TABLE OF CONTENTS PAGE NUMBER

Phytomedicine

Mankind has discovered the idea that certain plants had healing powers, and thus contain what we currently characterize as antimicrobial principles, long before the existence of microbes was ascertained (Ríos and Recio, 2005). The earliest evidence of humans' use of plants for healing dates back to the Neanderthal period (Kleiner, 1995). In the 16th century, botanical gardens were created to grow medicinal plants for medical schools (Akerele, 1993). Herbal medicine practice flourished until the 17th century when more "scientific pharmacological remedies were favoured (Trevelyan, 1993). Around the world, the history of herbal use begins when health care was provided by women in the home. Initially they used home-made botanical remedies and later purchased similar products as "patent medicines." In the early 19th century, scientific methods became more advanced and preferred, and the practice of botanical healing was dismissed as quackery. In the 1960s, with concerns over the iatrogenic effects of convectional medicine and desire for more self-reliance, interest in "natural health" and the use of herbal products increased (Trevelyan, 1993). Worldwide, herbal use again became popular and in 1974 the World Health Organization (WHO, Geneva, Switzerland) encouraged developing countries to use traditional plant medicines to "fulfill a need unmet by modern systems." (Trevelyan, 1993). Despite the availability of different approaches for the discovery of drugs, plants still remain the main reservoirs of natural medicines. It is estimated that about 30% of the drugs in the modern pharmacopeias were derived from plants and many others, which are synthetic analogues, were built on prototype compounds isolated from plants (Farnsworth, NR *et al.*, 1985; De Silva, 2005; Kim, 2005) and over the years there have been increasing interests in the use of herbal therapeutics worldwide. It is estimated that over 80% of the developing world's population still depend on herbal medicines, while half of the population in the industrialized countries, also use herbal formulations for health care (Bodeker and Kronenbger, 2002).

South Africa is exceptionally rich in plant diversity with some 21 000 species of flowering plants – almost 20% of the world's higher plants – of which 80 % are endemic (Fennel, CW *et. al.*, 2004). Many plants have been used for different purposes, such as food, drugs and perfumery. Yet, there has been limited research and support for the systematic understanding and development of the enormous wealth of medicinal plants in the country. Indeed, increasingly, more pharmacognostic investigations of plants are needed to be carried out to find novel drugs or templates for the development of new therapeutic agents.

Antimicrobial drugs have proved remarkably effective for the control of microbial infections. However, there is an increased attention on extracts and biologically active compounds isolated from plant species used in herbal medicine due to the side effects and the resistance that pathogenic micro-organisms build against conventional antibiotics (Essawi and Srour, 1999). According to Eloff (1998), the amount of resistant strains of microbial pathogens is increasing since penicillin resistant and multiresistant pneumococci caused a major problem in South African hospitals in 1977. The potential problem of emerging resistance to antimicrobial agents requires careful monitoring. Antimicrobial studies have shown that Gram-negative bacteria show a higher resistance to plant extracts than gram-positive bacteria (Palambo and Semple, 2001; Kudi, AC *et al*., 1999; Paz, 1995; Vlietinck, 1995). This can be as a result of the variation in the cell wall structures of Gram-positive and Gram-negative bacteria (Palambo and Semple, 2001). More specifically, Gram-negative bacteria have an outer membrane that is composed of high density lipopolysaccharides that serves as a barrier to many environmental substances including antibiotics (Kudi, AC *et al.*, 1999). New compounds inhibiting microorganisms such as benzoin and emetine have been isolated from plants (Cox, 1994). Eloff (1999) stated that the antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in the treatment of resistant microbial strains. As certain antibiotic treatments lead to the development of multiresistant organisms, it is now standard clinical practice to use a combination of two or more antibiotics with different mechanisms of action in an attempt to prevent the development of antibiotic resistance and improve the outcome of therapy (Beringer, 1999). Craig (1997) reported that infections caused by *Pseudomonas aeruginosa* are often difficult to treat and in an attempt to achieve an optimal outcome, clinicians would use combinations of antibiotic therapies with the aim of achieving antibiotic synergy. There is a continuous and urgent need to develop new antibiotic and immune modulating compounds with diverse chemical structures and novel mechanisms of action, because there has also been an alarming increase in the incidence of new and re-emerging infectious diseases (Rojas, R *et. al.,* 2003). The South African flora offers great possibilities for the discovery of new compounds with important medicinal applications in combating infection and strengthening the immune system.

