ANTIMICROBIAL ACTIVITY OF *HELICHRYSUM* SPECIES AND THE ISOLATION OF A NEW PHLOROGLUCINOL FROM *HELICHRYSUM CAESPITITIUM*

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ABBREVIATIONS

CD	Circular dichroism
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement of polarisation transfer
GCMS	Gas chromatography- mass spectrometry
HETCOR	Heteronucluear chemical shift correlation
HRMS	High resolution mass spectrometry
MS	Mass spectrometry
RT	Room temperature
TLC	Thin layer chromatography
TMS	Trimethylsilyl

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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CHAPTER 2

ANTIBACTERIAL ACTIVITY OF *HELICHRYSUM* SPECIES

CHAPTER 2

ANTIBACTERIAL ACTIVITY OF *HELICHRYSUM* SPECIES.

2.1 Introduction

South Africa has a rich flora of *Helichrysum* (everlastings / sewejaartjies) species that are widely distributed throughout the country. They produce many unusual secondary products, which are biologically active. *Helichrysum* species have been used in folk medicine for thousands of years in areas as far apart as Europe, Egypt, North America, China, Australia and South Africa (Balick, 1990; Cosar *et al.*, 1990; Farnsworth, 1988; Farnsworth, 1990; Jakupovic, 1989), and are included in many pharmacopoeias. South African species are generally used for infectious diseases and antibiotic activity has been demonstrated for a number of species (Hutchings and Van Staden, 1994; Watt and Breyer-Brandwijk, 1962). Extracts of *H. armenium, H. gaveolens and H. plicatum* have been reported to be active against *Staphylococcus albus* and *S. aureus* as well as a number of other Gram-positive and Gram-negative bacteria (Cosar and Cubukcu 1990; Mathekga and Meyer, 1998). Tomas-Barberan *et al.,* (1988, 1990) and Tomas-Lorente *et al.,* (1989) have respectively reported antibacterial activity of *H. decumbens* and *H. nitens*.

The antibacterial activity of 28 species reported to be used for various diseases in traditional Sotho, Xhosa and Zulu medicine are investigated in this study. Most of the plant preparations are snuffed or taken as decoctions.

The genus *Helichrysum* belongs to the tribe Inuleae in the Asteraceae family and is known for its aromatic and therapeutic properties. It is a large family of about 500 species worldwide, with 245 species indigenous to South Africa (Hilliard, 1983). The South African *Helichrysum* species display great morphological diversity and are, therefore, classified into 30 groups (Hilliard, 1983), occurring in or along forest margins of woodlands, while some species occur in drier regions or rocky outcrops or even in open grassland. There is a high concentration (83 species) in the Free State east of the Drakensberg escarpment in the former Basotho QwaQwa homeland, where they are

subjected to a high altitude, moist and warm climate, and conditions conducive for the proliferation of most microorganisms. *Helichrysum* species are annual or perennial herbs, with hairy leaves, and stems. Bright and variously coloured flowers, with a stereome (the thickened region in the lower part of an involucral bract), a structure which has been found to be of considerable value in the classification of the genera (Hilliard, 1983).

Some members of genus *Helichrysum* have been well characterized with respect to their secondary metabolites, largely dominated by alkaloids, flavonoids, phloroglucinols and tannins (Dekker *et al.*, 1983; Jakupovic *et al.*, 1989) with antibacterial properties.

In Table 2.1, the medicinal uses of some species are listed, as obtained from a review of the available literature and interviews with local traditional healers.

Not much information on the antibacterial activity of compounds isolated from these species is available. With the knowledge that antibacterial phloroglucinols and flavonoids have been found in *Helichrysum* species, this study was undertaken to screen as many indigenous species as available for antibacterial activity present in crude shoot extracts. In the present study 28 species were screened by the agar dilution bioassay method (Mitscher *et al.*, 1972) to determine the minimum inhibition concentration (MIC).

In vitro antibacterial screening provide the required preliminary observation to select among crude plant extracts those with potentially useful properties for further chemical and pharmaceutical investigation. In this investigation we studied the antibacterial activity of crude acetone extracts (epicuticular and homogenized) against five Gram-positive and five Gram-negative bacteria

Table 2.1. Medicinal uses of some *Helichrysum* species

Helichrysum species	Traditional use
H. caespititium (DC.) Harv.	Drunk by Bakwena and Bakgatla in the treatment of gonorrhoea. Basotho in addition to inhaling the smoke from burning the plant for the relief of head and chest colds, use the slightly warmed plant as wound dressing in male circumcision rites, and for 'internal wounds' (? intestinal ulceration) (Watt and Breyer-Brandwijk, 1962)
H. calocephalum Klatt.	Given to children suffering from diarrhoea (Hutchings, 1996)
H. imbricatum Less.	Taken as a tea, and an infusion as a demulcent in coughs and in pulmonary affections (Pappe, 1857)
H. kraussii Sch. Bip.	Smoked in a pipe for the relief of cough and as a remedy for pulmonary tuberculosis (Watt and Breyer-Brandwijk, 1962)
H. lepidissimum S. Moore	Basotho commonly use it as a body perfume in the form of a powder or an ointment (Hutchings, 1996)
H. nudifolium Less.	Basotho and Xhosa use the leaf as a remedy for cough and colds. Also as demulcent and an infusion used in catarrh, phthisis and other pulmonary affections, and in wound dressing (Pappe, 1857)

Helichrysum species	Traditional use
H. crispum Less.	Used in the Western Cape for heart trouble, backache, kidney disease, coronary thrombosis and hypertension (Petrie, 1913)
H. pedunculare DC.	Applied by Xhosa and Fengu as a dressing after circumcusion.The root is also used for coughs and colds (Hutchings, 1996)
H. psilolepis Harv.	Basotho use it as remedy for painful menstruation (Watt and Breyer-Brandwijk, 1962)
H. setosum Harv.	Basotho use it to fumigate huts. Zulu use a decoction of the leaf to swab the skin in acute dermatoses. The ash is dissolved in beer and taken as a cure for epilepsy (Hume, 1954, Hutchings, 1996).

2.2 Material and Methods

Plants were collected form the Drakensberg, Mont-aux-sources area in QwaQwa (Free State, South Africa). A taxonomist, Prof. R.O. Moffett, verified their identity and voucher specimens were deposited in the herbarium of the Department of Botany, University of the North, QwaQwa Branch, South Africa and the National Botanical Institute herbarium, Pretoria.

2.2.1 Extract Preparation

Shoots (excluding flowers) of the plants were air dried at room temperature. Each plant (80g) was shaken for five minutes in acetone and filtered through Whatman No 2 filter paper under suction to obtain an epicuticular extract. The residue was then homogenized

in acetone, and also filtered through Whatman No 2 filter paper under suction. Both extracts were concentrated to dryness under reduced pressure at 45^0C with a rotary evaporator. After determining the yields, extracts were stored at 4^0C until further use.

2.2.2 Bacterial strains

Ten bacteria species (Table 2.2) were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on nutrient agar (Biolab) and an inoculum was recovered for testing by growth in nutrient broth No 2 (Biolab) for 24 hours. Before streaking, each culture was diluted 1:10 with fresh sterile nutrient broth.

2.2.3 Antibacterial bioassay

The plant extracts (sterilized by filtering through a 0.22 mm filter) were added to 5 ml of nutrient agar medium in Petri dishes and swirled carefully before congealing. An aliquot of each extract was serially diluted (ten fold) to obtain a concentration range of 1.0 to 0.01 mg/ml in acetone. The organisms were then streaked in radial patterns on agar plates (Mitscher *et al.*, 1972). Plates were incubated at 37^0C and examined after 24 and 48 hours. Complete inhibition of growth was required for an extract to be declared bioactive. A blank containing only nutrient agar and a second containing nutrient agar and 2% acetone served as controls (Meyer and Afolayan, 1995).

2.3 Results

Twenty-three of the plant species tested exhibited significant antibacterial activity while, 17 out of the 28 plant extracts screened for antibacterial activity had a significant inhibitory effect on all Gram-positive bacteria tested (Table 2.2). Gram-negative bacteria were resistant to most extracts tested. Of the Gram-negative bacteria tested, five extracts significantly inhibited the growth of three bacteria, one extract inhibited two bacteria and six extracts inhibited only one bacterial strain. None of the plant extracts inhibited the growth of *Klebsiella pneumoniae* and *Serratia marscecens* at this range of testing. The epicuticular (shaken) extracts of *H. chionosphaerum*, *H. longifolium* and *H. chionosphaerum* showed no activity against *Bacillus cereus* and *Micrococcus kristinae*, respectively.

The epicuticular extracts of *H. candolleanum, H. herbaceum, H. melanacme, H. psilolepis, H. rugulosum, H. simillimum* and *H. umbraculigerum* and the homogenized extracts of *H. decorum* and *H. melanacme* significantly inhibited growth of the organisms at the low MIC of 0.10 mg/ml level. The shaken and homogenized extracts of *H. odoratissimum* did not inhibit the growth of *Escherichia coli., K. pneumoniae, P. aeruginosa* and *S. marscence,* all Gram-negative bacteria, but had a noticeably higher level of activity against the other bacteria, than most extracts tested, at the low MIC of 0.01 mg/ml, the highest dilution used in this study. The homogenized extracts inhibited the growth of four of the five Gram-positive bacteria at a MIC of 1.0 mg/ml. Epicuticular (shaken) extracts proved to be more bioactive when compared to the macerated (homogenized) extracts.

Table 2.2 Antibacterial activity (MIC) of the crude extracts of the aerial parts of *Helichrysum* species

Helichrysum Species (Voucher No.)		MIC (mg/ml)[a]									
		Gram-positive[b]					Gram-negative[c]				
Bacterial Species		*B. cer*	*B. pum*	*B. sub*	*M. kri*	*S. aur*	*E. clo*	*E. col*	*K. pne*	*P. ear*	*S. mar*
H. appendiculatum	S[d]	1.0	1.0	1.0	1.0	1.0	na[f]	na	na	na	na
(M5135)	H[e]	1.0	1.0	1.0	1.0	1.0	na	na	na	na	na
H. argyrosphaerum	S	na	1.0	0.01	1.0	1.0	na	na	na	1.0	na
(M5080)	H	na	1.0	0.01	1.0	na	na	na	na	1.0	na
H. aureonitens	S	1.0	1.0	1.0	1.0	1.0	na	1.0	na	na	na
(M5096)	H	1.0	1.0	1.0	1.0	1.0	na	1.0	na	na	na
H. bellum	S	1.0	1.0	1.0	1.0	1.0	1.0	na	na	1.0	na
(M5178)	H	1.0	1.0	1.0	1.0	1.0	1.0	na	na	1.0	na
H. caespititium	S	1.0	1.0	1.0	1.0	1.0	1.0	1.0	na	1.0	na
(M0011)	H	1.0	1.0	1.0	1.0	1.0	1.0	1.0	na	1.0	na

Helichrysum Species (Voucher No.)		MIC (mg/ml)[a]									
		Gram-positive[b]					Gram-negative[c]				
H. callicomum (M5054)	S	1.0	1.0	1.0	1.0	1.0	1.0	na	na	na	na
	H	1.0	1.0	1.0	na	na	na	na	na	na	na
H. candolleanum (M3078)	S	0.10	0.10	0.10	0.10	na	na	na	na	0.10	na
	H	na	1.0	1.0	1.0	na	na	na	na	na	na
H.chionosphaerum (M5111)	S	na	1.0	1.0	na	1.0	na	na	na	na	na
	H	na	na	na	na	na	na	na	na	na	na
H. decorum (A0006)	S	1.0	0.10	0.01	0.10	0.10	na	na	na	na	na
	H	0.10	0.10	0.10	na	na	na	na	na	na	na
H. glomeratum (M5055)	S	na	na	na	na	na	na	na	na	na	na
	H	na	na	na	na	na	na	na	na	na	na
H. herbaceum (M5272)	S	1.0	1.0	1.0	1.0	1.0	1.0	1.0	na	1.0	na
	H	1.0	1.0	1.0	1.0	1.0	1.0	1.0	na	1.0	na
H. hypoleucum (M5056)	S	1.0	1.0	1.0	1.0	1.0	1.0	1.0	na	1.0	na
	H	1.0	0.10	0.10	1.0	na	1.0	na	na	1.0	na
H. kraussii (M5173)	S	1.0	1.0	1.0	1.0	1.0	na	na	na	1.0	na
	H	na	1.0	1.0	1.0	na	na	na	na	1.0	na
H. longifolium (M5109)	S	na	1.0	1.0	1.0	na	na	na	na	1.0	na
	H	na	1.0	1.0	1.0	na	na	na	na	na	na
H. melanacme (M5110)	S	0.10	0.10	0.10	0.10	0.10	0.10	na	na	na	na
	H	0.10	0.10	0.10	0.10	0.10	0.10	na	na	na	na
H. microniifolium (5100)	S	1.0	1.0	1.0	1.0	1.0	na	na	na	na	na
	H	na	na	na	na	na	na	na	na	na	na
H. montanum (M3707)	S	na	na	na	na	na	na	na	na	na	na
	H	na	na	na	na	na	na	na	na	na	na
H. monticola (M5177)	S	na	na	na	na	na	na	na	na	na	na
	H	na	na	na	na	na	na	na	na	na	na
H. nudifolium (M3708)	S	1.0	1.0	1.0	na	1.0	1.0	1.0	na	1.0	na
	H	na	na	1.0	1.0	1.0	1.0	1.0	na	na	na
H. odoratissimum (M5061)	S	0.01	0.01	0.01	0.01	0.01	0.01	na	na	na	na
	H	1.0	1.0	1.0	1.0	na	na	na	na	na	na

Helichrysum Species (Voucher No.)		MIC (mg/ml)[a]									
		Gram-positive[b]					Gram-negative[c]				
H. oreophilum (M5097)	S	na	na	na	na	na	na	na	na	na	na
	H	na	na	na	na	na	na	na	na	na	na
H. pilosellum (M5059)	S	na	na	na	na	na	na	na	na	na	na
	H	na	na	na	na	na	na	na	na	na	na
H. psilolepis (M5081)	S	0.10	0.10	0.10	0.10	0.10	na	na	na	na	na
	H	1.0	1.0	1.0	1.0	1.0	na	na	na	na	na
H. rugulosum (M5060)	S	0.10	0.10	0.10	0.10	1.0	0.10	na	na	na	na
	H	1.0	1.0	na	na	na	1.0	na	na	na	na
H. simillimum (M0001)	S	0.10	0.10	0.10	0.10	0.10	na	na	na	0.10	na
	H	1.0	1.0	1.0	1.0	1.0	na	na	na	na	na
H. sutherlandii (M5179)	S	1.0	1.0	1.0	1.0	1.0	na	na	na	na	na
	H	na	na	na	na	na	na	na	na	na	na
H. trilineatum (M5172)	S	1.0	1.0	1.0	1.0	1.0	1.0	1.0	na	1.0	na
	H	1.0	1.0	1.0	1.0	1.0	1.0	1.0	na	1.0	na
H.umbraculigerum (M5174)	S	0.10	0.10	0.10	0.10	0.10	na	na	na	na	na
	H	1.0	1.0	1.0	1.0	1.0	na	na	na	na	na

[a] Minimum inhibition concentration

[b] *B. cer* (*Bacillus cereus*), *B. pum* (*Bacillus pumilus*), *B. sub* (*Baccilus subtilis*), *M. kri* (*Micrococus kristinae*), *S. aur* (*Staphylococcus aureus*)

[c] *E. clo* (*Enterobacter cloacae*), *E. col* (*Escherichia coli*), *K. pne* (*Klebsiella pneumoniae*), *P. aer* (*Pseudomonas aeruginosa*) and *S. mar* (*Serratia marcescens*)

[d] Shaken extract

[e] Homogenized extract

[f] Not active

herbaceum, H. melanacme, H. psilolepis, H. rugulosum, H. simillimum and *H. umbraculigerum* and the homogenized extracts of *H. decorum* and *H. melanacme* significantly inhibited growth of the organisms at the low MIC of 0.10 mg/ml level. The shaken and homogenized extracts of *H. odoratissimum* did not inhibit the growth of *Escherichia coli., K. Pneumoniae, P. aeruginosa* and *S. marscence,* all Gram-negative bacteria, but had a noticeably higher level of activity against the other bacteria, than most extracts tested, at the low MIC of 0.01 mg/ml, the highest dilution used in this study.

Discussion

Twenty-three (82%) of the *Helichrysum* species showed inhibition against the Gram-positive bacteria tested. The negative results obtained against Gram-negative bacteria were not unexpected as, in general, this class of bacteria are more resistant than the Gram-positive bacteria (Tomas-Barberan *et al.,* 1983). A novel phloroglucinol, isolated from the aerial parts of *H. caespititium* (Dekker *et al.,* 1983), showed significant inhibition against Gram-positive bacteria, but also had no observable effect against the Gram-negative bacteria.

Extracts are generally richest in antibacterial agents after the flowering (sexual) stage of their growth is complete, and plants taken from stressful environments were particularly active (Mitscher *et al.*, 1972). Antibacterial extracts from tested species can be assumed to be useful to the producing plant in warding off infectious diseases. The infecting microorganisms are usually the same as those infecting higher animals (Turnbull and Kramer, 1991), and there is therefore compelling reason to suppose that antiifective agents could be active against human or veterinary pathogens. It is comforting, to find that the spectrum of activity of these plant extracts is broad enough to include human pathogens, as was suggested by folkloric and historical accounts. A number of examples are included in Table 2.1 in which one sees a number of applications that could be interpreted as related to infectious disease.

These results are consistent with previous reports (Tomas-Barberan *et al.,* 1990, Dekker *et al.,* 1983) on related species against Gram-negative bacteria. Unlike Gram-positive bacteria, the lipopolysaccharide layer along with proteins and phospholipids are the major components in the outer surface of Gram-negative bacteria (Burn, 1988). Access of most

compounds to the peptidoglycan layer of the cell wall is hindered by the outer lipopolysaccharide layer. This explains the resistance of Gram-negative strains to the lytic action of most extracts exhibiting activity.

Infections caused by *P. aeruginosa* are among the most difficult to treat with conventional antibiotics (Levison and Jawetz, 1992). The growth of *P. aeruginosa* was inhibited at 0.1 mg/ml by three crude extracts. These plants may, thus, be a source which could yield drugs that could improve the treatment of infections caused by this organism.

The activity of most extracts against *S. aureus*, another human pathogen, qualify these plants for further investigation of their bioactive compounds. Strains of *E. coli* have been identified which are capable of colonizing the gastrointestinal tract and producing potent enterotoxins (Kwon-Chung and Bennett, 1992). The pathogenesis of the resulting illness resembles that of cholera. Outbreaks of *E. coli* are characterized by prolonged illness, high mortality and morbidity and by the ease and rapidity with which infection spreads (Turnbull and Kramer, 1991).

Bacillus species are common microbes found in most natural environments including soil, water, plant and animal tissues. While most *Bacillus* species are regarded as having little pathogenic potential, both *B. cereus* and *B. subtilis* have been known to act as primary invaders or secondary infectious agents in a number of diseases and have been implicated in some cases of food poisoning (Turnbull and Kramer, 1991). Some species of *Helichrysum* in the food and medicine of the indigenous people of South Africa may have helped to combat these microbes.

Different *Helichrysum* species produce different secondary metabolites (acetophenones, chalcones, flavonoids, phloroglucinols, tannins, etc) as a biochemical defence mechanism (Tomas-Barberan *et al.*, 1990). This indicates the use of different metabolic pathways to produce chemical barriers, which has a single ecological defence against bacteria and other pathogens. The antibacterial compounds harvested from these species may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in the treatment of resistant microbial strains.

2.5 Conclusion

On the basis of the results obtained, we conclude that the crude extracts of these *Helichrysum* species exhibit significant antibacterial activity and properties that support folkloric use in the treatment of some diseases as broad-spectrum antimicrobial agents. This probably explains the use of extracts from these species by the indigenous people of South Africa against a number of infections for generations. Consequently, we propose a detailed study of these plants in order to determine their pharmacological effects, active compounds as well as their mechanism of action.

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CHAPTER 3

ANTIFUNGAL ACTIVITY OF *HELICHRYSUM* SPECIES

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3.1 Introduction

Fungi differ from bacteria in possessing a number of chromosomes within a well-defined nuclear membrane, mitochondria and an endoplasmic reticulum. Like plants they have definite cell walls, but these are usually composed of chitin rather than cellulose. They lack chlorophyll, so they live either on dead organic material as saprophytes, or on living organic matter as parasites. The cells may live separately (yeasts) or more commonly, they form long multicellular filaments or hyphae which may contain cross-walls or septa. A mass of hyphae is a mycelium. Many species have both yeast and mycelial forms which are dependent on the cultural conditions, a process known as dimorphism (Alexopoulos *et al.*, 1996).

The classification of fungi is based on the form of their sexual reproductive apparatus but there is a large group, containing most of the human parasites, which have never been known to undergo sexual reproduction. These are the fungi imperfecti or deuteromycetes. Where the perfect stages has subsequently been identified, members of this group have been found to be either Ascomycetes or Basidiomycetes (Alexepoulos *et al.*,1996).

