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A STUDY OF THE ANTIMICROBIAL PROPERTIES OF SELECTED PLANTS GROWING IN THE FREE STATE

by

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"……..Their fruit will be for food, and their leaves for medicine."

Ezekiel 47:12 (NKJV)

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CHAPTER 1

INTRODUCTION

Plants are a good source of biologically active natural products that are biodegradable as well as renewable (Taniguchi & Kubo, 1993). The plant kingdom can be regarded as a largely untapped storage of phytochemicals (Aquino *et al.*, 1995) as evidenced by the following facts. Roughly 40 million square kilometres of the earth's surface is covered by forests, half of which are tropical forests and one-third rain forests (Farnsworth & Bingel, 1977 ; Rasoanaivo & Ratsimamanga, 1993). Philipson and Anderson (1987) as well as Hamburger and Hostettmann (1991) stated that, of the 250 000 to 500 000 species of higher plants on earth, less than 10% of them have been investigated for biological activities - in most cases only one activity has been studied. Tropical forests are home to most of the world's plant species but more than half of these are unknown. Moreover, the bioactivities and chemical composition of many more have never been studied (Rasoanaivo & Ratsimamanga, 1993). Tropical plants are a rich source of secondary metabolites possessing both antimicrobial as well as other biological activities because, due to the climate, they are always exposed to attack by various parasites such as bacteria, fungi and insects. Confronted with these harsh conditions for survival, they have developed efficient built-in defence mechanisms in the form of chemicals possessing a range of bioactivities (Kubo, 1995).

Many higher plants accumulate extractable organic substances in quantities sufficient to be economically useful as raw materials for various scientific, technological as well as commercial applications. Secondary metabolites are frequently accumulated by plants in smaller quantities than are primary metabolites. Some commercially useful plant secondary metabolites are nicotine, the pyrethrins and rotenone, which are used in limited quantities as pesticides (Balandrin *et al.*, 1985). These economically important primary and secondary metabolites tend to be relatively low in molecular weight. A number of biologically active compounds with medicinal properties has also been isolated from plants (Aquino *et al.*, 1995).

Throughout history, mankind has passed on information about efficacious and non-toxic medicinal plants (and occasionally toxicants) by word of mouth and through various writings. As a result of this continual refinement of knowledge, about 20 000 plant species, mostly in the form of crude extracts are now used for medicinal purposes around the world (Nigg & Seigler, 1992 ; Ishimaru & Shimomura, 1995). Chemical work, resulting in the isolation of active principles from crude aqueous or alcoholic plant extracts began in earnest in the 19th century. Activity directed isolation of plant compounds continues today in many industrial, academic and government laboratories where extracts, exhibiting a specific bioactivity of interest, are purified chromatographically, guided by periodic evaluation with one or more bioassay system(s) resulting in the eventual isolation of one or more bioactive constituent(s) (Nigg & Seigler, 1992 ; Sener, 1994). *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 (Mathekga & Meyer, 1998).

A number of research publications on the constituents and biological activity of African medicinal plants are available but the development of therapeutic agents from these plants has remained rather neglected despite the fact that modern antimicrobial chemotherapy has much of its origin in tropical medicine and chemotherapy (Ryley, 1995). Documentation of African medicinal plants has also not been done as fully as in other traditional societies, such as the Indian and Chinese societies, even though more than 80% of Africa's population still uses plant extracts to cure many forms of diseases (Iwu, 1993). Since prehistoric times man has been trying to find more useful plants and to improve the yield and the quality of the known ones. This has resulted in the knowledge of uses for numerous plants. However, industrialization has led to a decrease in the number of plants being used as well as a high probability of losing knowledge of the useful plants (Verpoorte, 1986).

The opinion frequently expressed in literature, that infectious diseases no longer pose a problem as a result of the development of antibiotics and vaccines, is not correct, though many pathogenic microorganisms can be controlled with currently available antibiotics (Zähner &

Fiedler, 1995 ; Kubo, 1995). Today, according to Zähner and Fiedler (1995), mankind is further away from mastering infectious diseases than it was 25 years ago. This has been attributed to changes in the spectrum of pathogens, examples being the HIV, atypical mycobacteria, aspergilli, *Cryptococcus neoformans, Listeria* and *Legionella*. It has been stated that antibiotic-resistant pathogens are on the increase, particularly in hospitals. In the past multi-resistant Gram-negative bacteria were the problem. Today it is the multi-resistant Gram-positive bacteria that are of concern, especially *Staphyloccoccus aureus* (MRSA-methicillin-resistant *Staphylococcus aureus*), enterococci and pneumococci. The resistance of these strains can be against up to over ten different antibiotics (Zähner & Fiedler, 1995). It is worth mentioning here that the development of resistance to antifungal drugs in medicine has been less of a problem than with antibacterial agents (Hunter, 1995), although this does not mean that there is no need to search for alternative cures of fungal infections (Russel *et al.*, 1995). For example, systemic infections caused by filamentous fungi, especially in patients with impaired host defence mechanisms, have become increasingly serious worldwide and control of many fungal diseases has not been achieved though many antifungal agents have been introduced (Kubo, 1995). Zähner and Fiedler (1995) also mentioned that the deterioration of social conditions in the developing, and increasingly in the industrialized countries, is a crucial factor to the renewed spread of infectious diseases such as tuberculosis. The other reason for research into antimicrobials of plant origin is the fact that known antimicrobial compounds still present drawbacks such as a narrow spectrum of activity, limited therapeutic usefulness and some degree of toxicity (Vanden Berghe & Vlietinck, 1991).

Although dermatophytes are among the commonest diseases in man and other animals, fungal infections in humans generally have had less impact on mankind than fungal-, bacterial- or viral infections on plants (Caceres *et al.*, 1991b ; Hunter, 1995). According to Hunter (1995), so far natural sources of antifungal agents have been microbial metabolites and, despite the fact that many novel compounds are described each year, very few have sufficient activity to be of interest and many more are toxic or are difficult to pursue.

An increase in bacterial diseases, like tuberculosis due to the AIDS epidemic and the fact that antiviral compounds of plant origin are more effective than their synthetic analogues (Van Den Berghe *et al.*, 1978 ; Vanden Berghe & Vlietinck, 1991 ; Elisabetsky & Posey, 1994), necessitates the development of plant derived antibacterial agents as safer and more effective alternatives. The fact that plants most interesting to the phytochemist or ethnopharmacologist grow in the tropics, has led to a renewed interest in phytochemistry and the value of natural plant metabolites in the development of new drugs is now being recognised (Bohm *et al.*, 1986 ; Rasoanaivo & Ratsimamanga, 1993).

Phytochemistry has been applied in chemotaxonomy with significant success for quite some time now. It would therefore be of great interest in the future to observe how ethnophamarcological data and chemotaxonomy can be used profitably in the search for new antibacterial, antifungal and antiviral compounds in plants growing in the Free State province of South Africa.

In recent years, improved chromatographic techniques have allowed the isolation of a number of new natural bioactive products, while advances in spectroscopy have allowed structure determinations to be executed quickly with small amounts of material. For example, with modern developments in nuclear magnetic resonance spectroscopy it is now possible to assign chemical structures to complex organic molecules rapidly, non-destructively and unambiguously (Tyler, 1986 ; Verpoorte, 1986 ; Jacobs, *et al.*, 1987 ; Roeder, 1990). At the same time, the proliferation of biochemical tests and *in vitro* methods has enabled rapid sensitive screening of small quantities of phytochemicals. These developments have also led to a resurgence in natural products research (Tyler,1986 ; Verpoorte, 1989).

The secondary metabolism of plants is of interest to researchers due to the fact that it provides among other substances, chemicals like pharmaceutical and antimicrobial compounds. The key to the study of these compounds has been and still is phytochemistry. In addition to providing

mankind with these compounds, secondary metabolites also protect plants from environmental threats in the form of microorganisms (Verpoorte, 1986).

A literature survey during this study revealed that secondary metabolites from plants have been more extensively used by the pharmaceutical industry than by the agricultural sector, hence the need to investigate the possibility of their extensive use in agriculture. A number of secondary plant metabolites have been shown to have some antiviral as well as other bioactivities *in vitro* and *in vivo*. These are alkaloids, flavonoids, terpenoids, steroids, phenols, tannins, coumarins, quinones, lignans and their glycosides. Also exhibiting antimicrobial activity are some lactones, peptide esters and polysaccharides (Philipson & Anderson, 1987 ; Farnsworth, 1994; Hudson & Towers, 1999). Some of these secondary metabolites with antimicrobial properties are also photosensitizers and are increasingly being studied due to their therapeutic potential (Hudson & Towers, 1991 ; Towers *et al.*, 1997 ; Hudson & Towers 1999) A brief account of some of the major compounds has been included in chapter 3.

CHAPTER 2

RATIONALE FOR THE STUDY

African medicinal plants provide a rich source of biologically active natural products (Marston *et al.*, 1993). They produce many secondary metabolites and constitute important sources and models for, among other chemicals, medicinal and agricultural raw materials that is, pharmaceutical drugs, microbicides and pesticides (Balandrin *et al.*, 1985). Plant produced compounds are of interest as a source of safer or more effective substitutes for synthetically produced antimicrobial agents (Heisey & Gorham, 1992; Maoz & Neeman, 1998). Hamburger and Hostettmann (1991) acknowledged that widespread ecological awareness and increased demands for non-classical therapies are the main reasons for the renewed interest in phytochemistry.

The growing interest in plant cell and tissue cultures as a potential alternative to traditional agriculture for the industrial production of secondary plant metabolites is of significant advantage to the natural products chemist for obvious reasons. Plant cell cultures provide a continuous, reliable source of natural products and the extraction of large amounts of the required chemicals from tissue cultures has been reported. Plant tissue cultures made from several medicinal plants have also been employed and resulted in the successful production of some useful secondary metabolites mainly alkaloids and terpenoids (Ishimaru & Shomomura, 1995). Ishimaru and Shomomura (1995) also reported that larger amounts (higher contents compared to those of the intact plant) of alkaloids and terpenoids have been isolated from tissue cultures as compared to isolation from intact plants. In addition, compounds from natural tissue cultures are more easily purified because of the absence of significant amounts of pigments, thus reducing production costs (Balandrin *et al.*, 1985 ; Van den Berg, 1988). At the same time, advances in chromatographic and spectroscopic techniques now permit the isolation and structural analysis of potent biologically active plant constituents with remarkable accuracy (Balandrin *et al.*, 1985 ; Tyler, 1986 ; Verpoorte, 1986 ; Jacobs, *et al.*, 1987 ; Roeder, 1990).

The Southern African subcontinent contributing 10% of all plant species in the world (Low & Rebelo, 1996), has a wealthy and diverse flora that could be of great benefit to the local agricultural, agrochemical and pharmaceutical industries if effectively tapped. Although a number of publications exists on the vegetation ecology of different regions of the Free State Province of South Africa (Venter, 1976 ; Kooij *et al.*, 1990a ; Fuls *et al.*, 1992b ; Du Preez., 1992 ; Malan *et al.*, 1995 ; Malan *et al.*, 1998), the plants of the Free State Province have never been mapped, collectively screened or documented for their medicinal properties, although much more work has been done in the past on plants growing in other provinces. This project was carried out with the hope of finding medicinal plants and plants that can be used in the agricultural sector as pesticides in the form of compounds, their synthetic analogues, crude extracts and/or even as composts in this case against soil-borne plant pathogens (Beautement, 1991).

One of the reasons why the screening of plants, growing in this province, for antimicrobial properties was done, is because data from traditional healers is not readily available as in other provinces, probably because it was not pertinently collected in the past. For this reason, it was decided to start by screening plants, for this activity, from the three districts namely Bloemfontein, Brandfort and Hoopstad (chapter 6). Sixteen families are represented in these areas and 27 representative species were collected for the initial screen.

The use of biodegradable synthetic analogues of antimicrobials of plant origin has been attempted by some researchers (Beautement, 1991). Other researchers have demonstrated the use of an epimeric mixture of naturally occurring plant products such as the diterpenes sclareol and 13-episclareol in the protection of plants against fungal infection. This mixture adheres to the surface of healthy tobacco (*Nicotiniana glutinosa*) leaves and prevents the germination of rust spores (Wain, 1986).

Since prehistoric times, people have used natural resources for medicinal purposes (Anesini & Perez, 1993). Today a large proportion (about 80%) of the world's population relies solely on the administration of plant derived preparations for the treatment of a variety of ailments

(Marston *et al.*, 1993; Aquino *et al.*, 1995). It is estimated that between 12 and 15 million South Africans still depend on traditional herbal medicine from as many as 700 indigenous plant species (Meyer *et al.*, 1996). This calls for a systematic study of these plants and the uninvestigated ones. Of the 45 or so plant derived compounds used in the industrialized countries by 1992, only some of the structurally simple, for example caffeine and papaverine, were manufactured synthetically with the rest produced more economically by cultivation and extraction (Nigg & Seigler, 1992). Currently, natural products and their derivatives represent more than 50% of all drugs in clinical use in the world, of which higher plants contribute no less than 25% of the total (Van Wyk *et al.*, 1997).

Despite the wide availability of clinically useful antibiotics of plant origin and their semi-synthetic analogues, a continuing search for new anti-infective agents remains indispensable. Some of the major antibiotics in use today are reported to have major drawbacks in terms of limited antimicrobial spectrum or serious side effects. The combination of the genetic versatility of the microbes and the widespread overuse of antibiotics has led to an increasing resistance of previously sensitive microorganisms as well as the emergence of previously uncommon (or unknown) infections (Vanden Berghe & Vlietinck, 1991). In view of this, the discovery of new molecules, either natural or synthetic, exhibiting prominent activity against infectious microorganisms such as toxogenic staphylococci, anaerobes, pseudomonas and various pathogenic fungi, showing cross-resistance with the existing antibiotics, would be of great benefit to primary health care. This has prompted an active research in this area.

Infections caused by *Pseudomonas aeruginosa* are among the most difficult to treat with conventional antibiotics. *Bacillus subtilis* has been known to be a primary invader or secondary infectious agent in a number of diseases, although many *Bacillus* species are regarded as having little pathogenic potential (Mathekga & Meyer, 1998). *Streptococcus mutans*, *Trichophyton rubrum* and *Candida albicans* cause common infections in humans that are difficult to control. Drugs currently available to control these species are limited, hence plant products that inhibit them without harming the host may have potential for use as therapeutic agents (Heisey & Gorham, 1992).

The wide spread occurrence of dermal infections caused by dermatophytes and the limited number of available drugs to effectively control them, have led investigators elsewhere to search for new antimycotic agents from various sources. There has been a recent increase in the frequency of skin mycoses world-wide. Here again, some of the modern antifungal therapies still cause considerable side effects in some of the treated patients and in some cases, resistance to treatment has been observed with some drugs (Maoz & Neeman, 1998). It is, therefore, imperative to search and develop new anti-infective agents that can inhibit the growth of these dermatophytes without causing harm to the host. The plant kingdom is a storage of untapped bioactive natural products that could be used as effective anti-infective drugs. The search for potential therapeutic agents could involve different strategies (Vanden Berghe & Vlietinck, 1991). One such possible strategy for finding new anti-infective drugs could involve the search for compounds with chemotherapeutic activities supplementary to- and structures significantly different from those in current use and these compounds can be extracted from higher plants as well.

As has been stated above, a world-wide ecological awareness is developing. Developments in agriculture have resulted in the destruction of the natural environment in many developing and developed countries. The use of synthetic agrochemicals, pesticides and fertilizers has polluted the natural environment. Both pesticides and fertilizers pollute food, soil as well as water sources even if used with utmost care. The ill effects of excessive use of pesticides on the environment and human health have been recognized for many years (Jansma *et al.*, 1993). This also necessitates the search for new compounds that are harmless to human health and the environment.

In the light of the above stated facts, it is quite obvious that the search for and the development of new non-toxic and environmentally friendly chemicals, both natural and synthetic, to effectively combat the threat of human and plant infectious diseases cannot be overemphasized. The current study, therefore, is an attempt to achieve these goals. It is aimed at:

- collecting leaves mainly from selected plant species growing in the Free State Province of South Africa, based on chemotaxonomy data. Leaves were concentrated on as we had to convince the authorities in the regions, in order to get permits, that the collection would not lead to the destruction of the plants.
- preliminary screening the crude extracts of these plant species against a number of common plant and one human pathogen,
- screening semi-purified extracts from the most potent species for antimicrobial activity against a number of plant and human pathogens,
- isolating and purifying compounds with antimicrobial activity from the most potent of the collected plant species,
- elucidating the chemical structures of the isolated compounds and
- mapping of the Free State Province for plant species with antimicrobial activity towards plant and human pathogens, based on the results from a preliminary screening of crude extracts above.

The importance of biodegradability and the possibility of renewal of plant derived bioactive compounds needs to be emphasized, because there is so much environmental degradation through soil and water pollution from the use of highly toxic pesticides and other agrochemicals (Lucas *et al.*, 1992). The ability of the plant sources of bioactive principles to renew themselves is necessary for the maintenance of a constant supply and much more urgently for the maintenance of the environment. For this reason, it was decided to apply a non-destructive collection procedure by mainly focussing on the leaf extracts from selected plant species growing in the Free State Province. Hopefully it will be possible to cultivate, on a large scale, the indigenous plant species that give the least toxic and most potent compounds in low doses for both the pharmaceutical and agrochemical industries, or even better still to use plant tissue cultures to produce these compounds. It is envisaged that, in the former instance, this may lead to the development of new crops in the future but this falls out of the scope of this monograph.

CHAPTER 3

LITERATURE SURVEY

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3.1 Some plant derived compounds with antimicrobial activity

Many phytochemicals vary in their distribution within the plant. The amount and composition of classes of compounds, e.g. flavonoids, alkaloids, essential oils and many others, are governed by the age of the plant or its parts and also by the plant's geographical as well as ecological (habitat) location (Ieven *et al.*, 1979). Antimicrobial properties have been observed with, among other phytochemicals, polyphenols, tannins, steroidal saponins, triterpenic saponins, sulphoxides, monoterpenic acids, sesquiterpenic alcohols and diterpene esters (Shibata,1977 ; Ieven *et al*,1979 ; Strack, 1997 ; Bramley, 1997 ; Dübeler, *et al.*, 1997). Many bioactive phytochemicals are photosensitizers, that is, their toxic activities against microorganisms, viruses, cells or insects are dependent on or augmented by light of certain wavelengths. The activities of photosensitizers have been observed to be selective and this has led to the possibility of their use in the chemotherapeutic control of infectious diseases, pests and cancer. Some of the main classes of photosensitizers are :- polyyines (polyacetylenes) and their thiophene and 1,2-dithiin derivatives, perylene naphtho- and anthraquinones (hypericin, hypocrellins), alkaloids based on tryptamine, phenylalanine and tyrosine or anthranilic acid, furanyl compounds (furocoumarins, furochromones), porphyrins and cinnamate derivatives - lignans, caffeic acids, tannins (Hudson & Towers, 1991 ; Towers *et al*, 1997 ; Hudson & Towers, 1997). Hudson and Towers (1991) also reported that reaction mechanisms usually involve singlet oxygen and radicals, which are thought to cause photodamage to membranes or macromolecules.

3.1.1 Alkaloids

These are nitrogen compounds of which about 5 500 are known. They form the largest single class of secondary plant metabolites (Harborne, 1984) and are usually basic, forming salts with mineral acids. Alkaloids are well known for their clinical usefulness ranging from antimicrobial to vasodilatory activity (Philipson & Anderson, 1987 ; Linskens & Jacobs, 1994). It is known that some of the biological activities of alkaloids are mediated by light (Towers *et al.*, 1997). The angiosperms are particularly rich in alkaloids e.g., the families Berberidaceae,

Leguminosae, Ranunculaceae and Solanaceae (Smith, 1976 ; Wink, 1997). Alkaloids that characterize species of a particular taxon are usually of the same chemical group. The quantity and proportions in plants are under genetic, environmental, seasonal, climatic and soil type influence. The content of these compounds changes as the plant matures and ages (Hegnauer, 1986). They are distributed throughout the tissues existing as salts in the vacuoles and are most concentrated in the storage organs (Smith, 1976).

In plants, alkaloids might have a protective or defence function, by repelling predators and pests due to their poisonous or unpalatable properties, e.g., the ragwort (Asteraceae) which is unpalatable to grazing animals (Smith, 1976). Steroid glycoalkaloids located in the peel of potato tubers have been found to form part of a multicomponent resistance mechanism in tubers (Kuc, 1992). The Amaryllidaceae is well known for alkaloid constituents which possess antitumour, antiviral and other biological activities (Antoun *et al.*, 1993). Indole has been found to inhibit the growth of Gram-negative bacteria:-*Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Escherichia coli* (Kubo *et al.*, 1992a). The cytotoxic alkaloids are invaluable in investigations aimed at finding anticancer drugs. Likhitwitayawuid *et al.* (1993) attributed the cytotoxicity of extracts from the tubers of *Stephania pierrei* (Menispermaceae) to the presence of aporphine alkaloids.

Many pharmacologically active monomeric and dimeric indole alkaloids have also been obtained from the Apocynaceae for the treatment of, among other illnesses, sore throat and fever (Mariee *et al.*, 1988). Also exhibiting antimicrobial properties are alkaloids from *Phyllanthus discoideus* (Euphorbiaceae) (Likhitwitayawuid *et al.*, 1993). Berberine from the dried rhizome of *Hydrastis canadensis* (Ranunculaceae) has been shown to be bacteriostatic at low doses and bactericidal at higher doses (Bruneton, 1995). According to this author, *in vitro*, the latter has also been found to be active against many microbes e.g. *Staphylococcus*, *Streptococcus*, *Salmonella*, *Proteus*, *Vibrio* and it is also a fungicide.

Widespread phototoxic alkaloids include the β-carboline alkaloids which are common in the Rutaceae and Simaroubaceae. They can also be found in the Cyperaceae, Fabaceae,

Polygonaceae, Rubiaceae, Sapindaceae, Passifloraceae, Solanaceae and Zygophylaceae (Hudson & Towers, 1991). The harmine related β-carboline alkaloids found in several species including *Peganum harmala* (Zygophyllaceae) are photosensitizers active against microorganisms, viruses and cells. Sanguinarine, a constituent of *Sanguinaria canadensis* (Papaveraceae), is reported to be phototoxic to microorganisms as well. It occurs together with berberine, another photosensitizing alkaloid, in plant species of the Papaverceae, for example, *Argemone glauca* (Towers *et al.*, 1997). In addition to the β-carbolines and sanguinarine, eudistomins and furanoquinoline dictamine were reported to be phototoxic to miciroorganisms (Hudson & Towers, 1991). The phototoxic isoquinoline alkaloids sanguinarine and berberine occur in at least nine families which include, Berberidaceae, Juglandaceae, Magnoliaceae, Menispermaceae, Ranuculaceae, Rubiaceae and Rutaceae (Hudson & Towers, 1991).

The furanoquinoline dictamine, found in the species of Rutaceae, was shown to have UVA-dependent effects on gram-positive bacteria and yeasts, and on several filamentous fungi such as *Mucor*, *Fusarium* and *Penicillium* species. The benzophenanthrene alkaloid, sanguinarine, was shown to inactivate *E. coli*. These alkaloids were found to be cytotoxic though, at the concentrations used (Hudson & Towers, 1991).

3.1.2 Phenolic compounds

Phenolic compounds are characterized by at least one aromatic ring (C_6) bearing one or more hydroxyl groups. Other than their structural role as cellular support materials, phenolics are of significant ecological importance along with various toxic nitrogen-containing compounds. The phenolics group is divided into a number of compound classes, an account of some of which has been attempted below.

Phenolics have been observed to accumulate in plants as post infection low-molecular-weight compounds (phytoalexins) as a result of microbial attack. Although normally present in plants at low concentrations, phytoalexins rapidly accumulate upon attack (Strack, 1997). Strack (1997) also stated that plants store pre-infection toxins in healthy tissues in the free or

conjugated form, for the protection of the plant against microbial attack. Among the phenolic phytoalexins and toxins, Strack (1997) and Walton (1997) cite hydroxycoumarins and hydroxycinnamates as major contributors to disease resistance in plants. Phytoalexins are examples of naturally occurring organic antimicrobials from the plant kingdom with possible agricultural uses. Such compounds can be synthesized as has been done, for example, with pisatin, rishitin, vignafuran and orchinol. This approach can also be used to prepare analogues closely related to the naturally occurring microbicides. These compounds definitely have the potential to be of agricultural and medicinal significance (Harborne, 1986 ; Wain, 1986). Polyphenols are important in that their antimicrobial properties are a result of complexing with the microbial proteins (Vanden Berghe & Vlietinck, 1991 ; Brantner & Grein, 1994).

Phenolic acids

Both chlorogenic and caffeic acid are widely distributed in plants (Kuc (1992). This author also stated that other widely distributed phenolics in plants include ferulic acid and ρ-coumaric acid which exhibit antifungal properties. Caffeic, chlorogenic, isochlorogenic as well as rosmarinic acids have been reported to be effective in inhibiting the replication of herpes viruses. Caffeic acid sugar esters have been shown to have antiviral, antibacterial as well as antifungal properties (Smith, 1976 ; Harborne, 1984 ; Ravn & Brimer, 1988).

Hydroxycinnamic acids are the most widespread phenylpropanoids important in, among other functions, disease resistance in plants. Hydroxycinnamic acids, being antimicrobial phenolics, are also found in plants together with some coumarin derivatives (Kuc, 1992 ; Strack, 1997). These compounds may also be of interest in plant pathology as natural plant protective agents (Smith, 1976 ; Harborne, 1984 ; Ravn, 1988). Methyl esters of hydroxycinnamic acids are known to be photobiologically active. Methyl-*cis (Z)* and trans *(E)-p*-methoxycinnamate were also reported to photosensitize *E. coli* cells (Towers *et al.*, 1997).

Endogenous compounds in potato peel and those produced at the site of injury have been found to be important in the resistance of tubers to scab and a positive correlation between the amount of chlorogenic acid and resistance to potato scab has been established (Kuc, 1992). Also reported is the fact that caffeic acid and oxidation products of chlorogenic and caffeic acids were produced after infection or tissue injury and that these products were even more fungitoxic, though transitory, than the parent phenols.

Lignans

Lignans are phenolic compounds widely distributed in the plant kingdom, including many medicinal plants. They accumulate in many parts of the plant, especially in wood, bark of trees as well as in resin as soluble components and some of them as glycosides. Lignans are dimers of cinnamic acids or their derivatives and some have been found to exhibit antiviral properties e.g., podophyllotoxin from *Podophyllum* species. The resin and extracts of this plant are used against Herpes viruses in the treatment of warts. More than 200 lignans have been identified. Antifungal activity of these compounds has also been reported (Hudson, 1994; Strack, 1997 ; Hudson & Towers, 1999).

Flavonoids

Flavonoids are phenolic compounds found mainly in cell vacuoles of higher plants. They constitute one of the largest groups of naturally occurring phenols and are excellent taxonomic markers. Flavonoids occur virtually in all plants and plant parts namely in leaves, roots, wood, bark, flowers and seeds and then mainly as flavonoid-O-glycosides (Markham, 1982 ; Harborne, 1984 ; Taniguchi *et al.*, 1993 ; Strack, 1997). They include, among other phenolics, anthocyanidins, flavones, flavonols, flavonones, chalcones, aurones as well as biflavonyls (Smith, 1976). Among the biological effects of flavonoids are antiinflammatory, antimicrobial, antioxidant as well as antiviral properties (Marston, *et al.*, 1984 ; Krol *et al.*, 1994 ; Rabe *et al.*, 1994 ; Aquino *et al.*, 1995 ; Müller *et al.*, 1998). *Thymus vulgaris* (Lamiaceae) essential oil is

rich in phenols and displays antibacterial and antifungal properties readily *in vitro*. Also exhibiting antiinflammatory activity is *Chamaemelum nobile* (Asteraceae).

Several isoflavonoids are formed as phytoalexins when species of Leguminosae are stressed or under fungal attack (Philipson & Anderson, 1987 ; Bruneton, 1995). Wang *et al.* (1989) reported that in a preliminary screening for biological activity, extracts from the leaves of *Psiadia trinervia* (Asteraceae) were found to be active against the plant pathogenic fungus *Cladosporium cucumerinum* as well as the Gram-positive bacterium *Baccillus cereus*. The use of flavonoids as substituents for conventional fungicides in the prevention of plant diseases has been considered by a number of authors. Along these lines, work was carried out by Weidenborner *et al.* (1989) to investigate the antifungal activity of isoflavonoids against the storage fungi of the genus *Aspergillus*. The findings of this study showed that isoflavonoids do possess antifungal activity towards this genus. Flavonone phytoalexins have also been observed to inhibit the growth of cariogenic bacteria, including *Streptococci* and *Lactobacilli* (Tsuchiya *et al.*, 1996).

Quinones

Many photosensitizing quinones are distributed among the higher plants and they have antimicrobial and antiviral activities that are enhanced in light. These are perylene naphtho- and anthraquinones. Hypericin and pseudohypericin, are the red anthraquinones found in *Hypercicum* species (Guttiferae), as well as the extracts from these species were shown to possess antibiotic activities (Hudson & Towers, 1991). These authors also reported that these two compounds are phototoxic to Gram-positive bacteria in visible light. Hypericin, although, has been observed to cause haemolysis of erythrocytes in the presence of ultra violet (A) radiation.

Tannins

These are complex polyphenolic compounds that occur widely in vascular plants. They usually exist as heterogenous collections of complex molecules that may be conjugated to various sugars. As a result, their structures and biological activities may be modified during extraction, leading to difficulties in their characterization. In angiosperms they are associated with woody tissues e.g. the bark of trees. The condensed tannins (flavolans) occur in ferns, gymnosperms and woody species of angiosperms. Hydrolyzable tannins are limited to a few families of dicotyledonous plants (Harborne, 1984). Tannic acid has been shown to inhibit the tobacco mosaic virus apparently due to a reaction between the virus and the polyphenol (Smith, 1976 ; Hudson & Towers, 1999). Some of the tannins are reported as possessing weak antiviral activity Hudson & Towers (1999).

3.1.3 Terpenoids

Terpenoids are isoprenoid compounds best known from higher plants but can also be found in some fungi and bryophytes. They are generally lipid soluble and are located in the cytoplasm of cells in higher plants, though some terpenoids in the form of essential oils are sometimes stored in special glandular cells of the leaf surface (Harborne, 1984). Many terpenoids have been found to exhibit antibiotic activity. Despite being one of the largest and most diverse group of plant secondary metabolites, few terpenoids exhibit photodynamic biological activity (Towers *et al.*, 1997). Variations in terpenoid content in plants are under genetic and temperature control as well as being influenced by altitude and mineral status of the soil. Terpenoids are classified according to the number of isoprene units from which they are derived, e.g., monoterpenes (2), sesquiterpenes (3), diterpenoids (4), triterpenes (6) (Bramley, 1997).

Monoterpenoids and sesquiterpenoids

Monoterpenoids and sesquiterpenes are found in many essential volatile oils. Particularly rich in these oils are the Asteraceae, Labiatae, Myrtaceae, Pinaceae, Rosaceae, Rutaceae, Umbelliferae and others (Bramley, 1997).

Kubo *et al.* (1992a) reported on the antimicrobial activity of the ten most abundant volatile components of green tea flavour. They observed that most of them inhibited the growth of *Streptococcus mutans*, one of the most important cariogenic bacteria. Nerilidol, a sesquiterpene, was found to be the most powerful while linalool, a monoterpene, was the least potent. These authors further stated that the activity of the sesquiterpene hydrocarbons δ-cadinene and caryophyllene against the dermatomycotic bacterium, *Propionobacterium acnes* was enhanced by indole, an alkaloid. Some sesquiterpenes act as active principles in numerous herbal plants, e.g. bisabolol, the antiinflammatory compound obtained from chamomile, *Chamomila recutita* (Smith, 1976 ; Philipson & Anderson, 1987). The phytochemical gossypol from *Gossypium* species is an aromatised bis-sesquiterpene exhibiting antiviral activity against viruses with membranes (Hudson, 1994).

Antifungal properties have been demonstrated for the sesquiterpene ipomeamarone in *Ipomoea batata* (sweet potato), and the compound is synthesized in quantity when the plant is attacked by the black rot fungus *Ceratocystis fimbriata*. Rishitin is another terpenoid phytoalexin produced by *Solanum tuberosum* when attacked by the fungus *Phytophthora infestans* (Smith, 1976 ; Philipson & Anderson, 1987). The sesquiterpenoid phytoalexin, 2,7-dihydroxycadelene was reported to be bactericidal towards *Xanthomonas campestris* in the presence of light (Hudson & Towers, 1991).

Iridoids are monoterpene cyclopentane lactones and they are active principles of a number of herbal plants used as skin and wound treatments. These compounds are found almost exclusively in the dycotyledonous plants, mostly as glycosides (Bramley, 1997). Biological

activities of iridoids include, among others, antibacterial activity. Bignoniaceae is one of the rich sources of iridoid glycosides (Philipson & Anderson, 1987 ; Iwagawa *et al.*, 1990).

Diterpenoids

Diterpenoids occur as bitter principles and as resin acids. They are of limited distribution, the only universally distributed one being phytol which forms part of the chlorophyll molecule. The seeds, leaves, wood bark and roots of about 15 species of the gymnosperm genus *Podocarpus* have been found to have diterpene dilactones that exhibit antitumour as well as antifungal properties (Kubo *et al.*, 1991).

Kubo *et al* (1992b) again reported the antimicrobial activity of six diterpenoids isolated from the bark of *Podocarpus nagi* (Podocarpaceae). They observed that totarol, one of these terpenoids, exhibited potent bacterial activity against Gram-positive bacteria:- *Propionobacterium acnes, Streptococcus mutans, Bacillus subtilis, Brevibacterium ammoniagenes* and *Staphylococcus aureus*. The aglycone of aucubin, a diterpenoid, which occurs widely in the Cornaceae and Scrophulariaceae, has also been found to have antimicrobial properties towards bacteria, yeasts and moulds (Davini, *et* al., 1986).

The Aristolochiaceae, a family of about ten genera and 600 species which are known to posses medicinal properties, are also a source of diterpenoids. Some species including *Aristolochia triangularis* are used in the treatment of wounds and skin diseases (Jones *et al.*, 1987 ; Lopes *et al.*, 1990). Lopes *et al.* (1990) also extracted diterpenoids, lignans and allantoin from *A. triangularis.*

Triterpenoids

The triterpenoids can be divided into four groups: the true triterpenes, steroids, saponins and cardiac glycosides. Saponins and cardiac glycosides are triterpenes or steroids which occur mainly as glycosides. Triterpenoids also form active components of several medicinal plants.

Some triterpenes have bitter tastes e.g. limonin, the lipid soluble bitter principle of *Citrus* fruits. It belongs to a series of bitter pentacyclic triterpenes- the limnoids and the quassinoids (Harborne, 1984).

Quassinoids are produced by degradation and rearrangement of triterpenes by species of Simaroubaceae, while the limnoids from the related families Rutaceae and Meliaceae are produced in a similar way too. The quassinoids have been found to posses stronger antiviral, antiinflammatory, antimalarial and amoebicidal activities than the limnoids (Harborne, 1984 ; Philipson & Anderson, 1987). Li *et al.* (1993) reported a new triterpene, suberosol, from the leaves and stem of *Polyalthia suberosa* (Annonaceae). They observed that suberosol has significant anti-HIV activity, raising hope for a discovery of an AIDS cure. Antiviral as well as antiinflammatory activity has also been noted with some triterpene esters as well (De Tommasi *et al.*, 1992). Triterpene lactones from the root bark of representatives from the Pinaceae are also known to exhibit antifungal properties (Chen *et al.*, 1993).

3.1.4 Polyynes (Polyacetylenes)

Polyynes are derived from the desaturation and chain shortening of fatty acids. The sulphur derivatives of the plyacetylenes include thiophenes and 1,2-dithiin (thiarunines) derivatives. Thiophenes are widely distributed in the Asteraceae as mono-, bi- or terthiophenes with a variety of side chains (Hudson & Towers, 1991 ; Hudson & Towers, 1999). They are found in the flowers, leaves, stems and roots. Hudson and Towers (1991) also reported that more than 700 polyyines have been isolated from plants, mainly in the families of Asteraceae, Apiaceae and Campanulaceae. The naturally occurring polyyne, 2-chlor-3,11-tridecadiene-5,7,9-triyn-1-ol was reported to be phototoxic to *Escherichia coli* under anaerobic as well as aerobic conditions (Towers *et al.*, 1997).

The thiophene α-terthienyl from *Tagetes* species (Asteraceae) is a plant photosensitizer shown to generate oxygen (Towers *et al.*, 1997). Caceres *et al.* (1993a) reported that *Tagetes lucida* exhibited activity against a number of enterobacteria. A number of strains of *Salmonella typhi*

and *Pseudomonas aeruginosa* were also inhibited *in vitro*. Also reported by Caceres *et al.* (1993a) was the fact that *T. lucida* contains thiophene derivatives among other compounds.

Thiarubrines are red polyynes isolated from the Asteraceae. These molecules are characterized by 1,2-dithiin or 1,2-dithiacyclohexa-3,5-diene ring coupled to two polyyne side chains at the 3 and 6 positions (Towers *et al.*, 1997). Thiarubrines are very active against pathogenic bacteria, fungi (including anti-*Candida*) activities in the dark. Thiarubrines absorb significantly in both the UVA and the visible light regions (Hudson & Towers, 1999).

3.1.5 Coumarins

The hydroxycoumarins umbelliferone, aesculetin and scopoletin were reported to exhibit antifungal properties (Kuc, 1992). This author further reported that umbelliferone, aesculetin and scopoletin are frequently found in glycoside forms which are hydrolyzed after tissue damage. Furocoumarins, are common constituents of many species of for example, the Rutaceae and Apiaceae (Towers *et al.*, 1997). Furocoumarins were also reported to exhibit phototoxicity to bacteria, among other organisms used in the investigation. Hudson and Towers (1999) stated that the furocoumarins are commonly phototoxic in UVA-light to bacteria, fungi, viruses and cells. These authors report that the potential disadvantage of furocoumarins is their phototoxicity to human cells.

3.1.6 Chromones

Furochromones like furocoumarins, are common constituents of many species of for example, the Rutaceae and Apiaceae. Their concentrations can be up to 1% by dry weight in plant tissues. They are normally phototoxic to bacteria, fungi, viruses as well as cells in UVA light (Hudson & Towers, 1999). The furochromones vinagin and khelin often occur in association with the coumarins, for example in species of *Ammi* (Apiaceae). Extracts of these plants and their purified compounds have a long history of use for medicinal purposes (Hudson & Towers, 1991).

3.2 Ethnopharmacology

To conclude this section, a brief summary of the ethnopharmacology of southern Africa is worth mentioning. The plants used in ethnopharmarcology against microbial infections could also be tested against plant pathogens. A number of plant families have provided popular remedies for a long time in southern Africa.

According to Watt and Gerdina (1932), *Acorus calamus* (Araceae) has its leaves used as a diarrhoea remedy while the rhizome decoction is used in chest infections. These authors further stated that some species of the genus *Asparagus* are used in the treatment of tuberculosis, for example, *Asparagus capensis, A. plumosus* and *A. striatus. Oxalis semiloba* (Oxalidaceae) is used as a topical treatment of fungal infections of the skin. Also for the treatment of skin diseases according to Watt and Gerdina (1932), are the twigs of *Leonitis leonurus* (Labiatae). A paste of the leaves of *Zizyphus mucronata* (Rhamnaceae) is applied to boils or slow healing wounds while the leaf infusion is used for colds, indicating the antimicrobial nature of the extracts (Watt & Breyer-Brandwijk,1962 ; Hutchings *et al.*,1996). Roberts (1992) and Hutchings *et al.* (1996) reported that the leaves of the following plants also exhibit these properties: *Matricaria globrata* (Asteraceae) is used for colds, bronchitis and coughs, while a leaf tea of *Lippia javanica* (Verbenaceae) cures coughs and fevers. These authors also mentioned that the leaf infusion of *Olea europeae* (Oleaceae) is generally used as a cure for sore throat. They further stated that the stems as well as leaves of *Lantana rugosa* (Verbenaceae) are used on sores, festering wounds and rashes. Also exhibiting antimicrobial properties are the leaves of *Adonsonia digitata* (Bombaceae) (Roberts, 1992).

Ethnopharmacological uses and information transfer from traditional healers to phytochemists have triggered the isolation, purification and identification of active substances from naturally occurring plants (Farnsworth, 1990). Currently used methods for separating and identifying active compounds arose in the 1980s from groups working on crude plant extracts and the chromatographic fractionation thereof. Crude extracts have been shown to perform well in *in vitro* assays, with the use of appropriate controls (Hamburger & Hostettmann, 1991).

According to these authors, the crude extract obtained is firstly tested for bioactivity. Fractionation of the crude extract is subsequently performed only when the dose-dependent effects are observed. The fractions from a chromatographic separation are retested and one that shows increased biological activity with respect to the original extract is chromatographed again. Hamburger & Hostettmann (1991) also mentioned that the process is repeated until the pure active compounds are isolated. Some plants though have been found to be more active as crude extracts than as purified compounds (Lozoya, 1994). This could be due to a synergy of effects, such that the pharmacological activity declines as purification proceeds (Barton & Ollis, 1985).

3.3 Bioactive plant derived compounds in medicine

The study of plants with antimicrobial properties can be used to improve traditional medicine which is practiced in many developing countries (Sindiga *et al.*,1995). Antibiotic activity is common among extracts of higher plants and therefore there is a high probability of finding chemotherapeutic agents (Ieven *et al.*, 1979). Use of traditional medicines through incorporation into national health care systems has been encouraged by the World Health Organization in the past 20 years, especially in the developing countries. This came up because the current health care systems were not coping with the existing levels of morbidity and mortality (Sindiga *et al.*, 1995).

One of the main causes of morbidity and mortality in Africa and the developing world in general, is respiratory infections such as pneumonia, tuberculosis and whooping cough, as well as water-borne diseases such as dysentery, typhoid and cholera caused by the enterobacteria. Enterobacteria have been showing increased resistance to some common antibiotics, thereby necessitating the need for, among other things, new antibiotics (Caceres *et al.*, 1993a). Fungal (mycotic) infections are also of concern as they are widespread in the tropical and subtropical countries, affecting mostly the skin and occasionally also internal organs (Caceres *et al.*, 1993b). Dermatophyte infections are chronic and require prolonged treatment with antimycotic drugs that are expensive and sometimes ineffective (Caceres *et al.*, 1991). At the same time,

health services are not accessible to everyone due to the large public and private health expenditure of between six and ten percent of the gross national product in many countries.

An approach formulated by the UNICEF and WHO since 1975, for the utilization of the community's local resources to provide primary health care, meant the inclusion of traditional practitioners in health care. The important role of medicinal herbs in health care systems of many developing countries was then highlighted at the World Health Assembly, under the WHO in 1978, while the Alm Ata Conference recommended that governments give priority to the use of traditional medicines in national drug policies and regulations. In 1987, at the 40th World Health Assembly, the resolutions and recommendations regarding the use of traditional herbal remedies were further reaffirmed (Sindiga *et al.*, 1995).

Traditional herbal remedies have a lot of support because of the following reasons : (a) herbal remedies are an integral part of many cultures and have been developed over many years, hence their effectiveness in curing many diseases, (b) they are socially acceptable (c) they produce the desired effect and are also wholesome, containing among other nutrients, minerals and vitamins (d) they are affordable and more readily available and (e) they are relatively safe at non-toxic doses.

