Characterization and biological activity of antifungal compounds present in *Breonadia salicina* (Rubiaceae) leaves

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DECLARATION

I **Salome Mamokone Mahlo,** hereby declare that this thesis submitted to the University of Pretoria for the degree of Doctor Philosophiae is the result of my own investigations in execution and has never been submitted at any other university or research institution. Any help I received is acknowledged in the thesis.

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DEDICATION

This work is dedicated to my daughter Dunisani Maashaba Mahlo and my parents Wilson and Maria Mahlo for their support throughout my studies.

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Psalm 23:4 Even though I walk through the valley of the shadow of death, I will fear no evil, for you are with me; your rod and your staff, they comfort me.

LIST OF ABBREVIATIONS

Amph B	Amphotericin B
BEA	Benzene: ethanol: ammonia
CC	Column chromatography
CEF	Chloroform: ethyl acetate: formic acid
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl
EIMS	Electron impact mass spectrometry
EMW	Ethyl acetate: methanol: water
EtOAc	Ethyl acetate
INT	p-Iodonitrotetrazolium violet
IPUF	Indigenous Plant Use Forum
LC_{50}	Lethal Concentration for 50% of the cells
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MS	Mass spectrometer
MTT	(3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear magnetic resonance
R_f	Retention factor
SDA	Sabouraud Dextrose Agar
TLC	Thin Layer Chromatography

ABSTRACT

The aim of this study was to investigate plant species to develop a product with the potential of protecting plants or plant products against plant fungal pathogens. Hexane, dichloromethane, acetone, and methanol leaf extracts of six plant species (*Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana*) were evaluated for antifungal activity against seven plant fungal pathogens (*Aspergillus niger*, *A. parasiticus*, *Colletotrichum gloeosporioides*, *Penicillium janthinellum*, *P. expansum*, *Trichoderma harzianum* and *Fusarium oxysporum*). These plant species were selected from more than 400 plant species evaluated in the Phytomedicine Programme that had good activity against two animal fungal pathogens. All the leaf extracts were active against at least one or more of the phytopathogenic fungi in a serial microdilution assay. Of the six plant species, *B. buceras* had the best antifungal activity against four of the fungi, with MIC values as low as 0.02 mg/ml and 0.08 mg/ml against *Penicillium expansum*, *P. janthinellum*, *Trichoderma harzianum* and *Fusarium oxysporum*.

The number of active compounds in the plant extracts was determined using bioautography with the above-mentioned plant pathogens. No active compounds were observed in some plant extracts against the fungal plant pathogens indicating possible synergism between metabolites responsible for the antifungal activity of the extract. *B. salicina* and *O. ventosa* were the most promising plant species, with at least three antifungal compounds.

The antioxidant activities of plant extracts were determined using the qualitative method by spraying TLC chromatograms developed in three eluent systems BEA, CEF and EMW with 1, l-diphenyl -2 picrylhydrazyl (DPPH). The plant extracts of five of these species did not have a strong antioxidant activity. The methanol extract of *X. kraussiana* was the most active radical scavenger in the DPPH assay amongst the six medicinal plants screened.

Based on good activity against *Aspergillus niger* and *A. parasiticus*, leaf extracts of the six plant species were also tested for antifungal activity against *A. fumigatus*, a very important animal fungal pathogen. The acetone extracts of *B. buceras*, *B. salicina*, *V. infausta* and *X. kraussina* had good antifungal activity against the animal pathogens, with MIC values ranging between 0.02 and 0.08 mg/ml. This indicates that crude extracts of these species may be more

valuable in combating *Aspergillus* infections in animals than in humans. Based on the results discussed above, *B. salicina* was selected for in-depth study.

Serial exhaustive extraction was used to extract plant material with solvents of increasing polarities namely, hexane, chloroform, acetone and MeOH. Amongst the four extractants, MeOH extracted the largest quantity of plant material 12.3% (61.5g), followed by acetone 5.6% (27.8 g), hexane 2.6% (12.8 g) and chloroform 2.1% (10.3 g). The chloroform fraction was selected for further work because it had the best antifungal activity against *A. niger*, *C. gloeosporioides*, *P. janthinellum* and *T. harzianum* and the bioautography assay showed the presence of several antifungal compounds in the chloroform fraction.

Column chromatography was used in a bio-assay guided fractionation and led to isolation of four compounds. The antimicrobial activity was determined against seven plant pathogenic fungi and three bacteria, including the Gram-positive *Staphylococcus aureus* (ATCC 29213) and the Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aureus* (ATCC 27853). The isolated compounds had good antifungal activity against *A. parasiticus* with an MIC of 10 µg/ml, while in other cases it ranged from 20 to 250 µg/ml. Amongst the four compounds tested, only three had a clear band, indicating that the growth of the pathogenic fungi was inhibited in the bioautography assay.

Nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS) were used for identification of isolated compounds. Only one compound was identified as the triterpenoid ursolic acid. Ursolic acid has been isolated from several plant species and has antifungal activity against *Candida albicans* (Shai et al. 2008). This is the first report on the isolation of antifungal compounds from leaves of *Breonadia salicina*. The other compounds isolated appeared to be mixtures of fatty acids based on mass spectroscopy and the structures were not elucidated.

The cytotoxicity of acetone extracts and the four isolated compounds were determined against Vero cells using a tetrazolium-based colorimetric (MTT) assay. The acetone extract was selected based on good *in vitro* antifungal activity and was used in an *in vivo* fruit experiment. The acetone extract was less toxic toward the Vero cells with an LC_{50} of 82 µg/ml than

ursolic acid and compound 4 which had LC_{50} values of 25 and 36 µg/ml respectively. Compounds 2 and 3 had low toxicity against the cells with LC_{50} values greater than 200 µg/ml.

The potential use of the extract or isolated compound(s) against three plant fungal pathogens *Penicillium expansum* and *P. janthinellum* as well as *P. digitatum* (isolated from infected oranges) were tested after treating the oranges with the extract and ursolic acid. The model used gave good reproducible results. The concentration that inhibited growth correlated reasonably well with MIC values determined by serial microplate dilution. There were substantial differences in the susceptibility of the different isolates tested. The activity of ursolic acid was in the same order as that of the crude acetone leaf extract of *B. salicina*. The LC_{50} of the extract varied from 1 to 1.8 mg/ml.

Penicillium digitatum was more resistant to amphotericin B in comparison to other *Penicillium* species. It has been reported that the fungus was resistant to the three fungicides: sodium *o*-phenylphenate (*o*-phenylphenol), imazalil, and thiabendazole used commercially in the fruit industry to reduce postharvest decay (Holmes and Eckert 1999).

The toxicity of the extract to Vero cells was in the order of 10 times lower than the LC_{50} of the extracts to the fungal pathogens. Although much work still has to be done, there is good potential that a commercial product can be developed from an acetone leaf extract of *B. salicina* leaves, especially if the activity of this extract can be improved by removing inactive compounds.

The results confirm the traditional use of *B. salicina* and demonstrate the potential value of developing biopesticides from plants.

CONFERENCES AND WORKSHOP

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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.

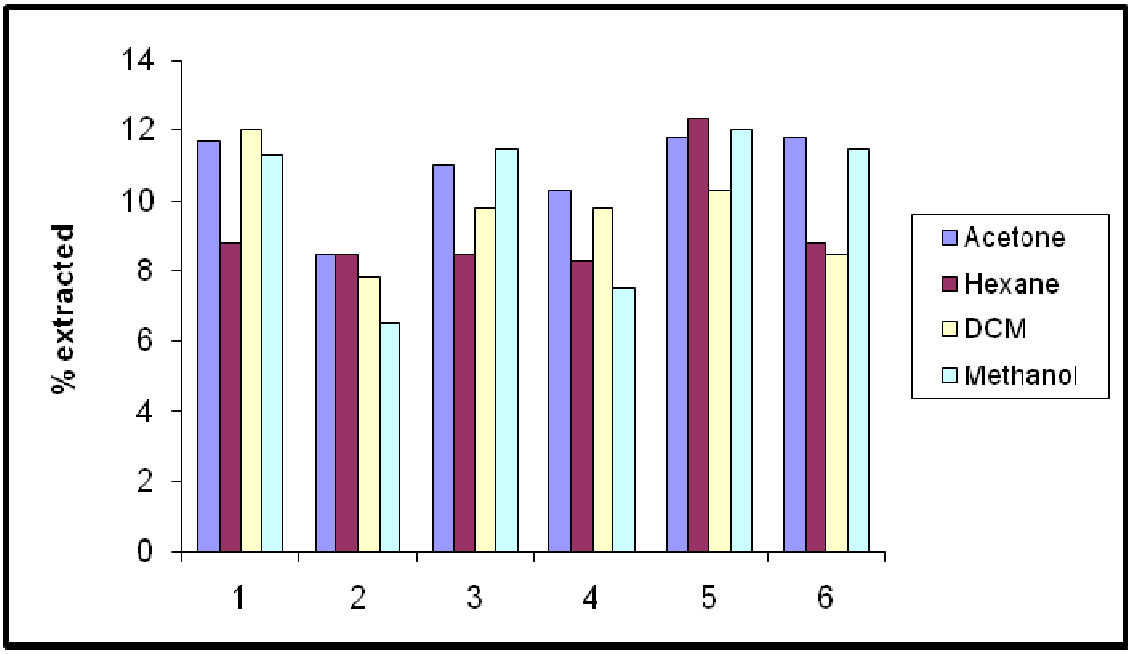

Figure 2-1 Percentage of material extracted from 4g of leaves of (1) *Bucida buceras*, (2) *Breonadia salicina*, (3) *Harpephyllum caffrum*, (4) *Olinia ventosa*, (5) *Vangueria infausta* and (6) *Xylotheca kraussiana* with different extractants

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.

