CHAPTER 1

INTRODUCTION

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Literature Review and objectives

1.1 A modern antibiotic era

More than 50 years have passed since the modern antibiotic era opened with the first clinical trial of penicillin in early 1941. In the intervening years medical practice has been transformed and the use of antibiotics has grown to enormous proportions. In 1985, for example, the world market for antibiotic drugs amounted to $15 billion (Farnsworth, *et al.* WHO, 1985). In the United States this is distributed among 120 antibiotics and antiinfective agents, 34 of which (17%) are listed among the top 200 most frequently prescribed drugs in the USA (Anon, 1987). The list does not give the total picture, as parenteral agents utilized in an institutional setting and those agents used in agricultural practice are not considered in the calculation. Unfortunately, no comparable statistics are available for South Africa at present.

It is estimated that between 5 000 and 10 000 natural antibiotics have been isolated and characterized and at least 50 000 to 100 000 analogues have been synthesised (Berdy, 1980). Clearly the vast majority fail to find medicinal use.

Most of the natural antibiotics have been isolated from soil microorganisms through intensive screening. In 1952, the bulk of the agents reported in the literature were derived from the streptomyces with most of the remainder coming from other bacteria and fungi. By 1985, the total number of new agents had increased to 220, but the percentage derived from the streptomyces had declined as had the number derived from other bacteria and fungi. One observed instead a dramatic increase in the use of rarer microorganisms (Mitscher & Raghar, 1984). The reason for this shift lie largely in the perception that the point of diminishing returns had been reached using classical methodology, and if newer agents were to be discovered, fishing in a different gene pool was more likely to prove useful. A clear look at the identity of the antimicrobial agents produced by the paradigm shift reveals that, whereas a significant number of structurally new agents were uncovered in this way, the newer agents still belonged primarily to the same chemical families as had

been seen in 1952. Thus, these agents are variants on a well-known theme rather than representatives of dramatically novel biological properties (Mitscher & Raghar, 1984).

Some other microbiology-based avenues explored in attempts to breathe significant novelty into antibiotic discovery include searches into novel environments (Okami, 1979), directed screening methodology (specific inhibition, comparative activity against resistant and supersensitive strains, addition of enzymes to the media, etc.) (Sykes, 1985), directed biosynthesis (including mutasynthesis) (Shier *et al.*, 1969; Kawasima *et al.*, 1986), biochemical screens directed towards a specific mode of action (Kirsh and Lai, 1986) and genetic engineering (Omura *et al.*, 1987). Other fruitful avenues under exploration include the search for antibiotics from sea organisms (Kaul, 1982) and from higher animals (Zasloff, 1985). In these cases quite novel findings are being made.

1.2 Written records of antimicrobial compounds

The use of higher plants for the treatment of infections predates written records. Some of the earliest accounts of medical practice (Pen Tsao of 3000 BC, the Ebers Papyrus of 1500 BC, and Calsius '*De Medicina*', Florey *et al.*, (1949) records such usage. From the vantage point of modern knowledge, most other reports seem full of fanciful nonsense. Man knew nothing reliable about the nature of infectious disease until the 1800s.

1.3 Ethnopharmacology

The last two decades have witnessed the growth of a new inter-disciplinary field variously termed ethnobotany, ecological biochemistry, phytochemistry, ethnopharmacognosy or ethnopharmacology, which is basically concerned with the biochemistry of plant and microbe interactions in correlation to their pharmacological effect. Its development has been due in no small measure to the increasingly successful identification of organic molecules in micro-quantities following the application of modern chemical techniques (spectroscopy and other elucidation techniques) to biological systems. It has also been due to the awareness of plant physiologists that we realise today that chemical substances and particularly secondary metabolites such as for example, alkaloids, tannins and phloroglucinols have a significant role in the complex interactions occurring between microbe, man, animal and plant in the natural environment. A further stimulation has been

the possible application of such new information in the control of insect pests and of microbial diseases in medicine, crop plants and in the conservation of natural communities. These new developments have enormously expanded our knowledge of plant, animal, man and plant interactions, and the field of ethnopharmacology.