The current problems with traditionally used plant drugs is that the doses are not measured and sometimes the side effects of combinations of some herbal remedies are not known. There is a need to investigate these plant remedies since all medicines must satisfy the following conditions: identity, safety and efficacy.

In modern medicine, plant derived bioactive molecules are used in four basic ways: (a) as sources of direct therapeutic agents; (b) as raw material for the preparation of more complex semi-synthetic drugs; (c) the chemical structures of plant metabolites can be used as models for new synthetic compounds; (d) plant metabolites can be used as taxonomic markers to establish the relationships between groups of plants and to forecast the presence of biologically

interesting compounds in families and genera (Aquino *et al.*, 1995). Logically, this can also be applied to the use of plant derived agrochemicals.

Literature on the possibilities of use of plants as sources of bioactive compounds with antimicrobial properties for controlling human pathogens is increasing all the time.

3.4 Some medically important microorganisms

Human pathogens can be broadly classified under viruses, bacteria and fungi. The presence of large numbers of microbes in the blood stream can lead to septicaemia and death. If this is survived, multiple lesions in various organs may develop. The localization of these lesions depends on the nature of the pathogen. Complete removal of the organisms from the blood stream by the reticulo-endothelial system, without the development of any secondary lesions, can occur spontaneously, but such an outcome is much more common now that the pathogens may be opposed by the lethal or inhibitory concentrations of antimicrobial drugs (Turk & Porter, 1969 ; Brock & Madigan, 1991).

A brief account of the microorganisms used in this study, the ailments they cause as well as plant species that show potential activity against them has been attempted below.

3.4. 1 Human pathogenic bacteria

Gram-positive cocci

Some of these cocci cause respiratory ailments which are important causes of morbidity and mortality in developing countries (Caceres *et al.*, 1991a).

The genus *Staphylococcus*

These are aerobic or facultatively anaerobic non-motile cocci found associated with the skin, skin glands and mucous membranes. *Staphylococcus aureus* may cause boils, abscesses, meningitis, osteomyelitis and food poisoning and is also a cause of respiratory infections (Turk & Porter, 1969 ; Caceres *et al.*, 1991a ; Brock & Madigan, 1991). Methicillin resistant *Staphylococcus aureus*, multi-resistant to various antibiotics, has been emerging worldwide as one of the major nosocomial pathogens.

It has been challenged with phytoalexins *in vitro* and with significant success (Tsuchiya *et al.*, 1996). The aqueous extract of *Asclepias curassavica* (Asclepiadaceae) was found to be active against *S. aureus* (Ieven *et al.*, 1979). At the same time, these authors observed that the aqueous extract of *Diospyros lotus* (Ebenaceae) as well as the aqueous, methanol and dichloromethane extracts of *Rhus tomentosa* (Anacardiaceae) were active against *S. aureus*.

El-Abyad *et al.* (1990) observed that the benzene and chloroform extracts of *Chenopodium murale* (Chenopodiaceae) and the alcohol extract of *Capsella bursa-pastoris* (Cruciferae) were active against *S. aureus* as well.

In a screening exercise, Caceres *et al.* (1991a) observed that ethanolic leaf extracts from *Thymus vulgaris* (Labiatae), *Buddleja americana* (Loganiaceae), *Eucalyptus globulus* (Myrtaceae), *Plantago major* (Plantaginaceae), *Theobroma cacao* (Sterculiaceae) as well as *Lippia alba* (Verbenaceae) were active against *S. aureus*. According to the authors, all these plants are used traditionally in Guatemala for the treatment of colds, coughs, bronchitis, sore throat and in the case of *P. major*, tuberculosis as well.

Naqvi *et al.* (1991) observed that the aqueous and the ethanolic extracts of *Lavandula stoechas* (Labiatae) and the same extracts from the leaves of *Ocimum sanctum* (Labiatae) significantly inhibited the growth of *S. aureus*.

A number of *Helichrysum* species (Asteraceae) are known to possess antimicrobial activity. The dichloromethane and chloroform extracts of *Helichrysum stoechas* also exhibited antimicrobial activity towards *S. aureus* (Rios *et al.*, 1991). In a study of antimicrobial activity of numerous *Helichrysum* species against a number of human pathogens, Mathekga and Meyer (1998) observed that the acetone extracts from the shoots of *H. callicomum*, *H. hypoleucum*, *H. odoratissimum* and *H. rugulosum* inhibited the growth of *S. aureus* among other microorganisms. Heisey and Gorham (1992) reported that the root bark extract (methanol/dichloromethane ; 1:1) of *Rhus glabra* (Anacardiaceae) also inhibited the growth of *S. aureus*.

Staphylococcus epidermidis is usually found on human skin and on mucous surfaces. It is normally non-pathogenic but can be responsible for the pathogenesis of acne and other minor skin lesions. *Staphylococcus epidermidis* is also reported to be one of the causes of bacterial endorcarditis (Turk & Porter, 1969 ; Brock & Madigan, 1991).

Hernandez-Perez *et al.* (1994) observed that the infusion, acetone and methanol leaf extracts of *Visnea mocanera* (Theaceae) were active against *S. aureus* as well as *S. epidermidis*, among other bacteria they tested. Braghiroli *et al.* (1996) observed that the aqueous extract of *Calluna vulgaris* (Ericaceae) inhibited the growth of *S. epidermidis*. The leaf extracts of *Tagetes minuta* (Asteraceae), used in Argentina for the treatment of stomach and intestinal diseases, were found to be active against *S. epidermidis* as well as *S. aureus*, among other bacteria (Tereschuk *et al.*, 1997).

The genus *Streptococcus*

The *Streptococci* are Gram-positive cocci and form part of the bacterial flora of the respiratory and alimentary tract (Turk & Porter, 1969 ; Clancy, 1974). The three clinically important groups are 1. Haemolytic streptococci 2. Viridans *streptococci* 3. *Enterococci* (Clancy, 1974).

Haemolytic *Streptococci*

Potentially pathogenic *Streptococcus pyogenes* species are carried, mainly, in the throats of healthy people. The commonest infection is acute sore throat, often involving inflammation of the tonsils and cervical lymphadenitis. This infection may spread to the middle ear and even meninges. Scarlet fever is also a *Streptococcus pyogenes* infection, usually of the throat. If untreated, *S. pyogenes* infections spread into the lymphatic system and into the blood stream causing septicaemia. *Streptococcus pyogenes* is also a another cause of respiratory infections especially in children and immuno-compromised individuals (Turk & Porter, 1969 ; Caceres *et al.*, 1991a). This organism is very sensitive to penicillin though (Turk & Porter, 1969). The problems arise when the patient is allergic to penicillin demanding the need for an alternative antibiotic.

Naqvi *et al.* (1991) observed that the aqueous root extract of *Convolvulus arvensis* (Convolvulaceae) was significantly active against *S. pyogenes*. These authors also reported that the aqueous and ethanolic leaf extracts of *Ocimum sanctum* (Labiatae) exhibited significant activity against *S. pyogenes*, while the ethanolic leaf extract of *Vitex negundo* (Verbenaceae) exhibited slight inhibition of the growth of the same bacterium.

Caceres *et al.* (1991a) reported that the leaf extract of *Satureja brownei* (Labiatae) was active against *S. pyogenes*. They also noted that the leaf extract of this plant is traditionally used as a cure for coughs, sore throat and sinusitis. These authors maintained that the leaf extract from *Matricaria recutita* (Asteraceae) as well as its inflorescence extract inhibited the growth of *S. pyogenes*. Also observed to be active against *S. pyogenes* were the inflorescence and leaf extracts of *Salvia officinalis* (Labiatae). According to Caceres *et al.* (1991a) *S. officinalis* is traditionally used in Guatemala as a cure for asthma, coughs, fever and sore throat.

Viridans *Streptococci*

Streptococcus pneumoniae are Gram-positive diplococci, the common cause of lobar pneumonia. These bacteria are found in the upper respiratory tract of healthy carriers. In the respiratory tract, *pneumococci* maintain and aggravate chronic diseases of the bronchi and paranasal sinuses. Other diseases caused by this species include otitis media, meningitis and peritonitis. *Pneumococci* are penicillin sensitive.

While screening selected plant species for antimicrobial activity, Caceres *et al.* (1991a) observed that the ethanolic leaf extract of *Mangifera indica* (Anacardiaceae), inhibited the growth of *S. pneumoniae*. The bark, bud, flower, fruit, and leaves of this plant are traditionally used in Guatemala for the treatment of bronchitis, coughs, colds, fever, sore throat as well as whooping cough.

The ethanolic leaf extract of *Acacia hindsii* (Fabaceae) was found to be active towards *S. pneumoniae* by Caceres *et al.* (1991a). This plant is traditionally used in Guatemala as a cure for coughs and fever. The authors also reported that the ethanolic leaf extract of *Cecropia obtusifolia* (Moraceae) was active against *S. pneumoniae*. The bud and leaves are traditionally used in Guatemala as a cure for asthma and whooping cough. Caceres *et al.* (1991a) also observed that the ethanolic leaf extract of *Psidium guajava* (Myrtaceae) was active against *S. pneumoniae*. According to these authors, the flowers, fruit and leaves of this plant are traditionally used as a cure for bronchitis, colds, coughs, fever, sore throat and tuberculosis. In the same series of investigations by these authors, the leaf extracts of *Lippia alba* (Verbenaceae) as well as that of *Lippia dulcis* were active against *S. pneumoniae* too. The flowers and leaves of *L. alba* are used as a cure for bronchitis, coughs, colds, fever, sore throat and chest problems. The flowers and leaves of *L. dulcis* are used as a cure for asthma, bronchitis, coughs and colds. Also reported by Caceres *et al.* (1991a), was that the fruit extract of *Physalis philadelphica* (Solanaceae) was active against *S. pyogenes* and *S. pneumoniae*, among other bacterial species. This plant is also used traditionally as a cure for bronchitis, colds and sore throat.

Gram-negative cocci

The genus *Moraxella*

Moraxella catarrhalis is an opportunistic pathogen found in the upper respiratory tract of humans. In healthy people, it is a commensal of the same habitat (Clancy, 1974 ; Collins *et al.*, 1989).

Deans and Svoboda (1990) reported that the essential oil of *Origanum majorana* markedly inhibited the growth of *Moraxella* species among other bacteria and fungi. The *Moraxella* species were also found to be sensitive to methanolic extracts from eight *Pelargonium* species (Lis-Balchin & Deans, 1996).

The genus *Acinetobacter*

Acinetobacter belong to the family Neisseriaceae together with the genus *Moraxella.* Just like the *Moraxella*, the *Acinetobacter* are Gram-negative cocci. They are found on the skin. Some species of *Acinetobacter* have been implicated in nosocomial infections. In our screening of plant extracts, *A. baumanii* was used.

Lis-Balchin and Deans (1996) stated that extracts from a number of *Pelargonium* species (Geraniaceae) inhibited the growth of *Acinetobacter calcoacetica*. These species were *P. zonale, P. inquinans, P. capitatum, P. acraem, P. scandens* and *P. hybridum*.

Gram-positive bacilli

The genus *Bacillus*

Most *Bacillus* species are regarded as having little pathogenic potential. However, *B. subtilis* has been known to act as a primary invader, or secondary infectious agent in a number of

diseases. This bacterium has also been implicated in food poisoning (Brantner & Grein, 1994 ; Mathekga & Meyer, 1998).

El-Abyad *et al.* (1990) reported that the benzene and chloroform extracts of *Ammi visnaga* (Apiaceae), the ether extract of *Anagallis arvensis* (Primulaceae), the benzene, ether and chloroform extracts of *Capsella bursa-pastoris* as well as the benzene and ether extracts of *Chenopodium murale* (Chenopodiaceae) were significantly active against *B. subtilis*. Also exhibiting significant activity towards this bacterium as observed by these authors was the ether extract of *Euphorbia geniculata* (Euphorbiaceae).

The alkaloid fraction of from the stem bark of *Ziziphus nummularia* (Rhamnaceae) also inhibited the growth of *B. subtilis* (Shah *et al.*, 1990). These authors reported that the bark of this plant is used in traditional medicine as a gargle for sore throat in Saudi Arabia.

Gundidza *et al.* (1993) stated that the essential oil of *Eucalyptus maidenii* (Myrtaceae) exhibited significant antibacterial activity against a number of bacteria that included *B. subtilis*.

Taniguchi and Kubo (1993) observed that the bark extract of *Hunteria zeylanica* (Apocynaceae) was active against *B. subtilis*. Active against this bacterium according to these authors was the leaf extract of *Hagenia abyssinica* (Rosaceae) as well as the root bark extracts of *Teclea trichocarpa* (Rutaceae) and *Indigofera paniculata* (Fabaceae).

Meyer & Afolayan (1995) detected antibacterial activity in *H. aureonitens* against *B. subtilis*. *Pelargonium* species (Geraneaceae) have also been found to be active against *B. subtilis* (Lis-Balchin & Deans, 1996).

The plant *Vahlia capensis* (Vahliaceae) was found to be active against *B. subtilis*. This plant is widely used in Botswana and also in Zimbabwe to treat eye infections. Vahlia biflavone and gallic acid were isolated and proved to be active against *B. subtilis* (Majinda *et al.*, 1997).

Mathekga & Meyer (1998) observed that the epicuticular extracts of a number of *Helichrysum* species were active against *B. subtilis*. Thy reported that the acetone shoot extracts of *H. callicomum, H. hypoleucum, H. odoratissimum* and *H. rugulosum* exhibited strong antibacterial activity against this bacterium.

Gram-negative bacilli

Enterobacteria

The Gram-negative enterobacteria are usually found in the intestine as commencals or as pathogens (Clancy, 1974).

The genus *Escherichia*

Escherichia coli is one of the species that form the normal flora of the lower part of the human intestine. A few antigenic types cause gastroenteritis in infants and young children. *E. coli* are involved in urinary tract infections, wound infections and intra-abdominal abscesses. Many of the *E. coli* strains are resistant to antibiotic treatment with ordinary levels of penicillins but may be sensitive to others (Brock & Madigan, 1991). Due to its resistance to antibiotics, *E. coli* is commonly used in antibacterial screening of plant extracts (Anesini & Perez, 1993 ; Brantner & Grein, 1994).

Ieven *et al.* (1979) found that he aqueous extract of *Asclepias curassavica* (Asclepiadaceae) possessed slight activity towards *E. coli*. Higher activity was observed towards this bacterium by these authors with the aqueous extract from *Rhus tomentosa* (Anacardiaceae), as well as with the aqueous, methanol and dichloromethane extracts from *Euphorbia neriifolia* (Euphorbiaceae).

El-Abyad *et al.* (1990) while screening plant extracts for antimicrobial activity made the following observations. The benzene, ether and chloroform extracts of *Capsella bursa-pastoris*

were all very active against *E. coli*. The alcohol extract of *Ammi visnaga*, the chloroform extract of *Euphorbia helioscopa* (Euphorbiaceae), the ether extract of *Plantago major* and the benzene extract of *Vicia sativa* (Fabaceae) also exhibited significant antimicrobial activity towards *E. coli*. The aqueous extract of *Ocimum sanctum* (Labiatae) was active against *E. coli* as well as a number of other organisms (Naqvi *et al.*, 1991).

The results of tests for antimicrobial activity in a number of extracts (Caceres *et al.*, 1993a) revealed that the methanol extract of the leaves of *Spondias purpurea* (Anacardiaceae) was the most active against enteropathogenic *E. coli*. Also exhibiting significant activity was the methanol leaf extract of *Psidium guajava* (Myrtaceae), *Diphysa robinioides* (Fabaceae) and *Lippia dulcis* (Verbenaceae) as indicated by large zones of inhibition. According to Zavala *et al.* (1997), the growth of *E. coli* was significantly inhibited by the methanol extract of *Eysenhardtia polistachia* (Fabaceae).

Among the organisms used by Mathekga and Meyer (1998) in the screening of *Helichrysum* species (Asteraceae) for antimicrobial activity was *E. coli*. Only the acetone shoot extract of *H. hypoleucum* was found to possess strong antibacterial activity against *E. coli* by these investigators. Methanolic extracts from *Pelargonium* species used to challenge bacteria were also active against *E. coli*. At the same time, Isogai *et al.* (1998) reported that the ethanolic extract of Japanese green tea inhibited the growth of this bacterial species.

The genus *Klebsiella*

This genus is also made up of Gram-negative bacilli. *Klebsiella pneumoniae* was one of the bacterial species used in this investigation. *K. pneumoniae* causes pneumonia, meningitis, otitis and sometimes sinusitis. Pandey and Devi (1990) stated that cyclopeptide alkaloids from Indian *Zizyphus* species *Z. rugosa*, *Z. nummularia* and *Z. xylopyra* significantly inhibited the growth of this bacterial species.

Gundidza *et al.* (1993) observed that the essential oil of *Eucalyptus maidenii* (Myrtaceae) possessed significant antibacterial activity against, among other organisms, *K. pneumoniae*. Also exhibiting antibacterial activity towards *K. pneumoniae* and other microorganisms, were essential oils from *Ziziphora taurica* ssp *cleonioides* and *Z. taurica* ssp *taurica*.

Extracts from several *Pelargonium* species were also found to be active against *K. pneumoniae*, among other bacteria (Lis-Balchin & Deans, 1996).

Pseudomonas species

The genus *Pseudomonas*

These are straight or curved rods found in soil and in water bodies. A representative of this species is *Pseudomonas aeruginosa*, a saprophyte inhabiting the intestine. It multiplies at room temperature in, for example, body lotions and eye ointments and usually in infants' incubators and respirators used in hospitals. *P. aeruginosa* can infect wounds, burns, urinary tract and many other places. The major problem with this bacterium is that it is resistant to many antibiotics (Porter & Turk, 1969 ; Mathekga & Meyer, 1998).

A number of authors have challenged *P. aeruginosa* with plant extracts with encouraging results. Ieven *et al.* (1979) observed that the aqueous extract of *Diospyros lotus* (Ebenaceaae) was active against this bacterium. The aqueous, methanol and dichloromethane extracts of *Euphorbia neriifolia* (Euphorbiaceae) as well as the aqueous extract of *Rhus tomentosa* (Anacardiaceae) were found by these authors to be active against *P. aeruginosa*. Mathekga & Meyer (1998) noted that the acetone extract of *Helichrysum hypoleucum* (Asteraceae) was also active against *P. aeruginosa*. Also reported here is that this herb is used traditionally by the Basotho as a cure for chest problems and also as an antibiotic. Extracts from *Pelargonium* species used to challenge other microorganisms were found to possess antibacterial activity towards *P. aeruginosa*.

Parvobacteria

The genus *Haemophilus*

These are Gram-negative facultatively, anaerobic coccobacilli or rods. They are strict parasites and require growth factors of blood, including hemin (X-factor). An example from this group is *Haemophilus infuenzae*, the influenza bacillus, a parasite normally carried in the nasopharynges of healthy people. These are small Gram-negative bacilli which cause paranasal sinusitis, epiglottitis, bronchitis and lobar pneumonia. These infections are generally treated with tetracyclines and ampicillin (Turk & Porter, 1969 ; Clancy, 1974).

3.4.2 Human pathogenic fungi

Fungal infections are very common worldwide and generally do not cause life threatening diseases. Fungi are a problem in that, generally, immunity does not follow infection though histoplasmosis and coccidiomycosis are exceptions. The increasing occurrence of opportunistic systemic mycoses associated with the use of immunosuppressive drugs and AIDS has led to new efforts in search of novel antifungal compounds (Hostettmann & Marston, 1994). Fungal diseases in man are among the most investigated with the use of plant derived extracts (Gordon *et al.*, 1980 ; Heisey & Gorham,1992 ; Irobi & Daramola, 1993 ; Fabry *et al.*, 1996).

The widespread occurrence of dermal infections caused by dermatophytes and the limited number of available drugs which are effective against them, has also led investigators to search for new antimycotic agents. Some of the modern antifungal therapies still cause considerable side effects in some patients. In other cases, resistance to treatment with some drugs is observed. Plant derived compounds are of interest in this context because they comprise safer or more effective substitutes for synthetically produced antimicrobial agents (Caceres *et al.*, 1991b ; Caceres *et al.*, 1993b ; Maoz & Neeman, 1998).

The few species that are pathogenic to human beings fall under the Fungi Imperfecti as their sexual phase is not clear (Turk & Porter, 1969). These medically important fungi will be discussed below according to the diseases they cause. As already stated above, in section 3.3, mycotic infections are a common problem in developing countries causing mainly skin diseases and on occasion, affecting internal organs as well and their treatment can be expensive.

The genus *Candida*

One of the causes of candidiasis, a systemic mycoses, is the yeast-like fungus of the genus *Candida*, a strongly Gram-positive microorganism. As an example, *Candida albicans* is a fungus normally present in healthy people as part of the normal flora of the upper respiratory tract, the skin and the alimentary tract among other organs. *C. albicans* is an opportunistic pathogen that produces a diverse range of mucosal and cutaneous infections as well as subcutaneous infections and systemic mycoses. It becomes pathogenic due to malnutrition, sickness, diabetes and disturbance of normal flora through antibiotic treatment. Drugs currently available to control these species are limited. Hence plant products that inhibit these fungi without harming the host may have potential for use as therapeutic agents (Turk & Porter, 1969 ; Heisey & Gorham, 1992).

Candidiasis is very common among AIDS sufferers. In its pathogenic state, it causes the following conditions: Thrush, a superficial infection of the mucous membranes of the mouth characterized by white patches of pseudomycelium which affects mainly small children, elderly patients and AIDS sufferers; Dermatitis, a condition usually associated with overweight individuals, affects warm, moist skin folds where the skin becomes exudative and itchy ; Bronchial candidiasis is a rare condition in which the fungus attacks the bronchial walls and lungs and may lead to a generalized infection and more serious infection in the internal organs (Turk & Porter, 1969 ; Brock & Madigan, 1991).

The aqueous extract of *Rhus tomentosa* (Anacardiaceae) was found to be active against, among other microorganisms, *Candida albicans* (Ieven *et al.*, 1979). Gordon *et al.* (1980) reported

that the isoflavonoid phytoalexins (+) pisatin from *Pisum sativum* (Fabaceae), (-) phaseolin, from *Phaseolus vulgaris* (Fabaceae), (-) phaseolin isoflavan and the phytoalexins maackian, medicarpin, sativan and vastitol possessed antifungal activity towards *C. albicans*.

El-Abyad *et al.* (1990) observed that the benzene and ether extracts of *Capsella bursa-pastoris*, the chloroform extract of *Plantago major* and the ether extract of *Vicia sativa* significantly inhibited the growth of *Candida albicans* among other micoorganisms. Also possessing this activity was the aqueous extract of the flowers from *Artemisia scoparia* (Asteraceae) as observed by Naqvi *et al.* (1991). Rios *et al.* (1991) noted that the chloroform and dichloromethane extracts of *Helichrysum stoechas* (Asteraceae) were active against *C. albicans* as well.

The results of investiations by Gundidza *et al.* (1993) indicated that the essential oil of *Eucalyptus maidenii* (Myrtaceae) exhibited significant activity against, among other microorganisms, *C. albicans*. Gundidza (1993) also reported that the essential oil of *Artemisia afra* (Asteraceae) was significantly active against *C. albicans*. The essential oils of *Ziziphora taurica* ssp *cleonioides* and *Z. taurica* ssp *taurica* (Rhamnaceae) growing in Turkey were also active against this yeast.

Irobi and Daramola (1993) observed that the ethanolic extracts of leaves and flowers of *Mitracarpus villosus* (Rubiaceae) produced definite antifungal activity towards *C. albicans*. Hiremath *et al.* (1994) also reported that the petrol, chloroform and ethanol successive extracts of *Striga lutea* (Scrophulariaceae) were active against *C. albicans*. The ethanolic extracts of the roots from *Agave lecheguilla* (Agavaceae) were active against *C. albicans* (Verastegui *et al.*, 1996).

Antifungal activity against *C. albicans* was also reported by Zavala *et al.* (1997) with the methanol extracts of *Eysenhardtia polistachia* (Fabaceae) and *Eryngium carlinae* (Apiaceae). In the same series of investigations, these authors reported that the chloroform, methanol and aqueous extracts of *Argemone mexicana* (Papaveraceae), *Cecropia obtusifolia* (Moraceae) as

well as those from *Bacccharis sordences* (Asteraceae) also showed antifungal activity towards *C. albicans*.

The genus *Cryptococcus*

A representative from this genus is the yeast *Cryptococcus neoformans* which causes systemic mycoses known as cryptococcosis. This yeast is found in soil throughout the world. Human infection begins as a cutaneous or pulmonary lesion but usually becomes generalized. The common feature of *C. neoformans* infection is subacute or chronic meningitis. Systemic yeast infections caused by *C. neoformans* are among the most common AIDS-related opportunistic infections. (Turk & Porter, 1969 ; Brock & Madigan, 1991).

The results of experiments by Gordon *et al.* (1980) revealed that the isoflavonoid phytoalexins (+) pisatin from *Pisum sativum*, (-) phaseolin, from *Phaseolus vulgaris*, (-) phaseolin isoflavan and the phytoalexins maackian, medicarpin, sativan and vastitol possessed antifungal activity towards *C. neoformans* as well as towards *Candida albicans*. The ethanolic extracts of roots from *Agave lecheguilla* (Agavaceae) were also active against *C. neoformans* as well as against *Candida albicans* (Verastegui *et al.*, 1996).

The genus *Aspergillus*

Aspergillosis is caused by the saprophytic moulds of the genus *Aspergillus* that grow on stored foods. Some diseases caused by fungi of the genus *Aspergillus* include (a) otomycosis, a chronic condition in which the fungus grows in the external auditory meatus usually of *Aspergillus niger*, and (b) pulmonary aspergillosis, the condition in which the fungus grows in the bronchi as it does in the ears. It may further invade the lung parenchyma (Turk & Porter, 1969).

The alkaloids from the three Indian *Ziziphus* species (Rhamnaceae) used by Pandey and Devi (1990) also exhibited significant activity against *A. niger*. Irobi and Daramola (1993) also

observed that the ethanolic extract of leaves and inflorescence of *Mitracarpus villosus* (Rubiaceae) showed significant activity against *A. niger*, in addition to the activity observed against *Candida albicans*. Matthews and Haas (1993) reported that the rhizomes of *Nelumbo nucifera* (Nelumbonaceae) possessed potent antifungal activity against *A. niger* too. The essential oil of *Eucalyptus maidenii* (Myrtaceae) (Gundidza *et al.*, 1993) and that of *Artemisia afra* (Asteraceae) (Gundidza, 1993) were active against *A. niger* as well as *C. albicans*.

3.5 Bioactive plant derived compounds in agriculture

Humans depend upon plants for their existence. Three-quarters of the total world food supply is drawn from cereal grains (grasses) and today only about 30 plant species make up 90% of mankind's total food supply. Some of the major crops today are: barley, citrus, maize, oats, potatoes, peanuts, wheat, sugarcane, soybean, rice, sorghum and pulses (beans & peas). Despite the improved cultivars and more efficient agricultural techniques, a number of plant diseases which are still a problem are bacterial wilt, downy mildew, ergot of rye, phytophthora root rot, potato late blight, root knot, smuts of cereals and wheat rusts (Lucas *et al.*, 1992).

Plant diseases do their damage in different ways. One major effect of diseases in plants is reduced plant vigour, resulting in decreased yields. The quality of crops produced is also reduced, for example, wheat rust infection reduces the size of grain kernels and lowers carbohydrate content (Lucas *et al.*, 1992).

The losses caused by plant diseases affect a wide range of people, large scale commercial farmers, people growing vegetables for food, people who market food and the consumer (Trench *et al.*, 1992).

It is estimated that by the year 2025, about 83% of the world's population will be living in the developing countries and a large proportion will be in the rural areas. For these people, agriculture is both a way of life and the primary means of earning a living (Swaminathan, 1993). Therefore it was quite appropriate that at the 'Earth Summit' and Global Forum in

Brazil in 1992 the issue of sustainability of food and agriculture was raised as an issue of unparalleled importance (Allen, 1993).

Agriculture also has a major impact on the environment. It threatens it with habitat loss, degradation and pollution (Jones, 1993). Mineral fertilizers as sources of nutrients as well as synthetic pesticides designed to kill insect pests, plant pathogens and weeds constitute two major sources of ecological problems, particularly with their excessive and sometimes inappropriate application (Swaminathan, 1993). Pests, under which the fungi, bacteria and viruses are grouped are a critical problem in agriculture because of the fact that they reduce crop yields (Trench *et al.*, 1992). To eradicate these pests so as to maintain or even improve crop yields, a lot of pesticides have been used and are still being used. These agrochemicals have boosted crop yields but their adverse effects on the environment and on human health cannot be overlooked. In addition to the good they have brought to agriculture, a lot of problems have arisen as well. Pesticide induced ill-health in humans as well as environmental pollution with pesticides and fertilizers, have reached unacceptable levels, threatening not only today's people, but also future generations (Jansma *et al.*, 1993 ; Waibel, 1993 ; Zadoks, 1993). Pesticides affect the natural resources such as plant and insect species, air and water in addition, of course, to human health (Waibel, 1993). It has been found that systematic application of synthetic pesticides often leads to pesticide resistance and the appearance of other diseases formerly unknown (Beautement *et al.*, 1991).

About 20% of the trade in pesticides takes place within the developing countries, which are mostly importers of these products, and often serve as dumping grounds for materials no longer permitted in the developed western countries. The type of pesticide compounds being traded with developing countries also causes concern. In many cases, pesticides of WHO class Ia and Ib that is, extremely or very hazardous pesticides, are imported by developing countries because they cost less (Waibel, 1993).

With increasing awareness of the environmental and health hazards of synthetic pesticides and the realization of the importance of sustainable agriculture, the concept of integrated pest

management is now well understood. Among the integrated pest management methods that are ecologically non-disruptive and stable is the use of plant products (Jayaraj & Rabindra, 1993 ; Steinhauer, 1999). According to these authors, Indian farmers used pesticides of plant origin long before synthetic chemical pesticides were discovered. The use of, for example, *Vitex negundo* (Verbenaceae) and *Azadirachta indica* (Meliaceae) products in India in pest management, both in the field and storage, is quite popular.

The above stated facts, coupled with the heightened public interest in and the demand for quality food products, cleaner environment, protection and preservation of the natural vegetation have prompted researchers to search for alternatives to synthetic pesticides. The potential source to target in this search is, undoubtedly, the plant kingdom, since it is a good storage of bioactive natural compounds that are both biodegradable and renewable. It is predicted that by the year 2000, biological methods of pest control will account for 10 percent of the pesticide market as compared to only one percent in use currently (Nigg & Seigler, 1992).

As it has already been proven that plants are good sources of biologically active compounds that are replaceable as well as biodegradable (chapter 1), we are joining other researchers in trying to find those plant compounds that can be used as natural pesticides with antimicrobial properties against plant pathogens. Use of plant compounds as antimicrobials will benefit mankind in every aspect as health and the environment will be well preserved.

3.6 Some agriculturally important microorganisms

3.6.1 Plant pathogenic viruses

Diseases of plants caused by viruses and virus-like organisms result in enormous economic losses especially in the tropics and subtropics, where ideal conditions for the transmission and survival of the viruses and their vectors exist (Varma, 1993). Unlike bacterial and fungal plant pathogens, viruses and viroids are totally dependent on their hosts. Some viruses are

transmitted from generation to generation of host plants via pollen or seeds or in plant parts of vegetatively reproducing species (Singh *et al.*, 1995b). According to these authors diseases caused by viruses are difficult to diagnose because the symptoms they present are similar to those resulting from other factors, e.g. by nutrient imbalance, insect damage or genetic mutation. Plant and human pathogenic viruses are not going to be included in this research project due to the lack of resources.

3.6.2 Plant pathogenic bacteria

Bacteria are classified into three general types, according to shape: *cocci*, *bacilli* and *spirilli*. Only non-spore forming bacilli are plant pathogens. Phytopathogenic bacteria are, in general, ordinary aerobic bacteria. They live in and around plants in which they cause infectious diseases. Most plants are susceptible to one or more such diseases, which range in economic importance from considerable to negligible (Lucas *et al.*, 1992).

Initial colonization of a plant can result from growth in contaminated soil, contaminated seed, transfer of bacteria in aerosols or raindrops driven by the wind from other infected plants, including weed hosts, and insect hosts in the case of mycoplasmas.

Generally, the aerial and root surfaces of plants are colonized by microbial flora that consist mostly of bacteria. Unlike fungi, only a few bacteria have evolved the ability to invade plants and cause disease. None of the phytopathogenic bacteria is biotrophic in a true sense. Their mode of parasitism is primarily necrotrophic except in root nodules forming *Rhizobium* species, where a symbiosis exists. Plant pathogenic bacteria induce symptoms like bacterial wilt, soft rot, blight, leaf spot, gall, malformation, mosaic, leaf scorch, yellows and rosette. (Daniels, 1992 ; Singh *et al.*, 1995a ; Perombelon & Salmond, 1995 ; Lucas *et al.*, 1992). Some of the major plant bacterial diseases are outlined below.

Soft rots

Soft rots in plants are caused by microorganisms that produce pecteolytic enzymes that dissolve the pectic substances which bind the cells together, particularly in the softer tissues. This leads to a falling apart of the cells and their destruction. The tissue becomes a mass of soft rot (Buchanan & Buchanan, 1956 ; Lucas *et al.*, 1992).

Bacterial wilts and blights

This disease is caused by the microorganisms invading the vascular system of the plant and preventing the flow of water and nutrients, resulting in the desiccation of the tissues being supplied (Buchanan & Buchanan, 1956 ; Daniels, 1992).

The blights resemble the wilts in some ways. The organisms penetrate through the intercellular spaces and vessels and cause the adjacent cells to die (Buchanan & Buchanan, 1956).

Galls

Galls are hypertrophies produced by organisms that cause the plant to develop new tissue rapidly at the point of invasion, leading to the formation of plant tumours, the plant analogues of cancer. Plant hormones, auxins and cytokinins produced by the bacteria are important in stimulating these growths (Buchanan & Buchanan, 1956 ; Daniels, 1992).

Canker

Bacterial canker is a seed borne disease characterized by browning or blackening of leaf margins, wilting, curling and shrivelling of lower leaflets (Lucas *et al.*, 1992).

Spots

In the spots the bacteria remain localized and kill a small area of tissue. Each of these localized lesions develops from a separate infection event (Buchanan & Buchanan, 1956 ; Daniels, 1992).

The host range of certain bacterial pathogens varies depending on the species, for example, *Agrobacterium tumefaciens* and *Pseudomonas solanacearum* have a wide host range while pathogens like *Pseudomonas syringae* and *Xanthomonas campestris* have pathovars that are restricted only to one plant or a few related species. Plant pathogenic bacteria are incapable of active penetration directly through the intact plant surfaces. They invade either through unprotected surfaces such as non-cutinized areas or wounds, or through stomata, leaf scars, lenticels, hydathodes and nectarthodes. After entry into a susceptible host, extensive multiplication of the bacteria occurs intracellularly, if the two are compatible. Saprophytes, under normal conditions of plant growth, do not multiply in the plant tissue. Like phytopathogenic fungi, phytopathogenic bacteria also penetrate the cells with the help of cell wall degrading enzymes, phytotoxins, extracellular polysaccharides and phytohormones in the case of galls induced by oncogens.

Genera of common bacteria causing plant diseases are *Erwinia, Pseudomonas, Xanthomonas Agrobacterium, Coryneform and Streptomyces* (Buchanan & Buchanan, 1956 ; Daniels, 1992 ; Trench *et al.*, 1992 ; Walton, 1997).

The genus *Erwinia*

Erwinia species are small Gram-negative rods that occur naturally in soil and water. They cause the soft rot of living plant tissues. The bacterium is a facultative anaerobe growing at an optimum temperature of about 25°C. They are the most important primary pathogens of growing plants as well as of the harvested crop, for example in potatoes. *Erwinia carotovora* spp. *carotovora* causes soft rot in a wide variety of crops and grows well at 28°C while *E.*

carotovora ssp. *atroseptica* causes soft rot in potatoes and grows well at lower temperatures, below 20°C (Starr, 1983 ; Daniels, 1992 ; Trench *et al.*, 1992).

Seeds infected by the bacteria rot before seedlings emergence from the ground. Infected plants develop soft, dark-green or black lesions on the stems. Leaves of the infected stem wilt, turn yellow and die and the stems collapse (Clancy, 1974 ; Lucas *et al.*, 1992 ; Trench *et al.*, 1992). The *Erwinias* are capable of producing large quantities of different pectic enzymes which enable them to macerate parenchyma tissue. They are widely distributed ecologically (Perombelon & Salmond, 1995). Individual species normally have a restricted geoclimatic distribution which, in some cases, is related to that of their host (Trench *et al.*, 1992).

Deans and Svoboda (1990) reported that *E. carotovora* was particularly sensitive to the essential oil from *Origanum majorana* (Labiatae). The growth of *E. carotovora* has also been found to be inhibited by rice plant extracts from the cultivar TKM6 (Dhaliwal *et al.*, 1990)

In addition to activity towards some bacteria, the extract (methanol/dichloromethane; 1:1) of the root bark of *Rhus glabra* (Anacardiaceae) inhibited the growth of *E. carotovora* as observed by Heisey and Gorham (1992).

Antibacterial activity towards this bacterium has also been reported by Alice and Siviprakasam (1995) They found that the clove extract of *Allium sativum* (Alliaceae) was effective in inhibiting growth and production of cell wall degrading enzymes by *E. carotovora* pv. *carotovora*. Lis-Balchin and Deans (1996) also stated that some *Pelargonium* species (Geraniaceae) and cultivars exhibited antibacterial activity against *E. carotovora*, among other microorganisms.

The genus *Pseudomonas* (*Ralstonia*)

Pseudomonas cells are not typically straight rods, but curved Gram-negative rods. In some plant pathogenic strains they can be very long (Clancy, 1974). In this genus, *Pseudomonas*

solanacearum is of major importance as it is the main cause of bacterial wilt. It is an aerobic microorganism. *In vitro*, it grows well at high temperatures of 30 -35°C. Bacterial wilt is a major problem with vegetable growers, especially tomato farmers in the tropics, subtropics and warm temperate regions of the world (Schroth *et al.*, 1983 ; Lucas *et al.*, 1992 ; Hayward, 1994 ; Hayward, 1995). Estimates of yield losses to the disease range from 15-95%. With the exception of *Agrobacterium tumefaciens*, the cause of crown gall in dicotyledonous plants, no other bacteria bacterial plant pathogen attacks as many different plant species as does *Pseudomonas solanacearum* (Hayward, 1994).

The destructiveness of *Pseudomonas solanacearum* is due to its widespread occurrence, the existence of different strains and its exceptional ability to survive in the soil and in the roots of non-host plants as well as its broad host range. Host plants of economic importance include tomato, potato, pepper, groundnut, eggplant, banana and tobacco. So far, the control of bacterial wilt has been ineffective (Daniels, 1992 ; Lucas *et al.*, 1992 ; Hayward, 1994). The disease is spread through infected planting material and through root-to-root transmission. In tobacco, rain splash dispersal of the bacteria from leaf surfaces of the plants has been reported as being common. Apart from the Solanaceae there are many host species classified under the Asteraceae and the Leguminosae. Most of the plants reported are dicotyledonous and relatively few are monocotyledonous these being banana plants and ginger (Lucas *et al.*, 1992). Infection occurs below ground level through wounds in the root system. The pathogen enters the plant at the point of emergence of lateral roots with the facilitation of pectinases and other extracellular enzymes. In plants affected by *Pseudomonas solanacearum* the leaves tend to grow rapidly on the upper side and adventitious roots appear, indicating an interference with auxin balance. Levels of indole acetic acid tend to increase greatly in affected plants (Lucas *et al.*, 1992 ; Hayward, 1995). The stems of infected plants quickly wilt, then the whole plant wilts, yellows and dies.

Inhibition of growth of *P. solanacearum* with extracts from the rice cultivar TKM6, among other organisms, was reported by Dhaliwal *et al.* (1990).

The genus *Xanthomonas*

These are Gram-negative straight rods common in South Africa and the rest of the world. *Xanthomonas* species can only survive in soil in association with plant material (Clancy, 1974 ; Starr, 1983 ; Daniels, 1992). As an example, *Xanthomonas campestris* pathovar (pv) *campestris* will be discussed. This is an economically important pathogen of cultivated crucifers, causing the disease 'black rot'. *X. campestris* pv. *campestris* infects primarily via the hydathodes that are openings at the leaf margins and once inside, is confined to the xylem, although entry through the wounds may occur. Characteristic symptoms are V-shaped chlorotic and necrotic lesions advancing along the veins from the leaf margins with eventual death of the leaf tissue adjacent to the veins. *X. Campestris* pv. *campestris* is seed-borne and plants derived from infected seeds may serve as a source of infection. This bacteria produces a range of plant cell wall degrading enzymes which most probably play a role in disease development (Osbourn, 1995).

Grange and Alvarez (1987) reported that the leaf extracts of *Artabotrys hexapetalus* (Annonaceae) reduced the growth of *X. campestris* pv. *campestris* from cabbage seeds. Dhaliwal *et al.* (1990) also observed that extracts from the rice plant cultivar TKM6 also inhibited the growth of *X. campestris* pv. *oryzae*.

The genus *Agrobacterium*

These are Gram-negative rods with a world wide distribution (Clancy, 1974). Pathogenic agrobacteria are common components of the microflora of soils where they grow saprophytically on or near plant roots or plant debris. They can survive for very long periods in soil. They are motile and exhibit chemotaxis towards nutrients exuding from plants or plant materials. The pathogenic process is initiated by chemotactic attraction of agrobacteria towards the wounded sites in the roots and underground stems of susceptible plants (Lippincott *et al.*, 1983 ; Daniels, 1992 ; Clare & McClure, 1995). The crops mainly attacked by these bacteria are grapes, stone fruits such as peach, apricot, nectarine, plum and cherry. The walnuts,

almonds, pears and apples as well as chrysanthemums and roses are affected too (Clare & McClure, 1995).

Agrobacterium species cause plant diseases known as crown gall (tumours) and hairy root. The galls appear mainly on the stems of the plant where they come out of the ground. The symptoms of both diseases result from induced hormonal imbalances within infected host tissues. In the case of crown gall, the result is the development of undifferentiated tumours called galls. These usually form just below soil level, on the crowns of plants, but also occur on roots and in some cases on aerial plant parts. With the hairy root disease, there is a proliferation of adventitious roots, usually associated with callus formation on crowns, basal stems or main roots. An example from this group is *Agrobacterium tumefaciens* (Clancy, 1974 ; Lucas *et al*, 1992).

The genus *Clavibacter*

Unlike most plant pathogenic bacteria, these are coryneform Gram-positive, aerobic, non-sporing rods many of which are club-shaped. (Clancy, 1974). *Clavibacter michiganensis* ssp *michiganensis*, a tomato pathogen and *Clavibacter michiganensis* ssp *sepedonicus* a potato pathogen are of the greatest international economic significance (Lucas *et al.*, 1992 ; Vidaver, 1995). *Clavibacter michiganensis* ssp *michiganensis* causes bacterial canker, a wide spread disease that causes severe losses where tomatoes are grown without rotation.