3.1.1 Fungi and man

Parasitic fungi cause many different diseases, which may be superficial, subcutaneous or deep inside man and animals. In the superficial mycoses, the fungus is limited to the horny layer of the skin and to structures derived from it, while in the subcutaneous and deep mycoses there is a deeper invasion of the tissues (Laks and Pruner, 1989, Kwon-Chung and Bennett, 1992). Generally speaking, fungal infections of humans are most common in tropical regions of the world. However, in recent years the number of individuals infected with fungi have increased drastically in all regions of the world. This is due primarily to the fact that there are more individuals who are predisposed to fungal infection than ever

before. Individuals with compromised immune systems (including HIV/AIDS patients) are most at risk.

Some species possess enzymes, which digest keratin. Geophilic species normally inhabit the soil and occasionally colonize animal hair and infect man. Zoophilic species are primarily parasitic on particular animal host species but can also infect many others, including man. Anthrophilic species are adapted for living on man and only occasionally infect other species. A well-adapted species such as *Trichophyton rubrum* evokes very little inflammatory reaction from the host, and its presence is therefore tolerated for very long periods (Kwong-Chung andBennett, 1992). Poorly adapted species, geophilic or zoophilic, evoke a fierce inflammatory response and the infection is eventually terminated (Kwong-Chung and Bennett, 1992).

There are a wide variety of clinical diseases produced by the filamentous, septate fungi of the genus Aspergillus. *A. fumigatus* is most commonly involved in human infections, followed by *A. niger* and *A. flavus* (Kwon-Chung and Bennett, 1992). These organisms are widely distributed in nature, being found in soil, vegetation, grain and mouldy hay. Aspergillosis is also a common infection of birds. In human systemic infections there is lung invasion with tissue destruction and a purulent granulomatous reaction (Kwon-Chung and Bennett, 1992). Aspergillus strains are important as they are responsible for most human systemic infections.

3.1.2 Epidemiology

Some species are cosmopolitan, while others are strictly limited to certain parts of South Africa. In zoophilic species, this may reflect dependence on a particular animal host, but it is not clear why many human pathogenic fungal species have a restricted distribution (Tomas-Barberan *et al.*,1990, Alexopoulos *et al.*, 1996). Changes in distribution have taken place in the last century. Some species have a particular affinity for one site. *Tinea pedis* (toe clefts), *T. manuum* (hands), *T. cruri* (intertrinous parts of the groin), *T. barbae* (beard). *T. corporis* (glabrous skin), and *T. unguium* (nail) are typical examples.

3.1.3 Fungi and plants

Fungi are, tremendously important to humans because of the plant diseases they cause. Most species of plants are subjected to attack by a number of different types of fungal pathogens. The consequences of such infections are varied but range from widespread death of individuals of a particular species to the development of insignificant symptoms associated with little, if any, damage to the host. In addition to serious economic losses caused to agronomically important species, fungi have literally altered the course of history and have affected social customs on both a regional and global scale (Alexopoulos *et al.*, 1996). Many fungi such as *Penicillium digitatum*, *Phytophthora citophthora*, *Aspergillus niger* and *Cladosporium cladosporioides*, are well known as major pathogens causing decay in fruit, vegetable and other agricultural products, especially during storage (Adikaram *et al.*, 1992). *Phytophthora infestans* causes a severe disease of potatoes known as ‘ late blight’. It not only kills the foliage but also infects the tubers, causing them to rot rapidly. Examples of a number of classical fungal diseases include: corn smut, black stem rust of wheat, foolish seeding disease of rice, ergot of rye, club root of crucifers and Dutch elm disease and Chestnut blight (Alexopoulos *et al.*, 1996), to mention a few.

Plant pathogenic fungi are, of course, not limited to desirable or agronomically important hosts. In fact, one understudied aspect of ecology is the influence of fungal pathogens on natural plant populations. All types of plants are attacked by parasitic fungi including weedy species that can pose serious problems to farmers, golf course managers, individuals involved in commercial lawn and landscape businesses, and individual home owners who want weed free yards and gardens. In this regard, scientists are actively involved in the development of certain plant pathogenic fungi as biological control agents for weeds (Kwong-Chung and Bennett, 1992).

Not all fungi associated with higher plants are detrimental to the plants. The hyphae of some fungi form specialized organs in the roots of plants known as mycorrhizae (Alexopoulos *et al.*, 1996). These structures provide significant benefits to both the fungi

and the host plants involved. An astonishing diversity of fungi known as endophytes has also been shown to be present in the leaves and stems of healthy plants ranging from conifers to grasses (Alexopoulos *et al.*, 1996). Many of these fungi appear to protect their hosts from pathogenic bacteria and fungi as well as insects and grazing mammals. Unfortunately several popular forage grasses such as *Festuca arundinacea* and ryegrass (*Lolium perenne*) often contain endophytes that produce such high levels of physiologically active alkaloids that they are toxic to domestic mammals, causing alarming physical and behavioural disorders (Clay, 1989). Fungi are also known to colonize optical instruments resulting in extensive and costly damage.

3.1.4 Exploitation of *Helichrysum* species for new antifungal agents

Plants have been used to treat human, animal and plant diseases from time immemorial. In traditional medicine, this empirical knowledge belongs to societies in general where those plants are found, or to a limited group of people, such as a family. Generally, only a few individuals inherit such knowledge from traditional healers and pass it from one generation to another, using their knowledge to improve the well-being of their kin.

As a result of the increasing need for new and better drugs to heal diseases, researchers from different disciplines are jointly attempting to study rationally and scientifically the resources of medicinal plants. This process includes the use of plants in their crude form or as starting material for drugs. However, the research focus differs from researcher to researcher and from country to country, due to differences that prevail in technological development and scientific level between countries. Whichever type they belong to, the starting point for their investigation generally follows the same intellectual process based on ethnopharmacology, or, on data from the literature (Cragg *et al.*, 1994). We can control many human and animal pathogens by currently available antibiotics. However, the need for new antibiotics still exits. For example, systemic infections caused by fungi, especially in patients with impaired host defence mechanisms, have become increasingly serious. Various antifungal agents have been explored, but the control of many of the fungal diseases has not yet been achieved. This study examines the role of *Helichrysum* species as another source of antifungal agents.

3.2 Material and Methods

3.2.1 Plant material

Shoots of *Helichrysum* species were collected from the Drakensberg in the Mont-aux-Sources area in Qwaqwa. A taxonomist, Prof. R.O. Moffett verified their identity and voucher specimens were deposited in the herbarium of the Department of Botany, University of the North, QwaQwa Branch, South Africa and the National Botanical Institute herbarium, Pretoria.

3.2.2 Preparation of extracts

Shoots (excluding flowers) of the plants were air dried at room temperature. Each plant (80g) was shaken for five minutes in acetone and filtered through Whatman No 2 filter paper under suction to obtain the 'shaken extract'. The residue was then homogenized in acetone, and also filtered through Whatman No 2 filter paper under suction. Both extracts were concentrated to dryness under reduced pressure at 45^0C with a rotary evaporator. After determining the yields, extracts were stored at 4^0C until further use.

3.2.3 Fungal strains

Six fungal species (Table 3.1) were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on nutrient agar (Biolab) and an inoculum was recovered for testing by growth on a potato dextrose nutrient agar (Biolab) for 24 hours.

3.2.4 Antifungal bioassay

The plant extracts (sterilized by filtering through a 0.22 mm filter) were added to 5 ml of nutrient agar medium in Petri dishes and swirled carefully before congealing. An aliquot of each extract was serially diluted (ten fold) to obtain a concentration range of 1.0 to 0.01 mg/ml in acetone. A negative blank containing only nutrient agar and a control containing nutrient agar and 2% acetone served as controls (Meyer and Afolayan, 1995). The prepared plates were inoculated with disks obtained from actively growing margins of the fungi plates (that is, before spore formation) and incubated at 25^0C in the

dark for two days. Plates were examined after 24 and 48 hours and complete suppression of growth was required for the extract to be declared bioactive. Three replications were used per treatment.

3.3 Results

Results of bioassays are summarised in Table 3.1. Of the 28 crude extracts tested, 27 (96.4%) showed varying degrees of antifungal activity. 21 (75%) extracts inhibited growth of all organisms tested, and in addition, six of these showed high activity in the bioassays, at 0.01 mg/ml, the highest dilution used in this investigation. The epicuticular extract of *H. pilosellum*, and 9 (32%) homogenized extracts did not show significant antifungal activity. Sixty-eight per cent of the homogenized extracts exhibited significant antifungal activity at the concentrations tested in this study. Results of the bioassays are summarized in Table 3.1. Of the 28 crude extracts tested, 27 (96.4%) showed varying degrees of antifungal activity. Twenty-one (75%) extracts inhibited growth of all organisms tested, and in addition, six of these showed high activity in the bioassays, at 0.01 mg/ml, the highest dilution used in this investigation. The been established that Helichrysum species can be divided into a number of chemical races, containing different compounds (Hilliard, 1983). However, some of the moderately active and least active plants were also reported to have similar and/or other active compounds but probably in smaller quantities. Different chemotypes of species could explain the observed variance in inhibitory activity.

3.4 Discussion

The present screening investigation has revealed a fairly high ‘hit’ rate for antifungal inhibition when selecting plants utilized in traditional medicines based upon the criteria given in Chapter 1. Some results obtained suggest the possible correlation between the folkloric uses of Helichrysum species and their activity.

For a biologically active compound like a fungicide to have activity 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 then partition itself onto the active site (Hansch, 1971). The rate of these

events will depend on the lipophilicity of the compound. Once at the active site, the compound has some chemical and physical effect that accounts for its activity. There is a growing consensus that, in most systems, antifungal agents exert their toxicity by some membrane-associated phenomenon (Laks and

Table 3.1 Antifungal activity (MIC) of the crude extracts of the aerial parts of *Helichrysum* species

Plant Species (Voucher specimen No.)	MIC (mg/ml)[a]						
		Fungi[b]					
		A. fla	*A. nig*	*C. cla*	*C. cuc*	*C. sph*	*P. cap*
H. appendiculatum	S[c]	1.0	1.0	na[e]	na	1.0	1.0
(M5135)	H[d]	na	na	na	na	na	na
H. argyrosphaerunm	S	1.0	1.0	na	na	1.0	1.0
(M5080)	H	na	na	na	na	na	na
H. aureonitens	S	1.0	1.0	na	na	1.0	1.0
(M5096)	H	na	na	na	na	na	na
H. bellum	S	1.0	1.0	0.10	0.10	1.0	1.0
(M5178)	H	na	na	na	na	1.0	na
H. caespititium	S	0.01	0.01	0.01	0.01	0.01	0.01
(M0011)	H	1.0	1.0	0.01	0.01	1.0	1.0
H. callicomum	S	0.01	0.01	1.0	0.01	0.01	0.01
(M5054)	H	0.01	na	na	na	na	na
H. candolleanum	S	1.0	na	1.0	1.0	1.0	na
(M3078)	H	na	na	1.0	1.0	1.0	na
H. chionosphaerum	S	1.0	0.10	0.10	1.0	0.10	1.0
(M5111)	H	na	1.0	1.0	na	1.0	na
H. decorum	S	1.0	1.0	1.0	1.0	1.0	1.0
(A0006)	H	1.0	0.10	0.10	0.10	0.10	na

Plant Species (Voucher specimen No.)		MIC (mg/ml)[a]					
		Fungi[b]					
		A. fla	*A. nig*	*C. cla*	*C. cuc*	*C. sph*	*P. cap*
H. glomeratum (M5055)	S	0.01	0.01	0.01	0.01	0.01	0.01
	H	na	na	1.0	1.0	na	na
H. herbaceum (M5272)	S	1.0	0.01	0.10	0.10	0.01	0.10
	H	1.0	1.0	0.10	1.0	1.0	0.10
H. hypoleucum (M5056)	S	1.0	0.01	0.01	0.01	0.01	0.01
	H	1.0	0.01	0.01	0.01	0.01	0.01
H. kraussii (M5173)	S	0.01	0.01	1.0	0.10	0.10	0.10
	H	1.0	1.0	1.0	1.0	1.0	na
H. longifolium (M5109)	S	0.10	0.10	0.10	0.10	0.10	0.10
	H	1.0	1.0	1.0	1.0	1.0	na
H. melanacme (M5110)	S	0.01	0.01	0.01	0.01	0.01	0.01
	H	0.01	0.01	0.01	0.01	0.01	0.01
H. microniifolium (5100)	S	1.0	1.0	na	na	1.0	1.0
	H	na	na	na	na	na	na
H. montanum (M3707)	S	1.0	1.0	1.0	1.0	1.0	1.0
	H	na	na	na	na	na	na
H. monticola (M5177)	S	1.0	1.0	1.0	1.0	1.0	na
	H	na	na	1.0	1.0	1.0	1.0
H. nudifolium (M3708)	S	0.10	0.10	0.10	0.10	0.10	0.10
	H	na	na	na	na	na	na
H. odoratissimum (M5061)	S	0.10	0.01	0.10	0.10	0.10	0.10
	H	na	1.0	na	na	na	na
H. oreophilum (M5097)	S	1.0	1.0	1.0	1.0	0.01	0.01
	H	na	na	na	1.0	0.01	0.01
H. pilosellum (M5059)	S	na	na	na	na	na	na
	H	na	na	na	na	1.0	na
H. psilolepis (M5081)	S	1.0	1.0	0.1	1.0	0.1	0.1
	H	na	na	1.0	1.0	1.0	1.0

Plant Species (Voucher specimen No.)	MIC (mg/ml)[a]						
		Fungi[b]					
		A. fla	*A. nig*	*C. cla*	*C. cuc*	*C. sph*	*P. cap*
H. rugulosum (M5060)	S	0.01	na	0.01	0.01	1.0	0.01
	H	0.01	na	na	0.01	na	0.01
H. simillimum (M0001)	S	1.0	na	na	1.0	1.0	na
	H	na	na	na	na	na	na
H. sutherlandii (M5179)	S	1.0	0.10	1.0	1.0	1.0	1.0
	H	1.0	1.0	na	na	na	na
H. trilineatum (M5172)	S	0.10	0.10	0.10	1.0	0.10	0.10
	H	na	na	na	na	0.10	na
H. umbraculigerum (M5174)	S	1.0	1.0	1.0	1.0	0.10	0.10
	H	1.0	1.0	1.0	1.0	0.10	0.10

[a] Minimum inhibition concentration

[b] A. fla (Aspergillus flavus), A. nig (Aspergillus niger), C. cla (Cladosporium cladosporioides), C. cuc (Cladosporium cucumericum), C. sph (Cladosporium sphaerospermum), and P. cap (Phytophthora capsici)

[c] Shaken extract

[d] Homogenized extract

[e] Not active

Pruner, 1989), again indicating the possible importance of lipophilicity for their activity.

The success of the ethnobotanical approach to drug discovery can no longer be questioned. Historical and current discoveries attest to its power (Cox, 1994). Medicinal plants are the 'backbone' of traditional medicine (Farnsworth, 1994). Focussing attention on those plants is the most effective way of identifying plants that contain bioactive compounds (Schultes, 1994). Internal uses predominate over external ones, but a decoction is the

primary form used. The types of diseases or complaints treated are ailments of the digestive tract, general pain, dermatological conditions, wound dressing and bronchiopulmonary disorders and inflammations (not necessarily in descending order of importance) but this natural antifungal activity can be rapidly lost because of seasonal changes, presumably due to chemical or enzymatic degradation of the active species (Prusky *et al*., 1983). The tested *Helichrysum* species represent a potential source of effective fungicides in food and medicine.

3.5 Conclusion

Helichrysum species have played an important role in the botanical pharmacopoeia of the indigenous people of South Africa. As described by Watt and Breyer-Brandwijk (1962), these plants have been used to treat a variety of ailments, many of which could have been caused or been complicated by fungal infection. In this investigation, the inhibitory effects produced by these Helichrysum species suggests that their agents may have played a medicinal role in the healing practice of the indigenous people of South Africa. All the fungal strains tested in this study were susceptible to most of the *Helichrysums* investigated.

It is not possible to make a direct correlation between the observed activity of the *Helichrysum* extracts *in vitro* and the actual effects when used *in vivo* for the diseases observed by the indigenous people and traditional healers. Therefore, it is important that the species which have demonstrated growth-inhibiting activity in this assay be further studied to evaluate the significance of these extracts' clinical role and, in the medical system of indigenous people. Additional research is also necessary to isolate and identify their active compounds for pharmacological testing.

Helichrysum species and the observations related to the use of these plants are open to extensive study. *Helichrysum* species not only function as important herbs, but also serve as nutritional and medicinal agents. It is certain, that, through observations made in this

study, *Helichrysum* species harbour many economically significant benefits awaiting 'discovery'.

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CHAPTER 4

AN ACYLATED PHLOROGLUCINOL WITH ANTIMICROBIAL PROPERTIES FROM *HELICHRYSUM CAESPITITIUM*

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An acylated phloroglucinol with antimicrobial properties from *Helichrysum caespititium*

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Abstract

A new acylated form of a phloroglucinol with significant antimicrobial properties was isolated by bioactivity guided fractionation from *Helichrysum caespititium* (Asteraceae). The structure elucidation, and conformation of the new phloroglucinol, 2-methyl-4-[2′,4′,6′-trihydroxy-3′-(2-methylpropanoyl) phenyl]but-2-enyl acetate, was established by high field NMR spectroscopic and MS data. The compound inhibited growth of *Bacillus cereus, B. pumilus, B. subtilis* and *Micrococcus kristinae* at the very low concentration of 0.5 μg/ml and *Staphylococcus aureus* at 5.0 μg/ml. Six fungi tested were similarly inhibited at low MICs, *Aspergillus flavus* and A. *niger* (1.0 μg/ml), *Cladosporium cladosporioides* (5 μg/ml), *C. cucumerinum* and *C. sphaerospermum* (0.5 μg/ml) and *Phytophthora capsici* at 1.0 μg/ml.

Keywords: *Helichrysum caespititium*; Asteraceae; Antimicrobial; Phloroglucinol

1. Introduction

Many *Helichrysum* (Asteraceae) species have been examined for their chemical components. These include 38 species from South Africa (Jakupovic, Kuhnke, Schuster, Metwally & Bohlmann, 1986; Jakupovic, Zdero, Grenz, Tsichritzis, Lechmann, Hashemi-Nejad, & Bohlmann, 1989b; Meyer, Afolayan, Taylor & Erasmus, 1997) eight from Madagascar (Randriaminahy, Proksch, White & Wray, 1992), several from Spain (Tomas-Baberan, Msonthi & Hostettmann, 1988; Tomas-Barberan, Iniesta-Sanmarin, Tomas-Lorente, & Rumbero, 1990) and many species from Australia (Jakupovic, Schuster, Bohlmann, Ganzer, King & Robinson, 1989a) The fact that different *Helichrysum* species produce different secondary metabolites (acetophenones, flavonoids, phloroglucinols) as a biochemical defence mechanism (chemical barrier) against bacteria and fungi is of great interest, since it indicates the use of different metabolic pathways. As part of a programme to investigate the medicinal potential of South African *Helichrysum* species we examined *H. caespititium* (DC.) Harv. for possible biological activity. The Southern Sotho inhale the smoke for relief of head and chest colds and also use it as a dressing for open wounds during circumcision rites. Caespitin (**1**) was previously isolated from this species (Dekker, Fourie, Snyckers & Van der Schyf, 1983) and shown to have antimicrobial properties. The antimicrobial activity guided fractionation of the acetone extract of the aerial parts of *H. caespititium* led to the isolation of the new phloroglucinol derivative 2-methyl-4-[2′,4′,6′-trihydroxy-3′-(2-methylpropanoyl)-phenyl]but-2-enyl acetate (**2**). Evaluation of the antimicrobial activity of compound **2** against ten bacteria showed significant biological activity against all the Gram-positive bacteria tested. In addition, the growth of the six fungi tested, was significantly inhibited at very low MIC values.

