CHAPTER 1

Medicinal Plants

1.1 Introduction

The study of medicinal plants has attracted many researchers, owing to the useful applications of plants for the treatment of various diseases in humans and animals. To date, medicinal plants have been used in all cultures as a source of medicine for the treatment of various diseases including stomach complaints, malaria, depression, cancer and AIDS (Hoareau and Da Silva 1999). Data has revealed that out of about 250 000 flowering plants in the world (Thorne 2000) more than 50 000 are used for medicinal purposes (Schippmann et al. 2002). According to the World Health Organization (WHO), more than 80% of the population in developing countries relies on medicinal plants as an integral part of their primary health care (Penso 1980). In South Africa several species of medicinal plants are used by many ethnic groups for the treatment of various ailments in both humans and domestic animals (Masika and Afolayan 2002). Up to 60% of the population consults one of an estimated 200 000 traditional healers, in preference to, or in addition to Western medical doctors, especially in rural areas (Van Wyk et al. 1997).

Access to free primary health care is important in South Africa's constitution and many rural communities now have access to mobile clinics and hospitals. However there is still, to a large extent, strong belief in herbal medicine, possibly due to an inherent distrust in anything "western". Moreover, the remoteness and lack of reliable modern health facilities in the rural communities also enhances the dependence on plants for medicine.

Despite several important publications in this field (Watt and Breyer-Brandwijk 1962, Hutchings et al. 1996, Van Wyk et al. 1997), many of the uses of plants for medicine have not been recorded yet in South Africa. The continued documentation of traditional knowledge, especially on the medicinal uses of plants is important, because it may provide mankind with new herbal remedies.

1.2 Literature review

1. 2.1 Importance of medicinal plants

Medicinal plants have an outstandingly long history of use on the African continent, especially in the manufacturing of remedies that are used for the treatment of easily diagnosed human and animal diseases. In South Africa, preparations based on plant species such as Cape aloes (*Aloe ferox*), buchu (*Agathosma betulina*) and devil's claw (*Harpagophytum*) have been produced commercially by pharmaceutical companies and other species have potential as a source of new herbal remedies (Van Wyk et al. 1997).

Plants are a primary source of new natural medicinal products (Hostettman 1999). Of 119 drugs still extracted from plants and used globally, 74% were discovered during an attempt to identify the chemical substances amongst medicinal plants responsible for combating human diseases (Farnsworth 1990). Drug discovery from medicinal plants led to the isolation of early drugs such as aspirin, vincristine, vinblastine, cocaine, digitoxin, quinine and morphine, of which some are still in use (Newman et al. 2000, Butler 2004, Samuelsson 2004, Gilani and Rahman 2005). Morphine isolated from *Papaver somniferum* is used as a painkiller, while aspirin, an acetyl salt of salicyclic acid from willow bark, is considered to be one of the most effective analgesic, antipyretic and anti-inflammatory agents commonly used in modern medicine (Gilani and Rahman 2005). Cocaine from the Coca plant, *Erythroxylum coca*, has served as a model for the synthesis of a number of local anaesthetics such as procaine, while quinine from *Cinchona* bark was used to treat the symptoms of malaria (Gilani and Rahman 2005, Gurib-Fakim 2006). Digitoxin is a steroidal glycoside obtained from the leaves of *Digitalis purpurea* and has been used for many years in the clinic, mainly for the treatment of cardiac congestion and some types of cardiac arrhythmias (López-Lázaro et al. 2006). Vinblastine from *Catharanthus roseus* is used in the treatment of patients with Hodgkin's disease, non-Hodgkin's lymphomas and renal, testicular, head and neck cancer (Hostettman 1999).

1.2.2 The use of plants against microbial infections

Bacterial and fungal infections may be fairly easy to diagnose by traditional healers and community members, therefore there is more chance of finding a successful traditional remedy from plant material used in treatment of such infections. Plant species such as *Rhoicissus tridentata*, *Cissus quadrangularis* and *Cyphostemma natalitium* have been reported to have antimicrobial activity and are regarded as potential plant remedies to be used for treatment of various diseases in human (Lin et al. 1999). Some researchers have also found antimicrobial activities from extracts of *Dicoma anomala*, *Leonotis leonorus* and *Gunnera perpensa* (Steenkamp et al. 2004) and from the Sterculiaceae family (Reid et al. 2005).

Previous studies have shown that plant species such as *Rhus javanica* L. have antifungal activity and has been used world wide as a source of natural drugs (Ahn et al. 2005). Extracts from *Alpinia galanga*, *Curcuma zedoaria* and *Zingiber purpureum* were reported to have antifungal activity against a wide variety of human pathogenic fungi (Ficker et al. 2003) while some researchers have also found antifungal activity from extracts of *Asclepia curassavica*, *Bixa orellana*, *Eupatorium aschenbornianum* and *Galpinia galuca* (García et al. 2003). These examples cited above reflect only a small representation of the work that has been carried out on the evaluation of plant extracts against microbial infectious agents.