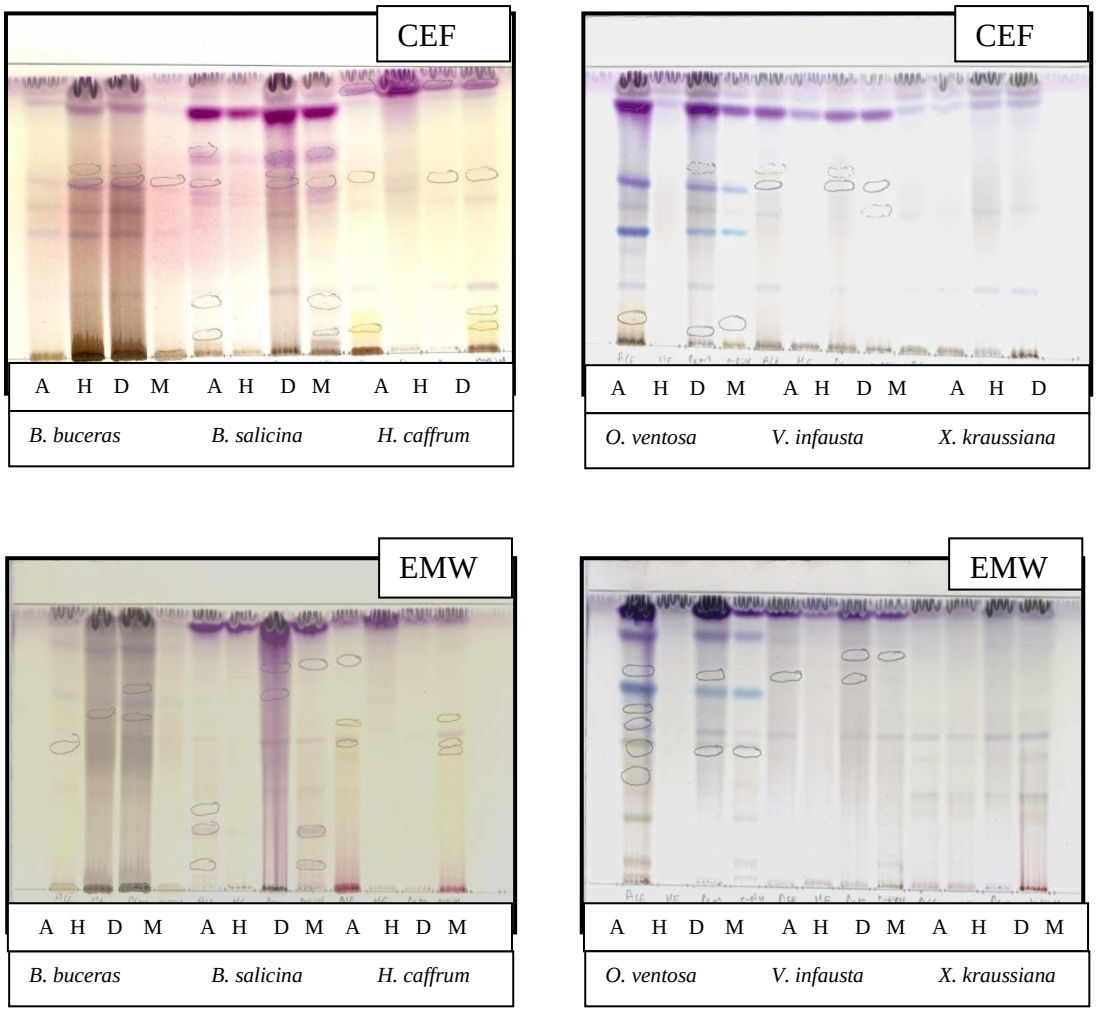

Figure 2-2 Chromatograms developed with CEF (top) and EMW (bottom) solvent systems, sprayed with vanillin-sulphuric acid.

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.

CHAPTER 3

Preliminary screening for antifungal activity of six selected plant species

3.1 Introduction

Antimicrobials are compounds that at low concentrations exert an action against microorganisms and exhibit therapeutic toxicity towards them (Goodyear and Threlfall 2004). These can be any substances of natural, synthetic or semi-synthetic origin that may be used to kill microorganisms including bacteria, fungi, protozoa and viruses (Yazaki 2004). The antimicrobial activity of different plant extracts can be detected by observing the growth of various microorganisms that have been placed in contact with extracts of the plants. If the plant extracts inhibit the growth of the test organism, and general toxic effects are not present, then the plant can potentially be used to combat diseases caused by the pathogens. The antimicrobial activities of plant extracts have formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Lis-Balchin and Deans 1997, Reynolds 1996).

There are several assays that can be used to determine antimicrobial activity in plant extracts, including agar diffusion, bioautography (direct, contact and overlay) and microplate assays (serial dilution assay). The agar diffusion assay is, in general, only suitable for aqueous extracts and can also be used to test up to six extracts per Petri dish against a single microorganism. However, the diffusion method is not suitable for testing non-polar samples or samples that do not easily diffuse into the agar (Cos et al. 2006).

The bioautography assay is used to detect active compounds in a crude plant extract (Cos et al. 2006). An inoculated layer of agar is poured over a developed thin layer chromatography (TLC) plate, and lack of bacterial or fungal growth in certain areas identifies the presence and location of antibacterial compounds on the TLC plate. On the other hand, TLC plates can also be sprayed with a fine suspension of bacteria or fungi and then sprayed with an indicator tetrazolium salt. The inhibition of fungal growth by compounds separated on the TLC plate is visible as white spots against a deep red background (Begue and Kline 1972). In the direct bioautography technique, the microorganism sprayed on the TLC plates will grow directly on

the chromatograms, while in contact bioautography, the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact. In overlay bioautography the agar is applied directly on the TLC plates and this can also be used with microorganisms that grow slowly (Hamburger and Cordell 1987, Rahalison et al. 1991). The advantage of using bioautography is that it can locate separated active compounds easily and also supports a quick search for antimicrobial agents through bioassay-guided isolation. However, there are problems associated with the assay, for example TLC eluent solvents with low volatility such as *n*-butanol (BUOH) and ammonia need to be allowed to evaporate completely so that they cannot inhibit the growth of the microorganism (Cos et al. 2006). The time taken for this may increase the risk of decomposition of active compounds.

In serial dilution assays, plant extract is mixed with water or broth in 96-well microplates and then fungal or bacterial cultures are added to the wells. The minimum inhibitory concentration (MIC) is recorded as the lowest concentration of plant extract resulting in inhibition of fungal growth, shown by a reduction in the red colour of the tetrazolium salt added as an indicator. Dilution techniques require a homogenous dispersion of the sample in water. They are used to determine, principally, the MIC values of an extract or pure compound. In the liquid dilution method, turbidity is often taken as an indication of growth, so where the sample is inactive against the microorganism tested, the liquid will appear turbid (Rios et al. 1998). The assay is quick, and works well with different microorganisms and non-aqueous extracts from different plant species. Moreover, it gives precise, reproducible results and requires just a small volume of extract to determine the minimal inhibitory concentration (MIC) for each bacterial test species against each plant extract or isolated compound. It suffers from one major drawback in that some compounds present in plant extracts may precipitate in the presence of the bacterial growth medium, making it difficult or impossible to use turbidity as a measure of microbial growth. This problem was resolved by adding *p*-iodonitrotetrazolium to the extract and microbial suspension. In the presence of microbial growth this compound is changed to a violet-coloured formazan (Eloff 1998b).

The above-mentioned assays differ in principle, and antimicrobial assay results are in general influenced by the type of assays used (Cos et al. 2006). It is necessary for bioassays to be as simple as possible; in this way sufficiently large numbers of different tests may be performed

so that many biological properties can be screened (Hostettman 1999). In this chapter, serial dilution and bioautography assays will be used to determine antimicrobial activity of the six plant species under investigation.

3.2 Materials and methods

3.2.1 Fungal strains

The seven test fungal species, *Aspergillus niger*, *A. parasiticus*, *Colletotrichum gloeosporioides*, *Trichoderma harzianum*, *Penicillium expansum*, *P. janthinellum* and *Fusarium oxysporum*, were obtained from the Department of Microbiology and Plant Pathology at the University of Pretoria. These fungi are among the most important pathogenic fungi of economic significance to plants. Fungal strains were maintained on Potato Dextrose (PD) agar. Fungal cultures were subcultured (1% inoculum) in PD broth at 35°C for at least two to four days before being used in the screening assays.