3.6.3 Plant pathogenic fungi

Fungi constitute the largest group of plant pathogens representing all types of parasitism like necrotrophic, semibiotrophic, biotrophic and symbiotic fungi. Pathogens such as *Rhizoctonia* spp. and *Sclerotium* spp. are hardy because, in addition to attacking tender plant tissues, they can also exploit mature tissues. They are necrotrophs which cause extensive damage to the host tissue by using cell wall degrading enzymes and secondary toxic metabolites and also have a wide host range.

Necrotrophs, like *Alternaria*, show more dependence on the host and less saprophytic characteristics. They rely more on toxins than on cell wall degrading enzymes. Although they cause extensive tissue necrosis, little or no tissue maceration occurs.

Semibiotrophs and biotrophs depend, to a lesser extent, on toxins and wall degrading enzymes. These enzymes are only used selectively and site-specifically to facilitate penetration or colonization of tissues rather than their maceration. For this purpose, phytohormones are used to a greater extent. The host range is restricted and there is an increased dependence of the parasite on living host cells and more and more synchronization of the physiological processes of host and parasite, to an extent that both host parasite start benefiting from each other (Kohmoto *et al.*, 1995).

Like fungi invading growing plants, fungi growing on stored grain greatly lower its quality and quantity and the secondary metabolites produced during fungal growth have been, in many cases, found to be toxic to both humans and animals. For a long time, low molecular weight acids have been used to protect grains from fungal infections, but nowadays there is a growing trend towards substituting them with natural plant extracts. This is evidenced by the growing research on antifungals from natural sources for food and grain preservation (Paster *et al.*, 1995).

Plant pathogenic fungi considered in this chapter are from the division Eumycotina because many of the plant pathogenic fungi belong to this division.

The genus *Rhizoctonia*

Rhizoctonia species belong to the subdivision Hymenomycetes in the class Basidiomycotina. As an example from the genus, *Rhizoctonia solani* is one of the most widely distributed plant pathogens. It is a soil borne fungus that infects seedlings of the crucifers, peas, beans, tomatoes, cereal grains and a number of other crops. It affects potatoes as black scurf and stem canker. Black scurf develops on the skin of mature tubers. These tubers if planted, produce

shoots which develop stem canker. Tubers with this scurf are down graded at markets while seed potatoes which show more than 1% black scurf can not be certified (Lucas *et al.*, 1992 ; Trench *et al.*,1992). *R. solani* produces different types of infections depending on the plant host. The infection sites of *R. solani* on the plant surface can be the epidermis, stomata or wounds. Stomatal infection of stems, cotyledons and leaves is quite common. The hyphae penetrate through the stomata, the branches fill the stomatal cavity and subsequently these hyphae invade the host tissue (Akino & Ogoshi, 1995).

Dhaliwal *et al.* (1990) reported that *R. solani* was among the microorganisms whose growth was inhibited by extracts from the rice plant, cultivar TKM6. Yegen *et al*, (1992) also observed the inhibition of this fungus by aqueous and essential oils from a number of plants.

Miles *et al.*, (1990) using the disc diffusion method noted that the methylene chloride extracts from the stems of *Wedelia biflora* (Asteraceae) also showed antifungal activity towards *R. solani*.

In a continuing search for natural compounds with antifungal activity, Steinhauer (1999) observed that, in the case of potatoes, the introduction of oil press cake obtained from *Azadirachta indica* (Meliaceae) seeds, also called neem cake, into the soil, reduced the damage caused by *R. solani*.

Qasem and Abu-Blan (1996) stated that the aqueous shoot extract of *Rumex crispus* (Polygonaceae) significantly inhibited the growth of *R. solani*. In their investigations, also significantly inhibiting the growth of *R. solani* was the flowering shoot extract of *Senecio vernalis* (Asteraceae).

The root extract of *Eupatorium fortunei* (Asteraceae) was also proven to be active against this bacterial species (Ushiki *et al.*, 1996). This plant, according to these authors, may be of value as an antagonist in mixed cropping systems.

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The genus *Fusarium*

Fusarium species are soilborne fungi of the Hyphomycetes in the Deuteromycotina that cause root and stem rots as well as wilts. They cause dry rot in potatoes during storage, as a post harvest decay or after planting as seed decay. Small brown to black, sometimes sunken spots, are visible on tubers. *Fusarium* species also cause stalk rot in sorghum. *Fusarium* species are often divided into *formae speciales* (f. sp.), a *formae speciale* being a special rank used principally in fungi based on the generic name of the host (Kolattukudy & Gamble, 1995). The fungus *F. oxysporum* f.sp. *lycopersici* affects tomatoes and the symptoms of the disease are yellowing of leaves which is followed by browning then wilting. *Fusarium graminearum* reduces yield and quality of wheat as the developed wheat dies early. *Fusarium oxysporum* f. sp. *cepae* is wide spread and a problem organism which causes onion bulb rot, with losses during both growth and storage. It is common in moist onions (Clarkson, 1992 ; Lucas *et al,* 1992 ; Trench *et al.*, 1992).

The inhibition of the growth of *F. oxysporum* f.sp. *lycopersici* was observed by Tariq and Magee (1990). They reported that the volatile components of crude aqueous extracts of garlic-*Allium sativum* (Alliaceae) bulbs inhibited the germination of microconidia and hyphal extension in *F. oxysporum* f.sp. *lycopersici*. Yegen *et al.* (1992) observed that *Fusarium moniliforme* is susceptible to a number of aqueous extracts and essential oils. On the other hand, Irobi and Daramola (1993) observed that the ethanolic leaf and flower extracts of *Mitracarpus villosus* (Rubiaceae) significantly inhibited the growth of *F. solani* as well as other fungi.

Neem cake was also found to significantly reduce the infection of tomatoes by *F. oxysporum* when applied to the soil (Steinhauer, 1999).

The genus *Sclerotium*

Sclerotium species are soilborne fungi that belong to the Hymenomycetes in the Basidiomycotina. They infect many vegetable crops, peanuts, soy bean, sun flower and forage crops among others. These fungi generally cause blights. An example is *Sclerotium rolfsii*, a fungus that grows on both dead and living plant material. It causes white mould also called sclerotium rot, basal stem rot or sclerotium blight. Infection starts on fallen leaves then spreads to the growing plant via the stem, leading to wilting which is characterized by leaves turning dark-brown, and eventually dropping. White moulds form on the diseased stem and crown. Tiny sclerotia form on infected parts or in the soil (Clarkson, 1992; Lucas *et al,* 1992 ; Trench *et al,* 1992).

The genus *Phytophthora*

These are airborne fungi belonging to the Oomycetes of the Mastigomycotina. *Phytophthora cinnamoni* causes phytophthora root rot. It is the most devastating disease of avocados in South Africa due to high rainfall and soil temperatures. It attacks and kills fine feeder roots which prevents normal water and mineral uptake by the trees. It also infects trunks causing canker on the lower parts. *P. cinnamoni* also infects grapes in vineyards causing root rots. Losses can be very high thereby affecting the wine industry (Clarkson, 1992 ; Lucas *et al.,* 1992).

P. infestans causes late blight in potatoes and tomatoes. This infection is characterized by dark green to brown lesions with a pale green area around them that appear on leaves. These lesions enlarge until the leaf dies. This disease also affects the other species of the Solanaceae (Clarkson, 1992 ; Lucas *et al.*, 1992).

The phytoalexin capsidiol from plants of the Solanaceae has been found to be highly active against *P. infestans* (Wain, 1985). A number of plant extracts have been found to be active against some species of this genus. For example, Yegen *et al.* (1992) observed that aqueous extracts and essential oils of a number of plants exhibited fungitoxicity against, among other

fungi, *Phytophthora capsici*. The essential oils were more toxic against this fungus than the fungicides carbendazim and pentachlornitrobenzene. Investigations with thin-layer chromatography implicated thymol and carvacrol as the active ingredients.

The genus *Botrytis*

Botrytis species are generally airborne fungi that belong to the Hyphomycetes of the Deuteromycotina. An example from this group is *B. cinerea*, a soil borne fungus that causes grey mould or botrytis blight. It is one of the most common diseases of field crops, fruits, ornamentals and vegetables throughout the world. It affects flowers, fruit or stems. Infection of flowers leads to the formation of infected fruits. The infected tissues become soft and watery and appear light brown. Infected fruit, for example grapes, darkens and shrivels. Infection occurs in cool wet weather, at temperatures less than 20°C and begins at soil level. The infected grapes are often covered by a greyish-brown mould (Clarkson, 1992 ; Lucas *et al.*, 1992).

The extracts of *Allium cepa* and *Allium porrum* (Alliaceae) have been reported to be inhibitors of the growth of *B. cinerea* (Favaron *et al.*, 1993).

The genus *Colletotrichum*

Colletotrichum species are airborne fungi that belong to the Pyrenomycetes of the Ascomycotina. They cause diseases collectively known as anthracnose. They have a wide host range which includes the Solanaceae (potatoes, tomatoes, peppers), cucurbits (pumpkins) and some legumes. Symptoms are black dots on the surface of tubers, roots and stems above and below the ground. These organs rot and dry out. *Colletotrichum acutatum* infects cotton bolls, leading to a disease called boll rot. *Colletotrichum truncatum* is a seed borne disease that infects soy beans, leading to defoliation and the development of mouldy, dark-brown shrivelled seeds (Clarkson, 1992 ; Lucas *et al.*, 1992).

The genus *Phoma*

Phoma species are part of the fungal species that belong to the Coleomycetes of the Deuteromycotina. They infect groundnuts and the resulting disease from *Phoma arachidicola* is called *phoma* leaf spot or web blotch. It is characterized by dark-brown to almost black blotches that appear on the upper surface of the leaves. *Phoma exigua* var *foveata* or *Phoma exigua* var *exigua* infects potato tubers and the infection results in the formation of web-like lesions. This fungus spreads on infected tubers which grow to produce diseased stems. Infection mostly occurs after harvest. Small, dark sunken lesions appear on the tuber usually at wounds, eyes or lenticels. *Phoma macdonaldii* infects sunflower stems (Clarkson, 1992).
Extracts from *Allium cepa* and *A. porrum* have been found to inhibit the growth of *Phoma terrestris* in addition to other microorganisms (Favaron *et al.*, 1993).

The genus *Aspergillus*

Aspergillus species belong to the class Plectomycetes of the subdivision Ascomycotina. In general they cause grain storage rots among other diseases. *Aspergillus niger* and *Aspergillus flavus* are representatives from this group. They are soil borne fungi that cause *aspergillus* seed decay and *aspergillus* crown rot and the yellow mould of pods and seeds in groundnuts. These fungi survive on organic debris in soil and on contaminated seed. Infection occurs under warm, moist conditions. *A. niger* produces masses of sooty, black spores, whereas *A. Flavus* produces masses of yellow-green spores. They both lead to poor quality seed (Clarkson, 1992 ; Trench *et al.*,1992).

Of the fungi challenged with the volatile oil from *Origanum majorana*, *A. niger* was the most susceptible (Deans & Svoboda, 1990). Hiremath *et al.* (1994) reported that the petrol, chloroform and ethanol successive extracts of *Striga lutea* (Scrophulariaceae) were active against *A. niger*, among other organisms. Because of the fact that low molecular weight fungi-inhibiting acids used for the preservation of stored grain have undesirable effects on the grain, Paster *et al.* (1995) attempted the use of oregon and thyme essential oils as fumigants against

Aspergillus niger and *A. flavus*. These authors found that the oregon essential oils were more effective in inhibiting the growth of mycelia and spores of these test organisms.

Steinhauer (1999) also reported that treatment of tomatoes with powdered leaves of *Azadirachta indica* (Meliaceae) followed by inoculation with *A. niger*, resulted in a significant reduction in fruit rot caused by the fungus compared with the controls.

A lot of established fungicides have been found to be less and less effective as time goes on due to resistance developed over time. Finding a new pesticide can also mean the finding of a fungicide that can control diseases currently uncontrollable (Beautement, 1991). According to this author, a plant derived antifungal compound can be used directly if produced in large quantities or may be used as a starting point for the synthesis of analogues with improved biophysical properties.

At this point it would be appropriate to mention how antibiotic substances inhibit the growth of microorganisms with a general emphasis on bacteria and fungi.

3.7 Action of antimicrobial agents on microorganisms

Antimicrobial agents are generally defined as those entities that adversely affect the viability (microbiocidal) or growth (microbiostatic) of an organism. However, the recognition that other adverse effects may be exerted on microorganisms at concentrations below the minimum growth inhibitory concentration (subMIC) of some agents, implies that the label 'antimicrobial' requires a broader definition. This is demonstrated, for example, by the ability of numerous antimicrobial agents to alter the adherence of microbes to surfaces. At the same time, a large number of substances which do not possess intrinsic microbiocidal or microbiostatic activity has been shown to exert an 'antimicrobial' effect through the interference with the microbial adhesion process (Gorman, 1991). The influence of antimicrobial agents on microbial adherence is an alternative possibility for prophylactic or therapeutic interference with the infectious process and the industrial control of microbial

biofilms. Microbial adherence can take place on human epithelial cells and also on non-clinical environments (Gorman, 1991).

Antimicrobial agents may exert different effects on the structure and functioning of microbial cells. The important targets of chemotherapeutic action are the cell wall, the cell membrane and the biosynthetic process of protein and nucleic acid synthesis. Fungi are differentiated from bacteria by the fact that the fungal cells are usually much larger and contain a nucleus, nuclear membrane and mitochondria, typical of eukaryotic cells, while the bacteria are prokaryotes. Therefore, inhibition of bacteria or fungi can be caused by inactivation or interference with one or more of the above mentioned processes (Brock & Madigan, 1991 ; Eklund & Nes, 1991). The degree to which interference with each of the above functions affects the microrganism depends on the value of that function to the species of the organism challenged.

Antibacterial and antifungal agents may exhibit both bacteriostatic and fungistatic as well as bactericidal and fungicidal effects. Bacteriostatic and fungistatic effects generally represent some form of metabolic inhibition which is released upon removal of the biocide, while bactericidal or fungicidal action is caused by irreversible or irreparable damage to a vital structure or function of the cell. Damage arising from interactions with biocides may, in many instances be repairable, but because of inhibition at another metabolic site, appropriate repair processes cannot be initiated and the cell dies (Denyer & Hugo, 1991b).

Many antimicrobial agents pass through the cell wall of by passive diffusion. The cytoplasmic membrane of microorganisms is a sensitive readily accessible (via the cell wall) target site of action for many antibiotics and antimicrobial agents. Interaction at this level may, therefore, lead to specific and non-specific permeability changes, leakage of intracellular material, osmotic lysis and the inhibition of membrane associated metabolic activity (Denyer & Hugo, 1991a & 1991b).

Although some lipophilic antimicrobial agents with an intracellular target can reach their site of action by passive diffusion, many others of a hydrophobic nature are excluded by the barrier function of the bacterial or fungal cytoplasmic membrane. This may not mean that no antibacterial or antifungal activity will result from their presence, but higher concentrations of the hydrophobic antimicrobial might be necessary to ensure sufficient intracellular levels. Uptake of antimicrobial compounds could also be facilitated through existing bacterial or fungal transport systems. This mechanism of transport is recognised to occur naturally in the action of certain antibiotics (Denyer *et al.*, 1991). Beautement *et al.* (1991) report that strobilurin A, strobilurin B, oudemansin A and myxothiazol are fungicides that inhibit mitochondrial respiration by blocking electron-transfer between cytochrome *b* and cytochrome c_1. According to Beautement *et al.* (1991) antibiotics, such as antimycins which also bind at cytochrome *c*, are part of the many other inhibitors that interfere with fungal respiration.

Chemical antimicrobial agents also exert their inhibitory effect on microbial cells either through irreversible covalent associations with biomolecular targets resulting in adjunct formation or through reversible interactions of a physico-chemical nature which alter the physical chemistry of the target molecule (Waigh & Gilbert, 1991).

The specific inhibition of enzyme action by antibacterial and antifungal agents is rarely a bactericidal or fungicidal event due to the often reversible nature of such inhibitions and the ability of the bacteria or the fungus to adopt alternative metabolic pathways. It is also known that residual activity which remains in the presence of the inhibitor may still allow the metabolic pathway to function at a useful level. Enzyme inhibition can make a significant contribution to the bacteriostatic or fungistatic activity of the biocides, thereby influencing microbial repair and recovery processes (Fuller, 1991).

The problem with many of the currently used antibiotics has been the development of resistance by the microorganisms due to overuse and the emergence of more resistant strains (chapter 1). There are two broad categories of microbial resistance: intrinsic and acquired. Intrinsic resistance means that inherent features of the cell are responsible for preventing

antimicrobial action. On the other hand, acquired resistance occurs when resistant strains emerge from previously sensitive bacterial populations, usually after exposure to the antimicrobial agent concerned. Acquired resistance can arise either by acquisition of plasmids and transposons or by chromosomal mutations (Chopra, 1991).

In our investigation of the antimicrobial properties of extracts from selected plant species growing in the Free State, a total of 26 plant species (chapter 4) were collected from the Bloemfontein, Brandfort and Hoopstad Districts. These species were tested for their antimicrobial activities against a number of fungi and bacteria, which included plant pathogens, in a preliminary screen in chapter 5.

CHAPTER 4

GENERAL MATERIALS AND METHODS

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4.1 Materials

4.1.1 Plant materials

Twenty six plant species, mainly leaves, were collected from the Free State between March 1997 and March 1998, to represent a total of 16 families growing here. As has been explained in Chapter 2, mainly leaves were collected since this is a non- destructive method allowed by the authorities in the collection areas. These plants were selected, mainly on the basis of taxonomy, from the Bloemfontein, Brandfort and Hoopstad Districts since information on the ethnopharmacological use of many plant species growing in the Free State Province is lacking as compared to other provinces. This is due to the fact that this information was not collected in the past. The National Museum in Bloemfontein facilitated the collection as well as authentication of all the plant species collected and a voucher specimen of each is currently kept at this museum. After collection, the plant material was air-dried at room temperature and then ground to a fine powder that was kept at -20°C until required for analysis.

Table 4.1: Plant species and families collected from the Free State for antimicrobial screening.

FAMILY & SPECIES	VOUCHER SPECIMEN No.	PARTS COLLECTED	TIME COLLECTED
Anacardiaceae			
Rhus ciliata Licht ex Schult.	NMB20 482	leaves	March, 1997
Rhus erosa Thunb.	NMB20 480	leaves	March, 1998
Rhus lancea L.f.	NMB17 047	leaves	March, 1997
Asclepiadaceae			
Asclepias fruticosa L.	NMB13 202	leaves	March, 1997
Asparagaceae			
Protasparagus laricinus (Burch.) Oberm	NMB17 652	leaves	March, 1997
Asphodelaceae			
Bulbine asphodeloides (L.) Willd	NMB19 490	inflorescence	October, 1997
Bulbine asphodeloides (L.) Willd	NMB19 490	roots	October, 1997
Asteraceae			
Senecio radicans (L.f.) Sch. Bip.	NMB12 965	leaves	March, 1997
Vernonia oligocephala (DC) Sch.Bip. ex Wolp.	NMB17 523	leaves	March, 1997
Boraginaceae			
Ehretia rigida (Thunb.) Druce	NMB17 526	leaves	March, 1997
Cucurbitaceae			
Coccinia sessilifolia (Sond.) Cogn.	NMB13 040	leaves	March, 1997
Ebenaceae			
Diospyros austro africana De Winter var. *microphylla* (Burch.) De Winter	NMB20 485	leaves	March, 1997
Euclea crispa (Thunb.) Guerke subsp. *crispa*	NMB20 483	leaves	March, 1997

Table 4.1 continued

FAMILY & SPECIES	VOUCHER SPECIMEN No.	PARTS COLLECTED	TIME COLLECTED
Fabaceae			
Acacia erioloba E. Mey	NMB20 479	leaves	March, 1997
Acacia hebeclada DC. subsp. *hebeclada*	NMB11 455	leaves	March, 1997
Acacia karroo Hayne	NMB20 478	leaves	March, 1998
Senna italica Mill. subsp. *arachoides* (Burch.)Lock	NMB12 882	leaves	March, 1997
Elephantorrhiza elephantina Burch. (Skeels)	NMB20 481	leaves	March, 1998
Loganiaceae			
Buddleja saligna Willd.	NMB20 477	leaves	March,1997
Oleaceae			
Olea europaea L.subsp. *africana* (Mill.) P.S. Green	NMB12 868	leaves	March, 1997
Polygonaceae			
Rumex lanceolatus Thunb.	NMB 202	leaves	March, 1997
Ranunculaceae			
Clematis brachiata Thunb.	NMB16 302	leaves	March, 1997
Ranunculus multifidus Forssk.	NMB17 066	leaves	March, 1997
Rhamannaceae			
Ziziphus mucronata Willd. subsp. *mucronata*	NMB20 475	leaves	March, 1997
Santalaceae			
Osyris lanceolata Hochst. & Steud.	NMB20 474	leaves	March, 1997
Tiliaceae			
Grewia flava DC.	NMB17 456	leaves	March, 1997
Grewia occidentalis L.	NMB13 207	leaves	March, 1997

4.1.2 Microorganisms

4.1.2.1 Plant pathogenic fungi

All plant pathogenic fungi and bacteria used in this investigation were provided by the Department of Plant Pathology, University of the Free State in Bloemfontein, Republic of South Africa.

Table 4.2: Plant pathogenic test fungi

FUNGUS	SUBDIVISION	CULTURE No.
Aspergillus niger	Ascomycotina	CCP 174
Botrytis cinerea	Deuteromycotina	CCP 8

Table 4.2 continued

FUNGUS	SUBDIVISION	CULTURE No.
Colletotricum acutatum	Ascomycotina	CCP 13
Fusarium oxysporum	Deuteromycotina	CCP 251
Fusarium oxysporum	Deuteromycotina	CCP 276
Phoma spp.	Deuteromycotina	*NA
Phytophthora cinnamoni	Deuteromycotina	CCP 137
Rhizoctonia solani	Basidiomycotina	CCP 159
Sclerotium rolfsii	Basidiomycotina	CCP 330

* not available

4.1.2.2 Plant pathogenic bacteria

Table 4.3 : Plant pathogenic test bacteria

BACTERIA	CLASS	GRAM STAIN
Agrobacterium tumefaciens	Methylococcaceae	negative
Clavibacterium michiganense pv. *michiganense*	Corynebacteriaceae	positive
Erwinia carotovora pv *carotovora*	Enterobacteriaceae	negative
Pseudomonas solanacearum	Pseudomonaceae	negative
Xanthomonas campestris pv. *phaseoli*	Pseudomonaceae	negative

4.1.2.3 Other microorganisms

Added to this list of test bacteria was the human opportunistic pathogen Gram-negative mirococcus *Moraxella catarrhalis* (previously known as *Branhamella catarrhalis*; Jones & Barry, 1990) as a taxonomic representative. *Moraxella catarrhalis* was also used in bioassay guided fractionation in the tests for antibacterial activity.

4.1.2.4 Antimicrobial controls

The quartenary ammonium compound dimethyl didecyl ammonium chloride (ZENECA Company, Paarl, RSA) was used as the positive control against both plant pathogenic bacteria

and fungi. This is a broad spectrum biodegradable biocide highly effective in the control of fungi, bacteria, algae, yeasts as well as viruses through contact with them in solution. A 5% solution (v/v) was used in antibacterial tests while a 15% (v/v) solution was used in antifungal tests as a control. These concentrations were chosen because they gave inhibition zones that were neither too big nor too small. Due to the sensitivity of *M. catarrhalis* to dimethyl didecyl ammonium chloride, a 2.5% solution was used as the control.

Two negative controls were also used. The first was the organism control and consisted of seeded Petri dishes to which paper disks saturated with a 10% (v/v) solution of DMSO in water were placed. This was done to check for the toxicity of DMSO to these organisms. In the second negative control plant extracts and dimethyl didecyl ammonium chloride were introduced to paper disks and placed on unseeded Petri dishes as a check for sterility of the plates (Brantner & Grein, 1994). Antifungal and antibacterial controls were run together with the other tests under identical conditions.

4.1.3 Chemicals

4.1.3.1 Solvents

All solvents used were of analytical grade, purchased from Merck:

acetone, chloroform, diethyl ether, ethanol, ethyl acetate, hexane, methanol and toluene

4.1.3.2 Spray reagents

Spray reagents were purchased from Sigma Chemicals:

potassium hydroxide, iron (III) chloride, potassium hexacyanoferrate, 3,5,-dinitrobenzoic acid, sodium hydroxide, vanillin, Dragendorff reagent, ammonium vanadate and ρ-anisidine

4.1.3.3 Reference compounds for thin layer chromatography

Reference compounds were purchased from Sigma Chemicals:

rutin, rutoside, chlorogenic acid, quercitrin, hypericin, dihydroquercetin, quercetin, kaempferol, reference compounds mixture (rutin, chlorogenic acid, hyperoside, quercetin and quercitrin)

All other chemicals used were of the purest grade available and purchased from Merck.

4.2 Methods

4.2.1 Preparation of crude extracts

The crude extracts were prepared from powdered material by percolating a weighed quantity of the powder with 95% MeOH in H_2O (v/v) at a ratio of 2ml MeOH $gram^{-1}$ powdered plant material at room temperature and by shaking overnight on a mechanical shaker. Ten extractions, with new solvent for each extraction, were carried out by shaking for 15 minutes. Each extract was filtered through Whatman filter paper No.1 on a Büchner funnel. The combined filtrate was evaporated under vacuum at temperatures below 40°C to leave a methanol-free extract. This aqueous crude extract was centrifuged at 3 000 rpm at room temperature for 10 minutes to separate the precipitated proteins and chlorophylls. The clear supernatant was used for the antimicrobial tests (Ganesan & Krishnaraju, 1995). The concentration of each resultant extract was determined in triplicate by drying 1 ml aliquots in an oven at 60-80°C to a constant weight (Brownleader & Dey, 1997). This dried material was discarded.

The aqueous crude extract was then reduced to an appropriate concentration for antimicrobial tests with a 10% (v/v) solution of DMSO in water. Alternatively, an aliquot from the methanol-free extract was dried to a constant weight in an oven equipped with a fan at 28-30°C. A specific dry weight, from here required for antimicrobial tests, was dissolved in a 10% (v/v) solution of DMSO in water. Aqueous, methanol-free extracts, the most frequent form of application in traditional medicine, were tested for their antimicrobial activity during the preliminary screening (Brantner & Grein, 1994). These extracts were stored at –20°C (Bloor, 1995).

4.2.2 Microbiological tests

4.2.2.1 Antifungal assays

The modified agar diffusion method described by Bauer *et al.* (1966) was used (Rios *et al.*, 1988).

This method is widely used for antibiotic susceptibility testing as well as for screening possible medicinal plants (Kavanagh, 1975 ; Gordon *et al.*, 1980 ; El-Abyad *et al.*, 1990 ; Irobi & Daramola, 1993). All plant pathogenic fungi were cultured on potato dextrose agar (PDA) with the antibiotic streptomycin to inhibit the growth of bacterial contaminants. PDA was prepared in the usual way and, on cooling to 45°C (in a water bath set at 45°C), 15 ml were aseptically poured into disposable plastic Petri dishes of 9 mm diameter to a depth of 2-3 mm and allowed to set in the laminar flow cabinet.

Plant pathogenic fungi, to be challenged with the extracts, were 7-10 day old cultures grown on PDA plates and were used as inocula on the control and test plates (March *et al.*, 1991). Plaques of agar with fungal mycelia were cut with a flame sterilized 10 mm diameter cork borer and placed in the centre of a sterile PDA plate in which the plant extracts were to be tested. Four wells into which extracts of known concentration were to be introduced were made equidistantly (about 1 cm) from the fungus plaque with a flame sterilized 10mm diameter cork borer.

During the preliminary screening of crude extracts for antifungal activity, the paper disk diffusion method was used instead of the hole-plate diffusion method. Sterilized assay paper disks of 10 mm diameter (Schleicher & Schuell) were used as reservoirs for the extracts on the agar instead of the wells. These disks were saturated with crude extracts by dipping in the extracts after which excess fluid was allowed to drip off (Holt, 1975 ; Kavanagh, 1975). All the tests were done in duplicate. The fungal cultures were incubated at 25°C for about seven days, depending on the rate of growth of each species. At the end of the incubation period, inhibition

zones were measured with a transparent ruler on two axes at right angles to each other to give two readings from each test. The measurement included the assay disk or well, depending on the method used (Pfaller *et al.*, 1992).

4.2.2.2 Antibacterial assays

The modified agar diffusion method of Bauer *et al.*, (1966) was also used in the testing of plants for antibacterial activity (Rios *et al.*, 1988). All the plant pathogens were cultured on plate count agar (PCA; Biolab) (Rasoanaivo & Ratsimamanga, 1993 ; Caceres *et al.*, 199a) for the screening of each of the extracts. PCA was prepared according to the manufacturer's instructions. On cooling to 45°C, it was aseptically poured into disposable plastic Petri dishes of 9 mm diameter to a depth of 2-3 mm and allowed to solidify in a laminar flow cabinet.

An overnight culture of each bacterium grown in a 1% (w/v) solution of nutrient broth (Biolab) in water (w/v) was diluted 1:100 with fresh, sterile nutrient broth of the same concentration (Meyer & Afolayan, 1995). Subsequently, a bacterial suspension of 100 µl was evenly spread over the agar surface using a flame sterilized glass spreader. During the preliminary screening of the crude extracts for antibacterial activity, sterile assay paper disks of 6 mm diameter (Schleicher & Schuell) were saturated with plant extracts of known concentration and firmly applied on the agar surface (Kavanagh, 1975 ; Rios, 1980 ; Rasoanaivo & Ratsimamanga, 1993). In later assays, plant extracts of known concentration were introduced into wells made on the seeded agar surface with a sterile 6 mm diameter cork borer. Every extract was tested in duplicate.

During the preliminary screening of plant extracts with plant pathogens, equilibration was done at 4°C to allow the extracts to diffuse into the agar before the bacteria started growing and multiplying. This also allowed for the development of clear zones of inhibition. *M. catarrhalis* was incubated at 35°C. The plant pathogenic bacteria were incubated at 30°C for 24 hours.

Inhibition zones were measured with a transparent ruler on two axes at right angles to each other to give two readings from each test. The measurement included the assay disk or well depending on the method used (March *et al.*, 1991 ; Pfaller *et al.*, 1992).

4.2.2.3 Maintenance of stock cultures

Fungal stock cultures were maintained on PDA slants and also on PDA suspended in distilled water at 5°C. Occasionally sporulation was observed among the cultures in water and as a result, cultures on agar slants were used if sporulation was evident.

Each plant pathogenic bacterium was maintained on nutrient agar (Biolab) slants at room temperature and was recovered for testing by growth in nutrient broth (Biolab) for 24 hours.

Before streaking, each culture was diluted 1:100 with fresh sterile nutrient broth (Meyer & Afolayan, 1995). *M. catarrhalis* was maintained as a suspension in a solution of nutrient broth (Biolab) prepared according to the manufacturer's recommendation and held at 5°C. It was subcultured every two months to limit the formation of mutants.

The results of the preliminary screening of crude plant extracts from the twenty six plant species are presented in chapter 6.

CHAPTER 5

MAPPING OF FAMILIES AND GENERA OF PLANTS WITH ANTIMICROBIAL PROPERTIES GROWING IN THE FREE STATE

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5.1 INTRODUCTION

The Free State Province of the Republic of South Africa occupies an area of 127 993 km^2. It lies between the latitudes 26° S and 31° S and longitudes 24° E and 30° E (Fig.5.1). It is situated on the central plateau of South Africa and consists largely of open rolling plains interrupted at irregular intervals by low rocky hills. The general altitude above sea level varies from 1000 m in the west to 2000 m in the east. Near the borders of Lesotho and Natal the landscape becomes more undulating and high peaks of up to 3000 m occur along the Maluti and Drakensberg Mountains near the junction of the Free State, Lesotho and Natal borders (Henderson, 1991 ; Du Preez *et al.*, 1991).

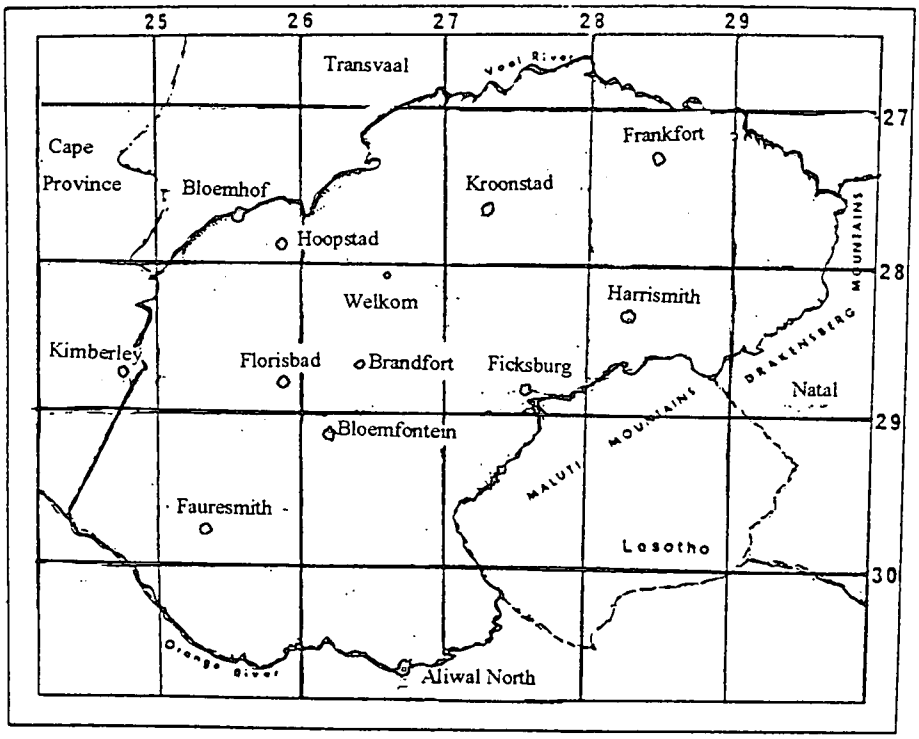

Figure 5.1 The Free State Province

Rain falls mainly in summer, in the months of November to April, with the mean annual rainfall more or less coinciding with altitude. It averages only 250-380 mm in the west and

increases gradually to 635-760 mm in the east with 890 mm or more near the border with Natal Province. This increase is due to the rising relief and decrease in average daily temperatures from west to east and hence evaporation (Henderson, 1991 ; Du Preez *et al.*, 1991b ; Malan, 1997).

Summer temperatures are generally high, sometimes exceeding 38°C during mid-summer. The hottest months of the year are from December to February. The resultant high rate of evaporation causes moisture deficit in all regions of the province, so that perennial streams and continuously moist soils occur only in very limited areas of the high rainfall eastern districts. Periods of drought frequently occur in spring and early summer (Henderson, 1991 ; Fuls *et al.*, 1992a ; Fuls *et al.*, 1993a).

Winters are generally cold and dry with June and July being the coldest and driest months, except in the mountainous eastern regions where mist and snow are common during these two midwinter months. Frost is moderate to severe over the entire province. Mean daily minimum temperatures for July range from about -2.5°C to about 2.5°C (Henderson, 1991).

Topography has a definite influence on temperature in the Free State especially along the rivers where species that tolerate more moisture grow. Woodland with dense bush is found in sheltered ravines (Du Preez & Venter, 1990b) while in the north eastern areas of the province, slopes facing the south are characterised by cooler conditions with increased and a well developed tree stratum in association with a sparse grass layer (Eckhardt *et al.*, 1993b).

The Free State is one of the major agricultural regions of the country (Kooj *et al.*, 1990b ; Malan, 1998). Extensive animal husbandry and intensive crop production are carried out within the province. The province is made up of roughly 72% Grassland Biome which is grazed upon by the cattle and sheep and as they graze, they continuously trample on other plant species as

well. Intensive crop production has also led to the removal of indigenous plant species to make way for cultivation of commercial crops (Kooij *et al.*, 1990b ; Kooij *et al.*, 1990c ; Fuls *et al.*, 1992a ; Fuls, *et al.*, 1992b). Also linked to the destruction of pristine vegetation in the province is urban development (Malan *et al.*, 1998). Together with the large scale cultivation of these crops is the use of agrochemicals which are in themselves environmental pollutants all over the world even if used with utmost care. The destruction of the indigenous species through farming practices has led to studies of the vegetation ecology of the province by a number of authors. As a result, literature on the vegetation ecology of the Free State is plentiful (Kooij, 1990b ; Henderson, 1991; Fuls *et al*, 1993b ; Eckhardt *et al.*, 1993b : Malan, 1997 ; Malan, 1998), but the plant communities here have not been classified according to their antimicrobial properties or any other bioactivities for that matter.

Sound ecological land-use planning, management and conservation can only be effective if the vegetation and habitat characteristics of the Free State are taken into consideration by everyone involved in land use (Eckhardt *et al.*, 1993b ; Fuls *et al.*, 1993d) and the necessity for the detailed plant ecological studies as a basis for sound land-use, planning, management, conservation and research is widely recognised (Fuls *et al.*, 1993b ; Fuls *et al.*, 1993e). Future land use planning must be based on sound ecological principles, so that further deterioration in the ecosystems of the province can be limited to a minimum and ultimate recovery as well as maintenance of the vegetation can be achieved (Eckhardt *et al.*, 1993a).

5.2 BIOMES OF THE FREE STATE

A biome or region is a broad ecological unit representing major life zones of large natural areas. In South Africa, a biome is defined mainly by vegetation structure and climate (Low & Rebelo, 1996). According to Low and Rebelo (1996), there are four biomes in the Free State. These are Grassland (71.7%), Nama-Karoo (21.62%), Savanna (6.64%) and the Forest biome, which

being the smallest, constitutes 0.05% of the province. Fourteen Acocks Veld Types are also recognised.

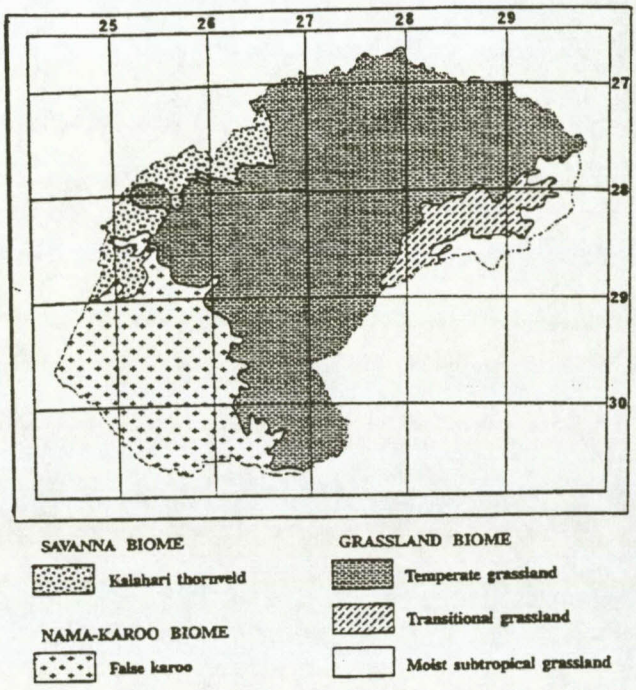

Figure 5.2 The biomes and broad veld type categories of the Free State province, after Rutherford and Westfall (1986) and Acocks (1988) (Henderson, 1991)

False Karoo {= Eastern mixed Nama Karoo (52)}; Kalahari thornveld {= Kimberley thorn Bushveld (32) }; Temperate Grassland {= Moist Cold Highveld Grassland (40)}; Temperate Grassland {= Grassland Biome }

The Free State province is generally undulating and broken country with indigenous scrub occurring on hillsides and ravine forests limited to deep mountain valleys (Henderson, 1991).

5.2.1 **Grassland Biome** (Low & Rebelo, 1996)

The Grassland Biome (Temperate grassland; Fig. 5.2), forming 71.7% of the Free State, is the largest biome and is limited to the summer rainfall areas with mean annual rainfall mostly between 400 and 2000 mm per annum (Malan, 1997). Altitude varies from near sea level to 2 850 m above sea level. The topography here is mainly flat to rolling, but can also be mountainous. The grasslands are characterised by a dominance of a single layer of grasses. Rainfall and grazing determine the amount of cover, in that where there is more rainfall, more grass flourishes and where a lot of grazing is taking place, only patches of grass cover are evident. Sweet and sour grasses are features of this biome. Sweet grasses are low in cellulose and therefore palatable to livestock while sour grasses are high in cellulose. Sour grasses, are dominant in high rainfall areas with soils of high acidity. Other grasses, and a number of woody species are also found in the Grassland Biome (Fuls *et al.*, 1992a ; Kooij *et al.*, 1990a ; Du Preez & Venter, 1992 ; Du Preez, 1992 ; Fuls *et al.*, 1993b). Moist subtropical grassland (Fig. 5.2) is limited to the high rainfall mountain region adjacent to the borders with KwaZulu-Natal and northern Lesotho. Patches of indigenous forest occur in valleys and on sheltered mountain slopes. Transitional grassland (Fig. 5.2) links the temperate and moist subtropical grasslands in the highest and wettest parts of the Free State. It is worth mentioning that indigenous woody species are scarce in this biome, being confined to occasional rocky hills (Henderson, 1991). The most common soil group in the Grassland Biome are the red-yellow-grey latosol plinthic catena (Malan, 1997). The Grassland Biome of the Free State is characterised by nine grassland types (Low & Rebelo, 1996).

5.2.1.1 Dry Clay Highveld Grassland (vegetation type 36) (Low & Rebelo, 1996 ; Malan, 1997)

This grassland type, constituting 1.66% of the province, covers the plains in the Wesselsbron area and the north-western Free State. Altitude ranges from 1 500 to 1 600 m. Summer rainfall averages 600 mm per year, lowest rainfall being in the western Free State. Temperatures vary between -8°C and 39°C.

The soils are dry, clayey, duplex derived from rocks of the Ecca Group sandstone and shale, with occasional dolerite sills.

Among the grass species present here, *Themeda triandra* is the most dominant.

5.2.1.2 Dry Sandy Highveld Grassland (vegetation type 37) (Low & Rebelo, 1996)

This grassland type make up 23.86% of the Free State province and is restricted to the plains in the dry western parts of the province and sandy areas west of Wesselsbron, extending south eastwards towards Bloemfontein. The landscape is flat to gently undulating, at an altitude of 1 70 to 1 460 m. Summer rainfall here is erratic with an average of 450 mm per year. Temperatures vary between -11°C and 41°C (Malan *et al.*, 1995).

Deep, red to yellow, apedal, aeolian sand covers limestone rocks. Vegetation, though mainly grassland, is characterised by a few *Acacia karroo* trees occurring occasionally along water courses. West of Bloemfontein, plant communities are dominated by dwarf shrubs, including *Rhus cilita*. The grassland merges with the bordering Kalahari Thornveld (Kimberly Thorn Bushveld) to the west.

5.2.1.3 Moist Clay Highveld Grassland (vegetation type 35) (Low & Rebelo, 1996)

Moist Clay Highveld Grassland constitutes 0.1% of the Free State province. This grassland type is limited to the north-eastern Free State on flat to slightly undulating plains, at altitudes of 1 00 to 1 700 m above sea level. The rainfall is 650-750 mm per year and occurs in summer. Temperatures vary between -13°C and 37°C.

The predominating soils are very clayey, black vertic or near vertic, mostly of montmorillonitic clays. *Themeda triandra* and other grasses predominate here.