1.2.3 Fungi as pathogens

Fungi are eukaryotic, filamentous, and mostly spore-bearing organisms, which exist as saprophytes or as parasites of animals and plants (Kurup et al. 2000). Many fungi are useful, for example edible mushrooms and antibiotic producers (e.g. *Penicillum notatum*). However, some fungi are plant, animal or human parasites and are harmful (Bordon-Pallier et al. 2004). For example, *Candida albicans* is a potentially pathogenic fungus often encountered as benign commensal yeast of the human digestive system and vaginal tract. Under certain conditions it behaves as an opportunistic pathogen, with the infections produced ranging from superficial to systemic (Doyle et al. 2006).

Aspergillus species, commonly found in soil, decaying organic matter, dust and air, are ubiquitous filamentous fungi that can cause severe opportunistic human diseases (Heinemann et al. 2004). Possible diseases caused by *Aspergillus candidus* are allergic (allergic alveolitis and asthma) or immunotoxic (mycotoxicosis, building-related disease), which are related to substances released in the lungs from inhaled spores and mycelium fragments (Ribeiro et al. 2005). *Trichophyton* species cause superficial mycoses commonly known as tinea infections in humans and other animals (Harris 2002, Patra et al. 2002, Shin 2004).

1.2.3.1 Antifungal drugs

There are few effective antifungal preparations currently available for the treatment of fungal diseases, for example, amphotericin B, 5-flucytosine, fluconazole, ketoconazole, and itraconazole (Raid and Mares 2003). Ketoconazole is one of the commonly used antifungal drugs administered orally for the treatment of both superficial and deep infections caused by *Trichophyton* (Pyun and Shin 2006). In addition the efficacy of ketoconazole is poor in immunosuppressed patients and in the treatment of meningitis (Craven and Graybill 1984). Many of the drugs have undesirable effects or are very toxic (amphotericin B), produce recurrence, show drug-drug interactions (azoles) or lead to the development of resistance (fluconazole, 5-flucytosine).

1.2.4 Resistance of fungi

Infectious diseases emanating from microorganisms such as bacteria, fungi, viruses and parasites are a major threat to public health care due to the growing resistance of many microorganisms to currently available antibiotics. The incidence of fungal infections has increased dramatically over the past few decades (Beck-Sague and Jarvis 1993, Georgopapadakou and Walsh 1994), mainly affecting immunocompromised or surgically treated patients, as well as the young and old (Georgopapadakou and Walsh 1994, Maertens et al. 2001). Immunocompromised patients with AIDS are commonly affected by fungal infections which cause morbidity and mortality. With the rise in infections caused by various fungi, and the development of resistance in fungal pathogens, it is important that novel antifungal agents be identified and developed (Alexander and Perfect 1997).

1.2.5 Food production and effects of fungal pathogens

Fungal pathogens cause major problems in food production and the safety of consumers is at risk due to food spoilage and poisoning by fungi and toxins produced by them, especially in developing countries. For example *Penicillium*, *Aspergillus* and *Fusarium* species are the most commonly known fungi causing spoilage of African food products (Nickelsen and Jakobsen 1997).

Fungal diseases cause a considerable loss of crop yields in agricultural industries worldwide. For example fungi such as *Fusarium* spp., growing on plants, are able to produce mycotoxins that can seriously harm consumers. Aflatoxin B_1 and B_2 and fumitoxins produced by *Aspergillus flavus* and *A. fumigatus* are some examples of mycotoxins (Singh et al. 1991). Antimycotics play an important role in agriculture; firstly, they are used to control fungal growth on plants and fruits. Secondly, they can be used to prevent or to ease the problem of post harvest spoilage of plants and fruits (Hof 2001).

Aspergillus spp. grow on a wide range of organic substrates, and often cause deterioration of stored food material (Barrios et al. 1997, Misra and Dubey 1994, Paster et al. 1990). There are also reports of *Aspergillus niger* inducing spoilage of mangoes (Prakash and Raoof 1989), grapes and tomatoes (Sinha and Saxena 1987). The presence and growth of this fungus in food and animal feed threatens human and animal health, respectively.

Some farmers use chemical fungicides in plant agriculture to control fungal diseases, for example, fire blight or blister spots in fruit trees (such as apple, pear and peach). However, many fungicides are toxic to humans and they can cause environmental contamination or may result in fungicide residues on food products (Moenne-Loccoz et al. 1998). On the other hand biological control, using microorganisms to repress plant disease, offers an alternative, environmentally friendly strategy for controlling agricultural phytopathogens (Chang et al. 2006). The screening of medicinal plants is another alternative that may produce fungicides that are relatively non-toxic and cost-effective.

1.2.6 Plants as antifungals

1.2.6.1 Previous related antimicrobial work in the Phytomedicine laboratory

Eloff (1999) reported on the antibacterial activity of 27 southern African members of the Combretaceae. The minimum inhibitory concentrations (MICs) of extracts of the plants were determined by a microplate serial dilution technique using *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* as the test organisms. It was found that all extracts inhibited the growth of the four test isolates, and the Gram-positive strains were slightly more sensitive than the Gram-negative species. Based on the MIC values and the total extract content of each plant, seven species with high antibacterial activity were discovered.