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2. Results and discussion

2.1. Structure elucidation of 2

The structure of **2** was established through the usual spectroscopic techniques including ^{1}H- and ^{13}C-NMR analysis. With the aid of DEPT, COSY and HETCOR pulse sequences, the multiplicity of the proton peaks, their relationship to one another and the identity of each carbon and hydrogen could be established, respectively. The process of identification was facilitated by the article (Dekker et al., 1983) on the compound caespitin; and the identification of acylphloroglucinols from *Helichrysum* species (Jakupovic et al., 1989b; Bohlmann & Mahanta, 1979). The presence of a free phloroglucinol nucleus was suspected on the grounds of three phenolic carbons being shifted far downfield in the ^{13}C-spectrum (C-2′, C-4′ and C-6′ at 164.0, 161.6 and 160.2 ppm, respectively). The single aromatic proton attached to C-5′, had the anticipated proton (∂5.98) and carbon (95.7 ppm) shifts. The ^{13}C-spectrum left no doubt that a carbonyl carbon and ester carbon were present (211.8 and 173.6 ppm, respectively). It remained to establish what other substituents were on the side chains. The 2-methylpropanoyl moiety at C-3′ showed the anticipated septuplet for the $CHMe_2$ group (∂3.96) and the doublet for the geminal dimethyl substituents (∂1.17). An initial problem was posed by the nature of substituents present on C-1′, but a closer examination of the proton NMR spectrum revealed that the side chain of **2** was not unlike that of the 3,3′-dimethylallyl group (Ar–$CH_2CH{=}CMe_2$) present in the compounds described by Bohlmann and Mahanta (1979). In case of compound **2**, however, the one terminal methyl group had been replaced by $–CH_2OCOCH_3$. The upfield ^{13}C-shift position of the methylene group at C-4 (benzylic to the aromatic ring and allylic to the side chain alkene) at 21.5 ppm was unusual, but in keeping with the findings of Tomas-Barberan et al. (1990), Dekker et al. (1983) and Bohlmann and Mahanta (1979) on similar moieties.

2.2. Significance of structure

The claim that **2** is a new compound is based on the finding that (Jakupovic et al., 1989b) describes an acylated phloroglucinol with molecular formula $C_{17}H_{22}O_6$ but ascribes an incorrect structure to it on p. 1120. The structure shown in that paper has an *n*-butyl group at C-3′ instead of the $–COCHMe_2$ group.

2.3. Antibacterial activity

The activity of compound **2** was examined against 10 bacteria by the agar dilution method (Turnbull & Kramer, 1991). The compound significantly inhibited the growth of all the Gram-positive bacteria tested (Table 1) at a concentration of between 0.5 and 5 μg/ml. This phloroglucinol had no activity against all the Gram-negative bacteria tested. These results are in accordance with previous reports (Tomas-Barberan et al., 1990; Dekker et al., 1983) of similar antimicrobial activity of related compounds against Gram-negative bacteria. 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 and have been implicated in some cases of food poisoning (Turnbull & Kramer, 1991). *Staphylococcus aureus*, is a human pathogen, whose infections are amongst the most difficult to combat with conventional antibiotics (Tomas-Barberan et al., 1988, 1990). This study provides a probable

Table 1
Antibacterial activity of the crude acetone extract of the aerial parts of *Helichrysum caespititium* and compound **2** isolated from the extract

Bacterial species	Gram (+/−)	MIC[a]	
		Crude extract (mg/ml)	**2** (μg/ml)
Bacillus cereus	+	1.0	0.5
B. pumilus	+	1.0	0.5
B. subtilis	+	1.0	0.5
Micrococcus kristinae	+	1.0	0.5
Staphylococcus aureus	+	1.0	0.5
Enterobacter cloacae	−	1.0	na[b]
Escherichia coli	−	1.0	na
Klebsiella pneumoniae	−	na	na
Pseudomonas aeruginosa	−	1.0	na
Serratia marcescens	−	na	na

[a] Minimum inhibitory concentration.
[b] Not active.

Table 2
Antifungal activity of the crude acetone extract of the aerial parts of *Helichrysum caespititium* and compound **2** isolated from the extract

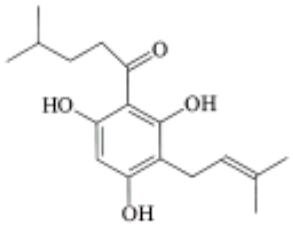
Fungal species	MIC[a]	
	Crude extract (mg/ml)	**2** (μg/ml)
Aspergillus flavus	1.0	1.0
A. niger	0.01	1.0
Cladosporium cladosporioides	0.01	5.0
C. cucumerinum	0.01	0.5
C. sphaerospermum	0.01	0.5
Physophthora capsici	1.0	1.0

[a] Minimum inhibitory concentration.

scientific explanation for the therapeutic potency attributed to *H. caespititium*, claimed by traditional healers in the Free State province of South Africa, for example, during wound treatment in male circumcision rites.

2.4. Antifungal activity

The growth of six fungi, *Aspergillus niger*, *A. flavus*, *Cladosporium cladosporioides*, *C. cucumerinum*, *C. sphaerospermum* and *Phytophthora capsici*, were significantly inhibited at very low MIC's by compound **2** (Table 2). *A. flavus* and *A. niger* are some of the most important fungi responsible for human systemic infections. These organisms were inhibited at 1.0 μg/ml. It is generally agreed that at least one acidic hydroxyl group and a certain degree of lipophilicity are required for biological activity compound (Tomas-Barberan et al., 1990). Lipophilicity is important because many antifungal metabolites exert their toxicity by some membrane associated phenomenon, and it is known that acidic hydroxyl groups may act by uncoupling oxidative phosphorylation. In this case, the antifungal compound isolated from *H. caespititium* bears three acidic hydroxyls (phenolic hydroxyls) and lipophilicity (3′-isobutyrylphenyl and but-2-enyl acetate residues). On the other hand, antibacterial activity, against Gram-positive bacteria seems to be related to the presence of phenolic hydroxyls (phenol itself is a well known antibacterial compound (Tomas-Barberan et al., 1990).



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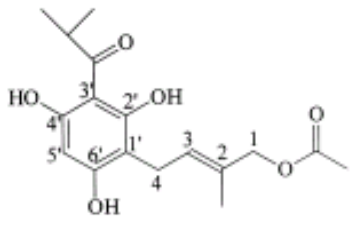

2

3. Experimental

3.1. Plant Material

Shoots of *H. caespititium* were collected from the Drakensberg in the Mount-aux-Sources area in QwaQwa, South Africa during August 1998. A voucher specimen (AM11) of the species was deposited in the herbarium of the National Botanical Institute of South Africa in Pretoria.

3.2. Preparation of extract

Air dried (80 g) plant material was immersed in acetone and shaken on a rotary shaker for 5 min without homogenising it. The extract was filtered and concentrated to dryness under reduced pressure at 40° with a rotary evaporator. After determining the yield (6.4 g (w/w)), the extract was stored at 4° until antibacterial assays commenced.

3.3. Antibacterial activity

An aliquot of the crude extract of *H. caespititium* was serially diluted (ten-fold) to obtain a range of 1.0–0.01 mg/ml in 2% acetone final concentrations. Compound **2** was diluted to final concentrations of 100.0, 10.0, 5.0 and 0.5 μg/ml in 2% acetone. The plant extract and isolated pure compound **2** (sterilised by filtering through a 0.22 μm filter) were added to 5 ml of sterilised nutrient agar in Petri dishes and swirled carefully before congealing. The organisms were streaked in radial patterns on agar plates (Mathekga & Meyer, 1998). Plates were incubated at 37° in the dark and examined after 24 and 48 h. Complete inhibition of growth was required for the extract to be declared bioactive. The controls consisted of Petri dishes containing only nutrient agar and others containing nutrient agar in 2% acetone. Each treatment was analysed in triplicate.

3.4. Antifungal activity

The acetone plant extract as well as compound **2** were subjected to the same treatment as noted above except that instead of streaking bacteria onto the agar, 48 h cultured fungal inoculum disks were carefully deposited at the centre of each Petri dish. Plates were incubated at 25° in the dark and examined after 24 and 48 h. Complete inhibition of growth was similarly required for the extract to be declared bioactive. Controls were likewise prepared containing only nutrient agar or nutrient agar in 2% acetone. Each treatment was analysed in triplicate.

3.5. Isolation and identification of 2

The crude acetone extract of *H. caespititium* was initially subjected to preparative TLC in $CHCl_3$–EtOAc (1:1). The targeted band was recovered and rechromatographed by column chromatography with 100% chloroform on silica gel 60. Direct TLC antibacterial bioassays of the fractions indicated the presence of several antibacterial compounds in the extract. The fraction with the highest antibacterial activity were finally isolated in a pure form by HPLC in H_2O–EtOH (1:1) on a reverse phase Phenomenex column (250 × 4.60 mm; 5 μ). NMR analysis of DEPT, COSY and HETCOR spectra were obtained using standard pulse sequences on a Varian 200 MHz spectrometer. Mass spectra were recorded on a Hewlett-Packard 5988 GC/MS instrument. High resolution mass spectra were obtained from a Kratos MS 80 RF double-focussing magnetic sector instrument.

3.5.1. Compound 2

2-Methyl-4-[2′,4′,6′-trihydroxy-3′-(2-methylpropanoyl)phenyl]but-2-enyl acetate mp 140°; ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.17 (6H, *d*, J = 6.7 Hz, $CH\underline{Me}_2$), 1.73 (3H, *s*, $CH_3C{=}$), 2.12 (3H, *s*, $\underline{CH_3}CO_2$), 3.40 (2H, *brd*, J = 7.1 Hz, H-4), 3.96 (1H, septuplet, J = 6.7 Hz, $\underline{CH}Me_2$), 4.79 (2H, *s*, H-1), 5.49 (1H, *brt*, J = 7.1 Hz, H-3), 5.98 (1H, *s*, H-5′), 7.90 (2H, *bs*, 2 × ArOH), 12.90 (1H, *bs*, ArOH on C-2′). ^{13}C-NMR (500 MHz, $CDCl_3$): 19.8 (di$\underline{Me}$), 21.5 (C-4), 21.7 ($\underline{Me}C{=}$), 21.7 ($\underline{CH_3}CO_2$), 39.6 ($\underline{CH}Me_2$), 64.8 (C-1), 95.7 (C-5′), 104.5 (C-3′), 106.5 (C-1′), 129.5 (C-3), 130.1 (C-2), 160.2 (C-4′), 161.6 (C-6′), 164.0 (C-2′), 173.6 (CO_2), 211.8 (C=O). GCMS m/z (rel. int.) : 262 (25, M-60), 219 (100, M-60-$CHMe_2$), 177 (8), 115 (6), 109 (7), 69 (10). Preparation of the trimethylsilyl derivative afforded a small peak (1%) at m/z 322 (M-trimethylsilyl ether). HRMS calculated for $C_{17}H_{22}O_6$ requires 322.14164; found 322.14363.

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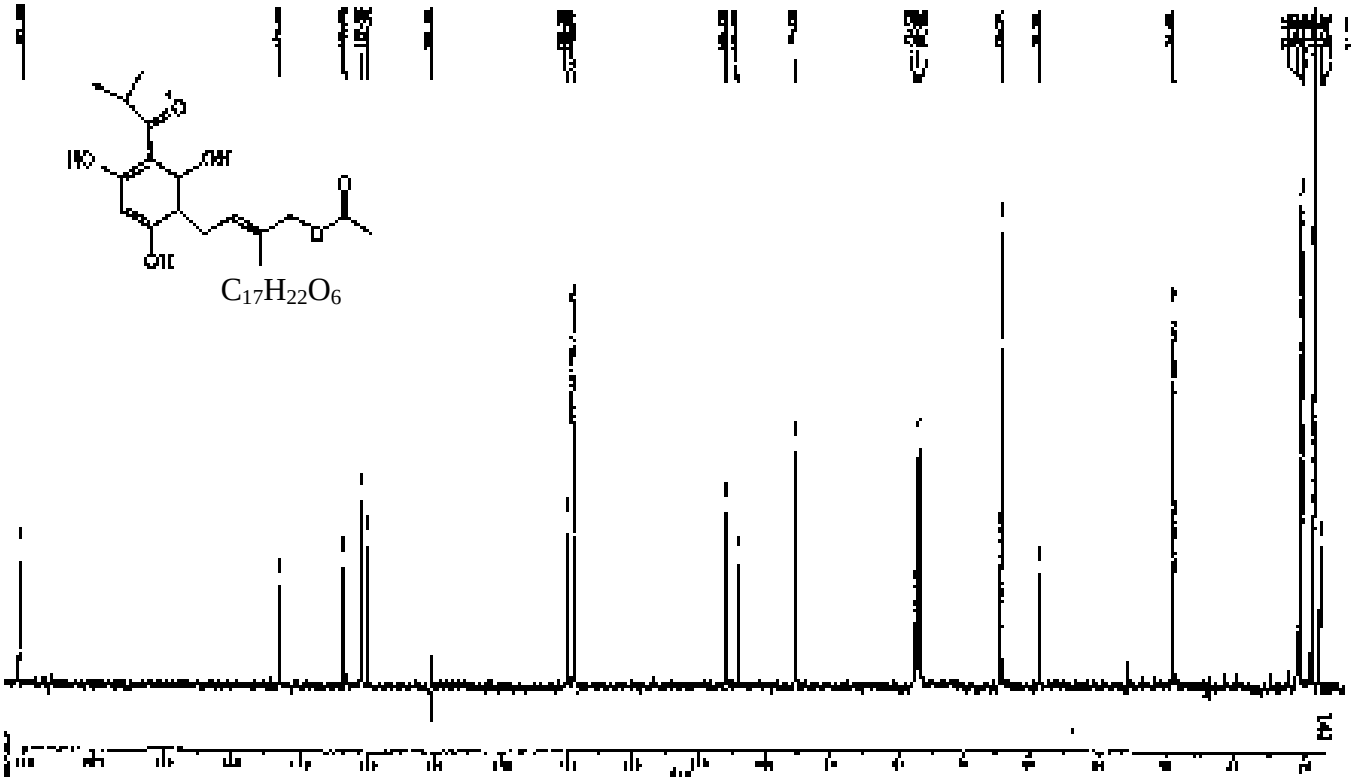

Figure 4.1 ^{13}CNMR of caespitate in $CDCI_3$. MW. 322.14 $C_{17}H_{22}O_6$

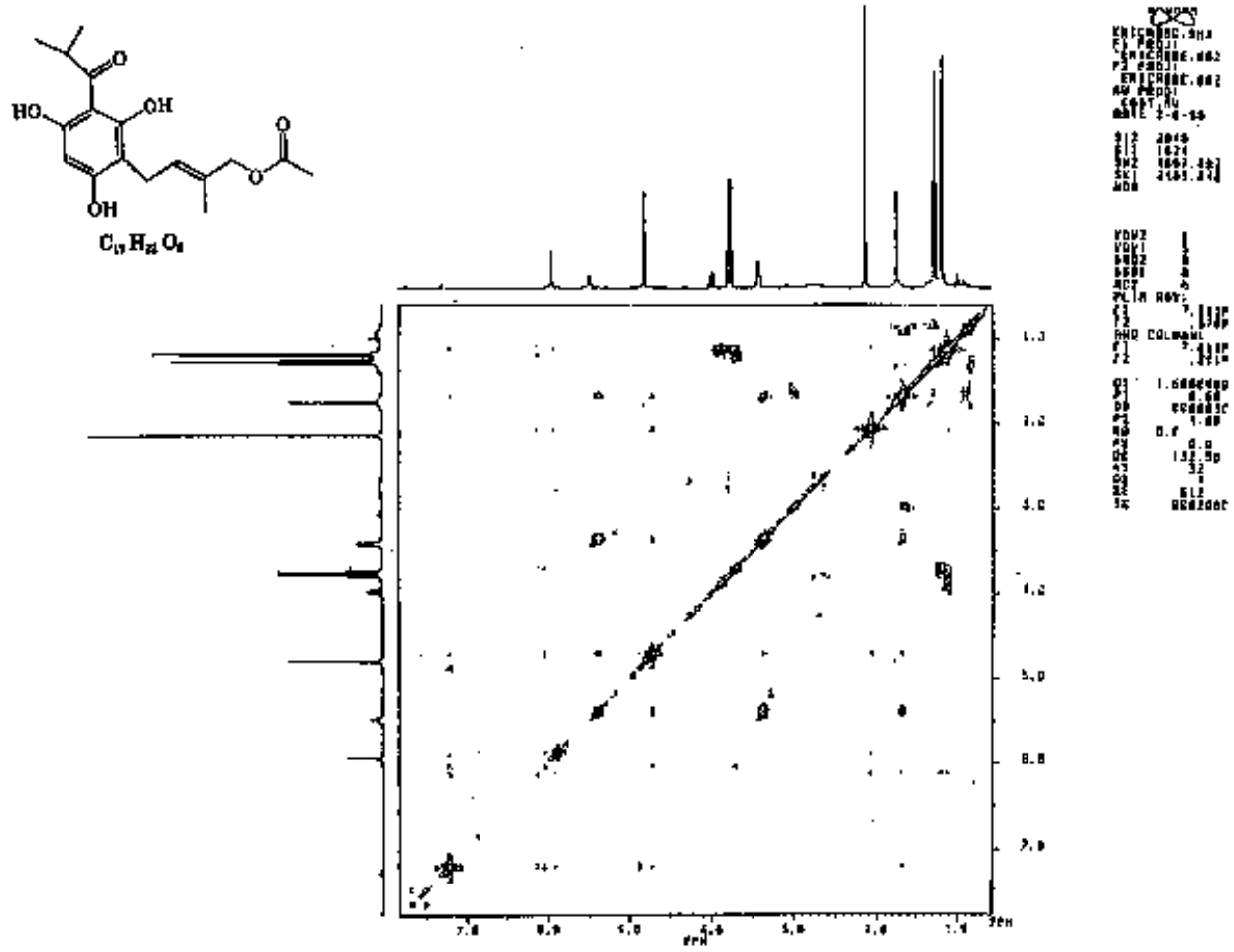

Figure 4.2: COZY of caespitate in $CDCI_3$ MW. 322.14 $C_{17}H_{22}O_6$

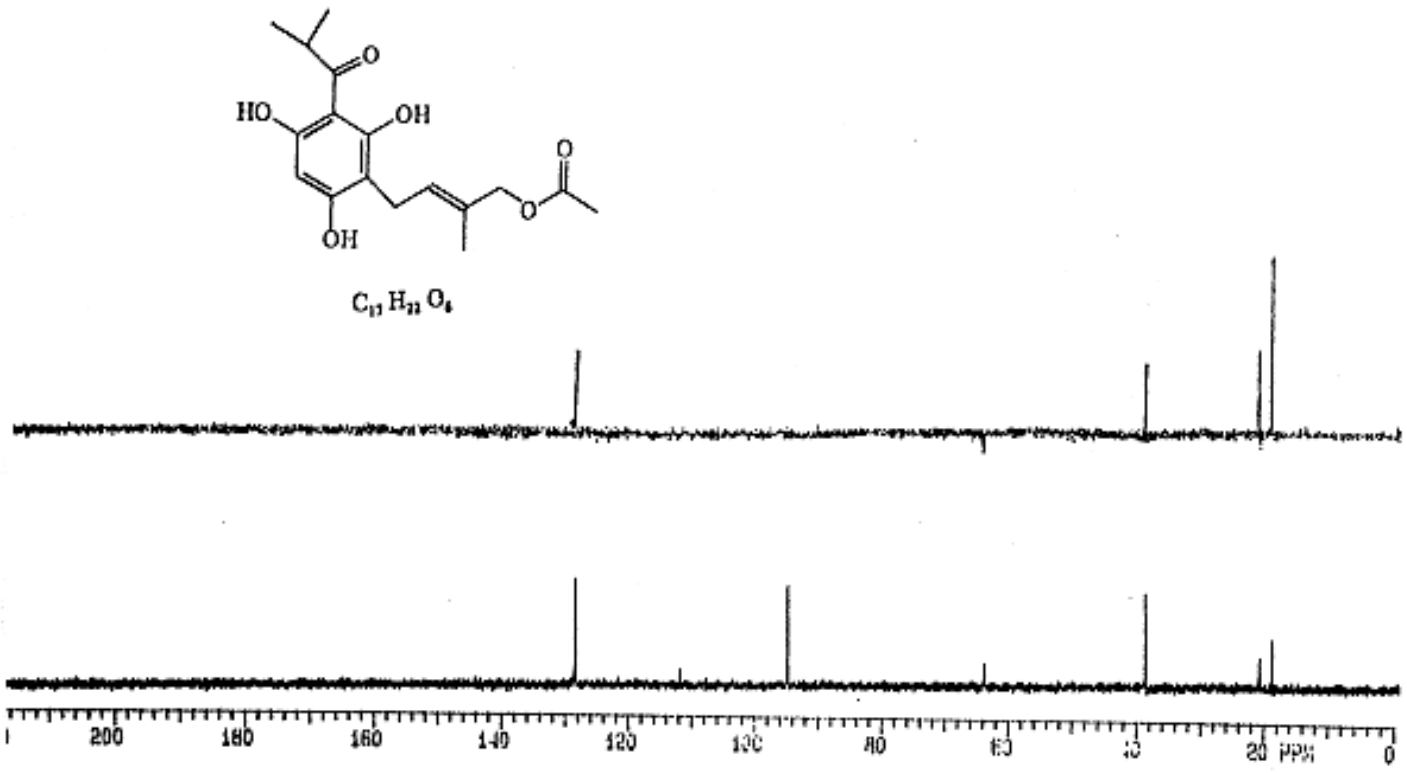

Figure 4.3: DEPT spectrum of caespitate in $CDCI_3$. MW. 322.14 $C_{17}H_{22}O_6$