5.2.1.4 Moist Cold Highveld Grassland (vegetation type 40) (Low & Rebelo, 1996)

This grassland occurs on the undulating plains immediately west of the high escarpment in the eastern Free State. It constitutes 9.42% of the province. This is a high altitude vegetation type from 1 350 to 2 000 m above sea level. Here, the rainfall ranges from 700-800 mm occurring mainly in summer. The region experiences severe frost, but little snow.

Deep, yellow and grey sandy-loam soils derived from sandstones and shales of the Beaufort Group in the south, and the Ecca Group in the north.

Moderately dense grassland dominated by *Cymbopogon plurinodis* predominates here. Other plant species include the non-woody *Helichrysum rugulosum* and *Senecio erubescens* among others.

5.2.1.5 Moist Cool Highveld Grassland (vegetation type 39) (Low & Rebelo, 1996; Malan, 1997)

This grassland type constitutes 30.28% of the province. It is widespread, covering the central-eastern part of the highveld in the Free State. Altitude varies from 1 400 to 1 600 m above sea level. Rainfall occurs in summer and varies from 600 to 700 mm per year. Temperatures vary from -11°C to 38°C.

Deep, red (Hutton) and yellow (Clovelly) soils, mostly on Karoo Sequence sediments but also on shale (Witwatersrand Supergroup), andestic lava (Ventersdorp Supergroup) predominate. Where the vegetation has not been disturbed, the grass *Themeda triandra* dominates entirely, and few other species, particularly non-woody dicotyledonous species occur. Among the dicotyledonous species growing here are *Ziziphus zeyheriana* and *Elephantorrhiza elephantina*. The grassland communities of, for example the Korannaberg, eastern Free State fall under this

grassland type and some of the woody species growing here are *Asclepias gibba*, *Senecio coronatus*, *Euclea crispa* and *Rhus divarica* (Du Preez, 1992 ; Du Preez & Venter, 1992).

5.2.1.6 Rocky Highveld Grassland (vegetation type 34) (Low & Rebelo, 1996)

Rocky Highveld Grassland type constitutes 1.49% of the Free State province. It is transitional between typical grasslands of the highland plateau and the bushveld of the lower inland plateau. It stretches from Lichtenburg to Middelburg in the east, including the southern slopes of the Magaliesberg, mainly between 1 500 and 1 600 m above sea level.

Rain falls in summer at an average of 650 to 750 mm per year. Temperatures vary between -12 and 39°C.

This area is made up of rocky mountains, hills, ridges and plains of quartzite, conglomerate, shale, dolomite and sometimes andesitic lava.

Grass species predominate, and some of them are *Trachypogon spicatus*, *Schizachyrium sanguineum* and *Andropogon schirensis*. Many dicotyledonous non-woody species that include *Senecio venosus* and *Vernonia oligocephala* also grow here. Afromontane non-woody species growing here include *Rhus rehmanniana*, *R. discolor*, *Vernonia natalensis* and *Helichrysum oreophilum*. Woody Afromontane vegetation appears as sheltered islands of temperate mountain bushveld within the Grassland Biome. Woody species growing here include *Acacia caffra*, *Rhus leptodictya*, *R. pyroides*, *R. rigida*, *R. zeyheri*, *Ehretia rigida*, *Euclea crispa*, *Ziziphus mucronata*, *Olea europaea* subsp. *africana* and *Grewia occidentalis*.

5.2.1.7 Wet Cold Highveld Grassland (vegetation type 41) (Low & Rebelo, 1996)

The Wet Cold Highveld Grassland type makes up 4.78% of the Free State province. This is mountain grassland with a typically cool, wet Drakensberg montane climate and severe frost. Snow and frequent burning influence this vegetation type. It is associated with rocky slopes and ravines of the lower slopes of the Drakensberg, at altitude greater than 1 750 m. The rains fall in summer at an average of about 900 m per year. Temperatures range from -5°C to 36°C.

Soils are typical of mountain landscape, being shallow lithosols, mainly representative of the Glenrosa and Mispah soil forms. Sandstones and mudstones of the Elliot and Molteno Formations (Karroo Sequence) and Beaufort Group are predominant rock types.

Vegetation here is structurally grassland, but a woody layer with an average height of 3 to 5 m form dense thickets in places. *Euclea undulata*, *Diospyros whyteana* and *Rhus dentata* are often present. The hot north-facing slopes are often dry and poor in species and dominated by grasses. On relatively moist, steep, cooler, south-facing slopes, species-rich, dense thickets with a sparse undergrowth are dominated by a non-grassy herbaceous layer. The non-woody species here are, among others, *Clematis oweniae*, *Leonotis dysophylla* and *Sutera polelensis*.

5.2.1.8 Alti Mountain Highveld Grassland (vegetation type 46) (Low & Rebelo, 1996)

The Alti Mountain Highveld Grassland type forms 0.09% of the Free State province. It occurs on the steep, treeless alpine Upper Mountain region bordering Lesotho and the KwaZulu-Natal Drakensberg, 2 500 to 3 480 m above sea level. The terrain includes plateau and steep slopes with small terraces. Summer rainfall exceeding 1 000 m per year is experienced here. Snow often falls in winter and temperatures range from -8°C to 32°C.

The geology is the massive Drakensberg basalts of the Stormberg Group, with shallow, acidic lithosols.

Vegetation in the Alti Mountain Highveld Grassland consists of tussock grasses, ericoid dwarf shrubs and creeping or mat forming plants. Non-woody species growing here include a wide range from the genus *Helichrysum*. Patches of Afromontane vegetation affiliated to Fynbos are scattered throughout the grassland, but large patches are restricted to the plateaux of the Drakensberg between Korannaberg and Thaba' Nchu, where altitudes range from 1 800 to 2 800 m above sea level. Species represented in this stretch include, *Erica maesta, Cliffortia nitidula* and *Euclea coricea*.

5.2.1.9 North East Mountain Highveld Grassland (vegetation type 43) (Low & Rebelo, 1996)

The North East Mountain Highveld Grassland makes up to 0.02% of the Free State. It occurs in the north-eastern parts of the province. Altitude ranges from 1 400 to 1 900 m. The rainfall is high, falling in summer, and ranges from 700 to 1 100 mm per year. Mist also plays an important role in the determination of plant species growing here. Temperatures vary from –8°C to 39°C. Soils are mostly shallow lithosols derived from a variety of rock types.

This grassland type contains many endemic plant species from the following families: Liliaceae, Iridaceae, Asteraceae, Lamiaceae and Orchidaceae. Typical species that are always present include the grasses *Loudetia simplex*, *Trachypogon spicatus* and *Aristida junciformis*. Non-woody species such as *Vernonia centaurioides*, *V. natalensis*, *Helichrysum wilmsii*, *H. cephaloideum*, *H. acutatum* and *H. pilosellum* also grow here. A number of species are, however, restricted to the mist belt zone on high mountain peaks and summits exceeding 1 700 m. These include *Helichrysum platypterum*, *H. umbraculigerum*, *H. mariepscopicum*, *Lobelia flaccida* and *Scilla nervosa*. At lower altitudes below 1 700 m, non-woody species such as *Helichrysum oreophilum* and *Indigofera sanguinea* and woody species such as *Faurea rochetiana* and *Protea caffra* grow. Forest related bush clumps occur on the Black Reef quartzites, situated at the edge of the escarpment in this area. Here woody species include *Psychotria capensis*, *Myrica pilulifera*, *Rhus tumulicola* and *Erica caffrorum*.

5.2.2 Nama –Karoo Biome (Low & Rebelo, 1996 ; Malan, 1997)

The Nama-Karoo Biome, making up 21.62% of the province, is the second largest biome, in the province after the Grassland Biome. This biome occurs on the central plateau of the western half of South Africa, at altitudes between 500 and 2 000 m above sea level, with most of the biome falling between 1 000 and 1 400 m. The rain falls in summer and varies between 100 and 520 mm per year.

The Nama-Karoo Biome consists mainly of extensive to undulating plains, interspersed with mesas, hills or an occasional mountain. The most common geological features here are of the Beaufort and Ecca groups. The main soil group is lime-rich and poorly developed, contributing up to 80% of the soils here. Other soil groups include sands, combinations of red clays and undifferentiated rocks and lithosols. The soils are generally alkaline with a pH range of 7.8 to 83 (Malan, 1997).

Vegetation of the Nama-Karoo Biome is dominated by chaemophytes and hemicryptophytes and can be described as a grassy, dwarf shrubland. The hemicryptophytes are characterised by C_4 graminoids. Plant species diversity in this biome is very low. Where overgrazing has occurred, many indigenous species like *Acacia karroo* proliferate.

5.2.2.1 Eastern Mixed Nama Karroo (vegetation type 52) (Henderson, 1991 ; Low & Rebelo, 1996)

This vegetation type (False karoo; Fig 5.2) reflects an extensive ecotone between the Nama Karroo Biome in the west and the Grassland Biome to the east constituting 20.95% of the Free State province. It occupies the western and south western arid regions of the province. Altitude varies from about 1 400 m for areas north of the escarpment to around 700 m for those below the escarpment. It is a landscape of mesas and buttes. Rainfall occurs mainly in late summer and

autumn, at an average of between 300 and 500 mm per year. Part of the Bloemfontein west district lies within this biome (Malan *et al.*, 1995).

Beaufort Group sandstones and shales dominate the landscape, with the flat-topped landscape shaped by many dolerite dykes and sills.

Vegetation is mainly a complex mix of grass- and shrub-dominated vegetation types, which are subject to changes in species composition dependent on seasonal rainfall regime. Common shrubs include *Pentzia incana*, *Eriocephalus ericoides* and *E. spinescens* while grasses such as *Aristida* spp. And *Aragrostis* spp. Dominate after good summer rains in the north-east. Only along the dry river beds are trees abundant. Here the most common tree species is *Acacia karroo*. In the Bloemfontein west district, the tree and shrub communities are restricted to the rock outcrops, whereas the grassland communities occur mainly on the low lying areas. The other common woody species here are *Olea europaea*, *Ziziphus mucronata*, *Osyris lanceolata*, *Buddleja saligna*, *Protasparagus laricinus*, *Rhus ciliata*, *Euclea crispa*, *Grewia occidentalis*, *Ehretia rigida* and *Diospyros lycioides* and they grow on the many low rocky hills (Malan *et al.*, 1995).

5.2.2.2 Orange River Nama Karroo (vegetation type 51) (Low & Rebelo, 1996)

This region occurs within the hot and arid drainage basin on the Orange River, extending westwards as far as Viooldrif. It constitutes 0.13% of the Free State. Altitude ranges from 350m in the mountains in the east to about 250 m near Viooldrif in the west. Rainfall is low, at 150 to 350 mm per year. Rains fall in late summer in the east and late autumn in the west.

Orange River Nama Karoo occurs on soils derived from the ancient basement granites and gneisses of the Namaqualand Mobile Belt on the south edge of the Richtersveld Craton. Red

and yellow apedal, freely draining, young soils dominate most of the area. Deep alluvial soils occur along the Orange River.

The complex geology and broken, rocky terrain result in a large number of distinct vegetation communities within the Orange River Nama Karoo. In rocky areas of the region, the trees *Aloe dichotoma*, *Euphorbia avasmontana* and *E. gregaria* grow on the steep slopes of mountains and hills. On the pediments *Acacia mellifera*, *Rhigozum trichotumum*, *Boscia albitrunca* and *B. foetida* are common trees and shrubs. The grasses *Stipagrostis uniplumis* often dominate the plains especially after good summer rains. The banks of the Orange River are characterised by lots of thickets. Trees growing among these thickets are *Tamarix usneoides*, *Ziziphus mucronata* and *Acacia erioloba*. These trees also grow on the dry river beds of the tributaries of the Orange River.

5.2.2.3 Upper Nama Karoo (vegetation type 50) (Low & Rebelo, 1996)

The Upper Nama Karoo vegetation type constitutes 0.54% of the Free State province. It occupies the central part of the upper plateau at an altitude of between 1 050 and 1 700 m. The topography is generally flat and stoney, but the area is dotted with hills and mountains. Rainfall is low, 200 to 250 mm per year, occurring mostly in autumn.

Karoo Sequence sandstones and shales which give rise to weak and structureless clayey and sandy soils are common. On the stoney plains *Eriocephalus ericoides*, *Plinthus karooicus* and *Rosenia humilis* are common among other shrubs. Grasses such as *Aristida congesta* and *Eragrotis lehmanniana* dominate after good rains. The more hilly areas are characterised by *Rhus undulata*, *Lycium spp*. And *Rhigozum trichotomum*, with *Merxmuellera disticha* on the tops of the higher mountains in the region.

5.2.3 Savanna Biome (Henderson, 1991 ; Low & Rebelo, 1996)

The Savanna Biome is characterised by a grassy ground layer and a distinct upper layer of woody plants. Where this upper layer is near the ground, the vegetation is referred to as Shrub, where it is dense, as Woodland and the intermediate stages as the Bushveld. The savanna biome occupies 6.64% of the province and is the third in size after the Nama-Karoo Biome.

The altitude of this vegetation type ranges from sea level to 2 000 m. Rainfall varies from 235 to 1 000 mm per year. Frost may occur from 0 to 120 days per year. Almost every major geological and soil type occurs in this biome.

A major factor delimiting this biome is the lack of sufficient rainfall which prevents the upper layer from dominating. The grass layer is dominated by C_4-type grasses, where the growing season is hot, but C_3-type grasses dominate where the rains fall predominantly in winter.

The shrub-tree layer varies from 1 to 20 m in height, but in Bushveld it varies from 3 to 7 m.

5.2.3.1 Kimberly Thorn Bushveld (vegetation type 32)

The Kimberly Thorn Bushveld (Kalahari thornveld; Fig. 5.2) occupies 6.64% of the Free State province. It is limited to the north-western parts of the province at altitudes from 1 000 to 1 200 m above sea level. The rains fall in summer at an average of 400-500 mm per year. Temperatures vary between –8°C and 41°C.

The Kimberly Thorn Bushveld is characterised by deep predominantly sandy to loamy soils, underlain by calcrete.

Vegetation is typically open *Acacia* Savanna with *Acacia tortilis* and *A. erioloba* being the dominant tree species. Also present are scattered species of *Acacia karroo* and *Boscia albitrunca*. The shrub layer is poorly to moderately developed in places and individual species of *Tarchonanthus camphoratus*, *Acacia mellifera*, *Grewia flava* and *Lycium hirsutum* occur

widely scattered. The grass layer is well developed with grasses such as *Themeda triandra*, *Enneapogon cenchroides*, *Eragrostis lehmanniana*, *Elionurus muticus* and *Cymbopogon plurinodis* being common.

The thornvelds of the Kroonstad area (northwestern Free State) are generally located along valleys, in ravines, small kloofs, plateaux, rocky ridges or hills and on outcrops of sandstone. These thornvelds are made up of *Acacia karroo*, *Protasparagus laricinus*, *Diospyros lycioides*, *Rhus pyroides* and *Themeda triandra* (Kooij *et al.*, 1991).

5.2.4 Forest Biome

Forests are restricted to the frost-free areas with mean annual rainfall of more than 525 mm in the winter rainfall region and more than 725 mm in the summer rainfall region. They occur from sea level to over 2 000 m above sea level. Forests occur in patches, a few of which cover areas greater than 1 km^2. The canopy cover of the forests is continuous, comprising mostly evergreen trees, and beneath it, the cover is multi-layered. The ground layer is almost absent due to the dense shade. In the Free State province, the Forest Biome is the smallest (Low & Rebelo, 1996).

5.2.4.1 Afromontane Forest (vegetation type 2) (Low & Rebelo, 1996)

The Afromontane Forest vegetation type makes up to 0.05% of the Free State province. Patches of these forests occur along the mountain chains at altitudes from sea level up to 1 500 m on the border with Lesotho and KwaZulu Natal Province. Forests here range from small to extensive patches on mountain-sides depending on locality and variation in aspect, geology and soils.

Rainfall is generally greater than 700 mm per year, and can exceed 2 000 mm in some regions, occurring throughout the year, or during winter or summer, depending on the region.

Temperatures can be extreme in some of the higher altitudes where snowfalls occur occasionally.

Soils are well developed and mature, and, in the higher-rainfall regions, may be leached. They tend to be shallow on steeper slopes, but may be fairly deep in valleys.

Trees can be up to 30 m or 40 m tall and distinct strata of emergent trees, canopy trees and shrub and herb layers are present. Tree species include *Podocarpus latifolius*, *P. falcatus*, *Trichocladus ellipticus*, *Rhus chiredensis*, *Curtisia dentata*, *Calodendrum capense*, *Ilex mitis* and *Ocotea bullata*. The common shrubs and climbers include *Maytenus heterophylla*, *Scutia myrtina*, *Carissa bispinosa* and *Zanthophyllum capense* among others. In the undergrowth grasses, herbs and ferns are also locally common. They include the grasses *Oplismenus hirtellus*, *Stipa dregeana* var. *elongata* and *Cyperus albostriatus*. Ferns, shrubs and small trees such *as Rapanea melanophloeos* are often abundant along the forest edges. Other woody species found in the Afromontane forests of the eastern Free State is the evergreen tree *Olea europaea* subsp. *africana* and also the deciduous shrubs *Grewia occidentalis* and *Rhus dentata* (Du Preez & Bredenkamp, 1991).

In the Afromontane forests of the eastern areas of the province, average annual precipitation increases from the west to the east. This is caused by the increasing relief from west to east and decrease of average daily temperatures from west to east. Approximately 550 mm or more of rain falls per annum, mainly during summer and autumn. The winters are cold and dry (Du Preez *et al.*, 1991).

Although rock type (geology) and soil type do influence the distribution pattern and extent of forest communities, climate is considered the major environmental determinant regarding the floristic composition and distribution of the Afromontane forest communities in the eastern parts of the Free State province (Du Preez *et al.*, 1991).

5.3 MATERIALS AND METHODS

The plant species collected in chapter 4 were entered on the map of the Free State province to indicate where they were collected.

5.4 RESULTS

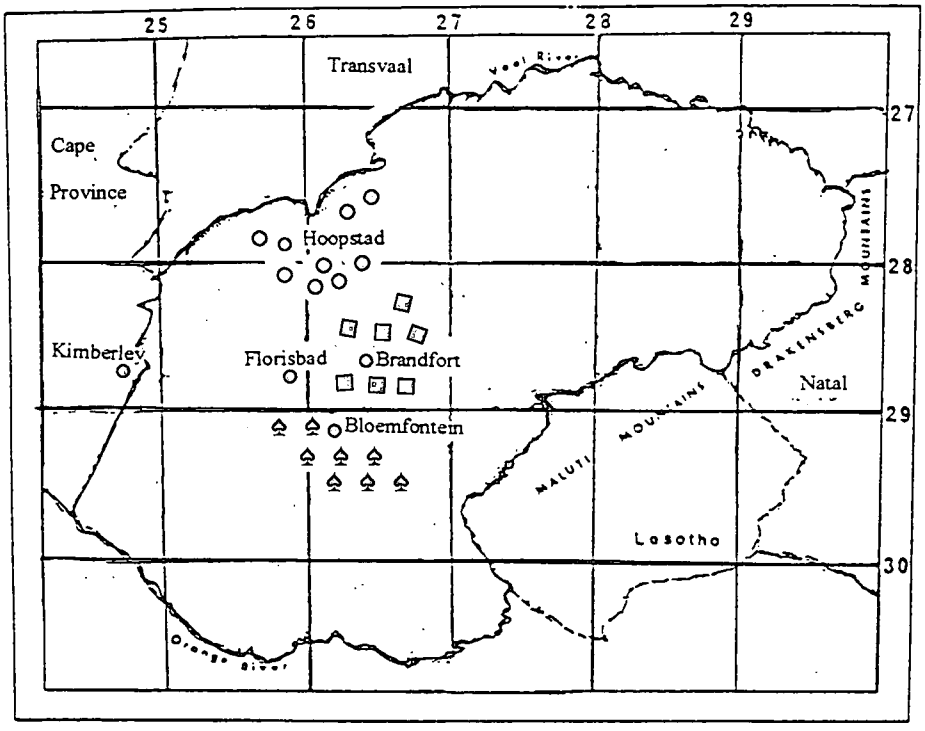

Figure 5.3 Collection areas of plant species used in the study

♠ Plant species collected from the Bloemfontein District: *Asclepias fruticosa, Vernonia oligocephala, Bulbine asphodeloides, Elephantorrhiza elephantina, Rhus erosa, Rhus ciliata, Rhus lancea, Diospyros austro-africana, Euclea crispa subsp crispa, Ehretia rigida, Grewia occidentalis, Osyris lanceolata, Ziziphus mucronata* subsp. *mucronata, Buddleja saligna, Olea europaea subsp. africana, Ranunculus multifidus, Senecio radicans.*

▫ Plant species collected from the Brandfort District : *Protasparagus laricinus, Rumex lanceolatus*

⊙ Plant species collected from the Hoopstad District : *Clematis brachiata, Acacia erioloba, Acacia hebeclada* subsp. *hebeclada, Acacia karroo, Coccinia sessilifolia, Senna italica* subsp. *arachoides, Grewia flava.*

5.5 DISCUSSION

Even though these plant species were collected from an area not covering the whole province, the collection area covered the major biomes, namely the Grassland Biome, the Savanna Biome and the Nama-Karoo Biome. Most of the plant material was collected from the Bloemfontein, Brandfort and Hoopstad districts. These plants were tested for their antimicrobial properties against a number of fungi and bacteria in chapter 6.

CHAPTER 6

PRELIMINARY SCREENING OF CRUDE METHANOLIC EXTRACTIVES FOR ANTIMICROBIAL PROPERTIES

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6.1 INTRODUCTION

Plants are a possible source of antimicrobial compounds for the control of human, animal and plant diseases caused by microbes. Compounds of plant origin are preferred to synthetic compounds, since they are environmentally safer, biodegradable and leave no harmful residues (Ganesan & Krishnaraju, 1995). Anti-infective activity of plant constituents is one of the major areas of research involving plants (Hamburger & Hostettmann, 1991).

According to the World Health Organization (WHO), about 80% of the inhabitants of the world rely mainly on traditional medicines for their primary health care requirements and the major part of traditional therapy involves the use of plant extracts or their active principles (Yadav & Dubey, 1996). In South Africa, about 33% of the population still depend on traditional herbal medicine from as many as 700 indigenous plant species (Meyer *et al.*, 1996).

World wide, recent changes in legislation tend to restrict or even eliminate the use of many synthetic crop protectants. Over the last years, increased research activity has been directed towards the development of effective alternative crop protectants. These are often sought from natural sources like plants, due to the fact that a large variety of antimicrobial compounds have been found to function in their natural defence systems (Gorris & Smid, 1995).

Although there is a large number of research publications available on the constituents and biological activity of medicinal plants from Africa, the development of therapeutic drugs and agrochemicals has remained neglected. The study of African medicinal plants has not been documented as fully as in other traditional societies, such as the Indian and the Chinese. Literature available (Watt & Gerdina, 1932 ; Watt & Breyer-Brandwijk, 1962) has shown that the Free State Province of South Africa is no exception to this situation and is probably the least documented. Due to the fact that folk medicine is a useful indicator of biologically active substances (Prance, 1994), many references are made in this study to the use of some plant species in traditional medicine.

Simple bioassays are used in phytochemical laboratories for investigating (a) antibacterial activity targeting human and plant pathogenic bacteria, and (b) antifungal activity targeting human and plant pathogenic fungi and yeasts (Hamburger & Hostettmann, 1991). The fact that crude plant extracts with medicinal properties can also be tested against plant pathogens (Miles, 1990) made appropriate the screening of human as well as plant pathogens with the same extracts in this investigation. It is known that *in vitro* antimicrobial screening methods are sufficient to provide the required preliminary observations for selecting, among crude plant extracts, those with potentially useful properties for further chemical and pharmacological investigations (Mathekga & Meyer, 1998).

Twenty six plant species from 16 Families growing in the Free State Province of South Africa were screened against a number of plant pathogenic fungi and bacteria. One human opportunistic bacterium was added to the list of microorganisms. The preliminary screening of these plant extracts was done as a search for plant species with the most potent antimicrobial properties as indicated by a broad spectrum of activity and large zones of inhibition.

6.2 MATERIALS & METHODS

6.2.1 Materials

Twenty six plant species representing 16 Families (Table 4.1; section 4.1.1, chapter 4) were screened against a number of microorganisms listed in Tables 4.2 and 4.3 and in section 4.1.2, chapter 4.

Chemicals

The solvent used for the preparation of crude extracts was methanol (analytical grade ; Merck).

Dimethyl sulphoxide (DMSO ; Sigma Chemicals) was used for the dissolution of crude extracts for antimicrobial tests.

6.2.2 Methods

6.2.2.1 Preparation of crude methanol extracts

The crude extracts were prepared according to the method described in section 4.2.1, chapter 4. Aliquots of 1 ml were dried at 60-80°C, until the weight remained constant, to determine their concentrations. These were then discarded. The crude extracts of known concentration were then diluted to 50 mg cm^{-3} with a 10% (v/v) solution of DMSO in water (Villarreal *et al.*, 1994). Zavala *et al.* (1997) used a range of concentrations from 0.5 –100 mg cm^{-3}. As a matter of convenience, a decision was made to record the results obtained with crude extracts at the concentration of 50 mg cm^{-3}.

6.2.2.2 Screening for antimicrobial properties

Antifungal and antibacterial controls were run together with the other tests under identical conditions. The modified agar diffusion method, a method described by Bauer *et al.* (1966) which is widely used for antibiotic susceptibility testing as well as for screening of medicinal and other plants for antimicrobial activity towards human and plant pathogens (Kavanagh, 1975 ; Rios *et al.*, 1988 ; El-Abyad *et al.*, 1990 ; Heisey & Gorham., 1992 ; Caceres *et al.*, 1993a).

6.2.2.2.1 Antifungal tests

Antifungal tests were done according to the method described in section 4.2.2.1, chapter 4.

6.2.2.2.2 Antibacterial tests

Antibacterial tests were carried out according to the method described in section 4.2.2.2, chapter 4.

6.3 RESULTS

With reference to Heisey and Gorham (1992), antimicrobial inhibition zones in the range of 6.0

- 6.5 mm were recorded as zero (no inhibition) where 6 mm paper disks were used. Where 10 mm paper disks were used, inhibition zones less than 11mm were also recorded as zero i.e., extracts were not considered active (Brantner & Grein, 1994).

6.3.1 Antifungal tests

Antifungal activity was determined against nine fungal test organisms listed in Table 4.2 (see section 4.1.2.1, chapter 4).

The results of the preliminary screening of crude extracts against nine plant pathogenic fungi showed that none of the 32 crude extracts (from 26 plant species) exhibited fungicidal properties against these pathogenic fungi, even at concentrations as high as 100 to 300 mg cm^{-3} (results not shown).

6.3.2 Antibacterial tests

In a series of tests, the antibacterial activity of all plant extracts was determined against five selected plant pathogenic bacteria listed in Table 4.3 (see section 4.1.2.2, chapter 4) to which *M. catarrhalis* was added. The results of these tests are shown in Tables 6.1.

Table 6.1: Preliminary screening of crude extracts for antibacterial activity at 50 mg cm^{-3}.

FAMILY & PLANT NAME	BACTERIA & MEAN INHIBITION ZONE DIAMETER (mm)					
	Agrobacterium tumefaciens	*Clavibacter michig.*pv *michiganense*	*Erwinia carotovora* pv *carotovora*	*Pseudomon. solanacear*	*Xanthomonas campest* pv. *phaseoli*	*Moraxella catarrhalis*
Anacardiaceae						
Rhus ciliata	0	7.0 ± 0.8	0	8.0 ± 0.0	0	9.0 ± 0.8
Rhus erosa	10.0 ± 0.8	10.0 ± 0.0	0	0	0	17.0 ± 0.8
Rhus lancea	0	7.0 ± 0.0	0	8.0 ± 1.0	0	14.0 ± 0.6
Asclepiadaceae						
Asclepias fruticosa	0	0	7.0 ± 1.0	0	0	0
Asparagaceae						
Protasparagus laricinus	0	0	7.0 ± 0.8	0	0	0
Asphodelaceae						
Bulbine-asphodeloides(inflorescence)	0	0	0	0	10.0 ± 0.0	21.0 ± 0.9
Bulbine asphodeloides (roots)	0	0	9.0 ± 0.8	0	10.0 ± 0.8	22.0 ± 0.0

Table 6.1 continued

FAMILY & PLANT NAME	BACTERIA & MEAN INHIBITION ZONE DIAMETER (mm)					
	Agrobacterium tumefaciens	*Clavibacter michig.*pv *michiganense*	*Erwinia carotovora* pv *carotovora*	*Pseudomon. solanacear*	*Xanthomonas campest* pv. *phaseoli*	*Moraxella catarrhalis*
Asteraceae						
Vernonia oligocephala	0	0	0	0	0	0
Senecio radicans	0	0	7.0 ± 0.6	0	0	0
Boraginaceae						
Ehretia rigida	0	0	7.0 ± 0.4	0	0	0
Cucurbitaceae						
Coccinia sessilifolia	0	7.0 ± 0.7	0	8.0 ± 0.6	0	0
Ebenaceae						
Diospyros austro-africana var. *microphylla*	15.0 ± 0.8	16.0 ± 0.0	0	15.0 ± 0.8	0	24.0 ± 0.4
Euclea crispa subsp. *crispa*	13.0 ± 0.4	13.0 ± 0.8	0	15.0 ± 0.4	14.0 ± 0.0	18.0 ± 0.0
Fabaceae						
Acacia erioloba	13.0 ± 0.4	11.0 ± 0.8	13.0 ± 0.4	12.0 ± 0.8	15.0 ± 0.4	22.0 ± 0.7
Acacia hebeclada subsp. *hebeclada*	0	0	7.0 ± 0.7	0	0	0
Acacia karroo	11.0 ± 0.4	11.0 ± 0.8	0	12.0 ± 0.8	11.0 ± 0.0	24.0 ± 0.4
Senna italica subsp. *arachoides*	7.0 ± 0.6	7.0 ± 0.6	7.0 ± 0.4	8.0 ± 0.0	8.0 ± 0.6	0
Elephantorrhiza-elephantina	11.0 ± 0.4	11.0 ± 0.8	0	12.0 ± 0.9	11.0 ± 0.9	14.0 ± 0.4
Loganiaceae						
Buddleja saligna	10.0 ± 0.0	10.0 ± 0.7	11.0 ± 0.4	9.0 ± 0.8	9.0 ± 0.4	21.0 ± 0.4
Oleaceae						
Olea europaea subsp. *africana*	0	0	7.0 ± 0.0	8.0 ± 0.8	7.0 ± 0.8	8.0 ± 0.7
Polygonaceae						
Rumex lanceolatus	0	15.0 ± 0.4	0	12.0 ± 0.4	8.0 ± 0.0	23.0 ± 0.8
Ranunculaceae						
Clematis brachiata	0	7.0 ± 0.8	7.0 ± 0.0	7.0 ± 0.6	0	9.0 ± 0.8
Ranunculus multifidus	0	9.0 ± 0.8	0	0	0	12.0 ± 0.8
Rhamnaceae						
Ziziphus mucronata subsp. *mucronata*	9.0 ± 0.8	9.0 ± 0.8	0	0	8.0 ± 0.7	21.0 ± 0.4
Santalaceae						
Osyris lanceolata	12.0 ± 0.8	12.0 ± 0.7	0	0	0	27.0 ± 0.8
Tiliaceae						
Grewia flava	0	10.0 ± 0.8	0	8.0 ± 0.6	0	12.0 ± 0.7
Grewia occidentalis	0	0	0	7.0 ± 0.0	0	14.0 ± 0.6
Control	20.0 ± 0.4	13.0 ± 0.7	13.0 ± 0.8	12.0 ± 0.8	13.0 ± 0.7	27.0 ± 0.6

The control was a 5% (v/v) solution of dimethyl didecyl ammonium chloride in water. The concentration of the control solution for assays with *M. catarrhalis* was a 2.5% (v/v) solution of dimethyl didecyl ammonium chloride in water.

Results in Table 6.1 indicate that of the 32 crude extracts (from 26 plant species) used in the preliminary screen, one extract (3.1% of extracts) from one plant species *Vernonia oligocephala* exhibited no antibacterial activity towards all test bacteria. Five crude extracts (15.6%) from *Asclepias fruticosa, Senecio radicans, Ehretia rigida* and *Acacia hebeclada* subsp. *hebeclada* and *Protasparagus laricinus* showed slight inhibition that was recorded as zone diameters of 7 mm only towards *Erwinia carotovora* pv. *carotovora* and no antibacterial activity towards all the other bacteria. The rest of the crude extracts (81.3%) exhibited some higher degree of antibacterial activity towards one or more test bacteria.

Generally considering the different degrees of growth inhibition of the bacteria by the different crude extracts, of the 32 extracts, 10 (31.3%) inhibited *A. tumefaciens*, 17 (53.1%) inhibited *C. michiganense* pv. *michiganense*, 5 (15.6%) inhibited *E. carotovora* pv. *carotovora*, 17 (53.1%) inhibited *P. solanacearum*, 10 (31.3%) inhibited *X. campestris* pv. *phaseoli* and 24 (75.0%) inhibited the growth of *M. catarrhalis*.

In order to eliminate the less potent crude extracts, it was found necessary to consider those extracts that produced significant zones of inhibition. A significant zone of inhibition was taken as 10 mm or more, i.e. ≥ 10mm (Naqvi *et al.*,1991). Using this criterion, significant activity observed with the 32 extracts had the following trend: 9 extracts (28.1%) inhibited *A. tumefaciens*; 13 extracts (40.6%) inhibited *C. michiganense* pv *michiganense*; 1 extract (3.1%) inhibited *E. carotovora* pv *carotovora*; 9 extracts (28.1%) inhibited *P. solanacearum*; 5 extracts (15.6%) inhibited *X. campestris* pv. *phaseoli* and 20 extracts (62.5%) inhibited the growth of *M. catarrhalis*.

The most inhibited organism was *M. catarrhalis* (62.5%) and the least inhibited was *E. carotovora* pv. *carotovora* (3.1%). With respect to pathogenic bacteria only, the most significantly inhibited pathogen was *C. michiganense* pv. *michiganense* (40.6%), followed by, in the decreasing order of inhibition: *A. tumefaciens* and *P. solanacearum* (both at 28.1%), *X.*

campestris pv. *phaseoli* (15.6%) and the most resistant was *E. carotovora* pv. *carotovora* (3.1%). It is interesting to note that the four crude extracts (12.5%) from *Asclepias fruticosa, Senecio radicans, Ehretia rigida* and *Acacia hebeclada* subsp. *hebeclada* that exhibited slight inhibition towards *Erwinia carotovora* pv. *carotovora* (recorded as 7mm inhibition) showed no activity at all towards other test bacteria.

The crude extract showing the most potent activity towards plant pathogenic bacteria, as indicated by significant inhibition zones ≥10 mm as well as by the broadest spectrum of activity, was from *Acacia erioloba*. This extract inhibited all (100%) of the bacteria tested, showing the largest zones of inhibition as compared to the rest of the extracts. *Acacia erioloba* was followed by *Euclea crispa* subsp. *crispa* which significantly inhibited the growth of 85.7% of the bacteria and showed large inhibition zones as well towards all the bacteria tested, with the exception of *Erwinia carotovora* pv. *carotovora*, towards which no activity at all was observed. *Acacia karroo* also significantly inhibited 85.7% of the bacteria. This was followed by *Diospyros austro-africana* var. *microphylla*, which inhibited 71.4% of the bacteria, though it gave larger zones than *E. crispa* subsp. *crispa*. Also inhibiting 71.4% of the bacteria was *Buddleja saligna* and *Elephantorrhiza elephantina*. *Rumex lanceolatus* exhibiting significant antibacterial activity towards 59.0% of the bacteria tested was chosen as the last among the most active crude extracts.

Considering plant pathogenic bacteria only *Vernonia oligocephala* showed no antibacterial activity at all. Slight inhibition (recorded as 7 mm zones only) was observed with *Protasparagus laricinus, A. fruticosa, S. radicans, E. rigida, A. hebeclada* subsp. *hebeclada, Clematis brachiata* and *Grewia occidentalis* . Small zones of inhibition, taken as low activity (between 7mm and 10mm), and narrow spectrums of activity were observed with *Coccinia sessilifolia, Senna italica* subsp. *arachoides, Olea europaea* subsp. *africana, Ranunculus multifidus, Ziziphus mucronata* subsp. *mucronata, Rhus ciliata, Rhus erosa, Rhus lancea* and *Bulbine asphodeloides*. *Buddleja saligna* inhibited the growth of all the five plant

pathogenic bacteria with inhibition zones in the range of 9.0-11.0mm. In summary, *Acacia erioloba* was followed by, in terms of potency, *Euclea crispa* subsp. *crispa*, *Elephantorrhiza elephantina* and *Acacia karroo*. These three species inhibited the growth of 80% of the plant pathogenic bacteria. *Diospyros austro-africana* var. *microphylla* and *Rumex lanceolatus* inhibited 60% of the plant pathogenic bacteria. Identical inhibition zones towards plant pathogenic bacteria were observed with *Acacia karroo* and *Elephantorrhiza elephantina* both from the Fabaceae.

Moreover, of the seven plant species showing the most promising antibacterial properties, i.e. *Acacia erioloba* (Fabaceae), *Euclea crispa* subsp. *crispa* (Ebenaceae), *Diospyros austro-africana* var. *microphylla* (Ebenaceae), *Acacia karroo* (Fabaceae), *Elephantorrhiza elephantina* (Fabaceae), *Buddleja saligna* (Loganiaceae) *and Rumex lanceolatus* (Polygonaceae), only *A. erioloba* and *B. saligna* inhibited the growth of *E. carotovora* pv *carotovora* (Gram-negative) the most resistant plant pathogenic bacterium in this investigation. The least resistant pathogen in this investigation, *C. michiganense* pv. *michiganense* (Gram-positive), was inhibited by all the seven most promising species that gave the most potent crude extracts.

6.4 DISCUSSION

The results of the preliminary screening of crude extracts from the 26 plant species growing in the Free State Province of the Republic South Africa collected as representatives from 16 Families, indicate that poor antifungal activity was present in these plant species. Before diluting to 50 mg cm^{-3}, the crude extracts from these plant species had been challenged with test fungi at concentrations ranging from 100 to 300 mg cm^{-3} and had also given negative results. This could have been due to the inadequate presence of biologically active compounds exhibiting antifungal properties (Farnsworth & Bingel, 1977 ; Gordon *et al.*, 1980) and also the presence of growth factors that stimulated the growth of fungi and hence negated the effect of inhibitory substances (Ieven *et al.*, 1979 ; Qasem & Abu-Blan, 1996). Some authors have

used lower concentrations of test extracts (10 mg cm^{-3}) with positive results (Naqvi *et al.*, 1991), while others have used very high concentrations of up to 100 mg cm^{-3} (Zavala *et al.*, 1997). Minimum inhibition concentrations of between 300 and 500 mg cm^{-3} of leaf extracts have been observed with the human pathogenic fungi *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* (Caceres *et al.*, 1993b). The absence of antifungal activity towards plant pathogens in this investigation can not be taken to mean that the plant species used in the investigation lack this particular activity. It would be necessary to do these tests in the future using a wider range of fungi so as to come to a definite conclusion on the antifungal status of these 26 plant species towards the plant pathogenic fungi.

Although doses of extracts required to inhibit the growth of microorganisms in this study are high in comparison with the control (dimethyl didecyl ammoium chloride), it should be considered that in this study crude extracts were used and obviously the compounds responsible for the antimicrobial activity are metabolites and normally found in small quantities (Hamburger & Hostettmann, 1991 ; Zavala *et al.*, 1997). However, the presented results (section 6.3) confirmed observations made by other investigators namely that pathogenic fungi are more resistant to plant extracts than pathogenic bacteria (Ieven *et al.*, 1979 ; Heisey & Gorham, 1992) and that plants used as antifungals are generally powerful antiinfective plants (King & Tempesta, 1994). As an example, Heisey & Gorham (1992) observed that in the primary screen of 60 crude extracts, 13 extracts (21.67%) inhibited the growth of bacteria, while only 5 extracts (8.33%) inhibited fungi. In this investigation, considering all forms of inhibition, except where zone diameters were 7-7.5 mm and recorded as zero, only 3 out of 26 plant species (11.54%) inhibited the growth of fungi, while 21 out of 26 plant species (80.77%) inhibited the growth of one or more bacteria. Two out of the 26 plant species (7.69%) exhibited no antimicrobial activity at all, exhibiting neither antifungal nor antibacterial properties. In summary, the results of this preliminary screen for antimicrobial activity showed that the plant species used in this investigation generally had good antibacterial properties but poor antifungal activity.

The results of investigations in this chapter clearly indicated that compounds inhibitory to all the test plant pathogenic and other bacteria were present in most of the plant species tested. Compounds with high antibacterial potency could therefore have been isolated from the most active extracts identified here. This investigation also emphasized the importance of screening local medicinal and other plants for antimicrobial activity before the loss of their habitat.

As has been mentioned above, the most potent crude extracts were from the following plant species: *Acacia erioloba*, *Buddleja saligna*, *Euclea crispa* subsp. *crispa*, *Diospyros austro-africana* var. *microphylla*, *Rumex lanceolatus*, *Acacia karroo* and *Elephantorrhiza elephantina*. These were also the most promising plants as indicated by marked or significant activity (zone diam. ≥ 10 mm) and broad spectrums of activity, especially towards the plant pathogenic bacteria. These results indicated the presence of either good antibacterial potency or of a high concentration of an active principle or principles in the crude extracts.

All of the seven tested plant species, exhibiting significant, antibacterial activity *in vitro*, are rich in polyphenolic compounds, catechins, tannins and polyphenolic acids (Hutchings *et al.*,1996). It is known that polyphenols can form heavy soluble complexes with proteins (Brantner & Grein, 1994). These authors also stated that polyphenols have the ability to bind to adhesins and by doing so they limit the availability of receptors on the cell surface. Literature indicates that antibacterial activity can also be due to tannins (Brantner & Grein, 1994 ; Bruneton, 1995 ; Van Wyk *et al.*, 1997). All the mentioned plant species also contain considerable amounts of essential oils (Hutchings *et al.*, 1996). When present in adequate concentrations, most of the essential oils are able to damage microorganisms (Brantner & Grein, 1994 ; Gorris & Smid, 1995 ; Tsuchiya *et al.*,1996). Some terpenes, being components of essential oils in many plant species, have been found to possess effective *in vivo* antimicrobial properties against plant pathogenic bacteria and fungi (Scortichini & Rossi, 1991). Antibacterial triterpenic saponins have also been identified in *Hedera helix* (Brantner & Grein, 1994). Investigations on the phytochemical profile of *E crispa* by Van der Vijver & Gerritsma (1973) and Sibanda *et al.* (1992) also revealed that this plant contains terpenoids.

Brief reviews of *Acacia erioloba*, *Buddleja saligna*, *Euclea crispa* subsp. *crispa*, *Diospyros austro-africana* var. *microphylla*, *Rumex lanceolatus*, *Acacia karroo*, and *Elephantorrhiza elephantina* are presented since they showed the most potent antimicrobial activity during the investigation.