3.2.1.1 Quantification of fungal inoculum

For quantification of fungi, the haemocytometer cell-counting method described by Aberkane et al. (2002) with some modifications was used for counting the number of cells for each fungal culture. The inoculum of each isolate was prepared by first growing the fungus on PD agar slants for 7 days at 35ºC. The slant was rubbed carefully with a sterile cotton swab and transferred to a sterile tube with fresh PD broth (50 ml). The sterile tubes were then shaken for five minutes and appropriate dilutions were made in order to determine the number of cells by microscopic enumeration using a haemocytometer (Neubauer chamber; Merck S.A.). The final inoculum size was adjusted to approximately 1.0×10^{6} cells/ml. To confirm the inoculum adjustment, 100 µl of serial dilutions of the conidial suspensions was spread onto PD agar plates. The plates were incubated at 35ºC and observation of the presence of fungal growth was done daily. The colonies were counted after the observation of visible growth and used to calculate the corresponding cells/ml.

3.2.2 Bioassays for antifungal activity

3.2.2.1 Dilution method

The serial microplate dilution method of Eloff (1998b), modified for antifungal activity testing by Masoko et al. (2005), was used to determine the MIC values for plant extracts of *B. buceras*, *B. salicina*, *H. caffrum*, *O. ventosa* and *V. infausta*. The plant extracts were tested in triplicate in each assay, and the assays were repeated once in their entirety to confirm results. Residues of different extracts were dissolved in acetone to a concentration of 10 mg/ml. The plant extracts (100 µl) were serially diluted 50% with water in 96 well microtitre plates (Eloff 1998c), and 100 µl of fungal culture was added to each well. Amphotericin B was used as the reference antibiotic and 100% acetone as the negative control. As an indicator of growth, 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet (INT) dissolved in water was added to the microplate wells. The covered microplates were incubated for three to five days at 35ºC at 100% relative humidity after sealing in a plastic bag to minimize fungal contamination in the laboratory. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth. The colourless tetrazolium salt acts as an electron acceptor and was reduced to a red-coloured formazan product by biologically active organisms (Eloff 1998b). Where fungal growth is inhibited, the solution in the well remains clear or shows a marked reduction in intensity of colour after incubation with INT.

In order to determine which plants can be used for further testing, not only the MIC value is important, but also the total activity. Since the MIC value is inversely related to the quantity of antifungal compounds present, the quantity of antifungal compounds present was calculated by dividing the quantity extracted in milligrams from 1g leaves by the MIC value in mg/ml. The total activity is used to determine to what volume an extract from 1 g of plant material can be diluted and still inhibit the growth of the test organism (Eloff 1999). It can also be used to evaluate losses during isolation of active compounds and the presence of synergism (Eloff 2004).

The total activity can be calculated as:

$$\text{Total activity} = \frac{\text{Quantity of material in mg extracted from 1 g of plant material}}{\text{Minimum inhibitory concentration (mg/ml)}}.$$

In the case of bioassay guided fractionation, the total activity in the crude extract and fractions can be calculated by dividing the mass in mg in the fraction with the MIC in mg/ml. Total activity in this case [x ml/fraction] provides an indication of the volume to which the crude extract or fraction can be diluted and still kill the microorganism.

3.2.2.2 Bioautography

TLC plates (10 × 10 cm) were loaded with 100 µg of each of the extracts with a micropipette. The prepared plates were each run using different mobile systems: CEF, BEA and EMW. The chromatograms were dried at room temperature under a stream of air overnight or up to five days until the remaining solvent were removed. Fungal cultures were grown on Potato Dextrose agar for 3 to 5 days. Cultures were transferred into PD broth from agar with sterile swabs. The developed TLC plates were sprayed with concentrated suspension containing c. 1.0×10^6 cells/ml of actively growing fungi. The plates were sprayed until they were wet, incubated overnight and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet and further incubated overnight or longer at 35ºC in a clean chamber at 100% relative humidity in the dark. White areas indicated where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the tested fungi. The plates were sealed in plastic to prevent the spreading of the fungi in the laboratory and to retain the humidity and then scanned to produce a record of the results.

3.3 Results and discussion

3.3.1 Quantification of fungal inoculum

The number of fungal cells in the two diagonally opposite corner grids of the haemocytometer were counted and averaged. If the cell number was more than 100, a calculated volume of fresh broth was added to obtain an approximate average of 100 cells. Hence, the cell

concentration for use in the bioassay was maintained at 100×10^4 cells/ml = 1.0×10^6 cells/ml. The same procedure was used for all other tested fungal species under study.

3.3.2 Microplate dilution assay

Plant pathogenic fungi were used as test organisms for testing antifungal activity of extracts of the six selected plant species (*B. buceras*, *B. salicina*, *H. caffrum*, *O. ventosa*, *V. infausta* and *X. kraussiana*). Extracts using solvents of different polarities (acetone, hexane, dichloromethane and methanol) were prepared from the six selected plants. Hexane, DCM and methanol extracts were re-dissolved in acetone since acetone was reported not to be toxic to microorganisms at the concentrations used in the assay (Masoko et al. 2007). The extracts were tested for antifungal activity against seven fungal species: *Aspergillus niger*, *Aspergillus parasiticus*, *Colletotrichum gloeosporioides*, *Penicillium janthinellum*, *Penicillium expansum*, *Trichoderma harzianum* and *Fusarium oxysporum*. The minimum inhibitory concentration (MIC) results presented in Table 3-1 indicate that plant pathogens are more susceptible than animal pathogens in this case. The plant extracts were tested in a preliminary screening test against two animal pathogens, *Candida albicans* and *Cryptococcus neoformans*, and the lowest MIC values obtained were 0.03 mg/ml.

Hexane and methanol extracts of *B. salicina* had the best activities against the three most sensitive organisms, *P. janthinellum*, *T. harzianum* and *F. oxysporum*. The acetone and methanol extracts of *H. caffrum* had good antifungal activity against the three most sensitive test organisms (with MIC values ranging between 0.02 and 0.08 mg/ml). The DCM extract of *H. caffrum* had lower activity against *T. harzianum* (MIC = 0.63 mg/ml). Four extracts of *O. ventosa* had the best activity against *T. harzianum* (MIC values of 0.04 and 0.08 mg/ml). The acetone and DCM extracts of *V. infausta* also had activity against *A. parasiticus* and *T. harzianum* (MIC values of 0.16 mg/ml). All extracts of *O. ventosa* had the highest activity compared to the other plant extracts against the tested fungi. On the basis of these results *B. salicina* and *O. ventosa* were selected for further investigation in the next chapter.

Table 3-1 Minimum inhibitory concentration (MIC) of six plant species against plant pathogenic fungi (values duplicate SD = 0)

Micr[1]	MIC (mg/ml)																									
	Bucida buceras					*Breonadia salicina*				*Harpephyllum caffrum*				*Olinia ventosa*				*Vangueria infausta*				*Xylotheca kraussiana*					Amp
	Time[2]	Extractant[3]																								
	(h)	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	Ave	
A. p	24	0.63	1.25	1.25	1.25	0.63	2.50	0.63	1.25	1.25	2.50	1.25	0.63	1.25	1.25	0.63	1.25	1.25	2.50	0.63	0.63	0.16	0.63	0.32	0.32	1.06	0.02
A. n	48	0.63	2.50	2.50	1.25	2.50	1.25	1.25	2.50	1.25	2.50	1.25	0.63	1.25	2.50	1.25	2.50	1.25	2.50	2.50	2.50	0.63	2.50	1.25	1.25	1.63	0.02
C. g	48	0.63	2.50	2.50	0.63	1.25	2.50	1.25	1.25	1.25	0.63	1.25	2.50	1.25	2.50	1.25	2.50	1.25	2.50	2.50	2.50	1.25	2.50	0.63	0.63	1.54	<0.02
P. e	24	0.08	0.63	0.32	0.32	1.25	2.50	2.50	2.50	2.50	1.25	2.50	0.63	0.32	1.25	1.25	0.63	2.50	1.25	1.25	1.25	0.63	2.50	1.25	1.25	1.27	<0.02
P. j	48	0.02	0.08	0.32	0.02	0.08	0.08	0.08	0.08	0.04	0.32	0.16	0.08	0.08	0.32	0.16	0.32	0.63	0.63	0.63	0.32	0.32	0.63	0.63	0.63	0.28	<0.02
T. h	48	0.02	0.08	0.63	0.02	0.63	0.32	0.63	0.63	0.08	0.63	0.63	0.08	0.04	0.04	0.04	0.08	0.32	0.32	0.32	0.32	0.16	0.32	0.16	0.32	0.28	<0.02
F. o	24	0.02	0.63	0.32	0.04	0.32	0.08	0.16	0.16	0.02	0.32	0.16	0.04	0.08	0.63	0.32	0.16	0.32	0.63	0.32	0.32	0.63	0.32	0.32	0.32	0.28	<0.02
Ave		0.29	1.02	1.05	0.5	0.92	1.25	0.89	1.12	0.88	1.09	0.99	0.62	0.61	1.14	0.70	0.99	1.04	1.37	1.09	1.05	0.54	1.23	0.65	0.67	0.91	

[1]Microorganisms: *A. p. = Aspergillus parasiticus*, *A. n. = Aspergillus niger*, *C. g. = Colletotrichum gloeosporioides*, *P. e. = Penicillium expansum*, *P. j. = Penicillium janthinellum*, *T. h. = Trichoderma harzianum* and *F. s. = Fusarium oxysporum*

[2]Time: MIC values after 24 h were sometimes not distinct, so the plates were left to incubate for a further 24 h before MIC was read

[3]Extractant: A = acetone, H = hexane, D = dichloromethane, M = methanol

The total activity values of the six plant species extracted with acetone, hexane, dichloromethane and methanol are given in Table 3-2. The highest total activity was found in the acetone extract of *H. caffrum* (22 000 ml/g) against *F. oxysporum*. The lowest total activity was observed in the methanol extract of *O. ventosa* (133 ml/g) against both *A. niger* and *C. gloeosporioides*. These observations are consistent with the variation of the MIC values in Table 3-2, that is, where the MIC value is low, the total activity is high.