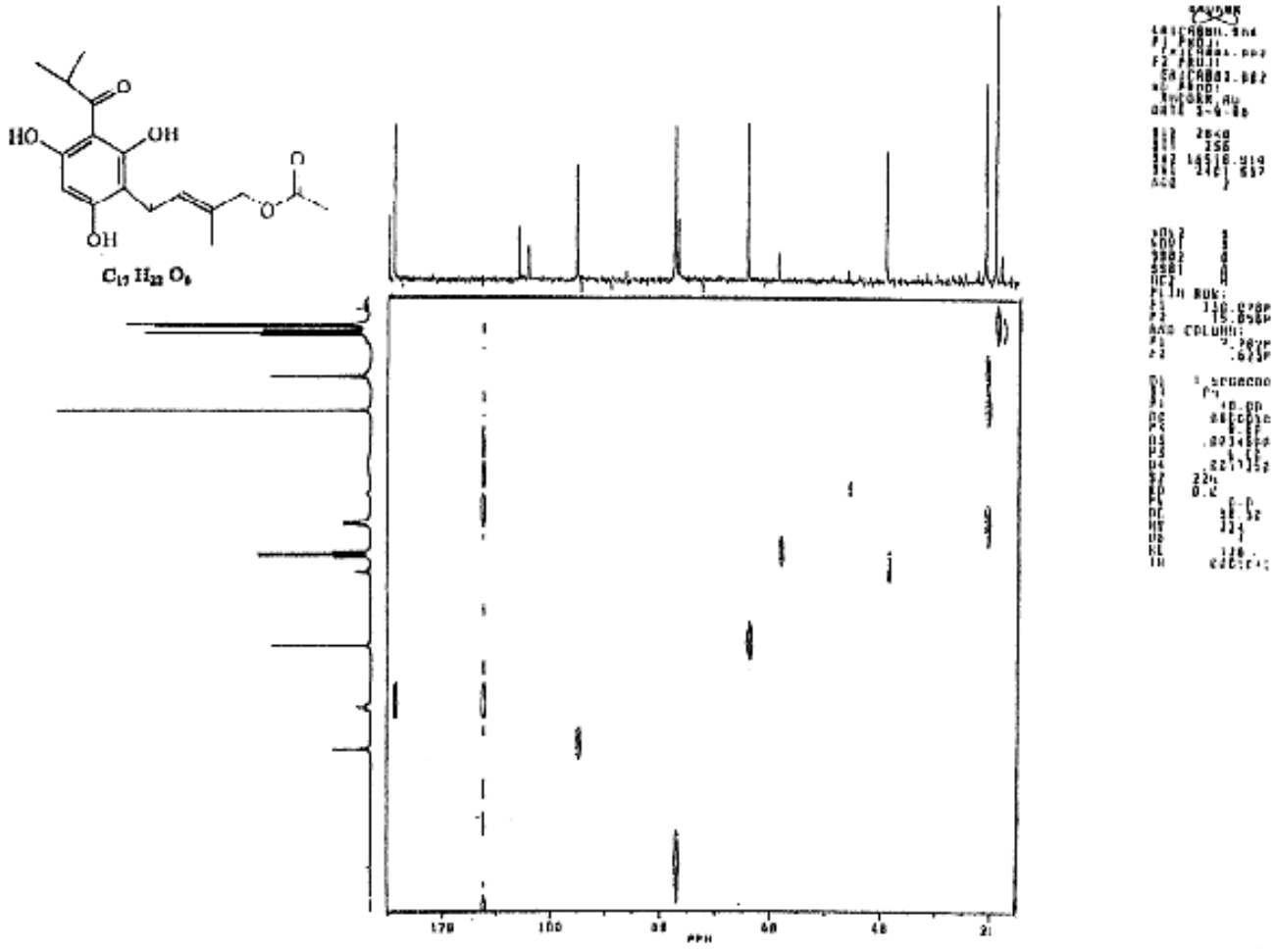

Fig 4.4: HETCOR of caespitate in $CDCI_3$. MW. 322.14 $C_{17}H_{22}O_6$

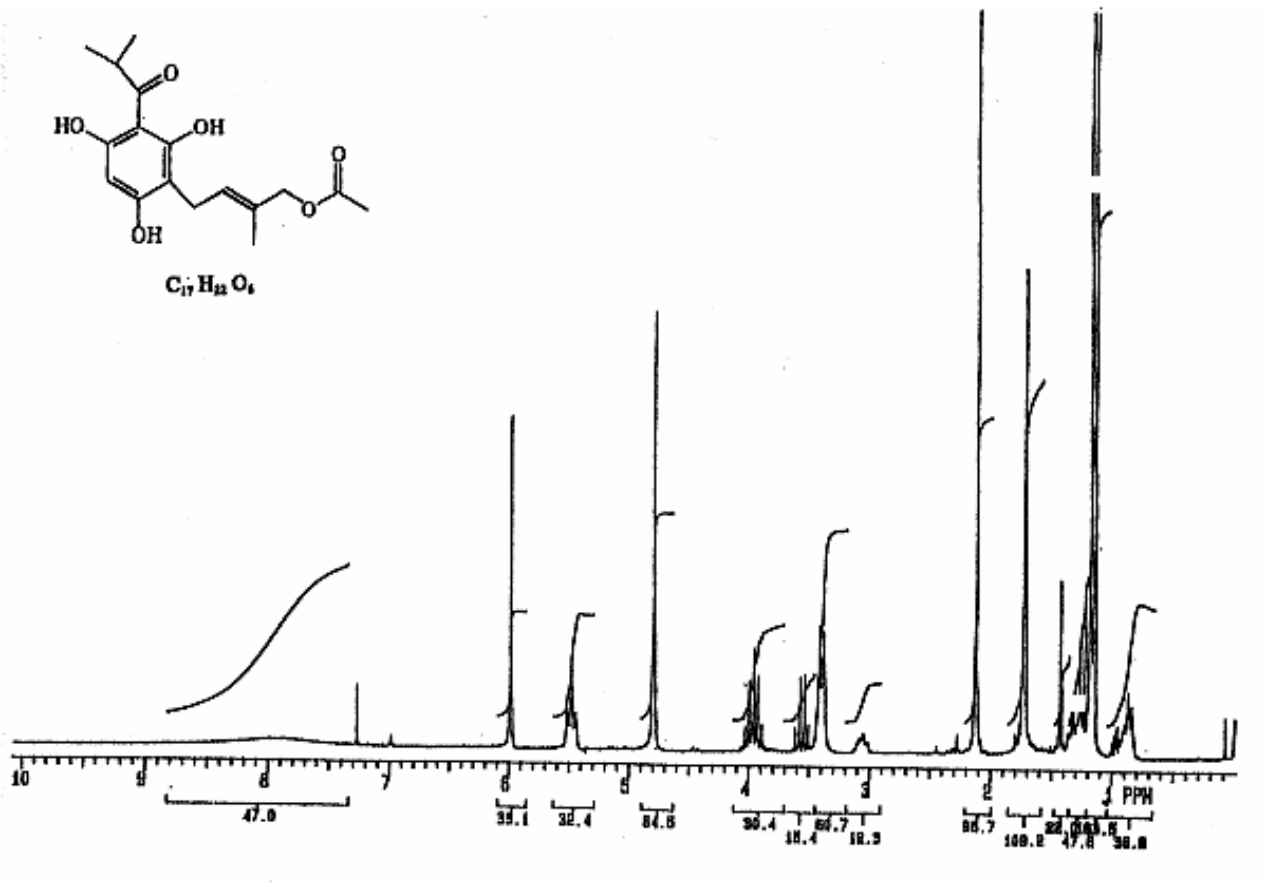

Figure 4.5: H of caespitate in $CDCI_3$ MW. 322.14 $C_{17}H_{22}O_6$

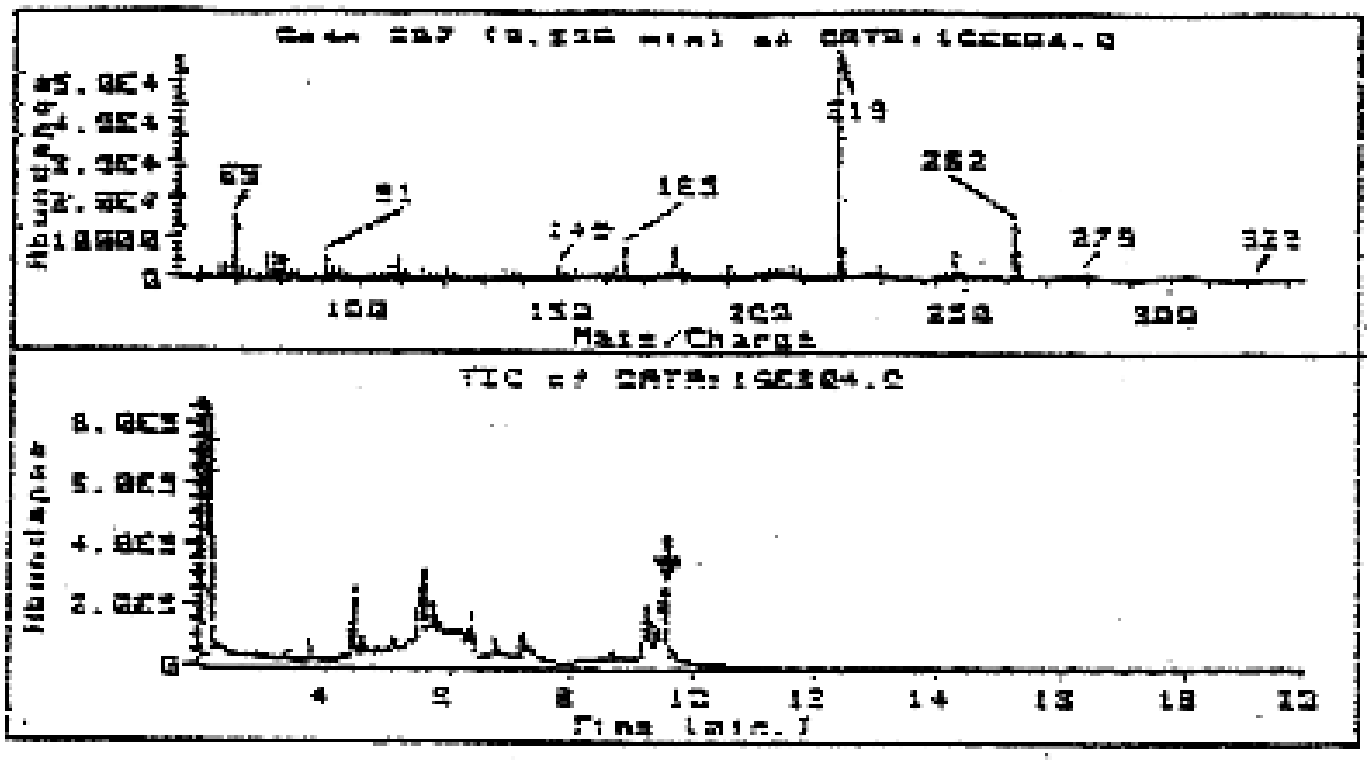

Figure 4.6 GCMS: TMS mass determination of caespitate. MW. 322.14 $C_{17}H_{22}O_6$

CHAPTER 5

CYTOTOXICITY OF CAESPITATE, A PHLOROGLUCINOL ISOLATED FROM *HELICHRYSUM CAESPITITIUM*

CHAPTER 5

CYTOTOXICITY OF CAESPITATE, A PHLOROGLUCINOL ISOLATED FROM *HELICHRYSUM CAESPITITIUM*

5.1 Introduction

Traditional medicine is widely used in South Africa with traditional healers treating over 75% of patients (Hutchings *et al.*, 1996). A decoction of *Helichrysum caespititium* (DC.) Harv., (*impepo* (Zulu), *seledu-sa-phooko* (South Sotho), *moriri-wa-naha* (Kwena), and *sephanyane* (Kgatla), is drunk in the treatment of, broncho-pneumonial diseases, sexually transmitted diseases, tuberculosis, ulceration and is used as a styptic wound dressing (Phillips, 1917; Watt and Breyer-Brandwijk, 1962). The importance of this indigenous herbal knowledge is therefore recognized and the use of the plant has been well documented (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996), however, caespitate, a new phloroglucinol (2-methyl-4-[2',4',6'-trihydroxy-3'-(2-methylpropanoyl) phenyl] but-2-enyl acetate) isolated from *H. caespititium* has not been analysed for cytotoxicity (Mathekga *et al.*, 2000). We demonstrated earlier that this plant exhibits significant potency against human bacterial and fungal pathogens (Chapters 2 and 3). Its antimicrobial spectrum is comparatively limited but its potency is reasonable.

It is interesting to note that *H. caespititium* contains more than one highly potent antimicrobial agent (Dekker *et al.*, 1983 and Mathekga *et al.*, 2000). The antimicrobial spectrum of caespitate (Table 5.1) seems to be limited to Gram-positive bacteria only but also, showed activity against fungi (Table 5.2) tested in this study. The information gained from studying caespitate might lead to the development and understanding of new molecular interactions, which in turn may lead to the development of new classes of therapeutic agents. With the rapid explosion of new molecular targets available for drug discovery and advances in automated high throughput screening technologies, there has been a dramatic increase in interest by the pharmaceutical and biotechnology industries in

sources of molecular diversity. The resources of the genus *Helichrysum* might play an important role in the discovery of novel lead structures for many of these new targets. The purpose of this study was to investigate the cytotoxicity of caespitate and to relate it to its folkloric use. The cytotoxicity and efficacy of caespitate was determined microscopically on vervet monkey kidney cells.

5.2. Materials and Methods

5.2.1 Plant material

Shoots of *H. caespititium* were collected from the Drakensberg in the Mont-aux-Sources area in QwaQwa, South Africa during August 1998. A voucher specimen (AM11) of the species was deposited in the herbarium of the National Botanical Institute of South Africa in Pretoria.

5.2.2 Preparation of extract

Air dried (80 g) plant material was immersed in acetone and shaken on a rotary shaker for 5 minutes without homogenizing it. The extract was filtered and concentrated to dryness under reduced pressure at 40 ^{0}C with a rotary evaporator. After determining the yield (8.4 g (w/w)), the extract was stored at 4 ^{0}C.

5.2.3 Preparation of caespitate

The antimicrobial activity guided fractionation of the acetone extract (Chapter 4) of the aerial parts of *H. caespititium* led to the isolation of the new phloroglucinol derivative, caespitate (2-methyl-4-[2',4',6'-trihydroxy-3'-(2-methylpropanoyl)-phenyl]but-2-enyl acetate). Caespitate was serially diluted in acetone to obtain a concentration range of 100.0 to 0.5 µg/ml.

5.2.4 Cytotoxicity

5.2.4.1 Stock solution

A stock solution of caespitate (60 mg/ml) was prepared in cell culture tested dimethyl sulfoxide (DMSO) purchased from Sigma.

5.2.4.2 Cell culture

Microtitres with vervet monkey kidney cells were prepared for testing caespitate cytotoxicity and cells were examined microscopically for pre-experimental infection and vitality. The multilayer cells in the tissue were rinsed three times with phosphate buffer saline (PBS) followed by 3 ml Trypsin EDTA. This facilitates dislodging cells adhering to the plate's bottom surface. The cell plates were incubated for 5 minutes at 37^{o}C. Eight ml of fresh maintenance medium (MM) were added to the tissue culture.

5.2.4.3 *In vitro* cytotoxicity assay

Determination of the ID_{50} of caespitate was carried out according to Geran *et al.,* (1972). Cell survival was measured microscopically (Grist *et al*., 1979) instead of using the methyl tetrazolium bromide (MTT) method described by Mosmann (1983) and Scudiero *et al.,* (1988). Briefly stated, cells in the exponential growth phase were harvested and centrifuged at 3000 x g for 5 minutes, re-suspended in Eagle's minimum essential medium (MEM) to 1.0 x 10^5 cells/ml and 180 ml of the cell culture was added to each well of a flat bottom 96 well plate with a multichannel pipette. After 24 hours incubation in a 5 % CO_2 humidified incubator at 37^{o}C, 20 ml of the test agent was added in 6 replicates to give final concentrations of 100.0, 50.0, 25.0, 12.5, 6.0, 3.0, 1.5, 0.7, 0.3, 0.1 and 0.05 mg/ml. The concentration of DMSO used to dissolve the compound was adjusted to 100.0 mg/ml and this concentration of solvent was used in control wells. The compound was tested for cytotoxicity by exposing the mono layers to the compound in MM at 37^{o}C. The cells were monitored over a period of six days for cytotoxicity effects. Mono layers of cells exposed to MM without the addition of the compound were used as controls. Cells were examined daily by light microscopy for the appearance of cytotoxicity. The ID_{50} was expressed as the compound concentration in mg/ml that caused a 50 % inhibition of growth compared with controls.

5.3 Results

The maximum non-toxic concentration of caespitate on the vervet kidney monkey cell cultures was 50 mg/ml. At this concentration, the cells did not exhibit altered morphology or growth characteristics indicative of cytotoxic effects. The cytotoxicity results from caespitate are shown in Table 5.1

5.4 Discussion

H. caespititium has long been used as a food spice and medicine by the Free State Basothos and other indigenous people and is therefore, probably not toxic to humans. This probably explains the continued use of the extract from this plant by the indigenous people of South Africa against a number of infections for generations.

Table 5.1 Cytotoxicity effects of caespitate on vervet monkey kidney cells. Each value represents the mean of six replicates.

TREATMENT (g/ml)	**TOXICITY**		
	DAY 3	**DAY 4**	**DAY 6**
Control (MEM medium)	100% growth	100% growth	100% growth
100.0 g/ml DMSO	100% growth	100% growth	100% growth
100.0 g/ml caespitate	100% toxic	100% toxic	100% toxic
50.0 g/ml caespitate	No toxicity	No toxicity	No toxicity
25.0 g/ml caespitate	No toxicity	No toxicity	No toxicity
12.5 g/ml caespitate	No toxicity	No toxicity	No toxicity
6.0 g/ml caespitate	No toxicity	No toxicity	No toxicity
3.0 g/ml caespitate	No toxicity	No toxicity	No toxicity
1.5 g/ml caespitate	No toxicity	No toxicity	No toxicity
0.7 g/ml caespitate	No toxicity	No toxicity	No toxicity
0.3 g/ml caespitate	No toxicity	No toxicity	No toxicity
0,1 g/ml caespitate	No toxicity	No toxicity	No toxicity

5.5 Conclusion

The cytotoxicity results obtained in these tests suggest that further studies to investigate the potential for anti-cancer activity of *H. caespititium* may be useful as antimicrobial compounds which exhibit non-toxicity at concentrations below 8.0 µg/ml have potential as anti-cancer agents (Balick ,1990). The antimicrobial and non-toxicity properties of *H. caespititium* as detected in this *in vitro* study may partly explain the popularity of this plant in folk medicine as a remedy for many diseases and skin infections.

Traditional medicine potions are mostly obtained from natural products. The advantage in some cases is that the concentration of active principles in the plant is usually small and it is further diluted when a decoction for traditional medicine is prepared. As a result, it is concluded that such work generates a gratifying promise of novel lead structures and the possibility of finding additional agents for human or agricultural use based upon the antimicrobial and cytotoxicity of caespitate. Additional scientific investigation in this field awaiting discovery, is recommended.

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CHAPTER 6

SYNERGISTIC ANTIBACTERIAL EFFECT OF CAESPITATE AND CAESPITIN, TWO PHLOROGLUCINOLS ISOLATED FROM *HELICHRYSUM CAESPITITIUM*

CHAPTER 6

SYNERGISTIC ANTIBACTERIAL EFFECT OF CAESPITATE AND CAESPITIN, TWO PHLOROGLUCINOLS ISOLATED FROM *HELICHRYSUM CAESPITITIUM*

6.1 Introduction

After a brief review of the present status of the field of antibiotics in general and the isolated compounds in particular, this chapter focuses upon the synergistic effects of the compounds isolated from *Helichrysum caespititium* (DC.) Harv. Antimicrobial tests demonstrated that caespitin (Dekker *et al*., 1983) and caespitate (Mathekga *et al.*, 2000) exhibit significant potency against human bacterial and fungal pathogens (Chapter 4). Caespitin and caespitate are readily obtainable by bioassay-directed isolation techniques. Their antimicrobial spectra are comparatively narrow and their potency is reasonable. Novel lead structures and the possibility of finding additional agents for human or agricultural use based upon these agents, is possible.

It is interesting to note that *H. caespititium* contains more than one antimicrobial agent. Caespitin and caespitate were both active against Gram-positive bacteria only at similar concentrations. In this study we investigated the synergistic effect of caespitate and caespitin on Gram-positive and Gram-negative bacteria.

6.2 Materials and Methods

6.2.1. Plant material.

Shoots of *H. caespititium* were collected from the Drakensberg in the Mont-aux-Sources area in QwaQwa, South Africa during August 1998. A voucher specimen (AM11) of the species was

deposited in the herbarium of the National Botanical Institute of South Africa in Pretoria.