In the developed countries of the world, plants have been widely accepted as the sleeping giants of the pharmaceutical industries that may provide an unlimited source of antibiotics and it is estimated that 27 million South Africans utilize traditional herbal medicines from more than 1020 plant species (Stafford, GI *et. al.,* 2004), more than 80% of those who live in the rural areas now depend on medicinal plants that have been used in folkloric medicine (Stafford, GI *et. al.,* 2004) and these include *H. pedunculatum* and *H. longifolium.* Reports of work done on the antimicrobial activities of *H. pedunculatum* and *H. longifolium* is scanty in available literature.

The genus *Helichrysum* Mill. derives its name from the Greek words *helios* (sun) and *chrysos* (gold) which is appropriate considering the attractive yellow flowers displayed by several species (Pooley, 2003). The genus belongs to the Asteraceae family, tribe Inuleae and subtribe Gnaphaliinae (Hilliard, 1983). This large genus consists of approximately 500–600 species and although *Helichrysum* species are also found in southern Europe, south-west Asia, southern India, Sri Lanka (previously Ceylon) and Australia, most species occur in Africa, including Madagascar (Hilliard, 1983). In South Africa and Namibia, 244–250 species are widely distributed; the tremendous morphological diversity displayed by these species resulted in their subdivision into 30 morphological groups, using the shape and size of the flower heads as differentiating characteristics (Hilliard, 1983). The flower heads are either solitary or occur in compact or spreading inflorescences. The aerial parts are usually hairy or woolly and plants occur as herbs or shrublets that are sometimes dwarfed and cushion forming. They are often aromatic (Pooley, 1998, 2003; Van Wyk, BE *et al.,* 2000). *Helichrysum* species are used extensively in ethnomedicine in South Africa and many of the uses are associated with the treatment of infections, e.g. it is used widely for treatment of respiratory diseases and wound dressing (Lourens, ACU *et al.,* 2008). The large morphological diversity of the genus is complemented by chemical diversity as illustrated by the range of novel compounds isolated from the genus. Despite the extensive past and present traditional uses, the unrivalled botanical diversity, and the chemical complexity, it remains ironic that explorations of the biological activities of indigenous species are comparatively poorly studied. The genus *Helichrysum* is notoriously challenging from a taxonomic perspective and several examples have been highlighted to emphasize the importance of correct botanical identification when embarking on ethnopharmacological and phytochemical studies. There is an interesting relationship between the morphological classification and the classes of chemical compounds isolated from a specific morphological group and there are certain classes of compounds, e.g. diterpenes, guaianolides, acylated phloroglucinols and α-pyrone derivatives, for which one can predict in which species they are most likely to occur (Lourens, ACU *et al.*, 2008). This may be important in the search of new plant-derived drugs, e.g. acylated phloroglucinols show potential as anti-staphylococcal drug leads (Gibbons, 2004) and α-pyrone derivatives have anti-HIV properties (McGlacken and Fairlamb, 2005; Appendino, G *et al.*, 2007). *Helichrysum* is an interesting genus from an ethnobotanical, phytochemical and pharmacological perspective but the biological data to correlate the ethnobotany to the chemistry are still lacking. To advance our knowledge on this fascinating genus a multidisciplinary approach involving microbiology, botany, chemistry and ethnopharmacology has been used to investigate some antibacterial potency of two species (*H. pedunculatum* and *H. longifolium*) employed in treating circumcision wounds and wounds infections.