Table 3-2 Total activity in ml/g of six plant species extracted with acetone, hexane, dichloromethane and methanol tested against seven fungi

Micr	Total activity (ml/g)																							
	Bucida buceras					*Breonadia salicina*				*Harpephyllum caffrum*				*Olinia ventosa*				*Vangueria infausta*				*Xylotheca kraussiana*				
	Time	Extractant[2]																						
	(h)	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	Ave
A. p	24	746	140	762	360	540	272	248	208	352	151	312	730	328	264	619	240	376	218	651	762	2938	556	1063	1438	595
A. n.	48	209	280	384	200	540	151	138	208	352	151	312	730	328	147	312	133	376	218	182	213	746	156	272	368	296
C g.	48	376	155	384	360	540	151	138	413	352	540	312	204	328	147	312	133	376	218	182	213	376	156	540	730	318
P. e.	24	376	156	213	200	4250	540	969	813	196	272	173	730	1281	264	312	476	209	392	328	384	746	156	272	368	587
P. .j	48	5875	4375	6000	5625	17000	4250	969	13000	11000	1063	2438	5750	5125	1031	2438	938	746	777	651	1500	1469	556	540	730	3910
T. .h	48	746	1094	762	714	17000	4250	492	13000	5500	540	619	5750	10250	8250	9750	3750	1469	1531	1281	1500	2938	1094	2125	1438	3993
F. o	24	1467	4375	3000	2813	17000	540	969	6500	22000	10625	2438	11500	5125	524	1219	1875	1469	778	1281	1500	746	1094	1063	1438	4222
Ave		1400	1510	1644	1467	8124	1451	560	4877	5679	1906	943	3628	3252	1518	2137	1078	717	590	651	868	1423	538	839	930	1989

[1]Microorganisms: *A. p.* = *Aspergillus parasiticus*, *A. n.* = *Aspergillus niger*, *C. g.* = *Colletotrichum gloeosporioides*, *P. e.* = *Penicillium expansum*, *P. j.* = *Penicillium janthinellum*, *T. h.* = *Trichoderma harzianum* and *F. s.* = *Fusarium oxysporum*.

[2]Extractant: A = acetone, H = hexane, D = dichloromethane, M = methanol

Acetone extracts in general had the lowest average MIC value of 0.71 mg/ml against the tested microorganisms, followed by DCM extracts (0.83 mg/ml), methanol extracts (0.89 mg/ml) and finally hexane extracts (1.19 mg/ml) as shown in Table 3-3. Plant extracts with low MIC values could be a good source of bioactive components with antimicrobial potency. In particular, acetone extracts have shown potentially interesting activity compared to extracts prepared using other solvents. Based on the MIC results, acetone was the best extractant, and additional positive features include its volatility, miscibility with polar and non-polar solvents and its relative low toxicity to test organisms (Eloff 1999).

In the current study, negative controls showed that acetone alone was not harmful to the plant pathogens at the highest percentage tested, confirming previous results (Eloff et al. 2007). However, plant extracts are traditionally prepared with water as infusions, decoctions and macerations. Therefore, it would be difficult for the traditional healer to be able to extract those compounds which are responsible for activity in the acetone and methanol extracts (Aliero and Afolayan 2005). Many traditional healers use water to extract plant material, since water is not toxic, not expensive and is the only extractant available. In some cases animal fat is mixed with plant material and under these conditions the non-polar compounds could become available.

Table 3-3 Average MIC values (mg/ml) of extracts prepared with different extractants on all test organisms

Extractants	MIC
Acetone	0.71
Hexane	1.19
Dichloromethane	0.89
Methanol	0.83

Out of the seven plant pathogens used, only three fungi (*P. janthinellum*, *T. harzianum* and *F. oxysporum*) had significant sensitivity to the plant extracts (average MIC values of 0.28 mg/ml, Table 3-4). *Fusarium oxysporum* was reasonably sensitive. This fungus has been reported to cause vascular wilt and damping off in plants which could result in substantial stand reduction and yield (Kishi 1974). The results showed that the other four fungi

(*Aspergillus niger*, *Aspergillus parasiticus*, *Colletotrichum gloeosporioides* and *Penicillium expansum*) were more resistant to all of the extracts (Table 3.4). *Aspergillus niger* was also reported to be resistant to DCM, aqueous and methanolic extracts of 14 plants used traditionally in Paraguay (Portillo et al. 2001).

Table 3-4 Average MIC values of acetone, hexane, dichloromethane and methanol extracts against seven plant pathogens

Microorganism	Average
Aspergillus parasiticus	1.06
Aspergillus niger	1.63
Colletotrichum gloeosporioides	1.54
Penicillium expansum	1.27
Penicillium janthinellum	0.28
Trichoderma harzianum	0.28
Fusarium oxysporum	0.28
Average	0.91

The average MIC values of each plant species using different extractants are shown in Table 3-5. Based on the results obtained, *B. buceras* was the most active plant extract (average MIC value 0.72 mg/ml), followed by *X. kraussiana* (0.78 mg/ml), *O. ventosa* (0.86 mg/ml), *H. caffrum* (0.89 mg/ml) and *V. infausta* (1.14 mg/ml).

In isolating antimicrobial compounds from plant extracts, bioautography plays an enormously important role to facilitate the isolation of antimicrobial compounds. Without good bioautography data it is very difficult to ensure success in isolating antimicrobial activity especially if more than one compound is required to express activity. The bioautography results will be discussed in the next section.

Table 3-5 The average MIC values (mg/ml) of plant extracts prepared using different extractants against seven plant pathogens

Extractants	Plant species					
	Bucida buceras	*Breonadia salicina*	*Harpephyllum caffrum*	*Olinia ventosa*	*Vangueria infausta*	*Xylotheca kraussiana*
A	0.29	0.92	0.88	0.61	1.04	0.54
H	1.02	1.25	1.09	1.14	1.37	1.23
D	1.05	0.89	0.99	0.7	1.09	0.65
M	0.5	1.12	0.62	0.99	1.05	0.67
Average	0.72	1.05	0.89	0.86	1.14	0.78

3.3.3 Bioautography assay

Bioautography was used to determine the number of active compounds in different plant extracts, and representative bioautograms are shown in Figures 3-1 and 3-2. Three solvent systems were used as eluents in the TLC separation, but only the results of CEF and EMW bioautography are given since the compounds in the extracts did not separate using BEA as expected from earlier results. However, the antifungal compounds separated well in both CEF and EMW solvent systems. Their R_f values were also calculated by dividing the distance moved by the compound of interest with the distance moved by the solvent front. The TLC chromatograms developed in CEF (Figure 3-1) of acetone, DCM and methanol extracts of *O. ventosa* showed the same number of active compounds (R_f value of 0.95). The antifungal compounds were clearly visible and more active compounds were observed against *A. parasiticus* than *T. harzianum* and *P. janthinellum*. Furthermore, three active compounds separated with CEF had the same R_f values (0.70, 0.85 and 0.95) in the acetone, hexane, DCM and methanol extracts of *B. salicina*. Three other active compounds with common R_f values of 0.54, 0.72 and 0.95 were visible in the acetone, DCM and methanol extracts of *O. ventosa* against *A. parasiticus* and *P. janthinellum* (Table 3-6).

Data for *B. salicina.*

In EMW (separates more polar compounds) bioautograms, the compound present in the acetone, hexane, DCM and methanol extracts (R_f 0.17) inhibited the growth of three fungi, i.e. *P. janthinellum, A. parasiticus* and *A. niger*. Acetone and hexane extracts had similar active compounds against *P. janthinellum* and *A. niger* with R_f value 0.17. Furthermore, active compounds with the same R_f value of 0.13 were observed in the acetone and hexane extracts against *C. gloeosporioides* and *A. parasiticus*. Bioautograms produced using *P. expansum* and *T. harzianum* showed active compounds in the acetone and hexane extracts, while in the case of DCM and methanol extracts, no active compounds were clearly visible.