Acacia erioloba (Fabaceae, Subfamily Mimosaceae)

This is an evergreen semi-decidious tree up to 3-10 m tall with a spreading crown. It is very common in the thorn veld of the western Free State Province (FSP) (Venter, 1976). The branches are characterized by stipular spines or recurved prickles. The young branches are prominently zig-zagged. The leaves are double compound with 2-5 pairs of pinnae, each bearing 8-15 pairs of leaflets (Venter & Venter, 1996). The plant produces bright yellow flowers. Some of the potential toxins known in this genus include anisaldehyde, anthraquinone, benzaldehyde, benzyl alcohol, butyraldehyde, cumic aldehyde, indole, hydrocyanic acid, linalool, nicotine, palmitic acid, quercitrin, rutin, saponins and tannic acid (Hutchings *et al.*, 1996). Some of the compounds mentioned here like tannic acid and other phenolic compounds in general, as well as indole, and saponins have known antimicrobial properties (Wang *et al.*, 1989 ; Kubo *et al.*, 1992a ; Stange, 1993 ; Paster *et al.*, 1995 ; Lis-Balchin & Deans,1996). Some members of this subfamily are used as antimicrobials in the treatment of various infections from colds to enterobacterial infections e.g. *Acacia gerrardii*, *Acacia karroo* and *Elephantorrhiza elephantina* (Hutchings *et al.*, 1996).

Acacia karroo (Fabaceae, Subfamily Mimosaceae)

Acacia karroo is a tree 3-10 m high in the FSP. It is widely distributed throughout the province, especially along water courses. The leaves are divided into about five pairs of leaflets and each again is divided into 10 or more pairs of smaller leaflets of about 5 mm long. The flowers are minute and borne in bright yellow, spherical heads (Venter, 1976 ; Van Wyk *et al.*, 1997). Leaves are used in different parts of Africa as emollients and astringents as well as for colds and enterobacterial infections. Tannins and gallic acid are

present in the leaves (Hutchings *et al.*, 1996 ; Van Wyk *et al.*, 1997).

Elephantorrhiza elephantina (Fabaceae, Subfamily Mimosaceae)

Elephantorrhiza elephantina occurs in the grassland biome of the FSP and mostly in the upper three-quarters of the province. The plant has several unbranched annual stems of about 1 metre in height, growing from a large underground rhizome of up to 8 metres long. The leaves are finely divided and have small narrow leaflets. Clusters of small, cream-coloured flowers are produced along the lower half of the aerial stem. This plant is used as a traditional remedy for enterobacterial infections, skin diseases and acne (Van Wyk *et al.*, 1997).

Buddleja saligna (Loganiaceae)

Buddleja saligna, in the FSP is a shrub of 2-4 m in height. It is common to abundant or even dominant in dry hilly veld of this province. Flowers are white to cream with a red centre. Leaves are long, opposite and simple with net venetion. The upper surface of each leaf is smooth and dark green while the lower surface is light coloured and scaley (Venter, 1976). Another member of this genus, *Buddleja americana,* has been found to have good antimicrobial properties towards a number of human pathogenic bacteria and is known to posses alkaloids as well as flavonoids (Caceres *et al.*, 1993). Extracts from the leaves of *B. globosa* possess antibacterial activity against *Staphylococcus aureus* and *Escherichia coli.* Verbascoside in leaves exhibits antimicrobial properties and is likely to be responsible for the reputed therapeutic properties of the species of this genus (Hutchings *et al.*, 1996).

Euclea crispa subsp.*crispa* (Ebenaceae)

In the FSP, *Euclea crispa* subsp. *crispa* is a tree of 2-7 m in height, it is abundant in cliff forests as well as the rocky habitat of the eastern Free State and common from the north to the

south. Leaf form and size varies form a few centimetres long in drier shrub to 10 cm long in moist mountain ravines. Leaf apex varies from round to acuminate (Venter, 1976). *Euclea crispa* subsp. *ovata* is a shrub or tree, 1-5 m high and is spread throughout the ridge veld of the southern FSP. It is distinguished from *Euclea crispa* subsp. *crispa* by the leaves which are less variable and by undulating leaf margins (Venter, 1976).

Dark coloured naphthaquinones or their derivatives are commonly produced in leaves of *Euclea* species (Hutchings *et al.*, 1996). These authors also reported that naphthaquinones have a vitamin K action, which justifies the application of leaves as wound dressings. They further mentioned that a total of twenty seven different naphthaquinone derivatives have been isolated from plant extracts of the Ebenaceae. Other isolated compounds include various pentacyclic triterpenoid alcohols and acids as well as phytosterols (Sibanda *et al.*,1992 ; Hutchings *et al.*, 1996). *Euclea* species are extensively used in traditional medicine for a wide range of ailments including dysentery, wounds, sores, leprosy and scabies (Khan & Rwekika, 1992). These authors isolated α-amyrin, lupeol and betulinic acid from the leaves of *Euclea natalensis*.

Diospyros austro-africana subsp. *microphylla* (Ebenaceae)

In the FSP, *Diospyros austro-africana* subsp. *microphylla* is a shrub, 1-3 m high, and is common on rocky ridges of the western half of the province. On the other hand, *Diospyros austro-africana* subsp. *rubriflora* is common on mountain slopes and ravines of the eastern parts of the FSP (Venter, 1976). Many species have been found to contain hydroxynaphthoquinones such as diospyrin, diospyrol and diosquinone (Hutchings *et al.*, 1996).

Rumex lanceolatus (Polygonaceae)

Rumex species are usually herbs, occasionally shrubby 0.3 to 0.5m in height, with erect leafy branches. The leaves are bright green and hairless. They are related to the medicinal plants called rhubarbs (*Rheum* species). Members of the genus *Rumex* are characterized by the

presence of anthraquinones and quinones (Hutchings *et al.*, 1996 ; Van Wyk *et al.*, 1997). Anthocyanin pigments, flavonones and flavonols are also common. Also mentioned by these authors is that the leaves of *Rumex* species contain large amounts of oxalic acid, tartaric acid as well as tannic acid.

The absence of, or low antimicrobial activity observed with some of the plant species used in this investigation does not mean that they are not important. They might have other forms of biological activities that were not relevant to the current study hence they are not discussed here. It is well documented that the habitat of each individual plant influences the phytochemical composition of its different parts due to the microclimate around it and the condition of the soil (Hegnauer, 1986). The time of the day at which the plant materials is collected also influences the concentrations of active substances in the tissues as it is known that these are under diurnal influence (Verpoorte, 1989 ; Gorris & Smid, 1995). Failure to obtain positive results with an extract containing active principles could have also been because the active compound(s) was (were) present in insufficient quantity in the crude extract from one collection or another (Farnsworth & Bingel, 1977) as a result of the above mentioned factors.

The conclusion that can be drawn from the observed results of this chapter is that test fungi used in this investigation were highly resistant to the crude extracts from the 26 plant species tested. It can also be concluded that the antifungal activity of the test plants was very poor due to the fact that even at concentrations higher than 100 mg cm^{-3} no activity had been observed. This confirmed the observations of other investigators (Ieven *et al.*, 1979 ; Heisey & Gorham, 1992) regarding the higher resistance of fungi to plant extracts as compared to bacteria. On the other hand, it can be safely concluded that most of the plant species growing in the FSP have good antibacterial properties.

The preliminary screening of crude extracts for antimicrobial activity was performed with the aim to find the most potent plant species that could be further tested against a wider range of microorganisms. It was also envisaged to determine the phytochemical profiles of the promising plant species as part of the investigation towards the identification of active

antimicrobial compounds as well as the cytotoxicity of the isolated compounds. Hence, an evaluation of the potential application of these plants as agrochemicals and/or chemotherapeutic agents was envisaged.

As was mentioned in the discussion above, the most potent crude extracts were from the following plant species: *Acacia erioloba*, *Buddleja saligna*, *Euclea crispa* subsp. *crispa*, *Diospyros austro-africana* var. *microphylla*, *Rumex lanceolatus*, *Acacia karroo* and *Elephantorrhiza elephantina*. These were also the most promising plants as indicated by marked or significant activity (zone diam. $\geq$ 10 mm) and broad spectrums of activity, especially towards the plant pathogenic bacteria. *A. erioloba* was the most promising plant among these. However, it was decided to continue investigations on *E crispa* subsp. *crispa* (chapters 7, 8 and 9), the second most promising and probably the less studied of the two instead.

From the preliminary screening of crude extracts for antimicrobial activity, the crude extract of *E. crispa* subsp. *crispa*, showing strong antimicrobial activity was subsequently separated by liquid-liquid chromatography into fractions of increasing polarity as an initial step towards its purification. The phytochemical profile of each of the semi-purified fractions from *E crispa* subsp. *crispa* was defined in chapter 7. In chapter 8, these semi-purified fractions (as well as the crude extract) were tested for antimicrobial activity with both plant and human pathogenic bacteria as well as human pathogenic fungi. The isolation of compounds with antimicrobial activity from the most active liquid-liquid separation fraction was attempted in chapter 9.

CHAPTER 7

PHYTOCHEMICAL PROFILE OF THE SEMI-PURIFIED (LIQUID-LIQUID FRACTIONATION) EXTRACTS FROM EUCLEA CRISPA SUBSP. *CRISPA (EBENACEAE)*

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7.1 INTRODUCTION

Today plants are still the almost exclusive source of drugs for the majority of the world's population. Major pharmaceutical companies are also showing renewed interest in higher plants as a source for new lead structures. As a result, plant metabolites are being investigated from a phytochemical as well as chemotaxonomic view (Hamburger & Hostettmann, 1991).

It is obvious that the key to further exploitation of nature for anti-infective and other chemicals is phytochemistry. The secondary metabolism of plants is not only of interest for the products themselves, but there is also an awareness that secondary metabolites play a major role in protecting the plant against microorganisms, insects and other plants (Verpoorte, 1989). Plants contain many metabolites and therefore any phytochemical investigation of a given plant reveals only a very narrow spectrum of its constituents (Hamburger & Hostettmann, 1991).

The results of the preliminary screening of crude extracts from 26 plant species growing in the Free State Province resulted in the observation that, of the 26, seven plant species were the most active crude extracts. Among the seven, was *E. crispa* subsp. *crispa* that was selected because of its ethnopharmacological application in some areas of South Africa like Kwa-Zulu Natal as well as its wide spread distribution throughout the country and in this province.

A search through literature revealed that some phytochemical analysis of *E. crispa* and few other species belonging to the Ebenaceae had been attempted. *Euclea divinorum* has been reported to contain triterpenoids and naphthaquinones (Costa *et al.*, 1976). Phytochemical analysis of four species of the Ebenaceae; *Diospyros greenwayi*, *Diospyros mafiensis*, *Diospyros natalensis* and *Euclea natalensis* (Khan and Rwekika, 1992) revealed the presence of triterpenoids in the leaves of these plants. All the four species contained lupeol and betulinic acid, while α-amyrin was detected only in the leaves of *D. mafiensis*, *D. natalensis* and *E.natalensis*. Sibanda *et al.* (1992) also isolated pentacyclic triterpenoids lupeol, betulin and oleanolic acid, from the root bark of *E. crispa*. Earlier, the naphthaquinones diospyrin,

isodiospyrin, 7-methyljuglone and 8,8'-dihydroxy-4,4'-dimethoxy-6,6'-dimethyl-2,2'-bisnaphthyl-1,1'-quinone were isolated from *E. crispa* (Van der Vijver & Gerritsma, 1973). Hutchings *et al.* (1996) also stated that roots of species from the genus *Euclea* have been found to be rich in naphthaquinones and that isodiospyrin, diospyrin and 7-methyljuglone are the major naphthaquinones which occur frequently within this genus. They reported that the plant parts rich in naphthaquinones and their derivatives are the leaves and stems of the species from the family Ebenaceae though no naphthaquinones yet had been found in the branch percolate of *E. crispa*. They also mentioned the presence of various pentacyclic triterpenoid alcohols and acids, as well as the phytosterols sitosterol and stigmasterol. The literature found on *E. crispa* gave a general indication that this plant contains mainly triterpenoids and naphthaquinones.

An investigation into the phytochemical profile of *E. crispa* subsp. *crispa* leaf extracts was done in this chapter in an effort to determine which of the compounds present in this plant are responsible for the antimicrobial activity (results presented in chapters 8 and 9). This approach was also used by El-Abyad *et al.* (1990). The investigation was also done as an initial guide towards isolation of the active compounds (results are in chapter 9). No literature was found detailing the phytochemical profile of *E. crispa* and its subspecies that grow in the Free State province. It was hoped that a wider range of compound classes and even novel compounds would be isolated during the course of the investigation (in chapter 9) to add to the existing knowledge on the phytochemical profile of *E. crispa*.

It is well known that the distribution of compounds in solvents depends upon the polarity of both the solvents and the solubility of the compounds in question. The separation and identification of the major classes of compounds present in the four liquid-liquid separation fractions (i.e. the semi-purified fractions) was done by analytical thin layer chromatography. Relevant spray reagents were also used. As has been stated in the preceding paragraph, liquid-liquid separation was carried out as a first step towards the isolation of active compounds from the leaf extracts of *E. crispa* subsp. *crispa*. Further investigations on the most active fraction led to the isolation of some of the compounds with antimicrobial activity in chapter 9.

7.2 MATERIALS AND METHODS

7.2.1 Materials

Silica gel silica gel 60 F_{254}, 0.25mm aluminium-backed thin layer chromatography (TLC) precoated sheets (Merck).

All the solvents used for TLC were of Analytical Grade (Merck).
Spray reagents were purchased from Sigma Chemicals, or prepared with chemicals purchased from Sigma Chemicals

7.2.2 Methods

7.2.2.1 Liquid-liquid fractionation

The crude extract was prepared by performing ten extractions on 1.73 kg of powdered material with 95% (v/v) methanol in water. The extract was filtered and the methanol evaporated under vacuum at 35°C to leave an aqueous extract. This was again filtered and the volume adjusted to 500 ml with distilled water.

Liquid-liquid fractionation was performed on the crude extract with the following four solvents of increasing polarity in the order *n*-hexane (dielectric constant (ε) = 1.9); diethyl ether, ε = 4.3; chloroform, ε = 4.8 and ethyl acetate, ε = 6.0, to separate the active components according to polarity (Harwood & Moody, 1989). A predetermined volume (500ml) of the methanol-free crude extract was successively treated with each of the above solvents starting with hexane, at a volume ratio of 1:2. The crude extract and the solvent were shaken ten times on a mechanical shaker with new solvent added for each time. The first shaking lasted 30 minutes and subsequent extractions lasted 15 minutes each. The four solvent extractives were collected separately and subsequently evaporated to dryness under vacuum at 35°C or below, depending on the solvent being removed in a Buchi rotavapor. This gave 30.46g of hexane fraction in the

form of a syrup, 42.04 g of diethyl ether fraction, 5.13 g of the chloroform fraction and 46.37 g of the ethyl acetate fraction. These same fractions were used for the antimicrobial tests in chapter 8, the results of which are presented in section 8.3.

7.2.2.2 Thin layer chromatography (TLC)

TLC was performed on precoated silica gel silica gel 60 F_{254}, 0.25mm aluminium-backed sheets (Merck). The sheets were generally cut into strips of about 5 x 10 cm. A sample from each of the four liquid-liquid separation fractions was diluted to a 1% (w/v) solution with the least polar component of the corresponding mobile phase. A volume of about 5 μℓ was spotted on the tlc plate for each phytochemical test. The TLC plates were developed in saturated chambers along the 10cm length. The chromatography solvent (mobile phase) was allowed to stand for 0.5-1 hour before immersion of the chromatogram for development to saturate the tank. Phytochemical analysis of extracts from *E. crispa* subsp. *crispa* was done according to the protocols outlined in Wagner & Bladt (1996).

7.2.2.3 Detection of phytochemicals

In all cases, detection was done at UV 254 nm (to detect substances that quench fluorescence) and at UV 365 nm (to detect substances that fluoresce in long wave UV-light) before spraying with the appropriate spray reagent.

7.2.2.3.1 Identification of anthraglycosides

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10) was used.

Spray reagent: Potassium hydroxide (KOH) reagent

The plate was sprayed with 10 ml of a 5% ethanolic solution of KOH. Evaluation was done in visible and in UV-365 nm, with and without warming.

7.2.2.3.2 Identification of cardiac glycosides

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Kedde reagent

A volume of 5 ml of freshly prepared 3% ethanolic 3,5-dinitrobenzoic acid was mixed with 5 ml of 2 M NaOH. The plate was sprayed with 5-8 ml of the reagent and evaluated under visible light.

7.2.2.3.3 Identification of bitter principles

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Vanillin-sulphuric acid reagent

Solution I: 1% ethanolic vanillin

Solution II: 10% ethanolic sulphuric acid

The plate was sprayed with 10 ml of solution I, followed immediately by 10 ml of solution II. After heating at 110°C for 5 minutes, the plate was evaluated in visible light.

7.2.2.3.4 Identification of alkaloids

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Dragendorff reagent

Solution (a): 0.5 g basic bismuth nitrate was dissolved in 10 ml glacial acetic acid and 40 ml water was prepared under heating. This solution was then filtered.

Solution (b): 8 g of potassium iodide was dissolved in 30 ml of water.

Stock solution: Solutions (a) and (b) were mixed 1:1

Spray solution: 1 ml stock solution was mixed with 2 ml glacial acetic acid and 10 ml water.

The plate was sprayed and viewed in visible light.

7.2.2.3.5 Identification of phenolic compounds

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Ammonium vanadate/ρ-anisidine spray reagent (Touchstone & Dobbins, 1978)

Solution (a): saturated, aqueous ammonium vanadate

Solution (b): A mass of 0.5g of ρ -anisidine was dissolved in 2 ml H_3PO_4. This solution was then diluted to 100 ml with ethanol and filtered. The plates were sprayed with solution (a), and

while still wet, they were then sprayed with solution (b) and heated at 80°C. Observation was done in visible light.

7.2.2.3.6 Identification of saponins (a)

Solvent system: Ethyl acetate : methanol : water (100 :13.5 :10)

Spray reagent: Vanillin-sulphuric acid reagent (as in section 8.4) and Anisaldehyde-sulphuric acid reagent.

A volume of 0.5 ml of anisaldehyde was mixed with 10 ml of glacial acetic acid followed by 85 ml of methanol and then 5 ml of concentrated sulphuric acid. The TLC plate was sprayed with about 10 ml, heated at 100°C for 5 min, then evaluated in visible light or UV-365 nm.

7.2.2.3.7 Identification of saponins (b)

Solvent system: Toluene : Ethyl acetate (93 : 7)

The test was done as in 8.2.2.6 above.

7.2.2.3.8 Identification of essential oils

Solvent system: Toluene : ethyl acetate (93 : 7)

Spray reagent: Vanillin-sulphuric acid (as in section 8.4)

Evaluation was done in visible light.

7.2.2.3.9 Identification of valepotriates

Solvent system: Toluene : ethyl acetate (93 : 7)

Spray reagent: Hydrochloric acid-glacial acetic acid

The plate was sprayed with a mixture of eight parts of concentrated hydrochloric acid and two parts of glacial acetic acid. After spraying, the plate was heated at 110°C for 10 min. Evaluation was done in visible light and under UV-365 nm.

7.2.2.3.10 Identification of coumarins

Solvent system: Toluene : ethyl acetate (93 : 7)

Spray reagent: Potassium hydroxide (KOH) reagent

The plate was sprayed with about 10 ml of a 10% ethanolic solution of KOH. Evaluation was done in UV-365 nm, before chemical treatment and after treatment as well as in visible light with and without and warming.

7.3 RESULTS

The tests indicated that *E. crispa* subsp. *crispa* possessed bitter principles, alkaloids, phenolic compounds, saponins and essential oils. All the liquid - liquid fractionation extracts tested negative for anthraglycosides, cardiac glycosides, valepotriates and coumarins (see appendix; pages 122 and 126; plates 7.1, 7.2, 7.9 and 7.10 respectively).

7.3.1 Separation and identification of bitter principles

The results of identification of bitter principles by tlc (see 7.2.2.3) are shown on Plate 7.3 (see appendix; page 123) and in Table 7.1.

Table 7.1: Comparative TLC profiles of the hexane, diethyl ether, chloroform and ethyl acetate fractions in the test for bitter principles.

Solvent system: EtOAc : MeOH : H_2O (100 :13.5 : 10)

R_f values of spots	Colours of spots with vanillin-sulphuric acid	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.99	bluish-violet	+	+	-	-
0.92	bluish-violet	+	-	-	+
0.91	bluish-violet	-	+	+	-
0.85	bluish-violet	-	+	-	-
0.77	yellowish-brown	-	-	-	+
0.76	yellowish-brown	-	+	-	-
0.74	bluish-violet	-	-	+	-
0.63	bluish-violet	-	-	-	+

Table 7.1 continued

R_f values of spots	Colours of spots with vanillin-sulphuric acid	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.58	bluish-violet	-	-	-	+
0.54	bluish-violet	-	-	-	+
0.50	bluish-violet	-	-	+	+
0.45	bluish-violet	-	-	-	+
0.36	bluish-violet	-	-	-	+
0.29	bluish-violet	-	+	+	-
0.20	bluish-violet	-	+	-	-
0.18	bluish-violet	-	-	+	-
0.15	bluish-violet	-	+	-	-
0.13	bluish-violet	-	+	-	-
0.09	bluish-violet	-	+	+	-

+ positive test for bitter principles; - negative test for bitter principles

Table 7.1 indicates that most of the spots reflecting bitter principles were observed in the diethyl ether and ethyl acetate fractions, which showed 9 out of 25 (36%) and 8 out of 25 (32%) spots respectively. The chloroform fraction showed 6 out of 25 (24%) and the hexane fraction 2 out of 25 (8%) of all the spots observed. The spots represented by R_f values of 0.92, 0.91 and 0.29 were very large, while the spots on R_f values of 0.77 and 0.76 were large as compared to the rest on the TLC plates. These large spots represented a high concentration of the particular compounds in the extracts.

7.3.2 Separation and identification of alkaloids

The results of the separation and identification of alkaloids are shown on Plate 7.4 (see appendix; page 123) and Table 7.2.

Table 7.2: Comparative TLC chromatograms of the hexane, diethyl ether, chloroform and ethyl acetate fractions in the test for alkaloids.
Solvent system : EtOAc : MeOH : H_20 (100 : 13.5 : 10)

R_f values of spots	Colours of spots with Dragendorff reagent	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0. 50	orange-brown	-	-	-	+
0.60	orange-brown	-	-	-	+
0.70	orange-brown	-	-	-	+
0.78	orange-brown	-	-	-	+

+ positive test for alkaloids ; - negative test for alkaloids

The only alkaloid spots observed were in the ethyl acetate fraction, and at R_f values which ranged between 0.50 and 0.78.

7.3.3 Separation and identification of phenolic compounds

The distribution of phenolic compounds in the liquid-liquid separation fractions is shown on Plate 7.5 (see appendix; page 124) and in Table 7.3.

The phenolic compounds were observed in the diethyl ether and chloroform fractions but mainly in the ethyl acetate fraction (Table 7.3).

Table 7.3: Comparative TLC chromatograms of the hexane, diethyl ether, chloroform and ethyl acetate fractions in the test for phenolic compounds.
Solvent system : EtOAc : MeOH : H_2O (100 : 13.5 : 10)

R_f values of spots	Colours of spots with ammonium vanadate/p-anisidine	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.74	bluish green	-	+	-	+
0.73	Bluish green	-	-	+	+
0.57	bluish green	-	-	-	+

Table 7.3 continued

R_f values of spots	Colours of spots with ammonium vanadate/p-anisidine	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.56	bluish green	-	-	-	+
0.53	bluish green	-	-	-	+
0.51	bluish green	-	-	-	+
0.48	bluish green	-	-	-	+
0.42	bluish green	-	-	-	+

+ positive test for phenolic compounds; - negative test for phenolic compounds

7.3.4 Separation and identification of saponins

Two different solvent systems were used to separate saponins on TLC plates. The distribution of saponins in the liquid-liquid separation fractions using the solvent system; EtOAc : MeOH : H_2O; (100 :13.5 :10) is shown on Plate 7.6 (see appendix; page 124) and Table 7.4a.

Table 7.4.a: Comparative TLC chromatograms of the hexane, diethyl ether, chloroform and ethyl acetate fractionsin the test for saponins.
Solvent system EtOAc : MeOH : H_2O (100 : 13.5 : 10)

R_f values of spots	Colours of spots with vanillin-sulphuric acid	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.90	blue	+	-	+	+
0.89	blue	-	+	-	-
0.84	blue	-	+	-	-
0.76	blue	-	+	-	-
0.56	blue	-	-	-	+
0.51	blue	-	+	-	-
0.49	blue	-	-	-	+
0.43	blue	-	-	-	+

+ positive test for saponins; - negative test for saponins

Table 7.4.a continued

R_f values of spots	Colours of spots with vanillin-sulphuric acid	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.31	blue	-	-	-	+
0.30	blue	-	+	-	-
0.29	blue	-	-	+	-
0.20	blue	-	-	+	-
0.16	blue	+	-	-	-
0.11	blue	+	-	-	-
0.10	blue	-	-	+	+

+ positive test for saponins ; - negative test for saponins

Using the solvent system, EtOAc : MeOH : H_2O (100 : 13.5 : 10), most spots representing saponins were observed with the diethyl ether (7 out of 18 ; 38.89%) and the ethyl acetate fractions (6 out of 18 ; 33.33%) fractions. The lowest number of spots representing saponins was observed with the chloroform, (4 out of 18 ; 22.22%) and the hexane fractions, (1 out of 18 ; 5.56%).

Susequently, Toluene : ethyl acetate (93 : 7) was used as the second solvent system and the distribution of saponins in the liquid-liquid separation fractions using this system is shown on Plate 7.7 (see appendix; page 125) and Table 7.4.b.

Table 7.4.b: Comparative TLC chromatograms of the hexane, diethyl ether, chloroform and ethyl acetate fractions in the test for saponins using the solvent system Toluene : ethyl acetate (93 : 7).

R_f values of spots	Colours of spots with vanillin-sulphuric acid	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.94	blue	+	+	+	-
0.30	blue	+	-	-	-
0.29	blue	-	+	-	-
0.18	blue	+	-	-	-
0.14	blue	+	-	-	-

Table 7.4.b continued

R_f values of spots	Colours of spots with vanillin-sulphuric acid	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.10	blue	+	+	-	-
0.09	blue	-	-	+	-
0.05	blue	-	+	-	-
0.04	blue	-	-	+	+
0.01	blue	-	+	-	-

+ positive test for saponins; - negative test for saponins

The solvent system Toluene : ethyl acetate (93 : 7), extracted more saponins into the hexane fraction. Table 7.4b shows that the hexane and the diethyl ether had the highest number of saponin spots, 5 out of 14 (35.71%) in both cases. On the other hand, the chloroform and the ethyl acetate fractions showed the lowest number of spots (3 out of 14 ; 21.43% and 1 out of 14 ; 7.14% respectively). Tables 7.4a and 7.4b indicate that all the liquid-liquid separation fractions contained saponins.

7.3.5 Separation and identification of essential oils

The distribution of essential oils in the liquid-liquid separation fractions is shown on Plate 7.8 (see appendix ; page 125) and Table 7.5.

Table 7.5: Comparative TLC chromatograms of the hexane, diethyl ether, chloroform and ethyl acetate fractions in the test for essential oils.
Solvent system: Toluene : ethyl acetate (93 : 7)

R_f values of spots	Colours of spots with vanillin-sulphuric acid	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.93	violet-blue	+	+	+	-
0.91	violet-blue	-	-	+	-
0.50	violet-blue	+	-	-	-
0.29	violet-blue	+	+	+	-
0.18	violet-blue	+	+	-	-

Table 7.5 continued

R_f values of spots	Colours of spots with vanillin-sulphuric acid	FRACTION			
		Hexane	Diethyl ether	Chloroform	Ethyl acetate
0.15	violet-blue	+	+	-	-
0.11	violet-blue	-	+	-	-
0.10	violet-blue	+	-	+	-
0.05	violet-blue	-	+	-	-
0.04	violet-blue	-	-	+	+
0.03	violet-blue	-	+	-	-
0	violet-blue	+	-	-	-

+ positive test for essential oils; - negative test for essential oils

The test for essential oils (Table 7.5) using the solvent system Toluene : ethyl acetate (93 : 7), indicated that most were located in the hexane and diethyl ether fractions, both showing 7 out of a total of 20 spots (35%) recorded. The number of spots observed on tlc of the chloroform fraction was 5 out of 20 (25%), while the ethyl acetate fraction showed 1 out of 20 (5%) of the spots.

A summary of the distribution of the different classes of compounds in the four liquid-liquid separation fractions is shown in Table 7.6 below.

Table 7.6: Summary of the distribution of phytochemical classes in the four liquid-liquid separation extracts of *E. crispa* subsp. *crispa*.

Class of compounds	Fraction and number of spots on tlc plates			
	Hexane	Diethyl ether	Chloroform	Ethyl acetate
Bitter principles	2	9	6	8
Alkaloids	0	0	0	4
Phenolic compounds	0	1	1	8
Saponins (a)*	1	7	4	6
Saponins (b)*	5	5	3	1
Essential oils	7	7	5	1

Saponins (a); saponins separated with the solvent system EtOAc : MeOH: H_20 (100 : 13.5 : 10)

Saponins (b) ; saponins separated with the solvent system Toluene : EtOAc (93:7)

The results in Table 7.6 show that the diethyl ether fraction had the highest proportion of bitter principles, followed by the ethyl acetate, choloroform and lastly the hexane fraction, with the lowest. Alkaloids were only detected in the ethyl acetate fraction. Phenolic compounds were present in all fractions, with the exception of the hexane fraction. The highest proportion of phenolics was in the ethyl acetate fraction. Saponin concentration was highest in the diethyl ether fraction, followed by the hexane, ethyl acetate and lastly, by the chloroform fraction. Only those results obtained with the solvent systems that gave the best separation of saponins are considered. Most of the essential oils were located in the hexane and diethyl ether fractions, followed by the chloroform fraction. The ethyl acetate faction had the lowest content of essential oils.

Summing up the proportions of all the classes of compounds, for each of the fractions, the trend from the fraction with the highest concentration of phytochemical classes to the fraction with the lowest was as follows; ethyl acetate > diethyl ether > chloroform > hexane.

7.4 DISCUSSION

The phytochemical investigation of the semi-purified extracts from *E. crispa* subsp. *crispa* confirmed that the plant possesses bitter principles, alkaloids, phenolic compounds, saponins and essential oils. The TLC profiles also indicated that significant amounts of bitter principles, saponins, essential oils and phenolic compounds are present in the leaves of this plant while alkaloids are less abundant. Alkaloids were identified only in the ethyl acetate fraction, but not in the other three liquid-liquid separation fractions. Literature on *E. crispa* subsp. *crispa*, or any of its other subspecies, in which the presence of alkaloids is documented, was not found.

Alkaloids are multipurpose compounds which, depending on the situation, may be active in more than one environmental interaction. As an example, quinolizidine alkaloids are important defense chemicals against bacteria, fungi and viruses. In these microorganisms, reduction of growth and antibiosis are usually the visible effects of alkaloid intoxication (Wink, 1997).

Bitter principles, saponins and essential oils are related in that they all are derivatives of terpenoids (Banthorpe, 1991 ; Wagner & Bladt, 1996). Hence, some of the compounds positive in the TLC test for bitter principles could also have been present in the TLC test for essential oils. Bitter principles in plants represent derivatives of monoterpenoids, sesquiterpenoids, diterpenoids and triterpenoids (Banthorpe, 1991).

Although the same spray reagent (vanillin-sulphuric acid) was used in the test for bitter principles and for essential oils, different solvent systems were employed. The solvent system EtOAc: MeOH : H_2O (100 :13.5 :10) was used to screen for bitter principles, while Toluene : EtOAc (93 : 7) was used for essential oils for better resolution. The bitter principles were concentrated mainly in the diethyl ether, chloroform and ethyl acetate fractions, while the essential oils were concentrated mainly in the hexane, diethyl ether and chloroform fractions.

It is well known that essential oils are one of the possible sources of plant derived antimicrobial compounds, especially broad spectrum antifungals for plant protection, since this class of antifungals constitutes one of the largest volumes of pesticides used (Gorris and Smid, 1995). Monoterpenoids, being components of essential oils are widely used in food preservation. *In vivo*, they have been found to possess antimicrobial properties against plant pathogenic bacteria and fungi (Scortichini and Rossi, 1991).

All the four liquid-liquid separation fractions showed significant amounts of saponins, depending on the extraction solvent and the TLC solvent system used. Both the solvent systems EtOAc : MeOH : H_2O (100 : 13.5 : 10) and Toluene : EtOAc (93 : 7) separated the saponins from the extracts well depending on the polarity of the fraction. The compounds in less polar extracts were best separated by the less polar solvent system containing toluene. The solvent system Toluene : EtOAc (93 : 7) was also used in the separation of essential oils which were detected with the same spray reagent but the colour reaction was different. Essential oils gave a violet blue colour while the saponins gave a blue colour.

Saponins are plant glycosidic triterpenoids whose parent compound is squalene. They are soluble in water giving stable foams. Three major classes found in plants are steroid glycosides, steroid alkaloid glycosides and triterpene glycosides. Triterpene glycosides form the largest group (Bramley, 1997). The presence of saponins in the semi-purified extracts of *E. crispa* subsp. *crispa* also indicated that triterpenoids are contained in the leaves of this plant. Saponins have been found to protect against fungal attack and they also have a low oral toxicity to humans, probably due to the low rates of absorption from the intestine (Bramley, 1997).

The general test for phenolic compounds indicated the presence of these phytochemicals in the diethyl ether, chloroform and ethyl acetate fractions with the solvent system EtOAc : MeOH : H_2O (100 :13.5 :10) and the spray reagent ammonium vanadate/p-anisidine. The role of phenolic compounds as antimicrobials is well known (Laks, 1987 ; Hutchings *et al.*, 1996 ; Robards & Antolovich, 1997 ; Strack, 1997).

Most of the classes of compounds present in the hexane, diethyl ether and chloroform fractions were also well represented in the ethyl acetate fraction. The only time the ethyl acetate extract showed fewer spots for any compounds was in the test for essential oils. This may have been due to the low polarity of the solvent system recommended for the test. The bitter principles though, being related to the essential oils by virtue of being made up of isoprene units, were plentiful in the ethyl acetate fraction.

The trend from the fraction having the highest concentration of phytochemicals to the fraction with the lowest proportion of compounds: ethyl acetate > diethyl ether > chloroform > hexane indicated that most of the compounds isolated from *E. crispa* subsp. *crispa* were concentrated in the ethyl acetate fraction. Each of these semi-purified fractions (including the crude extract) were further tested in chapter 8 against a number of plant pathogenic bacteria, human pathogenic bacteria as well as human pathogenic fungi. These antimicrobial tests were carried

out in order to locate in which of the four semi-purified (liquid-liquid separation) fractions most of the activity would be located.

The activity of an extract may be due to a number of factors like, the presence low quantities of highly active compounds, the presence of high quantities of compounds with low activity, the presence of high quantities of compounds with significant activity or even synergistic compounds. The most active semi-purified fraction would then be used in chapter 9 to isolate compounds with antimicrobial activity.

APPENDIX

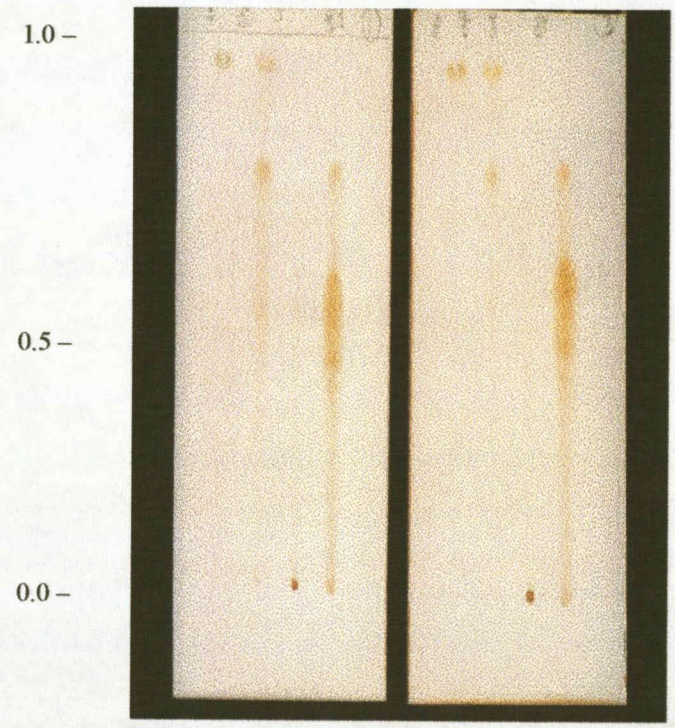

Plate 7.1 : Test for anthraglycosides Plate 7.2 : Test for cardiac glycosides
1= hexane fraction, 2 = diethyl ether fraction, 3 = chloroform fraction,
4 = ethyl acetate fraction
Solvent system : EtOAc : MeOH : H_2O (100 : 13.5 : 10)

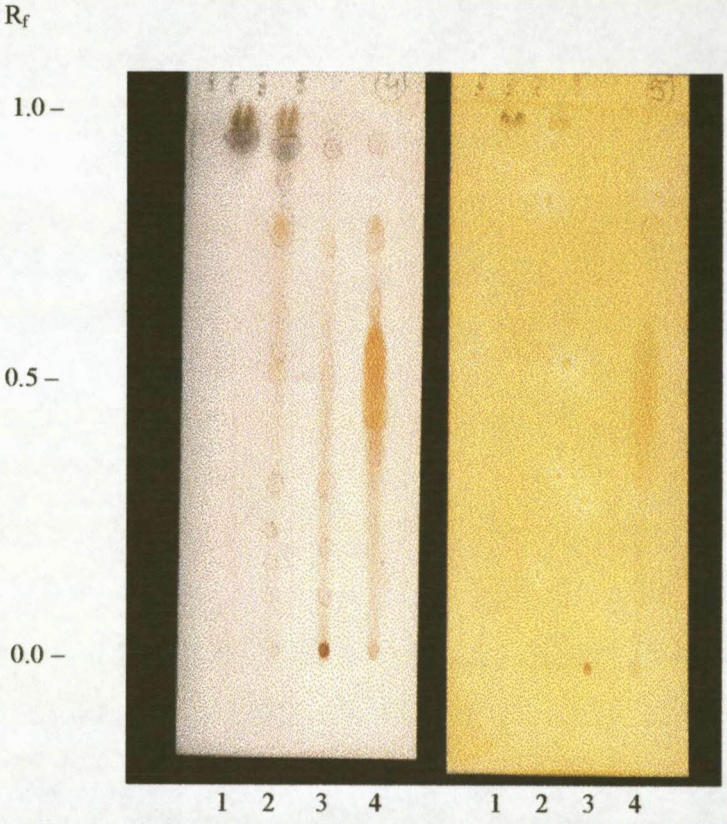

Plate 7.3 : Test for bitter principles Plate 7.4 : Test for alkaloids
1 = hexane fraction, 2 = diethyl ether fraction, 3 = chloroform fraction, 4 = ethyl acetate fraction
Solvent system : EtOAc : MeOH : H_2O (100 : 13.5 : 10)

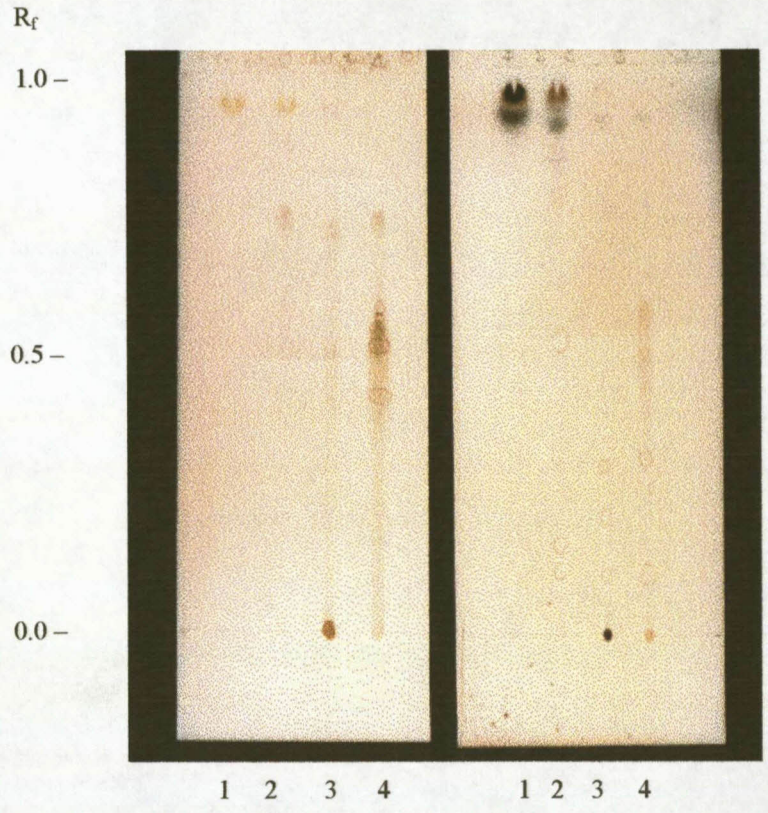

Plate 7.5 : Test for phenolic compounds Plate 7.6 : Test for saponins
1 = hexane fraction, 2 = diethyl ether fractiom, 3 = chloroform fraction,
4 = ethyl acetate fraction
Solvent system : EtOAc : MeOH : H_2O (100 : 13.5 : 10)

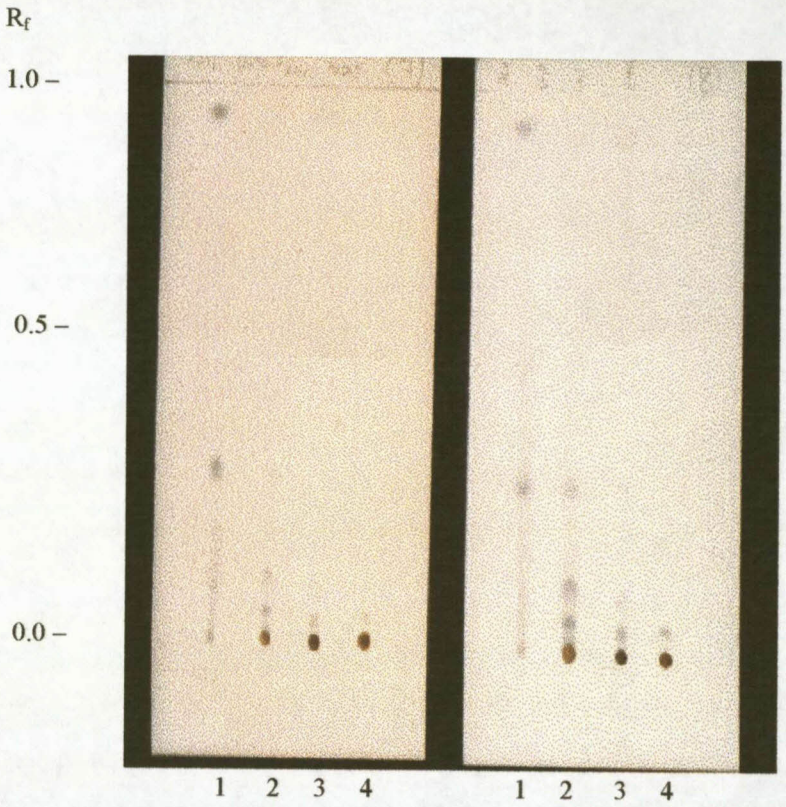

Plate 7.7 : Test for saponins Plate 7.8 : Test for essential oils
1 = hexane fraction, 2 = diethyl ether fraction, 3 = chloroform fraction, 4 = ethyl acetate fraction
Solvent system : Toluene : EtOAc (93 : 7)

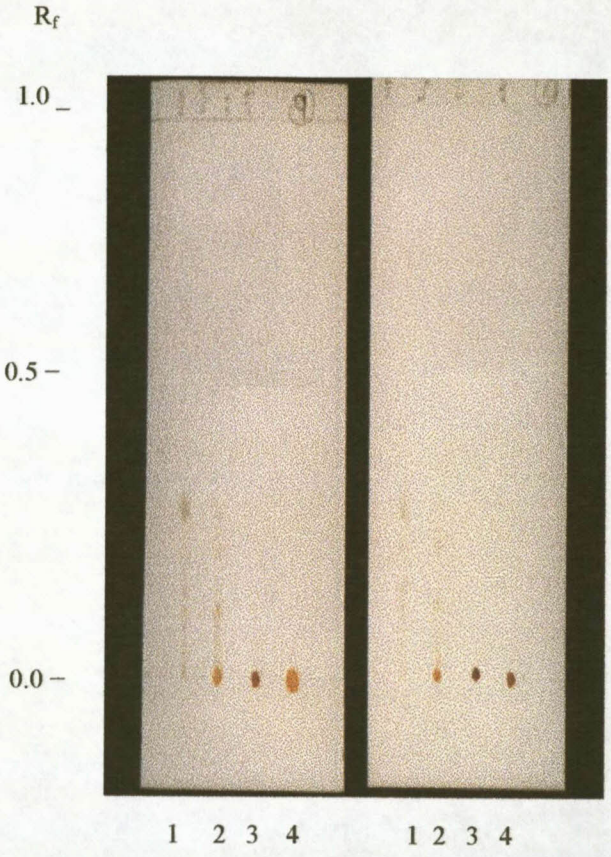

Plate 7.9 : Test for valepotriates Plate 7.10 : Test for coumarins
1 = hexane fraction, 2 = diethyl ether fraction, 3 = chloroform fraction,
4 = ethyl acetate fraction
Solvent system : Toluene : EtOAc (93 : 7)

CHAPTER 8

ANTIMICROBIAL PROPERTIES OF CRUDE AND SEMI-PURIFIED EXTRACTS FROM LEAVES OF EUCLEA CRISPA SUBSP. *CRISPA (EBENACEAE)*

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8.1 INTRODUCTION

The search for antimicrobial agents is still in progress in many phytochemical laboratories even though no plant derived compound has been found which can compete with clinically used antibiotics (Hamburger & Hostettmann, 1991). Treatments with immunosuppressive drugs and the spread of AIDS have meant that diseases caused by weaknesses in the immune system of humans are becoming more and more prevalent. Associated with these problems is the increasing predisposition to fungal attack. The infections commonly observed in the immuno-compromised host include candidiasis (caused by *Candida albicans* and other species) and cryptococcosis (caused by *Cryptococcus neoformans*) and also aspergillosis (caused by *Aspergillus flavus*, *A. fumigatus* and *A. niger*) (Hostettmann & Marston, 1994). Other diseases also observed are respiratory e.g. streptococcal, staphylococcal and *Haemophilus influenzae* infections, as well as systemic infections caused by e.g. *Streptococcus pneumoniae* and *S. pyogenes* and *Neisseria meningitidis* among other bacteria (Brock & Madigan, 1991).