1.4 Screening of antimicrobial plants for new pharmaceuticals

Plants are the oldest source of pharmacologically active compounds, and have provided humankind with many medically useful compounds for centuries (Cordell, 1981). Today it is estimated that more than two thirds of the world's population relies on plant derived drugs; some 7000 medicinal compounds used in the Western pharmacopoeia are derived from plants (Caufield, 1991). In the USA approximately 25% of all prescription drugs used contain one or more bioactive compounds derived from vascular plants (Farnsworth & Morris, 1976; Farnsworth, 1984). Thus, phytochemical screening of plants species, especially of ethnopharmaceutical use, will provide valuable baseline information in the search for new pharmaceuticals. Yet fewer than 10% of the world's plant species have been examined for the presence of bioactive compounds (Myers, 1984). Hence screening of antimicrobial plants for new agents poses an enormous challenge and are important especially with the emergence of drug resistant disease strains.

During the past 10 years there has been a substantial resurgence of interest and pursuit of natural products discovery and development, both in the public and private sectors. Explanation for this, possibly transient or at least cyclical revival, might include: the increasingly sophisticated science that can be brought to bear on the discovery and development processes (Meyer and Afolayan, 1995) and the very real threat of the disappearance of the biodiversity essential for such research. It has only been in the past two decades or so that interest in higher plant antimicrobial agents has been reawakened world wide, and the literature in this area is becoming substantial (Mistscher *et al.*, 1984).

1.5 Preformed antimicrobial compounds and plant defence against microbial attack

Plants produce a diverse array of secondary metabolites, many of which have antimicrobial activity. Some of these compounds are constitutive, existing in healthy plants in their biologically active forms. Others such as cyanogenic glycosides and glucosinolates, occur

as inactive precursors and are activated in response to tissue damage or pathogen attack. This activation often involves plant enzymes, which are released as a result of breakdown in cell integrity. Compounds belonging to the latter category are still regarded as constitutive because they are immediately derived from pre-existing constituents. Mansfield (1983) and Van Etten *et al.*, (1995) have proposed the term 'phytoanticipin' to distinguish these preformed antimicrobial compounds from phytoalexins, which are synthesised from remote precursors in response to pathogen attack, probably as a result of *de novo* synthesis of enzymes. In recent years, studies of plant disease resistance mechanisms have tended to focus on phytoalexin biosynthesis and other active responses triggered after pathogen attack (Hammond-Lassack & Jones, 1996). In contrast, preformed inhibitory compounds have received relatively little attention, despite the fact that these plant antibiotics are likely to represent one of the first chemical barriers to potential pathogens.

1.6 Phytoalexins (postinfectional agents)

There have been numerous attempts to associate natural variation in levels of preformed inhibitors in plants with resistance to particular pathogens, but they have failed to reveal any positive correlation. However, whereas preformed inhibitors may be effective against a broad spectrum of potential pathogens, successful pathogens are likely to be able to circumvent the affects of these antibiotics by avoiding them altogether or by tolerating or detoxifying them (Schonbeck & Schlosser, 1976; Fry & Myers, 1981; Van Etten *et al.*, 1995). The biology associated with these classes, [the constitutive (preinfective) agents and the phytoalexins (postinfectional agents)] is strikingly similar, and in some cases, the same compound is a constitutive agent in some species and a phytoalexin in others (Osbourn, 1996).

Phytoalexins are antimicrobial compounds that are either not present or are present only in very small quantities in uninfected plants (Van Etten *et al.*, 1995). After microbial invasion, however, enzymes, which catalyse the formation of phytoalexins that are toxic to the invading organism, become activated. In plants, phytoalexin production and field resistance to infection is often a consequence of this feature of their biosynthetic

machinery. Also, the quantity of phytoalexins is often very small even in infected plants when compared with the amount of constitutive agents.

1.7 Efficacy of traditionally used plants

The search for natural products to cure diseases represents an area of great interest in which plants have been the most important source. In South African traditional medicine, the use of plants is a widespread practice, and the persistence in the use of medicinal plants among people of urban and rural communities in South Africa could be considered as evidence of their efficacy (Meyer and Afolayan, 1996). Although there is an important local ethnobotanical bibliography describing the most frequently used plants in the treatment of conditions consistent with sepsis and other diseases, there are very few experimental studies, which validate the therapeutic properties of these plants.