In another study, the isolation and characterization of antibacterial compounds from *Combretum erythrophyllum* (Burch) Sond was performed (Martini and Eloff 1998). This study yielded seven antibacterial flavonoids from the same fraction, possibly due to different extraction techniques. Three of these compounds were flavones, i.e. apigenin, genkwanin and 5-hydroxyl-7, 4'-dimethoxyflavone and four flavonols were identified i.e. kaempferol, rhamnocitrin, rhamazin and quercetin-5, 3'-dimethylether. Although all these compounds are fairly common flavonoids, they were all reported for the first time in *Combretum erythrophyllum*, and in some cases for the first time in the family Combretaceae.

The process of selecting plants to work on was examined by Eloff (1998a), where an analysis was made of approaches to be followed towards selecting plants for research and gene banking. Plants used as phytomedicines in Africa were also analysed and of these, the Combretaceae made up a major group.

Selection of the best extraction procedure was also done by Eloff (1998b), where several extractants were tested and evaluated on many different parameters. Acetone was found to be the best extractant. Selection of the best purification procedure was done by Eloff (1998c), where the solvent-solvent fractionation procedure used by the USA National Cancer Institute was tested and refined and several TLC separation procedures were also developed. By developing a novel way of determining antibacterial activity, it could be shown that the

traditional agar diffusion assays for determining activity of plant extracts did not work in all scenarios, such as for non-water soluble extracts. A new serial dilution microplate assay using INT (iodonitrotetrazolium violet) was developed (Eloff 1998d).

In investigations of other biological activities of *Combretum* species, the anti-inflammatory, anthelmintic and antischistosomal activity of 20 *Combretum* species was determined. There was very little antischistosomal activity, low to medium anthelmintic activity and medium to strong anti-inflammatory activity in extracts of the different species (McGaw et al. 2001).

Most of the work in the Phytomedicine laboratory to date has been based on antibacterial activity, and a new approach is to concentrate on antifungal activity of plant extracts. The microplate method of Eloff (1998b) modified for antifungal activity testing by Masoko et al. (2005) was used against five animal pathogens (*Candida albicans*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Aspergillus fumigatus* and *Microsporum canis*) to determine the MIC values for extracts of *Terminalia* and *Combretum* species. Most of the crude extracts had MIC values of 0.02 to 0.08 mg/ml against *C. neoformans*, *S. schenckii* and *M. canis*.

Masoko and Eloff (2005) used a bioautography method to screen for antifungal compounds in extracts of different *Terminalia* species. Acetone, hexane, dichloromethane and methanol leaf extracts of six *Terminalia* species (*T. prunioides*, *T. brachystemma*, *T. sericea*, *T. gazensis*, *T. mollis* and *T. sambesiaca*) were tested against five fungal animal pathogens. Hexane and dichloromethane extracts had at least three times more antifungal compounds than the other extracts, indicating the non-polar character of the antifungal compounds.

1.2.7 Selection of plants for study

1.2.7.1 Ethnobotanical information on six selected species

The six selected species for the current study are *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana*. These species were selected from leaf extracts of more than 400 tree species that have been screened for antifungal activity in the Phytomedicine laboratory against *Cryptococcus neoformans* and *Candida albicans* (Pauw and Eloff, unpublished data).

Bucida buceras L. is an evergreen tree and it belongs to the family Combretaceae. The plants in this family are used for the treatment of various diseases in humans and this includes abdominal pains, chest coughs, colds, conjunctivitis, diarrhoea, earache, fever, infertility in women, leprosy, pneumonia, scorpion bite, swelling caused by mumps, syphilis, heart diseases, sore throat and nose bleeds (Hutchings et al. 1996, Van Wyk et al. 1997). In southern Florida the leaves of *B. buceras* are used for the treatment of gonorrhea (Adonizio et al. 2006). In South Africa no information on the ethnomedicinal use of this particular species was found.

Breonadia salicina (Vahl) Hepper and J.R.I Wood belongs to the family Rubiaceae and is found in Limpopo, Mpumalanga and KwaZulu-Natal provinces (De Moor et al. 1977, Moll 1978, Moll and White 1978, Furness and Breen 1980). Rubiaceae are mainly tropical woody plants and consist mostly of trees and shrubs, less often of perennial to annual herbs, as in Rubieae (subfamily Rubioideae) which are found in temperate regions (Mongrand et al. 2005), and there are a few arctic species. In South Africa, Zulu people use the bark for stomach complaints and the Vhavenda use root decoctions for the treatment of tachycardia (Arnold and Gulumian 1984). The bark of *B. salicina* is reported to be astringent (Doke and Vilakazi 1972).

Harpephyllum caffrum Bernh. ex Krauss belongs to the family Anacardiaceae (mango family), which is the fourth largest family in southern Africa, boasting approximately 80 tree species and many shrubs (Dlamini 2004). *Harpephyllum caffrum* grows from the Eastern Cape northwards through KwaZulu-Natal, Swaziland, southern Mozambique, Limpopo and Zimbabwe. The stem bark of *Harpephyllum caffrum* is used traditionally in African folk medicine to manage, control and/or treat an array of human ailments, including diabetes mellitus and hypertension (Ojewole 2006).