Choice of *Helichrysum pedunculatum* and *Helichrysum longifolium* for this study

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value and their acceptance in recent times as an alternative form of healthcare, especially with increasing trend of development of microbial resistance to available antibiotics, has led to extensive investigation on the antimicrobial potentials of medicinal plants. Our study candidates – *H. pedunculatum* and *H. longifolium* have been recognized as important plants, being extensively used in South Africa where they grow widely for the healing of circumcision wounds. Although the use of these plants in male circumcision practices in South Africa has been age long, many incidences of alarmingly high numbers of death caused by post-circumcision infections have been reported which questions the efficacy of the plants or its application in wound infection control and healing. There has not been any detailed study of the antimicrobial potentials of these plants against most of the wound associated microbial pathogens know to science. Neither has there been any study to elucidate the mechanisms of actions of the active compounds of these plants, nor their potential in combination antimicrobial chemotherapy.

Fig 1: *Helichrysum pedunculatum* in its natural vegetation

Fig 2: *Helichrysum longifolium* in its natural vegetation

JUSTIFICATION OF THIS RESEARCH

We now have the global problems of multiple antibiotics resistance, emergence of new and resurrection of previously eradicated diseases, considerable drawbacks in the use of existing antibiotics and in the developing countries, an inability of the average family to control infections using orthodox medicine (Lupetti, A *et al.,* 2003). Concurrent or sequential treatment for invasive infections has been typically considered as an option to improve results of monotherapy; however, combination therapy could be an alternative to monotherapy for patients with invasive infections that are difficult to treat, such as those due to multi-resistant species and for those who fail to respond to standard treatment. Antimicrobial compounds used in combination might promote the effectiveness of each agent, with efficacy being achieved using a lower dose of each drug. Pharmacological benefits would accrue, with one drug clearing infection from one body system while the other clears it from a different site. In addition, synergism in antimicrobials could be utilized in an attempt to prevent or delay the emergence *in vivo* of resistant populations of the pathogenic organisms. Both literature reports and ethnobotanical records indicate a general consensus on the use of antimicrobially active medicinal plants to provide cheaper drugs that may complement existing supplies from orthodox medicine in the Primary Health programme and/or provide novel or lead compound that may be employed in controlling infections in our communities (Lupetti, A *et al.,* 2003). Several studies have confirmed the antimicrobial efficacy of plants of South Africa origin, however there is still a serious dearth of information regarding the synergistic potentials of these plants, which is the primary focus of this research (Afolayan, AJ *et al.,* 2002, Van Wyk, BE *et al.*, 1997, WHO, 1995).

AIMS AND OBJECTIVES

BROAD AIM

The overall objective of this project was to investigate the antibacterial properties of extracts of *Helichrysum pedunculatum* and *Helichrysum longifolium* found in the Eastern Cape Province, South Africa and which are commonly used in the treatment of circumcision wounds.

SPECIFIC OBJECTIVES

A: Identification of plants with antimicrobial property.

Information on traditional herbal practice in the Eastern Cape Province of South Africa is passed from one generation to the other through oral tradition (Grierson and Afolayan, 1999). Considering the rapid rate of deforestation and loss of biodiversity, there is a need for accurate scientific documentation of the knowledge and experience of these herbalists. Therefore the objective of this study was to investigate the use of traditional plants for the treatment of circumcision wounds. *H. pedunculatum* and *H. longifolium* were identified as the most used species in the province for the treatment of circumcision wound and other wounds infections. These species were repeatedly mentioned as the prominent plants used for the treatment of circumcision wounds and were therefore chosen as our candidate plants.

B: Determination of the antibacterial properties of plants

With the increasing acceptance of herbal medicine as an alternative form of healthcare, the screening of plant extracts for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents. The antibacterial activity of *H. pedunculatum* and *H. longifolium* is scanty in available literature prior to this study. Another objective of this study was to investigate the antibacterial activities of *H. pedunculatum* and *H. longifolium* by preliminary bioassay screening of their extracts against a wide range of pathogenic microorganisms implicated in wounds and wound infections including referenced, environmental and clinical strains.

