus* by approximately 1 Log_{10} unit after 24 hr which by convention is regarded as bacteriostatic. With *P. vulgaris*, there is no apparent bactericidal activity at the MIC as well but at 2×MIC, although there is an initial increase in viable cells for the first 2 hr, there is rapid decrease of viable cells by about 6 Log_{10} in 6 hr showing considerable bactericidal activity (Figure 4.2). *Staph. aureus* OKOH1 decreases steadily throughout the incubation period and the extract achieves an almost 5 Log_{10} decrease of the viable cells in 24 hr (Fig. 4.3.) exhibiting bactericidal activity comparable to the comparator drug. The extract exhibited dose/concentration-dependent bactericidal activity for all the test organisms because increasing

the extract concentration to 2×MIC improved its bactericidal activity. In concentration-dependent killing the killing capacity of the antimicrobial is directly proportional to increasing concentration and maximum effect is achieved by increasing concentration to almost 10×MIC. This is in contrast to time-dependent kinetics were the killing capacity is not affected by increasing concentrations but by the amount of time the bacteria is exposed to the antimicrobial. Many pharmacodynamics and clinical studies have evaluated either dose elevation or prolonged infusion for time-dependent antimicrobials as promising therapeutic approaches (Kiffer *et al*., 2005).

A bactericidal activity is defined as a 3 $\log_{10}$ decrease in the cfu/ml or a 99.9 % kill over a specified time (Yagi and Zurenko, 2003). The time it took to reduce the initial inoculum by 3 $\log_{10}$ in 24 hr ($T_{99.9\%}$) for *B. cereus* was greater than 24 hr, 8 hr for *P. vulgaris* and 24 hours for *Staph. aureus* OKOH1. An important observation to note which seems like an anomaly is that *P.vulgaris,* a Gram negative bacterium, was eliminated faster than *B.cereus* and *Staph. aureus* OKOH1, both Gram positive organisms. This shows probable species specific bactericidal activity of the methanol extract of *H. pedunculatum*.

An important problem often observed in time kill studies but rarely addressed in data analysis is microbial regrowth after initial reduction in the starting inoculum. Some authors have conceptualized a bacterial population as consisting of two distinct and discrete subpopulations with drastically different susceptibility (Jumbe *et al*., 2003). Regrowth is then attributed to the preferential killing of the susceptible sub-population, coupled with selective amplification of the resistant sub-population. Given that typical time kill studies are performed over 24 hr, there may not be adequate time for the resistant sub-population to take over the entire population in the study time period. After 24 hr of exposure, bacterial burdens can be identical in a slow

bactericidal agent or a rapid killing agent followed by regrowth (due to emergence of resistance). In contrast, some authors attribute regrowth to adaptation with the drug exposure representing a selective pressure eliminating the susceptible subpopulations leaving the resistant subpopulations (Tam *et al*., 2005). Also, some species are able to withstand adverse conditions better than others (Oshodi *et al*., 2004).

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). This has found application in the use of plant extracts in combination with antibiotics to overcome microbial resistance to those particular antibiotics. Plant extracts are believed to inhibit microorganisms by mechanisms different from those of conventional antibiotics and this could be useful in the development of novel, efficient drugs that either block or circumvent resistance mechanisms or those that attack new targets (Oluwatuyi *et al*., 2004; Samie *et al*., 2005). This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment (Nascimento *et al*., 2000).

The understanding of synergism mechanism is fundamental to development of pharmacological agents to treat diseases using medicinal plants (Betoni *et al*., 2006). While the effect of the combinations on the bacteria was species specific, increasing the concentrations of plant extract in combinations with chloramphenicol, mostly resulted in improved antibacterial activity. The opposite is true for most combinations with penicillin where increasing the concentration resulted in reduced antibacterial activity.