Data for *O ventosa.*
In CEF bioautograms, acetone extracts inhibited the growth of fungi i.e *P. janthinellum* with R_f value of 0.17 while no compound were observed in hexane, DCM and methanol extracts. Surprisingly, the acetone extract of *B. salicina* and *O. ventosa* showed similar antifungal compound (R_f 0.13 and 0.17) which were active against *A. parasiticus* and *P. janthinellum*. All extracts of *O. ventosa* did not inhibit the growth of the other three fungi, *A. niger*, *C. gloeosporioides* and *P. expansum.*

Breonadia salicina and *O. ventosa* had the most promising number of antifungal compounds in all four extracts (acetone, hexane, DCM and methanol) because they showed compounds that inhibit the fungi. In summary, the results obtained showed that there were more active compounds separated by EMW (total of 35) than by CEF (total of 17).

Data for other plant extracts.
The remaining four plant species (*B. buceras, H. caffrum, V. infausta* and *X. kraussiana*) showed no activity in the bioautography screening against the seven test organisms, thus the results are considered not significant. However, these plant extracts had good activity in the microplate assay. Possible reasons may be that some of the active compounds were volatile and evaporated during the drying period of the TLC chromatograms prior to bioautography. Biological activity synergism between different compounds in the extracts is also a possible reason.

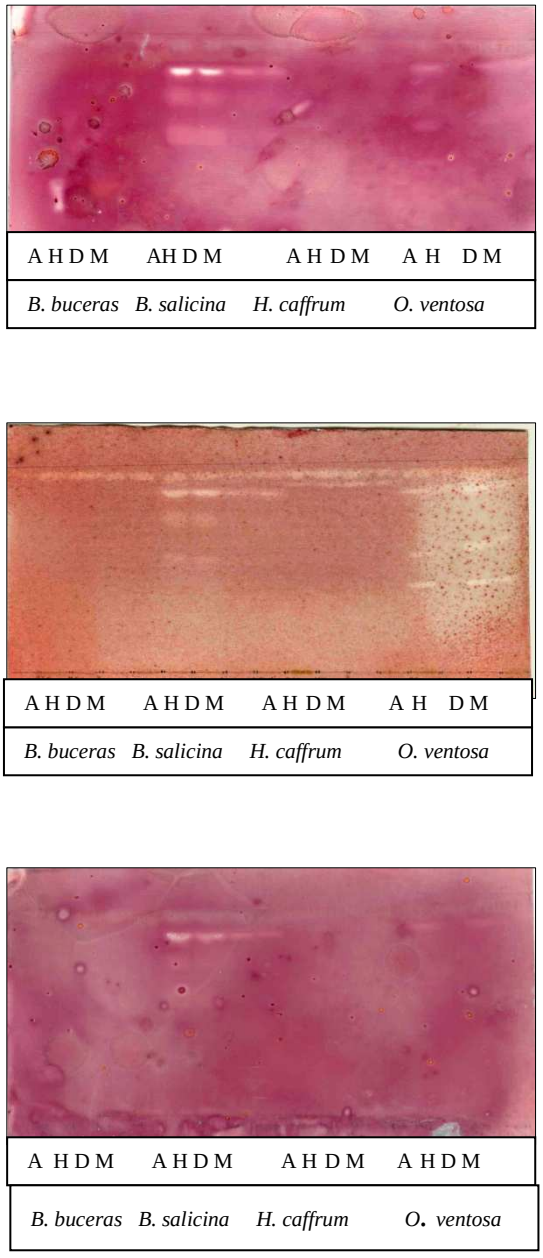

Figure 3-1 Bioautograms of extracts of *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum* and *Olinia ventosa*. Chromatograms were developed in CEF and sprayed with *Penicillium janthinellum* (top), centre (*Aspergillus parasiticus*), bottom (*Trichoderma harzianum*). White areas indicate inhibition of fungal growth. **Lanes from left to right:** acetone (A), hexane (H), DCM (D) and methanol (M)

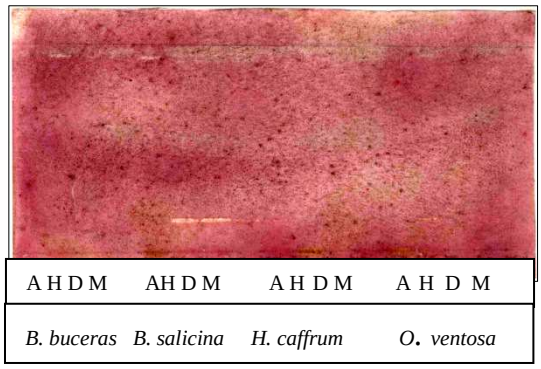

Figure 3-2 Bioautograms of extracts of *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum* and *Olinia ventosa*. Chromatograms were developed in EMW and sprayed with (*Aspergillus niger*) top, centre (*Colleototrichum gloeosporioides*) and bottom (*Penicillium janthinellum*). White areas indicate inhibition of fungal growth. **Lanes from left to right:** acetone (A), hexane (H), DCM (D) and methanol (M)

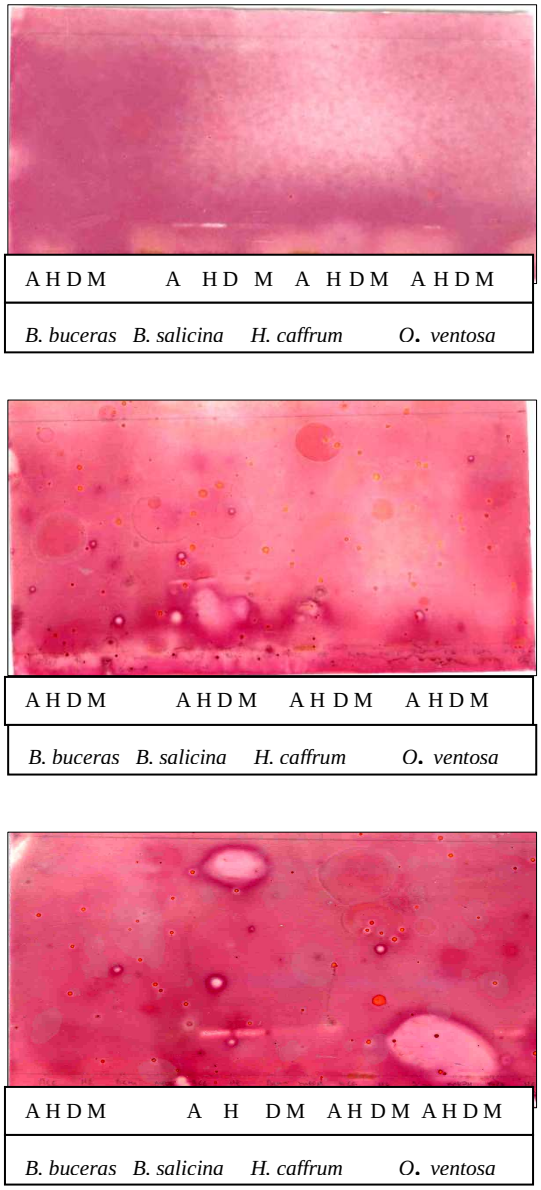

Figure 3-2 (continuation) Bioautograms of extracts of *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum* and *Olinia ventosa*. Chromatograms were developed in EMW and sprayed with (*Aspergillus parasiticus*) top, centre (*Penicillium expansum*) and bottom (*Trichoderma harzianum*). White areas indicate inhibition of fungal growth. **Lanes from left to right:** acetone (A), hexane (H), DCM (D) and methanol (M).

Table 3-6 The inhibition of fungal growth by bioautography of different plant extracts separated by TLC (CEF solvent system). R_f values of active compounds are shown.

R_f values	Extractants	Microorgansims							TOTAL
		A. p	*A. n*	*C. g*	*P. e*	*P. j*	*T. h*	*F. o*	
Breonadia salicina									
0.70	A	1				1			2
0.85		1				1			2
0.95		1				1	1		3
0.70	H	1				1			2
0.85		1				1			2
0.95		1				1	1		3
0.95	D	1				1	1		3
0.95	M	1				1	1		3
Olinia ventosa									
0.54	A	1							1
0.72		1							1
0.95		1				1	1		3
	H								
0.54	D	1							1
0.72		1							1
0.95		1				1	1		3
0.54	M	1							1
0.72		1							1
0.95		1				1	1		3
Total									35

Microorganisms: *A. p. = Aspergillus parasiticus, A. n. = Aspergillus niger, C. g. = Colletotrichum gloeosporioides, P. e. = Penicillium expansum, P. j. = Penicillium janthinellum, T. h. = Trichoderma harzianum* and *F. s. = Fusarium oxysporum*.

Table 3-7 The inhibition of fungal growth in the bioautography assay of different plant extracts separated by TLC (EMW solvent system). R_f values of active compounds are shown.

R_f values	Extractants	Microorgansims							**TOTAL**
		A. p	*A. n*	*C. g*	*P. e*	*P. j*	*T. h*	*F. o*	
Breonadia salicina									
0.13	A	1	1	1					3
0.17						1			1
0.22							1		1
0.36					1				1
0.13	H	1	1	1	1				4
0.17						1			1
0.22							1		1
0.36					1				1
	D		1						1
	M								
Olinia ventosa									
0.13	A	1							1
0.17						1			1
0.22							1		1
	H								
	D								
	M								
Total									17

Microorganisms: *A. p. = Aspergillus parasiticus, A. n. = Aspergillus niger, C. g. = Colletotrichum gloeosporioides, P. e. = Penicillium expansum, P. j. = Penicillium janthinellum, T. h. = Trichoderma harzianum* and *F. s. = Fusarium oxysporum*.