1.8 Criteria for the choice of *Helichrysum* species

There are 500 *Helichrysum* species worldwide of which 245 occur in South Africa. The South African species display great morphological diversity and therefore, are classified into 30 groups (Hilliard, 1983). They are confined to ecological and geographical niches resulting in specificity of plant and product. *Helichrysum* species have been reported for their antimicrobial activities (Rios *et al.*, 1988, Tomas-Barberan *et al.*, 1988; Tomas-Barberan *et al.*, 1990; Tomas-Lorente *et al.*, 1989, Mathekga & Meyer; 1998, Mathekga *et al.*, 2000). Not much information on the bioactivity of compounds isolated from these species is available. *In vitro* antimicrobial screening methods may produce the required preliminary observations to select among crude plant extracts those with potentially useful properties for further chemical and pharmacological investigations.

In the constant effort to improve the efficacy and ethics of modern medical practice, researchers are increasingly turning their attention to folk medicine as a source of new drugs (Haslam, 1989). When selecting a plant for the screening of bioactivity, four basic methods are usually followed, (1) random choice of plant species; (2) choice based on ethnomedical use; (3) follow up of existing literature on the use of the species and (4) chemotaxonomic approaches (Suffness & Douros, 1979). Comparison of the four methods showed that the choice based on folklore has given about 25% more positive leads than

other methods (Vlietnick & Vanden Berghe, 1991). The genus *Helichrysum* with 245 species in South Africa (Hilliard, 1983) constitutes a major group of angiosperms exploited for their efficacy and medicinal value by the indigenous people of South Africa (Phillips, 1917).

The development of resistance by pathogens to many of the commonly used antibiotics provides sufficient impetus for further attempts to search for new antimicrobial agents to combat infection and overcome the problem of resistance and side effects of the currently available antimicrobial agents.

The choice of *Helichrysum* species is aimed at screening available and selected South African species for their antimicrobial activity, evaluating their potential use in treating infection caused by bacteria and fungi and to determine whether their prolonged and continuing use in folklore medicine is justified or validated.

1.9 *Helichrysum caespititium*

H. caespititium (DC.) Harv. (commonly known as one of the everlastings/sewejaartjies) is a prostate, perennial, mat-forming herb that is profusely branched and densely tufted (Figure 1.1). Branchlets are about 10mm tall and closely leafy. Leaves are patent, on average 5-10 x 0.5mm, linear and obtuse with a broad base, clasping branches. Margins are revolute with both surfaces and stems enveloped in a silver Atissue-paper-like@ indumentum, breaking down to wool. The leaves are dotted with orange glands. The flowers are silvery white with yellow centres and a pale furry underneath. The plant flowers in late summer. Exudates of this herb are claimed to be effective against broncho-pneumonial diseases, sexually transmitted diseases, tuberculosis, ulceration and is used as a styptic wound dressing (Phillips, 1917; Watt & Breyer-Brandwijk, 1962).

1.10 Chemotaxonomic relationship

Related plant taxa tend to produce similar chemical compounds (Harborne, 1984). The closer the taxonomic relationship, the better are the chances that similar compounds may occur in these taxa (chemical race). When such a compound(s) is (are) of medical or pharmaceutical importance, attempts are made to search for similar or related compounds

in related taxa (for example, other varieties within the same species, other species within the same genus (Afolayan, 1996), even other genera within the same families). Such knowledge is the basis of chemotaxonomy and our point of departure in this study.

Figure 1.1. H. caespititium.(Everlasting). Inflorescence structure and other diagnostic features are: capitulate in dense racemes; pale furry below; leaves velvety and small. Flower colour silvery white with yellow centres and a pale furry underneath. Flowers in late summer. Habitat preference and altitude range are rocky areas in short grassland, common from about 1800 m and summits. H. caespititium is endemic to South Africa.