The MIC range of the methanol extract of *H. pedunculatum* is relatively of magnitude higher than those of common broad spectrum antibiotics or even other plant extracts and in this

regard could be of little use as an effective antimicrobial. However it exhibited promising activity when combined with antibiotics and could be useful combined therapy with antibiotics to overcome microbial resistance which may help to extend the use of these antibiotics or to reduce doses in order to prevent adverse effects. The trend for antimicrobial drugs has been to relate an *in vitro* index of antimicrobial action such as the MIC with pharmacokinetic action *in vivo* but conditions in broth culture measurements of MIC will not mimic precisely conditions occurring in living systems. Furthermore some therapeutic agents are converted to active metabolites that may possess significant antimicrobial activity *in vivo* (Lees and Aliabadi, 2000). There is need to explore other extracts of this plant to make a conclusion on its effectivess to treat wound infections. On the other hand, the strength of this plant, which could justify its use by the Xhosa, could be more as an anti-inflammatory agent than as an antimicrobial agent.

REFERENCES

Afolayan AJ, Meyer JJM (1997). The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. Journal of Ethnopharmacology 57: 177-181.

Ahn JW (1994). Cytotoxic Limonoids from *Melia azedarach* var. *japonica*. Phytochemistry 36(1): 1493-1496.

Al-Bakri AG, Afifi FA (2007). Evaluation of antimicrobial activity of selected plant extracts by rapid XTT colorimetry and bacterial enumeration. Journal of Microbiological Methods 68: 19-25.

Al-hebshi N, Al-haroni M, Skaug N (2006). In vitro antimicrobial and resistance-modifying activities of aqueous crude khat extracts against oral microorganisms. Archives of Oral Biology 51:183-188.

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

Betoni JEC, Mantovani RP, Barbosa LN, Di Stasi LC, Fernandes Junior A (2006). Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases *Mem Inst Oswaldo Cruz* 101(4): 387-390.

Briskin DP (2000). Medicinal plants and Phytomedicines. Linking Plant Biochemistry and Physiology to Human Health. Plant Physiology 124: 507-514.

Clark AM (1996). Natural products as a resource for new drugs. Pharm Res. 13.

Cowan MM (1999). Plant products as antimicrobial agents. Clinical Microbial Review 12(4): 564-582.

Daily Sun, Friday 7 July 2006. South Africa.

Day C (1990). Hypogycaemic compounds from plants. In New antidiabetic drugs. CJ Bailey and Flatt PR editors. London: Smith Gordon.

Dilika F, Afolayan AJ, Meyer JJM (1996). Comparative antibacterial activity of two *Helichrysum* species used in male circumcision in South Africa. South African Journal of Botany 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 71(4):450-452.

Dilika NF, Nikolova RV, Jacobs TV (1995). Plants used in the circumcision rites of the Xhosa tribe in South Africa. International Symposium on medicinal and Aromatic plants 426.

Dwivedi SK, Singh KP, (1999). Fungitoxicity of some higher plant products against *Macrophomina phaseolina* (Tassi) Goid. Flavour and Fragrance Journal 13(6).

Eloff JN (1998a). A sensitive and quick microplate method to determine the minimum inhibitory concentration of plant extracts for bacteria. Planta Medica 64: 711-713.

Eloff JN (1998b). Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology 60: 1-8.

EUCAST Definitive Document (2000). Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. Clinical Microbiology and Infection 6(9): 503-508.

EUCAST Discussion Document (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clinical Microbiology and Infection 9(8): 1-7.

Foster S (1997). Phytomedicine - harnessing the healing power of plants: 5 herbs for the next millenium. Better Nutrition, 1997.

Gibbons S (2003). An overview of plant extracts as potential therapeutics. Expert Opinion on Therapeutic Patents 13(4): 489-497.

Gibbons S, Oluwatuyi M, Veitch NC, Gray AI (2003). Bacterial resistance modifying agents from *Lycopus europaeus*. Phytochemistry 62:83-7.

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. Journal of Ethnopharmacology 66: 103-106.

Hammer KA, Carson CF, Riley T.V (1999). Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86(6): 985.

