BIOLOGICAL ACTIVITY OF TRADITIONAL MEDICINAL PLANTS USED AGAINST VENEREAL DISEASES IN SOUTH AFRICA

by

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"When you get to the end of your courage and there's nothing more you can do- Try folding your hands for a moment and letting God's guidance get through. Life can't be altered by worry. Stop trying to push it your way. Just let the good Lord take over. You'll find that things change when you pray". Patience Strong
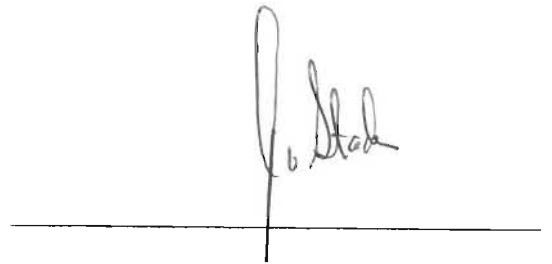
DECLARATION

The experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg, from June 2003 to December 2005, under the supervision of Professor J. van Staden.

These studies are the result of my own investigations, except where the work of others is acknowledged, and have not been submitted in any other form to another University.

Lisa Valencia Buwa

I declare the above statement to be correct

Professor J. van Staden

(Supervisor)

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ABSTRACT

Throughout the history of mankind, many infectious diseases have been treated with plant extracts. Venereal infections are one such group and are regarded as conditions that are highly responsive to traditional treatment. Aqueous, ethanol and ethyl acetate extracts of 13 plants used in South Africa for the treatment of venereal diseases were screened for *in vitro* antibacterial, antifungal, mutagenic and antimutagenic activities.

Antibacterial activity was evaluated using the disc-diffusion and microdilution assays to determine the minimal inhibitory concentration (MIC) values of the extracts. The extracts were tested against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, and the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae*. Among the plants tested, *Gunnera perpensa*, *Harpephyllum caffrum*, *Hypoxis latifolia* and *Ledebouria ovatifolia* showed the best antibacterial activity. The aqueous rhizome extract of *Gunnera perpensa* displayed good activity against Gram-negative bacteria with an MIC value of 0.78 mg/ml, and against *S. aureus* (0.78 mg/ml). Aqueous and ethanol extracts of *H. caffrum* bark were active against both Gram-positive and Gram-negative bacteria. *Hypoxis latifolia* aqueous corm extracts exhibited very good MIC values against *K. pneumoniae* (0.78 mg/ml), *E. coli* and *S. aureus* (1.56 mg/ml). Ethanol and ethyl acetate bulb extracts of *Ledebouria ovatifolia* displayed good activity against *Bacillus subtilis* bacteria with MIC values of 0.78 mg/ml and 0.39 mg/ml respectively.

Antifungal activity was evaluated using the microdilution bioassay. Good activity was shown by the ethanolic bark extracts of *Bersama lucens* and *Harpephyllum caffrum* against *Candida albicans*. Only in the case of *Harpephyllum caffrum* did aqueous extracts have activity against *Candida albicans*. In the Ames test, all plant extracts showed a negative genotoxic response except for ethanol and ethyl acetate bulb extracts of *Cyrtanthus obliquus* which induced mutations in TA98.

Moderate antimutagenic activity was observed with the ethyl acetate extract of *G. perpensa* and the ethanolic extract of *H. latifolia*.

High antibacterial and antifungal activity detected with *Harpephyllum caffrum* bark extracts resulted in an investigation on seasonal and geographical variation of this inhibitory activity. Seasonal variation in antibacterial and antifungal activities was investigated in order to determine the best collection time to ensure potential high medicinal activity in plant preparations. The highest inhibitory activity was detected with plant material collected in June and December 2003, with a decline in activity when collections were made in September 2004. The chemical profiles of TLC chromatograms were compared and little variation was found, particularly in the case of plant material obtained from the Botanic Garden of the University of KwaZulu-Natal and a 'Muthi' Shop in Pietermaritzburg.

Identification of active compounds from *G. perpensa* and *H. caffrum* was not successful due to insufficient amounts of isolated fractions.

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LIST OF ABBREVIATIONS

AIDS	-	Acquired Immune Deficiency Syndrome
ATCC	-	American Type Culture Collection
DMSO	-	Dimethyl sulfoxide
EA	-	Ethyl acetate
E	-	Ethanol
INT	-	p-Iodonitrotetrazolium Violet
MeOH	-	Methanol
MH	-	Mueller-Hinton
MIC	-	Minimum Inhibitory Concentration
NMR	-	Nuclear Magnetic Resonance
4-NQO	-	4-Nitroquinoline-N-oxide
R_f	-	Relative front
STDs	-	Sexually Transmitted Diseases
TLC	-	Thin Layer Chromatography
UV	-	Ultraviolet
VLC	-	Vacuum Liquid Chromatography
WHO	-	World Health Organisation

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Throughout the history of mankind, many infectious diseases have been treated with plant extracts. According to ARNOLD and GULUMIAN (1984) and MARTINEZ et al. (1996), a good portion of the world's population, particularly in developing countries, relies on plants for the treatment of infectious and non-infectious diseases. Plants have been selected and used empirically as drugs for centuries, initially as traditional preparations then as pure active principles (KAMIL, 1993). The estimated 250 000 higher plant species known, form a vast potential pool for the further investigation and exploration of plant products for future use in medicine. It has been estimated that only 5-15% of the identified higher plant species have been systematically surveyed for the presence of biologically active compounds (BALANDRIN et al., 1993). Today, natural products and their derivatives represent more than 50% of all the drugs in clinical use, with higher plant-derived natural products representing 25% of the total (BALANDRIN et al., 1993).

A drug is a substance used in the treatment or prevention of a disease as a component of a medication. COUZINIER and MAMATAS (1986) and TAYLOR et al. (2001) defined it as a chemically identified substance, either derived from plants or animals or produced by synthesis. HAMBURGER and HOSTETTMANN (1991) placed medicinal plant drugs into two broad categories- firstly, those included in complex mixtures containing a wide variety of compounds (e.g. infusions, essential oils, tinctures or extracts), and secondly those used as pure, chemically defined active principles. Pure compounds are used when the activity is strong and specific, and/or has a small therapeutic index. Where plants show weaker and less specific

pharmacological activity, or if the activity has not been fully categorised, the use of general plant extracts is appropriate (HAMBURGER and HOSTETTMANN, 1991).

Until the development of chemistry, medicinal plants and herbs were the sole source of active principles capable of curing man's ailments. They continue to be important to people who do not have access to modern medicines and, moreover, modern pharmaceuticals rely heavily on the same active principles, be they natural or synthetic. NJAU (1991) stated that half a century ago there were relatively few useful drugs available and that today there are nearly 1400 drugs in use, which are derived from both natural and synthetic sources. FARNSWORTH (1984) and LEWINGTON (1990) estimated the cost of making a new drug to be from 50 to 100 million dollars, and that approximately 10 years is needed to develop it. Apart from Egypt, most of the other countries in Africa still depend on imported synthetic drugs, while only a few produce up to 20% of the drugs they need locally (SOFOWORA, 1981).

Many currently used drugs are expensive or not readily available. A major set back to their continued usage is the development of drug resistance. There is thus an urgent need for new, inexpensive drugs that will be able to act for longer periods before resistance sets in.

1.2 TRADITIONAL MEDICINE

Traditional medicine has been used by the majority of the world's population for thousands of years. Until the beginning of the 19th century, all medicine was traditional (KIOTA, 1991). The use of herbal medicine in seeking relief from illness can be traced back over 5 millennia to documents produced by the early civilizations in China, India and the Near East, but it stretches indisputably even earlier into the history of mankind (HAMBURGER and HOSTETTMANN, 1991). The World Health Organization (WHO) estimates that 4 billion people - 80% of the world population - use herbal medicine for some aspects of primary health care (FARNSWORTH et al., 1985).

In Africa and most developing countries traditional medicine still forms the backbone of rural medical practices. Medicinal herbs are extensively used for various ailments in these countries. Developing countries are often subject to shortages of funds, medical facilities and newly developed medicine, which make them more dependent on their natural resources (MAMMEN and CLOETE, 1996; SHALE *et al.*, 1999). The rural population of the country is more disposed to traditional ways of treatment because of its availability and cheaper cost (BANQUAR, 1993). Ethnobotanical studies throughout Africa confirm that native plants are the main constituents of traditional African medicines (KOKWARO 1976; HERDBERG *et al.*, 1983; OLIVER-BEVER, 1987; CUNNINGHAM, 1993). With 70-80% of Africa's population relying on traditional medicines, the importance of the role of medicinal plants in the health care system is enormous. Medicinal plants are now being given serious attention, as is evidenced by the recommendation by the WHO in 1970 (WONDERGEM *et al.*, 1989) that traditional remedies should be incorporated within national drug policies. Moreover, recent moves towards a greater professionalism within African medicine (LAST and CHAVUNDUKA, 1986), and the increased commercialisation and pharmaceutical production using traditional medicinal plants with known efficacy (SOFOWORA, 1981; CUNNINGHAM, 1993) have caused medicinal plants to be seriously considered.

The study of African medicinal plants in the past has not been taken as seriously, or documented as fully, as Indian and Chinese Traditional Medicine. Over 5000 plants are known to be used for medicinal purposes in Africa, but only a few have been studied (TAYLOR *et al.*, 2001). Of those African medicinal plants investigated for their chemical components, some have been found to contain compounds with interesting biological activity, which have proved therapeutically active in controlled clinical evaluations (IWU, 1993). Indigenous people have discovered the therapeutic action of a range of wild plants, over centuries (HUTCHINGS *et al.*, 1996). The major role played by plants as sources of pharmacologically active substances is beyond dispute. Some have questioned the future need for plants as sources of these substances, but new and important plant-based drugs are still being introduced, with considerable economic significance (PRINCIPE, 1989). However, the use of such medicinal plants in their crude forms

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without establishing scientifically their efficacy and safety could, in the long run, be harmful to the health of mankind. Therefore, there is an urgent need to carry out scientific evaluations of these medicines because this could lead to new drug discoveries.

1.3 TRADITIONAL MEDICINE IN SOUTH AFRICA

Today, almost 80% of the world's population relies on plants as an essential part of their primary health care. An increasing worldwide interest in natural medicines has caused pharmaceutical companies from abroad to exploit African, and particularly South African medicinal plants, due to its great biodiversity (LOUW et al., 2002). Medicinal plants are an important part of South African culture. Studies undertaken by VAN WYK et al. (1997) suggest that over 30 000 species of higher plants may be found in southern Africa. With the remarkable plant and cultural diversity in South Africa, it is not surprising that approximately 3000 plant species are used as medicines. Of these, about 350 species are commonly used and traded medicinal plants (VAN WYK et al., 1997).

Traditional healing plays an integral part in black African culture and according to WHO's estimates, it provides for the primary health care needs of a large majority of the black population in South Africa (JÄGER et al., 1995). It is estimated that 27 million South Africans depend on traditional herbal medicines from as many as 1020 plant species (DAUSKARDT, 1990; MEYER et al., 1996; WILLIAMS, 1996; MANDER, 1997). The country's vast variety of indigenous species suggests a high potential for the discovery of novel bioactive chemicals (DUNCAN et al., 1999). In a country where access to medical doctors is limited, the value of traditional healing cannot be overestimated. Up to 60% of the South African population consult one of an estimated 200 000 traditional healers, in preference to, or in addition to, Western medical doctors, especially in the rural areas (VAN WYK et al., 1997). The South African Medical Association has estimated that in the cities there is one doctor for every 700 people, but in rural areas, one doctor for every 10 000 people (SUNDAY TIMES BUSINESS TIMES, 2001; McGAW, 2001). With the acute shortages of Western

medical doctors and health clinics in rural areas, people in these districts often have to rely solely on traditional healers for treatment.

South Africa has a huge diversity of tribes which is reflected in the systems of medicine practised (VAN WYK *et al.*, 1997). Traditional healers in South Africa are commonly known as "inyanga" and "sangoma" Zulu, "ixhwele" and "amagqirha" Xhosa, "nqaka" Sotho, "bossiedokter" and "kruiedokter" in the Western and Northern Cape (VAN WYK *et al.*, 1997). Practical knowledge regarding the healing powers of plants is passed on to next generations by word of mouth and experience.

There is a need for detailed documentation on the use of medicinal plants in this country. A few books have been published on South African medicinal plants (WATT and BREYER-BRANDWIJK, 1962; ROBERTS, 1990; HUTCHINGS *et al.*, 1996; VAN WYK *et al.*, 1997). There are also numerous publications on the healing values and bioactivity of these plants. The increasing numbers of publications appearing on work related to the screening for, and isolation of, bioactive compounds from plants used by traditional healers, and traditional medicine has provided an increasing solid scientific foundation for potential genotoxic effects (ELGORASHI *et al.*, 2002). There have been many validations of traditional remedies through scientific research (MEYER and AFOLAYAN, 1995; JÄGER *et al.*, 1996; AFOLAYAN and MEYER, 1997; DILIKA *et al.*, 1997; RABE and VAN STADEN, 1998; ELOFF, 1999; GRIERSON and AFOLAYAN, 1999; LIN *et al.*, 1999; LINDSEY *et al.*, 1999; KELMANSON *et al.*, 2000; McGAW *et al.*, 2000; SPARG *et al.*, 2000; STEENKAMP *et al.*, 2004; TSHIKALANGE *et al.*, 2004). The evaluation and recognition of traditional medicine in South Africa aims to improve its efficacy, safety, availability and wider application at low cost. During the last century, significant correlation was found between traditional remedies and scientific proof of their pharmacological action (THEUNIS *et al.*, 1992).

A major problem is the conservation of medicinal plant resources. Wild medicinal plant resources are increasingly under threat from habitat destruction caused by agricultural, forestry, industrial and urban/housing encroachment. Traditional and professional herb gatherers thus have an exaggerated impact on the

remaining wild stocks. In the past, plants were not subject to over-harvesting as traditional healers collected and stored their medicinal plants in accordance with traditions and taboos (VAN WYK *et al.*, 1997). Today, the urbanized healers purchase their plants from street markets and stores, providing an economic incentive for the destructive harvesting of vulnerable medicinal plants (VAN WYK *et al.*, 1997). The exponential population growth in South Africa has resulted in more people demanding medicinal plants. A study undertaken by SINDIGA (1995a) suggests that medicinal plants are under threat from population pressure, destruction by indiscriminate harvesting and foreign export. The author further suggests that traditional medicines require specific protection, enhancement and development, including the legal recognition of this medical system. VAN STADEN (1999) suggested that the only real solution would be to develop the valuable medicinal plants as crops through small-scale farming. The cultivation of medicinal plants, as suggested by CUNNINGHAM (1988) and MANDER *et al.* (1996), is one way in which to improve the intensive harvesting of wild medicinal plants, and could reduce the impact on biodiversity. The cultivation of important medicinal plant species by farmers, gatherers and traditional healers is an important strategy for the conservation of endangered species. This would provide a means of employment and income, and an alternative source of medicinal plants which are in high demand (CUNNINGHAM, 1991). The propagation of medicinal plants by traditional healers and small-scale farmers is receiving increasing attention and has the potential to alleviate some of the pressure on wild populations (JÄGER and VAN STADEN, 2000).

Drugs used by traditional healers are mostly prepared by some form of aqueous extraction, as they do not usually have access to other more lipophilic solvents (KELMANSON *et al.*, 2000). Alcohols, such as ethanol or methanol are also used as extraction solvents. These solvents are relatively inexpensive and freely available (FOUKARIDIS *et al.*, 1995). Methods of preparation commonly used in traditional medicine include boiling (root and bark), soaking in cold water (crushed leaves or small herbaceous plants), burning of dried leaves or herbs (use of ash), chewing and heating or roasting (succulent leaves for poultices).

The bark, leaves and roots are generally harvested from trees, whereas with herbs, the leaves are normally used to prepare medicine (VAN WYK and GERICKE 2000). In the case of bulbous, tuberaceous or rhizomous plants, the storage organs are normally regarded as the most valuable material (LIKHITWITAYAWUID et al., 1993; ZSCHOCKE et al., 2000). Herbalists use underground plant parts most frequently, believing that they contain the highest concentration of potent healing agents (SHALE et al., 1999). Leaves or flowers of bulbous plants are used less frequently in traditional practice (BANGANI et al., 1999; CROUCH et al., 1999; ZSCHOCKE et al., 2000; KELMANSON et al., 2000; LOUW et al., 2002).

Some medicinal plants or plant parts are only used fresh, while others may be stored in a dried state. The dried material is usually stored whole (or sliced) or in powder form. Storage containers include glass jars, brown paper bags, newspaper or tin cans (VAN WYK et al., 1997). Storage of plant material dry and in the dark favours the preservation of active compounds. However, recent investigations by STAFFORD et al. (2005) have showed some variation of activity in a species due to treatment after collection, i.e. storage and preparation. This study showed that there was chemical breakdown during storage in certain species.

1.4 VENEREAL DISEASES

Plant extracts have been used for centuries to treat infectious diseases. Venereal diseases are among those infectious diseases which have been treated for a long time by using plants and their extracts. As a result, venereal diseases are regarded as conditions that are highly responsive to traditional treatment. Venereal diseases, also known as sexually transmitted diseases (STDs), are infections that are usually acquired during sexual intercourse. WHO estimated that 340 million new cases of STDs have occurred throughout the world in 1999 (WHO, 2001), with at least, 111 million occurring in young people under 25 years of age. The largest number of new infections occurred in South and Southeast Asia, followed by sub-Saharan Africa and Latin America and The Caribbean (WHO, 2001). In the developing world, venereal diseases rank among the top five diseases for which adults seek

health care and are the second most important cause for the overall disease burden in young adult women (DALLABETTA, 1996). There are approximately 11 million cases of STDs that are treated annually in South Africa, with approximately five million of these managed by general practitioners (HIV/AIDS/STD STRATEGIC PLAN FOR SOUTH AFRICA, 2000). It is estimated that on average, about 17% of antenatal patients and between 49% and 90% of women attending family planning clinics have at least one STD (PHAM-KANTER et al., 1996). There is substantial evidence demonstrating that the presence of other STDs increase the chances of both acquiring and transmitting HIV (WASSERHEIT, 1992; FLEMING and WASSERHEIT, 1999) and they may be partly responsible for the growing HIV epidemic in Africa (O'FARRELL et al., 1991; GROSSKURTH et al., 1995). A number of studies undertaken (GROSSKURTH et al., 1995, GILSON et al., 1997, MAYAUD et al., 1997) have shown that a proper therapy for other STDs, be they ulcerative or non-ulcerative, is an important strategy for HIV control.

Five diseases that are usually known as venereal diseases include gonorrhoea, syphilis, chancroid, lymphogranuloma veneteum, and granuloma inguinale. In the 1960s up to 20 other diseases were recognised as being transmitted by sexual contact. Some of the more common of these are cystitis, vaginal infection, bed-wetting, groin, human lice, abscess and hernia. The most common or most reported venereal diseases are gonorrhoea and syphilis.

Venereal diseases affect men and women of all backgrounds and economic levels. They are most prevalent among teenagers and young adults. Nearly two-thirds of all STDs occur in people younger than 25 years of age (NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, 1999). Most STDs are treatable. However, even the once easily cured gonorrhoea has become resistant to many of the older traditional antibiotics. Other STDs, such as herpes, AIDS, and genital warts, all of which are caused by viruses, have no cure. Some of these infections are very uncomfortable, while others can be deadly. Syphilis, AIDS, genital warts, herpes, hepatitis, and even gonorrhoea have all been known to cause death.

Venereal diseases are caused by more than 25 infectious organisms (HEALTH INFORMATION FOR INTERNATIONAL TRAVEL, 2003-2004). The organisms that cause these diseases are very fragile and cannot survive outside the body. They thrive in warm, moist dark areas including the genital area, anus and mouth. These organisms include bacteria, fungi and viruses and they produce a variety of manifestations. Common pathogens include *Escherichia coli*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterobacter* species and *Candida albicans*. The symptoms include penile and vaginal discharge, vaginal itching, genital ulcers, painful urination, and pelvic and abdominal pain. *Staphylococcus aureus* has been reported to cause vaginal infections. The three most common types of vaginal infections are vulvovaginal candidiasis, trichomoniasis and bacterial vaginosis. These diseases can be classified on the basis of either their causes or their clinical manifestations. Although other microorganisms do not cause venereal diseases, those with compromised immune systems such as occurs with HIV+ patients, have been known to get skin infections caused by *Micrococcus luteus* and *Staphylococcus aureus*. Moreover, if venereal diseases are left untreated, they can lead to other diseases, e.g. conjunctivitis which is caused by *Bacillus subtilis*.