Olinia ventosa (L.) Cufod belongs to the Oliniaceae family and is an evergreen forest tree. It occurs mainly along the southern and eastern coastal regions of South Africa, from the Cape Peninsula to just above the borders of the Transkei into southern KwaZulu-Natal (Jaffe 2006). No information on the ethnomedicinal use of this species was found.

Vangueria infausta (Burch.) belongs to the family Rubiaceae (Bohrer et al. 2003) and is a common indigenous fruit tree that grows in Botswana. In Tanzania the root and fruit are used to treat parasitic worms and east coast fever (De Boer et al. 2005). Anthelmintic activity (Teichler 1937) and antiplasmodial activity (Nundkumar and Ojewole 2002) has been previously detected in extracts of *V. infausta.* No antifungal activity has been reported.

Xylotheca kraussiana Hochst belongs to the family Flacourtiaceae and is a multi-stemmed shrub or small tree. *Xylotheca* species occur naturally in the eastern region of southern Africa, from Transkei to Mozambique, in coastal bush and forest, but also in sand forest and bushveld. *Xylotheca kraussiana* is the only species of *Xylotheca* in South Africa (Williams 2004). No information on the ethnomedicinal use of this species was found.

1.2.7.2 Phytochemical data available on selected species

Hayashi et al. (2002) isolated four new clerodane diterpenes, bucidarasins compounds from extract of *B. buceras*. The chemical structures of the new isolated compounds were also determined in detailed by NMR analyses using COSY, HMQC, HMBC and NOESY techniques. Cytotoxicity of the isolated compounds was determined and the compounds had activity against human tumour cell lines with IC_{50} values ranging between 0.5 and 1.9µM.

McGaw et al. (2000) used the disc diffusion assay and the microdilution method to determine the antibacterial activity of *H. caffrum* against four bacteria (*Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*). The results obtained showed that *H. caffrum*, particularly the ethanol extract, had antibacterial activity with the best MIC value of 98 µg/ml against the Gram-positive *B. subtilis*. To the best of my knowledge no chemical work has been reported from extracts of the other four plant species, *B. salicina*, *O. ventosa*, *V. infausta* and *X. kraussiana*.

1.2.8 Motivation

There are few different classes of effective antifungal drugs available for the treatment of fungal diseases of plants, animals and humans. Therefore, it is important to develop new sources of antifungal agents. Further development of antifungal compounds with diverse chemical structures and novel mechanisms of action is necessary because there has been an alarming increase in the incidence of new and re-emerging infectious diseases as well as resistance to currently used drugs. The investigations on new antifungal substances should therefore be continued and all possible strategies and techniques need to be explored further. Plants produce a diverse array of secondary compounds that may be effective in combating fungal pathogens. Plants therefore, are a good source of investigation for potential antifungals.

In previous work done in the Phytomedicine Programme (Angeh 2002, Mdee et al. 2009, Meela 2008) substantial activity was found against plant fungal pathogens and, because it is so much easier to do *in vivo* experiments involving field trials with plants this work focussed on plant fungal pathogens.

Based on a survey of the literature a number of plant pathogens commonly known to cause diseases in fruits and vegetables were selected to work on. If used on plants and not therapeutically in humans or animals then cytotoxicity of antifungal preparations is slightly less of a problem. If active against plant fungi, the plants may be further investigated for efficacy against animal and human pathogens. However, further development for these purposes would require more comprehensive toxicity and *in vivo* efficacy tests.

1.2.9 Aim

The aim of this study is to identify plant species with good antifungal activity and to isolate and characterize antifungal compounds or extracts with strong antifungal activity, which could be used to develop a product with good activity against plant fungal pathogens.

1.2.10 Objectives

- To select and identify plant species active against plant fungal pathogens for further phytochemical investigation based on the proven activity of extracts against animal fungal pathogens.
- To determine the antifungal activity of leaf extracts of the selected plant species against the important animal fungal pathogen *Aspergillus fumigatus*.
- To screen leaf extracts of plant species for qualitative antioxidant activity as an additional parameter for selecting the most promising species for in depth investigation.
- To isolate antifungal compounds from the selected plant species and to determine the structure of these compounds.
- To determine the biological activity of the crude extract and the isolated compounds in antimicrobial and cytotoxicity assays.
- To evaluate the potential use of the extract or isolated compound(s) against a plant fungal pathogen.

CHAPTER 2

Extraction and phytochemical investigation of selected plant species

2.1 Introduction

Fresh, frozen and dried plant material can be used as a source of plant material for isolation of secondary components. Although freezing was reported by the FAO/IAEA (2000) to be the preferred option, thawing of the plant material could cause rupturing of cell membranes. However, this may be an advantage in making plant secondary compounds available for extraction. Dried plant material is mostly used in preference to fresh material, since there are fewer problems caused by contaminating fungal growth on fresh material as a result of time delays between collecting and processing plant material. Furthermore, it is difficult to work with fresh material because differences in water content may affect solubility or separation of extracted components by liquid-liquid extraction (Eloff 1998a). Traditional healers in practice prefer dried plant material to prepare their medicine since they can grind the plants and store the material in bottles for a long time.