C: Determination of rate of kill of the extracts of these plants

The effectiveness of an antibacterial agent is measured by its ability to inhibit or kill bacteria (Nostro, A *et al.,* 2001). *In vitro* time-kill assays are expressed as the rate of killing by a fixed concentration of an antimicrobial agent and are one of the most reliable methods for determining tolerance (Nostro, A *et al.,* 2001). Generally, the effect of anti-infective agents on the test bacteria is time and concentration dependent, as it is always evident from the data presented. At higher concentration and longer duration of interaction more organisms will be affected. Prior to this study, the *in vitro* time-kill activities of *H. pedunculatum* and *H. longifolium* have never been reported in the literature. In the present investigation extracts of these plants were screened *in vitro* for time-kill studies. This was carried out in order to validate the use of these plants for the healing of circumcision wounds.

D: Investigation of the synergistic potentials of these plants extracts and some antibiotics.

The screening of crude plant extracts for synergistic interaction with antibiotics is expected to provide leads for the isolation of multiple drug resistance (MDR) inhibitors. The use of *Catha edulis* extracts at subinhibitory levels, has been reported to reduce the minimum inhibitory concentration (MIC) values of tetracycline and penicillin G against resistant oral pathogens, *Streptococcus oralis, Streptococcus sanguis* and *Fusobacterium nucleatum* (Al- hebshi, N *et al*., 2006). There have been worrisome incidences of high death rate resulting from circumcision wounds infection even after treating such wounds with *H. pedunculatum* and *H. longifolium* leaves. Perhaps the plant(s) could be of more relevance in combination therapy and a source of resistance modifying principles. Hence, another objective of the study was to investigate the synergistic potentials of various combinations of these plants extracts as well as some standard antibiotics.

E: Determination of phytochemicals and the anti-oxidant activities of these plant extracts

Free radicals have been implicated in the causation of several chronic diseases such as diabetes, cancer and atherosclerosis. Many complications of wound healing, including inflammation, retinopathy and atherosclerotic vascular diseases, have been linked to oxidative stress (McCune and Johns, 2003). Bioactive compounds and plant extracts that can scavenge free radicals have a great potential in preventing the progression of these diseases (Sabu and Kuttan, 2002). Despite the wide uses of *H. pedunculatum* and *H. longifolium* for circumcision wound healing in the Eastern Cape Province of South Africa, there is no information on their antioxidant activities, yet such information is important in the understanding of the justification for utilizing these plants as sources of medicine. Thus, one of the objectives of this project was to investigate the antioxidant activity of both extracts of these plant species. To achieve this, the DPPH and ABTS radical scavenging experiments as well as $Fe^{3+} \rightarrow Fe^{2+}$ transformation assay were used to determine the antioxidant capacity of the extracts. Catechin and Butylated hydroxyltoluene (BHT) were used in the experiments as standard antioxidant compounds.

F: Isolation and identification of pure compounds from candidate plants

Secondary metabolites derived from both microorganisms and higher plants have provided a wide range of drugs that are used for the treatment of infectious diseases. Many species of the genus *Helichrysum* are known for their antiinfective properties. They are rich in different compounds like phenolics e.g. flavonoids and chalcones, phthalides, α-pyron derivatives, terpenoids, essential oils, volatiles and fatty acids (Czinner, N *et al*., 2001) . However, considering the various pharmacological activities of this species, many other bioactive compounds are yet to be isolated and characterized in the plant. For example, before the commencement of this study, there were only few reports on the pure compounds isolated from *H. pedunculatum,* with virtually no reported compound ever isolated from *H. longifolium* in the literature (Czinner, N *et al*., 2001). One of the objectives of this project, therefore, was to perform a bioactivity-guided isolation and identification of compounds from the leaves of *H. pedunculatum* and *H. longifolium.* To achieve this, several chromatographic techniques coupled with the use of 1D and 2D, NMR spectroscopy such as ^{1}H, ^{13}C, COSY, HMQC and HMBC spectroscopy were used in structure elucidation.