The development and practical implementation of plant derived antimicrobials in crop protection is a high priority area in relation to both food preservation and crop protection. Interest in the use of chemicals of plant origin, especially as crude plant extracts, is increasing and larger agrochemical industries are partaking in this development because of the increased awareness of dangers involved in the use of synthetic agrochemicals. One commercial preparation resulting from this is marketed as Milsana™, a dried extract of *Reynoutria sachalensis* (Polygonaceae), which inhibits powdery mildews (Gorris & Smid, 1995).

E. crispa subsp. *crispa* is one of the most common trees throughout the Republic of South Africa (Venter, 1976 ; Malan *et al.*, 1998) but the least documented of the subspecies. *Euclea* species are extensively used in traditional medicine for a wide range of ailments, including gonorrhoea, wounds, leprosy, scabies and dysentery (Khan & Rwekika, 1992). In the Republic of South Africa, an infusion of the root bark of *Euclea crispa* is used as a cough remedy, as well as for the treatment of measles and melanomas (Sibanda *et al.*, 1992).

Antimicrobial properties of crude and semi-purified extracts from leaves of *Euclea crispa* subsp. *crispa* were investigated in an effort to find out in which of the liquid-liquid separation fractions antimicrobial activity was concentrated. The most active fraction was then analyzed in chapter 9 in an effort to isolate the active compounds.

8.2 MATERIALS AND METHODS

8.2.1 Materials

The plant material was collected in March, 1997 and treated as mentioned in chapter 4 (sections 4.1.1 and 4.2.1).

8.2.1.1 Plant pathogenic bacteria

These were the same as those listed in chapter 4 (section 4.1.2.2).

8.2.1.2 Human pathogenic bacteria

Human pathogenic bacteria were clinical isolates kindly provided by the Faculty of Health Sciences, Department of Microbiology at the University of the Free State, Bloemfontein. These are listed in Table 8.1

Table 8.1: Human pathogenic test bacteria

BACTERIA	DESCRIPTION	GRAM STAIN
Acinetobacter baumanii	bacilli	negative
Bacillus subtilis	bacilli	positive
Escherichia coli	bacilli	negative
Haemophilus influenzae	bacilli	negative
Klebsiella pneumoniae	bacilli	negative
Moraxella catarrhalis	cocci	negative
Pseudomonas aeruginosa	bacilli	negative
Staphylococcus aureus	cocci	positive
Staphylococcus epidermidis	cocci	positive
Streptococcus pneumoniae	cocci	positive
Streptococcus pyogenes	cocci	positive

8.2.1.3 Human pathogenic fungi

Human pathogenic fungi were also clinical isolates provided by the Faculty of Health Sciences, Department of Microbiology at the University of the Free State in Bloemfontein. These are

listed in Table 8.2.

Table 8.2: Human pathogenic test fungi

FUNGUS	DESCRIPTION
Candida albicans	yeast
Cryptococcus neoformans	yeast

8.2.1.4 Chemicals

Dimethyl sulphoxide (DMSO) and Tween-80 were purchased from Merck (Germany) and were of the highest purity available.

8.2.2 Methods

The crude extract was prepared as explained in section 6.2.2.1; chapter 6, while the semi-purified extracts were prepared as described in section 7.2.2; chapter 7.

The dried crude extract, the chloroform and ethyl acetate fractions were dissolved in a 10% (v/v) solution of DMSO in water, while the hexane and diethyl ether extractives were dissolved in a 10% (v/v) solution of Tween-80 in water for antimicrobial tests. A stock solution of each of the extractives was prepared to a concentration of 50 mg cm^{-3}.

8.2.2.1 Antibacterial tests

8.2.2.1.1 Tests with plant pathogenic bacteria

Plate count agar (PCA) was used for tests with plant pathogenic bacteria using the hole-plate diffusion method. The agar was prepared according to the manufacturer's instructions. About 15 ml were poured into each plate to a depth of approximately 3 mm.

Antibacterial tests against plant pathogenic bacteria were done as indicated in chapter 4, section 4.2.2.2, with the positive control being dimethyl didecyl ammonium chloride (DDAC). Overnight cultures of the plant pathogens grown at 30°C in a 1% (w/v) solution of nutrient broth (Biolab) in water were diluted 1:100 with fresh sterile nutrient broth (Meyer & Afolayan, 1995). A 100µl bacterial suspension was transferred and streaked onto the surface of a sterile PCA plate using a flame sterilized glass spreader. Holes of 6 mm diameter, into which the

crude extract or the liquid-liquid fractionation extractives were introduced were made on the agar surface using a cork borer. Inhibition zones were measured and recorded as diameter in millimetres as described by Pfaller *et al.* (1992). The concentration of stock solutions of the crude and semi-purified extracts was 50 mg cm^{-3}. Initial tests were done with 1mg of extract per hole. This concentration was increased to 2 mg per hole in the next set of tests. The plant pathogens were incubated at 30°C for 24 hours.

The first set of experiments was done at 1 mg per hole of 6 mm diameter and the second set at 2 mg per hole of the same diameter.

A third series of experiments was carried out with the five plant pathogenic test bacteria. The crude (50 mg cm^{-3}) and the liquid-liquid separation extractives (50 mg cm^{-3}) were diluted with a solution of 10% (v/v) solution of DMSO in water. A 10% (v/v) solution of Tween-80 in water was used to dilute the less polar fractions. The dilutions were made to give the following concentrations: 90, 80, 70, 60, 40, 30, 20, 15, 10, 7.5, 5, 2.5 and 1.0% (Brantner & Grein, 1994). A volume of 20 µl of each dilution was loaded per 6 mm diameter hole. Two 20 µl volumes of a solution of 10% (v/v) solution of DMSO in water, and another two 20 µl volumes of a solution of 10% (v/v) solution of Tweeen-80 in water were used as negative controls, while DDAC was used as the positive control. The concentration of the DDAC stock was 2.50 mg cm^{-3}. It was diluted just like the other extracts. All experiments were repeated twice. The degree of bacterial inhibition by the extracts was determined using the agar hole-plate diffusion method as described above. Antibacterial activity of each extract was evaluated by measuring the inhibition zone diameters observed. The inhibition zone diameter was then plotted against log. Concentration of each extract (Anesini & Perez, 1993). Linear regression analysis was applied to each experiment.

Stock cultures of plant pathogenic bacteria were maintained as indicated in chapter 4; section 4.2.2.2.

8.2.2.1.2 Tests with human pathogenic bacteria

The agar hole-plate diffusion method, with Müller-Hinton agar prepared according to the manufacturer's specifications, was used in antibacterial tests against human pathogenic bacteria. Modifications were made depending on the nutritional demands of the different organisms.

Overnight cultures of human pathogenic bacteria were diluted 1:100 with Müller-Hinton broth. The bacterial suspension was streaked evenly on Müller-Hinton agar (MHA) plates with a sterile cotton bud. In the case of *Streptococcus pneumoniae* and *Streptococcus pyogenes*, sheep blood was added to MHA (Caceres *et al.*, 1991a). Holes of 6 mm diameter were made on the surface of the agar with a flame sterilized cork borer. Crude and semi-purified extracts of specified concentrations were introduced at 1 mg of extract per hole while the stocks of all the extracts were at 50 mg cm^{-3}. The plates were incubated at 37°C for 24 hours. The non-aerobes were incubated in an atmosphere with carbon dioxide.

Three types of negative controls were used. Blank wells were filled with 20µl of a 10% (v/v) solution of DMSO in water, 10% (v/v) solution of Tween-80 in water and pure distilled water. The positive controls were different antibiotic standards. After equilibration at room temperature (Vanden Berghe & Vlietinck, 1991), the plates were incubated overnight at 37°C and the resulting zones of inhibition measured on two axes at right angles to each other (Pfaller *et al.*, 1992). Each experiment was repeated twice.

The second set of experiments was done with *M. catarrhalis*. The crude extract (50 mg cm^{-3}) and the liquid-liquid separation extractives (50 mg cm^{-3}) were diluted with a 10% (v/v) solution of DMSO in water. Alternatively, a 10% (v/v) solution of Tween-80 in water was used with the less polar fractions. The dilutions were done to give the following concentrations: 90, 80, 70, 60, 40, 30, 20, 15, 10, 7.5, 5.0, 2.5 and 1.0% (Brantner & Grein, 1994). A volume of 20 µl of each dilution was loaded per 6 mm diameter hole. Two 20 µl volumes each of a solution of 10% (v/v) solution of DMSO and 10% (v/v) solution of Tween-80 were used as negative controls, while DDAC was used as the positive control. DDAC was used instead of the

traditional antibiotic due to its availability at the time. The concentration of the DDAC stock solution was 2.50 mg cm^{-3}. It was diluted just like the other extracts. All experiments were repeated twice. The degree of bacterial inhibition by the extracts was determined using the agar hole-plate diffusion method as above. Antibacterial activity of each extract towards the test organism *M. catarrhalis* was evaluated by measuring the inhibition zone diameters. The inhibition zone diameter was also plotted against log. Concentration of each extract (Anesini & Perez, 1993). Linear regression analysis was applied to each experiment as well.

Stock cultures of human pathogenic bacteria were maintained on nutrient agar slants (Biolab) at 4°C during the screening. They were recovered for antibacterial tests by growth in nutrient broth (Biolab) for 24 hours. Before streaking, each culture was diluted 1:100 with fresh sterile nutrient broth (Meyer & Afolayan, 1995).

8.2.2.2 Antifungal tests with human pathogens

Sabouraud agar containing chloramphenicol was used for antifungal tests using the hole-diffusion method. The agar was prepared according to the manufacturer's specifications, autoclaved and cooled to about 45°C after which the antibiotic was added to exclude the growth of bacteria. A volume of 15 cm^{-3} on average was poured into each plate to give a depth of 2-3 mm. The agar was allowed to set in a sterile atmosphere before use.

An overnight growth of each fungus in Sabouraud broth was diluted 1:100 with the same fresh sterile broth. This was then evenly spread on a sterile Sabouraud agar plate with a sterile cotton bud. Holes of 10 mm diameter were made on the agar surface. A volume of 100 µl (equivalent to 20 mg) of crude or semi-purified extract was introduced into each hole. The extracts were allowed to diffuse into the agar for 1 hour at room temperature before the plates were incubated at 37°C for 24-48 hours (Verastegui *et al.*, 1996). Each experiment was repeated twice. The resulting zones of inhibition were measured on two axes at right angles to each other.

Negative controls for each experiment were: (I) unseeded Sabouraud agar plates, (ii) seeded

plates with 100 µl 10% (v/v) solution of Tween-80 in water per well, (iii) seeded plates with 100 µl 10% (v/v) solution of DMSO in water. The positive control was nystatin (Difco) (30µg disks). The human pathogenic fungi were stored on Sabouraud's dextrose agar slants at 4°C prior to use (Irobi & Daramola, 1993).

8.3 RESULTS

8.3.1. Antibacterial activity of crude and semi-purified extracts against plant pathogenic bacteria

The results of antibacterial tests with plant pathogenic bacteria are shown in Table 8.3.

At 1 mg per hole, the crude and the ethyl acetate extracts showed significant antibacterial activity (Table 8.3), as indicated by inhibition zone diameters ≥10 mm (Naqvi *et al.*, 1991). These two extracts were also active against all the test bacteria and, in most cases, compared favourably with the DDAC control. The hexane, diethyl ether and chloroform extractives showed no activity against the plant pathogenic bacteria.

Table 8.3: Antibacterial activity of crude and semi-purified extracts of *Euclea crispa* subsp. *crispa* against plant pathogenic bacteria. 1 mg extract was loaded per hole using the hole-diffusion method.

BACTERIA	EXTRACT AND MEAN INHIBITION ZONE DIAMETER (mm)					
	Crude	Hexane	Diethyl ether	Chloroform	Ethyl acetate	*DDAC
A. tumefaciens	16.0 ± 0.6	0	0	0	13.0 ± 0.0	23.0 ± 0.4
C. michiganense pv. *michiganense*	15.0 ± 0.7	0	0	0	15.0 ± 0.4	16.0 ± 0.4
E. carotovora pv. *carotovora*	10.0 ± 0.4	0	0	0	14.0 ± 0.6	17.0 ± 0.8
R. solanacearum	15.0 ± 0.0	0	0	0	11.0 ± 0.7	16.0 ± 0.4
X. campestris pv. *phaseoli*	15.0 ± 0.8	0	0	0	20.0 ± 0.8	15.0 ± 0.0

*The DDAC control was used at a concentration of 2.50 mg cm^{-3}. A volume of 20 µl was loaded per 6 mm diameter well.

The antibacterial tests were repeated at 2 mg per hole in order to establish whether the differences in activity observed for different extracts were due to a very low test concentration or due to inherent differences (Table 8.4).

Table 8.4: Antibacterial activity of crude and semi-purified extracts of *Euclea crispa* subsp. *crispa* against plant pathogenic bacteria. 2 mg extract was loaded per hole using the hole-diffusion method.

BACTERIA	EXTRACT AND MEAN INHIBITION ZONE DIAMETER (mm)					
	Crude	Hexane	Diethyl ether	Chloroform	Ethyl acetate	*DDAC
A. tumefaciens	16.0 ± 0.4	0	0	0	20.0 ± 0.7	23.0 ± 0.8
C. michiganense pv. *michiganense*	16.0 ± 0.4	0	0	0	17.0 ± 0.8	16.0 ± 1.0
E. carotovora pv. *carotovora*	15.0 ± 1.0	0	11.0 ± 0.0	0	18.0 ± 0.8	17.0 ± 0.8
R. solanacearum	16.0 ± 0.6	0	0	0	19.0 ± 0.8	16.0 ± 0.0
X. campestris pv. *phaseoli*	16.0 ± 0.7	0	0	0	18.0 ± 0.6	15.0 ± 0.8

*The DDAC control was used at 2.50 mg cm^{-3}. A volume of 20 µl was loaded per 6 mm diameter well.

At 2 mg per hole the crude extract and the ethyl acetate fraction showed enhanced and significant antibacterial potency towards all the test bacteria. The ethyl acetate fraction gave larger zones of inhibition than the crude extract. Significant activity was also observed with the diethyl ether fraction towards *E. carotovora* pv. *carotovora.*

8.3.2 Inhibition curves of crude and semi-purified extracts towards plant pathogenic bacteria.

The inhibition curves of crude and semi-purified extracts towards plant pathogenic bacteria are shown in Figures 8.1 to 8.5.

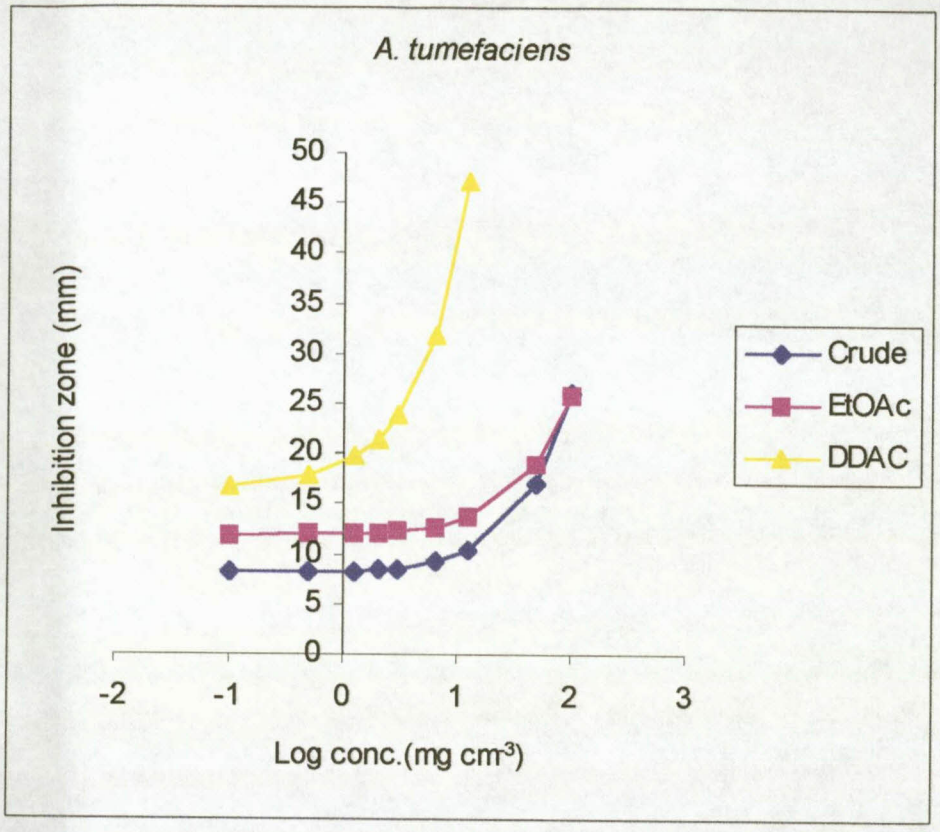

Fig 8.1: Inhibition curves of crude and semi-purified extracts of *E. crispa* subsp. *crispa* towards A. *tumefaciens*.

Comparing the extracts from *E. crispa* subsp. *crispa*, the ethyl acetate fraction (semi-purified) elicited higher responses in the form of larger zones of inhibition as compared to the crude extract. Inhibition curves of the crude extract and ethyl acetate towards *A. tumefaciens* (Fig.8.1), showed dampened responses to the ethyl acetate and the crude extracts between log. conc. –1 (0.1 mg cm^{-3}) and log. conc. 0.8 (6.31 mg cm^{-3}). Here the slopes were close to zero. After log conc. 0.8, both the ethyl acetate fraction and the crude extract inhibition curves started rising exponentially. The inhibition curves of the crude extract and the ethyl acetate fraction intersected at log. conc. 2 (100 mg cm^{-3}) when tested with *A. tumefaciens*. The inhibition curve of DDAC (positive control) towards *A. tumefaciens* was exponential throughout.

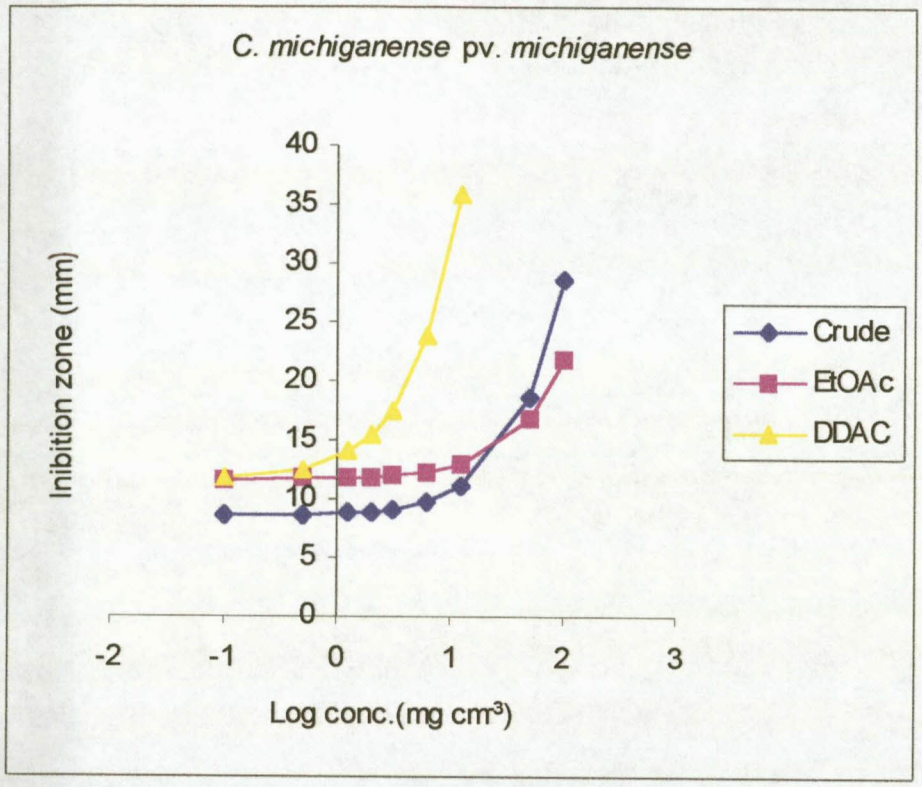

Fig. 8.2: Inhibition curves of crude and semi-purified extracts of *E. crispa* subsp. *crispa* towards *C. michiganense* pv. *michiganense*.

The inhibition curves of the crude extract and ethyl acetate fraction (semi-purified) towards *C. michiganense* pv. *michiganense* (Fig. 8.2) were also dampened between log conc. –1 (0.10 mg cm^{-3}) and log conc. 0.5 (3.16 mg cm^{-3}), with a gradient close to zero. After log conc. 0.5, both curves started rising exponentially. The response inhibition curves of the crude extract and the ethyl acetate fraction merged at log. conc. 1.5 (12.59 mg cm^{-3}). The responses of the bacteria to the ethyl acetate fraction were slightly higher than the responses to the crude extract, till after the point of intersection, log. conc. 1.5. After that, the crude extract seemed to elicit more inhibitory ressponses from this bacterium. The inhibition curve of DDAC towards *C. michiganense* pv. *michiganense* was also exponential throughout.

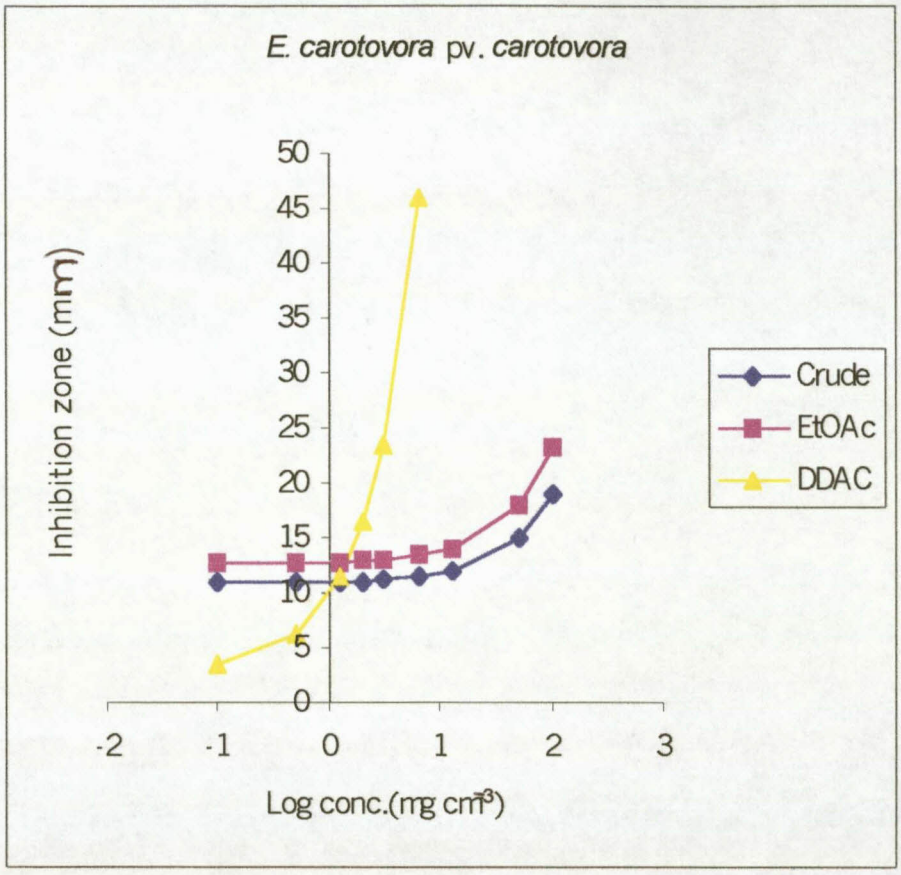

Fig. 8.3: Inhibition curves of crude and semi-purified extracts of *E. crispa* subsp. *crispa* towards *E. carotovora* pv. *carotovora*.

The inhibition curves of extracts from *E. crispa* subsp. *crispa* with *E. carotovora* pv. *carotovora*, indicated that the ethyl acetate fraction (semi-purified) elicited higher responses in the form of larger zones of inhibition as compared to the crude extract. Inhibition curves of the crude extract and ethyl acetate towards *E. carotovora* pv. *carotovora* (Fig. 8.3) showed dampened responses to the ethyl acetate and the crude extracts between log. conc. –1 (0.1 mg cm^{-3}) and log. conc. 0.8 (6.31 mg cm^{-3}). Here the gradient was close to zero as well. After log. conc. 0.8, the slopes rose steadily towards exponential. The inhibition curve of DDAC towards *E. crotovora* pv. *carotovora* was exponential throughout.

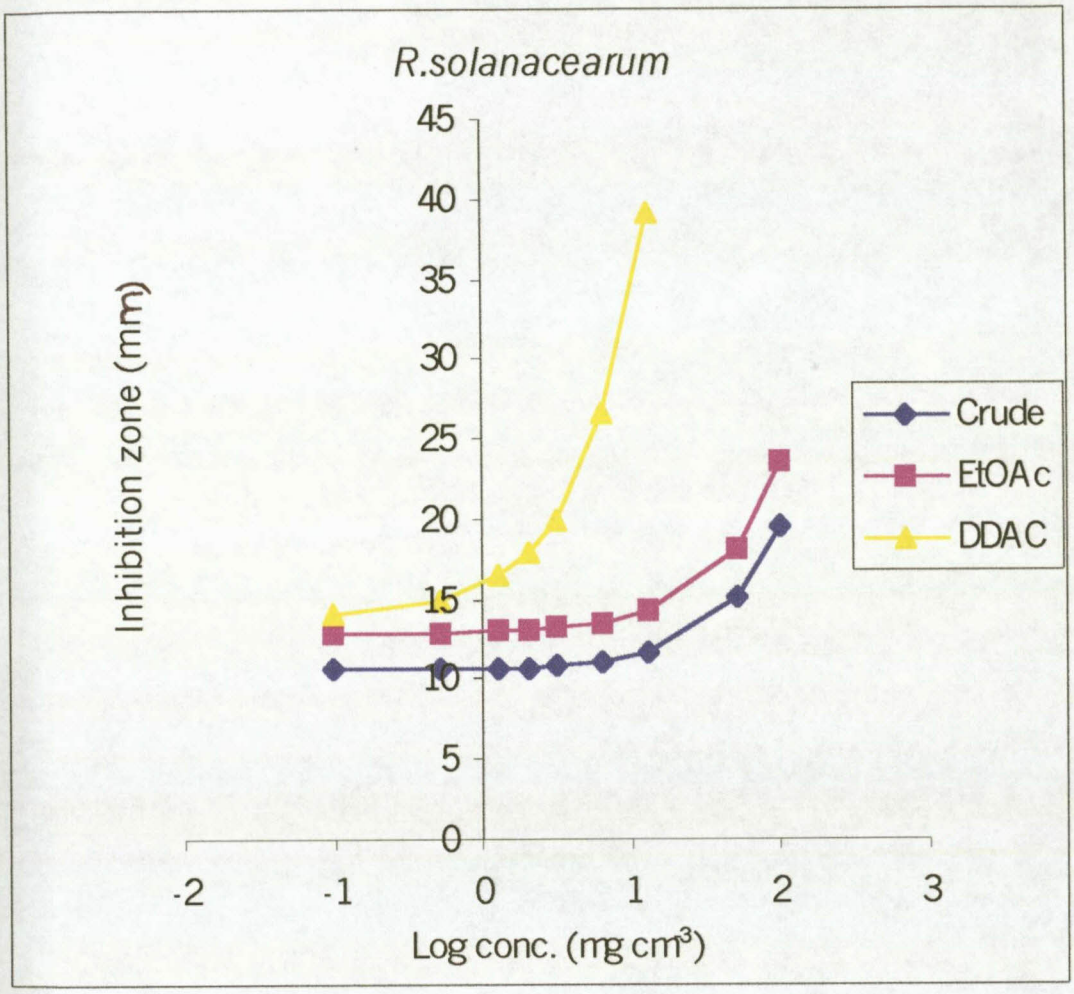

Fig. 8.4: Inhibition curves of the crude and semi-purified extracts of *E. crispa* subsp. *crispa* towards *R. solanacearum*.

Comparing the extracts from *E. crispa* subsp. *crispa*, the ethyl acetate fraction (semi-purified) elicited higher responses in the form of larger zones of inhibition as compared to the crude extract. Inhibition curves of the crude extract and ethyl acetate fraction towards *R. solanacearum* (Fig. 8.4) showed dampened responses to the ethyl acetate and the crude extracts between log. conc. –1 (0.1 mg cm^{-3}) and log. conc. 0.8 (6.31 mg cm^{-3}), also with gradient also nearly zero. After log. conc. 1.0, the inhibition curves of both the ethyl acetate fraction and the crude extract started rising exponentially. The inhibition curve of DDAC towards *R. solanacearum* also was exponential.

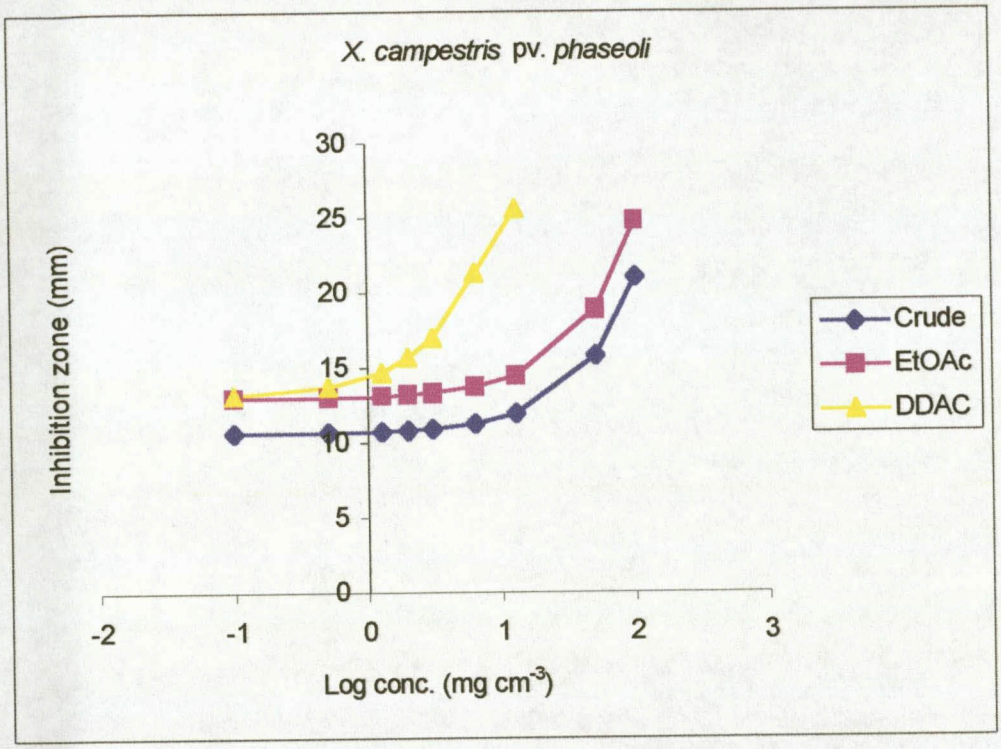

Fig. 8.5: Inhibition curves of crude and semi-purified extracts of *E. crispa* subsp. *crispa* towards *X. campestris pv. phaseoli*.

The inhibition curves of the crude extract and ethyl acetate towards *X. campestris* pv. *phaseoli* (Fig. 8.5) were also dampened between log conc. –1 (0.10 mg cm^{-3}) and log conc. 0.5 (3.16 mg cm^{-3}), with a gradient close to zero. After log. conc. 0.5, the slopes started rising steadily. Just after log. conc. 1.0, the inhibition curves of both the crude and the ethyl acetate fraction also started rising exponentially. The responses of the bacteria to the ethyl acetate fraction were slightly higher than the responses to the crude extract. The inhibition curve of DDAC towards *X. campestris* pv. *phaseoli* was also exponential as in the inhibition of the other plant pathogenic bacteria.

The inhibition curves for all the plant pathogens were exponential at one point or another. Dimethyl didecyl ammonium chloride (DDAC) gave the highest exponential responses with respect to all the bacteria used in the tests. A 1% increase in concentration of the control

(DDAC) lead to a more than 1% increase in the zone of inhibition with all plant pathogens tested. Inhibition zones of DDAC control against plant pathogens were very responsive to changes in concentration.

The minimal inhibition concentrations in disk (MICD) of extracts that showed some form of inhibition of growth of the test bacteria (Caceres *et al.*, 1993a) are shown in Table 8.5.

Table 8.5: Minimal inhibition concentrations in disk (MICD) of crude and semi-purified extracts of *Euclea crispa* subsp. *crispa* inhibiting the growth of selected plant pathogenic bacteria.

BACTERIA	MINIMUM INHIBITION CONCENTRATION		
	Crude	Ethyl acetate	*DDAC
	mg cm^{-3}	mg cm^{-3}	µg cm^{-3}
A. tumefaciens	15.00	5.00	6.25
C. michiganense pv. *michiganense*	10.00	3.50	12.50
E. carotovora pv. *carotovora*	5.00	2.50	12.50
R. solanacearum	5.00	3.50	6.25
X. campestris pv. *phaseoli*	3.50	1.25	6.25

*DDAC control

The most sensitive organism to the crude extract, as indicated by the lowest concentration that resulted in some degree of inhibited growth, was *X. campestris* pv. *phaseoli* (2.50 mg cm^{-3}), while the most resistant organism to the crude extract was *A. tumefaciens* (15 mg cm^{-3}). The general trend from the least sensitive to the most sensitive was as follows: *A. tumefaciens* > *C. michiganense* pv. *michiganense* > *R. solanacearum* = *E. carotovora* pv. carotovora > *X. campestris* pv. *phaseoli*.

With reference to the ethyl acetate fraction, the most sensitive organism indicated by the lowest concentration that resulted in a response in the form of inhibited growth was *X. campestris* pv. *phaseoli* (1.25 mg cm^{-3}), while the most resistant organism to the crude extract was *A. tumefaciens* (5 mg cm^{-3}). The general trend from the least sensitive to the most sensitive was as follows: *A. tumefaciens* > *C. michiganense* pv. *michiganense* = *R. solanacearum* > *E. carotovora* pv. *carotovora* > *X. campestris* pv. *phaseoli*.

8.3.3 Antibacterial activity of crude and semi-purified extracts against human pathogenic bacteria.

The results in table 8.6 show that where there was inhibition of the growth of the human pathogenic bacteria, all the zones of inhibition observed were significant, i.e. greater than or equal to 10 mm (Naqvi *et al.*, 1991). The ethyl acetate fraction gave the largest zones of inhibition and the broadest spectrum of activity towards human pathogenic bacteria. *M. catarrhalis* and *P. aeruginosa* were inhibited by the crude and all the four semi-purified extracts; though larger zones of inhibition were observed with *M. catarrhalis* than with *P. aeruginosa*. The least resistant bacterium to the extracts was therefore taken to be *M. catarrhalis*, followed by *P. aeruginosa*. On the other hand, none of the five extracts showed any inhibitory effects on *H. influenzae* and *K. pneumoniae*. These two species were the most resistant pathogens, resistant to all the five extracts as evidenced by the lack of zones of inhibition. *A. baumanii* and *E. coli* were also resistant to all the extracts except the ethyl acetate fraction, with which 11.0 ± 0.7 mm and 12.0 ± 0.4 mm inhibition zones were observed respectively.

Table 8.6: Antibacterial activity of crude and semi-purified extracts of *Euclea crispa* subsp. *crispa* against human pathogenic bacteria. 1mg extract was loaded per hole using the hole diffusion method.

BACTERIA	EXTRACT AND MEAN INHIBITION ZONE DIAMETER (mm)					
	Crude	Hexane	Diethyl-ether	Chloroform	Ethyl-acetate	Antibiotic control
Acinetobacter baumanii	0	0	0	0	11.0± 0.7	17.0 ± 0.0 [a]
Bacillus subtilis	12.0 ± 0.4	0	0	10.0 ± 0.0	15.0 ± 0.4	35.0 ± 0.4 [c]
Escherichia coli	0	0	0	0	12.0 ± 0.4	18.0 ± 0.4 [c]
Haemophilus influenzae	0	0	0	0	0	29.0 ± 0.4 [c]
Klebsiella pneumoniae	0	0	0	0	0	33.0 ± 0.6 [c]
Moraxella catarrhalis	27.0 ± 0.8	20.0 ± 0.4	25.0 ± 0.6	20.0 ± 0.8	34.0 ± 0.6	18.0 ± 0.6 [a]
Pseudomonas-aeruginosa	14.0 ± 0.6	13.0 ± 0.8	13.0 ± 0.8	20.0 ± 0.8	21.0± 0.4	32.0 ± 0.0 [c]
Staphylococcus aureus	16.0 ± 0.6	0	0	0	25.0 ± 0.4	32.0 ± 0.4 [c]
Staphylococcus-epidermidis	18.0 ± 0.4	0	0	0	24.0 ± 0.4	33.0 ± 0.0 [c]

Table 8.6 continued

BACTERIA	EXTRACT AND MEAN INHIBITION ZONE DIAMETER (mm)					
	Crude	Hexane	Diethyl-ether	Chloroform	Ethyl-acetate	Antibiotic control
Streptococcus-pneumoniae	0	19.0 ± 0.4	17.0 ± 0.0	22.0 ± 0.8	0	21.0 ± 0.6 [c]
Streptococcus pyogenes	0	13.0 ± 0.0	14.0 ± 0.4	12.0 ± 0.8	10.0 ± 0.0	28.0 ± 0.4 [p]

[a] ampicillin (10 µg disks) ; [c] chloramphenicol (30 µg disks) ; [p] penicillin (10 units)

S. aureus and *S. epidermidis* gave similar response patterns to the extracts of *E. crispa* subsp. *crispa* in that both were inhibited by the crude extract and ethyl acetate fraction only (Table 8.6). The resulting zones of inhibition were almost identical, differing only by 1 unit with both the crude and the ethyl acetate extracts. These two pathogens were resistant to the hexane, diethyl ether and chloroform extractives, with which no zones of inhibition were observed.

S. pneumoniae and *S. pyogenes* were both resistant to the crude extract, although they were both inhibited by the hexane, diethyl ether, and chloroform extractives. Larger zones of inhibition were observed with *S. pneumoniae* than with *S. pyogenes*. Of the two species, only *S. pyogenes* was inhibited by the ethyl acetate fraction.

The growth of *B. subtilis*, a spore forming bacterium, was inhibited by the crude extract, chloroform and ethyl acetate fractions. The ethyl acetate extractives gave the largest zones of inhibition with this bacterium as well.

The following is a summary of the results of tests with human pathogenic bacteria. A total of 11 human pathogenic bacteria were used in the antibacterial tests. Five (45.5 %) of the bacteria were inhibited by the crude extract. Four (36.4%) of the bacteria were inhibited by the hexane extractives and also four (36.4%) of the pathogens were inhibited by the diethyl ether fraction. Five (45.5%) of the pathogens were inhibited by the chloroform extractives while 8 (72.7%) of the test bacteria had their growth inhibited by the ethyl acetate fraction. The two bacterial species whose growth was not inhibited by any of the extracts constituted 18.2% of all the

human pathogenic test bacteria. The other two, *M. catarrhalis* and *P. aeruginosa*, inhibited by all the extracts, also constituted 18.2% of all the species.

8.3.4 Inhibition curves of crude and semi-purified extracts towards *M. catarrhalis*.

Linear regression analysis was applied only to the inhibition of *M. catarrhalis* in the case of human pathogenic bacteria.