When aiming to extract a particular compound, or class of compounds, the polarity of the solvent should be close to that of the target compound. For hydrophilic compounds, polar solvents such as water, methanol, ethanol or ethyl acetate can be used to extract plant material, while for the extraction of lipophilic compounds, dichloromethane as an example can be used (Cos et al. 2006). Acetone can be used to extract both hydrophilic and lipophilic compounds, and is a very useful extractant (Eloff 1998b). In some instances, extraction with hexane prior to the main extraction procedure is used to remove chlorophyll and fatty acids, which may have non-specific biological activities.

The extraction solvent selected also depends on the purpose of preparing the extract. If the aim is to screen plants for the presence of antimicrobial compounds, the extractant should not inhibit the bioassay procedure. Acetone, methanol, dichloromethane, ethyl acetate, ethanol, hexane, and other solvents can be used for preparation of plant extracts for antimicrobial assays. The extracts can be re-dissolved in acetone, DMSO, or another solvent which is not toxic to the microbes at the concentrations tested. Acetone is often preferred for use in

antimicrobial assays due to its volatility, miscibility with polar and non-polar solvents, and its lack of toxicity to many microorganisms at the concentrations used in commonly-employed assays such as the serial micro-dilution assay for antibacterial activity (Eloff et al. 2007). Acetone, hexane, dichloromethane (DCM) and methanol are four solvents of varying polarities selected to be used as extractants for the present study, following considerations of availability of reagents, and cost and safety concerns (Yu et al. 2002b). This series of solvents was chosen for its potential ability to extract a range of active plant components from the plant material of interest. Moreover, extracts prepared using these solvents were tested during a preliminary screening of different plant species including *Terminalia* species (*T. pruniodes*, *T. sericea*, *T. gazensis* and *T. mollis*) and *Combretum* species (*C. erythrophyllum*, *C. molle* and *C. petrophilum*), yielding good results in antifungal assays (Masoko et al. 2005).

2.2 Materials and methods

2.2.1 Plant selection

The six species selected for the current study are *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana*. These plants were selected on the basis of good antifungal activity of acetone leaf extracts from the list of close to 400 tree species that have been screened for antimicrobial activity in the Phytomedicine laboratory (Pauw and Eloff, unpublished data). In this preliminary screening procedure, the acetone extracts of these six plant species had the best activity against the animal fungal pathogens *Candida albicans* and *Cryptococcus neoformans (*Table 2-1). The MIC values ranged between 0.03 and 0.08 mg/ml for *Breonadia salicina*, for example.

Table 2-1 Minimum inhibitory concentrations (MIC) of acetone extracts of six plant species against two animal fungal pathogens (Pauw and Eloff unpublished data)

Plant species	Family	Microorganism: average MIC (mg/ml)	
		Candida albicans	*Cryptococcus neoformans*
Bucida buceras L.	Combretaceae	0.07	0.13
Breonadia salicina (Vahl) Hepper and J.R.I	Rubiaceae	0.03	0.08
Harpephyllum caffrum Bernh.ex Krauss	Anacardiaceae	0.32	0.64
Olinia ventosa (L.) Cufod	Oliniaceae	0.05	0.64
Vangueria infausta (Burch.)	Rubiaceae	0.64	1.25
Xylotheca kraussiana Hochst	Flacourtiaceae	0.08	0.43

2.2.2 Plant collection

Plant leaves were collected from labelled trees growing in the Lowveld National Botanical Garden in Nelspruit, Mpumalanga during the summer. The tree labels indicated year of planting as well as the collection number from which the origin of the plants could be determined from the herbarium database. To ensure efficient drying, leaves were collected in open mesh orange bags and kept apart as long as possible.

2.2.3 Plant storage

Collected fresh plant material was examined and the old, insect- and fungus-infected leaves were removed. Leaves were dried at room temperature (c. 25ºC) in a forced air draught in a purpose-built drying machine. The dried plant material was ground to a fine powder using a laboratory grinding mill (Telemecanique/ MACSALAB model 200) and stored in airtight bottles in the dark until extraction.

2.2.4 Extraction Procedure

2.2.4.1 Laboratory extraction method

Separate samples of finely ground plant material (4 g) were extracted with 40 ml of solvents of different polarities: hexane, dichloromethane, acetone and methanol (technical grade-MERCK) in polyester plastic tubes, while shaking vigorously for 3-5 minutes on a Labotec model 20.2 shaking machine at high speed. After centrifuging at 3500 rpm (2310 x g) using a Hettich 32A centrifuge for 5 minutes, the supernatants were decanted into labelled, weighed glass vials. The process was repeated 3 times on the marc and the extracts were combined. The solvent was removed under a stream of cold air at room temperature. Upon re-suspending the extracts to the desired concentration prior to analysis and bioassay testing, the samples were sonicated in an ultrasonic bath (Bransonic 220) at room temperature for 5 minutes, enabling the solvents to dissolve the crude extracts.