The structure of the thesis

This thesis consists of contributions in the form of reprints of published papers, accepted articles and some manuscripts submitted for publication. The thesis is structured as follows: Chapter 2 presents a vast review of literature on the use of bioactive plant products; it reveals an overview of the use of bioactive plant products in combination with standard antibiotics and its implications in antimicrobial chemotherapy. Chapter 3 presents a study on the *in vitro* time-kill assessment of crude methanol extract of *H. pedunculatum*, while chapter 4 as well presents studies on the *in vitro* time-kill assessment of crude aqueous and acetone extracts of *H. pedunculatum*. The *in vitro* antibacterial time-kill studies of leaves of *H longifolium* are reported in chapter 5. Chapters 6, 7 and 8 reports on the *in vitro* evaluation of the interactions between crude extracts from the leaves of *H. pedunculatum* and *H. longifolium* as well as some antibiotics. The phytochemical properties and antioxidative properties of the crude extract are presented in chapters 9 and 10, while chapter 11 reports on isolated compounds from the crude leaves extracts of these plants which were obtained in small amounts. The general discussion and conclusion from this study are presented in chapter 12, in an attempt to consolidate the results obtained from the study of these valuable medicinal plants.

References

Afolayan AJ, Grierson DS, Kambizi L, Madamombe IT, Masika PJ (2002). *In vitro* antifungal activity of some South African medicinal plants. *South Afri. J. Bot.* 68: 72–76.

Akerele O (1993). Nature's medicinal bounty: don't throw it away. *World Health Forum*, 14: 390-395

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

Appendino G, Ottino M, Marquez N, Bianchi F, Giana A, Ballero M, Sterner O, Fiebich BL, Munoz E (2007). Arzanol, an anti-inflammatory and anti-HIV-1 phlroglucinol α-pyrone from *Helichrysum italicum* ssp. *microphyllum*. *J. Nat. Prod*. 70: 608–612.

Beringer PM (1999). New approaches to optimizing antimicrobial therapy in patients with Cystic fibrosis. *Curr. Opin. Pul. Med*. 5: 371-377.

Bodeker G, Kronenbger F (2002). A public health agenda for traditional, complementary, alternative medicine. *Ame. J. Pub. Health*. 92, 1582-1591.

Cox PA (1994). The ethnobotanical approach to drug discovery: strengths and limitations. In: Prance G.T. (Ed.), Ethnobotany and the Search for New Drugs. *Wiley, Chichester*, pp. 25-41.

Craig WA (1997). The future-can we learn from the past? *Diag. Microbiol. Infect. Dis*. 27: 49-53.

Czinner E, Hagmasi K, Blazovics A, Kery A, Szoke E, Lemberkovics E (2001). The *in vitro* effect of *Helichrysum flos* on microsomal lipid peroxidation. *J. Ethnopharmacol*. 77: 31-35.

De Silva T (2005). Industrial utilization of medicinal plants in developing countries, chemical institute branches, industrial sectors and environment division. *United Nation Industrial Development Organization*, pp. 1-11, http://www.fao.org/docrep/W7261e07.

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

Eloff JN (1999). It is possible to use herbarium specimens to screen for antibacterial components in some plants. *J. Ethnopharmacol.* 67: 355-360.

Essawi T, Srour M (1999). Screening of some Palestinian medicinal plants for antibacterial activity. *J. Ethnopharmacol*. 70: 343-349.

Farnsworth NR, Akerele O, Bingel AS, Soejarta DD, Eno Z (1985). Medicinal plants in therapy. *Bull. WHO* 63: 226-230.

Fennel CW, Light ME, Sparg SG, Stafford GI, Van Staden J (2004). Assessing African medicinal plants for efficacy and safety: agricultural and storage practices. *J. Ethnopharmacol.* 95: 113-121.

Gibbons S (2004). Anti-staphylococcal plant natural products. *Nat. Prod. Reports.* 21: 263–277.

Grierson DS, Afolayan AJ (1999). An ethnobotanical study of plants used for the treatment of wounds in the Eastern Cape Province, *South Afri. J. Ethnopharmacol*. 67: 327-332.