Table 1.1: Venereal diseases and the organisms responsible (NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, 1999)

ORGANISM	DISEASE
BACTERIA	
Neisseria gonorrhoeae	Gonorrhoea
Chlamydia trachomatis	Chlamydial infections, Lymphogranuloma venereum
Mycoplasma hominis	Postpartum fever, salpingitis
Treponema pallidum	Syphilis
Hemophilus ducreyi	Chancroid
Calymmatobacterium granulomatis	Donovanosis (granuloma inguinale)
Gardnerella vaginalis	Bacterial vaginosis
Shigella sp.	Shigellosis in homosexual men
Campylobacter sp.	Enteritis, proctocolitis
Group B Streptococcus	Neonatal sepsis, neonatal meningitis
VIRUSES	
Herpes simplex virus	Initial and recurrent genital herpes, aseptic meningitis, neonatal herpes, cervical dysplasia and carcinoma, carcinoma *in situ* of the vulva
Hepatitis B virus	Acute hepatitis B, chronic active hepatitis, persistent (unresolved) hepatitis, polyarteritis nodosa, chronic membranous glomerulonephritis, mixed cryoglobulinemia, polymyalgia rheumatica, hepatocellular carcinoma
Hepatitis A virus	Acute hepatitis A

Table 1.1 (Continued...)

ORGANISM	DISEASE
VIRUSES	
Cytomegalovirus	Heterophil-negative infectious mononucleosis, congenital infection, gross birth defects and infant mortality, cognitive impairment (e.g., mental retardation, sensorineural deafness), cervicitis, protean manifestations in the immunosuppressed host
Human papillomavirus	Genital (venereal) warts
Human immunodeficiency virus	Acquired Immune Deficiency Syndrome (AIDS)
Molluscum contagiosum virus	Genital molluscum contagiosum
PROTOZOA	
Trichomonas vaginalis	Trichomonal vaginitis
Entamoeba histolytica	Amebiasis in homosexual men
Giardia lamblia	Giardiasis in homosexual men
FUNGI	
Candida albicans	Vaginal yeast infections
ECTOPARASITES	
Phthirius pubis	Pubic lice
Sarcoptes scabiei	Scabies

1.5 SCREENING FOR ANTIMICROBIAL ACTIVITY

1.5.1 Antibacterial activity

Globally, plant extracts are used for their antibacterial, antifungal and antiviral activities (MEYER et al., 1996; VERASTEGUI et al., 1996). Some plant decoctions are of great value in the treatment of diarrhoea or gastrointestinal disorders, urinary tract infections, cervicitis, vaginitis, skin infections, infertility, wounds and cutaneous abscesses (CACERES et al., 1990; OLUKOYA et al., 1993; MEYER et al., 1996).

The discovery and application of penicillin and other antibiotics against infectious diseases promoted great interest in the discovery and development of chemotherapeutic agents in the twentieth century (LAURENCE and BENNETT, 1980), and has led to the development of a pharmaceutical industry in the second half of the last century that has done much to combat disease in man (ELOFF, 2000). Programmes for the screening of fungi, actinomycetes and bacteria for antibiotic production were conducted following the success of penicillin for the treatment of casualties during the Second World War. These screening programmes led to the successful isolation of streptomycin, tetracyclines, erythromycin and other antibiotics (LAURENCE and BENNETT, 1980). The term chemotherapy refers to the drug treatment of various infections (bacteria, viruses, protozoa, fungi, nematodes), in which the agent of infection is removed or destroyed without injuring the host or affecting host cells.

Due to the development of resistant bacterial strains, there is an increase in the number of papers published on antibacterial activity of plant extracts. Previous in vitro studies have revealed activities of several South African medicinal plants against a good number of bacteria and viruses pathogenic to man (MEYER et al., 1996; SALIE et al., 1996; RABE and VAN STADEN, 1997; ELOFF, 1999; LALL and MEYER, 2000; McGAW et al., 2000; ELOFF et al., 2001).

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1.5.2 Antifungal activity

Candida, which was discovered more than a century ago as a causative organism of oral thrush (PRASAD, 1991), can now be claimed to infect practically every tissue of the human body. The ever-increasing literature on various aspects of infections caused by *Candida* is, in itself, an indication of the concerted efforts of leading scientists in unravelling the mysteries of its pathogenicity and treatment (CUTLER and HAZEN, 1983; BODEY and FANSTEIN, 1985; SHEPHERD *et al.*, 1985; ODDS, 1988; PRASAD, 1991). Human mycosis, including infections of the skin and mucosal surfaces, constitute a serious problem in developing countries. KULBERG (1997) observed fungal infections as the primary cause of mortality in patients with severely impaired immune mechanisms. According to PORTILLO *et al.* (2001), SILVA *et al.* (2001), AFOLAYAN *et al.* (2002), there has been an increase in the number of reported cases of immuno-compromised patients which develop opportunistic and superficial mycoses, such as candidiasis and aspergillosis, in patients with AIDS in recent years. Despite several available antimycotic drugs, the treatment of immuno-compromised patients is still limited due to a number of factors such as low drug potency, poor solubility, emergence of resistant strains and drug toxicity (McCUTCHEN *et al.*, 1994; LI *et al.*, 1995; NWOSU and OKAFOR, 1995). The increase in AIDS-related fungal opportunistic pathogens and the emergence of resistant strains in recent years have lent additional urgency to antifungal studies (SILVA *et al.*, 2001; AFOLAYAN *et al.*, 2002; MOTSEI *et al.*, 2003).

1.6 TOXICITY OF TRADITIONAL MEDICINE

According to ERNST (1998) the amount of information about the relative safety of herbal remedies is limited, compared to synthetic drug treatments. The adverse effects of widely used plants are not well documented in the literature. There is a lack of use of standard/measured doses, and the large volumes of the doses used are difficult to manage (CAMPBELL, 1990). The plants used in traditional medicine are assumed to be safe, due to their long-term use by humans. However, several studies undertaken by HIGASHIMOTO *et al.* (1993); SCHIMMER *et al.* (1988, 1994); KASSIE

et al. (1996); ELGORASHI *et al.* (2002, 2003) have revealed that many plants used as food or in traditional medicine have mutagenic effects *in vitro*. This raises concern about the potential mutagenic hazards resulting from the long-term use of such plants.

Many widely used medicinal plants have been implicated as possible causes of long-term disease manifestations such as liver and kidney diseases (ADDAE-MENSAH, 1992). The use of herbal enemas is considered the primary cause of some of the poisonings as well as cases of liver damage recorded in hospitals (HUTCHINGS, 1989a).

A history of traditional use is not a reliable guarantee of safety. Delayed effects (e.g. mutagenicity), rare adverse effects, and adverse effects arising from long-term use may not be detected by traditional practitioners (ERNST, 1998). There is, therefore, a need to test for toxicity of medicinal plants used in traditional healing.

1.7 ISOLATION OF THE ACTIVE COMPOUND

Isolation of biologically active compounds is often limited because they may be present in very minute quantities. Furthermore, such substances may be thermally or hydrolytically unstable or have unfavourable solubility properties (KINGHORN, 1992). The isolation of an active constituent from a crude plant extract may seem somewhat discouraging in light of the fact that a plant may contain hundreds or even thousands of compounds with a variety of different biological properties (HOSTETTMANN, 1986). However, despite the difficulties which may be encountered, there are numerous separation techniques which can be employed to obtain pure compounds.

Various types of solid phase chromatography (thin layer chromatography, column chromatography, low, medium and high pressure chromatography) and liquid-liquid chromatography are among some of the systems which are today used in

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compound isolation. Each method has advantages and limitations, and usually several separation steps involving different techniques, when used in combination, make the isolation of pure compounds possible (HOSTETTMANN, 1986; HOSTETTMANN *et al.*, 1995). This is done in conjunction with bioassays, which is important in determining which fractions have the activity.

1.8 AIMS AND OBJECTIVES

The principal objectives of this study were:

- To screen medicinal plants used by South African traditional healers for the treatment of venereal diseases for the presence of antibacterial and antifungal activities.

- Many of the plants used in traditional medicine have solid scientific support with regard to their efficacy. A study done by ELGORASHI *et al.* (2003) raised questions about the safety of medicinal plants used in South African traditional medicine and their continued extensive use in primary health care in the rural areas of South Africa. Therefore, this study also aimed to investigate the potential harmful genotoxic effects of plants used.

- To attempt to isolate and identify compounds exhibiting antibacterial and antifungal activities.

CHAPTER 2

PLANT SELECTION, COLLECTION, IDENTIFICATION AND EXTRACTION

2.1 INTRODUCTION

The discovery of biological agents from natural sources has been an essential quest of mankind since prehistoric times because of their relative ease of collection, their potential for sustainable development and the structural and biological diversity of their constituents. A rational strategy for drug discovery is required, since it is currently not reasonable to investigate all available plants for even a single biological activity. It is generally recognized that there are five systematic approaches for the selection of plants that may contain new biological agents from plants: the ethnopharmacological, the chemotaxonomic, the random, the taxonomic, and the information-managed approach (CORDELL *et al.*, 1993).

Ethnopharmacology has been defined as the study of the use of local materials for medicinal purposes by ethnic groups, usually designated as those indigenous to a geographical area (HOUGHTON, 1999). Many bioactive constituents have been discovered through scientific investigations of traditional medicinal plants using the 'ethnopharmacological approach' (FARNSWORTH, 1984). The 'chemotaxonomical approach' relies upon correlations between plant taxonomy and the occurrence of specific chemical constituents (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). In the 'random selection approach', a variety of plants (and/or plant parts) are submitted to routine extraction and bioassay without preconceived selection on the basis of ethnobotanical knowledge or

chemotaxonomical data (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). In the taxonomic approach, plants of selected families or genera considered to be of interest are sought from diverse locations. If it is known that a certain class of chemical compound is more promising biologically for activity of interest to the scientist or pharmaceutical firm than are other classes, plants can be selected that are predicted to contain that type of compound using the principle of chemotaxonomy, e.g. indole alkaloids in the Apocynaceae, coumarins in the Rutaceae and sesquiterpene lactones in the Compositae (FARNSWORTH, 1994). In the information-managed approach, ethnopharmacological, biological and chemical information is collated and prioritised to afford a list of plants for specific collection (CORDELL et al., 1993).

The ethnobotanical approach to drug discovery has been successful on many occasions (COX, 1994). A study undertaken by KHAFAGI and DEWEDAR (2000) has shown that plant sampling based on the ethnobotanical approach produced a greater number of plants showing antimicrobial activity, compared to plants collected using the random method. However, the random selection approach may be valuable for identifying the presence of bioactive compounds from plants with unknown medicinal uses (KHAFAGI and DEWEDAR, 2000).

Following collection, plant material is dried and extracted with different kinds of solvents. Several organic solvents with varying elution effects find common usage in phytochemical research for extracting plant metabolites (GEORGE et al., 2001). The choice of the solvent depends on the type of chemical you are looking for. If the extraction aims at screening for antimicrobial activity, it is essential that the solvent by itself should not inhibit the bioassay procedure (ELOFF, 1998). The extractants petroleum ether, chloroform, ethanol, methanol and water (SALIE et al., 1996), 80% ethanol (VLIETINCK et al., 1995), methanol (TAYLOR et al., 1995), acetone and methanol-chloroform-water mixtures (ELOFF, 1998) have been used with varying success. Some solvents, like water, take longer and, if not properly evaporated, could result in microbial contamination (GEORGE et al., 2001).

Herbal medicines are prepared and administered in different ways. Usually, herbal medicines may be given in the form of infusions, crude liquid extracts or hot decoctions of the whole herb or its parts, such as flowers, seeds, leaves, roots, bark or gum (SINDIGA *et al.*, 1995). The whole plant or plant parts may be soaked in water overnight or pounded and boiled. The crude extract or decoction thus obtained may be given internally or applied externally as a poultice to affected body parts such as skin eruption, lesions or wounds.

Most of the medicinal herbs are poisonous if taken in large quantities. Therefore, the dosage should be prepared with great care and accuracy. Unless the chemical analysis is done, it may not be possible to estimate the quantity of the extract that can be used as the correct dose; any additional amount may cause deleterious symptoms or even death. However, traditional healers, with their long practical experience, are well versed in estimating dosage.

In this study, the ethnopharmacological approach was chosen because venereal diseases are readily recognized by traditional healers and are among the infectious diseases which have been treated for a long time by using plants and their extracts. Also, they are regarded as conditions that are highly responsive to traditional treatment. This approach is a proven method of plant selection for active biological compound screening and isolation, and, importantly, it is also a means of validating the work of South African traditional healers.

2.2 MATERIALS AND METHODS

2.2.1 Plant collection

Some plants were collected through consultations with a traditional healer and herbalist from the Eastern Cape and others were collected on the basis of a literature survey from the KwaZulu-Natal region of South Africa. The interviewed traditional healers work as full-time traditional doctors. One has a consultation room at the back of his house and the other one has an herbal shop with the consultation room in town. Voucher specimens for each plant were prepared and deposited in the herbarium of the University of KwaZulu-Natal Pietermaritzburg.

2.2.2 Plant preparation

The collected plant material was dried in an oven at a temperature of 50°C until dry, whereafter it was ground to fine powders using a blender. The material was then stored in sealed clear-plastic honey jars in the dark at room temperature until further processing.

2.2.3 Extraction

Three separate samples of 1 g each were extracted with 10 ml of distilled water, ethanol and ethyl acetate, respectively. Extraction was performed by sonication for 30 min in a Julabo ultrasound bath. The plant extracts were filtered through Whatman No. 1 filter paper into pill vials. The filtrates were taken to dryness in a stream of air and residues stored at 10°C.

2.3 RESULTS AND DISCUSSION

The plants investigated are listed in Table 2.1. Fresh or dried plant material may be used as a source for secondary plant components (ELOFF, 1998a). In this study, dried plant material was used to eliminate the possibility of water content of different plants influencing the amount of residue resulting from the extraction. Most scientists have tended to use dried material because there are fewer problems associated with the large scale extraction of dried rather than fresh plant material (ELOFF, 1998a). The time delay between collecting and processing the plant material makes it difficult to work with fresh material because differences in water content may affect solubility (ELOFF, 1998a). Also, the secondary metabolites should be relatively stable if they are to be used as antimicrobial agents, and most plants are used in the dried form (or as aqueous extract) by traditional healers (ELOFF, 1998a).

SILVA *et al*. (1998) recommended drying plant material at room temperature or in an oven away from direct sunlight, because ultraviolet radiation may produce chemical reactions giving rise to compound artefacts. The plant material should be well ventilated to avoid fungal infestation and elevated temperatures by fermentation (SILVA *et al*., 1998). Oven drying is generally poor because of the difficulty in regulating accurate temperature, and if done improperly, the oils evaporate and the plant material becomes scorched. But when appropriately controlled, artificial drying is possible, and this quick drying process will retain a great deal of value.

The dried plant material was ground to powders as grinding assists in the penetration of the solvent to the cellular structure of the plant tissues, thereby helping to dissolve the secondary metabolites and increase the yields of extraction (SILVA *et al*., 1998). Generally, it has been found that the smaller the particle size of the plant material the more efficient the extraction (SILVA *et al*., 1998).

The extracts were stored in a cold room at a temperature of 10°C to prevent decomposition of natural products before screening was undertaken. Incorporate

Table 2.1 South African medicinal plants used for the treatment of venereal diseases

Family and botanical name (voucher numbers[a]) and collection site	Vernacular names in Xhosa (X) and Zulu (Z)	Plant part used	Traditional uses and administration
Amaryllidaceae			
Cyrtanthus obliquus Ait. (BUWA 6 UKZN) Eastern Cape, Grahamstown	Umathunga (X/Z)	Bulbs	Decoctions taken for venereal diseases
Anacardiaceae			
Harpephyllum caffrum B. (BUWA 8 UKZN) Eastern Cape, Grahamstown	Umgwenya (X/Z)	Stem bark	Decoctions taken orally for gonorrhoea
Asclepiadaceae			
Xysmalobium undulatum R. Br. (BUWA 12 UKZN) Eastern Cape, Grahamstown	Ishongwe (Z)	Roots	Decoctions taken for syphilis
Capparidaceae			
Capparis tomentosa Lam. (BUWA 5 UKZN) Eastern Cape, Grahamstown	Intsihlo (X)	Roots	Infusion used as steam bath against lice
Gunneraceae			
Gunnera perpensa Linn. (BUWA 7 UKZN) KwaZulu-Natal, Pietermaritzburg	Iphuzi (X)	Roots	Decoctions used for gonorrhoea, syphilis and urinary infections

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Table 2.1 (continued...)

Family and botanical names (voucher numbers[a]) and collection site	Vernacular names in Xhosa (X) and Zulu (Z)	Plant part used	Traditional uses and administration
Hyacinthaceae			
Bowiea volubilis Harv. (BUWA 4 UKZN) KwaZulu-Natal, Pietermaritzburg	Umagaqana (X)	Bulbs	Poultice for the treatment of syphilis
Ledebouria ovatifolia (Bak.) Jessop (BUWA 11 UKZN) KwaZulu-Natal, Pietermaritzburg	Icubudwana (Z)	Bulbs	Decoctions taken orally for venereal diseases
Hypoxidaceae			
Hypoxis latifolia Hook. (BUWA 9 UKZN) Eastern Cape, Grahamstown	Ilabathela (X)	Roots and corm	Decoctions used as steam bath against lice and taken orally for urinary infections
Leguminosae			
Albizia gummifera (J.F. Gmel.) C.A. Smith (BUWA 1 UKZN) KwaZulu-Natal, Pietermaritzburg	Umgadankawu (Z)	Stem bark	Decoctions used for venereal diseases
Liliaceae			
Albuca nelsonii N.E. Br (BUWA 2 UKZN) Eastern Cape, Grahamstown	Inqwebeba (X/Z)	Bulbs	Decoctions used for gonorrhoea

Table 2.1 (continued…)

Family and botanical name (voucher number[a]) and collection site	Vernacular names in Xhosa (X) and Zulu (Z)	Plant part used	Traditional uses and administration
Melianthaceae			
Bersama lucens (Hochst.) Szyszyl. (BUWA 3 UKZN) KwaZulu-Natal, Pietermaritzburg	Isindiyandiya/ Undiyaza (X/Z)	Stem bark	Decoctions used against lice
Ranunculaceae			
Knowltonia bracteata Harv. Ex Zahlbr (BUWA 10 UKZN) Eastern Cape, Grahamstown	Umvuthuza (X)	Roots and Leaves	Decoctions used as steam bath and/ or taken orally against lice
Rutaceae			
Zanthoxylum capense (Thunb.) Harv. (BUWA 13 UKZN) Eastern Cape, Grahamstown	Umlungumabele (X)	Leaves	Infusions used for syphilis

[a]Voucher number: Herbarium of School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg.

CHAPTER 3

PHARMACOLOGICAL SCREENING

3.1 INTRODUCTION

Bacteria and fungi are responsible for a variety of diseases of plants and animals. The continuous evolution of bacteria resistant to currently available antibiotics has necessitated the search for novel and more effective antibacterial compounds. Efforts in this venture have focused on plants due to historical experience and the fact that a good portion of the world's population, particularly in developing countries rely on plants for the treatment of infectious and non-infectious diseases (ARNOLD and GULUMIAN, 1984; MARTINEZ *et al.*, 1996). Globally, plant extracts are employed for their antibacterial, antifungal and antiviral activities (MEYER *et al.*, 1996; VERASTEGUI *et al.*, 1996). Fungal diseases of man were recognised in some of the earliest medical literature.

3.1.1 Antibacterial activity

Bacteria are amongst the most abundant microorganisms on earth. They are both useful and harmful to humans. Infections caused by bacteria are widespread and often cause death. For this reason antibacterial drugs are among the most important therapeutic discoveries of the twentieth century (PAGE *et al.*, 1997). An increasing problem with antibiotics is the development of resistance by a variety of bacteria.