Hansh C and Lien EJ (1971). Fungal steroids and the mode of action of the polyene antibiotics. Journal of Medical Chemistry 14: 653-670.

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. Planta medica 58(2): 176-183.

Hess S, 1998. Traditional healers in South Africa. Health Systems Trust Update 37:6-7.

Hilliard OM (1983). Asteraceae.ln: Flora of Southern Africa. Ed. Loeistne O.A. National Botanical Institute of South Africa 61.

Hughes I (2002). Science in Africa Magazine. Issue 56

Hutchings A, 2006. Zulu Medicinal Plants. University of Natal Press. Pietermaritzburg

Iwu MW, Duncan AR, Okunji CO (1999). New antimicrobials of plant origin. In:J.Janick(ed), Perspectives on new crops and new uses. ASHS Press. Alexandria 457-462.

Joyce LF, Downes J, Stockman K, Andrew JH (1992). Comparison of five methods, including PDM Epsilometer test (E-test), for antimicrobial susceptibility testing of *Pseudomonas aeruginosa*. Journal of Clinical microbiology 30(10): 2709-2713.

Jumbe N, Louie A, Deziel MR, 2003. Application of a mathematical model to prevent in-vivo amplification of antibiotic resistant bacterial populations during therapy. Journal of Clinical investigation 112:275-285.

Kiffer CRV, Sampaio JLM, Sinto S, Oplustil CP, Koga PCM, Arruda AC, Turner PJ, Mendes C (2005). In vitro synergy test of meropenem and sulbactam against clinical isolates of *Acinetobacter baumannii*. Diagnostic Microbiology and Infectious disease 52: 317-322.

Krizek TJ and Robson M.C (1975). Evolution of quantitative bacteriology in wound management. American Journal of Surgery 130(5): 579-584.

Lee JY, Oh WS, Ko KS, Heo ST, Moon CS, Ki HK, Kiem S, Peck KR, Song JH (2006). Synergy of arbakacin-based combinations against vancomycin hetero-intermediate *Staphylococcus aureus*. Journal of Korean Medical Science 21: 188-192.

Lees P and Aliabadi FS (2000). Rationalizing dosage regimens of antimicrobial drugs: a pharmacological perspective. Journal of Medical Microbiology 49:943-945.

Lis-Balchin M and Deans SG (1997). Bioactivity of selected plant essential oils against *Listeria monocytogenes*. Journal of Applied Bacteriology 82: 759-762.

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. Journal of Ethnopharmacology 95: 253-258.

Mahady GB (2001). Global Harmonization of Herbal Health Claims. Journal of Nutrition 131: 1120S- 1123S.

Maoz M, Neeman A (1998). Antimicrobial effects of aqueous plant extracts on the fungi *Microsporium canis* and *Trichophyton rubrum* and on bacterial species. Letters in Microbiology 26 (1): 61-63

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.

Mathekga ADM and Meyer JJM (1998). Antibacterial activity of South African *Helichrysum spp.* South Africa Journal of Botany 64(5): 293-295.

Mathekga ADM, Meyer JJM, Horn MM, Drewes S.E (2000). An acylated phloroglucinol with antimicrobial properties from *Helichrysum caespititium*. Phytochemistry 53: 93-96.

Matkowski A, Wolniak D (2005). Plant phenolic metabolites as free radical scavengers and mutagenesis inhibitors. Biomed Central Plant Biology 5(1).

May J, Chan CH, King A, Williams L, French GL (2000). Time kill studies of tree tea oils on clinical isolates. Journal of Antimicrobial Chemotherapy 45(5): 639-643.

Mbata TI, Lu Debiao, Saikia A (2006). Antibacterial activity of the crude extract of Chinese Green Tea (*Camellia sinensis*) on *Listeria minocytogenes*. The Internet Journal of Microbiology 2(2).

Meckes M, Calzada F, Amparo Tapia-Contreras, Roberto Cedillo-Rivera (1999). Antiprotozoal properties of *Helianthemum glomeratum*. Phytotherapy Research 13(2**)**: 102 – 105.