6.2.2 Preparation of extract

Air dried (80 g) plant material was immersed in acetone and shaken on a rotary shaker for 5 minutes without homogenizing it. The extract was filtered and concentrated to dryness under reduced pressure at 40 ^{0}C with a rotary evaporator. After determining the yield (8.4 g (w/w)), the extract was stored at 4 ^{0}C until antibacterial assays commenced.

6.3 Preparation of caespitate

6.3.1 Isolation and identification of caespitate .

The crude acetone extract of *H. caespititium* was initially subjected to preparative TLC in $CHCl_3$-EtOAc (1:1). The targeted band was recovered and rechromatographed by column chromatography with 100% chloroform on silica gel 60. Direct antibacterial bioassays on TLC of the fractions collected indicated the presence of several antibacterial compounds in the extract. The fraction with the highest antibacterial activity was finally isolated in a pure form by HPLC in H_2O-EtOH (1:1) on a reverse phase Phenomenex column (250 x 4.60 mm; 5 µm). NMR analysis (DEPT, COSY and HETCOR spectra) were obtained using standard pulse sequences on a Varian 200 MHz spectrometer. Mass spectra were recorded on a Hewlett-Packard 5988 GC/MS instrument. High resolution mass spectra were obtained from a Kratos MS 80 RF double-focussing magnetic sector instrument.

6.3.2 Preparation of caespitate and caespitin solutions

Caespitin (obtained from Noristan Laboratories, South Africa) was previously isolated from *H. caespititium* (Dekker *et al.*, 1983) and shown to have antibacterial properties. Equal aliquots of caespitin and caespitate were well mixed and then serially diluted to give a concentration range of 0.1 to 0.001µg/ml in 2% acetone.

6.3.3 Antibacterial activity of caespitate and caespitin

The combined test solutions (sterilised by filtering through a 0.22 µm filter) were added to 5ml of sterilised nutrient agar in Petri dishes and swirled carefully before congealing. The organisms were streaked in radial patterns on agar plates (Mathekga and Meyer, 1998).

Plates were incubated at 37 ^{0}C in the dark and examined after 24 and 48 hours. Complete inhibition of growth was required for the extract to be declared bioactive. The controls consisted of Petri dishes containing only nutrient agar and others containing nutrient agar in 2% acetone. Each treatment was analyzed in triplicate.

6.4 Results

6.4.1 Antibacterial activity

Caespitate exhibited antimicrobial activity at a range of 5.0 to 0.5 mg/ml (Table 6.1) against Gram-positive bacteria only. The bioassay demonstrated that the combination of caespitate and caespitin not only maintained their original broad spectrum antibacterial activity against Gram-positive bacteria but that their synergistic effect enhanced activity down to a range of 0.1 to 0.05 mg/ ml. In addition, the growth of four Gram-negative bacteria, *E. cloacae, E. coli, K. pneumoniae* and *P. aeruginosa* were significantly inhibited. *Serratia marcescens* was not susceptible to the synergistic effect of caespitate and caespitin.

Table 6.1 Synergistic effect of the antibacterial activity of caespitin and caespitate isolated from *H. caespititium.*

		MIC [a]		
	Gram	caespitin	caespitate	caespitate and caespitin
Bacterial species	+/-	(µg/ml)	(µg/ml)	(µg/ml)
Bacillus cereus	+	1.0	0.5	0.05
B. pumilus	+	1.0	0.5	0.05
B. subtilis	+	1.0	0.5	0.05
Micrococcus kristinae	+	1.0	0.5	0.05
Staphylococcus aureus	+	1.0	0.5	0.05
Enterobacter cloacae	-	na[b]	na	0.10
Escherichia coli	-	na	na	0.10
Klebsiella pneumoniae	-	na	na	0.05
Pseudomonas aeruginosa	-	na	na	0.05
Serratia marcescens	-	na	na	na

a Minimum inhibitory concentration

b Not active

6. 5 Discussion.

The rationale for this enhanced effect may be based probably, on an increase in the permeability of the antibiotic through the plasma membrane by the combination of caespitate and caespitin (Kato *et al*., 1990). Hydrophobicity is not the sole determinant for the active stability of a membrane structure. It seems very likely, therefore, that many other molecular elements other than hydrophobicity are involved such as protein flexibility

(Kato *et al.*, 1985), surface charge (Adam *et al.*, 1971), conformational stability (Kato *et al.*, 1990, Song and Damodaran, 1987), solubility (Haling, 1981, Chobert *et al.*, 1988), and molecular size (Kato *et al*., 1990). Therefore, to enhance the antibacterial activity of caespitate, it was combined with caespitin. Here, the strategy is designing an effective anti Gram-negative agent to enable it to fuse into the outer membrane, an amphitropic compound approach. Amphitropic compounds are lipid-binding compounds, such as a-actinin and vinculin, that can traverse through the cell membrane reversibly (Burn, 1988). The combination treatment of caespitate and caespitin provides a simple functional molecule conjugate (Norden, *et al.*, 1979; Nakamura, *et al*., 1990, Masaki, *et al*., 1992), not necessarily target-specific, which can access the outer membrane lipopolysaccharide layer by linking the active molecule to a hydrophobic ligand to facilitate its delivery to the site of action (cytoplasmic membrane) to perform its task (Ibrahim *et al.*, 1991) .

Of the many different types of proteins found capable of passing through the outer membrane of *E. coli*, for example, all appear to possess a hydrophobic sequence (e.g. hemolysin) or contain a covalently bound fatty acid (amphitropic proteins such as a-actinin and vinculin) (Kato *et al.*, 1990). It appears, therefore, that when the synergised molecules are fused into the cell membrane, the positively charged molecules caespitate and caespitin come into contact with or approached the negatively charged phospholipid bilayer of the inner membrane, most probably via zones of adhesion between the outer and inner membranes (Adam *et al.*, 1971). As a result there may be an electrostatic interaction between the positively charged groups of the moderately modified caespitate and caespitin and negatively charged head groups of phospholipids, thus leading to localization of caespitate and caespitin in the vicinity of the site of action (Kato *et al.*, 1990). Therefore, it is rational to expect the results obtained in this study as they are consistent with similar findings by other researchers on synergistic effects.

6. 6 Conclusion

The high activity of crude extracts of the shoots of *H. caespititium* (Chapters 2 and 3) is attributable to the presence of at least two phenolic components, caespitate and caespitin . They are individually less potent than their combined synergistic effect. Combining caespitin and caespitate enhanced the antibacterial activity. This approach heralds fascinating opportunities for engineering potentially active compounds such as caespitate and caespitin that are lethal to Gram-negative and Gram-positive bacteria.

The antimicrobial enhancement (synergistic effect) and non-toxicity of *H. caespititium* as detected in this *in vitro* study may partly explain the popularity of this plant in folk medicine as a remedy for many diseases and skin infections.

Traditional medicine is a potential source of new drugs, a source of cheap starting products for the synthesis of known drugs, as has recently been shown with drugs such as reserpine from *Rauwolfia* species, vinblastine from *Catharanthus reus* and the discovery of a contraceptive in the Zoapatle (*Montanoa tomentosa* (Hahn *et al.*, 1981)).

The traditional practitioner’s potions often come in multi-component preparations (similar to multi-drug therapy in TB treatment) aimed at healing several ailments simultaneously, probably simulating the results obtained by the synergistic effect of caespitin and caespitate and a possible explanation of the efficacy of the traditional practitioner's potion. However, further study is needed to elucidate the mechanism underlying the behaviour of caespitin and caespitate. The combinations made by traditional healers pose an additional scientific investigation challenge in the field of drug discovery.

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CHAPTER 7

TRICHOME MORPHOLOGY AND ULTRASTRUCTURE OF *HELICHRYSUM CAESPITITIUM*

CHAPTER 7

TRICHOME MORPHOLOGY AND ULTRASTRUCTURE OF *HELICHRYSUM CAESPITITIUM.*

7.1 Introduction

Trichomes are appendages of diverse form, structure and function (Upfold, 1962). Despite the variety of systems that exist for the classification of trichome types, they are ultimately classified as being either glandular with a secretory function or covering hairs (non-glandular) without a secretory function (Cutter, 1978). Developmental and structural studies of trichomes can shed light on the nature of the secreted material and the functional significance of the glands (Franceshi and Giaquinta, 1983). The development of trichomes from the epidermis results from differential enlargement and subsequent divisions of epidermal cells and their derivatives (Carlquis, 1958). In his classification of different trichome types, Upfold (1962) used the plane of division of the initial epidermal cell as a distinctive characteristic. Where more than one trichome type occurs in a single species, each apparently has a special development pathway, as the different structural forms are not one type which is arrested at different stages in a common pathway (Hammond and Mahlberg, 1973). The morphology and ultrastructure of these trichome features have not been reported in *Helichrysum caespititium* (DC.) Harv.

The source of epicuticular secondary metabolites has been attributed to glandular trichomes (Wollenweber, 1984). Production of epicuticular phloroglucinols with antimicrobial properties have been eported in other species of *Helichrysum* (Tomas-Barberan *et al.,* 1988; 1990; Tomas- Lorente *et al.,* 1989; Dekker *et al.,* 1983; Mathekga *et al.,* 2000). Some of these species are in common use in African traditional medicine for the treatment of various ailments. For example, *H. aureonitens* is used against herpes simplex virus type 1 (Meyer *et al*., 1996); *H. melanacme* against drug resistant TB (Lall and Meyer, 1998). Secretions of *H. caespititium* are believed to be effective against

broncho-pneumonial diseases, sexually transmitted diseases, tuberculosis, ulceration and is used as a styptic wound dressing (Phillips, 1917; Watt and Breyer-Brandwijk, 1962).

H. caespititium is usually sold on local markets in a fragmentary state. This situation renders this crude drugs highly susceptible to adulteration and substitution. Thus, wrong plant material could easily be used in many herbal preparations with potentially dangerous consequences. The problem of accurate identification and dearth of reliable information about the medicinal plant species found in a region whose flora is incompletely known, have hampered the optimal utilization of these crude drugs and diminished their general acceptability.

In this investigation we examined the morphology and ultrastructure of foliar appendages of *H. caespititium* with a transmission (TEM) and scanning (SEM) electron microscopy. The aim is to evaluate the epicuticular morphology and ultrastructural features of the leaf for reliable taxonomic characters that may facilitate an accurate and rapid identification of the plant sample, and to relate our observation to their, possible functional role in the production of antimicrobial compounds.

7.2 Materials and Methods

7.2.1 Plant material

Plants used in this study were collected in the Mont-Aux-Sources area in QwaQwa, South Africa during August 1998. A voucher specimen (AM11) of the species was deposited in the herbarium of the National Botanical Institute of South Africa in Pretoria. *H. caespititium* 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 and clasping branches. Margins are revolute with both surfaces and stems enveloped in a silver 'tissue-paper-like' indumentum, breaking down to wool. The leaves are dotted with orange glands.

7.2.2 Transmission electron microscopy

Fresh leaves were sectioned into tip, middle and base portions and immediately fixed in a 2.5% glutaraldehyde: 2% formaldehyde (50:50) mixture in 0.075 M phosphate buffer (pH 7.4), on the collection site. Leaf sections were rinsed three times in the laboratory in 0.075 M buffer and post-fixed in 1% aqueous osmium tetroxide for four hours. The leaf material was then rinsed three times for 10 minutes per rinse in distilled water, and then dehydrated in an ethanol series (50% x 3, 70% x 3, 90% x 3 and 100% x3), for 15 minutes per rinse.

7.2.3 Scanning electron microscopy

Cross and longitudinal sections of leaves dehydrated in a graded ethanol series were dried to a critical point with a Bio-Rad E3000 critical point drying apparatus for 24 hours to allow for the substitution of ethanol with CO_2. The dried material was mounted, secured by a double-sided adhesive tape (Figure 2A), and carefully examined before suitably representative parts were selected for photography. Each sample was photographed at a magnification of x 750 to reveal the general surface micromorphology and x2500 to show details. Samples were examined with a JEOL 840 SEM at 5kV

7. 3 Results

Abaxial and adaxial surfaces as well as cross and longitudinal sections of *H. caespititium* were investigated . Representative scanning electron and transmission micrographs of leaf sections are shown in Figures 7.1 to 7.3.

Observations with the SEM revealed the presence of two types of trichomes. The non-secreting type are abundant and responsible for the silvery 'tissue-paper-like' indumentum covering aerial shoot surfaces. Secreting hairs are club-shaped orange glandular structure of variable size and density found scattered on both surfaces of the leaf. The cuticle is designed to keep water and solutes in, but to keep invaders out. Light microcopy revealed

that the glandular trichomes contain typical secretory cell organelles, including, numerous endoplasmic reticulum, golgi bodies, scattered mitochondria, plastids, ribosomes, and a dense cytoplasm.

The indumentum of long non-glandular trichomes forms a dense covering that completely obscures the epidermal surface and characters (Figure 7.1A-E and G) of the lamina. The non-glandular hairs consists of uniseriate cells displaying a single morphological form (Figure 7. 1A-E and G-H)whereas mature non-glandular trichomes consist of four cells, namely, a base and two stalk living cells and a long dead head cell (Figure 7.1E, 7.2H). A secreting trichome consists of four cells, namely, a base and two stalk cells and a dome shaped secreting head cell. The base and two stalk cells are characterized by dense cytoplasm, numerous organelles and scattered vacuoles, whereas the dome-shaped apical cell is in addition visibly highly vacuolated 7.3E-G. Patterns of variation in abaxial and adaxial epidermal characters of *H. caespititium* cannot be studied exclusively by LM observations for finer details without the aid of clearing and magnifying devices, because of the dense, protective indumentum

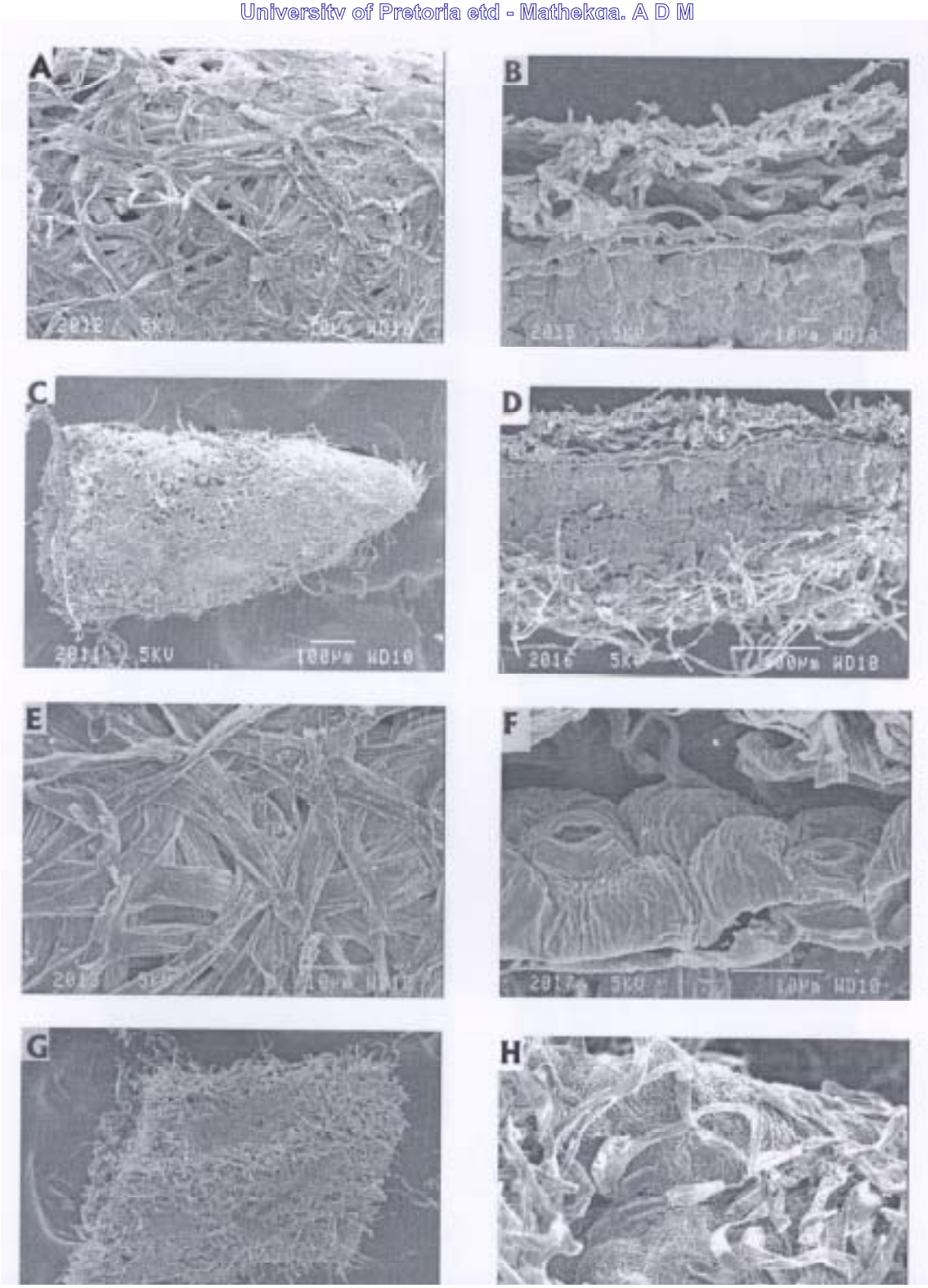

Figure 7.1 Electron micrographs of leaf epidermal cells of H. caespititium. A. Adaxial indumentum of non-glandular trichomes. B. Cross-section of leaf revealing palisade and mesophyl tissues. C. Leaf tip in indumentum. D. Longitudinal section revealing adaxial and abaxial mesophyls. E. Abaxial indumentum. F. Striated cuticle. (Note that the cuticle is devoid of wax covering and the elevated stomata). G. Middle section of leaf in indumentum. H. Partially cleared abaxial epidermis.

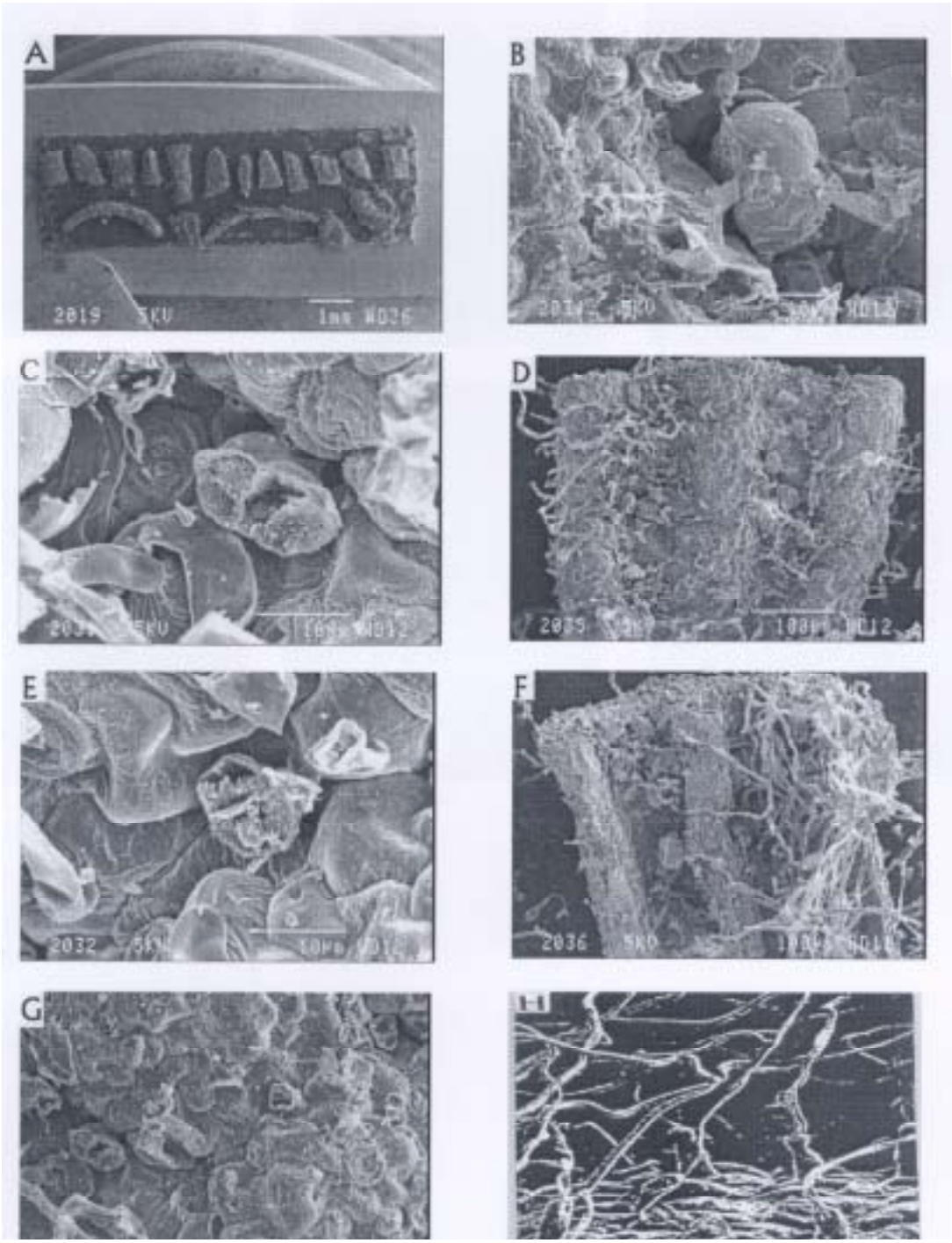

Figure 7.2 SEM dried mounted leaf sections of *H. caespititium*. A. Cross, epidermal and longitudinal sections of tip, middle and base leaf parts mounted for examination. B. Revolute leaf margins and midrib of leaf. C. Abaxial leaf surface revealing bifid nature of trichomes. D. Leaf revealing relationship between revolute margins, glandular trichomes and midrib. E. Torn cuticle subtends stalked basal cells. F. Sparsely distributed trichomes. G. Base cells enchored in epidermal cells. H. High magnification of abaxial surface revealing the morphology of the non-glandular trichomes.