2.2.5 Phytochemical analysis

Chemical constituents of the extracts were analyzed by thin layer chromatography (TLC) using aluminium-backed TLC plates (ALIGRAM®SIL g/UV 254-MACHEREY-NAGEL, Merck). The extracts were dried and weighed and then re-suspended in their extracting solvent to a known concentration (10 mg/ml). One hundred micrograms (100 µg) of plant extract were loaded in bands of approximately 1 cm in length on the TLC plates.
Duplicate TLC plates were developed using each of the three eluent systems developed by Kotze and Eloff (2002):

- Ethyl acetate: methanol: water = 40:5.4:4 [EMW] (polar)
- Chloroform: ethyl acetate: formic acid = 5:4:1 [CEF] (intermediate polarity/acidic)
- Benzene: ethanol: ammonia hydroxide = 90:10:1 [BEA] (non-polar/basic)

Development of the chromatograms was done in a closed tank in which the atmosphere had been saturated with the eluent vapour. Samples were applied quickly onto the TLC plates and developed without delay (as soon as the bands were dry) to minimize the possibility of photo-oxidative change. The separated components were visualized under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600). For the detection of chemical

compounds not visible under UV light, vanillin-sulphuric acid spray reagent (1 g vanillin: 28 methanol: 1 ml sulphuric acid) was used for identification (Stahl 1969, Wagner and Bladt 1996).

2.2.6 Retention factor (R_f) values of compounds

Characterization of different compounds can be facilitated by measuring R_f values in different TLC systems. Before calculating the R_f values of separated compounds for each plant species, the solvent front was marked on the TLC plate and the plate allowed to dry before visualizing the bands relating to different compounds under UV light. The R_f value is given by the following equation:

$$R_f \text{ value} = \frac{\text{distance moved by the component from the origin to spot centre}}{\text{distance moved from origin to solvent front}}.$$

2.3 Results and discussion

2.3.1 Extraction using different solvents

The total percentages of plant material extracted from the six selected plant species using four different extractants (acetone, hexane, dichloromethane, and methanol) are given in Figure 2-1. Dichloromethane was the best solvent in terms of mass extracted from *B. buceras*, extracting 12.0% compared to acetone (11.75%) and methanol (11.3%). Hexane was the least effective, extracting 8.8% from the same plant. Acetone and hexane extracted the most material from *B. salicina* (8.5%), while DCM and methanol extracted less with 7.8% and 6.5%, respectively. On the other hand, methanol extracted 11.5% of *H. caffrum*, followed by acetone (11%), DCM (9.8%) and hexane with 8.5%. Acetone extracted more of the material (10.3%) of *O. ventosa* followed by DCM (9.8%), hexane (8.3%) and methanol (7.5%). Hexane extracted most material from *V. infausta* (12.3%), followed by methanol (12%), DCM (10.3%) and acetone (11.7%). Acetone extracted the most material (11.7%) from *X. kraussiana* followed by methanol (11.5%), hexane (8.8%) and DCM (8.5%). Acetone extracted the same percentage of material from *B. buceras*, *V. infausta* and *X. kraussiana* with

11.7%, and methanol extracted 11.5% from *H. caffrum* and *X. kraussiana*. However, methanol extracted the least quantity from *B. salicina* (6.5%) compared to the other plants. The different quantities extracted by the different solvents reflect the presence of non-polar relative to polar compounds in the different plants.

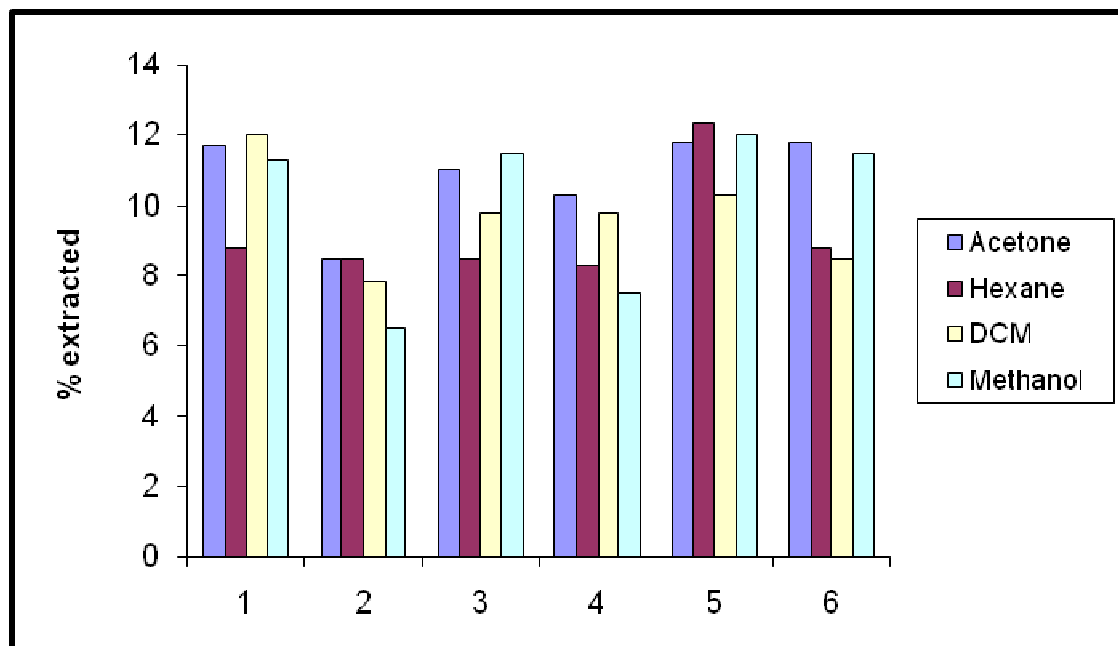

Figure 2-1 Percentage of material extracted from 4g of leaves of (1) *Bucida buceras*, (2) *Breonadia salicina*, (3) *Harpephyllum caffrum*, (4) *Olinia ventosa*, (5) *Vangueria infausta* and (6) *Xylotheca kraussiana* with different extractants