Bacteria are generally classified as either Gram-positive or Gram-negative. This classification is based on whether the organisms do or do not stain with Gram's stain. Gram-positive and Gram-negative bacteria are different in several respects. RANG and DALE (1987) suggested that these differences play an important role, with regard to the action of antibiotics.

The cell wall of Gram-positive bacteria is a relatively simple structure, which forms no appreciable barrier to the entry of antibiotics, whereas Gram-negative organisms have a more complex cell wall structure. From the plasma membrane outwards, the Gram-negative bacteria consists of: (1) a periplasmic space containing enzymes and other components; (2) a 2 nm peptidoglycan layer, which is often linked to outward projecting lipoprotein molecules; (3) an outer membrane consisting of a lipid bilayer with protein molecules and complex lipopolysaccharides on its outer surface. Many antibiotics are less active against Gram-negative than Gram-positive bacteria, probably as a result of the complex outer layer which is more difficult to penetrate (RANG and DALE, 1987).

Antibacterial agents can be generally divided into two groups, those which act primarily by stopping bacterial growth (bacteriostatic) and those which act by killing the bacteria (bactericidal). However, some drugs are bacteriostatic or bactericidal according to their concentration, and most bacteriostatic drugs can be shown to be bactericidal at high concentration. These differences do have relevance in relation to combined therapy (LAURENCE and BENNETT, 1980).

Antimicrobial activity of medicinal plants has been evaluated previously using various methods, which are classified into three groups, i.e. the disc-diffusion methods, dilution methods and bioautographic assays (RIOS et al., 1988). The choice of technique to be used will depend on the extract or compound to be tested, and it is important to understand the advantages and limitations of each method. Studies undertaken by JANSSEN et al., (1987) and THOMAS (1989) have shown that there

are many factors which can influence the results obtained and the great variation often observed in testing crude plant extracts in antibacterial assays. These include the assay technique, the culture medium, the strain of bacteria used for testing, the botanical source of the plant, the age of the plant, the state of the plant material used (fresh or dried) and the quantity of extract tested.

The disc-diffusion method enables several extracts to be screened against various microorganisms at the same time, and is therefore suitable for preliminary bioassay scanning (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). A filter paper disc, impregnated with extract, is placed on an inoculated medium. There are some variations of this method which use a hole in the agar medium, or a cylinder for the sample reservoir. After incubation the inhibition zone around the disc, hole, or cylinder is measured.

The dilution methods are used to determine more precisely the antibacterial activity of extracts, giving the Minimum Inhibitory Concentration (MIC) values of test extracts for a given microorganism (RASOANAIVO and RATSIMAMANGA-URVERG, 1993).

The use of bioautography is a useful procedure for testing antibacterial activity and it allows a rapid identification of bioactive constituents of plant extracts (RASOANAIVO and RATSIMAMANGA-URVERG, 1993) and is valuable in the bioassay-guided fractionation of active extracts. Developed thin layer chromatography (TLC) plates are allowed to dry before being sprayed with a mixture of the test bacteria and liquid nutrient medium (direct bioautography), or the plate may be covered with an agar medium containing the test bacteria (overlay assay). The plates are incubated in a humid atmosphere, resulting in inhibition zones where growth is prevented by the active components. These inhibition zones are usually visualized more clearly by the use of tetrazolium salts such as iodonitrotetrazolium chloride (INT) or methylthiazoyl-tetrazolium chloride (MTT) (BEGUE and KLINE,

1972; HOSTETTMANN *et al.*, 1995; GIBBONS and GRAY, 1998). It is often useful to include a known antibiotic in the assay as a positive control. This is useful for establishing the sensitivity of the test organism (RIOS *et al.*, 1988).

3.1.2 Antifungal activity

Fungi are responsible for a variety of diseases of plants and animals. Fungal diseases of man were recognized in some of the earliest medical literature. *Candida albicans* is a yeast-like microorganism with fungal characteristics (SALTARELLI, 1989). *Candida albicans* can live on many other living organisms without inducing problems; however, it may invade and cause infections and death in humans and animals (SALTARELLI, 1989). In humans it is mainly found in warm, dark, moist areas of the body. Antibiotics, steroid-hormones, birth control pills, excessive stress and too much sugar in the diet cause the yeast to proliferate. When *C. albicans* becomes active, it can affect every part of the body. It is estimated that one out of three people in the Western world have *Candida* infections. This disease affects women, men, boys, girls and even babies, showing mild symptoms or even chronic conditions (BURTON, 1989).

Candida was discovered more than a century ago as a causative organism of oral thrush (PRASAD, 1991). Clinically the fungal infection is identified as creamy-white, curd-like patches on the tongue or other oral mucousal surfaces which are removed by scrapings (DROUHET and DUPONT, 1989). Progress to more severe oesophageal candidiasis is associated with difficulty in swallowing and hence causes dehydration, non-compliance with medication regimes and general malnutrition (CLARK and HUFFORD, 1993), which is sometimes associated with severe diarrhoea (DROUHET and DUPONT, 1989; DUBE and MUTLOANE, 2001). Hence, those who suffer from oral candidiasis often lose a lot of weight due to a sore throat, which prevents them from eating (SANNE, 2001). The infection causes lesions in a

wide variety of morphological sites: mouth, bronchi, lungs, intestines, skin and vagina. It is mostly observed on the mouth and oesophagus. *Candida* and other infections deplete the immune system. Treatment of these infections in an early stage can therefore help reduce the deterioration in the health of an individual (SANNE, 2001).

There has been extensive research on the development of antifungal drugs, but only six of these antifungal agents were licensed in 1995. These include polyene amphotericin B, the azoles, imiazoles, miconazole and ketoconazole, triazole and itraconazole and the pyrimidine synthesis inhibitor flucytosine (5-FC) (ESPINEL-INGROFF and PFALLER, 1995). However, new broad-spectrum antifungal agents have entered the clinical stage (MASCHMEYER, 2002). Among them, voriconazole, caspofungin and intravenous itraconazole have been demonstrated to be at least as effective as, yet significantly better tolerated than, Amphotericin B (MASCHMEYER, 2002). It has been found that the treatment with these drugs, especially for extended periods of time, can lead to problems with toxicity to the patients (amphotericin B) or lead to the development of resistant fungal organisms during the course of therapy (5-fluorocystine) (BOONCHIRD and FLEGEL, 1982). Moreover, SANGLARD (2002) reported on resistance of fungal pathogens to antifungal drugs such as polyenes, pyrimidine analogues (5-fluorocytosine) and the azoles.

The increase in the STD-related fungal opportunistic pathogens and emergence of resistant strains in recent years has lent additional urgency to antifungal studies (SILVA *et al.*, 2001). The ongoing threat of synthetic agent resistance to yeast infections has led to researchers concentrating on naturally acquired agents. Many researchers in the world have investigated antifungal properties, involving the screening of plants against *C. albicans*.

3.1.3 Mutagenic and antimutagenic activities

Plants produce a variety of toxic substances, some of them in significant amounts, as a defence against microorganisms (bacteria, fungi, insects and viruses) and animals. The random use of medicinal plants, many of which are toxic, can have health risks because a dosage threshold exists for each medicinal plant as it does for synthetic pharmaceuticals (ALMEIDA, 1993). Toxic compounds in medicinal plants may favour mutational events in somatic or germ cells, such events possibly leading to the development of somatic diseases or teratogenic, mutagenic or carcinogenic effects (D'OLIVEIRA, 1998). Some substances produced by medicinal plants have been studied and characterized (SALVADORI *et al.*, 1991; SIMOES *et al.*, 2000; YOUNES *et al.*, 2000 and CARVALHO *et al.*, 2001) but insufficient toxicological and genotoxicological studies have been done, although there has recently been growing interest in the possible toxic, genotoxic and/or mutagenic effects of those plant metabolites which are used therapeutically.

Several plants and plant infusions rich in pyrrolizidine alkaloids have been reported as having genotoxic, mutagenic, carcinogenic and teratogenic effects (AMES, 1983), some of these plants being commonly used in human diets and herbal medicines. However, many natural or synthetic antimutagenic substances also occur and these can modulate the effects of toxic compounds by acting as antagonists to mutagenic agents (HAYATSU *et al.*, 1988).

3.2 MATERIALS AND METHODS

3.2.1 Antibacterial screening

The microorganisms that were used in this study were: *Bacillus subtilis* (ATCC No. 6051), *Escherichia coli* (ATCC No. 11775), *Klebsiella pneumoniae* (ATCC No.

13883), *Micrococcus luteus* (ATCC No. 4698) and *Staphylococcus aureus* (ATCC No. 12600), obtained from the American Type Culture Collection.

3.2.1.1 Disc-diffusion assay

The disc-diffusion assay (RASOANAIVO and RATSIMAMANGA-URVERG, 1993) was used in the antibacterial screening procedure. Residues of plant extracts were resuspended in their extracting solvents at a concentration of 100 mg/ml. MH agar (Biolab) base plates were prepared using sterile 90 mm Petri dishes. MH agar at 48°C was inoculated with MH broth culture of each bacterial species and poured over the base plates to form a homogeneous layer. Filter paper discs (Whatman No. 3, 6 mm diameter) were sterilized by autoclaving. One milligram of plant residue (10 µl of 100 mg/ml suspension) was applied to each filter paper disc and allowed to air-dry. The dry-discs were placed on the seeded MH agar plates. Each extract was tested in quadruplicate. Each plate also contained a reference antibiotic, neomycin (500 µg/ml), as a positive control. The plates were incubated at 37°C overnight, after which the zones of inhibition around each disc were measured. The ratio of the inhibition zone (mm) produced by the plant extract and the inhibition zone around the neomycin reference (mm) was used to express antibacterial activity (VLIETINCK *et al.*, 1995).

3.2.1.2 Microdilution assay

The microplate method of ELOFF (1998b) was used to determine the minimal inhibitory concentration (MIC) values for plant extracts with antibacterial activity. Residues of plant extracts were dissolved at 50 mg/ml with the extracting solvents in the case of water and ethanol. Ethyl acetate extracts were dissolved in ethanol. All extracts were initially tested at 12.5 mg/ml in 96-well microplates and serially diluted twofold to 0.098 mg/ml, after which 100 µl bacterial culture were added to each well.

The antibiotic neomycin was included as a standard in each assay. Extract-free solution was used as a blank control. The microplates were incubated overnight at 37°C. As an indicator of bacterial growth, 40 µl p-iodonitrotetrazolium violet (INT) dissolved in water were added to the wells and incubated at 37°C for 30 min. MIC values were recorded as the lowest concentration of the extract that completely inhibited bacterial growth, i.e. a clear well. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms (ELOFF, 1998b). Where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT.

3.2.2 Antifungal screening

A standard strain of *Candida albicans* (ATCC 10231) was obtained from the South African Bureau of Standards. The water extract residues were redissolved in water and the organic solvent extract residues were dissolved in dimethyl sulfoxide (DMSO). All extracts were dissolved to a concentration of 100 mg/ml.

A modification of the NCCLS proposed method (M27-P) broth microdilution test was performed (ESPINEL-INGROFF and PFALLER 1995; MOTSEI *et al.*, 2003). Four millilitres of sterile saline were added to approximately 400 µl of 24-h-old *Candida* cultures. The absorbance was read at 530 nm and adjusted with sterile saline to match that of a 0.5 McFarland standard solution. From the prepared stock yeast culture, a 1:1000 dilution with broth (e.g. 10 µl stock yeast culture: 10 ml broth) was prepared.

One hundred microlitres of broth were added to each well of a 96-well microplate. One hundred microlitres of the water extract were added to well (A) and serially diluted from (A) by taking 100 µl into (B). This two-fold dilution was continued down the plate and 100 µl from the last well (H) were discarded. In the case of

organic solvent extracts 25 µl of the extracts were added to 175 µl broth and serially diluted. Three replicates were prepared for each extract. All the wells were then filled with 100 µl of stock yeast culture. Amphotericin B was used as a standard for this experiment and the following controls were prepared: wells containing broth only, fungal strain with no extract, and serial dilutions of Amphotericin B with the fungi at the recommended inhibitory concentrations. The plates were then read at 630 nm in an ELISA reader, covered with parafilm and incubated at 33°C overnight, whereafter their absorbance was reread. An increase in absorbance indicated the loss of activity.

3.2.3 Mutagenicity and antimutagenicity screening

The extract residues were dissolved in ethanol to a concentration of 50 mg/ml and the following dilutions were prepared: 5.0, 0.5 and 0.05 mg/ml. The Ames assay was performed with *Salmonella typhimurium* strain TA98. The standard plate incorporation assay as described by MARON and AMES (1983) was used.

Two hundred microlitres of bacterial stock were incubated in 15 ml of Oxoid Nutrient broth for 16 h at 37°C on a rotative shaker. The bacterial cultures (100 µl) were added to 100 µl of the plant extract in 500 µl phosphate buffer and 2 ml of agar containing biotin-histidine (0.5 mM). The mixture was poured on a minimal agar plate and incubated at 37°C for 48 h. 4-Nitroquinoline-N-oxide (4-NQO) was used as a positive control at a concentration of 2 µg/ml, and water was used as negative control. After incubation the number of revertant colonies (mutants) was counted. Samples were tested in triplicate.

For the purpose of antimutagenicity testing, 100 µl of overnight cultures were added to 2 ml of top agar (containing traces of biotin and histidine) together with 50 µl of test solution (plant extract, solvent control, positive control), 50 µl mutagen and 0.5

ml phosphate buffer. The mixture was poured on a minimal agar plate and incubated at 37°C for 48 h. The percentage inhibition of the test solutions was calculated by using the following formula:

Inhibition (%) = [1-T/M] X 100

T = revertants per plate in presence of mutagen and plant extract;

M = revertants per plate in positive control (i.e. contains mutagen, no plant extract).

3.3 RESULTS AND DISCUSSION

3.3.1 Antibacterial activity

The results for the general screening for antibacterial activity are shown in Table 3.1. A total of 45 extracts belonging to 13 plant species were investigated.

In the disc-diffusion assay, out of the 13 tested plant species, only 7 showed activity. Extracts were considered highly active if their inhibition zone was between 0.70 and 1.00, minimum inhibitory activity with an inhibition zone between 0.30 and >0.70, and low or no inhibitory activity with an inhibition zone between 0.00 and >0.30. Plants exhibiting low or no antibacterial activity were *Albuca nelsonii*, *Bowiea volubilis*, *Capparis tomentosa*, *Cyrtanthus obliquus*, *Gunnera perpensa*, *Hypoxis latifolia*, *Knowltonia bracteata*, *Xysmalobium undulatum* and *Zanthoxylum capense*. Good antibacterial activity was detected against *S. aureus* with the ethanolic and ethyl acetate extracts of *Albizia gummifera*, *Bersama lucens* and *Ledebouria ovatifolia*. The *Harpephyllum caffrum* bark extract had high inhibitory activity. Water and ethanol extracts of *H. caffrum* were the most active against both Gram-positive and Gram-negative bacteria. The extracts inhibited most of the tested bacteria including *E. coli*, a Gram-negative bacterium. According to VLIETINCK *et al.* (1995), Gram-positive bacteria (*B. subtilis* and *S. aureus*) are significantly more susceptible to extracts than the Gram-negative bacteria (*E. coli* and *K. pneumoniae*). Due to the

outer membrane structure of Gram-negative bacteria, the effectiveness of a drug is somewhat challenging (PAGE *et al.*, 1997). This is often the case with plant extracts as well. Only three extracts showed activity against *E. coli*, while only one extract was active against *K. pneumoniae*. Concerning results against Gram-positve bacteria, 9 extracts were active against *B. subtilis*, 15 against *S. aureus* and only 7 against *M. luteus*.

MIC values of active extracts are shown in Table 3.1. Among the plants tested, *G. perpensa*, *H. caffrum*, *H. latifolia* and *L. ovatifolia* showed the best antibacterial activity. Poor inhibitory activity was detected against Gram-negative bacteria with most of the plant extracts tested. The aqueous and ethanolic extracts of *G. perpensa* had the highest inhibitory activity against both the Gram-negative bacteria. The MIC values in water extracts were high for both *E. coli* and *K. pneumoniae* (0.78 mg/ml), and one Gram-positive bacteria *S. aureus* (0.78 mg/ml).

H. caffrum plant extracts showed the best MIC values for the ethanolic extracts, where the extract showed activity against the four bacterial strains used. Good antibacterial activity was also detected with aqueous extracts. The MIC values in aqueous extracts were high, especially against *B. subtilis* and *S. aureus* (0.39 mg/ml). The ethyl acetate extract showed slight activity. *H. caffrum* is reported to contain phenolic compounds which may be responsible for its biological activity (EL SHERBEINY and EL ANSARI, 1976).

H. latifolia aqueous corm extracts exhibited very good MIC values against *K. pneumoniae* (0.78 mg/ml), *E. coli* and *S. aureus* (1.56 mg/ml). The ethanolic leaf extract, and water and ethyl acetate root extracts of *K. bracteata* had an MIC value of 1.56 mg/ml against both Gram-negative bacteria. The ethyl acetate extracts of *A. gummifera*, *C. obliquus* and *L. ovatifolia* also showed good antibacterial activity with MIC values of 1.56 mg/ml.

Table 3.1 Determination of the antibacterial activity of South African medicinal plants using the disc-diffusion and microdilution assays (MIC recorded in mg/ml)

Plant	Plant part extracted	[a]Extraction solvent	[b]Bacteria tested									
			B.s.		E.c.		K.p.		M.l.		S.a.	
			[c]DIF	[d]MIC	DIF	MIC	DIF	MIC	DIF	MIC	DIF	MIC
Albizia gummifera	Bark	W	0	>12.5	0	>12.5	0	>12.5	0	-	0	>12.5
		E	0.11	3.125	0	3.125	0	3.125	0	-	0.5	0.39
		EA	0.08	1.56	0	3.125	0	3.125	0	-	0.39	1.56
Albuca nelsonii	Bulb	W	0	>12.5	0	>12.5	0	>12.5	0	-	0	>12.5
		E	0	6.25	0	3.125	0	3.125	0	-	0	6.25
		EA	0	3.125	0	3.125	0	3.125	0	-	0	6.25
Bersama lucens	Bark	W	0	>12.5	0	>12.5	0	>12.5	0	-	0	>12.5
		E	0	3.125	0	3.125	0	3.125	0	-	0.64	3.125
		EA	0.06	-	0.19	-	0	-	0	-	0.59	-
Bowiea volubilis	Bulb	W	0	>12.5	0	>12.5	0	>12.5	0	-	0	>12.5
		E	0.08	3.125	0	3.125	0	3.125	0	-	0	3.125
		EA	0.14	-	0	-	0	-	0.17	-	0.22	-
Capparis tomentosa	Roots	W	0	>12.5	0	>12.5	0	6.25	0	-	0	>12.5
		E	0	3.125	0	3.125	0	3.125	0	-	0	6.25
		EA	0	6.25	0	6.25	0	3.125	0	-	0	12.5

Species	Part	Extract										
Cyrtanthus obliquus	Bulb	W	0	>12.5	0	12.5	0	>12.5	0	-	0	>12.5
		E	0	6.25	0	6.25	0	6.25	0	-	0	6.25
		EA	0	1.56	0	3.125	0	3.125	0	-	0	1.56
Gunnera perpensa	Roots	W	0	12.5	0	0.78	0	0.78	0	-	0	0.78
		E	0	3.125	0	1.56	0	1.56	0	-	0.28	6.25
		EA	0	3.125	0	6.25	0	6.25	0	-	0	3.125
Harpephyllum caffrum	Bark	W	0.13	0.39	0.75	1.56	0	1.56	0.44	-	0.75	0.39
		E	0.5	0.098	1	0.78	0	1.56	0.54	-	0.75	0.195
		EA	0	3.125	0.43	3.125	0	3.125	0.23	-	0.21	3.125
Hypoxis latifolia	Roots	W	0	>12.5	0	>12.5	0	>12.5	0	-	0	>12.5
			0	3.125	0.17	3.125	0.34	3.125	0.16	-	0.29	3.125
		E	0	3.125	0	6.25	0	6.25	0	-	0	6.25
	Tuber	EA	0	>12.5	0	1.56	0	0.78	0	-	0	1.56
		W	0	6.25	0	6.25	0	3.125	0	-	0	12.5
		E	0	-	0	-	0	-	0	-	0	-
		EA										
Knowltonia bracteata	Leaves	W	0	>12.5	0	3.125	0	3.125	0	-	0	3.125
		E	0	3.125	0	1.56	0	1.56	0	-	0	3.125
		EA	0	3.125	0	3.125	0	3.125	0	-	0	3.125
	Root	W	0	>12.5	0	1.56	0	1.56	0	-	0	1.56
		E	0	6.25	0	6.25	0	6.25	0	-	0	6.25
		EA	0	3.125	0	1.56	0	1.56	0	-	0	3.125

Species	Part	Solvent[a]	B.s. Dif[c]	B.s. MIC[d]	E.c. Dif	E.c. MIC	K.p. Dif	K.p. MIC	M.l. Dif	M.l. MIC	S.a. Dif	S.a. MIC
Ledebouria ovatifolia	Bulb	W	0	12.5	0	6.25	0	12.5	0	-	0	12.5
		E	0.11	0.78	0	3.125	0	3.125	0.13	-	0.28	1.56
		EA	0.11	0.39	0	1.56	0	1.56	0.20	-	0.32	0.78
Xysmalobium undulatum	Root	W	0	>12.5	0	12.5	0	12.5	0	-	0	12.5
		E	0	3.125	0	3.125	0	3.125	0	-	0	3.125
		EA	0	-	0	-	0	-	0	-	0	-
Zanthoxylum capense	Leaves	W	0	>12.5	0	12.5	0	12.5	0	-	0	>12.5
		E	0	6.25	0	3.125	0	3.125	0	-	0	6.25
		EA	0	-	0	-	0	-	0	-	0	-
Neomycin				9.8×10^{-2}		3.9×10^{-1}		3.9×10^{-1}		-		2.0×10^{-1}

[a]Extraction solvent: W = water; E = ethanol; EA = ethyl acetate

[b]Bacteria tested: *B.s.* = *Bacillus subtilis*; *E.c.* = *Escherischia coli*; *K.p.* = *Klebsiella pneumoniae*; *M.l.* = *Micrococcus luteus*; *S.a.* = *Staphylococcus aureus*.