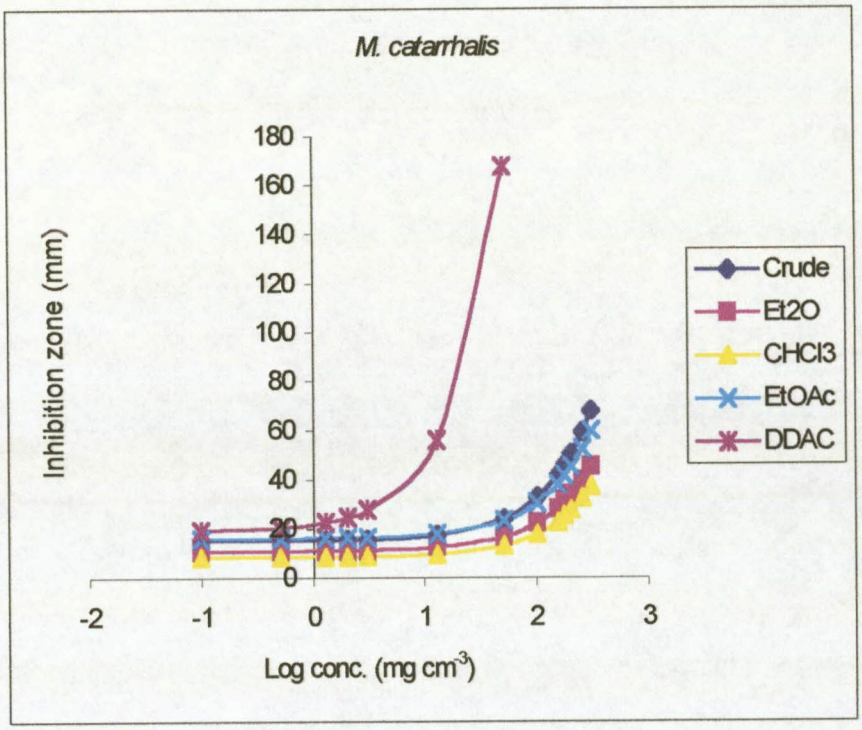

Fig. 8.6: Inhibition curves of crude and semi-purified extracts of *Euclea crispa* subsp. *crispa* towards *M. catarrhalis*.

As with plant pathogenic bacteria, a good fitting of data to a linear function (antibacterial activity vs. logarithm of concentration) at lower concentrations (form log. conc. –1.0 up to about log. conc. 0.5) was observed with *M. catarrhalis*. At higher concentrations (from about log. conc. 0.6), an exponential function was also evident (Figure 8.6).

At lower concentrations up to log conc. 0.50 (3.16 mg cm^{-3}) *M. catarrhalis* showed dampened responses to the crude extract, diethyl ether, chloroform and ethyl acetate fractions. At concentrations ranging from log. conc. 0.5 onwards, all the inhibition curves of the crude extract and the three liquid-liquid separation (semi-purified) fractions became exponential. The ethyl acetate fraction gave the highest response curve, followed closely by the response curve of the crude extract. The ethyl acetate and crude extract response curves merged at log conc. 1.1 (12.59 mg cm^{-3}). The lowest response curve was given by the chloroform fraction followed by the diethyl ether fraction.

The minimal inhibitory concentrations in disk (MICD) of the extracts towards *M. catarrhalis* are given below. The term MICD was taken from Caceres *et al.* (1993a).

Table 8.7: Minimal inhibitory concentrations in disk (MICD) of crude and semi-purified extracts of *Euclea crispa* subsp. *crispa* inhibiting the growth of *M. catarrhalis*.

MICD OF EXTRACTS				
Crude	Diethyl ether	Chloroform	Ethyl acetate	*DDAC
mg cm^{-3}	mg cm^{-3}	mg cm^{-3}	mg cm^{-3}	µg cm^{-3}
2.50	3.50	10.00	3.50	6.25

*DDAC control

DDAC being a pure compound, and acting as the positive control, gave the lowest value for the minimum concentration inhibiting the growth of this bacterium and hence *M. catarrhalis* had the highest sensitivity to DDAC. The hexane fraction (not shown) was active at 50 mg cm^{-3} and at 45 mg cm^{-3}, but at lower concentrations, no activity was observed.

The minimum concentrations of extracts that inhibited the growth of *M. catarrhalis*, from the most potent to the least potent were as follows: crude extract (2.50 mg cm^{-3}) > diethyl ether = ethyl acetate (each at 3.50 mg cm^{-3}) > chloroform fraction (10 mg cm^{-3}) > hexane fraction (45.0 mg cm^{-3}; not shown on graph and table). These results were expected in that liquid-liquid separation with hexane (ε = 1.9) was mainly to remove chlorophylls and lipids. The solvent chloroform (dielectric constant (ε) = 4.8) was used in order to ascertain that all substances

soluble in hexane and diethyl ether (ε = 4.3) had been removed and hence no much activity from this fraction was expected. Ethyl acetate (ε = 6.0) extracted substances of higher polarity than the first three solvents. The crude extract was prepared from methanol (95% (v/v) solution in water). The polarity of methanol (ε = 32.7) allowed for the extraction of both polar and non-polar substances and probably this is why *M. catarrhalis* gave the highest sensitivity to the crude extract as compared to the other extracts.

8.3.5 Antifungal activity of the crude and semi-purified extracts towards human pathogenic fungi.

All the extracts, with the exception of the hexane fraction, showed some degree of inhibition on the growth of both *Candida albicans* and *Cryptococcus neoformans* (Table 8.8). The largest zones of inhibition of the two fungi were obtained with the crude extract. *C. albicans* was more susceptible to the active extracts than *C. neoformans*, resulting in the formation of larger zones of inhibition. In the case of *C. albicans*, inhibition zone diameters decreased with the following general trend: crude extract > ethyl acetate > chloroform > diethyl ether > hexane fraction. The trend with *C. neoformans* was as follows: crude extract > ethyl acetate > chloroform = diethyl ether > hexane fraction.

Table 8.8: Antifungal activity of crude and semi-purified extracts of *Euclea crispa* subsp. *crispa* against two human pathogenic fungi. 20 mg extract was loaded per hole using the hole-diffusion method.

EXTRACT	FUNGUS AND MEAN INHIBITION ZONE DIAMETER (mm)	
	Candida albicans	*Cryptococcus neoformans*
Crude	25.0 ± 0.4	20.0 ± 0.6
Hexane	0	0
Diethyl ether	14.0 ± 0.4	12.0 ± 0.0
Chloroform	15.0 ± 0.4	12.0 ± 0.4
Ethyl acetate	18.0 ± 0.8	15.0 ± 0.8
*Nystatin	33.0 ± 0.4	30.0 ± 0.6

*Nystatin (100 units, Difco) antibiotic control

8.5 DISCUSSION

Of all the four liquid-liquid separation fractions, the ethyl acetate fraction possessed most of the antimicrobial activity towards both plant pathogenic bacteria, human pathogenic bacteria and human pathogenic fungi (Tables 8.3, 8.4, 8.6 and 8.8). The ethyl acetate fraction differed from the rest of the semi-purified fractions in that it contained most of the alkaloids and phenolic and the least amount of essential oils. The bitter principles and saponins were present in significant amounts as well, comparable to the chloroform and diethyl ether fractions respectively (see Table 7.6, chapter 7).

The fact that the ethyl acetate fraction exhibited the highest activity towards plant pathogenic bacteria while the hexane, diethyl ether and chloroform fractions exhibited virtually no activity towards these same bacteria (Table 8.3) could be explained as follows. The activity of the ethyl acetate fraction towards the plant pathogens could have been due to (a) the higher content of alkaloids and phenolic compounds, or (b) higher concentration of all compounds put together, or even (c) a combination of both (a) and (b).

Other authors have also compared the activities of extracts prepared with solvents of different polarities as well as the inhibition trends of the test organisms. A number of examples are given here. Caceres *et al.* (1993a) sequentially extracted plant material by reflux with solvents of different polarity (n-hexane, acetone and ethanol). They reported that the solvent which best extracted the antibacterial activity was ethanol. These authors also went on to compare the susceptibility trends of the different test organisms towards the extracts.

Ali-Shtayeh *et al.* (1997) compared the antimicrobial activity of organic and aqueous solvent extracts and fractions of *Micromeria nervosa* (Lamiaceae) and observed that the ethanolic extract was the most active, followed by the ethyl acetate and petroleum ether extracts. They observed that the least active extract was the aqueous one. These authors also considered the susceptibility of the test organisms to the extracts. They reported that the most susceptible test organism in their tests was *Proteus vulgaris* to carvacrol. They reported that the most susceptible test organisms to both carvacrol and thymol were *P. vulgaris* and *Candida*

albicans. The least susceptible organism to both carvacrol and thymol was found to be *Pseudomonas aeruginosa*.

Kwo *et al.* (1996) used a disc diffusion susceptibility test to screen extracts from the bark of three plants for antimicrobial activity. Solvents with different polarity were used for the extraction (methylene chloride, ethyl acetate, 95% ethanol and acetonitrile). The patterns of inhibition were studied and found to vary with the plant extract, solvent used for extraction as well as with the test organism. The ethanol extracts of *Kigelia africana* (Bignoniaceae) gave the largest zones of inhibition against *Staphylococcus aureus* and *Pseudomonas aureginosa*.

Ekpendu *et al.* (1994) tested the petroleum ether and methanol leaf extracts of *Mitracarpus scaber* (Rubiaceae) for antimicrobial activity *in vitro*. The order of sensitivity they observed was *Pseudomonas aeruginosa* > *Klebsiella pneumoniae* > *Staphylococcus aureus* > *Escherichia coli*. They also reported that the methanol extract exhibited higher activity than the petroleum ether extract.

Desta (1993) prepared a petroleum ether, dichloromethane, methanol, residual aqueous and direct aqueous extracts for each of the plant species the author screened for antimicrobial activity using the agar well diffusion method. Susceptibility of the test microorganisms to the extracts as reported by the author decreased in the order: *Staphylococcus aureus* > *Pseudomonas aureginosa* > *Candida albicans* > *Salmonella gallinarun* > *Escherichia coli* >*Klebsiella pneumoniae* > *Proteus vulgaris*. The author also reported that the highest antimicrobial activity was found in the direct aqueous extracts, while the lowest activity was in the petroleum ether extracts.

Iqbal *et al.* (1998) tested a number of plant extracts (aqueous, hexane and alcoholic) for their antibacterial activity using the agar well diffusion method. They reported that, on the whole, the alcoholic extracts showed greater activity than their corresponding aqueous and hexane extracts.

It is interesting to note that the patterns of inhibition of human pathogenic bacteria (Table 8.6) by the liquid-liquid separation fractions; hexane, diethyl ether and chloroform were identical, the only difference being that the chloroform fraction inhibited *B. subtilis*, which the other two fractions did not inhibit. These three semi-purified fractions were richer in essential oils than the ethyl acetate fraction that had a proportion of 5%, as compared to the other three fractions with an average of about 30%. It is probable therefore that the human pathogenic bacteria whose growth was inhibited by these three fractions were more sensitive to the presence of essential oils in the extracts. The human pathogenic bacteria whose growth was not inhibited by the hexane, diethyl ether and chloroform fractions, but inhibited by the ethyl acetate fraction were probably not sensitive to the presence of higher concentrations of essential oils in the first three fractions, but sensitive to the higher concentrations of the alkaloids and phenolic compounds in the ethyl acetate fraction. There is also the probability that the presence of a wider range and higher concentration of compounds in the ethyl acetate fraction in synergism resulted in this fraction being the most potent.

No particular trend was observed in terms of susceptibility to the extracts of both the Gram-negative and the Gram-positive test bacteria in the tests.

All the fractions except the hexane fraction were active against the human pathogenic test fungi. This implies that antifungal principles were extracted with the solvents methanol (for the crude extract), diethyl ether, chloroform and ethyl acetate. In this case though, the crude extract gave the largest inhibition zones followed by the ethyl acetate extractives. The diethyl ether and chloroform extractives gave similar zones of inhibition, meaning that the solvents employed possibly extracted similar antifungal principles. Like human pathogenic fungi, human pathogenic bacteria were inhibited by different extracts, depending on the organism in question.

The concentration of the antimicrobial activity in the ethyl acetate fraction (in the case of bacteria) and crude extract (in the case of human pathogenic fungi) implies that the active principles are quite polar. All the extracts used in these tests were mixtures of compounds.

Large inhibition zones may have been caused by (a) highly active substance(s) present in small amount(s) or (b) by a substance(s) of low activity present in high concentration(s) in the extracts (Vanden Berghe & Vlietinck, 1991).

Antibacterial agents may exert different effects on the structure and functioning of bacterial cells. The important targets of chemotherapeutic action are the cell wall, the cell membrane and the biosynthetic process of protein and nucleic acid synthesis (Brock & Madigan, 1991).

The results of the investigations in this chapter are evidence that extracts of *E. crispa* subsp. *crispa* contain antimicrobial properties, hence the use of the plant in traditional medicine for the treatment of diseases caused by microorganisms. General antimicrobial activity was observed with the less polar as well as with the more polar extracts. The extracts used in the treatment of ailments are usually in the form of infusions, decoctions and tinctures (Van Wyk *et al.*, 1997), which are quite polar themselves.

Although doses of the extracts required to inhibit the growth of microorganisms tested in this study seems high in comparison with antibiotics normally used (and DDAC), it should be considered that in this study crude and semi-purified extracts were used and obviously the compounds responsible for the antimicrobial activity are metabolites and normally found in small quantities (Onah *et al.*, 1994 ; Zavala *et al.*, 1997). This low activity might also have been due to weakly active compounds present in substantial concentrations.

The variations in minimum concentrations of extracts inhibiting the growth of the test bacteria may be due to permeability barriers peculiar to each of the four isolates from *E. crispa* subsp. *crispa* and also to the nature of the individual bacterial species that determines their sensitivity to the extracts. Impermeability to drugs or chemical substances can confer resistance on microorganisms (Onah *et al.*, 1994).

The crude and ethyl acetate fractions, being more polar, contained antimicrobial compounds that were active towards the plant pathogenic bacteria. This implies that the 95% solution of

methanol in water and the ethyl acetate extracted most of this antibacterial activity from *E. crispa* subsp. *crispa*. In the case of human pathogenic bacteria, all of the five fractions showed some activity towards the pathogens. This could be due to the different nature of each species and hence the site of inhibition of each. On the other hand, compounds with activity towards human pathogenic fungi (*C. albicans* and *C. neoformans*) were extracted with the solvents diethyl ether, chloroform and ethyl acetate. Most of the activity, though, was extracted by the solvent ethyl acetate.

The plant *Euclea crispa* subsp. *crispa* seems to be a good source of antimicrobials for the treatment of both bacterial and fungal infections. The results of this investigation confirm its use in traditional medicine. Literature mentioning the use of this plant against plant pathogens was not found though our results show effective antibacterial activity against plant pathogenic bacteria in the crude and the ethyl acetate fractions. Isolation of the active compounds from the ethyl acetate fraction of *E. crispa* was attempted in chapter 9; due to the fact that it possessed the highest antimicrobial activity as indicated by the results in this chapter.

Because of the localization of most of the antimicrobial activity in the ethyl acetate fraction, it was decided to do further isolation and purification of compounds with antimicrobial activity from this fraction in chapter 9. Data on the phytochemical profile of *E. crispa* subsp. *crispa* obtained in chapter 7 gave an indication of the classes of compounds likely to be present in this fraction.

CHAPTER 9

PURIFICATION, IDENTIFICATION AND ANTIMICROBIAL ACTIVITY OF COMPOUNDS IN THE ETHYL ACETATE FRACTION OF EUCLEA CRISPA SUBSP. *CRISPA (EBENACEAE)*

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9.1 INTRODUCTION

It is well known that plant extracts are used for therapeutic purposes as either complex mixtures or pure compounds depending on their therapeutic indices. It is, therefore, necessary to determine the chemical composition of the complex mixture so as to identify the compounds responsible for the antimicrobial, or any other, bioactivity. Isolation and identification of the active constituents is essential for the study of their toxicity, stability and effects on metabolism and physiology, for dosage purposes, for structure-activity investigations (Aquino *et al.*, 1995) and hence their value as therapeutic agents. Toxicological data for natural antimicrobials is often lacking (Beautement *et al.*, 1991) and it is not proper to assume that all plant derived compounds are non-toxic. Isolation of individual compounds would also benefit pesticide research where the discovery of compounds with novel taxophores and new modes of action is still a challenge.

Compounds with antimicrobial activity are isolated all the time. These compounds may be used directly as agrochemicals or chemotherapeutic substances, if isolated in sufficient quantities, if not, they can also be synthesized. Alternatively, they may serve as starting points for the synthesis of analogues with improved biological and physical properties (Beautement *et al.*, 1991 ; Tyman, 1995). The classic example of the use of natural products as leads for the design and synthesis of new agrochemicals are the pyrethroids. Pyrethroids are synthetic insecticides whose ancestors are the natural pyrethrins (Beautement *et al.*, 1991). These authors also reported that several fungicidal natural products have prompted research which has led to active synthetic analogues like griseofulvin, hadacin and thiolutin that have been worth patenting. Beautement *et al.* (1991) also stated that investigations on the natural product pyrrolnitrin have led to the development of fenpiclonil which is currently used as a fungicidal seed treatment. Carvone, the major monoterpenoid in the essential oil of *Carum carvi* L. (caraway) exhibits antifungal activity on, for instance, the potato tuber pathogen *Fusarium sulphureum*, that causes dry rot of potato tubers. However, due to its volatility there is a need to develop slow-release formulations (Gorris & Smid, 1995). New types of compounds isolated

from plant extracts can be manufactured and sold without having to apply to manufacture them, and can themselves be patented (Beautement *et al.*, 1991).

It is also known that some compounds are active in synergism while others are potent as individuals. Synergism is characterized by the fact that, as separation and purification of the constituents progresses in bioassay guided fractionation, the degree of activity of the isolates falls. Where synergism is not the case, isolation and purification of the compounds is not accompanied by a drop in bioactivity, but by increased activity (Barton & Ollis, 1985).

Today, natural products chemists have a wide range of highly sophisticated instrumentation available to perform structure elucidation. Of the powerful technologies, mass spectrometry (MS), gas chromatography (GC), GC-MS, high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy are the most widely used. A combination of different forms of ^{1}H NMR and ^{13}C NMR spectroscopy can provide, in most cases, sufficient information for characterization of new structures (Boykin, 1995).

It was decided to further investigate the ethyl acetate fraction of *E. crispa* subsp. *crispa* i.e., to locate the compounds with antimicrobial activity as well as attempt to purify and identify them for the following reason. The ethyl acetate fraction showed the highest antimicrobial activity as compared to the other semi-purified liquid-liquid separation fractions (see section 8.3, chapter 8). The ethyl acetate fraction, as was reported in section 7.3 (chapter 7) contained saponins, bitter principles, essential oils, phenolic compounds and alkaloids. An attempt was made to purify at least one bioactive compound from each of these classes.

Preparative tlc was applied in the separation of compounds from the ethyl acetate fraction. The bands were scraped off from the plates under ultra violet light, eluted with acetone and tested for antimicrobial activity against the test organism *M. catarrhalis*, used in our bioassay guided

fractionation. Only the active bands from the preparative TLC plates were further purified and the structures of the compounds therein determined.

Analytical TLC of the biologically active isolated compounds was carried out in order to determine their classes.

9.2 MATERIALS AND METHODS

9.2.1 Materials

Precoated silica gel 60 F_{254}, 0.25mm aluminium-backed TLC plates (Merck) were used for analytical TLC.

Preparative TLC, was performed on precoated silica gel $F_{1500/LS}$ glass-backed, 20 x 20 cm plates of 0.5 mm thickness with luminescer (254nm) (Merck All solvents used for analytical and preparative TLC were of Analytical Grade (Merck).

Microcolumns were used for eluting isolated compounds from the silica.

9.2.2 Methods

9.2.2.1 Preparative thin layer chromatography (P-TLC)

The main advantage of P-TLC is the unmatched simplicity with which it can be carried out. It is advantageous for the separation of substances with sufficiently different R_f values. In the case of mixtures with very close R_f values, much depends on the quality of sample application (Mikes & Chalmers, 1979).

P-TLC was performed on 20 x 20 cm silica gel $F_{1500/LS}$ (0.5 mm) plates. A 20% solution of the ethyl acetate fraction was used. Approximately 15 mg were loaded per 20 x 20cm plate of 0.5 mm thickness by streaking across with the aid of a glass capillary. The plates were dried with a fan and then developed in a saturated chamber (Mikes & Chalmers, 1979) in the appropriate solvent systems. Detection and isolation of compounds was done in UV-light (254 nm).

The individual compounds were isolated from the layer by scraping off the detected zones of the sorbent layer from the plate and then transferring the material mechanically into a micro-column packed with cotton wool. The substances were freed from the silica by elution with acetone and then tested for antimicrobial activity. Only the active isolated compounds were again tested for purity in the original analytical TLC system (Touchstone & Bobbins, 1978; Mikes & Chalmers, 1979). Acetone was evaporated off under reduced pressure at 35°C in a rotary evaporator. The pure compounds were further dried in a vacuum oven in preparation for structure elucidation by proton nuclear magnetic resonance spectroscopy (NMR).

9.2.2.2 Analytical thin layer chromatography

Analytical thin layer chromatography was initially done to determine the classes of the active compounds isolated by preparative TLC from the ethyl acetate fraction. It was also used to verify the purity of the isolated compounds.

9.2.2.3 Nuclear Magnetic Resonance Spectroscopy (NMR) (Harborne, 1984 ;Verpoorte, 1986; Harwood & Moody, 1989)

In our experiments, ^{1}HNMR spectroscopy was performed on a Bruker 300MHz DRX 300 spectrometer at 296K (23°C). The solvents used were deuteriochloroform ($CDCl_3$), or deuterioacetone $[(CD_3)_2CO]$ as indicated. Samples for NMR were dissolved in $CDCl_3$ and placed in glass tubes (5 mm outside diameter) of varying lengths (up to 15 cm long). Only 3-5 cm of the length of the tube were filled with 0.4-0.7 ml solvent. Once the sample was in the tube, the tube was sealed with a plastic cap and mounted in a turbine assembly before being placed in the spectrometer. The tube was spun at high speed (300 Hz) about its vertical axis, which helped to even out inhomogeneities in the magnetic field. Chemical shifts were reported in parts per million (ppm) on the δ-scale against tetramethylsilane (TMS) = 0.0 ppm (Harwood & Moody, 1989). The coupling constants were given in Hz. The relative number of protons contributing to the absorption was given to the nearest interger, together with any multiplicity

of the absorption and related coupling constants. The following abbreviations were used: s – singlet; d- doublet, dd- doublet of doublets, m- multiplet, J – coupling constant.

9.2.2.4 Antibacterial tests

The isolated compounds were tested for antimicrobial activity towards the following bacteria as indicated in chapter 7: *Bacillus subtilis*, *Escherichia coli*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, *Acinetobacter baumanii*, *Moraxella catarrhalis*. Tests were done with the isolated compounds at 5 mg cm^{-3} in a 10% (v/v) solution of DMSO in water. A volume of 20 µl was loaded per 6 mm diameter hole.

9.3 RESULTS

9.3.1 Isolation of compounds by preparative TLC

As has been mentioned above, determination of phytochemical classes in the ethyl acetate fraction (section 7.3, chapter 7) indicated that this fraction was rich in terpenoid derivatives (essential oils, bitter principles, saponins) alkaloids as well as phenolic compounds.

Preparative TLC of the ethyl acetate fraction in the solvent system ethyl acetate: methanol: water (100:13.5:10) led to the isolation of a terpenoid with a cholesterol-like structure and R_f value of 0.61. This compound tested positive for bitter principles and saponins with the appropriate spray reagents. The structure of this terpenoid was determined by GC-MS. The identity of this compound, that is, its real name was not confirmed (see Appendix for GC-MS results). It possessed significant antimicrobial activity towards *M. catarrhalis* but was not tested against any other microorganisms due to the lack of its true identity. Some triterpenoids and tetraterpenoids though have been reported to have cholesterol-like skeletons (Tedder *et al.*, 1972).

An attempt was equally made to isolate alkaloids as well as other subclasses of phenolic compounds present in this fraction. We were able to successfully isolate by preparative TLC, and the solvent system ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26) five flavonoids. These compounds are presented in (Plates 9.4-9.6; Table 9.1: catechin (compound No.16), epi-catechin (compound No. 17), gallo-catechin (compound No.15), hyperoside (compound No.11) and quercitrin (compound No.14). The flavonoid glycosides hyperoside and quercitrin were positively identified by analytical TLC (Plates 9.1-9.6) through comparison with authentic samples (Sigma Chemicals) because their quantities were inadequate for structure elucidation by ^{1}HNMR. The three catechins had their structures determined by ^{1}H NMR. There were three unidentified compounds (Plates 9.4-9.6; compounds 10, 12, 13). Their concentrations were too low for structure determination by NMR spectroscopy.

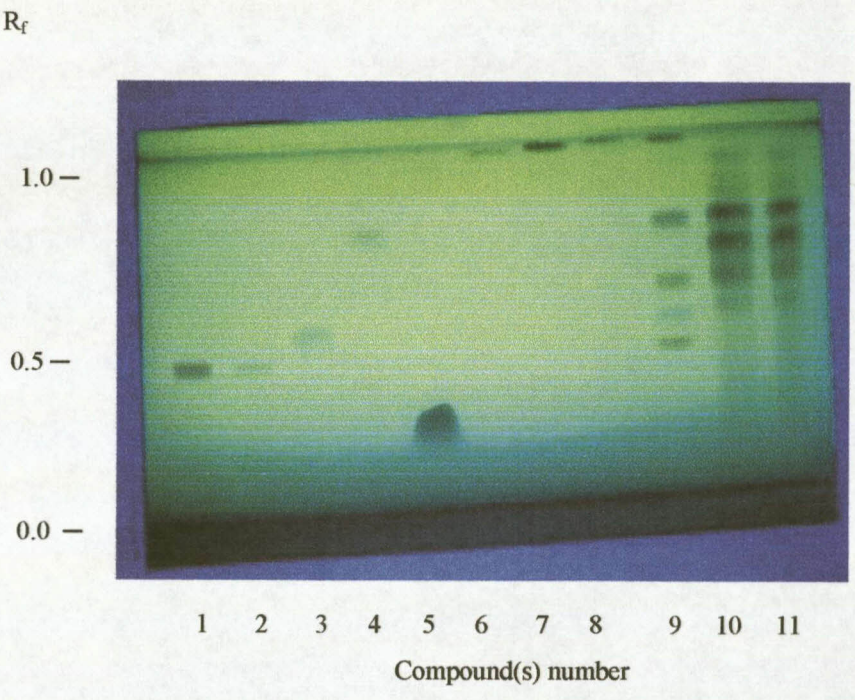


Plate 9.1: TLC profile of the reference compounds, their mixture and EtOAc fraction of *E. crispa* subsp. *crispa* under UV-254nm before spraying.

1= rutin, 2 = rutoside, 3 = chlorogenic acid, 4 = quercitrin, 5 = hypericin, 6 = dihydroquercetin, 7 = quercetin, 8 = kaempferol, 9 = reference compounds mixture (rutin, chlorogenic acid, hyperoside, quercetin, quercitrin), 10 = EtOAc fraction (5µl spot), 11= EtOAc fraction (2 µl spot).

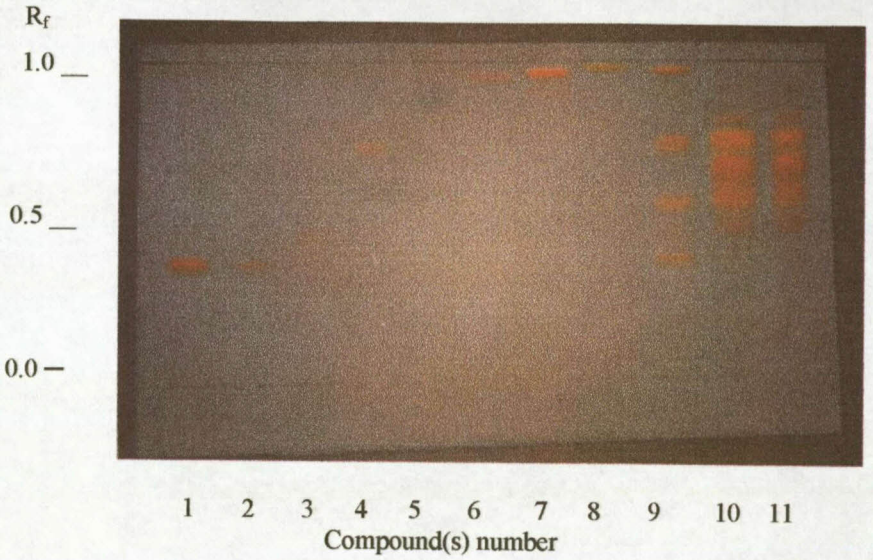


Plate 9.2 : TLC profile of the reference compounds, their mixture and EtOAc fraction *E. crispa* subsp. *crispa* under visible light after spraying.

1= rutin, 2 = rutoside, 3 = chlorogenic acid, 4 = quercitrin, 5 = hypericin, 6 = dihydroquercetin, 7 = quercetin, 8 = kaempferol, 9 = reference compounds mixture (rutin, chlorogenic acid, hyperoside, quercetin, quercitrin), 10 = EtOAc fraction (5µl spot), 11= EtOAc fraction (2 µl spot).

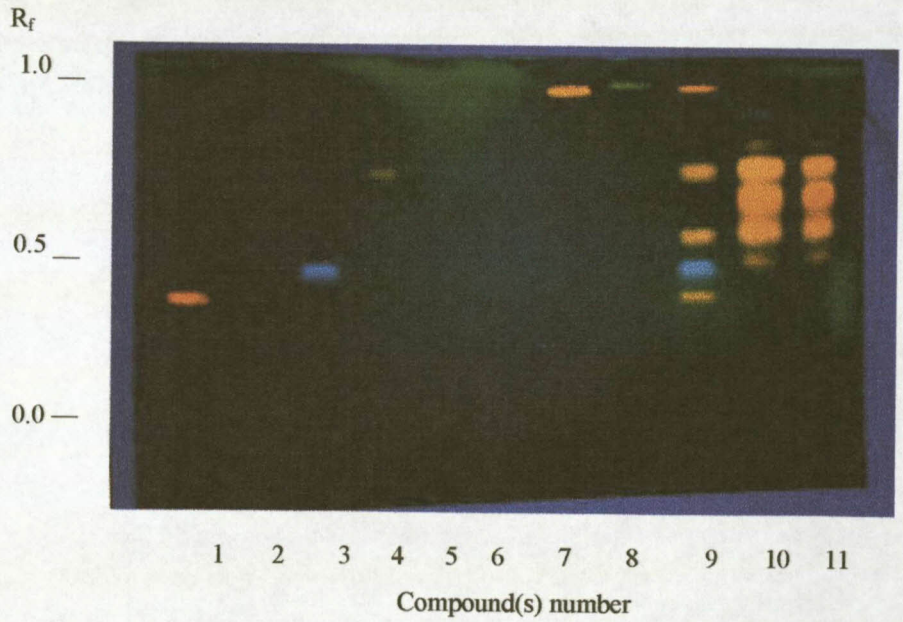


Plate 9.3: TLC profile of the reference compounds, their mixture and EtOAc fraction of *E. crispa* subsp. *crispa* under UV-365 nm after spraying.

Plate 9.3 continued...

1= rutin, 2 = rutoside, 3 = chlorogenic acid, 4 = quercitrin, 5 = hypericin, 6 = dihydroquercetin, 7 = quercetin, 8 = kaempferol, 9 = reference compounds mixture (rutin, chlorogenic acid, hyperoside, quercetin, quercitrin), 10 = EtOAc fraction (5µl spot), 11= EtOAc fraction (2 µl spot). Spray reagent: NEP/PEG.

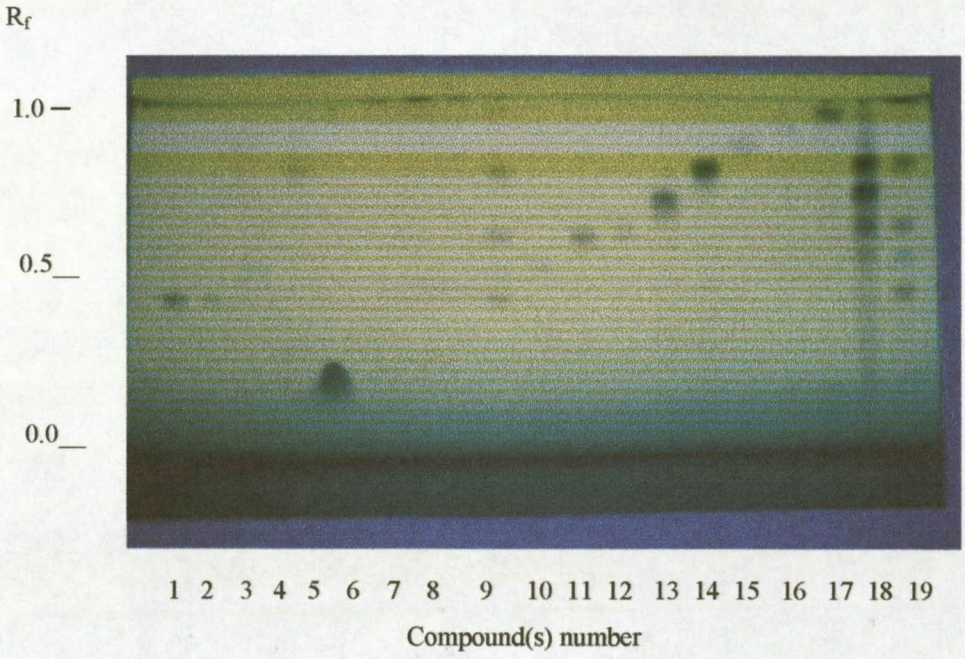


Plate 9.4 : TLC profile of the reference compounds, reference compounds mixture, isolated compounds from the EtOAc fraction of *E. crispa* subsp. *crispa* as well as the intact EtOAc extract under UV-254 nm, before spraying.

1= rutin, 2 = rutoside, 3 = chlorogenic acid, 4 = quercitrin, 5 = hypericin, 6 = dihydroquercetin, 7 = quercetin, 8 = kaempferol, 9 = reference compounds mixture (rutin, chlorogenic acid, hyperoside, quercetin, quercitrin), 10-17 = isolated compounds, 18 = EtOAc fraction, 19 = reference compounds mixture (as in spot 9)

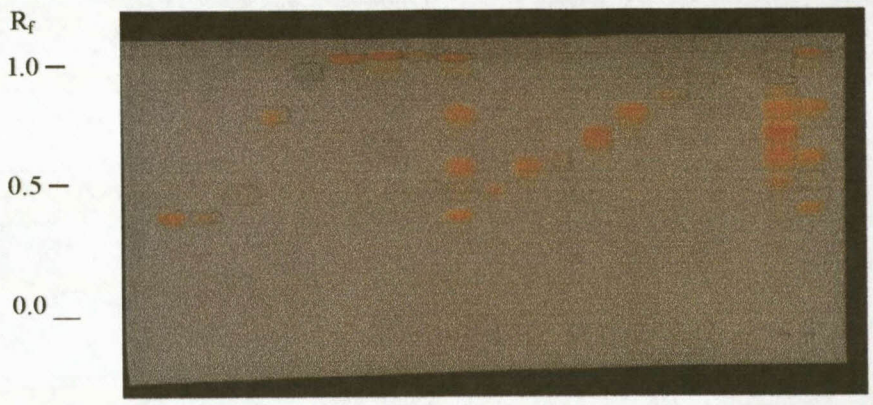


Plate 9.5 TLC profile of the reference compounds, reference compounds mixture, isolated compounds from the EtOAc fraction of *E. crispa* subsp. *crispa* as well as the intact EtOAc extract in visible light after spraying. 1= rutin, 2 = rutoside, 3 = chlorogenic acid, 4 = quercitrin, 5 = hypericin, 6 = dihydroquercetin, 7 = quercetin, 8 = kaempferol, 9 = reference compounds mixture (rutin, chlorogenic acid, hyperoside, quercetin, quercitrin), 10-17 = isolated compounds, 18 = EtOAc fraction, 19 = reference compounds mixture (as in spot 9)

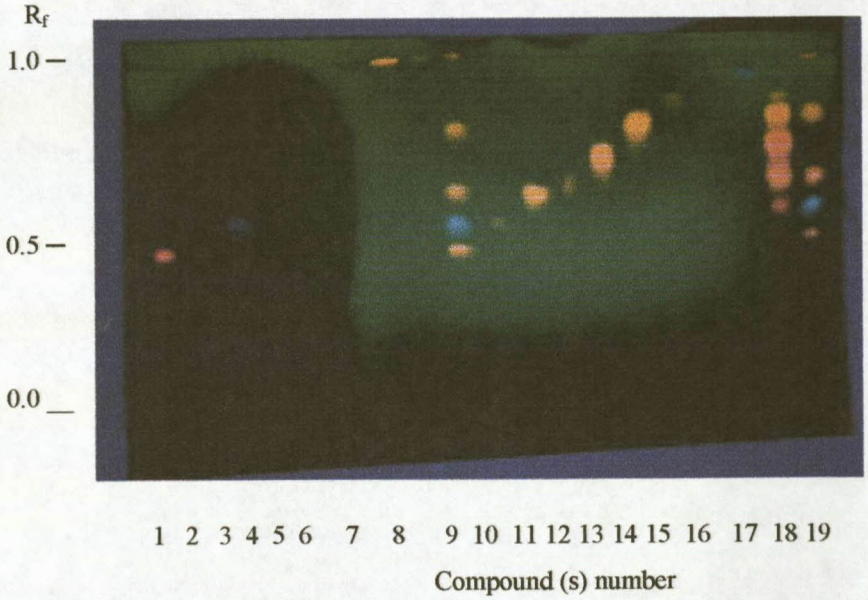


Plate 9.6 : TLC profile of the reference compounds, reference compounds mixture, isolated compounds from the EtOAc fraction of *E. crispa* subsp. *crispa* as well as the intact EtOAc extract under UV-365 nm, after spraying. Profile is the same as in Plates 9.5 and 9.6. 1= rutin, 2 = rutoside, 3 = chlorogenic acid, 4 = quercitrin, 5 = hypericin, 6 = dihydroquercetin, 7 = quercetin, 8 = kaempferol, 9 = reference compounds mixture (rutin, chlorogenic acid, hyperoside, quercetin, quercitrin), 10-17 = isolated compounds, 18 = EtOAc fraction, 19 = reference compounds mixture (as in spot 9). Spray reagent: NEP/PEG.

The flavonoid profile of the standard mixture was (rutin, R_f 0.40; chlorogenic acid, R_f 0.50; Hyperoside, R_f 0.58; quercetin, R_f 0.96 and quercitrin, R_f 0.72).

Table 9.1: Flavonoid TLC profile of the ethyl acetate fraction of *Euclea crispa* subsp. *crispa* on silica gel 60 F_{254} and solvent system ethyl acetate : formic acid : glacial acetic acid : water (100:11:11:26).

R_f values of spots shown by EtOAc fraction	Compound No.	Colours of spots with NP/PEG spray reagent under uv-light after spraying	R_f values of reference compounds	Reference compound
-	-	-	0.98	kaempferol (blue fluorescence)
-	-	-	0.96	quercetin (orange)
-		-	0.95	dihydroquercetin (orange)
0.93	17 (epicatechin)	yellow	-	-
-	-	-	0.82	hypericin (violet)
0.81	16 (catechin)	blue	-	-
0.76	15 (gallocatechin)	blue	-	-
0.72	14	orange	0.72	quercitrin
0.71	13	orange	-	-
0.66	12	orange-yellow	-	-
0.58	11	orange-yellow	0.58	hyperoside
		orange-yellow	0.51	quercetin
0.50	10	orange	0.50	chlorogenic acid (yellow)
-	-	-	0.44	rutoside
-	-	orange	0.4	rutin (orange)

The flavonoid glycoside reference compounds that matched the compounds from the ethyl acetate fraction were quercitrin (R_f 0.72) and hyperoside (R_f 0.58) (see Appendix; Plate 9.10).

The compound with an R_f value of about 0.50, had the same R_f value as chlorogenic acid but the colour reaction was different. Chlorogenic acid gave a light yellow colour reaction with the flavonoid (NP/PEG) spray reagent and on viewing in visible light (Plate 9.2), while the compound from the ethyl acetate fraction gave an orange colour reaction.

9.3.2 Structure determination by ^{1}H NMR.

The ^{1}H NMR spectra of these compounds are shown in Plates 9.7, 9.8 and 9.9 (see Appendix).

9.3.2.1 Catechin (Appendix; Plate 9.7)

The ^{1}HNMR spectrum ($CDCl_3$) of catechin displayed an aromatic ABX system for the B-ring. (H-2', δ 6.89 , d, J = 2.5 Hz ; H-5', δ 6.79 , d,J = 10.5 Hz ; H-5', δ 6.73, dd, J = 8.5 Hz ; H-6' , δ 6.73, dd, J = 9.5 Hz).

A-ring (H-6, δ 6.02, d,J = 2.5 Hz ; H-8, δ 5.85, d,J = 2.5 Hz)

C-ring (H-2, δ 4.51, d,J = 8.0 Hz ; H-3, δ 3.69, m,J = 25 Hz ; H_{eq}-4, δ 2.89, dd, J = 16.0 Hz ; H_{ax}-4, δ 2.495 , dd, J = 16.0 Hz)

9.3.2.2 Epicatechin (Appendix; Plate 9.8)

The 1HNMR also displayed an ABX system for the B-ring (H-2', δ 7.49, d,J = 2.5 Hz ; H-6' , δ 7.33, dd, J = 8.5 Hz ; H-5', δ 7.08 , d,J = 8.5 Hz)

A-ring (H-6, δ 6.49, d,J = 2.5 Hz ; H-8, δ 6.21, d,J = 2.5 Hz)

C-ring (H-2, δ 5.41, d,J = 2.5 Hz ; H_m-3, δ 4.22, d,J = 8.5 ; H-4, δ 3.37, m,J = 12.0 Hz)

9.3.2.3 Gallocatechin (Appendix; Plate 9.9)

An ABX system for the B-ring was displayed here just like with the other two flavan-3-ols. The B-ring also displayed two proton singlets at positions H-2' and H-6', δ 6.45

A-ring (H-6, δ 6.01, d,J = 4.0 Hz ; H-8, δ 5.89, d,J = 4.0 Hz)

C-ring (H-2, δ 4.47, d,J = 8.0 Hz ; H-3, δ 3.98, d,J = 25.0 Hz ; H_{eq}-4, δ 2.85, dd, J = 16 Hz ; H_{ax}-4, δ 2.85, dd,J = 16 Hz).

9.3.3 Antibacterial properties of the isolated compounds

No antibacterial activity was observed with all the five compounds towards the following bacterial species: *E. coli*, *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *K. pneumoniae*, *S. pyogenes*, *A. baumanii* tests at a concentration of 5 mg cm^{-3} and 20 µl per $hole^{-1}$ 6 mm in diameter. The table shows results of tests against organisms whose growth was inhibited by the isolated compounds.

Table 9.2: Antibacterial properties of the isolated flavonoids and terpenoid at 5 mg cm^{-3}; 20 µl per 6mm diameter $hole^{-1}$.

	BACTERIA & MEAN ZONE DIAMETER (mm)			
Compound	*B. subtilis*	*H. influenzae*	*S. pneumoniae*	*M. catarrhalis*
catechin	0	0	0	12.0 ± 0.6
epi-catechin	12.0 ± 0.4	0	12.0 ± 0.4	14.0 ± 0.4
gallo-catechin	0	0	0	11.0 ± 0.8
hyperoside	8.0 ± 0.4	8.0 ± 0.6	0	11.0 ± 0.4
quercitrin	0	0	0	10.0 ± 0.7
terpenoid	n/t	n/t	n/t	15.0 ± 0.4

n/t : not tested

Antibacterial tests revealed activity was much lower as compared to that of the ethyl acetate fraction (chapter 7:3.3) from which these compounds were isolated. Most of the activity was against *M. catarrhalis*. Epicatechin and hyperoside were the most active, inhibiting three bacterial species each. They both inhibited *B. subtilis* and *M. catarrhalis*. In addition to these bacteria, epicatechin inhibited *S. pneumoniae* while hyperoside inhibited the growth of *H. influenzae*. The lowest activity was observed with catechin, gallo-catechin and quercitrin; these three compounds only inhibiting *M. catarrhalis*.