3.4 Conclusion

The serial microdilution and bioautography assays were used to determine antifungal activity and number of active compounds present in the plant extracts respectively. The results obtained in this preliminary work showed that extracts of *O. ventosa* and *B. salicina* possess the best antifungal activity with MIC values of 0.04 and 0.08 mg/ml compared to the other four plant species tested.

Based on MIC results, acetone was the best extractant. It is also low in toxicity to the test organisms and further studies will be carried out using acetone as extracting solvent. In bioautography, several active compounds were visible in acetone, hexane, dichloromethane and methanol extracts of *B. salicina* while only one antifungal compound was observed in the acetone and hexane extracts of *O. ventosa*. The other four plant species (*B. buceras, H. caffrum, V. infausta* and *X. kraussiana*) showed no activity in the bioautography screening against the test organisms *Aspergillus niger, A. parasiticus, Colletotrichum gloeosporioides, Penicillium janthinellum, P. expansum, Trichoderma harzianum* and *Fusarium oxysporum*. In TLC chromatograms developed in CEF (non-polar) more active compounds were observed (total of 35) than in the chromatograms developed using EMW (total of 17). No active compounds were visible in the TLC chromatograms developed in BEA for all four extracts of *V. infausta* and *X. kraussiana*. In summary, *B. salicina* was selected as the best plant species for comprehensive investigation and only two solvent systems, CEF and EMW were recommended for further studies. In the next chapter I will investigate the qualitative antioxidant activity from leaf extracts of six selected plant species.

CHAPTER 4
Antioxidant activity

4.1 Introduction

Plants contain a wide variety of free radical scavenging molecules such as flavonoids, anthocyanins, carotenoids, vitamins and endogenous metabolites. More importantly, these natural products are rich in antioxidant activities (Hertzog et at. 1992). Free radicals can be defined as species with unpaired electrons (Wettasinghe and Shahidi 2000). Antioxidants are known as free radical scavengers and they tend to retard or prevent the oxidation of other molecules by capturing free radicals (Breton 2008). They have various biological activities such as anti-inflammatory, anticarcinogenic and anti-atherosclerotic, antibacterial, antiviral, antimutagenic, antiallergic and antiulcer activity activities (Ikken et al. 1999, Noguchi et al. 1999). These activities may be due to their antioxidant activity (Chung et al. 1998).

Antioxidant compounds also help delay and inhibit lipid oxidation. They play an important role in the maintenance of health and prevention of several diseases. The best way to help prevent these diseases is consumption of an optimal diet containing natural antioxidants. When these constituents are added to foods they tend to minimize rancidity, retard the formation of toxic oxidation products, help maintain the nutritional quality and increase their shelf life (Fukumoto and Mazza 2000). The consumption of food such as fruit, vegetables, red wines and juices helps protect the body from being afflicted with diseases such as cancer and coronary heart disease. This protection is due to the capacity of antioxidants in the plant foods to scavenge free radicals, which are responsible for the oxidative damage of lipids, proteins, and nucleic acids.

Many medicinal plants contain large amounts of antioxidants such as polyphenols, which plays an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Djeridane et al. 2006). A polyphenol antioxidant is a type of antioxidant containing a polyphenolic substructure. Polyphenol antioxidants are found in a wide array of phytonutrient-bearing foods. For example, most legumes, fruits (such as apples, grapes, pears, plums, raspberries and strawberries), vegetables (such as broccoli,

cabbage and onion) are rich in polyphenol antioxidants (Breton 2008). Previously, it has been reported that polyphenolic compounds have antioxidant activity, free-radical scavenging capacity, coronary heart disease prevention, and anticarcinogenic properties (Satora et al. 2008).

Two free radicals, 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) are commonly used to determine antioxidant activity in plant extracts and isolated compounds. The ABTS assay measures the relative ability of antioxidant to scavenge the ABTS generated in aqueous phase, as compared with a Trolox (water soluble vitamin E analogue) standard (Miller and Rice Evans 1997). The method is rapid and can be used over a wide range of pH values (Arnao 1999, Lemanska et al. 2001), in both aqueous and organic solvent systems. On the other hand, it also preferred since it has good repeatability and is easy to perform. In this chapter, a qualitative DPPH method will be used to determine the number of antiradical/antioxidant compounds in plant extracts. The advantage of this qualitative method is that it has good repeatability and is used frequently.

Phenolic compounds are commonly found in both edible and non-edible plants and they have been reported to have multiple biological effects, including antioxidant activity (Kähkönen et al. 1995) and they are considered to provide a major contribution to the total antioxidant activity. This activity of phenolics is mainly due to their redox properties, which can function as hydrogen donors, and singlet or triplet oxygen quenchers. In addition, they have a metal chelation potential (Rice-Evans et al. 1995).

Flavonoids are one of the well known groups of polyphenols. They are found in edible plant products, especially fruits and vegetables (Bravo 1998). Previously, it has been reported that no less than 1-2 g of polyphenols (including flavonoids) should be consumed daily. Food products that contain flavonoid compounds are green leafed, yellow and red vegetables (e.g. onion, cabbage, tomatoes and peppers), fruit (e.g. grapefruits, oranges, dark grapes and apples), red wine and also green tea (Oleszek et al. 1988, Pelegrinin et al. 2000).

4.2 Materials and methods

4.2.1 Extraction

Six plant species were extracted with the following solvents of varying polarities: acetone, hexane, DCM and methanol. The extraction procedure is explained in detail in **section 2.2.4**

4.2.2 Assay for free radical scavenging (DPPH)

The antioxidant activities of plant extracts were determined using the qualitative method 2, 2-diphenyl-1-picrylhydrazyl (DPPH). This assay is preferred because it is used to provide stable free radicals (Fatimi et al. 1993). The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability.

4.2.2.1 TLC fingerprint and antioxidant activity

The TLC chromatograms were developed in three eluent systems BEA, CEF and EMW as described in **section 2.2.4**. The prepared TLC chromatograms were visualized under UV light 254 and 364 and the compounds were identified and highlighted by light pencil circles. A solution of 0.2% DPPH in methanol was prepared and then sprayed on the plates (until it became wet) and allowed to dry in a fume cupboard. The presence of antioxidant compounds was indicated by yellow bands which showed radical scavenger capacity against a purple background. The intensity of the yellow band depends on the quantity and nature of the radical scavenger present in the plant extracts.

4.3 Results and discussion

Figure 4-1 shows the TLC chromatograms of plant extracts sprayed with DPPH. The chromatograms developed in BEA had no zones with antioxidant activity in most of the extracts, probably because antioxidant compounds are usually too polar to be separated well with the BEA solvent system (Figure 4-1). Only the methanol extract of *X. kraussiana* showed a yellow band with an R_f value of 0.14. However, the activity was not strong since the yellow band is not very clear.

In general, the methanol extract had a higher antioxidant activity than the acetone, hexane and DCM extracts. This is again due to the polar nature of most antioxidant compounds, as methanol extracts largely polar compounds. In comparison to members of the Combretaceae (Masoko and Eloff 2007) the extracts of these species contain very few antioxidant compounds and inhibition of microbial infections by stimulating the immune system of the host does not appear to be a realistic mechanism for its activity and traditional use.