The exploitation of *Helichrysum* species provide a good example for modern-day chemotaxonomically based field search of bioactive compounds (Dekker *et al.*, 1983, 1987) . In general, when one is searching for species that may yield similar or related compounds with the same biological activity, but in yields that would be higher than the original species, chemotaxonomically based field searching could provide a productive return. However, when one is looking for compounds with different biological activities (therapeutic categories), chances of finding novel structures are considered too low to merit serious consideration (Soejarto & Farnsworth, 1989).

Positive and promising laboratory test (*in vitro*) results for an extract provide a strong basis to go back to the field to recollect samples of the same taxon of plant in a larger quantity for further studies (chemical isolation and other bioassays). In fact, results of biomedical screening of plant extracts against a wide spectrum of biological activities (antibacterial, antifungal, antidiabetic, antimalarial, antituberculosis, anticancer, etc.) continually appear in the literature (Taitz, 1999).

1.11 Sequestration of antimicrobial compounds in *Helichrysum* species

The distribution of antimicrobial compounds in plants is often tissue specific (Price *et al.*, 1987; Fenwick *et al.*, 1992). There is a tendency for these compounds to be concentrated in the outer layers of plant organs, suggesting that they may indeed act as deterrents to pathogens and pests (Bennett & Wellsgrove, 1994, Afolayan, 1995). In general, however, antimicrobial compounds are commonly sequestered in vacuoles or organelles in healthy plants. Trichomes and other foliar epidermal characters are of wide occurrence in plants. Though the taxonomic value of trichomes has been recognised for a long time, till today they have not been used for identification purposes (Sasikala & Narayanan, 1998). The

nature and level of antimicrobial compounds will also vary depending on factors such as genotype, age, and environmental factors (Price, 1987; Davis, 1991).

1.12 Alternative (traditional) primary health care services

South Africa is a country of about 48 million people, where modern medical services are insufficient to provide the population with basic curative medical attention. Traditional medical treatment, supported mainly by the use of medical plants, represents the main alternative method which has its basis in indigenous knowledge gained from ancestral experience. This knowledge is mainly undocumented scientifically and is still communicated verbally from one generation to the next. Many leads for further investigation could be discovered here. So far few species of Helichrysum have been recorded in South Africa with antimicrobial activity, of which a small percentage represents ethnomedical contributions from different parts of the country. Hutchings and Van Staden (1994) provide a list of detailed uses of a few *Helichrysum* species. However, similar studies particularly on *Helichrysum* species in other regions have not been conducted. Such information is expected to be useful in maintaining the equilibrium between utilization and conservation of plant resources, as well as help development activities, which will provide local benefits.

1.13 Significance of antimicrobial activity in *Helichrysum* species

Plants in general are among the primary producers on which all other members of an ecosystem depend. Because of the central importance of their hosts, pathogens drive many ecological and evolutionary processes in natural ecosystems. Disease causing organisms can regulate host populations and/or modify their genetic composition, restrict host distribution at various spatial scales, promote or reduce community diversity, mediate plant-herbivore, plant-man or animal and plant-plant interactions. They may reduce host growth or reproduction, and thus affect the availability of food for man and animals. They also may drive evolution of species, sex, and host defences (Barbosa, 1991; Burdon, 1991; Dickman, 1992; Herms & Mattson, 1992). For all these reasons, the role of human and plant diseases in natural ecosystems deserves greater attention in conservation and health care services.

Most of the plants collected in this study have been reported in the literature to be used as medicinal plants. Previous chemical investigations of *Helichrysum* species (Asteraceae) have revealed that they are rich sources of acetophenones, flavonoids, sesquiterpenoids, and phloroglucinols (Hilliard, 1983) used as chemical defence mechanisms (chemical barriers) against bacteria and fungi. In the present research, we have evaluated the bacterial and fungal effects of Helichrysum species determining the minimal inhibitory concentration (MIC) in order to widen our knowledge about the range and potency of their bioactivity.

The outcome of any research is dependent on the success of the exploitation. However, in order to recuperate the researcher's effort as well as the funds invested in new drug development, a patent should be filed for the protection of the discovery in line with the legislation of the country. A patent has been filed (Appendix 2) with the South African Registrar of patents, concerning the discovery of a new phloroglucinol from *H. caespititium*. This invention relates to the novel phloroglucinol compound and its derivatives, their use in the treatment and control of sensitive and resistant strains of tuberculosis caused by *Mycobacterium tuberculosis* as well as the treatment of other pathogenic bacteria and fungi.