9.4 DISCUSSION

Euclea species are widely used in traditional medicine in some African countries for the treatment of some infectious diseases (Khan & Rwekika, 1992 ; Sibanda *et al.*, 1992). Khan &

Rwekika (1992) also reported the isolation of triterpenoids the leaves of *E. natalensis*. A number of terpenoids are known to possess antimicrobial activity (Kubo *et al.*, 1992b ; Li *et al.*, 1993 ; Chen *et al.*, 1993). The terpenoid isolated in this project, due to its steroid-like structure might have been one of the triterpenoids characteristic of the *Euclea* species.

Of the isolated flavonoids, epicatechin seems to have been the most potent as compared to the other four compounds. This is evidenced by the broader spectrum of activity and larger inhibition zones. Activity of these compounds was lower in comparison to that of the intact ethyl acetate fraction (chapter 7, section 7.3). This low activity could have been due to the possibility that these compounds function in synergy with a range of other compounds. As has been stated earlier, medicinal plant extracts are used either as complex mixtures or as pure compounds depending on their therapeutic indices. Considering that the ethyl acetate fraction was the most active liquid-liquid separation fraction, in comparison with the hexane, diethyl ether, and chloroform fractions, it seems that extracts from *E. crispa* subsp. *crispa* are more effective in the form of complex mixtures. The activity of the crude methanol extract was even closer, and in some cases higher, than that of the ethyl acetate fraction for example in the tests against human pathogenic fungi (chapter 7, section 7.3.3). Therefore, it is clear that the more complex extracts of *E. crispa* subsp. *crispa* are more potent than the pure compounds due to a synergy of effects. This does not mean that there is no need to isolate and study the constituents of plant extracts showing similar tendencies like *E. crispa* subsp. *crispa*. As was mentioned in section 9.1, there are a number of reasons why constituents of an extract should be investigated, for example, to determine toxicity and dosage. These isolated compounds were not tested, though, against plant pathogenic bacteria for the purposes of crop protection.

A literature search on the antimicrobial properties of flavonoids revealed that other than antioxidant (Watanabe, M. 1998 ; Heinonen *et al.*,1998 ; Gardner *et al.*, 1998 ; Meyer *et al.*, 1998 ; Huang *et al.*, 1998) and antiinflammatory activity (Ibewuike *et al.*, 1997), some flavonoids do possess antimicrobial activity as well (Markham, 1989). The ethyl acetate

fraction of the methanol extract from *Calliandra haematocephala* (Mimosaceae) was found to be active against *Staphylococcus aureus* and *Bacillus subtilis* by Nia *et al.* (1999). From this ethyl acetate fraction, these authors reported the isolation of the following flavonoids: p-hydroxybenzoic acid, caffeic acid, protocatechuic acid, catechin-3-rhamnoside and epicatechin-3-rhamnoside as well as quercetin. The flavonol glycosides kaempferol-3-O-glucoside (astragalin), quercetin-3-O-galactoside (hyperoside), quercetin 3-O-rutinoside (rutin) together with kaempferol-3-O-rutinoside (nicotiflorin) are known to be the main flavonol glycosides of *Eupatorium guayanum* (Compositae) widely used in Peru for the treatment of colds, among other illnesses (Aquino *et al.*,1995). These authors also stated that the flavonoids quercetin-3-O-galactoside (hyperoside) and rutin isolated from the methanol extract of *Arcytophyllum nitidum* (Rubiaceae) have been shown to be effective against a number of Gram negative bacteria including *E. coli*. They also attributed the antibacterial action of flavonoids to their action on cell membrane permeability. Catechins have been found to possess potentially valuable properties as they inhibit the growth of both Gram-negative and Gram-positive bacteria, including *E. coli*. The mode of bactericidal action of catechins is through damage of the lipid bilayer (Isogai *et al.*, 1998). These authors also reported that catechins are effective in the inhibition of canine periodontal disease *in vivo*. Fukunaga *et al.* (1989) observed that quercitrin has antibacterial activity towards *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeureginosa* and *Klebsiella pneumoniae*, with MICs greater than 400 μg cm^{-3} for the first three bacterial species and only 400 μg cm^{-3} for *K. pneumoniae*. In our investigation, activity towards these pathogens was not observed probably due to lower doses applied in our experiments.

Quercitrin and catechin have been found to be effective against herpes simplex virus type 1 (HSV-1), parainfluenza virus type 3 (Pf-3) and Sindbis virus (SV-1) by affecting one or more of the biochemical processes involved in the intracellular replication of each of these viruses. The compound (-)-epicatechin has also been found to exhibit pronounced anti HIV-1 activity

in vitro. Differences between the isomers (-)-epicatechin and (+)-catechin were noted in the lower antiviral activity of (+)-catechin with respect to (-)- epicatechin (Aquino *et al.*, 1995).

In addition to the above mentioned properties of flavonoids as biologically active compounds, they possess the following characteristics that make them ideal chemotherapeutic agents. Flavonoids are (a) wide spread, (b) their patterns seem to be species specific (c) are easy to detect, chromatograph and identify (d) they are relatively stable (e) their biosynthesis and accumulation is independent of environmental influence (Markham, 1989). The fact that one of the functions of flavonoids in plants is to protect them against diseases caused by microorganisms means that they have the potential to be used in crop protection as well. Since flavonoids are phenolic compounds, they react with proteins, and thus they can interact with enzymes and biological processes in the cells. This consequently makes them toxic to some microorganisms and animals (Grayer, 1989 ; Markham, 1989).

Literature on the isolation of flavonoids, or other phenolic compounds from *E . crispa* subsp. *crispa*, was not found. As was mentioned earlier (chapter 7), only literature on the isolation of triterpenoids from this plant was come across. It can therefore be concluded that together with the other compounds found in *E . crispa* subsp. *crispa*, catechin, epicatechin, gallocatechin, hyperoside and quercitrin also contribute to the antimicrobial activity of extracts from this plant.

APPENDIX

Plate No.	Compound	Page
Plate 9.7	Catechin	169
Plate 9.8	Epicatechin	170
Plate 9.9	Gallocatechin	171
Plate 9.10	Hyperoside & Quercitrin	172
	GC-MS results	173

Plate 9.7 [$(CD_3)_2CO/D_2O$, 296K)]

CATECHIN

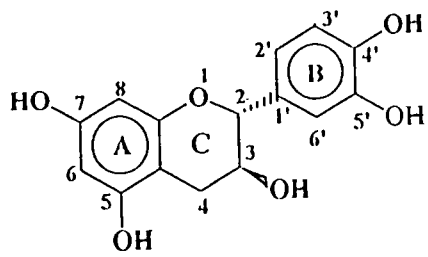
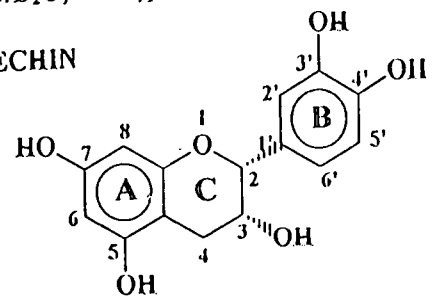
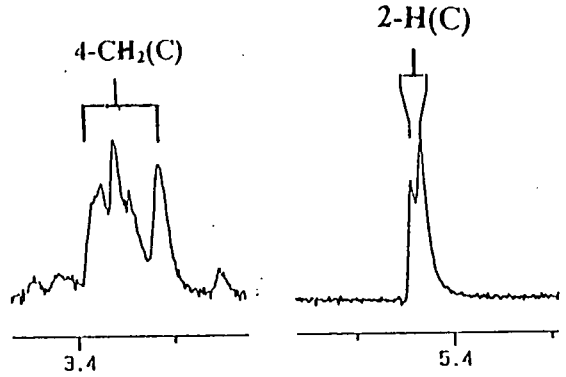
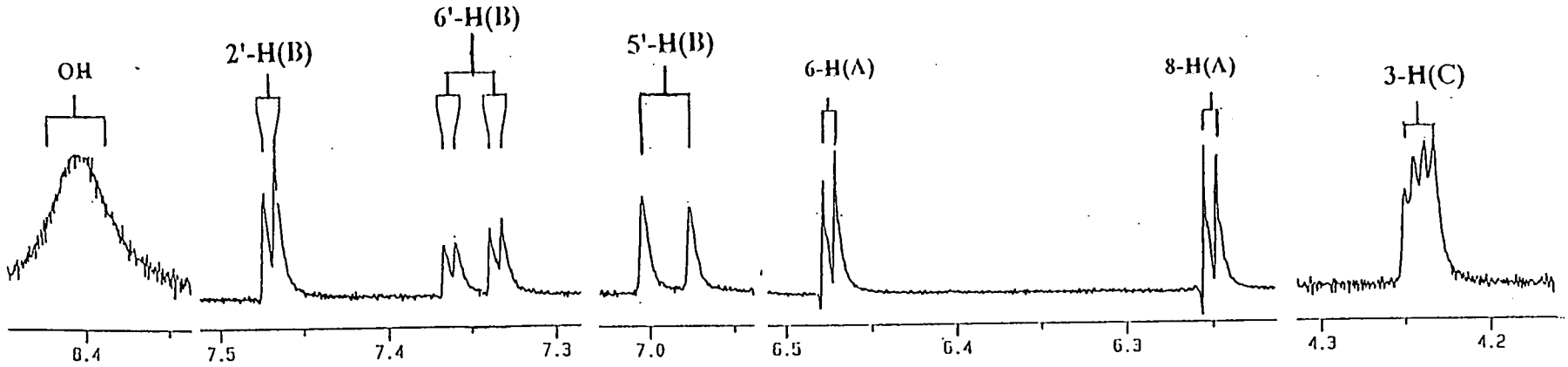


Plate 9.8 [$(CD_3)_2CO/D_2O$, 296K)]

EPI-CATECHIN







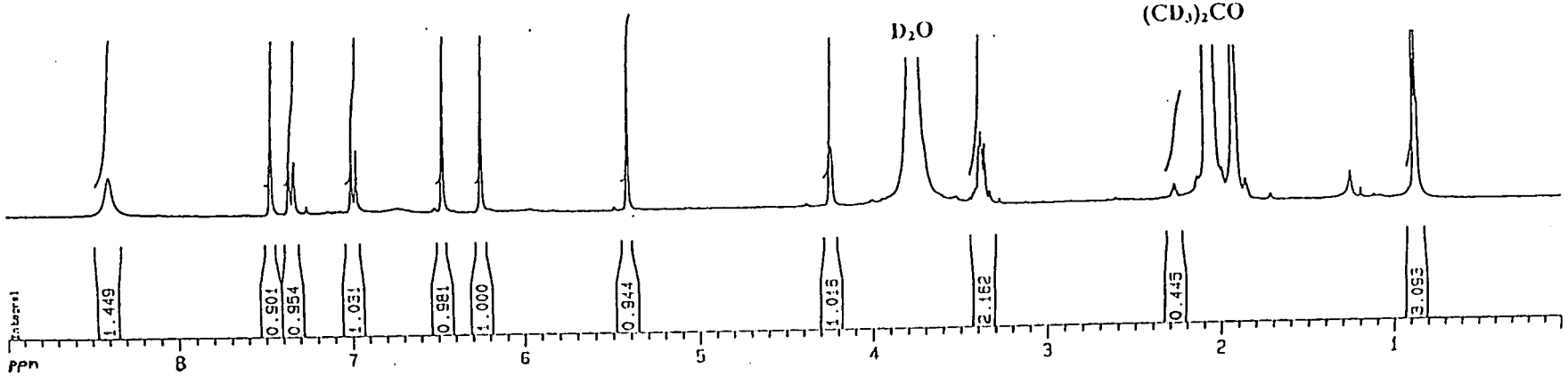
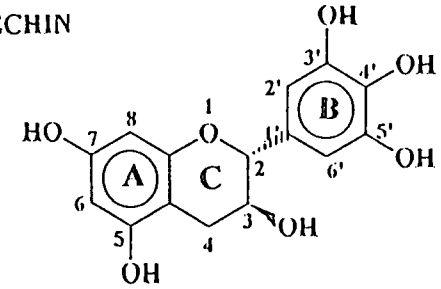
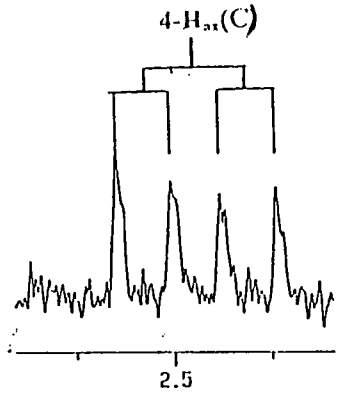
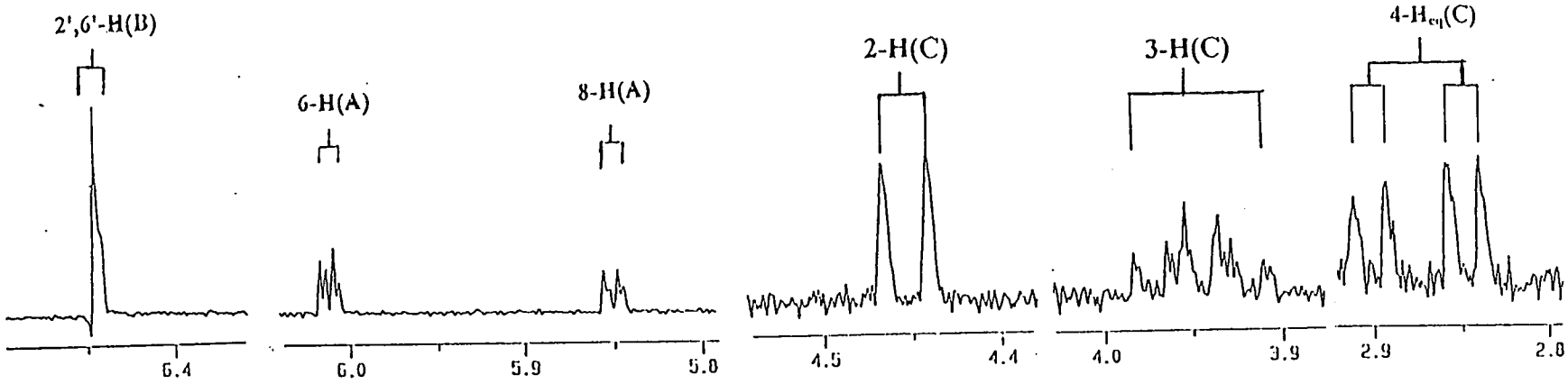


Plate 9.9 [$(CD_3)_2CO/D_2O$, 296K)]

GALLOCATECHIN







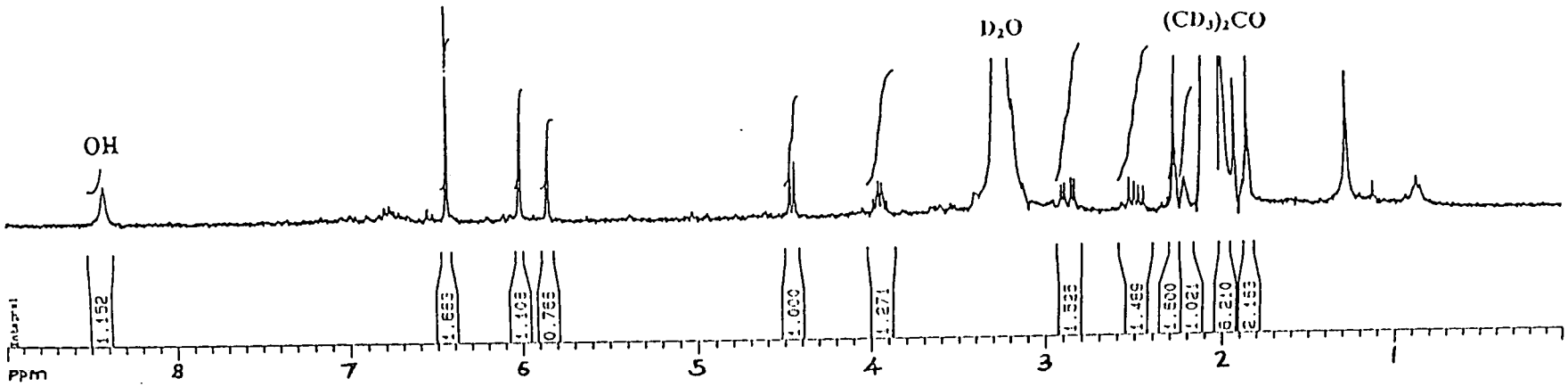
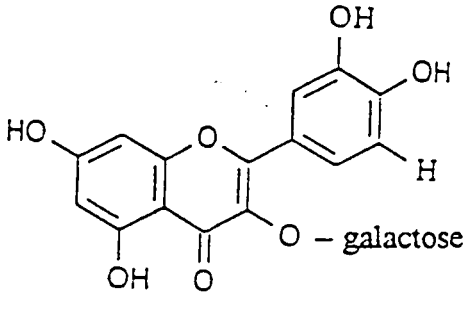
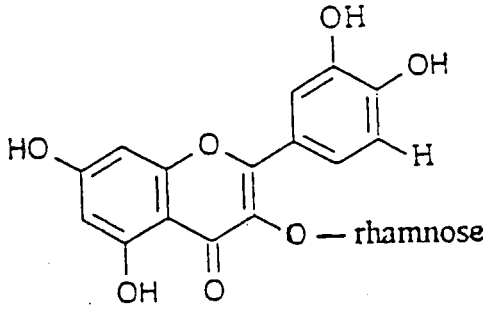


Plate 9.10

Hyperoside (quercetin-O-galactoside)



Quercitrin (quercetin 3-O-rhamnoside)



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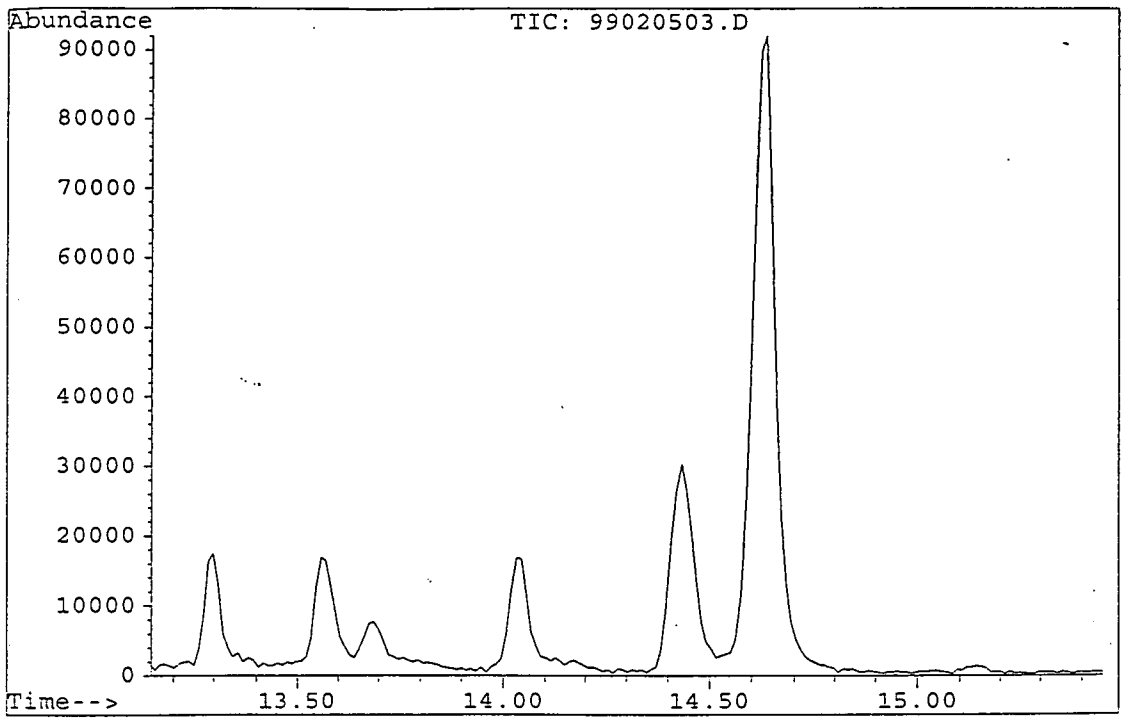
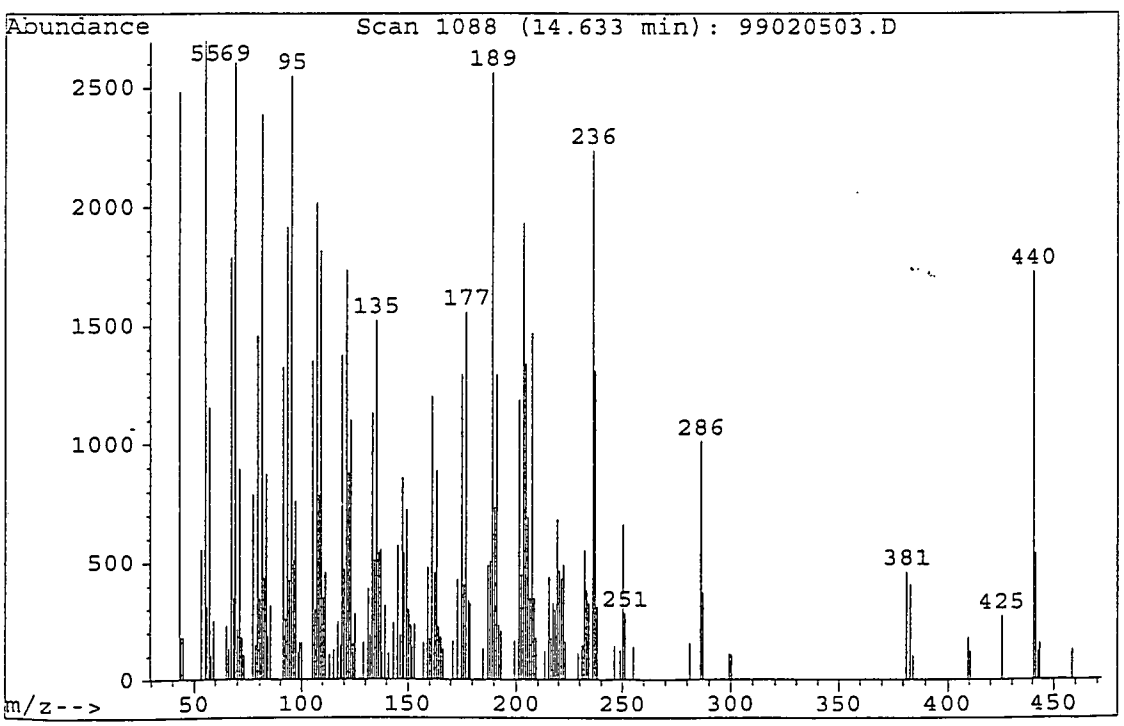
Vial Number: 3





CHAPTER 10

GENERAL DISCUSSION

The Free State Province (FSP), being located in a summer rainfall area and containing a number of geological features as well as biomes, is home to a diverse selection of plant species many of which are reported to possess antimicrobial properties. The soils here are generally fertile. The existence of extensive literature describing the vegetation ecology of this province is evidence that the FSP is endowed with a rich diversity of plant species (Venter, 1976 ; Kooij *et al.*,1990 ; Fuls *et al.*, 1992a ; Du Preez & Bredenkamp,1991 ; Du Preez *et al.*,1991 ; Malan *et al.*, 1998), many of which are of ethnopharmacological value (Van Wyk *et al.*, 1997 ; Hutchings *et al.*, 1996).

All plant material used in this study was collected basically from the three districts of Bloemfontein, Brandfort and Hoopstad. Most of the collection area is within the Dry Sandy Highveld Grassland (37) in the east and the Eastern Mixed Nama Karoo (52) in the west. Dolerite outcrops are conspicuous in the general topography. In the low lying areas the soils are deep and clayey. On slopes the soils are shallow and mainly of the Mispah formation (Malan *et al.*, 1995).

The geographical area of collection determines which plant species grow there, while the habitat determines the type and amounts of compounds in the plant species. The general phytochemical composition of the plant is all the same, determined by the genetic code of the plant (Harborne, 1984).

A study of plants that grow in this province and possess antimicrobial activity has never been done before. Due to a number of factors, that included the need to have these plant species documented, these investigations were done. Screening of the plant extracts for antimicrobial activity was done with the agar hole plate diffusion method, a method, though qualitative, is widely used in the screening of plant extracts and other antibiotics for antimicrobial activity

(Kavanagh, 1975 ; Bloomfield, 1991 ; March *et al.*, 1991 ; Heisey & Gorham, 1992 ; Anesini & Perez, 1993 ; Brantner & Grein, 1994).

Of the 26 plant species screened for antimicrobial activity during this study (chapter 6), 21 (80.8%) inhibited the growth of one or more test bacteria. The seven species giving the most potent extracts were *Acacia erioloba* (collected in the Sandveld Nature Reserve; Hoopstad district), *Euclea crispa* subsp. *crispa* (Bloemfontein district), *Acacia karroo* (Sandveld Nature Reserve; Hoopstad district), *Elephantorrhiza elephantina* (Brandkop, Bloemfontein District), *Buddleja saligna* (Botanical Gardens, Bloemfontein District), *Rumex lanceolatus* (Florisbad, Brandfort District) and *Diospyros austro-africana* var. *microphylla* (Brandkop, Bloemfontein District).

The plant pathogenic bacterium inhibited by most crude extracts was *C. michiganense* pv. *michiganense* and the least inhibited was *Erwinia carotovora* pv. *carotovora*. *Moraxella catarrhalis* was the most inhibited of all the bacteria put together by the crude extracts (chapter 6). *E. carotovora* pv. *carotovora* is one of the most difficult plant pathogens to control. On the other had, *M. catarrhalis* is an opportunistic human pathogen which, under normal circumstances, does not give rise to any sickness. Throughout the study, *M. catarrrhalis* was the most susceptible organism to the plant extracts.

Considering that the number of species screened for antimicrobial activity is a small representation of all the plants growing in this province, there is a lot of potential for the development of plant derived antimicrobials from plants growing here. The apparent absence of antifungal activity towards plant pathogenic fungi (chapter 6, section 6.3) does not necessarily dismiss these plant species as having no antifungal activity. The results in chapter 8, section 8.3.3, indicated that *E. crispa* subsp. *crispa* exhibited antifungal activity towards *Candida albicans* and *Cryptococcus neoformans* despite the absence of activity towards plant pathogenic fungi. Antifungal activity towards other human pathogenic fungi is therefore a significant possibility. Other authors have observed antifungal activity towards plant as well as

human pathogens with a number of species from some of the families included in this study (Matthews, 1993 ; Irobi & Daramola,1993). The fact that plant species belonging to the same family or genus are phytochemically related, allows for the assumption that antifungal activity towards other plant pathogenic fungi not used in our study could be observed with our plant collection as well.

E. crispa subsp. *crispa* exhibited a broad spectrum of antibacterial activity towards a number of both plant and human pathogenic bacteria in addition to antifungal activity mentioned in the preceding paragraph towards the human pathogenic fungi.

Semi-purification of the crude extract prepared from the leaves of *E. crispa* subsp. *crispa* by liquid-liquid separation (chapter 7) made it possible to determine the general classes of compounds present in the four semi-purified fractions. Screening of these four fractions for antimicrobial activity (results in chapter 8) showed that most of the activity among these semi-purified extracts was concentrated in the ethyl acetate fraction. This fraction inhibited the growth of all the plant pathogenic bacteria used in the tests. The three human pathogenic bacteria not inhibited by the ethyl acetate fraction were *Haemophilus influenzae*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae*. The phytochemical profile of the ethyl acetate fraction indicated the presence of mainly terpenoid derivatives; the essential oils, saponins, bitter principles as well as alkaloids and phenolic compounds.

Preparative thin layer chromatography of the ethyl acetate fraction (chapter 9) led to the isolation of a terpenoid and phenolic compounds with antimicrobial activity towards *M. catarrhalis*, the test organism used during this project for bioassay guided fractionation. The isolated terpenoid tested positive for bitter principles and saponins with the appropriate spray reagents. Structure analysis of this terpenoid by GC-MS revealed that it was a steroid-like. The name of the terpenoid was not available in the database of the GC-MS. Some tri- and tetra-terpenoids are reported to have steroid-like structures (Tedder *et al.*, 1972). Since saponins are

triterpenoids, a conclusion was made that this might have been one of the many triterpenoids found in species of *Euclea crispa* subsp. *crispa* and its other subspecies.

Further, attempts to isolate different classes of compounds present in the ethyl acetate fraction by preparative thin layer chromatography led to the successful isolation of only a number of phenolic compounds with antimicrobial activity. The use of different spray reagents for the identification of the subclasses of phenolics revealed that these compounds were flavonoids. NMR structure analysis led to the identification of catechin, epicatechin and gallocatechin. The two other compounds, due to their inadequate quantities for analysis by NMR, were identified by analytical thin layer chromatography with the use of authentic samples as being hyperoside and quercitrin. As has been mentioned in the preceding paragraph, the intact ethyl acetate fraction could not inhibit the growth of *Haemophilus influenzae*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae*. The compounds isolated from this fraction; hyperoside inhibited the growth of *H. influenzae* while epi-catechin inhibited the growth of *S. pnumoniae*, which are some of the most problematic human pathogenic bacteria. These two flavonoids were the most active compounds isolated. It is noted that *K. pneumoniae* was neither inhibited by the intact ethyl acetate fraction nor by any of the isolated compounds. Actually, *K. pneumoniae* was not inhibited by any of the extracts from *E. crispa* subsp. *crispa*. That is, the crude extract and all the four semi-purified liquid-liquid separation fractions did not inhibit the growth of this pathogen. This implied that *K. pneumoniae* was the most resistant human pathogen used in the screening of the extracts from *E. crispa* subsp. *crispa*. All the isolated flavonoids are well known for their biological activity that also includes antimicrobial activity. They are very common in the so-called green teas and exhibit antiinflammatory, antimicrobial as well as antioxidant activity.

The activity of the individually isolated compounds towards human pathogenic bacteria was generally not as high as that of the complex ethyl acetate fraction. It was suspected that this observation was due to the possibility that the isolated compounds function in synergy in the complex mixture (ethyl acetate fraction) and that purification therefore led to a decrease in

antimicrobial activity. Most extracts from plant species used in ethnopharmarcology are used in the form of infusions or tinctures, and these are complex mixtures. In cases where the activity of an extract falls as purification progresses such mixtures should be used in their complex form. Determination of the chemical composition is, therefore, in such situations done to determine the toxicity of the individual compounds and how the presence of other compounds in synergy lowers this toxicity due to chemical interactions. Toxicity is a major factor that should be considered whether one is working on agrochemical antimicrobial pesticides or antimicrobials for chemotherapeutic purposes. The fact that the same extracts were tested against plant and human pathogens with positive results, in chapter 8, implies that plant derived antimicrobials can be equally applied in both cases. Being a province with a lot of agricultural activity going on, the Free State would benefit from the endemic species with antimicrobial properties.

Uncontrolled overgrazing and destruction of indigenous plant species through cutting down of trees, except in protected areas is a major threat to the rich flora of the province (Malan, *et al.*,1998 ; Kooij *et al.*, 1990b ; Fuls *et al.*, 1992b). Initial large scale cultivation of the species with antimicrobial activity would also benefit the environment by countering the green house effect of gases as well as reducing soil erosion. The harvesting of leaves is a cheap, non-destructive method of the use of plant derived materials in comparison with the use of the roots or stem bark. In cases where a larger number of species is needed for industrial scale isolation of active compounds, as was stated in earlier chapters, the use of tissue and cell cultures would be beneficial to both the pharmaceutical and the agrochemical industries.

CHAPTER 11

SUMMARY

The Free State province has a rich diversity of indigenous woody plant species that exhibit antimicrobial activity towards both plant and human pathogens. Most of the province (about 72%) is located within the Grassland Biome, only about 21% is in the Nama-Karoo Biome, 6.64% in the Savanna Biome and with the Forest Biome making up only 0.05% of the province. The location of the province in what is mostly the Grassland Biome has resulted in extensive animal husbandry and intensive crop production. Grazing by animals, especially cattle and sheep, continues to have detrimental effect on the local pristine vegetation. On the other hand, intensive crop production, though done with utmost care in most cases, is also resulting in environmental degradation through water pollution by herbicides, both antimicrobials and insecticides. Chances are that some of these pesticides might end up in the food consumers buy, and may lead to health problems.

There is great awareness in the Free State province for the need to conserve the environment and the fact that conservation can only be done through the knowledge of the vegetation ecology of the province. Towards this end, a lot of studies on the vegetation ecology of the different areas of the province have been attempted by a number of authors. Despite the existence of information on the vegetation ecology, the Free State still had to be mapped for plants with biological activities, in our case, antimicrobial activity. This exercise was successfully done.

Antimicrobial screening of the crude extracts was done as a preliminary screen to determine which plant species exhibited this activity. Bench top assays were used due to their reliability, speed and cost effectiveness. The screen gave valuable information on the antimicrobial properties of these selected plants. By referring to their phytochemical compositions in literature, we were able to conclude why they are used in ethnopharmacology, as well as determine the active principles. Antibiotic plant species with ethnopharmacological value can

also be used as agrochemicals against plant pathogenic miroorganisms with success. The extracts of selected plant species in our investigations were tested against both plant and human pathogens with encouraging results. The general absence of antifungal activity towards plant pathogenic fungi was attributed to lower doses of the extracts used, the crude nature of the extracts as well as the the probable presence of growth promoters for the fungi or the general absence of active antifungal substances by nature.

The crude extracts of seven plant species out of a total of twenty six exhibited the most outstanding antibacterial activity as evidenced by broad spectrums of activity and larger inhibition zones in comparison with the rest of the plant species. The most resistant bacterial pathogen towards the crude extracts was *Erwinia carotovora* pv. *carotovora* while the least resistant was *Moraxella catarrhalis*. From the seven most active plant species, it was decided to do further investigations on *Euclea crispa* subsp. *crispa*. This was due to the fact that it is widely used in ethnopharmacology in areas like Kwa-Zulu Natal and also that it is one of the most common plant species in the province as well as in the country as a whole.

The crude and semi-purified (liquid-liquid separation) extracts of *E. crispa* subsp. *crispa* showed antibacterial activity towards human pathogenic bacteria to varying degrees. The ethyl acetate fraction was the most active however, since only three human pathogenic bacteria were not inhibited by it. *Klebsiella pneumoniae* was the most resistant human pathogenic bacterium. With regards to plant pathogenic bacteria, generally, only the ethyl acetate fraction and (the crude extract) showed activity against all the bacteria. Antifungal screening of the semi-purified extracts with human pathogenic fungi also showed that most of the antifungal activity was located in the ethyl acetate fraction. No antifungal activity was observed with the hexane fraction. Due to the more complex nature of the crude extract, further phytochemical studies were carried out only with the ethyl acetate fraction, the most active semi-purified fraction.

Investigations into the general phytochemical profile of the ethyl acetate fraction of *E. crispa* subsp. *crispa* revealed that the plant extract contained essential oils, bitter principles, saponins,

alkaloids and phenolic compounds; the first three being terpenoid derivatives. Bitter principles are in some cases reported as being mixtures of different classes. A terpenoid that tested positive for bitter principles and for saponins as well as possessing a steroid-like structure was isolated from the ethyl acetate fraction. This compound was active against *M. catarrhalis*. Also isolated from the ethyl acetate fraction and showing antimicrobial activity were five flavonoids identified as hyperoside, quercitrin, catechin, epicatechin and gallocatechin. The most active flavonoids were epicatechin and hyperoside which inhibited the growth of *Streptococcus pneumoniae* and *Haemophilus influenzae* respectively.

The activities of the isolated flavonoids towards human pathogenic bacteria were lower than that that of the more complex ethyl acetate fraction. The conclusion arrived at was that these compounds function in synergy in the latter more complex fraction. Since most of the plant extracts are used in the form of complex mixtures in ethnopharmacology, a suggestion was made to use the ethyl acetate fraction in its complex form. It was further acknowledged that the use of complex mixtures instead of pure compounds does not eliminate the need to determine the chemical composition of the complex mixtures. This is necessary to determine the dosages and toxicity of the constituents of the extracts.

In conclusion, it can be said that valuable information on the antimicrobial properties of selected plant species growing in the Free State Province was obtained during the course of our investigations. Also of value is the information on the distribution of these species in relation to biomes, geology and soil types. The availability of literature on the vegetation ecology of different parts of the province was also of immense help to us during this investigation.

HOOFSTUK 11

OPSOMMING

Die Oranje Vrystaat beskik oor 'n ryk diversiteit van inheemse houtagtige plant spesies wat antimikrobiese aktiwiteit openbaar teenoor bëide plant- en mens patogene. Die grootste gedeelte van die provinsie (ongeveer 72%) is geleë binne die Grasland Bioom, slegs 21% is in die Nama-Karoo Bioom, 6.64% in die Savanna Bioom terwyl die Bos Bioom slegs 0.05% van die provinsie uitmaak. Die lokaliteit van die provinsie, hoofsaaklik Grasland Bioom, het aanleiding gegee tot ekstensiewe vee – en intensiewe gewasproduksie. Weiding deur diere, veral skape en beeste, het 'n vernietigende effek op die pristien vegitasie. Aan die ander kant gee intensiewe gewasproduksie, alhoewel met sorg beoefen, ook aanleiding tot die agteruitgang van die omgewing as gevolg van die gebruik van onkruiddoders, antimikrobiese middels en insekdoders. Die kans bestaan dat van hierdie chemikalieë in voedsel wat deur verbruikers gekoop word mag opeindig en potensieël tot gesondheidsprobleme mag aanleiding gee.

Daar is egter 'n bewustheid vir die behoefte om die omgewing van die Oranje Vrystaat provinsie te bewaar en die feit dat bewaring alleen moontlik is as ons oor die nodige vegitasie ekologie kennis van die provinsie beskik. Om hierdie doel te verwesenlik, is baie vegitasie ekologie studies reeds onderneem deur verskeie outeurs. Ten spyte van die bestaan van hierdie inligting, moes die provinsie nog vir plante wat biologiese aktiwiteit toon, in ons geval antimikrobiese aktiwiteit, gekarteer word. Hierdie oefening is met 'n redelike mate van sukses uitgevoer.

Die toetsing van ru-ekstrakte vir anti-mikrobiese aktiwiteit was as 'n voorlopige studie uitgevoer ten einde vas te stel watter plante in hierdie kategorie val. Laboratorium essaheringstegnieke is gebruik vanweë hulle betroubaarheid, koste-effektiwiteit en spoed waarteen vinnige resultate bekom kan word. Hierdie voorlopige toetsing het waardevolle inligting met betrekking tot die antimikrobiese potensiaal van plante opgelewer. Deur ook na

die plante se fitochemiese samestellings, soos in die literatuur vervat te verwys, was ons instaat om vas te stel waarom baie van hierdie plante etnobotaniese gebruike het asook om die aktiewe bestanddele hiervoor verantwoordelik te identifiseer. Plant spesies met antibiotiese en dus etnofarmakologiese potensiaal kan ook as agrochemikalieë met sukses teen plantpatogeniese mikroorganismes ingespan word. In hierdie studie is ekstrakte van selektiewe plantspesies teen beide plant- en menspatogene getoets en met belowende resultate. Die algemene afwesigheid van anti-fungale aktiwiteit by hierdie plante kan moontlik toegeskryf word aan die lae dosisse van toets ekstrakte deur ons gebruik, die moontlike aanwesigheid van fungale groei stimuleerders of selfs die natuur-like afwesigheid van antifungale aktiewe bestandele by hierdie plante.

Sewe plantspecies uit 'n totaal van 26 het bo-gemiddelde antibakteriese potensiaal getoon soos aangedui deur die breë spektrum van beheer asook groter inhibisie sones in vergelyking met die res. Die mees weerstandbiedende bakteriese patogeen was *Erwinia carotovora* pv. *carotovora* terwyl die mees sensitiewe een *Moraxella catarrhalis* was. Daar is egter besluit om verdere studies slegs op *Euclea crispa* subsp. *crispa* uit te voer angesien dit die meeste etnofarmakologiese toepassing geniet maar ook omdat dit so wyd versprei in die provinsie en die land aangetref word.

Die ru- en semi-gesuiwerde ekstrakte van *E. crispa* subsp. *crispa* het antibakteriese aktiwiteit met variërende intensiteit teen mensbakterieë getoon. Geen besondere patroon is waargeneem nie maar die etielasetaat fraksie was die mees aktiewe een. Met betrekking tot plantpatogeniese bakterieë het slegs die ru- ekstrak en etielasetaat fraksie enige aktiwiteit getoon. Antifungale toetsing op menslike swamme het ook aktiwiteit in slegs die ru-ekstrak en etielasetaat fraksie uitgewys. Geen aktiwiteit is met die nie-polêre heksaan fraksie waargeneem nie. Vanweë die meer komplekse aard van die ru-ekstrak, is verdere fitochemiese studies slegs met die etielasetaat fraksie uitgevoer wat naas die ru-ekstrak, as die mees aktiewe semi-gesuiwerde fraksie uitgestaan het.

'n Ondersoek na die algemene fitochemiese profiel van die etielasetaat fraksie van *E. crispa* subsp. *crispa*, het daarop gedui dat die plant ryk is aan essensiële olies, bitter bestanddele, saponiene en fenoliese verbindings. Die eerste drie is almal terpenoïed derivate. Vyb flavonoïede is geïsoleer en geïdentifisser naamlik hiperosied, quersitrien, catachien, epicatechien en gallocatechien. Die aktiwiteit van die geïsoleerde flavonoïede teen menspatogeniese bakterieë was laer as dié van die meer komplekse etielasetaat fraksie. Die gevolgtrekking hieruit is dat die verbindings waarskynlik sinergisties funksioneer in laasgenoemde meer komplekse fraksie. Aangesien meeste plantekstrakte etnofarmakologies in die vorm van komplekse mengsels gebruik word, is 'n aanbeveling dan ook gemaak dat die etielasetaat fraksie net so in sy komplekse vorm gebruik word. Dit is ook verder erken dat die gebruik van komplekse mengsels eerder as suiwer komponente nie die behoefte vir die bepaling van die afsonderlike komponente in die mengsel se chemiese strukture wegneem nie. Laasgenoemde is steeds belangrik ten einde die dosisse en toksisiteit van afsonderlike konstituente te bepaal. In die toekoms behoort hierdie flavonoïede meer intensief teen plantpatogeniese bakterieë en swamme getoets te word.

Ten slotte kan die steling gemaak word dat waardevolle inligting met betrekking tot die antimikrobiese eienskappe van geselekteerde plante in die Oranje Vrystaat met hierdie studie bekom is. Ook van waarde is die inligting oor die verspreiding van hierdie spesies in verhouding tot die bioom, die geologie en die grond tipes van die provinsie. Die beskikbaarheid van literatuur oor die vegitasie ekologie van die verskillende streke van die provinsie was egter van groot hulp tydens hierdie studie.

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