Hilliard OM (1983). Asteraceae. In: Leistner, O.A. (Ed.), Flora of Southern Africa, vol. 33, part 7 (Inuleae). Botanical Research Institute of South Africa, Pretoria, pp. 7,2:61–7,2:317.

Kim HS (2005). Do not put too much value on conventional medicines. *J. Ethnopharmacol.* 100: 37-39.

Kleiner SM (1995). The true nature of herbs. *Phys. Sport Med*. 23: 13-14

Kudi AC, Umoh JU, Eduvie LO, Gefu J (1999). Screening of some Nigerian medicinal plants for antibacterial activity. *J. Ethnopharmacol*. 67: 225-228.

Lourens ACU, Reddy D, Bas er, KHC, Viljoen AM, Van Vuuren SF (2004). *In vitro* biological activity and essential oil composition of four indigenous South African *Helichrysum* species. *J. Ethnopharmacol*. 95: 253–258.

Lupetti A, Nibbering PH, Campa M, *et al* (2003). Molecular target treatment for fungal infections: the role of drug combinations. *Trend Mol. Med.* 9: 269-76.

McCune LM, Johns T (2002). Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous people of the North American arboreal forest. *J. Ethnopharmacol*. 82: 197-205.

McGlacken GP, Fairlamb IJS (2005). 2-Pyrone natural products and mimetics: isolation, characterization and biological activity. *Nat. Prod. Reports*. 22: 369–385.

Nostro A, Cannatelli MA, Grisafi G, Alonszo V (2001). The effect of *Nepata cataria* extract on adherence and enzyme production of *Staphylococcus aureus*. *Int. J. Antimicrob. Agents.* 18: 583-585.

Palambo EA, Semple SJ (2001). Antibacterial activity of traditional Australian medicinal plants. *J. Ethnopharmacol*. 77: 151-157.

Paz EA, Cerdeiras MP, Fernandez F, Mona P, Soubes M, Vazquez A, Vero S, Zunino L (1995). Screening of Uruguayan medicinal plants for antimicrobial activity. *J. Ethnopharmacol*. 45: 67-70.

Pooley E (1998). A Field Guide to the Wild Flowers. KwaZulu-Natal and the Eastern Region, 1st ed. *Natal Flora Publications Trust, Durban*, pp. 82, 212–215, 310–317, 442–443.

Pooley E (2003). Mountain Flowers: A Field Guide to the Flora of the Drakensberg and Lesotho, 1st ed. *The Flora Publications Trust, Durban*, pp. 44, 102–110, 146–157, 222–225.

Ríos JL, Recio MC (2005). Medicinal plants and antimicrobial activity. *J. Ethnopharmacol*. 100: 80-84.

Rojas R, Bustmante B, Bauer J, Fernandez I, Alban J (2003). Antimicrobial activity of selected Peruvian medicinal plants. *J. Ethnopharmacol*. 88: 199-204.

Sabu MC, Kuttan R (2002). Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. *J. Ethnopharmacol*. 81: 155-160

Stafford GI, Jager AK, Van Staden J (2004). Effect of storage on the chemical composition and biological activity of several popular South African medicinal plants. *J. Ethnopharmacol*.

97: 107-115.

Trevelyan J (1993). Herbal medicine. *Nursing Times.* 89: 36-38

Van Wyk BE, Van Oudtshoorn B, Gericke N (1997). Medicinal Plants of South Africa. Briza, Pretoria, pp. 1–304.

Van Wyk BE, Gericke N (2000). People's Plants. A Guide to Useful Plants of Southern Africa, 1st ed. Briza, Pretoria, p. 166.

Vlietinck AJ, Van Hoof L, Totte J, Lasure A, Vanden Berghe D, Rwangabo PC, Mvukiyumwani J (1995). Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *J. Ethnopharmacol*. 46: 31-47.

WHO (1995). Resolution, Promotion and Development of Training and Research in Traditional Medicine. *WHO Document No. WHA* 30:49.