[c]Dif: Results obtained in the disc-diffusion assays. Antibacterial activity is expressed as the ratio of the inhibition diameter around the extract to the inhibition zone around the reference neomycin antibiotic. 0- indicates no activity, i.e. no inhibition zone around the extract discs.

[d]MIC: Results obtained in the dilution assays. Antibacterial activity is expressed as the minimum inhibitory concentration (mg/ml). -, MIC not determined.

3.3.2 Antifungal activity

The results of the antifungal screening are presented in Table 3.2. MIC values were recorded as the lowest concentration of the extract that completely inhibited fungal growth. Among the plants tested, the ethanolic bark extracts of *B. lucens* and *H. caffrum* displayed the best activity against *Candida albicans* with an MIC value of 0.78 mg/ml. The aqueous bark extract of *H. caffrum* was the only aqueous extract that had the best activity amongst all the plants screened. It was the only one that showed promising results against *Candida* with an MIC value of 1.56 mg/ml. The candidal strain was found to be resistant to most plant extracts screened.

Most of the plant extracts tested showed some level of antibacterial activity. This supported the observations made by other investigators that plant pathogenic fungi are more resistant to plant extracts than plant pathogenic bacteria (LEVEN *et al.*, 1979, NAQVI *et al.*, 1991, HEISEY and GORHAM, 1992). HEISEY and GORHAM (1992) observed that 13 extracts inhibited the growth of bacteria, while only 5 extracts inhibited fungal growth.

It is worth noting that only in the case of *G. perpensa* and *H. caffrum* did aqueous extracts have activity. Drugs used by traditional healers are mostly prepared with water, as the healers do not usually have access to other more lipophilic solvents. This is of concern, as it is possible that healers do not extract all the active compounds that might be present in the plant. Dosage is important with regard to which solvent is being used. If water is used, the dosage would be higher, whereas the same dosage using a lipophilic solvent may be dangerous.

Table 3.2: Minimum Inhibitory Concentration of plant extracts against a standard strain of *Candida albicans* (ATCC 10231)

Plant	Plant part extracted	[a]Extraction solvent	[b]MIC (mg/ml)
Albizia gummifera	Bark	W	>25
			3.125
		E	>6.25
		EA	
Albuca nelsonii	Bulb	W	12.5
		E	6.25
		EA	6.25
Bersama lucens	Bark	W	12.5
		E	0.78
		EA	3.125
Bowiea volubilis	Bulb	W	>25
		E	-
		EA	3.125
Capparis tomentosa	Roots	W	>25
		E	6.25
		EA	3.125
Cyrtanthus obliquus	Bulb	W	>25
		E	6.25
		EA	6.25
Gunnera perpensa	Roots	W	25
		E	-
		EA	6.25
Harpephyllum caffrum	Bark	W	1.56
		E	0.78
		EA	3.125

Table 3.2 (Continued...)

Plant	Plant part extracted	[a]Extraction solvent	[b]MIC (mg/ml)
Hypoxis latifolia	Roots and corm	W	12.5
		E	6.25
		EA	6.25
Knowltonia bracteata	Roots	W	12.5
		E	-
		EA	-
Ledebouria ovatifolia	Bulb	W	25
		E	3.125
		EA	3.125
Xysmalobium undulatum	Roots	W	12.5
		E	-
		EA	-
Zanthoxylum capense	Leaves	W	-
		E	-
		EA	-
Amphotericin B			0.195

[a]Extraction solvent: W = water; E = ethanol; EA = ethyl acetate
[b]MIC: Results obtained in the dilution assays. Antibacterial activity is expressed as the minimum inhibitory concentration (mg/ml). -, MIC not determined.

3.3.3 Mutagenic and antimutagenic activities

Mutagenicity screening was used to evaluate the mutagenic potential of plant extracts by studying their effect on one or more histidine requiring strains of *Salmonella typhimurium*. The mutagenic response of different plant part extracts is shown in Table 3.3. Plant extracts were considered positive for mutagenic activity if the number of colonies was at least twice or more the revertant control number. All plant extracts showed a negative genotoxic response except for ethanol and ethyl acetate bulb extracts of *C. obliquus* which induced mutation in *Salmonella typhimurium* strain TA98. Amaryllidaceae species are an exclusive source of Amaryllidaceae alkaloids (VILADOMAT *et al.*, 1997). Phytochemical investigations within the genus *Cyrtanthus* led to the isolation of Amaryllidaceae alkaloids from *Cyrtanthus elatus* (HERRERA *et al.*, 2001), *Cyrtanthus obliquus* (BRINE *et al.*, 2002) and *Cyrtanthus falcatus* (ELGORASHI and VAN STADEN, 2003). Quercetic, furoquinoline alkaloids and isothiocyanates are considered to be among the possible mutagens of plant origin (SCHIMMER *et al.*, 1994; KASSIE *et al.*, 1996).

Antimutagenicity was used to evaluate the potential of plant extracts to inhibit mutation. Results of antimutagenic tests are shown in Table 3.4. Extracts were considered weak or negative if the percentage coefficient was <25%, moderate if it was between 25-40%, and strong if it was >40%. Plants exhibiting weak or negative results were *Albuca nelsonii*, *Albizia gummifera*, *B. lucens*, *C. tomentosa*, *C. obliquus*, *H. caffrum* and *K. bracteata*. Moderate antimutagenic activity was observed with the ethyl acetate extract of *G. perpensa* and the ethanolic extract of *H. latifolia*. No strong activity was detected with any of plant extracts tested, the percentage coefficient was <40%.

Table 3.3: Table showing summary of the results obtained with the bacterial Ames assay, where - = negative and + = genotoxic response

Plant	Plant part extracted	Extract tested	
		Ethanol	**Ethyl acetate**
Albizia gummifera	Bark	-	-
Albuca nelsonii.	Bulb	-	-
Bersama lucens	Bark	-	-
Capparis tomentosa	Roots	-	-
Cyrtanthus obliquus	Bulb	+	+
Harpephyllum caffrum	Bark	-	-
Hypoxis latifolia	Corm	-	-
Knowltonia bracteata	Leaves	-	-

Table 3.4: Results obtained with the antimutagenic assay for plant extracts used against venereal diseases

Plant	Plant part extracted	[a]Extraction solvent	Concentration tested (mg/ml)	Rep 1	Rep 2	Rep 3	Average	Coeff. (%)
Albizia gummifera	Bark	W	5.00	136	122	115	124	-15
			0.50	117	98	93	123	-14
			0.05	100	114	104	106	2
		E	5.00	128	130	125	128	-19
			0.50	151	138	101	130	-20
			0.05	135	88	101	108	0
		EA	5.00	105	124	106	112	-12
			0.50	99	92	93	95	5
			0.05	124	114	111	116	-16
Albuca nelsonii	Bulb	W	5.00	114	88	87	96	11
			0.50	106	90	99	98	9
			0.05	86	102	89	92	15
		E	5.00	156	144	168	156	-44
			0.50	124	142	134	133	-23
			0.05	141	138	154	144	-33
		EA	5.00	102	86	106	98	2
			0.50	117	93	87	99	1
			0.05	98	111	110	106	-6

Bersama lucens	Bark	W	5.00	162	188	164	171	-58
			0.50	171	132	159	154	-46
			0.05	142	134	117	131	-21
		E	5.00	NR	114	143	129	-19
			0.50	155	113	81	116	-7
			0.05	NR	104	112	108	0
		EA	5.00	146	140	142	145	-45
			0.50	102	132	128	121	-21
			0.05	152	109	155	139	-39
Capparis tomentosa	Roots	W	5.00	145	159	129	144	-33
			0.50	145	136	125	135	-25
			0.05	149	153	128	143	-32
		E	5.00	124	105	NR	115	-6
			0.50	NR	111	103	107	1
			0.05	NR	96	101	99	8
		EA	5.00	NR	145	122	134	6
			0.50	144	119	146	136	5
			0.05	136	140	190	155	-8

Cyrtanthus obliquus	Bulb	W	5.00	152	159	158	156	-44
			0.50	155	138	139	144	-33
			0.05	126	131	132	130	-20
		E	5.00	114	NR	107	111	-3
			0.50	NR	168	153	161	-49
			0.05	130	NR	148	139	-28
		EA	5.00	145	150	138	144	-1
			0.50	164	153	137	151	-6
			0.05	102	123	143	123	14
Gunnera perpensa	Rhizome	W	5.00	115	116	110	114	-6
			0.50	87	90	75	84	22
			0.05	85	87	93	88	19
		E	5.00	170	204	170	181	-27
			0.50	200	184	232	205	-43
			0.05	172	168	174	171	-20
		EA	5.00	97	112	132	114	20
			0.50	109	125	105	113	21
			0.05	114	106	96	105	27
Harpephyllum caffrum	Bark	W	5.00	100	107	89	99	8
			0.50	127	103	109	113	-5
			0.05	125	109	103	112	-3
		E	5.00	176	190	162	176	-23
			0.50	174	202	156	177	-24
			0.05	188	170	172	177	-24

		EA	5.00	201	194	187	194	-94
			0.50	168	153	167	163	-63
			0.05	122	126	126	125	-25
Hypoxis latifolia	Corm	W	5.00	125	123	108	119	-10
			0.50	112	124	119	118	-9
			0.05	101	115	133	116	-7
		E	5.00	124	99	98	107	25
			0.50	128	90	105	108	24
			0.05	106	115	82	101	29
		EA	5.00	157	163	142	154	-54
			0.50	125	149	130	135	-35
			0.05	115	116	101	111	-11
Knowltonia bracteata	Roots	W	5.00	145	153	190	163	-51
			0.50	141	122	NR	132	-22
			0.05	108	122	132	121	-12
			5.00	110	105	115	110	23
			0.50	105	107	113	108	24
		E	0.05	126	132	103	120	16
			5.00	135	146	129	137	-37
			0.50	117	131	131	126	-26
		EA	0.05	141	142	125	136	-36

[a]Extraction solvent: W = water; E = ethanol; EA = ethyl acetate. NR- indicates no results

3.4 CONCLUSIONS

Evaluation of plants used by traditional healers to treat venereal diseases is essential in South Africa, since venereal diseases are regarded as conditions that are highly responsive to traditional treatment and traditional healers are relied upon to provide efficient treatment. Scientific exploration of plants commonly used may lead to the isolation of novel compounds with different mechanisms of action to those presently in use. With the indisputable issue of rising drug-resistant pathogens, Western medicine is searching for new and effective antimicrobial substances to fight this worldwide threat.

The screening of crude extracts made from medicinal plants have shown that some of the screened plants are potentially rich sources of antibacterial and antifungal agents. Determining the antibacterial and antifungal properties of plants used in traditional medicine is helpful to the rural communities and informal settlements.

Although plant extracts have been used in the treatment of diseases according to knowledge accumulated over centuries, scientific research has shown some substances present in these medicinal plants to be potentially toxic or carcinogenic. However, information about the safety and efficacy of medicinal plants is difficult to find due to the lack of rigorous clinical studies and limited toxicological data available (MELO *et al.*, 2001). The discovery of mutagenic effects for ethanol and ethyl acetate extracts of *Cyrtanthus obliquus* revealed that plants used in traditional medicine should be administered with caution and after thorough toxicological investigations. Some attempts have been made to screen medicinal plants used in South African traditional medicine for genotoxic effects using *in vitro* assays (ELGORASHI *et al.*, 2003). The investigations revealed that many plants used as food or in traditional medicine have mutagenic effects (ELGORASHI *et al.*, 2003). Therefore, thorough

screening for potential harmful genotoxic effects of plants used in traditional medicine is recommended before long-term usage.

CHAPTER 4

SEASONAL AND GEOGRAPHICAL VARIATION IN MEDICINAL ACTIVITIES OF *HARPEPHYLLUM CAFFRUM*

4.1 INTRODUCTION

An important aspect of bioactivity of medicinal plants is that plants do not consistently produce the same chemicals in the same quantities. The effectiveness of medicinal plants is affected by a number of factors, such as the biochemical factors within the individual species and external factors such as climate, geographical location, season and other ecological and growth conditions (PRANCE, 1994). The variation of activity in a species which occurs due to its genetics, growth conditions, location, stage in its life cycle or the part of it which is being investigated is known as intraspecific variation (HOUGHTON, 1999). Consequently, bioassays of extracts made from other plant parts, or of plant parts collected at a different time or from a different specimen, may yield novel active compounds.

Variation of the activity can also occur due to treatment after collection, i.e. storage and preparation (COLEGATE and MOLYNEUX, 1993; HOUGHTON and RAMAN, 1998, STAFFORD *et al.*, 2005). Studies have shown that the therapeutic products from field-collected plants frequently differ from those obtained from the nursery. A compound from a chemical reactor inside a factory is identical batch by batch, but the amount of products in living plants vary within the same plant species due to the effects of sunlight, temperature, humidity, plant husbandry and packaging (SRIVASTAVA *et al.*, 1996). Time of harvest may affect composition of secondary

metabolites in different plant species. Variation in plant products can affect the medicinal activity of plants.

Thin Layer Chromatographic (TLC) fingerprinting is an efficient way of characterising analytical fingerprints of plant extracts (WAGNER and BLADT, 1996). This technique clearly illustrates differences in chemical composition of plant extracts. A chromatogram of a species may vary depending on the area the collection took place and time of year of harvesting. Plants adapt their ability to function adequately through various seasons and thus a variety of components may be produced by the plant during each season.

High antibacterial and antifungal activity detected with the bark of *H. caffrum* resulted in it being used as an indicator plant for the investigation on seasonal variation of its inhibitory activity.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Three collections of *H. caffrum* bark from the Eastern Cape were made in June 2003, December 2003 and September 2004 from the same plant. Other plant material was collected from different geographical locations in KwaZulu-Natal, i.e. Silverglen Nature Reserve (June 2001), National Botanic Garden Pietermaritzburg (June 2001), Botanic Garden of the University of KwaZulu-Natal Pietermaritzburg (December 2001) and a Muthi Shop in Pietermaritzburg (February 2002) were investigated.

The collected plant material was dried at 50°C, ground to a powder and stored in sealed plastic jars in the dark at room temperature until further processed. The ground plant material was extracted with water and ethanol and taken to dryness, as described in Section 2.2.3.

4.2.2 Testing for seasonal variation in antibacterial activity

H. caffrum bark collected from the Eastern Cape was tested for antibacterial activity using the microdilution bioassay as described in Section 3.2.1.2. The residues from the plant extracts were redissolved in water and ethanol at a concentration of 50 mg/ml. Each extract was tested against *B. subtilis*, *S. aureus* (Gram-positive) and *E. coli*, *K. pneumoniae* (Gram-negative).

4.2.3 Testing for seasonal variation in antifungal activity

Dried residues from the extracts were redissolved at a concentration of 100 mg/ml as described in Section 3.2.2. The microdilution bioassay was used to test for antifungal activity using *Candida albicans*.

4.2.4 TLC Fingerprinting

TLC fingerprints of the different extracts were prepared by loading 0.5 mg of each extract in a 1 cm band onto 10 X 10 cm plastic-backed TLC plates (silica gel 60 F_{254}, 0.25 mm, Merck). The plates were developed in petroleum ether: ethyl acetate: chloroform: formic acid (8:7:5:1), over a distance of 7.5 cm. Once the extract had been separated by TLC and the solvent evaporated, the separated components were

visualised under visible and ultraviolet light (UV_{254nm} and UV_{366nm}). The TLC plates were then stained with anisaldehyde spray reagent and heated at 110°C for 5-10 min, allowing for the colour development of various components previously not visible.

Data was recorded by scanning (anisaldehyde and bioautography) and taking photographs of different fingerprints of the various extracts under UV light of 366 nm (long wavelength exposing blue light) and 254 nm (short wavelength exposing green light). UV light detection is non-destructive (GIBBONS and GRAY, 1998) making it a favourable technique for compound detection. R_f values determine the locality of the various compounds. This value was calculated by determining the ratio of the distance travelled by the band maximum to the distance travelled by the leading edge of the mobile phase.

4.2.5 Bioautography

Ethanolic extracts made from plant material (0.125 mg) were spotted onto a large TLC plate (silica gel 60 F_{254}, 0.25 mm, 20 cm x 20 cm, Merck). The plate was developed in petroleum ether: ethyl acetate: chloroform: formic acid (8:7:5:1), over 17 cm. The solvent front was marked and the plate was allowed to dry before visualisation under UV_{254} and UV_{366}. For the bioautographic assay, S. aureus (ATCC 12600) was used as the test organism (MARTINI and ELOFF, 1998). An overnight culture was prepared in MH broth medium (2 x 20 ml). The cultures were centrifuged at 3000 g for 10 min and the supernatant medium discarded. The pellets of bacterial cells were combined and resuspended in 10 ml of fresh MH broth. This broth culture was then sprayed onto a TLC plate and incubated overnight at 37°C in 100% humidity. After incubation the plate was allowed to dry slightly before spraying with a 2 mg/ml solution of INT (Fluka). The plate was then re-incubated for a further 30-60 min to allow for colour development. Zones of inhibition appeared as white spots against a pink coloured background (BEGUE and KLINE, 1972).

4.3 RESULTS

Seasonal variation in antibacterial and antifungal activities of *H. caffrum* was investigated in order to determine the best time to collect medicinal plants to ensure high medicinal properties.

4.3.1 Seasonal variation in antibacterial activity

The results of seasonal variation in antibacterial activity are presented in Table 4.1. The ethanolic extracts displayed the highest activity against both Gram-negative and Gram-positive bacteria. The highest inhibitory activity was detected with the material collected during winter (June 2003) and summer (December 2003), with a decline in activity when collection was made during spring (September 2004).