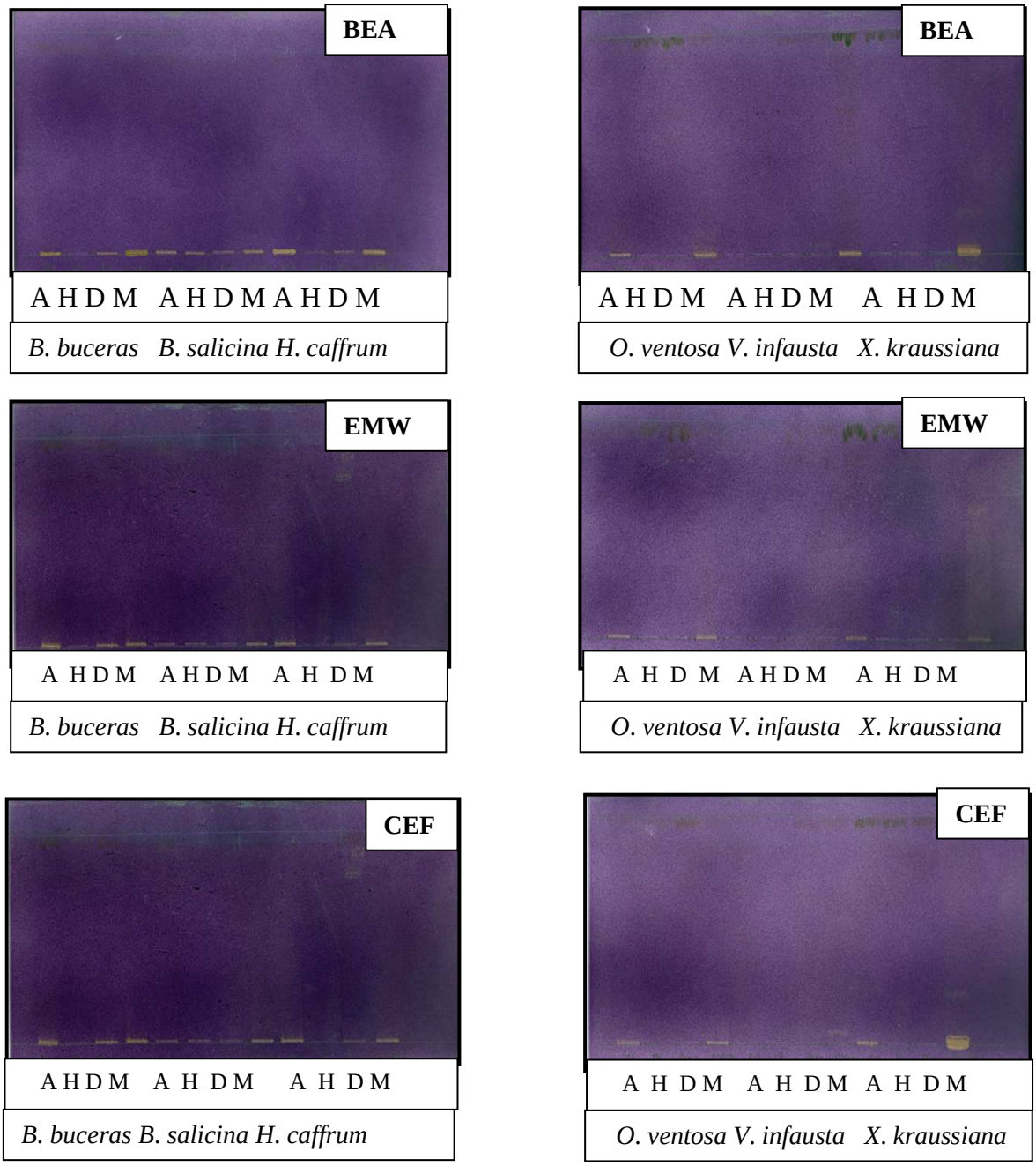

Figure 4-1 TLC chromatograms of six plant species (left to right: *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana*) extracted with acetone, hexane, DCM and MeOH (left to right), developed in BEA, CEF and EMW, and sprayed with DPPH solution.

4.4 Conclusion

The plant extracts of five of the six medicinal plants did not possess strong antioxidant activity. As expected, the polar methanol extracts of *X. kraussiana* showed the antioxidant activity. Due to the limited activity visualised in the qualitative assays, it was deemed unnecessary to include quantitative antioxidant assays in the screening procedure.

It appears that the compounds with antifungal activity, shown using bioautography techniques did not have any antioxidant activity. If they did have such activity, it would have been easier to isolate the active compounds by using the DPPH assay rather than the more complicated and time consuming antifungal bioautography assay for bioassay fractionation to isolate the antifungal compounds. Although *Olinia ventosa* extracts gave very promising results in the earlier antifungal results, the plant was not readily available for collection of test material. In the next chapter the species selected for further work, *Breonadia salicina*, will be investigated in more depth.

CHAPTER 5

Activity of crude leaf extracts of plant species against *Aspergillus fumigatus*

5.1 Introduction

We have recently shown that a crude plant extract can be as effective in treating animals infected with *Aspergillus fumigatus* as the commercially used fungicide (Suleiman 2009). Aspergillosis is a very important disease especially affecting birds. Because the plant extracts had good activity against *Aspergillus niger* a plant pathogen (see Chapter 3), the activity against the animal pathogen *Aspergillus fumigatus* will be determined in this chapter.

Aspergillus fumigatus is a mold that causes various infectious diseases in humans and animals. Molds are fungi that form a threadlike filament and they are produced by spore formation. The spores of molds are usually coloured and can be seen on the surface of the substrate as a sign of food growth. Molds prefer dark, moist, aerobic environments and organic matter in order to grow (Todar 2006).

Aspergillus fumigatus is an asexual fungus that propagates via highly dispersible conidia. This fungus has adapted to survive and grow under a broad range of environmental conditions, contributing to ubiquity of the species. One of the most important distinguishing characteristics of *A. fumigatus* from other *Aspergillus* species is its ability to survive and grow at higher temperatures of 52 to 55°C. Since *A. fumigatus* can survive at higher temperatures, they are considered to be thermo-tolerant fungi (Chang et al. 2004).

Aspergillus fumigatus causes various diseases such as aspergillosis, for example allergic aspergillosis and invasive pulmonary aspergillosis in humans. Aspergillosis is acquired by inhalation of air-borne conidia and invasive pulmonary aspergillosis is one of the leading causes of life-threatening fungal diseases among immunosuppressed patients (Denning, 1998). The treatment of aspergillosis diseases with Western medicine is limited due to a lack of information on the toxicity of the drugs and in some instances, the medication is very expensive. More importantly, the percentage mortality rate is very high (80-90%) despite the

current available antifungal drugs such as amphotericin B, to which most diseases are resistant, and triazole drugs (Denning 1996, Gigolashvili 1999).

Invasive aspergillosis (IA) is the leading cause of infectious death in bone marrow transplant recipients and patients with hematologic malignancies (Kontoyiannis and Bodey 2002). Two commonly known antifungal agents have been used, itraconazole and caspofungin, which is a novel echinocandin that inhibits fungal cell wall biosynthesis. Previously, it has been reported that the drug has antifungal activity against *Aspergillus* species and it can be used for the treatment of invasive aspergillosis (Groll et al. 1998).

5.2 Materials and methods

5.2.1 Fungal strain

Aspergillus fumigatus was obtained from the culture collection of the Department of Microbiology at the University of Pretoria. The fungus was isolated from a chicken which suffered from aspergillosis. Fungal strains were maintained on Sabouraud Dextrose (SD) agar at 4ºC and incubated at 37 ºC for four to five days before use.

5.2.2 Quantification of fungal inoculum

The method is described in detail in chapter 3, section **3.2.1**.

5.2.3 Bioassays for antifungal activity

The methods are described in detail in chapter 3, section **3.2.2.1** and **3.2.2.2**.

5.3 Results and discussion

5.3.1 Dilution method

Amongst all of the extracts tested, only acetone extracts of *B. buceras*, *B. salicina*, *V. infausta* and *X. kraussiana* had good antifungal activity against the animal pathogen. Their MIC values ranged between 0.02 and 0.08 (Table 5-1). Similarly, the hexane, DCM and MeOH extracts of the two plant species, *B. buceras* and *V. infausta*, had activity with the same MIC value of 0.16 mg/ml. It is interesting to note that all of the extracts of *B. salicina* possess a very strong antifungal activity (MIC = 0.08 mg/ml) against the tested fungus. Four extracts of *X. kraussiana* had the best antifungal activity with MIC values ranging between 0.02 and 0.08 mg/ml. Of the four extracts, acetone and hexane extracts of *H. caffrum* and *O. ventosa* were active against the tested microorganism with MIC values of 0.16 and 0.32 mg/ml for the acetone and hexane extracts respectively. *Harpephyllum caffrum* is reported to contain phenolic compounds which may be responsible for its biological activity (El Sherbeiny and El Ansari 1976).

The acetone extracts had the lowest average MIC value (0.72 mg/ml) while the highest were observed in the MeOH extracts (Table 5-1). This confirms that acetone was the best extractant and is also not toxic to the tested animal pathogen. These results are consistent with those obtained for plant pathogens, as discussed earlier in Chapter 3 (Table 3-1).

The crude acetone, hexane and MeOH extracts of *B. buceras* had the highest antifungal activity against the four plant pathogenic fungi, *P. janthinellum, P. expansum, Trichoderma harzianum* and *Fusarium oxysporum* with MIC values ranging between 0.02 and 0.08 mg/ml. When the four extracts were tested against *A. fumigatus* it was discovered that only the acetone extract had a strong antifungal activity with MIC = 0.04 mg/ml. However, in the case of *B. salicina*, all of the four extracts had a strong antifungal activity against the animal pathogen with MIC ranging between 0.04 and 0.08 mg/ml. More importantly, hexane, DCM and MeOH extracts had the same MIC value of 0.08 mg/ml that was observed against *P. janthinellum*. On the other hand, the acetone extract of *V. infausta* had activity with MIC = 0.08 mg/ml while the extracts of *O. ventosa* had a moderate antifungal activity against *A.*

fumigatus with MIC ranging between 0.16 and 0.32 mg/ml (Table 5-1). More surprisingly, the extracts of *X. kraussiana* possess strong antifungal activity (MIC between 0.02 and 0.08 mg/ml) against *A. fumigatus*, in contrast to extracts tested against plant pathogens, where all of the four extracts had a moderate activity with MIC ranging between 0.16 and 2.50 mg/ml. In the current study, extracts of *H. caffrum* were particularly active against *A. fumigatus*. All of the extracts did not show the best antifungal activity against *Aspergillus* species. Previously, the water, ethanol and ethyl acetate extracts of *H. caffrum* were tested against the yeast, *Candida albicans* (Buwa and Van Staden 2006). Their findings revealed that the extracts were not active against the animal pathogenic fungus *C. albicans* since their MIC value was very high (6.25 mg/ml).