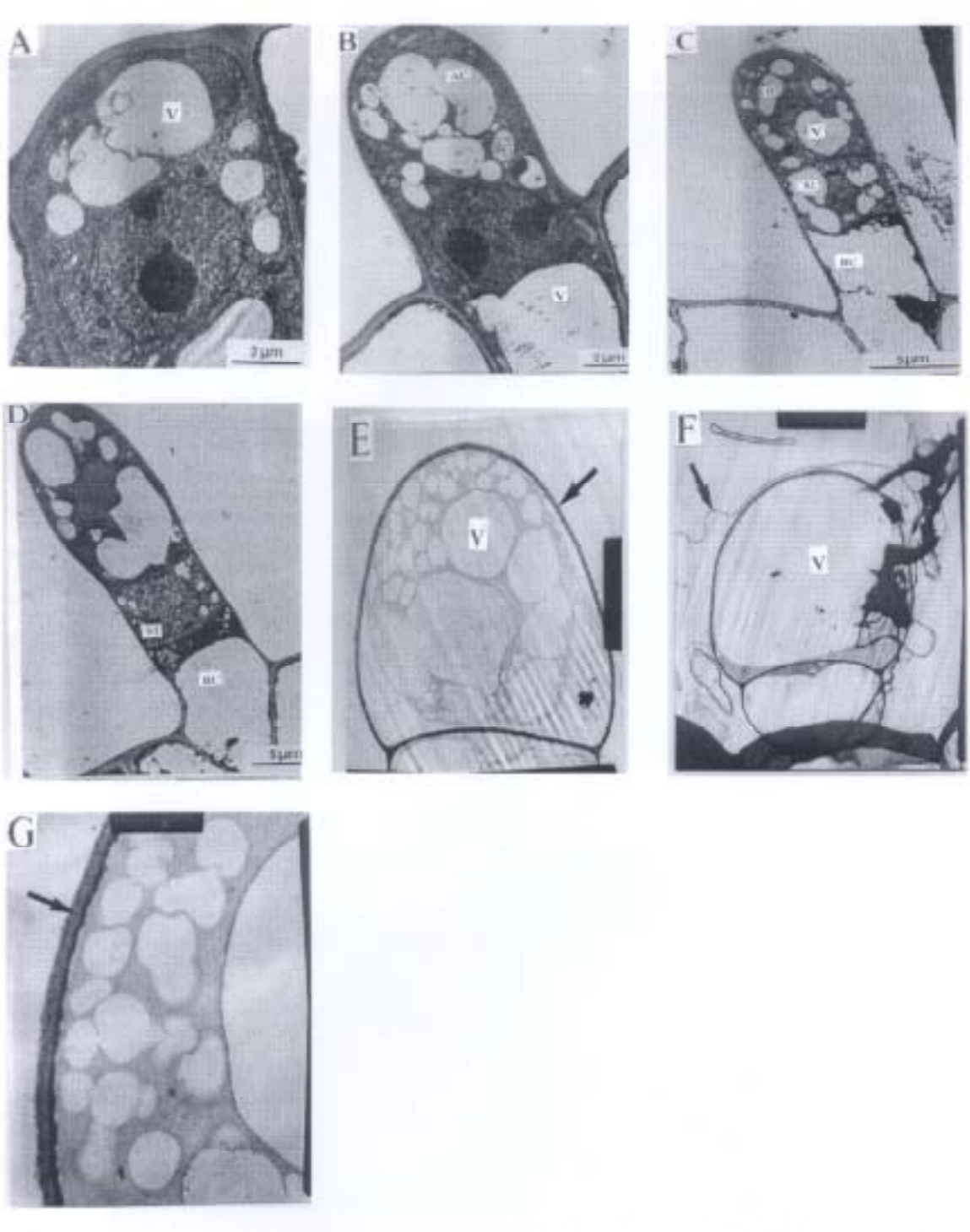

Figure 7.3 Transmission electron micrographs of various stages in the development of the glandular hairs of *H. caespititium.* A. Unicellular stage: papillate outgrowth of epidermal cell representing glandular hair initial. Note that the cytoplasm is dense with an apparent lack of chloroplasts. B. Two-celled glandular hair stage, consisting of an apical cell (AC) and a basal cell (BC). C. Three-celled glandular hair stage, consisting of a head (H), stalk cell (SC) and a

vacuolated, elongated basal cell (BC). D. Four-celled glandular hair stage, consisting of a head (H), two stalk cells (S1 and S2) and a vacuolated, elongated basal cell (BC). E-G. Light micrographs of longitudinal sections of trichomes of *H. caespititium*. E. Oval shaped head. Note smooth cuticle (arrow). F. Globular head. Note that the cuticle has raptured to release a secretory product (arrows). G. Initiation of cell wall and cuticle prior to secretion (arrow).

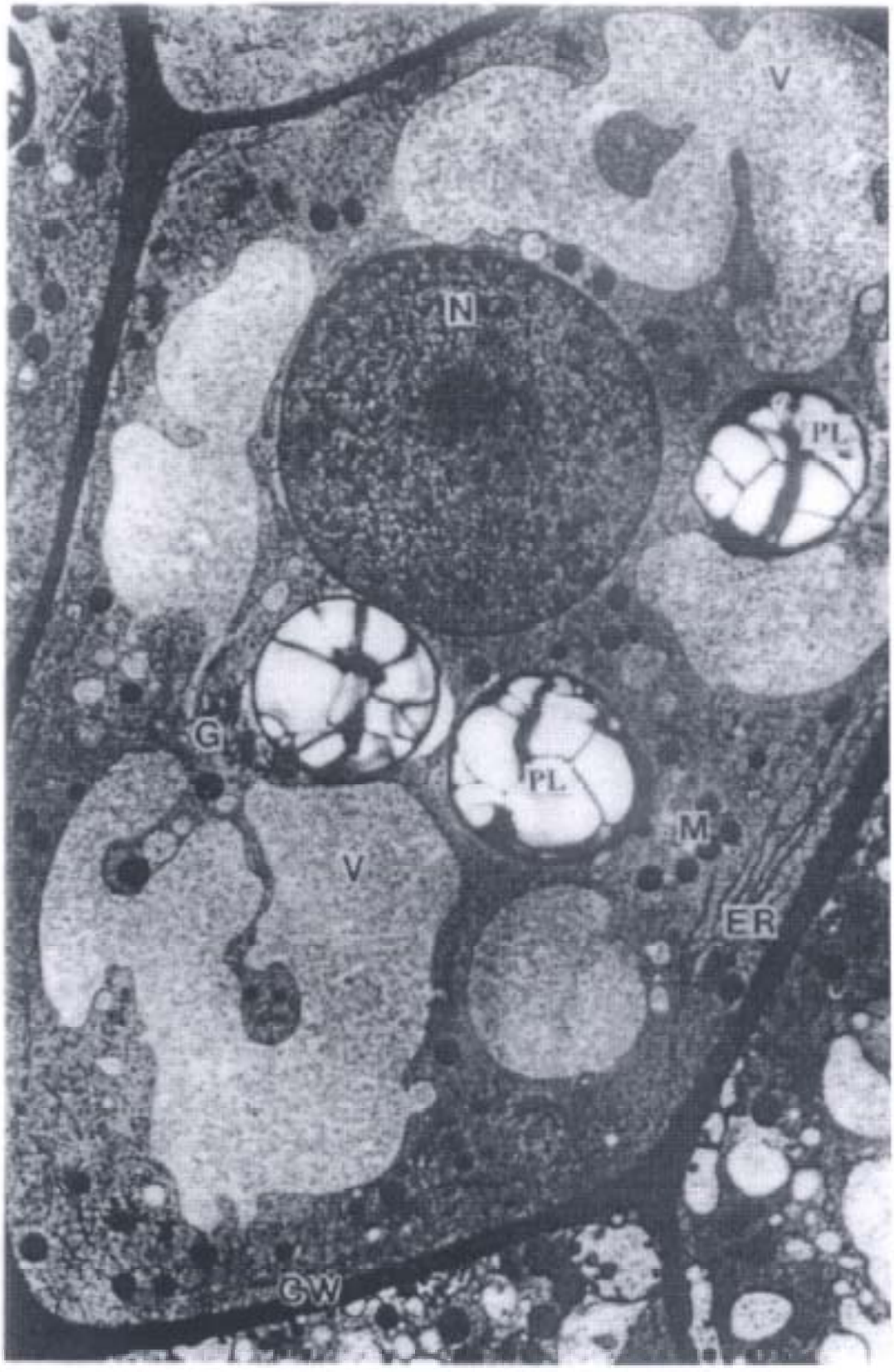

Figure 7.4 Ultrastructure of secretory trichome cell of *H. caespititium.* Cross section of secreting cell. Scale bar 2.5 m. CW= cell wall; ER= endoplasmic reticulum (part of endomembrane system). Note that the proximity of the ER to the cell wall may probably serve to export secretory products to neighbouring cells or secretory cavity; GA= Golgi apparatus; M= mitochondria; N= large nucleus with nucleolus; PL= degenerated plastid; V= vacuole.

A carefully cleaned (de-indumented) abaxial and adaxial leaf surface reveals a striated cuticle devoid of a wax covering. Epidermal cells are isodiametric and polygonal (Figure 7.1F). Glandular trichomes are bifid (Figure 7.2C, 2E and 2G) and glabrous (Figure 7.2 B, 2D and 2F). In this species, trichomes are sparsely distributed between the midrib and revolute margins (Figure 7.2D and 2F). Single guard-cell stomata occur in *H. caespititium* (Figure 7.1F). Stomata are limited to the lower surface. Each stoma has a thick and prominent outer stomatal ledge. Stomata are superficial or slightly raised, with narrow but long cuticular rims (Figure 7.1F). Stomatal frequence, size, and numbers of cells per unit area were not considered in this study. Adaxial anticlinal cell walls consist of irregular ridges of varying height and thickness (Figure 7.1 B and D). Periclinal walls are slightly concave (Figures 7.1 B and D). Wax was not found on the leaf surface. Abaxial cell boundaries are indicated by shallow grooves of varying width and depth (Figure 7.1F). The leaf epidermis is characterized by distinct oriented striae (Figures 7.1F and H), an indication of no wax on the leaf surface. Non-secretory trichomes are unicellular and usually taper gradually from base to apex (Figure 7.2C, 2E and 2G). Cells eventually break down to wool forming the dense indumentum. (Figures 7.1A-E and G-H). No secretory pores were observed in the cuticle of the secreting trichomes head cell (Figure 7.3E-G) Ultrastructure of secreting trichomes reveals the presence and concentration of mitochondria, endoplasmic reticulum, golgi bodies, vacuoles, plastids, a comparatively

large nucleus with prominent nucleolus, a dense cytoplasm and the other usual cell components (Figure 7.4).

7. 4. Discussion

The glandular hairs originate from papillate outgrowths of a single epidermal cell with a relatively large nucleus (Figure 7.3A). This epidermal cell is delimited from the other epidermal cells by its dense cytoplasm, and from the stoma guard cells, which also possess a dense cytoplasm, by the apparent absence of chloroplasts. The initial glandular hair cell elongates markedly and polarization into apical and basal parts occurs by vacuolization of the basal part of the cell (Figure 7.3B). The first cell division is transverse and gives rise to a vacuolated basal cell and apical cell with dense cytoplasm (Figure 7.3B). The basal cell elongate and does not take part in any further cell division so that the head and two stalk cells develop from the apical cell (Figure 7.3C). A transverse division of the apical cell gives rise to a four celled glandular hair consisting of two stalk cells and a head cell as well as a basal cell with dense cytoplasm (Figure 7.3D). The oval head cell enlarges to a dome shape while the basal cell elongates to anchor the trichome (Figure 7.3B-D)

Secretions of compounds probably occur from the young three-celled stage (Figure 7.3C). The young secreting glandular head cell has a smooth surface (Figure 7.3E) but with the accumulation of compounds in the subcuticular space between the cell wall and cuticle a protrusion is formed on top of the head cell (Figure 7.3E-H). No pores occur in the cuticle (Figure 7.3E-G) , therefore it ruptures to release the secretory product (Figure 7.3F). The accumulation process is then repeated since a new cuticle is apparently formed under the ruptured one (Figure 7.3F). Secretion of compounds thus occurs repeatedly in young and old three-celled glandular hairs.

Observations of the repeated secretion of compounds, despite the absence of pores in the cuticleand the rupture of the cuticle with secretions, led to the conclusion that a new cuticle must be formed repeatedly during secretion. In *Inula viscosa*, where lipids are produced continuously throughout the life of the hair, materials are secreted directly through the cell

wall, without the obstacle of the cuticle, once the cuticle has ruptured (Werker and Fahn, 1981). Evidence of formation of a second cuticle is also seen in Figure 3F and G. In the literature no evidence of similar observations in this species and possibly genus could be found and this phenomenon may have implications in the formation of the cell wall and cuticle.

The preceding observations indicate that microscopic features of the leaf may be useful in identifying plants or leaf fragments of *H. caespititium* which are otherwise indistinguishable. Taxa can be separated from each other by a combination of other characters such as a stomatal index, epidermal cell size and pubescence. The isodiametric cells of a species distinguishes it from other taxa of the genus and more importantly, from varieties to which it is most closely related (Olowokudejo, 1990). Trichome density in *H. caespititium* constitutes an important distinguishing feature. The absence of wax on the cuticle can be readily distinguished on by its striated pattern. An artificial identification key may be constructed based on observations made with the SEM and TEM allowing separation of taxa.

These distinguishing epidermal features may be of taxonomic significance because they are reasonably constant in this investigated species. The reliability of epidermal characteristics as taxonomic indicators varies from one group of plants to another. While Mueller (1966) and Cutler and Brandham (1977) have shown that these characters are under strong genetic control, and therefore little affected by the environment, Stace (1965) and Dilcher (1974) indicate that the characters vary according to environmental conditions. The tall order, however, lies in the scanning of the entire genus to verify or achieve this. It can however, be assumed that most of the genetically determined leaf differences are the result of natural selection and are related to a particular combination of (and perhaps trade-off between) various functions. Some properties of the leaf may even have been formed in response to evolutionary pressures different from those encountered by the extant plant (Kerstiens, 1996).

Trichome secretory cells characterized by a dense cytoplasm indicate a well-developed endomembrane system (ES) (Figure 7.4) (Campbell, 1999). The ES includes the nuclear envelope, endoplasmic reticulum (ER), Golgi apparatus (GA), mitochondria, various kinds of vacuoles and the plasma membrane related to the ER and other internal membranes (Figure 7.4). The ER manufactures membranes and performs many other biosynthetic functions.

Trichome secretory cells are rich in smooth ER (Figure 7.4), a standard feature that fits the functions of these cells (Campbell, 1999). The smooth ER functions in diverse metabolic processes, including synthesis of lipids, metabolism of carbohydrates and detoxification of drugs and poisons (Campbell, 1999). Enzymes of the smooth ER help detoxify drugs and poisons. Detoxification involves adding hydroxyl groups to drugs (*H. caespititium* has three hydroxyl groups), making them more soluble and easier to flush from cells (Campbell, 1999). The smooth ER is possibly the location for phloroglucinol synthesis in secretory trichomes.

Alcohol and many other drugs induce the proliferation of smooth ER and its associate detoxification enzymes (Campbell, 1999). This in turn increases tolerance to the drug, meaning that higher doses are required to achieve a particular effect, such as sedation. Also, because some of the detoxification enzymes have relatively broad action, the proliferation of smooth ER in response to one drug can increase tolerance to other drugs as well (broad spectrum). Hence drug abuse, for example, may decrease the effectiveness of certain antibiotics and other useful drugs.

Many types of specialized cells in trichomes secrete various proteins produced by ribosomes attached to the rough ER. Oligosaccharides are covalently bonded to carbohydrates in the rough ER. The ER membrane expands and can be transferred in the form of transport vesicles to other components of the ES (Campbell, 1999).

The GA (Figure 7.4) finishes, sorts and ships cell products. It is a centre of manufacturing, warehousing, sorting and shipping (Campbell, 1999). Products of the ER are modified, according to supply and demand, stored and then sent to other destinations. GA is specifically extensive in trichome cells. The GA has a distinct polarity (*cis* and *trans* faces), with the membranes of cisternae at opposite ends of a stack differing in thickness and molecular composition. The GA removes some sugar monomers and substitutes others, producing oligosaccharides and other compounds (Campbell, 1999).

H. caespititium is a mat-forming herb (Figure 1.1), subject to infection by a diversity of soil borne pathogenic viruses, bacteria and fungi that have the potential to damage tissues and even kill the plant. It seems to have a defence system that prevents infection and counters pathogens that do manage to infect the plant.

The first line of defence is the physical barrier of the plant's outer cover, the epidermis of the primary plant body. In addition, *H. caespititium* has a well-developed indumentum of dead non-secreting trichomes. The indumentum undoubtedly plays a significant role in the protection of the aerial parts of the plant. The first line of defence, however, is not impenetrable. Viruses, bacteria and the spores and hyphae of fungi can enter the plant through natural openings in the epidermis such as stomata. Once a pathogen invades, the plant mounts a chemical attack as a second line of defence that kills the pathogens and, prevents their spread from the site of infection. This second line of defence is enhanced by the plant's inherited ability to recognize certain pathogens (Harborne, 1992). This explains the narrow range in activity of some of the plant extracts.

7.5 Conclusion

H. caespititium's defence is based on both physical and chemical factors. Physical defence against herbivores are readily appreciated: tough epidermis, cuticular deposits and indumentum. Defence may be purely strategic, as in this case by growing close to the soil

and by vegetative reproduction under the soil surface. Nevertheless, chemical defences are also very important provided by toxins and repellent substances of one type or another within the plant itself. These toxins have had and continue to have a key role in protecting the plant from overgrazing. The ER is possibly the localization of phloroglucinol synthesis in the trichomes of *H. caespititium*. In the leaf and epidermal studies of *H. caespititium*, the most useful morphological and anatomical characters are: the indumentum, the cuticle, palisade ratio, pattern of anticlinal walls, density and type of trichomes. These characteristic features have been used in framing a key to the species to make it possible to identify the species either in the vegetative or fragmented state (Ogundipe, 1992).

The indumentum and cuticle have jointly a wider perspective of structure-function relationships, namely, to form a mechanical barrier against penetration by fungal hyphae and insect mouth-parts; reduce the uncontrolled loss of water and apoplastic damage; and protect the tissues from mechanical damage. In addition, they act as an accommodation compartment of exudate compounds on the leaf surface.

The structure of the cuticle suggests that its main function is to act as a medium for plant signals perceived from insects or microbes arriving on the leaf surface, hence the absence of wax . The range of chemical compounds located on the surface and found to be involved in processes such as host recognition and herbivore deterrence is bewildering. Some of them, such as natural pesticides and the antimicrobial compounds found in *H. caespititium* also may be exuded by the biseriate glandular trichomes. Probably the majority, however, reach the surface after diffusing across the cuticle (Kerstiens, 1996).

The wide diversity of functions fulfilled by the appendages of the leaf of *H. caespititium* indicate the possible structural links or overlaps between different functions including secretion of antimicrobial compounds. It is not surprising that not a single obvious conflict between the requirements of different functions, forcing a trade-off, could be identified.

The way the leaf and its appendages have evolved seems to be extraordinarily well suited to playing many different roles at a time.

Leaf morphological and ultrastructural trichome studies in the genus *Helichrysum* are rare or non-existent. An intensive morphological and ultrastructural study of the genus to construct a leaf key to the species which would make it possible to identify the species either vegetatively or in a
fragmented state is imperative for purposes of easy identification, classification and comparative studies.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

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GENERAL DISCUSSION AND CONCLUSION.

8.1 Screening of plants for bioactive agents

The screening of bioactive agents from plants is one of the most intensive areas of natural product research today, yet the field is far from exhausted. Sandberg and Bruhn (1979) reported that only around 10% of all plants had been investigated in detail for bioactive agents. For this reason alone it could be argued that further investigation on *Helichrysum* species is worthwhile.