Table 4.1: MIC values (mg/ml) of seasonal variation in antibacterial activity of *H. caffrum* bark

Collection time	[a]Extracting solvent	[b]Bacteria tested			
		Bs	Ec	Kp	Sa
2003 June	W	0.29	0.49	0.29	0.29
	E	0.195	0.098	0.098	0.15
2003 December	W	0.39	0.39	0.39	0.29
	E	0.195	0.098	0.098	0.098
2004 September	W	0.195	1.56	1.56	0.26
	E	0.098	1.56	1.56	0.098

[a]Extracting solvent: W= water; E= ethanol
[b]Bacteria: Bs= *Bacillus subtilis*; Ec= *Escherichia coli*; Kp= *Klebsiella pneumoniae*; Sa= *Staphylococcus aureus*

4.3.2 Seasonal variation in antifungal activity

H. caffrum bark extract exhibited seasonal variation in antifungal activity as shown in Table 4.2. The highest inhibitory activity was detected with plant material collected during the warmer summer month (December 2003), with a decline in activity when collections were made during the cooler months (June 2003/September 2004). Little or no variation was detected with ethanol extracts.

Table 4.2: MIC values (mg/ml) of seasonal variation in antifungal activity of H. caffrum bark

Collection time		[a]Extracting solvent	[b]MIC (mg/ml)
2003	June	W	2.8
		E	1.5
2003	Dec	W	0.9
		E	1.5
2004	September	W	2.6
		E	1.4

[a]Extracting solvent: W= water; E= ethanol
[b]MIC: Antifungal activity is expressed as the minimum inhibitory concentration (mg/ml)
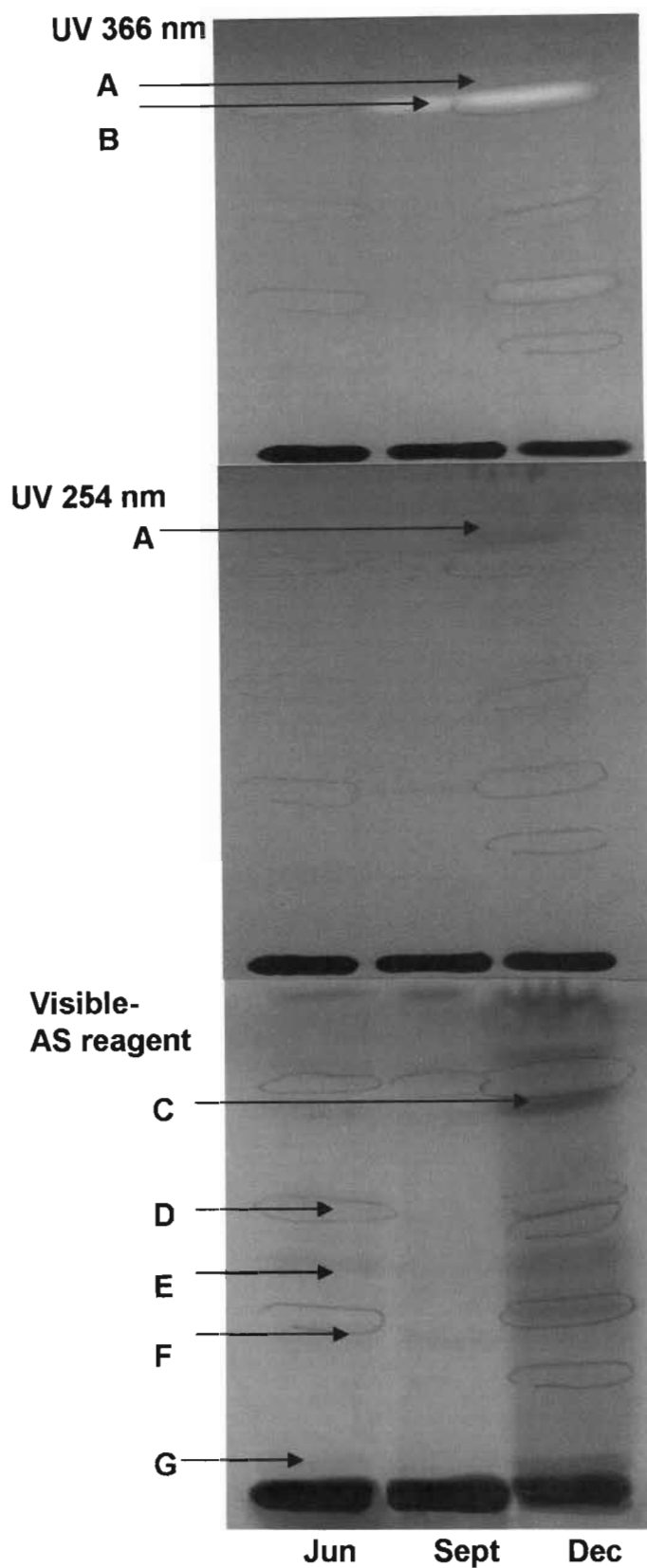
4.3.3 TLC Fingerprinting

The TLC fingerprints for extracts prepared from the *H. caffrum* bark collected at different geographical locations and seasons, and the corresponding bioautographic assays are shown in Figures 4.1-4.4. TLC analysis was used to compare the chemical composition of the crude ethanol extracts of *H. caffrum* where the R_f values and colours of the various components were compared. The same substances indicate the same R_f values and colours under the same experimental conditions. On viewing at UV 366 nm components were clear and distinct, while at UV 254 nm, many components were undetected.

TLC fingerprints of ethanol extracts prepared from the three collections of plant material from the Eastern Cape at different times of the year are shown in Figure 4.1. The ethanolic bark extract made from the plant material collected in cooler months (June 2003/September 2004) showed a loss of photosynthetic pigments compared with the ones collected in summer. Examples of this can be seen in bands A (R_f 0.86) and C (R_f 0.69), which are only present in the last lane (December 2003). The white fluorescing band B (R_f 0.83) appeared brighter in the bark extract collected in spring and summer as compared to the plant material collected in winter.

The bioautography showed a number of clear zones against a pink background (Figure 4.2). Inhibition zones were visible in all the tested extracts. Extract from the material harvested in December 2003 gave the most inhibition zones: band I (R_f 0.75) which corresponds with band C on the reference plate (visible AS reagent), band II (R_f 0.43) which corresponds with band E, band III which corresponds with band F on the reference plate, and a large clear zone IV.

Figure 4.1 TLC fingerprints of *H. caffrum* bark collected in Jun= June, Sept= September and Dec= December. TLC plates developed in petroleum ether: ethyl acetate: chloroform: formic acid (8:7:5:1).
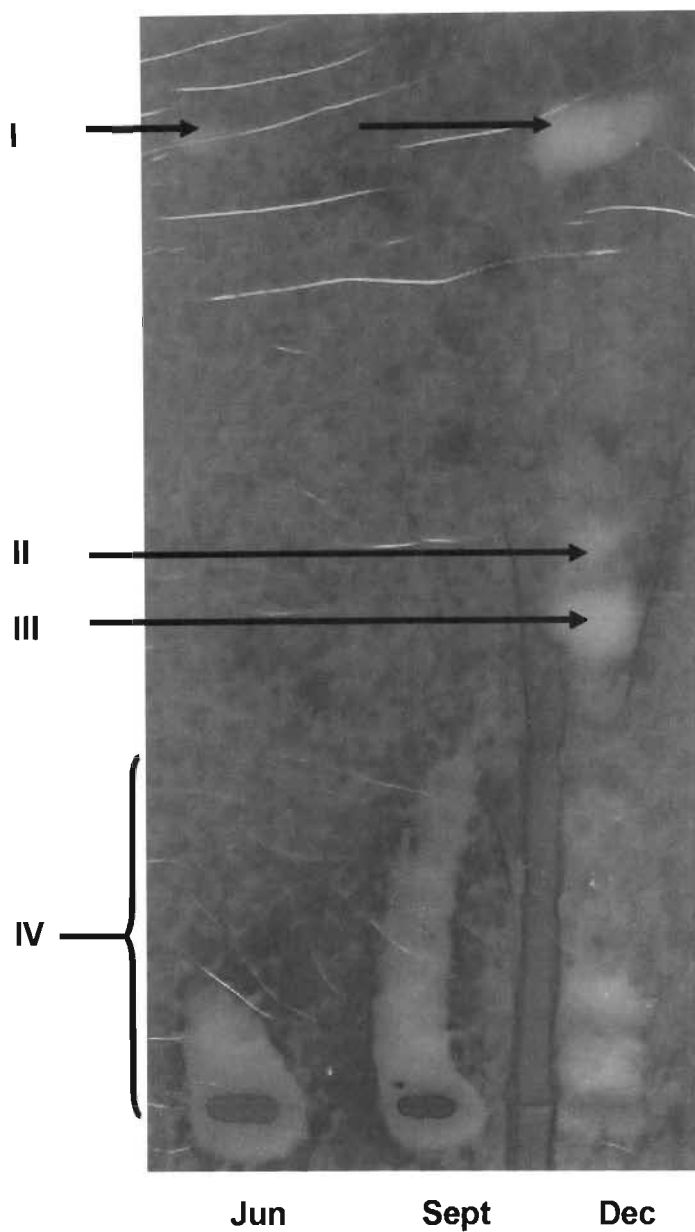
Figure 4.2 Bioautographic assay of extracts obtained from the bark of *H. caffrum* collected from the Eastern Cape in Jun= June, Sept= September and Dec= December. TLC plate developed in petroleum ether: ethyl acetate: chloroform: formic acid (8:7:5:1). Arrows indicate white areas representing zones of bacterial inhibition.

*4.3.3.1 Seasonal and geographical TLC fingerprints*

TLC fingerprints of ethanol extracts made from bark collected from the Eastern Cape (June 2003), Silverglen Nature Reserve (June 2001), National Botanic Garden Pietermaritzburg (June 2001), Botanic Garden of the University of KwaZulu-Natal Pietermaritzburg (December 2001), and a Muthi Shop Pietermaritzburg (February 2002) were investigated.

Collection of plant material from different locations and different seasons showed differences in the chemical composition of plant extracts (Figure 4.3). Loss of photosynthetic pigments A (R_f 0.86), C (R_f 0.81) and D (R_f 0.72) was observed with the bark collected from the Eastern Cape and National Botanic Garden Pietermaritzburg. The white fluorescing band B (R_f 0.83) appeared brighter in the material collected from the Botanic Garden of the University of KwaZulu-Natal and from the Muthi Shop but was fading in the extracts made from material collected from the Eastern Cape, Silverglen and National Botanic Garden Pietermaritzburg. The plant material obtained from the Botanic Garden of the University of KwaZulu-Natal and Muthi Shop appear to produce the same chemicals, perhaps due to the plant material being collected in summer. Bands E and F were seen in all the extracts although the fluorescence was more intense in the material collected in the Botanic Garden of the University of KwaZulu-Natal. All the extracts had band G (R_f 0.08) although it was more faded in the material from the Eastern Cape, Botanic Garden Pietermaritzburg and the Muthi Shop.

Duplicates of the anisaldehyde stained plate were prepared for bioautography with *S. aureus*. The inhibition of *S. aureus* on this plate was clearly seen by the contrast of white zones corresponding to separated bands of the extracts against a red background of the plate (Figure 4.4). Clear zones were visible in the material obtained from the Eastern Cape, Botanic Garden of the University of KwaZulu-Natal and a Muthi Shop: band I which corresponds with band D on the reference plate

(visible AS reagent). A clear zone was also visible at the origin of the TLC chromatogram.

Figure 4.3 TLC fingerprints of *H. caffrum* bark collected from EC= Eastern Cape, SIL= Silverglen Nature Reserve, NBGP= National Botanic Garden of Pietermaritzburg, UKZNP= Botanic Garden of the University of KwaZulu-Natal and MS= Muthi Shop. TLC plates were developed in petroleum ether: ethyl acetate: chloroform: formic acid (8:7:5:1). A-G shows differences in the chemical profile of plant extracts after developing TLC plates.
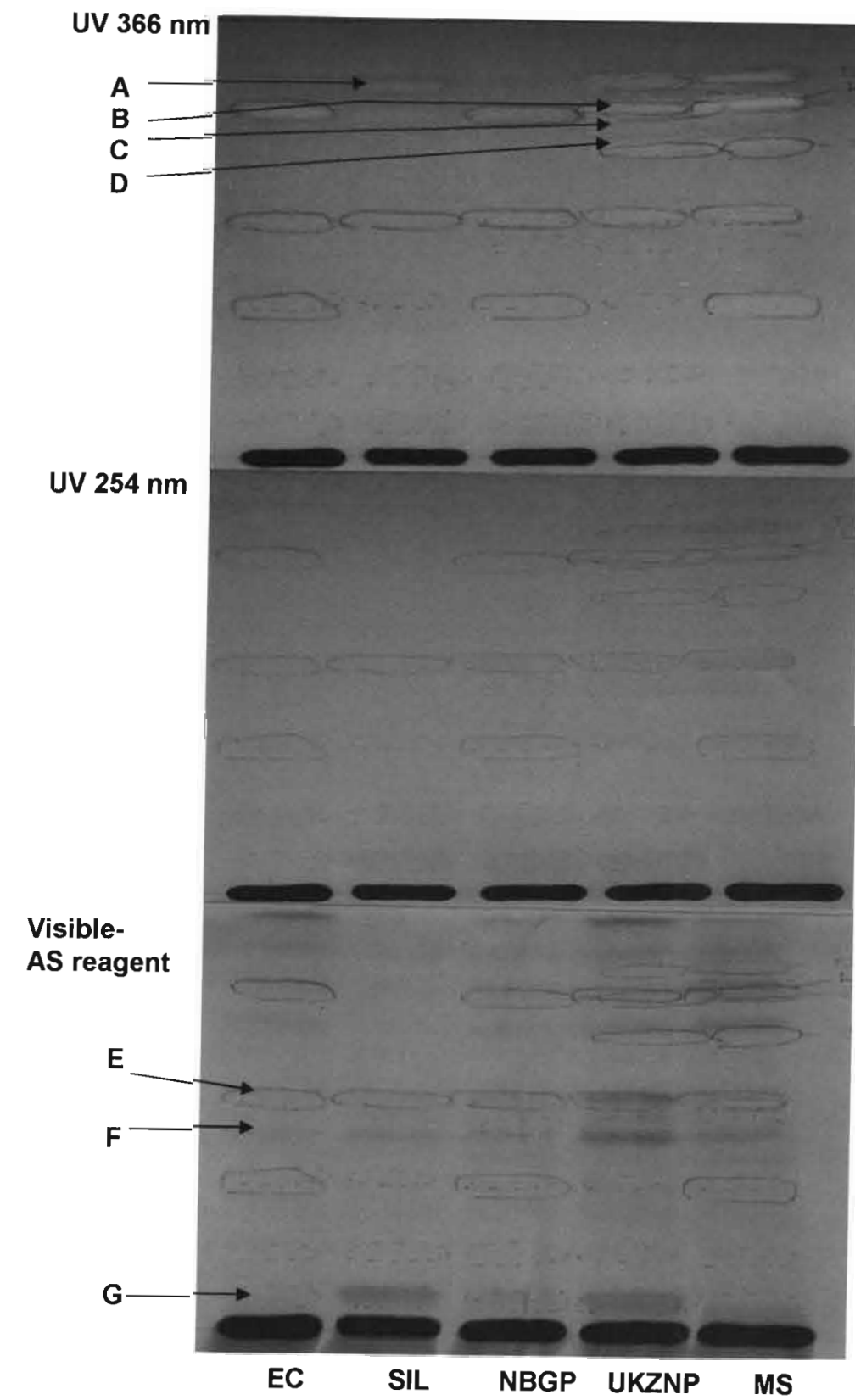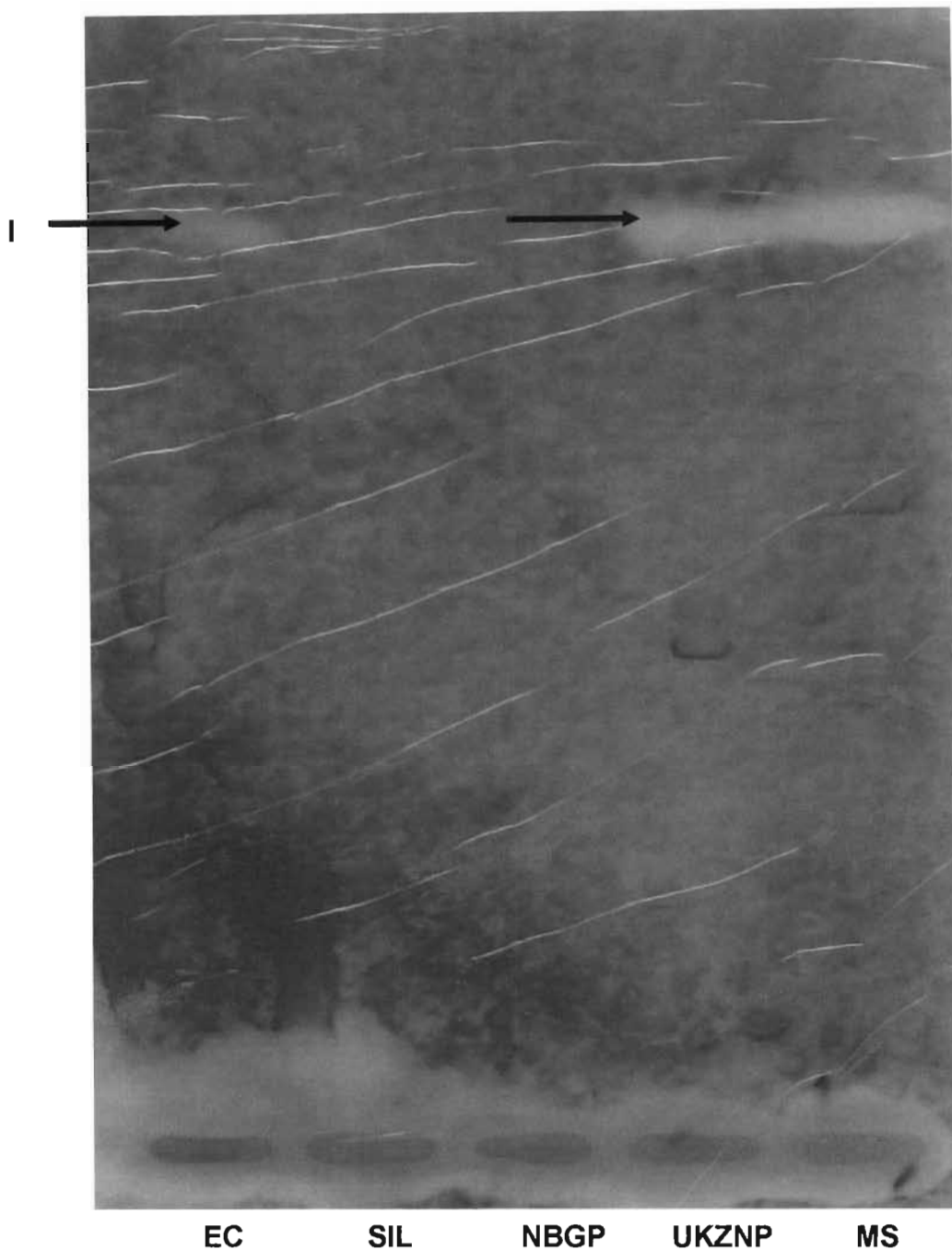
Figure 4.4 Bioautographic bioassay of bark extracts of *H. caffrum* collected from EC= Eastern Cape, SIL= Silverglen, NBGP= Botanic Garden Pietermaritzburg, UKZNP= Botanic Garden of the University of KwaZulu-Natal and MS= Muthi Shop. TLC plates developed in petroleum ether: ethyl acetate: chloroform: formic acid (8:7:5:1).

4.4 DISCUSSION AND CONCLUSIONS

Depending on the plant, the ailment and disease it is used for, the time of harvest and the state of the material for extraction may only have minimal influence on the degree of activity (LIGHT *et al.*, 2002). The results from this work suggest that *H. caffrum* can be collected during the warmer months since the highest inhibitory activity was detected with plant material collected during December 2003. In June/December 2003, antibacterial activity increased against Gram-negative bacteria whereas it declined against Gram-positive bacteria. Likewise, an increase was observed with antifungal activity in summer. Furthermore, an increase in activity was observed with plant material collected in September 2003, against Gram-positive bacteria whereas it declined against Gram-negative bacteria. The bufadienolide concentration has been found to fluctuate substantially during the year in *Tylecodon wallichii* (BOTHA *et al.*, 2001). It tended to be higher in the winter and increased again in spring and early summer (BOTHA *et al.*, 2001).