Meyer JJM, Afolayan AJ (1995). Antibacterial activity of *Helichrysum aureonitens* (Asteraceae). Journal of Ethnopharmacology 47(2): 109-111.

Meyer JJM, Dilika F (1996). Antibacterial activity of *Helichrysum pedunctulatum* used in circumcision rites. Journal of Ethnopharmacology 53(1): 51-54.

Mitscher LA, Leu R, Bathala MS, Wu W, Beal JL (1972). Antimicrobial agents from higher plants. Introduction, rational, and methodology. Lloydia 35:157-166.

Mukherjee PK (2003). GMP for Indian Systems of Medicine. In: Mukherjee PK and Verpoorte R eds GMP Botanicals: Regulatory and Quality issues on Phytomedicines. Business Horizons, New Dehli. pp 99-112. ISBN 81-900788-5-2.

Nascimento GGF, Locatelli J, Freitas PC, Silva GL (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Brazilian Journal of Microbiology 31 (4): 247-256.

Natal Witness, 2002. Pietermaritzburg, South Africa, Wednesday, 26 June 2002.

National Nosocomical Infections Surveillance (NNIS) System Report (1996). American Journal of Infections Control 24(5): 380-388.

Ngemenya MN, Mbah JA, Pierre T and Vincent PKT (2006). Antibacterial effects of some Cameroonian medicinal plants against common pathogenic bacteria. African Journal of Traditional, Complementary and Alternative Medicines 3(2): 84-93.

Nostro A, Germano M.P, D'Angelo V, Marino A, Cannatelli MA (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Letters in Microbiology 30(1): 379-384.

Ogunleye DS, Ibitoye SF (2003). Studies of antimicrobial activity and chemical constituents of Ximenia Americana. Tropical Journal of Phramaceutical Research 2(2): 239-241.

Okemo PO, Mwatha WE, Chhabrab SC and Fabryc W (2001).The kill kinetics of *Azadirachta indica* A. JUSS. (Meliaceae). Extracts on *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. African Journal of Science and Technology Science and Engineering Series 2(2): 113-118.

Okoli S and Iroegbu CU (2005). In vitro bactericidal activity of *Synclisa scabrinda* whole root extracts. African Journal of Biotechnology 4(9): 946-952.

Oluwatuyi M, Kaatz GW, Gibbons S (2004). Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochemistry 65: 3249-3254.

Osbourne AE (1996). Preformed antimicrobial compounds and plant defense against fungal attack. The plant cell 8: 1821-1831.

Oshodi AA, Amoo IA, Eleyinmi AF (2004). The antimicrobial activity of Nigerian medicinal plants potentially usable as hop substitutes. Master Brewers Association of the Americas 41(4): 398-402.

Pankey G, Ashcraft D and Patel N (2005). In Vitro Synergy of Daptomycin plus Rifampin against *Enterococcus faecium* Resistant to both Linezolid and Vancomycin. Antimicrobial Agents and Chemotherapy 49(12): 5166–5168.

Parekh J, Jadeja D, Chanda S (2005). Efficacy of Aqueous and Methanol Extracts of Some Medicinal Plants for Potential Antibacterial Activity. Turkish Journal of Biology 29: 203-210.

Parekh J, Karathia N, Chanda S (2006). Screening of some traditionally used medicinal plants for potential antibacterial activity. Indian Journal of Pharmaceutical Sciences 68(6): 832-834.

Rojas JJ, Ochoa VJ, Ocampo S.A, Monoz JF (2005). Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in treatment of non-nosocomial infections. BMC Complementary and Alternative Medicine 6:2.

Salie F, Eagles PFK and Lens HMJ (1996). Preliminary antimicrobial screening of four South African Asteraceae species. Journal of Ethnopharmacology 52(1): 27-33.

Salvat A, Antonnacci L, Fortunato RH, Suarez EY and Godoy HM (2001). Screening of some plants from Northern Argentina for their antimicrobial activity. Letters in Applied Microbiology 32: 293-297.