In general, acetone extracted the largest quantity of plant material (average 108.3 mg), followed by methanol (100.4 mg), DCM (96.7 mg) and then hexane (91.7 mg) (Table 2-2). Acetone extracted the same amount of plant material (117.5 mg) from *B. buceras*, *V. infausta* and *X. kraussiana*. Methanol and DCM extracted more plant material from *B. buceras* and *V. infausta* (120 mg) compared to the other four plants. However, DCM extracted the same amount of plant material from *H. caffrum* and *O. ventosa* (97.5 mg). Acetone was therefore the best extractant compared to the other solvents based on the quantity of plant material extracted. Methanol was the second best extractant in terms of quantity of plant material extracted despite recording only 65.0 mg from the extract of *B. salicina* compared to 85.0 mg of acetone extract.

Table 2-2 Quantity in mg extracted from 1 g of powdered leaf material of different species by acetone [A], hexane [H], dichloromethane [D] and methanol [M]

Plant species	Quantity extracted (mg)				
	A	H	D	M	Average (mg)
Bucida buceras	117.5	87.5	120.0	112.5	109.4
Breonadia salicina	85.0	85.0	77.5	65.0	78.1
Harpephyllum caffrum	110.0	85.0	97.5	115.0	101.9
Olinia ventosa	102.5	82.5	97.5	75.0	89.4
Vangueria infausta	117.5	122.5	102.5	120.0	115.6
Xylotheca kraussiana	117.5	87.5	85.0	115.0	101.3
Average	108.3	91.7	96.7	100.4	99.3

2.3.2 Phytochemical analysis of extracts

2.3.2.1 TLC analysis of plant extracts for preliminary screening

In this study, the TLC chromatograms were developed in three solvent systems of different polarity, BEA (non-polar solvent system), CEF (intermediate polar) and EMW (polar). Thin layer chromatography is a rapid and effective means of obtaining a characteristic analytical fingerprint of a plant extract (Wagner and Bladt 1996). This technique is used to show the differences in chemical composition of plant extracts.

The TLC chromatograms of extracts of the six plant species under study are shown in Figure 2-2. Comparing the chemical profiles, TLC chromatograms developed in BEA and sprayed with vanillin-sulphuric acid showed no compounds in all extracts of *B. buceras*, *B. salicina*, *H. caffrum*, *O. ventosa*, *V. infausta* and *X. kraussiana*. However, from TLC chromatograms developed in CEF, compounds with R_f value of 0.60 were observed in the acetone, dichloromethane and methanol extracts of *B. buceras*, *B. salicina* and *H. caffrum*. In the EMW separation system, only compounds with R_f values of 0.08 and 0.21 were visible under UV light with wavelength of 254 and 365 nm in the acetone and methanol extracts of *B. salicina*, while the acetone extract of *O. ventosa* and *V. infausta* showed a compound with the

same R_f value of 0.73. No compounds were observed in TLC chromatograms developed in CEF and EMW solvent systems for extracts of *X. kraussiana* under UV light. However, after spraying with vanillin-sulphuric acid, compounds were visible in all four extracts (Figure 2-2). In the next chapter, bioautography will be used to determine the number and properties of active compounds present in the extracts discussed above.