Earlier studies have shown that plants display variation in the concentration of their bioactive phytochemicals depending on intrinsic factors like the age of the plant, its parts used and extrinsic factors like the geographical climate, the nature of the soil, season and processing. The phytochemicals in the essential oil of *Salvia libanotica* have been reported to change quantitatively with season (FARHAT *et al.*, 2001). The oil extracted from plants collected in winter contained higher levels of camphor, α, β-thujone and camphene than the summer, autumn and spring extracts. The winter extract was the most toxic and exhibited powerful convulsant properties, because it had higher levels of camphor, α-thujone, β-thujone and camphene, while the spring extract was the least toxic due to decreased contents of these chemicals (FARHAT *et al.*, 2001). The flavonoid and phenolic contents in spinach have also been reported to vary with season (HOWARD et al., 2002). Seasonal variation in the quantity of the phytochemicals in the plants with anticancer activity like *Crinum macowanii*, *Taxus baccata*, *Taxus wallichiana* and *Taxus brevifolia*, have also been reported (WHEELER *et al.*, 1992; VANCE *et al.*, 1994; GLOWNIAK *et al.*, 1999; MUKHERJEE

et al., 2002). The testing of plants to determine the loss of biological activity over time will allow traditional healers and consumers to make informed decisions with regard to the collection and use of plant material.

TLC provides information on the major active constituents of a drug, thus enabling an assessment of drug quality, in addition to its qualitative detection (WAGNER *et al.*, 1984). It is suitable for monitoring the identity and purity of drugs against adulteration and substitution, as it provides a chromatographic drug fingerprint, and can also be used to analyse drug combinations and phytochemical preparations (WAGNER *et al.*, 1984).

The TLC analysis revealed some variation in chemical composition of each of the *H. caffrum* extracts tested. TLC chromatograms of different samples of the same species from varying geographical locations showed a high level of consistency, for *Combretum* species (CARR and ROGERS, 1987) and *Maytenus* species (ROGERS *et al.*, 2000). The TLC fingerprints of *H. caffrum* bark collected from the Botanic Garden of the University of KwaZulu-Natal Pietermaritzburg and a Muthi Shop were very similar. ZSCHOCKE *et al.* (2000) successfully used TLC analysis to compare the chemical composition of various plant parts of four threatened South African medicinal plants.

CHAPTER 5

ISOLATION OF ANTIBACTERIAL COMPOUNDS FROM

GUNNERA PERPENSA

5.1 INTRODUCTION

Multiple drug resistance has become a very real issue in pharmacotherapeutics as there is an increasing number of diseases which are exhibiting various levels of drug resistance, including bacterial infections (HENRY, 2000). The search for new drugs to combat this problem is receiving much attention (COATES *et al.*, 2002). Plants used in traditional medicine have the potential to provide pharmacologically active natural products which can be used to treat various ailments.

The family Gunneraceae comprises a single genus found growing in the southern hemisphere from tropical regions to Antarctica (MABBERLY, 1987). A few species are grown as ornamentals and are therefore known to gardeners and horticulturists in the northern hemisphere. The peeled young leaf stalks of some species are used as food. *Gunnera perpensa*, also known as river pumpkin, "uGhobo" in Zulu or "iPhuzi" in Xhosa, is a vigorous perennial herb which grows in marshy areas and along stream banks in many parts of South Africa. Large round leaves on long, thick stalks arise from the thick tuberous rhizomes below the ground. They superficially resemble pumpkin leaves, hence the common name (Figure 5.1). Traditionally the finely ground roots of the plant are boiled in water and administered orally to an animal (cow) after calving in order to expel the retained placenta. Human uses of the aqueous decoction are relief of pain in rheumatic fever, to ensure an easy

childbirth, and to treat infertility in women (HUTCHINGS, 1996). In preliminary tests, crude decoctions of the rhizome showed definite uterotonic activity (KAIDO *et al.*, 1997).

Figure 5.1   *Gunnera perpensa* Linn.

The aim of this part of the study was to try to isolate and identify the active antibacterial compound(s) in *G. perpensa* which was found to have antibacterial activity against *E. coli*, *K. pneumoniae* and *S. aureus* in the present screening (Chapter 3).

5.2 MATERIALS AND METHODS

5.2.1 Bulk extraction of *G. perpensa*

Dried, ground plant material (300 g) obtained from the botanic garden of the University of KwaZulu-Natal Pietermaritzburg (altitude 762 m;29°37'S:30°24'E) was

extracted with 3 X 2 L of ethanol by sonicating for 2 h and leaving in a shaker overnight. The extract was filtered through Whatman No. 1 filter paper and dried in front of a fan. A dark brown oily extract was obtained (34.12 g dry weight).

5.2.2 Bioassay-guided fractionation

The isolation of an active compound from *G. perpensa* was conducted according to the outline shown in Figure 5.2. The antibacterial activity of the fraction was tested after each purification step using the bioautographic bioassay with *S. aureus* as described in Section 4.2.5.

5.2.2.1 Vacuum liquid chromatography

Dry silica gel 60 (0.040-0.063 mm, Merck) was placed in a large vacuum liquid chromatography (VLC) column (5 X 27 cm). The extract ($\approx$34 g) was adsorbed onto a small amount of the silica gel and allowed to dry before being placed as a top layer on the silica gel in the column. A dichloromethane: ethanol gradient (dichloromethane proportions: 100%; 95%; 90%; 85%; 80%; 75%; 70%; 65%; 60%; 55%; 50%; 0%) was used and the solvent mixtures (400 ml) were eluted on the column under vacuum. The fractions were collected and dried in front of a fan.

Figure 5.2 Flow diagram outlining the procedure followed in the isolation of antibacterial compounds from a *G. perpensa* rhizome ethanolic extract. The crossed-out fractions mean there was no bacterial inhibition against *S. aureus* in bioautography.
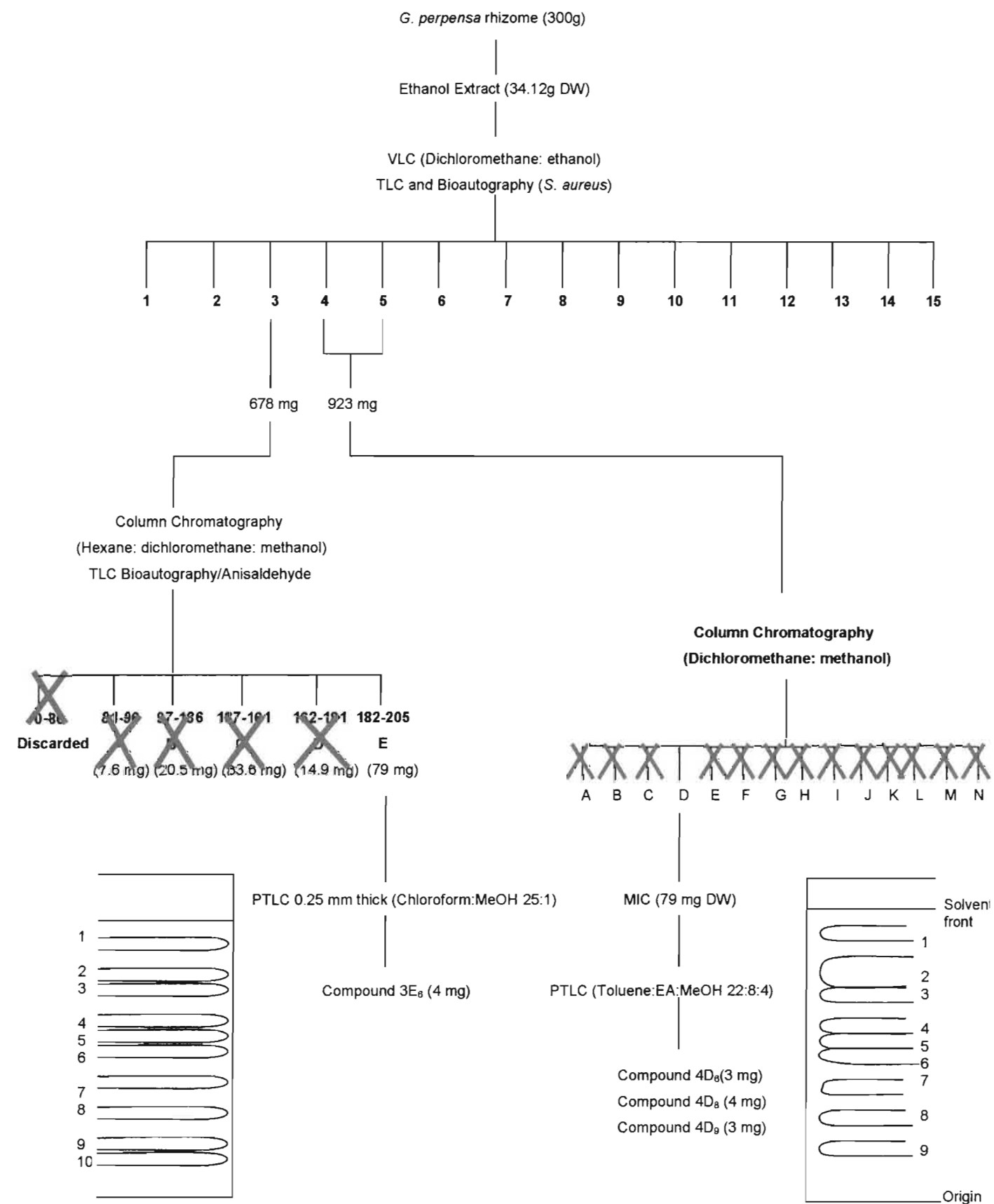
5.2.2.2 Evaluation of VLC fractions

Fractions 1-15 (0.125 mg) were spotted onto 2 TLC plates (silica gel 60 F_{254}, 0.25 mm, 20 cm x 20 cm, Merck). The plates were developed simultaneously in dichloromethane: methanol (15:1), over 17 cm. The solvent front was marked and the plates were allowed to dry before visualisation under UV_{254} and UV_{366}.

For the bioautography assay, *S. aureus* (ATCC 12600) was used as a test organism as described in Section 4.2.5, Chapter 4.

5.2.2.3 Column chromatography

From the results obtained with the bioautographic bioassay of the VLC fractions, fraction 3 and fraction 4-5 (which were combined to produce fraction 4) were selected for further purification using column chromatography.

Silica gel (200 g, particle size 0.063-0.200 mm, Mesh ASTM) was suspended in minimal volume of hexane, stirred thoroughly to remove air bubbles and quickly poured into the column (3.5 cm internal diameter). The extract was redissolved in 5 ml hexane and slowly added to the column. The solvent was allowed to run out of the column until the extract was almost at the surface of the silica gel. Five ml of the solvent was gradually added and allowed to run through the column until the solvent above the silica was clear. The column was carefully filled up with the eluent by placing a filled reservoir at the top of the column. The extract formed bands along the column and fractions were collected at 5 min intervals. These fractions were spotted onto a TLC plate and developed in dichloromethane: methanol (15:1) as described in Section 5.2.2.2. Fractions that showed similar spots were combined, dried in front of a fan and tested in the bioautography assay.

5.2.2.4 Preparative TLC

From the results obtained with column chromatography fractions, fraction 3E and fraction 4D were selected for further purification and compound isolation using preparative TLC.

Fractions 3E and 4D were applied onto TLC plates (silica gel 60 F_{254}, 0.25 mm, 20 cm x 20 cm, Merck) with approximately 25 mg of the fraction applied in a long band onto each plate. A number of plates were developed simultaneously in a solvent system, which gave better separation. The solvent front was marked and the plates air dried before visualisation under UV_{254} and UV_{366}. A Bioautography assay was performed to test for antibacterial activity, and the active fractions were scraped off the TLC plates and eluted from the silica with methanol. The extracts were filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica.

5.2.2.5 MIC determination

The MIC values of the isolated compounds were determined using the microdilution assay as described in Section 3.2.1.2, Chapter 3. The compounds were dissolved in 25% ethanol at a concentration 4 mg/ml (giving a starting concentration of 1 mg/ml). The isolates were sent for Nuclear Magnetic Resonance Spectroscopy analysis to the Institute of Organic Chemistry and Biochemistry, Czech Republic.

5.3 RESULTS

5.3.1 Bulk extraction

The bulk extraction of the rhizomes of *G. perpensa* with ethanol yielded a brown and oily residue of 34.12 g.

5.3.2 Bioassay-guided fractionation for isolation of active compounds

5.3.2.1 Vacuum liquid chromatography

The TLC separation of the VLC fractions and the corresponding bioautographic assay can be seen in Figure 5.3. The chromatogram which was stained with anisaldehyde reagent gave a clear indication that the VLC was a successful method of initial separation of the bulk extract.

Extracts 1-6 showed little overlap of similar compounds, whereas fraction 7-15 were quite similar in nature, this is also evident in the bioautography. In bioautography, a number of clear zones were evident against a pink background. Fraction 3 and fraction 4, which had large zones, were chosen for further separation using gravity column chromatography.

5.3.2.2 Gravity-assisted Column Chromatography

A total of 205 (for the first column) and 200 (second column) fractions were collected during the running of the gravity column, and grouped according to their

TLC patterns. The fractions were assayed for antibacterial activity and fractions 3E and 4D showed large clear zones (Figure 5.4, Figures 5.6 and 5.7).

5.3.2.3 Preparative TLC

The results of preparative TLC are presented in Figures 5.5 and 5.8. Following preparative TLC on fraction 3E, the mass of the isolated compound $3E_6$ (identified as a navy-blue band under UV_{366}) was 4 mg. Preparative TLC on the residue of fraction 4D resulted in isolation of antibacterial compounds $4D_6$, $4D_8$ and $4D_9$ with a mass of 3 mg, 4 mg and 3 mg respectively.

5.3.2.4 MIC determination

The MIC (mg/ml) results obtained for the isolated compounds are presented in Table 5.1. The isolated compounds exhibited good antibacterial activity against all the tested bacteria when compared with the results obtained for the crude extract. NMR analysis results of the isolated compounds showed that the compounds were not clean and were not sufficient for further purification and identification.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
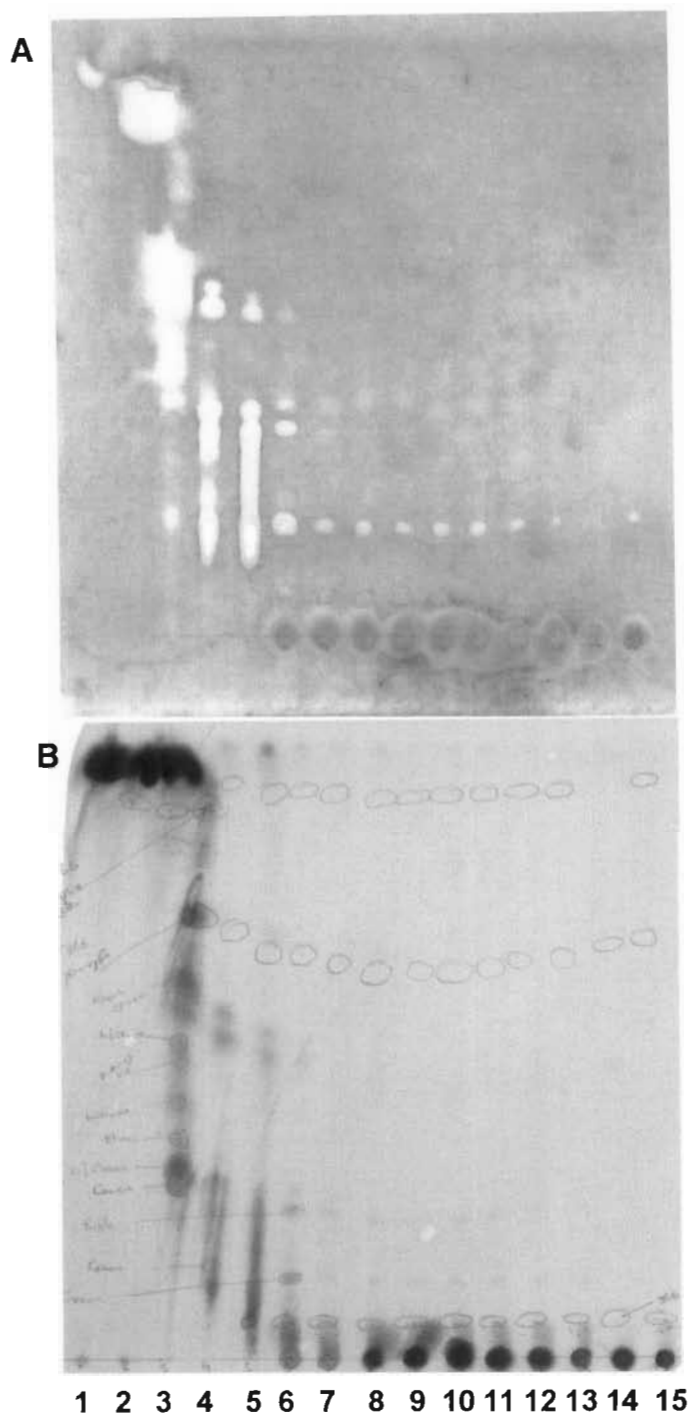
Figure 5.3 TLC plates of *G. perpensa* ethanol extract from the rhizome. VLC fractions (1-15) were spotted on TLC plates and developed with a dichloromethane: methanol (15:1) solvent system. (A) TLC plate with *S. aureus* bacterial overlay. Clear zones of bacterial inhibition. (B) Reference TLC plate stained with anisaldehyde-sulphuric acid reagent.