In their computer analysis of data on worldwide research on plant derived drugs, Farnsworth and Bingle (1977) reported that 1650 compounds of novel structure were reported from plants in the year 1975 alone, while, 3077 compounds of known structure were also reported from other plants in the same year. In the same year, more than 400 patents were issued for substances isolated from higher plants. 325 compounds were reported for having one or more types of biological activity in systems having relevance to their potential use as drugs. Out of the 325 compounds, 93 were of novel structures reported for the first time and 232 had previously known structures.

Another reason for screening *Helichrysum* species for bioactive agents is that by isolating such agents it is possible to demonstrate that the reported medicinal activity of the plant is a reality. The fact that the antimicrobial activity of *H. caespititium* has been shown to be due to a particular chemical compound makes detailed pharmacological and other academic studies possible. It is not always possible, however, to isolate the bioactive agent in a plant and cases are known where attempts at such isolation have proved fruitless, even though an extract of the plant may be active, for example, a plant containing highly unstable compounds (Harborne,1992). Nevertheless, such attempts should continue as characterization of the active agent enables structure-related activity studies to be carried out, leading to the possible synthesis of a more potent drug with reduced toxicity. The

mode of action of the whole plant or plant part producing the biological effect can also be better investigated if the active principle is characterized. Often such studies lead to a better application of the drug, a better formulation into appropriate dosage forms, and may even lead to a better understanding of the disease itself.

The results of this study confirm that the co-occurrence of acidic phenolic hydroxyls and lipophilic residues is an important chemical feature for the expression of antifungal activity (Tomas-Barberan et al., 1990). The same requirements for a hydroxyl group and a degree of lipophilicity are also found in the simpler commercial phenolic fungicides such as p-pentyl phenol, dinitrophenol and pentachlorophenol (Laks and Pruner, 1989). These compounds exert their toxicity through the acidity of the hydroxyl group by uncoupling oxidative phosphorylation (Laks and Pruner, 1989). It is evident from Table 3.1 that over 96% of the species tested are worthy of closer investigation and isolation of their active compound.

Some drug registration bodies, like the Food and Drug Administration of the USA, the Dunlop Committee of the UK and the Republic of South Africa Patent Act of 1978, require the information on the structure(s) of the active agent(s) in a vegetable drug before it can be approved for general administration (Farnsworth, 1980). The pure compound is required to assess the possible lethal toxicity or side-effects (chronic and acute toxicity) of the drug. It was recently reported in the Los Angeles Times that medicines kill about 100 000 people each year:

> "More than 100,000 Americans are inadvertently killed every year by prescription drugs– one of the leading causes of death in the country. Some people die of drug reactions that are completely unexpected, the stuff of dramatic headlines and heavy lawsuits. But the majority of such deaths are preventable, the result of mistakes or confusion about dosage, dangerous drug interactions from mixing medications or known allergic reactions. Some patients, especially the elderly, die because their liver or

> kidneys are so weakened by other illnesses that they cannot effectively process new drugs....
>
> Over the past two years, the Food and Drug Administration has recalled five drugs and moved to re-evaluate several others, including the diabetes drug Rezulin, and the Parkinson's medication Tasmar, both of which have caused instances of liver failure."

Academically, the isolation of bioactive agents helps to provide chemotaxonomic evidence for the classification of genera or species, especially those whose classification on morphological grounds alone is unclear. When two plants are taxonomically identical but do not produce the same chemical constituents (either quantitatively or qualitatively) they are classified into chemical races. This difference can be due to genetic variation and is not merely because of a change in the environment or a difference in their geographical location (Soforowa, 1982; Harborne, 1992; Gillian *et al.*, 1998). For example, at least three chemical races of *Ocimum gratissimum* have been established from the major constituent of the volatile oil they produce: these are the thymol type, the eugenol type, and the citral type (Soforowa, 1982). The same plant may have been given different names by different taxonomists. For example: *Hunteria umbellata* Roxb = *Picralima nitida* Th. and Hel. Durr. and *Catharanthus roseus* G.Don = *Vinca rosea*. L.

The introduction of chemotaxonomy into plant systematics also complicates this problem further because of the repeated change of plant names or their families along with the change in name to the appropriate taxonomist. Phytochemists also often fail to confirm the identity of the plant they are investigating and the wrong plant names therefore sometimes get published along with their chemical findings. As a result, the chemical agent reported cannot be tallied in future with the biological activity claimed for the plant. This latter problem is generally disappearing, however, because it is now accepted practice to deposit a voucher specimen (with a voucher number) of any plant investigated in a recognized herbarium for future reference.

It is important to realize that the products of primary metabolism are usually innocuous, except for some toxic proteins, and were therefore of little interest to us investigating drug activity in *Helichrysum* species. Secondary metabolites such as alkaloids, acetophenones, phloroglucinols, flavonoids etc., are usually biologically active in animals and man. The isolation of bioactive agents from plants in general, can be grouped into two broadly fundamental procedures, namely:

(1) Biological screening, i.e. searching for a specific physiological effect.

(2) Phytochemical screening, i.e. randomly searching for bioactive compounds

8.2 Scope of research

Research in the field of Helichrysum species, was multi-disciplinary in approach and objectives were

set. Our objectives, among others were, to:

(a) Identify the agents in Helichrysum species which may possibly be used to produce useful drugs and,

(b) Quantify (mg/ml) the compounds and determine the MIC

The problems to be solved will depend on the overall objective to be achieved. It must be borne in mind, however, that in traditional medicine, medicinal plants are esteemed for their occult powers as well as their therapeutic effect.

Two major approaches were made in the investigation of the Helichrysum species studied:

(a) Performing a purely scientific exercise which may or may not result in the isolation of bioactive agents and,

(b) Recommending further investigations into the incorporation of useful and harmless Helichrysum species (pharmacological and therapeutical) into the modern health care system.

8.3 Acceptance of the hypotheses

The following hypotheses of this study may be accepted, namely, that:

(a) Crude extracts *Helichrysum* species exhibit significant antimicrobial activity and properties that support folkloric use in the control of bacterial and fungal related infections as broad spectrum agents. Secretions from leaf trichomes exhibit signicant antibacterial activity and properties that support folkloric use.

(b) Epicuticular extracts of *Helichrysum* species exhibit a relatively higher antimicrobial activity (minimum inhibition concentration (MIC)) compared to homogenized extracts. Antimicrobial compounds are probably sequestered in trichomes in *H. caespititium*. Shaken extracts proved to be more active than homogenized extracts.

(c) The previously studied *H. caespititium*, may in addition to the compound isolated (caespitin) by Dekker *et al*., (1983) contain novel constituents that can be discovered by bioassay directed fractionation methodology. This hypothesis can be accepted on the basis that a new phloroglucinol derivative, caespitate, was isolated in addition to the previously isolated caespitin.

(d) Mixtures of several closely related structures of the same class are produced by the plant and it is likely that synergism might occurs. A synergistic antibacterial bioassay demonstrated that the combination of caespitate and caespitin enhanced activity. The hypothesis can therefore, be accepted.

(e) 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. The hypothesis can be accepted as caespitate proved to be non-toxic at biologically active concentrations.

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CHAPTER 9

SUMMARY

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SUMMARY

ANTIMICROBIAL ACTIVITY OF *HELICHRYSUM* SPECIES AND THE ISOLATION OF A NEW PHLOROGLUCINOL FROM *HELICHRYSUM* CAESPITITIUM

by

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Department of Botany

Doctor of Philosophiae.

There are 500 *Helichrysum* (Asteraceae) species world wide of which 245 occur in South Africa.The South African species display great morphological diversity and are, therefore classifiedinto 30 groups (Hilliard, 1983). *Helichrysum* species have been reported for their antimicrobial activities (Rios *et al.*, 1988; Tomas-Barberan *et al.*, 1990; Tomas-Lorente *et al.*, 1989; Mathekga, 1998, Mathekga *et a*l., 2000). Not much information on the bioactivity of compounds isolated from these species is available. *In vitro* antimicrobial screening methods provide the required preliminary observations to select among crude plant extracts those with potentially useful properties for further chemical and pharmaceutical investigations. In this study we investigated the antimicrobial activities of crude acetone extracts (shaken and homogenized) of twenty-eight Helichrysum species on ten bacteria species and six fungal species.

A new phloroglucinol with significant antimicrobial properties was isolated by bioactivity guided fractionation from *Helichrysum caespititium*. The structure elucidation, conformation and stereochemistry of the new phloroglucinol, 2-methyl-4-[2',4',6'-trihydroxy-3'-(2-methylpropanoyl) phenyl] but-2-enyl acetate (caespitate), was established by high field NMR spectroscopic, crystallographic and MS data. The compound inhibited

growth of *Bacillus cereus, B. pumilus* and *Micrococcus kristinae* at the very low concentration of 0.5 µg /ml and *Staphylococcus aureus* at 5.0 µg/ml. Six fungi tested were similarly inhibited at low MICs: *Aspergillus flavus* and *A. niger* (1.0 µg /ml), *Cladosporium cladosporioides* (5 µg/ml), *C. cucumerium* and *C. sphaerospermum* (0.5 µg /ml) and *Phytophthora capsici* at 1.0 µg/ml.

The cytotoxicity of most currently used drugs has become a serious problem and efforts are being directed to obtaining new drugs with different structural features. One option favoured is the search for new plant derived non-toxic drugs, as was investigated in this study. Caespitate proved to be non-toxic at biologically active concentrations.

Development of resistance to synthetic chemotherapeutic agents is known to occur in modern medicine; for example, resistance to some antibiotics of certain strains of microorganisms. A synergistic antibacterial bioassay demonstrated that the combination of caespitate and caespitin enhanced activity from a concentration range of 5 µg /ml to 0.5 µg /ml down to 0.1 µg /ml to 0.05 µg /ml on Gram-positive bacteria. The synergistic effect was in addition displayed against Gram-negative bacteria.

The study of the morphology and ultrastructure of the epicuticular trichomes revealed that trichomes in *H. caespititium* originate from papillate cell outgrowths which elongate, develop and later polarise into apical, stem and basal parts and that repeated secretions of compounds probably occur from the young three-celled stage, enable us to characterise and relate our observations to their possible functional role in the production of the antimicrobial and other compounds on the leaf surface.

South African *Helichrysum* species are a potentially good source of antimicrobial agents worthy of further investigation as efficient therapeutic compounds and in assisting the primary health care in this part of the world.

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CHAPTER 10

ACKNOWLEDGEMENTS

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TO GOD BE THE GLORY.

APPENDIX 1

APPENDIX 1

APPENDIX 1

CRYSTAL DATA AND DETAILS OF THE STRUCTURE DETERMINATION.

2-Methyl-4-[2',4',6'-trihydroxy-3'-(2-methylpropanoyl) phenyl] but-2-enyl acetate.

1. **Crystal data**.

Empirical formula $C_{17}H_{22}O_6$

Formula weight 322.35

Crystal system Monoclinic

Space group C2/c (No. 15)

a, b, c, [Angstrom] (a) 13.9411(9); (b) 17.4233 (11); (c) 15.6427 (10)

alpha, beta, gamma [deg.] 90 112.9050 (10) 90

V [Ang ** 3] 3500.0 (4)

Z 8

D (obs), D (calc) [g/cm ** 3] 0.000, 1.224

F (000) 1376

Mu (Moka) [/mm] 0.092

Crystal size 0.30 x 0.30 x 0.60

2. **Collection data**

Temperature (K) 296.2

Radiation [Angstrom] Moka 0.71073

Theta Min-Max [Deg] 5.92, 28.30

Scan, (Type and Range) [Deg] 0.00 + 0.35 Tan (Theta)

Hor. and Vert. Aperture [mm] 0.00 and 0.00

3. **Reference Reflection(s)**

Dataset 18: 18:23:23; 20:20

Tot., Uniq. Data, R (int) 19052, 4294, 0.030

Observed data [I > 2.0 sigma (I)] 3063

4. **Refinement**

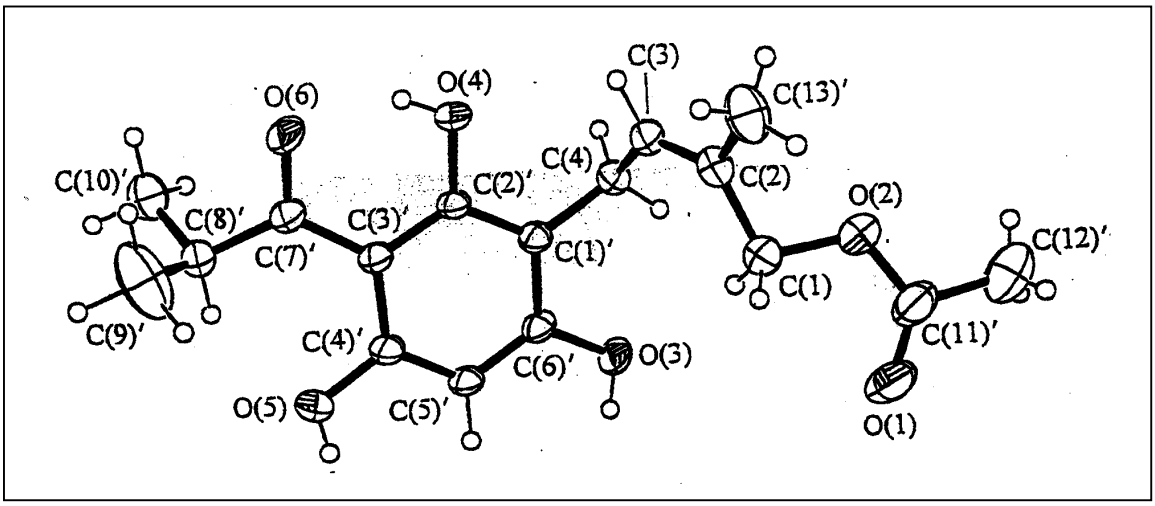
Nref, N par 0, 220

R, wR, S 0.0588, 0.1654, 1.053

w = 0.0961

Max. and Av. Shift/Error 0.05 0.00

Min. and Max resd. Dens. [e/Ang ^ 3]. 0.27, 0.40



Appendix 1 (Figure). The X-ray structure and molecular stereochemistry of the acylated phloroglucinol derivative,caespitate (C_{17} H_{22} O_6), showing the numbering scheme employed.

5. **Structure Solution**

The structure was solved in the monoclinic space group C2/c with the direct methods program SHELXS-97 [1] as implemented by the crystallographic program OSCAIL [2]. The E-map lead to the location of all non-hydrogen atoms; these were refined anisotropically with the program SHELXL-97. A difference Fourier synthesis led to location of all methine, methylene, and methyl hydrogens. All were included as idealized contributors in the least-squares process with standard SHELXL-97 [1] idealization

parameters. No evidence (difference Fourier map) for the inclusion of solvent in the lattice could be found. The final refinement converged to values of: R1 = 0.0588 and wR2 = 0.1654 for the observed 3063 unique reflections [I > 2.0 sigma (I)] and R1 = 0.0823 and wR2 = 0.1829 for all 4294 unique reflections. The maximum and minimum electron densities on the final differences Fourier map were 0.40 and. 0.27 e/A ^ 3, respectively. The final model was plotted using the program ORTEP [3].

Caespitate has a *cis*- double bond in the side chain (App.1. Figure). This is unusual stereochemistry in plant products and may be responsible for the observed activity of the compound (Drewes, personal communication).

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APPENDIX 2

APPENDIX 2

APPENDIX 2

SPOOR AND FISCHER

JOHANNESBURG

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Provisional Patent Specification

COUNTRY : **SOUTH AFRICA**

APPLICATION NUMBER : **99/6242**

NAME OF APPLICANT : **UNIVERSITY OF PRETORIA**

DATE OF FILING : **30 SEPTEMBER 1999**

NAME OF INVENTORS : **JACOBUS JOHANNES MARION MEYER**

ABBEY DANNY MATOME MATHEKGA

TITLE OF INVENTION : **PHLOROGLUCINOL COMPOUNDS**

FILE REF : JP/U 082/MK/acm

DATE : 8 October 1999

1 BACKGROUND OF THE INVENTION

THIS invention relates to the treatment and control of tuberculosis caused by *Mycobacterium tuberculosis*, as well as to the treatment and control of other bacteria an fungi and in particular to the use of phloroglucinol derivatives for use in such treatment and control.

Tuberculosis (TB) remains a serious health problem in many regions of the world, especially in developing nations. It is a contagious disease and is becoming epidemic in some parts of the world. It is estimated that 30-60% of adults in developing countries are infected with *Mycobacterium tuberculosis*. Approximately 8-10 million individuals develop clinical TB and 3 million die of TB each year (WHO/IUATLD, 1989).

In South Africa, over 3 in every thousand people die of TB, the highest rate in the world, while one out of every 200 people suffers from active tuberculosis. Tuberculosis is the most commonly notified disease in South Africa and the fifth largest cause of death among the black population (South African Tuberculosis Association, 1998).

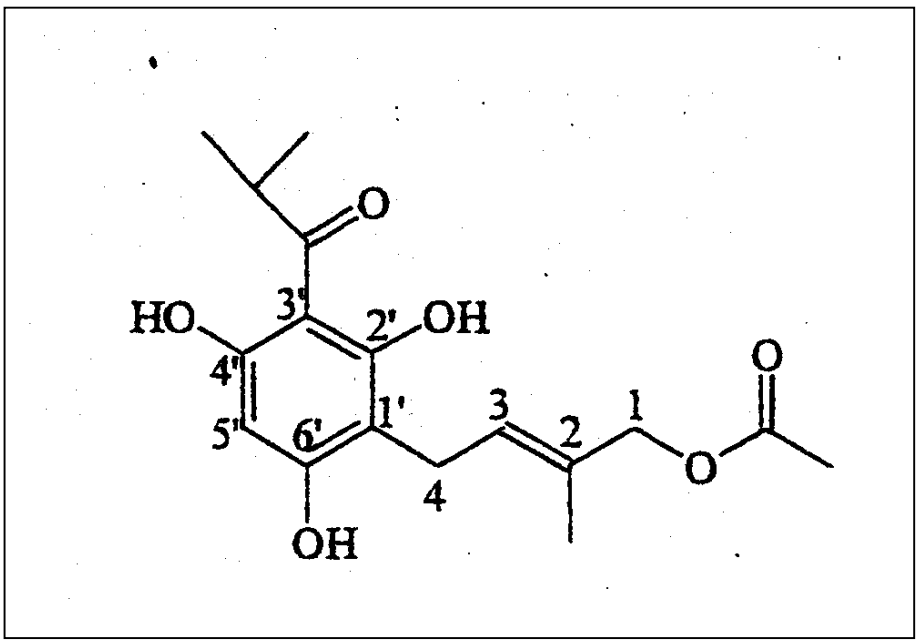
In the United States, the number of TB cases steadily decreased until 1986 when an increase was noted. Since then TB cases have continued to rise. Ten million individuals are infected in the U.S.A., with approximately 26000 new cases of active disease each year (National Jewish Medical and Research Centre, 1994).

Individuals infected with Human Immunodeficiency Virus (HIV) are very susceptible to tuberculosis and often develop this disease before other manifestations of AIDS become apparent (Grange and Davey, 1990). Control of the TB epidemic linked with HIV infection will depend largely on the adequate treatment of TB, and possibly of effective chemoprophylaxis, not just for HIV-infected persons but for communities as well (WHO/IUATLD, 1989).

TB therapy has been revolutionized and the present treatment regimes for TB are based on multidrug therapy with usually 3 or 4 antituberculosis drugs. However, the problem of multidrug resistant tubercle bacilli is emerging for various drugs such as isoniazid,

ethambutol, rifampicin and streptomycin, for example (Girling, 1989; Grange and Davey, 1990). Drug-resistant TB is very difficult to treat requiring greater numbers and varieties of medications for a longer period of treatment. The need for new antituberculosis agents is urgent due to the increasing resistance of mycobacteria to these classic antituberculosis drugs. A recent WHO report states that, globally, 2% of all cases of tuberculosis are multidrug resistant-by definition, resistance to rifampicin plus isoniazid (plus/minus other resistances). Such cases can be treated in the USA and other high resource regions but at a great cost (> US$ 250,000 per case!) and using very long courses of rather toxic drugs, thereby raising serious problems of compliance (WHO, 1997). South Africa is witnessing an explosion in the number of cases of drug-resistant tuberculosis. In some parts of South Africa, 1 in 10 cases of TB is resistant to treatment (*New Scientist*, March 1997). It is essential to have new antituberculosis agents, preferably those that can readily and simply be produced from some local source.

The present invention is directed at the use of phloroglucinol derivatives in the treatment and/or control of tuberculosis caused by *Mycobacterium tuberculosis* and infection caused by other pathogenic bacteria and fungi. In particular, phloroglucinol derivatives of the general Formula 1 have been found to be effective against *Mycobacterium tuberbulosis* and infection caused by other pathogenic bacteria and fungi.