Samie A1, Obi C.L, Bessong PO, Namrita L (2005). Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. African Journal of Biotechnology 4 (12):1443-1451.

Scheck AC, Perry K, Hank NC, Clark WD (2006). Anticancer activity of extracts derived from the mature roots of *Scutellaria baicalensis* on human malignant brain tumor cells. Biomed Central Complementary and Alternative medicine 6:27.

Shiota S, Shimizu M, Mizushima T, Ito H (1999). Marked reduction in the minimum inhibitory concentration (MIC) of ß-lactams in methicillin-resistant *Staphylococcus aureus* produced by epicatechin gallate, an ingredient of green tea (*Camellia sinensis*). Biol. Pharm.Bull. 22(12):1388-1390

Sikkema J, De Bont JAM, Poolman B (1995). Mechanisms of membrane toxicity of hydrocarbons. Microbial Rev 59: 201-22

Silva MTG, Simas S.M, 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).

Silva O, Duarte A, Cabrita J, Pimentel M, Diniz A and Gomez E (1996). Antimicrobial activity of Guinea-Bissau traditional remedies. Journal of Ethnopharmacology 50: 53-59.

Simons V, Morrissey JP, Latijnhouwers M, Csukai M, Cleaver A, Yarrow C, and Osbourn A (2006). Dual Effects of Plant Steroidal Alkaloids on *Saccharomyces cerevisiae*. Antimicrobial Agents and Chemotherapy 50(8): 2732-2740.

Singhal H (2006).Wound infection article. Department of Surgery, Imperial College. School of Medicine.UK.

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, and Lewis K (2000). Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a

multidrug pump inhibitor. Proceedings of the National Academy of Sciences of the United States of America 97(4): 1433-1437.

Tam VH, Schilling AN, Nikolaou M (2005). Modeling time-kill studies to discern the pharmacodynamics of merepenem. Journal of antimicrobial chemotherapy 55(5): 699-706.

Tanaka JCA, da Silva CC, de Oliveira AJB, Nakamura CV and Dias Filho BP (2006) Antibacterial activity of indole alkaloids from *Aspidosperma ramiflorum.* Brazilian Journal of Medical and Biological Research 39(3): 387-391.

Taylor JLS, Rabe T, McGaw LJ, Jäger AK and van Staden J (2001). Towards the scientific validation of traditional medicinal plants. Plant growth regulation 34(1): 324-327.

Tegos G, Stermitz FR, Lomovskaya O, and Lewis K (2002.) Multidrug Pump Inhibitors Uncover Remarkable Activity of Plant Antimicrobials. Antimicrobial Agents and Chemotherapy 46(10): 3133-3141.

Tepe B, Donmez E, Unlu G, Polissiou M, Sokmen A (2004). Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *Salvia cryptantha* (Montbret et Aucher ex Bench.) and *Salvia multicaulis* (Vahl). Food Chemistry 84(4): 519-525.

Tomas-Barberan F, Iniesta-Sanmartin E, Tomas-Lorente F, Rumbero A (1990). Antimicrobial phenolic compounds from three Spanish helichrysum species. Phytochemistry 29: 1093-1095.

Wakdikar S (2004). Global health care challenge: Indian experience and new prescriptions. Electronic Journal of Biotechnology. 7(3). ISSN: 0717-3458.

Wallace JR (2004). Antimicrobial properties of Plant Secondary Metabolites. Proceedings of the Nutrition Society 63: 621-629.

World Health Organisation (2002). WHO Policy Perspectives on Medicines- Traditional Medicine Growing Needs and Potential

Yagi BH and Zurenko GE (2003). An in vitro time-kill assessment of linezoid and anaerobic bacteria. Anaerobe 9(1): 1-3

Yamaji K, Ishimoto H, Usui N, Mori S (2005). Organic acids and water soluble phenolics produced by *Paxillus* species. Mycorrhiza 15(1): 17-23