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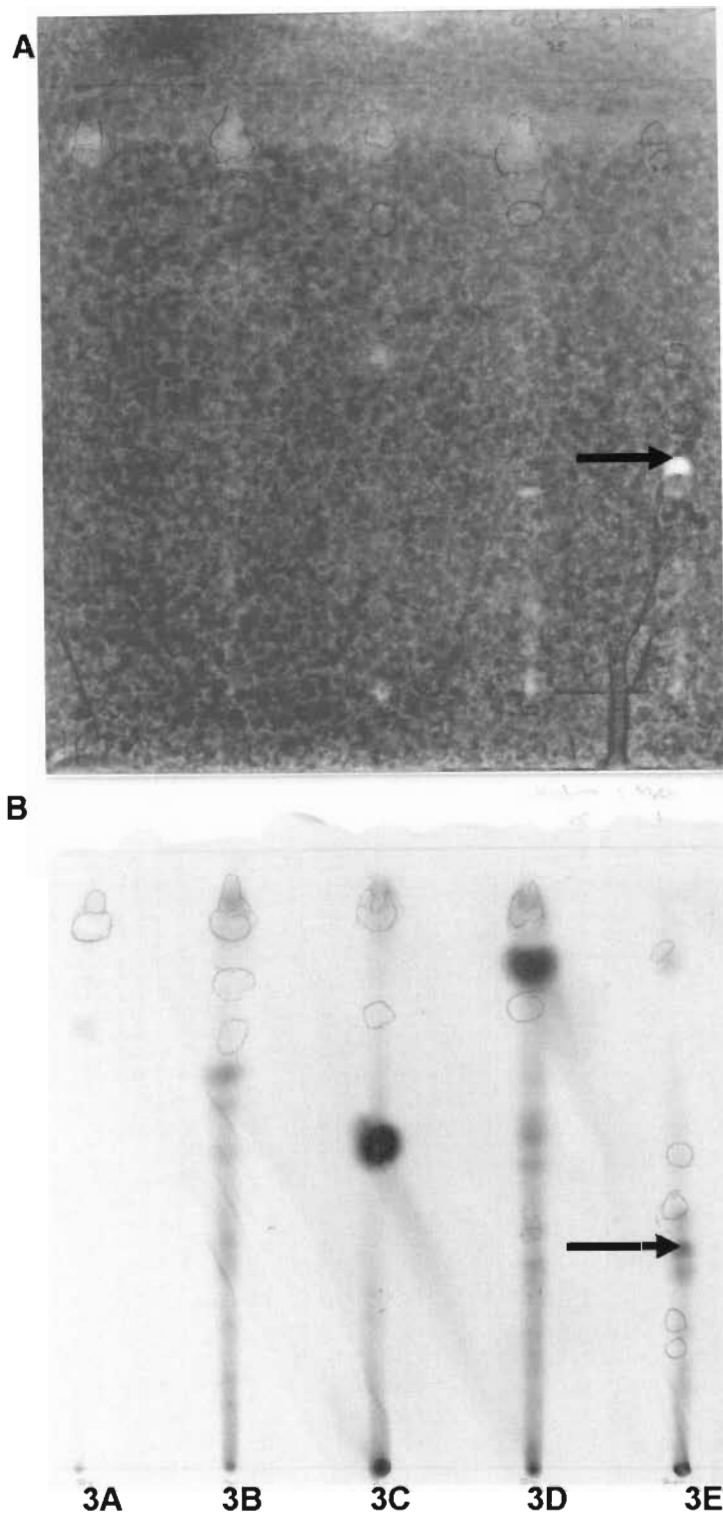
Figure 5.4 TLC chromatograms of *G. perpensa* ethanol extract from rhizome. Fractions collected from Silica column chromatography were spotted on
TLC plates and the plates were developed with chloroform: methanol (25:1) solvent system. (A) TLC plate with *S. aureus* bacterial overlay. The arrow points to the zone of bacterial inhibition. (B) Reference TLC plate stained with AS reagent.
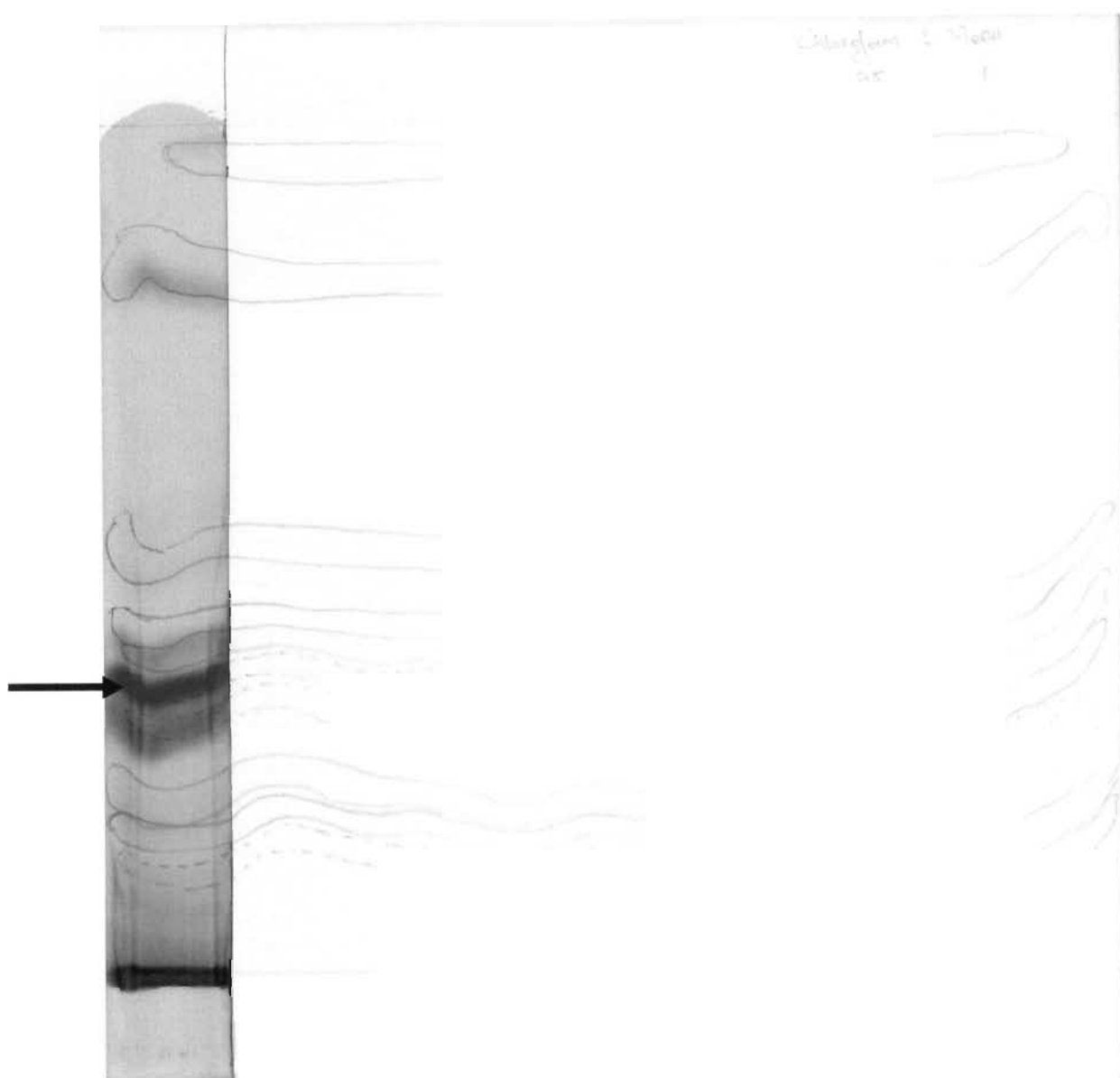
Figure 5.5 Isolation of antibacterial compounds from *G. perpensa* rhizome extract. Representative TLC chromatogram from fraction 3E, indicating colours and zones scraped by preparative TLC methods. The arrow points to the scraped zone. The plate was developed with chloroform: methanol (25:1) solvent system.
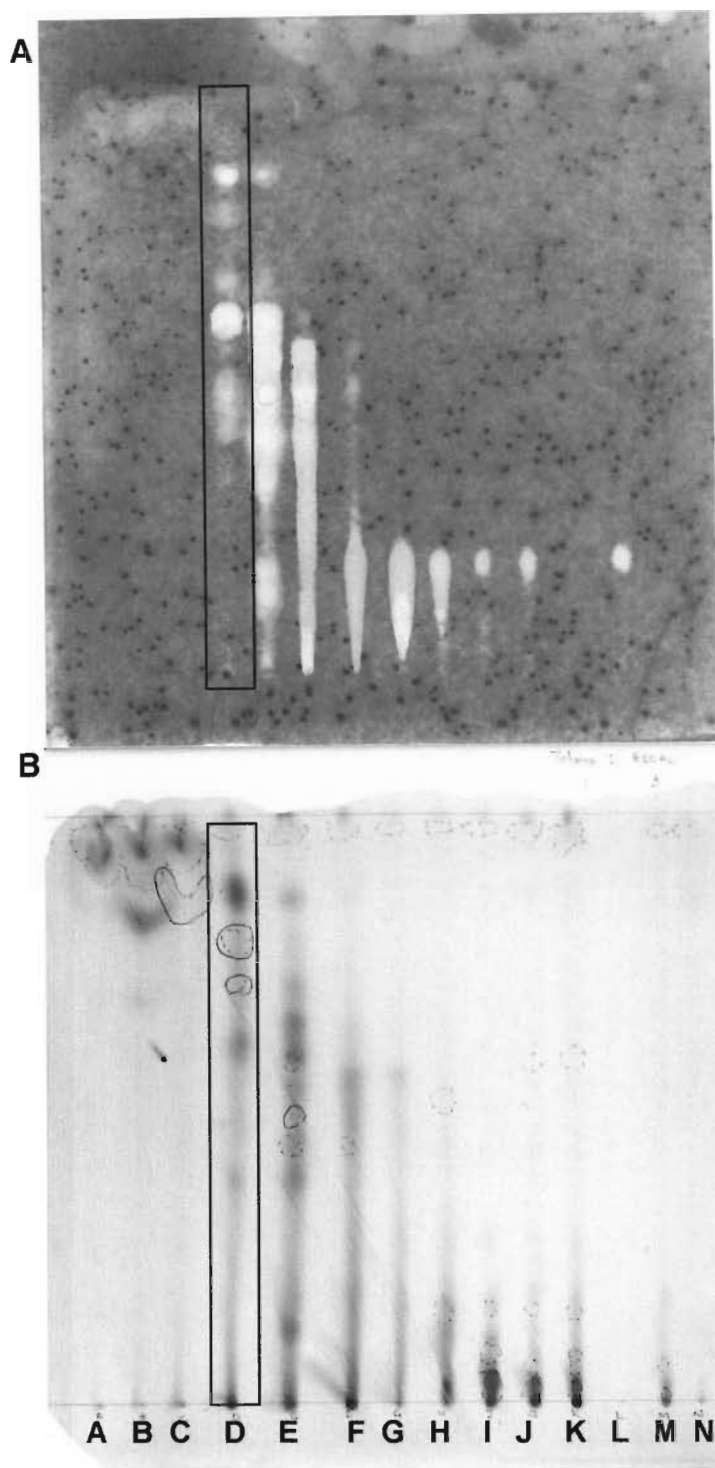
Figure 5.6 TLC chromatograms of *G. perpensa* ethanol extract from rhizome. Fractions collected from gravity-assisted column chromatography were spotted on TLC plates and developed with toluene: ethyl acetate (1:3) solvent system. (A) TLC plate with *S. aureus* bacterial overlay. (B) Reference TLC plate stained with AS reagent. The rectangle represents area of interest which was concentrated on because of high antibacterial activity.
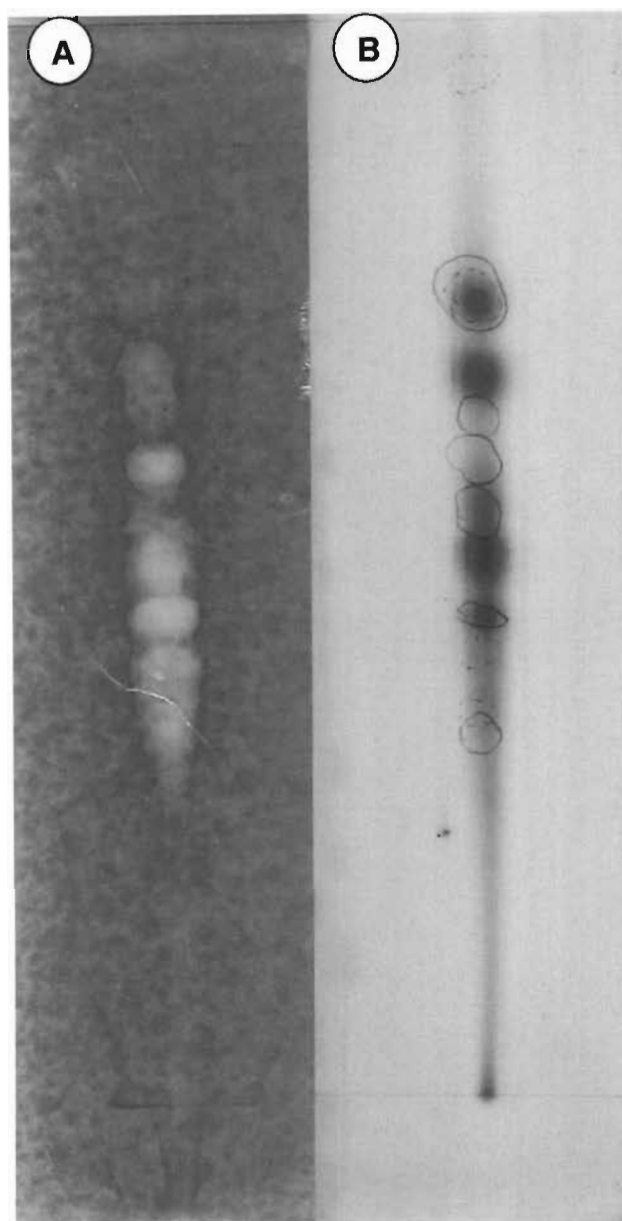
Figure 5.7   Fraction 4D spotted on TLC plates and the plates developed with a toluene: ethyl acetate: methanol (22:8:4) solvent system. (A) TLC plate with *S. aureus* bacterial overlay. (B) Reference TLC plate stained with AS reagent.
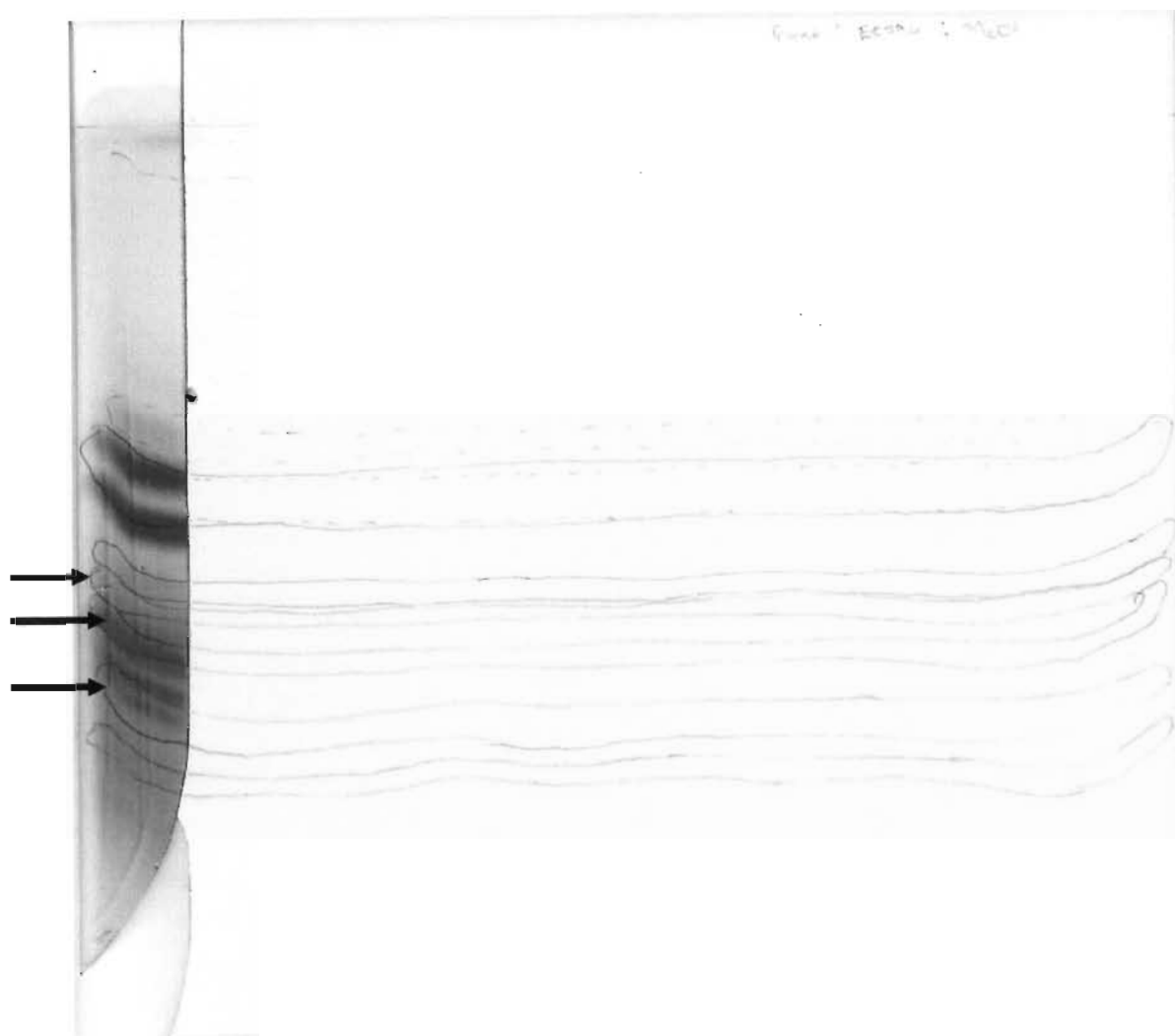
Figure 5.8 Isolation of antibacterial compounds from *G. perpensa* rhizome extract. Representative TLC chromatogram from fraction 4D, indicating colours and zones scraped by preparative TLC methods. The arrows point to the scraped zones. The plate was developed with a toluene: ethyl acetate: methanol (22:8:4) solvent system.

Table 5.1: MIC values (mg/ml) of fractions isolated from *G. perpensa*

Bacteria tested	MIC values of fractions			
	$3E_6$	$4D_6$	$4D_8$	$4D_9$
B. subtilis	0.5 (bacteriostatic)	>1	>1	1
E. coli	0.25	>1	>1	>1
K. pneumoniae	0.25	0.5	1	>1
S. aureus	0.5	0.5	>1	0.25

5.4 DISCUSSION AND CONCLUSIONS

In this study, bioautographic and MIC bioassays were used to determine antibacterial activity. A TLC bioautographic technique was used to track activity through the separation process. This assay is sensitive and gives accurate localization of active compounds. However, its limitation is that it does not distinguish between bacteriocidal and bacteriostatic metabolites. The bioautography results showed a number of clear zones. These clear zones give an indication of antibacterial activity by compounds present in these areas. However, lipophilic compounds may also result in clear zones because the inoculated nutrient broth may not sufficiently wet the area due to the hydrophobic nature of the compounds (JORK et al., 1990). A liquid broth assay (e.g. microdilution assay) needs to be employed for efficient results.

Although the active antibacterial compounds from G. perpensa have not been successfully isolated and identified during this study, an early report mentioned the presence of a bitter principle, celastrin (WATT and BREYER-BRANDWIJK, 1962), and a recent study undertaken by KHAN et al. (2004) has reported on compounds isolated from this species. The minor components, such as succinic acid, lactic acid, pyrogallol and the trimethyl ether of ellagic acid glucoside were isolated from the aqueous extract of the dry rhizomes of G. perpensa (KHAN et al., 2004). The major constituent was identified as Z-venusol, phenylpropanoid glucoside. Z-venusol has been previously isolated by PROLIAC et al. (1981) and PAGANI (1990). Both the Z and E isomers were obtained recently by VIORNERY et al. (2000) where the plant source was Umbilicus pendulinus or Umbilicus ruperstris (Crassulaceae).

Studies undertaken by KAIDO et al. (1997) and KATSOULIS et al. (2000) have shown that the aqueous Gunnera extract exhibits direct smooth activity on the uterus only, whereas tests on isolated uterine smooth muscle from rats showed that the whole extract directly stimulated a contractile response for both the ileum and uterus

muscle (KHAN *et al.*, 2004). Furthermore, a study undertaken by KHAN *et al.* (2004) has shown that Z-venusol induced a state of spontaneous contractile response for both the ileum and uterus muscle. It is possible that venusol exerts its action in conjunction with substances present in the whole extract (KHAN *et al.*, 2004).

Although attempts on isolation and structure elucidation of the active compound(s) were not successful, high inhibitory activity of the compounds obtained highlighted some possible active compounds that may be found in *G. perpensa*. The presence of antibacterial active compounds in *G. perpensa* supports the use of the plant by traditional healers and it also substantiates the claims by traditional healers.

CHAPTER 6

ISOLATION OF ANTIFUNGAL COMPOUNDS FROM

HARPEPHYLLUM CAFFRUM

6.1 INTRODUCTION

Harpephyllum caffrum Bernh. commonly known as wild plum, belongs to the family Anacardiaceae (mango family), which is the fourth largest tree family in southern Africa. The natural distribution is restricted to southern Africa (PALMER and PITMAN, 1972; VON BREITENBACH, 1986). The *H. caffrum* grows from the Eastern Cape northwards through KwaZulu-Natal, Swaziland, southern Mozambique, Limpopo and into Zimbabwe. It is a popular tree in frost-free areas. The generic name *Harpephyllum* is of Greek derivation, meaning sickle-like leaves, referring to the shape of the falcate leaflets. The specific name *caffrum* is derived from its place of origin, Kaffraria, now part of the Eastern Cape. This word also means 'indigenous'.