The inventors of the present application undertook an extensive research program in order to identify antituberculosis, antibacterial and antifungal agents that can readily and simply be produced from local resources.

Twenty-eight South African medicinal plants used to treat pulmonary diseases were screened by the inventors for activity against drug-resistant and sensitive strains of *M. tuberculosis*. A preliminary screening of acetone and water plant extracts, against a drug-sensitive strain of *M. tuberculosis* H37Rv, was carried out by the agar plate method. Fourteen of the 20 acetone extracts showed inhibitory activity at a concentration of 0.5 mg/ml against this strain. Acetone as well as water extracts of *Cryptocarya latifolia, Euclea natalensis, Helichrysum caespititium, Nidorella anomala* and *Thymus vulgaris* inhibited the growth of *M. tuberculosis*.

the agar plate method a further study was carried out employing the rapid radiometric method to confirm the inhibitory activity. These active acetone extracts were screened against the H37Rv strain as well as a strain resistant to the drugs, isoniazid and rifampin. The minimal inhibitory concentration of *Croton pseudopulchellus, Ekebergia capensis, Euclea natalensis, Nidorella anomala* and *Polygala myrtifolia* was 0.1 mg/ml against the H37Rv strain by the radiometric method. Extracts of *Chenopodium ambrosioides, Ekebergia capensis, Euclea natalensis, Helichrysum caespititium, Nidorella anomala* and *Polygala myrtifolia* were active against the resistant strain at 0.1 mg/ml. Eight plants showed activity against both the strains at a concentration of 1.0 mg/ml.

The following procedure was developed by the applicant for the isolation of caespitate from *H. caespititium* and other species in this genus, as well as any other plants that may synthesise caespitate or other compound of formula 1 acylated phloroglucinol derivatives.

2. Identification of plant species

The aerial plant parts of *H. caespititium* were collected near Harrismith and identified at the HGWJ Schweickerdt Herbarium of the University of Pretoria and also at the herbarium of the National Botanical Institute, Pretoria.

2.1 Extraction

Dried aerial plant parts of *H. caespititium* were shaken in acetone for 5 minutes, filtered and concentrated to dryness at reduced pressure on a rotary evaporator.

2.2. Thin layer chromatography

A direct antibacterial bioassay (Dilika and Meyer 1996) on TLC-plates was employed to speedup the activity guided isolation of the antimicrobial compound. *M. tuberculosis* cannot be tested in this way because of its very slow growth rate. The direct antibacterial bioassays of the acetone extract were done on TLC plates (Merck) developed with chloroform-ethylacetate (1:1). After development, the TLC plates were dried and sprayed with a 24 hr old *Staphylococcus aureus* culture in nutrient broth. After 24 hr incubation, the plates were sprayed with an aqueous solution of 2mg/ml p-iodonitrotetrazolium violet to visualise the bacterial cells. The plates were then reincubated at 37^0C for 2-3 hours.

Four zones of bacterial growth inhibition could be seen on TLC plates sprayed with *S. aureus*. Activity was more pronounced in the R_f 0.57 zone (chloroform-ethylacetate (1:1)) than in the other 3 zones.

2. 3 Column chromatography

The crude extract of the plant was dried, its mass determined and resuspended in chloroform. Column chromatography was performed on silica gel 60 using chloroform as eluent. The antibacterial fractions collected were then again tested for antibacterial activity on TLC to detect the fraction containing the active compound of R_f 0.57.

2.4 High performance liquid chromatography

The compound was further purified by HPLC utilising an analytical Phenomenex reverse phase 250x4.60 mm column, at a flow rate of 1.0 ml/min, oven temp. 40^0C and a wavelength of 206nm. An ethanol-water (50:50) solution was employed as mobile phase. The pure compound was collected from the eluent. The chemical

structure was confirmed by ^{1}H-, ^{13}C-, COSY-, DEPT- and HETCOR-nmr, MS and crystallography.

3 ANTIBACTERIAL ACTIVITY

The activity of caespitate was examined against ten bacteria by the agar dilution method (Turnbull and Kramer, 1991). Caespitate significantly inhibited the growth of all the Gram-positive bacteria tested at a concentration of between 0.5 and 5µg/ml (Table 1).

Caespitate had no activity against all the Gram-negative bacteria tested. These results are in accordance with previous reports (Tomas-Barberan, Iniesta-Sanmarin, Tomas-Lorente, and Rumbero, 1990; Dekker, Fourie, Snyckers and Van der Schyf, 1983) of similar antimicrobial activity of related compounds against Gram-negative bacteria. 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 and have been implicated in some cases of food poisoning (Turnbull and Kramer, 1991). *Staphylococcus aureus,* is a human pathogen, whose infections are amongst the most difficult to combat with conventional antibiotics (Tomas-Barberan, Msonthi and Hostettmann, 1988; Tomas-Barberan, Iniesta-Sanmarin, Tomas-Lorente, and Rumbero, 1990).

TABLE 1. Antibacterial activity (MIC) of the crude acetone extract of the aerial parts of *H. caespititium* and caespitate isolated from the extract.

Bacteria	***Gram* +/-**	***Crude extract (mg/ml)***	***Caespitate MIC(µg/ml)***
Bacillus cereus	+	1	0.5
B. pumilus	+	1	0.5
B. subtilis	+	1	0.5
Micrococcus kristinae	+	1	0.5
Staphylococcus aureus	+	1	5
Enterobacter cloacae	-	1	na[b]
Escherichia coli	-	1	na
Klebsiella pneumoniae	-	na	na
Pseudomonas aeruginosa	-	1	na
Serratia marcescens	-	na	na

[a] Minimum inhibitory concentration

[b] Not active

This study provided a probable scientific explanation for the therapeutic potency attributed to *H. caespititium*, claimed by traditional healers in the Free State province of South Africa, for example, during wound treatment in male circumcision rites.

4 ANTIFUNGAL ACTIVITY

The growth of six fungi, *Aspergillus niger, A. flavus, Cladosporium cladosporioides, C. cucumerium, C. sphaerospermum* and *Phythophthera capsici,* were significantly inhibited at very low MIC's by caespitate (Table 2). *A. flavus* and *A. niger* are some of the most important fungi responsible for human systemic infections. These organisms were inhibited at 1.0 µg/ml. It is generally agreed that at least one acidic hydroxyl group and a certain degree of lipophilicity are required for biological activity compound (Tomas-Barberan, Iniesta-Sanmarin, Tomas-Lorente, and Rumbero, 1990).

Lipophilicity is important because many antifungal metabolites exert their toxicity by some membrane associated phenomenon, and it is known that acidic hydroxyl groups may act by uncoupling oxidative phosphorylation. In this case the antifungal caespitate isolated from *H. caespititium* bears three acidic hydroxyls (phenolic hydroxyls) and lipophilicity (3'-isobutyrylphenyl and but-2-enyl acetate residues). On the other hand, antibacterial activity, against Gram-positive bacteria seems to be related to the presence of phenolic hydroxyls (phenol itself is a well known antibacterial compound (Tomas-Barberan, Iniesta-Sanmarin, Tomas- Lorente, and Rumbero, 1990).

TABLE 2. Antifungal activity of the crude acetone extract of the aerial parts of *Helichrysum caespititium* and caespitate isolated from the extract.

	MIC [a]	
Fungal species	Crude Extract mg/ml	Caespitate mg/ml
Aspergillus flavus	1.0	1.0
A. niger	0.01	1.0
Cladosporium cladosporioides	0.01	5.0
C. cucumerium	0.01	0.5
C. sphaerospermum	0.01	0.5
Phytophthora capsici	1.0	1.0

[a] Minimum inhibition concentration.

5 ANTITUBERCULOSIS ACTIVITY

The effect of caespitate on the growth of the sensitive strain (H37Rv) and resistant strains of *Mycobacterium tuberculosis* as determined by the radiometric method are set out in Table 3. The results show that caespitate controls the *Mycobacterium tuberculosis* bacterium effectively. As far as the applicant has been able to establish, caespitate has never been synthesised in a laboratory.

TABLE 3. Inhibition of *Mycobacterium tuberculosis* strains by caespitate

Mycobacterium tuberculosis strains	MIC (mg/ml)	DGI[a] values of plant extracts (mg/ml)	DGI values of the control vial (mg/ml)
H37 sensitive strain	0.1	7.33 ± 4.93	25 ± 4
2 drug resistant strain (res. to Isoniazid and rifampicin).	0.1	7 ± 2	26 ± 3.2
3 drug resistant strain (res. to streptomycin, isoniazid and ethambutol),	0.1	3 ± 1.73	17.33 ± 3.05
4 drug resistant strain (res. to streptomycin, isoniazid, rifampicin and ethambutol).	0.1	8.66 ± 1.52	23 ± 3.5
5 drug resistant strain.(res to isoniazid, streptomycin, rifampicin, thiacetazone and cycloserine).	0.1	8.3 ± 2.88	23.3 ± 3.51
6 drug resistant strain (res. to isoniazid, rifampicin, ethionamide, terizidone, thiacetazone and ofloxacin).	0.1	10 ± 3.60	27 ± 5.56
7 drug resistant strain.(res to isoniazid, steptomycin, ethambutol, kanamycin, rifampicin, and ethionamide)	0.1	10.3 ± 2.52	26.33 ± 7.09

[a] DGI values are means ± s.d.

It is believed that caespitate and related acylated phloroglucinol derivatives are viable alternatives to conventional drugs in the treatment and control of tuberculosis in humans.

DATED THIS _____ DAY OF _____ 1999
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APPLICANT'S PATENT ATTORNEYS

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APPENDIX 3

APPENDIX 3

Antibacterial activity of South African *Helichrysum* species

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Acetone extracts of *Helichrysum callicomum, H. glomeratum, H. hypoleucum, H. odoratissimum, H. pilosellum* and *H. rugulosum* were investigated for antibacterial activities against ten bacteria using the agar diffusion method. Epicuticular (shaken) and homogenized extracts of *H. hypoleucum, H odoratissimum* and *H. rugulosum* significantly inhibited the growth of *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae* and *Staphylococcus aureus* (all Gram-positive bacteria) and *Enterobacter cloacae* (Gram-negative) at a concentration range of 0.01 to 1.0 mg/ml. In addition, the epicuticular extract of *H. hypoleucum* was active against *Escherichia coli* and *Pseudomonas aeruginosa* whereas the homogenized extract only had activity against *P. aeruginosa*. None of the other six extracts inhibited the growth of *E. coli, Klebsiella pneumoniae, P. aeruginosa* and *Serratia marcescens*, all Gram-negative bacteria. The extracts of *H. glomeratum* and *H. pilosellum* had no activity against any of the organisms tested. Shaken extracts proved to be more bioactive than homogenized extracts.

Keywords: Antibacterial, Asteraceae, *Helichrysum*, traditional medicine.

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Introduction

There are 500 *Helichrysum* species of which 245 occur in South Africa. The South African species display great morphological diversity and are, therefore, classified into 30 groups (Hilliard 1983). *Helichrysum* species have been reported to have antimicrobial activities (Rios *et al.* 1988; Tomas-Barberan *et al.* 1988, Tomas-Barberan *et al.* 1990; Tomas-Lorente *et al.* 1989; Cosar & Cubukcu 1990; Meyer & Afolayan 1995; Meyer & Dilika 1996). Not much information on the bioactivity of compounds isolated from these species is available. *In vitro* antimicrobial screening methods provide the required preliminary observations to select among crude plant extracts those with potentially useful properties for further chemical and pharmacological investigations. In this study we investigated the antibacterial activities of crude acetone extracts (shaken and homogenized) of six *Helichrysum* species against ten bacteria species.

Materials and Methods

Plant material

Plants were collected from the Drakensberg Mount-aux-Sources area in QwaQwa (Witsieshoek, South Africa). Their identity was verified by a taxonomist, Prof. R.O. Moffett, and voucher specimens were deposited in the herbarium of the Department of Botany, University of the North, QwaQwa Branch, South Africa.

H. callicomum Harv., (herbarium voucher specimen M5054) Group 2 (Hilliard 1983), is a tufted perennial herb with short woody stems and greyish-white clustered leaves, often forming large patches in overgrazed grasslands. Basothos in the QwaQwa district use leaf infusions for colds, body pains and as ingredients in an enema for colic (Watt & Breyer-Brandwijk 1962).

H. odoratissimum (L.) Sweet., (M5061) Group 4 (Hilliard 1983), is a much branched aromatic perennial herb with greyish-white leaves, forming large patches on grassy or rocky slopes. It is used to relieve abdominal pains, heartburn, coughs, colds, and treats female sterility, eczema and wounds (Van Puyvelde *et al.* 1989).

H. glomeratum Klatt, (M5055) Group 6 (Hilliard 1983), is a rhizomatous perennial herb with densely rosetted leaves, found in large colonies in open grassland. Leaf infusions are administered to children with stomach ache and burnt parts used as an ingredient in a colic remedy by the Basothos (Watt & Breyer-Brandwijk 1962).

H. rugulosum Less., (M5060) Group 9 (Hilliard 1983), is a perennial herb with a creeping rootstock and a tufted flowering stem. Plants grow in poor sand or stony grasslands, invading over-grazed and road-side areas. A decoction of the root is administered to children suffering from diarrhoea and to fumigate huts. It is similarly used as an ingredient in an enema for colic (Watt & Breyer-Brandwijk 1962).

H. hypoleucum Harv., (M5056) Group 16 (Hilliard 1983), is a soft-wooded shrub with white, hairy leaves, growing in tangled clumps on forest margins on steep grassy mountain slopes. It is used by Basothos as a tea, emetic and for chest problems. It is also used as a poultice for swelling and as an antibiotic (Hutchings & Van Staden 1994).

H. pilosellum (L.f.) Less., (M5059) Group 23 (Hilliard 1983) is a perennial herb with silky hairy leaves and is widespread in the grasslands. Leaf infusions are administered for stomach ache, coughs and colds and is also used as an ingredient in colic remedies by the Basothos (Hutchings & Van Staden 1994).

Preparation of extracts

Shoots (excluding flowers) of the plants were air dried at room temperature. Each plant (80 g) was shaken for five minutes in acetone and filtered through Whatman No 2 filter paper under suction. This filtrate will be referred to as the 'shaken extract'. The residue was then homogenized in acetone, also filtered through Whatman No 2 filter paper and both extracts concentrated to dryness under reduced pressure at 40°C, with a rotary evaporator. After determining the yields, the extracts were stored at 4°C until further use.

Bacteria

Ten bacterial species (Table 1) were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on nutrient agar (Biolab) and an inoculum was recovered for testing by growth in nutrient broth No 2 (Biolab) for 24 hours. Before streaking, each culture was diluted 1:10 with fresh sterile nutrient broth.

Preliminary screening for antibacterial activities

The plant extracts (sterilized by filtering through a 0.22 µm filter) were added to 5 ml of nutrient agar medium in Petri dishes and swirled carefully before congealing. An aliquot of each extract was serially diluted (ten fold) to obtain a concentration range of 1.0 to 0.01 mg/ml in 2% acetone. The organisms were then streaked in radial patterns on agar plates (Mitscher *et al.* 1972). Plates were

Table 1 Antibacterial activity (MIC) of *Helichrysum* extracts

		MIC (mg/ml)									
	Yield	Gram-positive bacteria[b]					Gram-negative bacteria[b]				
Extracts[a]	% w/w[c]	*B.cer*	*B. pum*	*B. sub*	*M. kri*	*S. aur*	*E. clo*	*E. col*	*K. pne*	*P.aer*	*S. mar*
H. cal S[d]	3.5	1.0	1.0	1.0	1.0	1.0	1.0	na[f]	na	na	na
H[e]	1.9	1.0	1.0	1.0	na	na	na	na	na	na	na
H. glo S	4.0	na	na	na	na	na	na	na	na	na	na
H	1.5	na	na	na	na	na	na	na	na	na	na
H. hyp S	2.3	0.10	1.0	1.0	1.0	1.0	1.0	1.0	na	1.0	na
H	1.2	0.10	0.10	0.10	1.0	na	1.0	na	na	1.0	na
H. odo S	6.0	0.01	0.01	0.01	0.01	0.01	0.01	na	na	na	na
H	4.0	1.0	1.0	1.0	1.0	na	na	na	na	na	na
H. pil S	3.0	na	na	na	na	na	na	na	na	na	na
H	1.8	na	na	na	na	na	na	na	na	na	na
H. rug S	4.2	0.10	0.10	0.10	0.10	1.0	0.10	na	na	na	na
H	1.2	1.0	1.0	na	na	na	1.0	na	na	na	na

[a] *H. cal (Helichrysum callicomum), H. glo (H. glomeratum), H. hyp (H. hypoleucum), H. odo (H. odoratissimum), H. pil (H. pilosellum* and *H. rug (H. rugulosum)*

b *B. cer (Bacillus cereus), B. pum (B. pumilus), B. sub (B. subtilis), M. kri (Micrococcus krtistinae, S. aur (Staphylococcus aureus), E. clo (Enterobacter cloacae), E. col (Escherichia coli), K. pne (Klebsiella pneumoniae), P.aer (Pseudomonas aeruginosa)* and *S. mar (Serratia marcescens)*

[c] % w/w (% residue mass/fresh mass)

[d] S (shaken extract)

[e] H (homogenised extract)

[f] na (not active)

incubated at 37°C and examined after 24 and 48 hours. Complete inhibition of growth was required for an extract to be declared bioactive. A blank plate containing only nutrient agar and a second containing nutrient agar and 2% acetone served as controls (Meyer & Afolayan 1995). Each treatment was performed in triplicate.

Results and Discussion

Four of the plant species tested exhibited some degree of antibacterial action (Table 1). Their extracts were active against all the Gram-positive bacteria tested. *H. glomeratum* and *H. pilosellum* had no activity against any of the organisms.

The epicuticular extract of *H. callicomum* had no effect on *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Serratia marcescens* (all Gram-negative bacteria), but was active against *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae* and *Staphylococcus aureus* (all Gram-positive) as well as *Enterobacter cloacae* (Gram-negative). The homogenized extract was active against *B. cereus, B. pumilus* and *B. subtilis*. The MIC of all the extracts of this species was 1.0 mg/ml.

The epicuticular extract of *H. hypoleucum* showed strong activity against all Gram-positive bacteria tested and in addition inhibited the growth of the Gram-negative bacteria *E. cloacae, E. coli* and *P. aeruginosa*. The shaken extract had an MIC of 1.0 mg/ml whereas the homogenized extract was active at 0.10 mg/ml against *B. cereus, B. pumilus* and *B. subtilis*; it was in addition active against *M. kristinae, E. coli* and *P. aeruginosa* at 1.0 mg/ml.

Both extracts of *H. odoratissimum* did not inhibit the growth of *E. coli, K. pneumonia, P. aeruginosa* and *S. marcescens* all Gram-negative bacteria, but had activity against all Gram-positive bacteria at the low MIC of 0.01 mg/ml, the highest dilution used in this study. The homogenized extract inhibited the growth of four of the five Gram-positive bacteria at a MIC of 1.0 mg/ml.

None of the extracts of *H. rugulosum* showed activity against *E. coli, K. pneumoniae, P. aeruginosa* and *S. marcescens*, but the shaken extract was active against *E. cloacae* at a MIC of 0.1 mg/ml and the homogenized extract at 1.0 mg/ml. The shaken extract was active against all Gram-positive bacteria at a MIC of 0.10 mg/ml, except for *S. aureus* which had an 1.0 mg/ml MIC. The homogenized extract was only active against *B. aureus, B. pumilus* and *E. cloacae*.

Infections caused by *P. aeruginosa* are amongst the most difficult to treat with conventional antibiotics (Levinson & Jawetz 1992). The growth of *P. aeruginosa* was inhibited by extracts of *H. hypoleucum* at a MIC of 1.0 mg/ml and this plant may, thus, be a source which could yield drugs that could improve the treatment of infections caused by this organism.

The activity of *H. callicomum, H. hypoleucum, H. odoratissimum* and *H. rugulosum* against *S. aureus*, another human pathogen, qualifies these plants for further investigation of their bioactive compounds.

Most *Bacillus* species are regarded as having little pathogenic potential, however, both *B. cereus* and *B. subtilis* have been known to act as a primary invader or secondary infectious agents in a number of diseases and have been implicated in some cases of food poisoning (Turnbull & Kramer 1991). Four of the *Helichrysum* species investigated in this study inhibited the growth of these bacilli.

In view of the fact that Gram-negative organisms are in general terms more resistant than Gram-positive ones to anti-

microbial agents from plants (Stickler & King 1992), it is significant that *E: cloacae, E. coli* and *P. aeruginosa* were inhibited at concentrations of 1.0 mg/ml or lower by some of the plant species investigated. These are much lower MIC's than those found by authors investigating other plant species (Rios *et al.* 1990; Meyer & Dilika 1996; Navarro *et al.* 1996; Salie *et al.* 1996).

Research is underway to isolate and identify the active compounds from some of the species that showed high inhibitory activity.

Acknowledgements

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