1.14 Hypotheses tested during this investigation.

The following research hypotheses were tested in this study:

a) Crude extracts of *Helichrysum* species exhibit significant antimicrobial activity and properties that support folkloric use in the control of bacterial and fungal related infections.
b) Antimicrobial compounds are sequestered in trichomes in *H. caespititium* plants. Epicuticular extracts of *Helichrysum* species exhibit a relatively higher antimicrobial activity (minimum inhibition concentration (MIC)) compared to homogenized extracts.
c) *H. caespititium* may in addition to the compound caespitin isolated previously, contain other novel constituents that can be discovered by bioassay directed fractionation methodology.

d) Mixtures of several closely related structures of the same class are produced by the
e) plant and it is likely that synergism might occur.
f) Persistence on the use of *H. caespititium* among people of urban and rural communities in South Africa is good evidence of its non-toxicity and efficacy.

1.15 The structure of the thesis.

This thesis consists of ten chapters , including a reprint publication of a new phloroglucinol isolated from *H. caespititium*. In addition, there are two appendices.

Chapter 1.

As part of a continuing program to exploit the medical potential of South African genus Helichrysum species in general and *H. caespititium* in particular, we have examined 28 species for possible biological activity. The pain relieving, anti-infective and anti-inflammatory properties quoted for *H. caespititium* and other *Helichrysum* species in the folk medical context instigated this study. A detailed background of our rationale and research approach are described**.**

Chapters 2 & 3

Many pharmaceuticals used today are of botanical origin and are based on herbal remedies from folk medicines of indigenous South African (Watt & Breyer-Branwijk, 1962) plants. The literature of South African traditional medicine includes many of the 245 *Helichrysum* species from which the claimed therapeutic remedies are prepared for many ailments. Chapter 2 describes the investigation of 28 *Helichrysum* species tested for antibacterial activity by the agar dilution method, while Chapter 3 describes the antifungal activity of these species. In addition, the methodology to obtain the MIC of their crude extracts is described.

Chapter 4

This Chapter is in the form of a reprint publication written in the format of Phytochemistry and deals with the isolation, identification and elucidation of a novel phloroglucinol compound with interesting antimicrobial properties, established through the usual spectroscopic techniques including ^{1}H and ^{13}C NMR analysis, as well as with DEPT,

COSY and HETCOR pulse sequences. The antimicrobial activity of the novel compound is also described.

Chapter 5

A detailed account on how the purified compound (caespitate*)* was tested for cytotoxicity by exposing monolayers of vervet monkey kidney cells to dilutions of the sterilized compound, is outlined.

Chapter 6

In this Chapter**,** the biological activity of the novel compound isolated in Chapter 4 and its synergistic effect with caespitin, another phloroglucinol derivative produced by *H. caespititium*, is described.

Chapter 7

In this Chapter we report on the morphology and ultrastructure of *H. caespititium* examined by electron microscopy for the presence of secretory structures (secretory or non-secretory trichomes). The objective of the research was to describe the morphology and ultrastructure of the epicuticular structures (trichomes) of *H. caespititium* to enable us to characterize and relate our observations to their possible functional role in the production of the antimicrobial and other compounds on the leaf surface.

Chapter 8

The general discussion presents a coordination of all the chapters, presenting a holistic and coherent overview and to relate all the outcomes of this research. The expansion of knowledge on the South African Helichrysum species, and local production of pharmaceuticals based on the derivatives from such plants, offering an affordable alternative to Western medicine for the indigenous people, is reviewed.

Chapter 9

This chapter is a summary of the research in general, presenting our conclusions on the research topic.

Chapter 10

This chapter contains the acknowledgments**.**

Appendix 1 and 2

Appendix 1 describes the crystallographic analysis and data of the novel phloroglucinol compound, indispensable complementary knowledge necessary for the comprehensive understanding of the molecular biology and the stereochemistry of caespitate for the complete appreciation of its activity and expression. Appendix 2 is a reprint of the provisionally registered patent on the antimicrobial activity of caespitate.

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