The wild plum is a large evergreen tree that grows up to 15 m tall (PALMER and PITMAN, 1972; COATES PALGRAVE, 1977; VAN WYK *et al.*, 1997). The dark green, shiny leaves are divided into several leaflets. The whitish green flowers are borne near the ends of the branches with male and female flowers on separate trees (VAN WYK *et al.*, 1997), throughout summer (November to February). The tasty plum-like fruits first appear green and then turn red when they ripen in autumn. The fruit of *H. caffrum* contains a single seed and is widely utilized by birds, animals, insects as well as humans. They are commonly used for making jam and jellies. With their sour taste, they are also good to make rosé wine. The bark is a popular traditional medicine. The decoctions are used as blood purifiers or emetics (WATT

and BREYER-BRANDWIJK, 1962; PUJOL, 1990). It may also be used for facial saunas and skin washes, and to treat skin problems such as acne and eczema (PUJOL, 1990). Powdered burnt bark is used to treat sprains and bone fractures (HUTCHINGS, 1996). In some parts of the Eastern Cape, root decoctions are traditionally taken for paralysis

Figure 6.1   *Harpephyllum caffrum* Bernh. Inset shows
the flowers and berries.

The aims of this study were to isolate and identify the active antifungal compound(s) in *Harpephyllum caffrum*.

6.2 MATERIALS AND METHODS

6.2.1 Bulk extraction of *H. caffrum bark*

All solvents used in the isolation of the biologically active compounds were first distilled under vacuum to remove impurities.

Dried, ground plant material (584 g) was extracted with 2 X 3 L ethanol by sonicating for 2 h and leaving in a shaker overnight. The extract was filtered through Whatman No. 1 filter paper and dried in front of a fan. A brown powdered extract was obtained (74.23 g dry weight).

6.2.2 Bioassay-guided fractionation

The isolation of an active compound from *H. caffrum* was conducted according to the outline shown in Figure 6.2. The antifungal activity of the fraction was tested after each purification step using the microdilution bioassay with *Candida albicans* as described in Section 3.2.2, Chapter 3.

6.2.2.1 Vacuum Liquid Chromatography

The maximum amount of extract that could be loaded onto the VLC was 10 g. Therefore, to prevent overloading, seven separate columns were run simultaneously. The columns and the extract were prepared as described in Section 5.2.2.1, Chapter 5. A toluene: ethanol gradient (toluene proportions: 100%; 95%; 90%; 85%; 80%; 75%; 70%; 65%; 60%; 55%; 50%; 0%) was used and the solvent mixtures (400 ml) were run through each column. Fractions were collected from seven columns- where the same ratio of solvent was used- combined and dried *in vacuo*.

Figure 6.2 Flow chart outlining the procedure followed in the isolation of antifungal compounds from a *H. caffrum* bark extract. The crossed-out fractions mean no activity was detected with the fractions against *C. ablicans*.

Harpephyllum caffrum bark (584 g)

Ethanol extract (74.23 g DW)

7 X VLC columns (about 10 g/column),
Eluted with toluene: ethanol: methanol,
Microdilution assay

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

2 X Gravity columns (1.008g & 675 mg),
Eluted with hexane: ethyl acetate: ethanol: methanol,
Microdilution bioassay

| 1-15 | 16-45 | 46-75 | 76-120 | 121-145 | 146-180 | 181-205 | 206-215 | 216-281 |
| A | B | C | D | E | F | G | H | I |

Preparative TLC plate (107 mg DW)
Solvent system (hexane: ethyl acetate 3:2)

Compounds:
B1 (?? oily)
B6 (1 mg)
MICs

Preparative TLC plate (66 mg DW)

Compounds:
C4 (1 mg)
C5 (1.9 mg)
C6 (0.5 mg)
C7 (1.1 mg)
C8 (0.7 mg)
MICs

Solvent front
1
2
3
4
5
6
7
8
Origin

6.2.2.2 Evaluation of VLC fractions

Fractions 1-15 (0.125 mg) were spotted onto a TLC plate (silica gel 60 F_{254}, 0.25 mm, 20 cm x 20 cm, Merck). The plate was developed in toluene: ethanol (2:1) over 17 cm. The solvent front was marked and the plate was allowed to dry before visualisation under UV_{254} and UV_{366}.

For the evaluation of VLC fractions, single spots were scraped off the TLC plate and assayed for antifungal activity using the microdilution assay. A modification of the NCCLS proposed method (M27-P) broth microdilution test was performed as described in Section 3.2.2, Chapter 3.

6.2.2.3 Gravity-assisted Column Chromatography

From the results obtained with the microdilution bioassay of the VLC fractions, fractions 4-9 were combined and further purified using gravity-assisted column chromatography.

Two separate columns were run simultaneously since the maximum amount of extract that could be loaded onto the column was 1 g. Dried residue was applied to gravity-assisted column chromatography as described in Section 5.2.2.3, Chapter 5. A hexane: ethyl acetate gradient (hexane proportions: 100%; 90%; 80%; 60%; 50%; 40%; 30%; 20%; 10%; 0%) was used. The collected fractions were spotted onto a TLC plate and developed in hexane: ethyl acetate (1:2). Fractions that showed similar spots were combined, dried in front of a fan and tested in the microdilution bioassay.

From the results obtained with column chromatography fractions, fraction B and fraction C were selected for further purification and compound isolation using preparative TLC.

Fractions B and C were applied onto TLC plates and developed in hexane: ethyl acetate (3:2) as described in Section 5.2.2.4, Chapter 5. Separated fractions were scraped off the TLC plates and tested for antifungal activity using the microdilution bioassay. The active compound in ethanol was filtered through Millipore filters (0.45 μm and 0.22 μm) to remove the silica. The purity of the isolated compound was confirmed by TLC using various solvent systems. The isolates were sent for NMR analysis to the School of Chemistry, University of KwaZulu-Natal Pietermaritzburg Campus.

6.3 RESULTS

6.3.1 Plant extraction

The bulk extraction with ethanol of the dried and powdered bark (584 g) of *Harpephyllum caffrum* yielded 74.23 g of crude extract.

6.3.2 Bioassay-guided fractionation for isolation of active compound(s)

6.3.2.1 Vacuum liquid chromatography

The VLC of ethanolic crude extract yielded 15 fractions. These fractions were pooled according to their TLC profile and tested for antifungal activity using the microdilution bioassay. Fractions were considered highly active if the MIC value was below 1 mg/ml. Fractions collected between toluene: ethyl acetate 85:15 to 60:40 all yielded high antifungal activity against *C. albicans* (Table 6.1). The fractions were combined and further separated using gravity-assisted column chromatography.

Table 6.1: MIC values of single spots scraped off the TLC plate from VLC fractions against *C. albicans*

Fraction tested	MIC (mg/ml)
Fraction 1	>1
Fraction 3A	>1
Fraction 3B	>1
Fraction 3C	0.93
Fraction 4	0.7
Fraction 6	0.93
Fraction 10	0.47
Amphotericin B	0.006

6.3.2.2 Gravity-assisted Column Chromatography

This procedure yielded 281 eluents that were grouped into 9 fractions according to their TLC profiles. They were again assayed for antifungal activity using the microdilution bioassay. The results are presented in Table 6.2. Fractions 16-45, 46-75 and 181-205 (which were combined to produce fractions B, C and G respectively) showed the highest antifungal activity. Due to insufficient amount of fraction G, only fraction B (107 mg) and fraction C (66 mg) were further purified using preparative TLC.

Table 6.2: MIC values of fractions obtained from column chromatography against *C. albicans*

Fractions tested	Label	MIC (mg/ml)
Fraction 1-15	A	3.125
Fraction 16-45	B	0.78
Fraction 46-75	C	1.56
Fraction 76-120	D	>12.5
Fraction 121-145	E	>12.5
Fraction 146-180	F	6.25
Fraction 181-205	G	0.3
Fraction 206-215	H	3.125
Fraction 216-281	I	>12.5
Amphotericin B		0.006

6.3.2.3 Preparative TLC

Preparative TLC of fraction B yielded one active pure fraction (fraction B_6), and fraction C yielded 5 active pure fractions (fractions C_4, C_5, C_6, C_7 and C_8). The MIC (mg/ml) results obtained for the isolated compounds are presented in Table 6.3. The isolated fractions displayed good antifungal activity against *C. albicans*.

Table 6.3: MIC values (mg/ml) of fractions isolated from *H. caffrum*

Fractions tested	Fraction yield (mg)	MIC (mg/ml)
B_6	1	0.315
C_4	1	0.315
C_5	1.9	0.47
C_6	0.5	0.94
C_7	1.1	0.94
C_8	0.7	0.78

6.3.2.4 NMR Spectroscopy

Due to insufficient quantities of fractions B_6, C_4, C_6, C_7 and C_8, only fraction C_5 could be chemically elucidated. Inspection of the ^{1}H NMR (Figure 6.3) of fraction C_5 showed a mixture of two compounds- a major compound and a minor compound. The major compound contained a long chain with two double bonds and hydroxyl

functionality. The activity of the isolated major compound might be related to the functional group.

A literature search revealed the isolated major compound to be equivalent to cyclopentenyl fatty acids, which have been reported to be antibacterial and leprostatic (BLAISE *et al.*, 1997). The minor compound was suspected to contain a fragment such as:
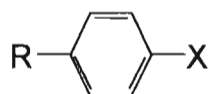
Further identification could not be performed since the compounds were only available in small quantities.
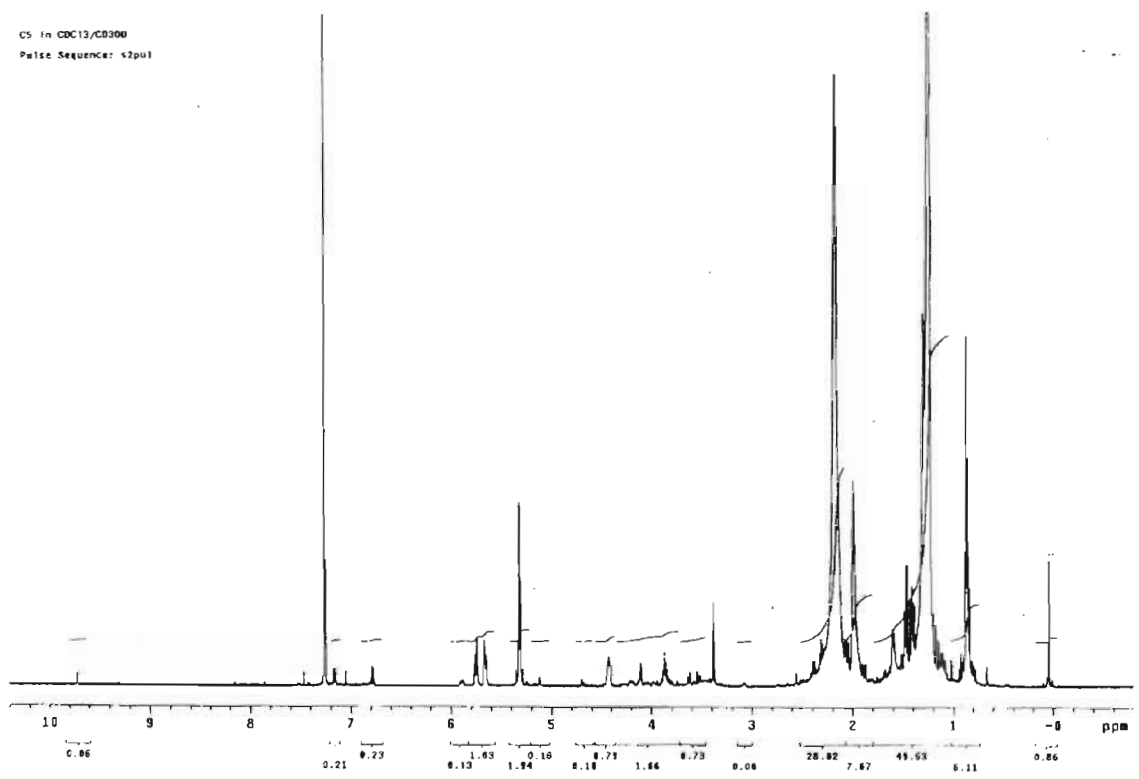
Figure 6.3 ^{1}H NMR Spectrum of antifungal compounds isolated from *H. caffrum* bark.
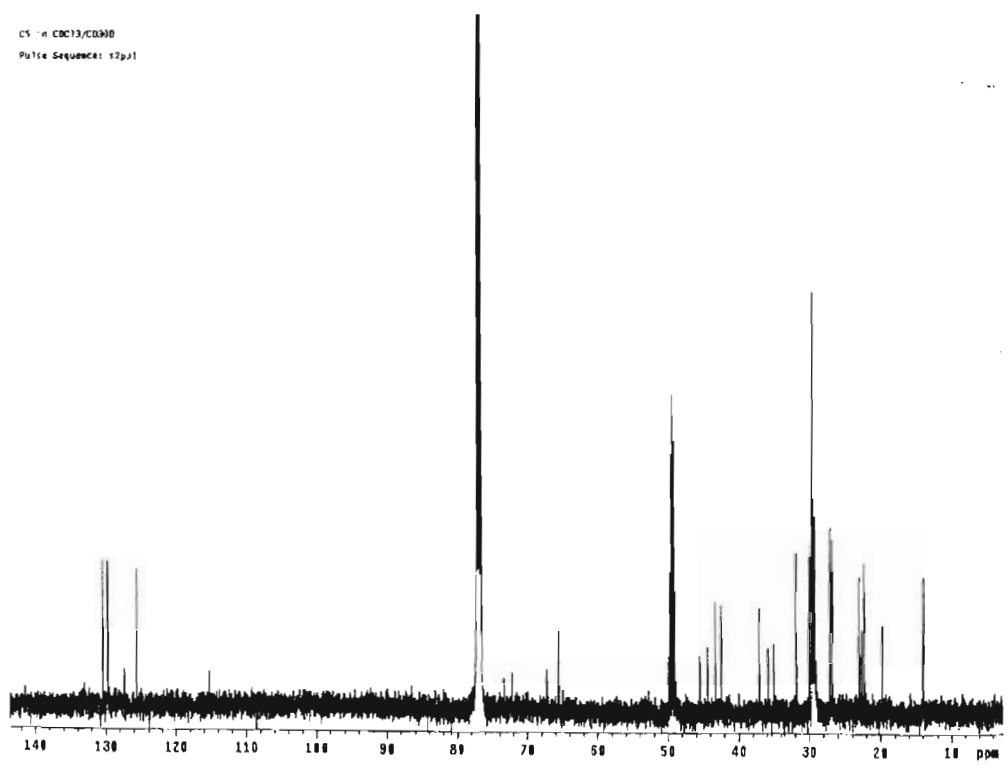
Figure 6.4 ^{13}C NMR Spectrum of antifungal isolates from *H. caffrum* bark.

6.4 DISCUSSION AND CONCLUSIONS

In the chemistry of natural products, the separation of large or small quantities of complex mixtures is seldom efficiently, rapidly and inexpensively achieved. In most cases, many naturally occurring compounds tend to interfere with the isolation and purification of a desired bioactive plant constituent. The long route from a crude plant extract, containing hundreds of constituents, to a pure compound is very tedious and often requires several separating steps involving various techniques (HOSTETTMANN, 1986; SILVA *et al.*, 1998).

Although the isolation and purification of the active antifungal compound(s) from the ethanolic bark extract of *H. caffrum* were not successful, a study undertaken by EL SHERBEINY and EL ANSARI (1976) reported on compounds isolated from this species. The study of the polyphenolics and flavonoids of *H. caffrum* resulted in the isolation of protocatechuic acid, gallic acid, methyl gallate, kaempferol-3-rhamnoside, kaempferol-3-galactoside, apeginin-7-glucoside, quercetin-3-rhamnoside, quercetin-3-glucoside, quercetin-3-arabinoside and the free aglycones quercetin and kaempferol (EL SHERBEINY and EL ANSARI, 1976) from the ethanolic leaf extracts. Of these compounds, flavonoids are known to have antibacterial activity (BRUNETON, 1995). These compounds may be responsible for the observed activities investigated in this study.

H. caffrum has been reported to contain tannins (EL SHERBEINY and EL ANSARI, 1976), with its leaves and bark containing 11% and 18% tannins respectively (EL SHERBEINY and EL ANSARI, 1976). Tannins are commonly found in high concentrations in plant extracts. They are reported to give false-positive results in many biological assays due to their tendency to precipitate proteins through multipoint hydrogen bonding and by reacting with flavonoids (SILVA *et al.*, 1998). Such occurrences may explain the high activities observed with this species. The results showed different levels of activities against *C. albicans*. The antifungal activity

exhibited by the isolated fractions was considerably much higher than that of the crude extract.

CHAPTER 7

GENERAL CONCLUSIONS

7.1 INTRODUCTION

Since ancient times higher plants, as sources of medicinal compounds have played and continue to play a dominant role in the maintenance of human health. Plant-derived natural products have long been and will continue to be extremely important as sources of medicinal agents and models for the design, synthesis and semi-synthesis of novel substances for treating diseases. Traditional knowledge of plants and their medicinal value, combined with patterns of harvest developed over generations, has ensured the sustainable use of many natural resources.

Traditional medicine is widespread within South Africa. The prime advantage of traditional medicine is that it is there as an immediate, existing source of health care for people where they live at minimal cost.

The work presented in this thesis covered three major lines of study focused around traditional medicinal plants used against venereal diseases in South Africa. The first part centered on the screening of plant extracts for potential antibacterial, antifungal, mutagenic and antimutagenic activities. The second part entailed the investigation of seasonal and geographical variation in medicinal activities of *Harpephyllum caffrum*. The third part attempted the isolation of active compound(s) that exhibited high antibacterial and antifungal activities in different bioassays used during the screening for bioactivity.

7.2 PHARMACOLOGICAL SCREENING

The screening of plant extracts as described in Chapter 3 showed that many plants have antibacterial activity and only few have antifungal activity. However, negative results do not mean absence of bioactive constituents nor that the plant is inactive. Active compound(s) may be present in inadequate quantities in the crude extracts to show activity with employed dose levels (TAYLOR *et al.*, 2001). On the other hand, if the active principles are present in high quantities, there could be other constituents exerting antagonistic effects or opposing the positive effects of the bioactive agents (JÄGER *et al.*, 1996). With no antibacterial or antifungal activity, extracts may be active against other bacterial or fungal species which were not tested. In addition, they may act in other ways by stimulating the immune system of the patient, or by creating internal conditions unfavourable for the multiplication of the pathogen. Although most of the investigated plants did not show any potential mutagenic effects, this does not necessarily mean absence of harmful genotoxic effects that rationalize their use as a safe traditional medicine. Toxicity tests are necessary to gain a full understanding of the effectiveness and possible toxic nature of traditional medicine. Furthermore, scrupulous screening for potential harmful effects of plants used in traditional medicine is recommended before long-term usage.

Pharmacological screening of medicinal plants remains important to provide a scientific basis for the continued use of medicinal plants. Furthermore, it provides the society with potential sources of new, effective and safe drugs.

7.3 SEASONAL AND GEOGRAPHICAL VARIATION IN MEDICINAL ACTIVITIES OF *HARPEPHYLLUM CAFFRUM*

Seasonal and geographical variation is a factor to take into consideration in the investigation of medicinal plants, as this may influence the results of any research into biological activity. In Chapter 4, the effects of time of collection on antibacterial and antifungal activities and on the chemical composition as visualized using TLC fingerprints of *H. caffrum* were compared. It was evident that a seasonal effect is important with respect to activity. This study suggests warmer months to be the best time for the collection of *H. caffrum* bark.

7.4 ISOLATION OF ACTIVE COMPOUND(S)

Although the active antibacterial and antifungal compounds have not been successfully isolated and identified, high inhibitory activity of the isolates obtained showed the existence of active compounds that may potentially be isolated from *Gunnera perpensa* and *Harpephyllum caffrum*.

7.5 CONCLUSION

Plants have played, and will continue to play, an important role in the research and development of pharmaceuticals. In turn, the pharmaceutical industry's reliance on natural products also has an immense impact on certain plant species and their natural habitats. As demands for herbs, phytotherapies, and naturally derived pharmaceuticals increase, however, both traditional cultures and their biological resources become increasingly vulnerable to the pressures of market economies.

The scientific investigation of the potential biological activity of plants used by traditional healers against venereal diseases is important. The selected plants used against venereal diseases in South Africa have demonstrated significant antibacterial and antifungal activities which may explain and justify the usage of these plants by traditional healers. If these activities are high enough and if the compounds responsible for toxicity are stable and do not pose too great a risk to non-target organisms, plant-derived natural products can be of immense potential for prevention and treatment of venereal diseases.

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