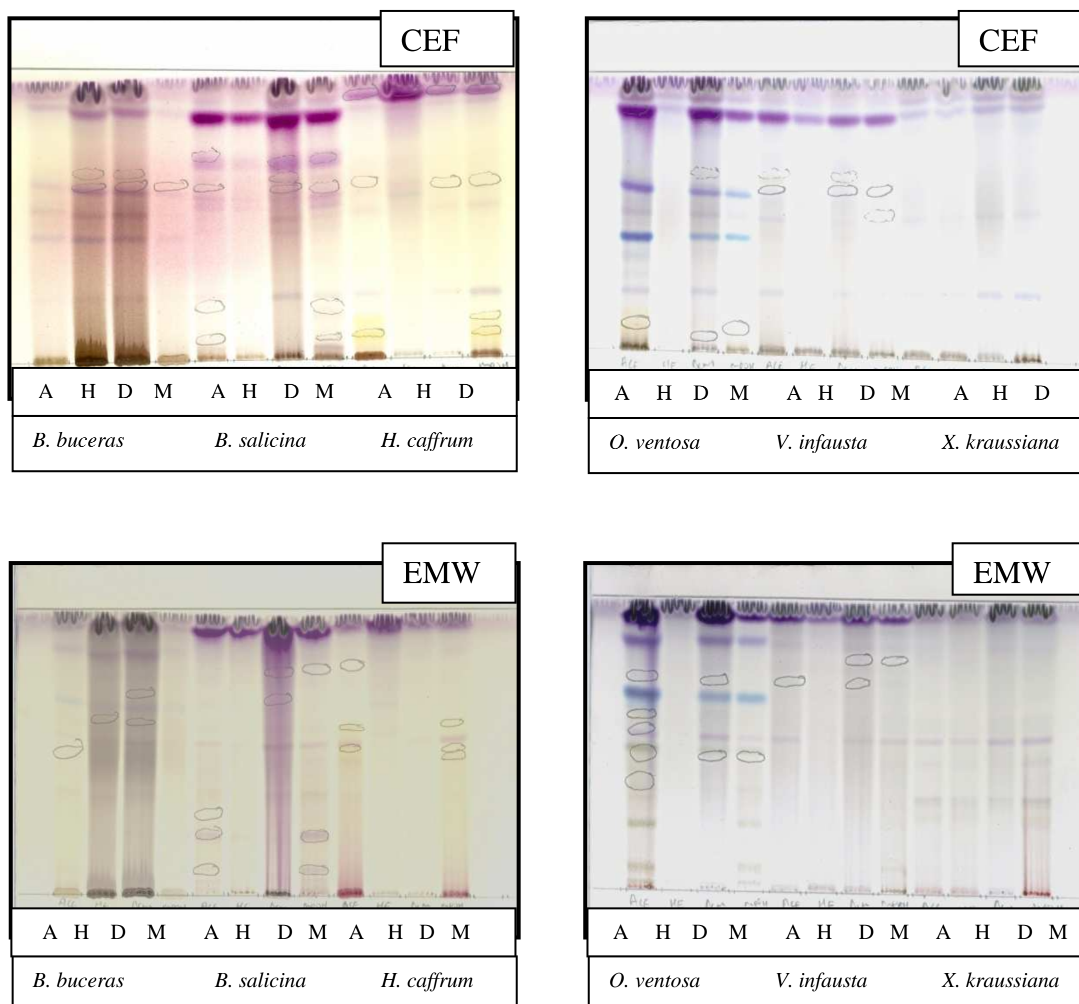

Figure 2-2 Chromatograms developed with CEF (top) and EMW (bottom) solvent systems, sprayed with vanillin-sulphuric acid.

Table 2-3 R_f values of compounds separated in CEF and EMW, extracted by A (acetone), H (hexane), D (dichloromethane) and M (methanol). The compounds were visualized under visible light, UV light 254 or 365 and sprayed with vanillin-sulphuric acid.

Plant species	Solvent system	Solvents	R_f values of compounds
	CEF		
B. buceras		A	0.48
		H	0.60 0.64
		D	0.60 0.64
		M	0.60
	EMW	A	0.48
		H	0.61
		D	0.61 0.70
		M	-
B. salicina	CEF	A	0.06 0.18 0.60 0.69
		H	-
		D	0.60 0.64 0.68
		M	0.06 0.18 0.60 0.64
	EMW	A	0.08 0.21 0.27
		H	-
		D	0.69 0.78
		M	0.08 0.21 0.78
H. caffrum	CEF	A	0.06 0.18 0.60 0.89
		H	-
		D	0.60

Table 2-3 continued. R_f values of compounds separated in CEF and EMW, extracted by A (acetone), H (hexane), D (dichloromethane) and M (methanol). The compounds were visualized under visible light, UV light 254 or 365 and sprayed with vanillin-sulphuric acid

Plant species	Solvent system	Solvents	R_f values of compounds
	CEF	M	0.06 0.13 0.60 0.89
	EMW	A	0.52 0.58 0.78
		H	-
		D	-
		M	0.48 0.52 0.59
O. ventosa	CEF	A	0.08
		H	-
		D	0.07 0.61
		M	0.08
	EMW	A	0.37 0.46 0.54 0.61 0.73
		H	-
		D	0.46 0.73
		M	0.46
V. infausta	CEF	A	0.57 0.64
		H	-
		D	0.57 0.64
		M	0.49 0.57
	EMW	A	0.73
		H	-
		D	0.73 0.79
		M	0.79

2.4 Conclusion

Acetone and methanol were the best extractants with respect to the amount of plant material extracted (averages of 108.3 and 104 mg respectively). The number of compounds present in some of the plants was also the highest in extracts prepared from acetone and methanol. Further studies will be carried out using these two solvents. In BEA (a non-polar solvent system), no compounds were visible in the TLC separations of the extracts of the six plant species but separated compounds were observed in CEF and EMW (non-polar and polar). The lack of visible compounds in the BEA system is probably because the active compounds were relatively polar, since they did not separate well in BEA (non-polar). The results showed that TLC chromatograms separated with CEF and EMW shared the same number of compounds (total of ten) in extracts of *B. buceras* and *V. infausta* while more compounds (a total of nineteen) were visible in extracts of *B. salicina* (CEF separated 11 and EMW 8 compounds). Further TLC studies will be carried out using these two solvent systems (CEF and EMW). In the next chapter I will investigate in-depth the antifungal activity from leaf extracts of six plant species against seven plant pathogenic fungi.