The highest total activity was observed in the MeOH extract of *B. salicina* (2781 ml/g) and the lowest was found in the hexane extract after 24 hours (Table 5-1). More importantly, all of the extracts did not possess a strong antifungal activity after 48 hours. When we compared the total activity obtained from the plant and animal pathogens it was found that the highest total activity was obtained in the acetone extract of *H. caffrum* (22 000 ml/g) against *F. oxysporum* while the lowest was observed in the methanol extract of *O. ventosa* against *A. niger* (133 mg/l) (Table 3-2). However, in the case of the animal pathogen, it was discovered that the highest total activity was found in MeOH extract of *B. salicina* (2781 ml/g) and the lowest was observed in hexane extract of *H. caffrum*. This total activity value means that the methanol extract from 1 g of *B. salicina* leaves diluted to 22 000 ml will still inhibit the growth of the fungus.

Table 5-1 Minimum inhibitory concentration (MIC) of six plant species against *Aspergillus fumigatus* using different extractants (A = acetone, H = hexane, D = dichloromethane, M = methanol). The results are the average of three replicates and the standard deviation was zero (0).

Plant species	Time	Extractants									
		A		H		D		M		Average	
		MIC	TA	MIC	TA	MIC	TA	MIC	TA	MIC	TA
Bucida buceras	24	0.04	875	0.32	159	0.16	314	0.16	797	4.94	434
	48	1.25	28	1.25	41	2.5	20	2.5	51	11.1	38
Breonadia salicina	24	0.08	1769	0.08	507	0.08	1250	0.08	2781	4.86	1266
	48	1.25	57	1.25	32	2.5	40	2.5	89	11.1	53
Harpephyllum caffrum	24	0.16	407	0.32	78	0.63	68	0.63	282	5.15	172
	48	1.25	52	2.5	10	2.5	17	2.5	71	11.4	40
Olinia ventosa	24	0.16	567	0.32	127	0.16	255	0.32	1219	5.0	438
	48	1.25	73	2.5	16	2.5	16	2.5	156	11.4	62
Vangueria infausta	24	0.08	1134	0.16	252	0.16	316	0.32	283	4.94	402
	48	2.5	36	2.5	16	1.25	40	1.25	72	11.1	42
Xylotheca kraussiana	24	0.02	1250	0.04	500	0.04	750	0.08	2344	4.84	974
	48	0.63	40	0.63	32	0.63	48	2.5	75	10.5	49
Average		0.72	524	0.99	148	1.09	261	1.28	685	1.02	404

5.3.2 Bioautography assay

In BEA, one antifungal compound was observed in all extracts (R_f 0.08) of *B. salicina*, while the extractants of *X. kraussiana* had three antifungal compounds with R_f 0.02, 0.04, 0.04 and 0.08 in acetone, hexane, dichloromethane and methanol extracts, respectively. Similarly the acetone extract of *V. infausta* also had an antifungal compound with an R_f value of 0.08 (Figure 5-1). The active compound (with R_f 0.08) observed in the above plant extracts is the same since the chromatograms were developed in the same solvent system, BEA. In CEF, three antifungal compounds were found in acetone, DCM and methanol extracts of *B. salicina* with R_f values of 0.70, 0.85 and 0.90. On the other hand, one active compound with the R_f value of 0.70 was visible in hexane extract. The DCM extract of *O. ventosa* had active compounds while no clear bands were observed in acetone, hexane and DCM extracts of *V. infausta* against *A. fumigatus*. Most of the antifungal compounds were visible in CEF, where at least three compounds were observed in acetone, one in hexane, and two in each of the DCM and MeOH extracts. In general, acetone extracts showed more of the active compounds (total of 9) in CEF (Figure 5-1). However, no antifungal compounds were observed in chromatograms developed in EMW in any of the plant extracts. The non-activity of some of the plant extracts used in the current study could be due to the disruption of synergism between active compounds or a very low concentration of the compounds present in the crude extracts that are active against *A. fumigatus*.

In the current study, it was found that for chromatograms separated using CEF, three antifungal compounds with R_f values 0.70, 0.85 and 0.90 in acetone extracts of *B. salicina* were active against three plant pathogens, *P. janthinellum*, *A. parasiticus* and *T. harzianum* (Chapter 3, Figure 3-1). Three antifungal compounds were also observed in acetone extract of *B. salicina* against *A. fumigatus*. There was a very distinct clear active band with R_f value of 0.90. Although there were three active compounds in hexane extracts against the three plant pathogens above (*P. janthinellum*, *A. parasiticus* and *T. harzianum*), only one compound was observed against *A. fumigatus*. In the case of DCM and MeOH extracts, only one antifungal compound was found against *P. janthinellum* and *A. parasiticus*. However, it was different in the case of the animal pathogen, where only two active compounds were visible in DCM and MeOH. In general, all extracts of *B. salicina* showed several antifungal compounds compared

with extracts of the remaining five plant species. In bioautography using BEA, three extracts (acetone, DCM and MeOH) of *O. ventosa*, *V. infausta* and *X. kraussiana* showed one antifungal compound on bioautograms screening against *A. fumigatus*. This was not observed in all extracts against plant pathogens (Figure 3-1). In CEF, acetone, DCM and MeOH extracts of *O. ventosa* showed antifungal compounds with R_f values of 0.54, 0.72 and 0.95 against *A. parasiticus*. Surprisingly, these compounds were not observed in extracts tested against *A. fumigatus* (Figure 5-1). The total number of antifungal compounds found in extracts against plant pathogens was 35 while 19 were visible against the animal pathogenic *A. fumigatus*.

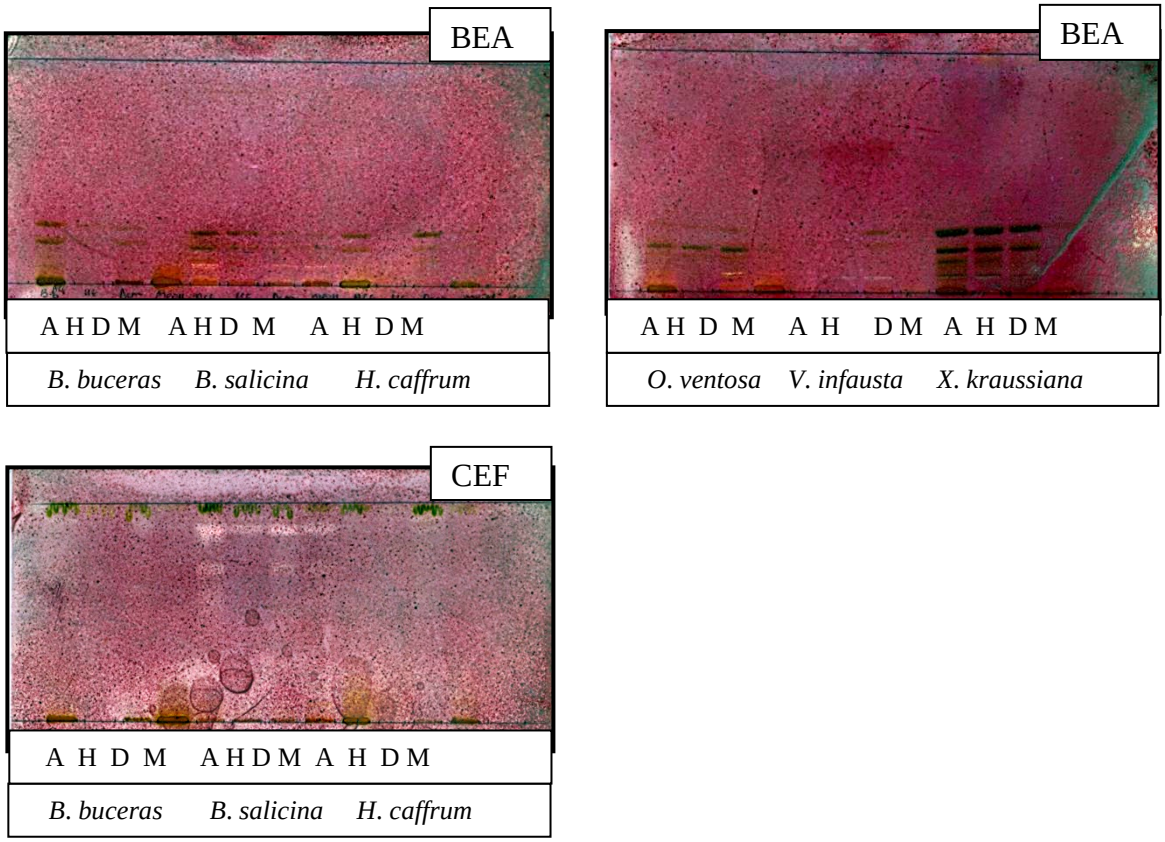

Figure 5-1 Bioautograms of six plant species (left to right: *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana*) extracted with acetone, hexane, DCM and MeOH (left to right), developed in BEA and CEF, and sprayed with *Aspergillus parasiticus*. Clear zones on the bioautograms indicate inhibition of fungal growth.

5.4 Conclusion

Antifungal compounds were observed in all extracts of *B. salicina* while in some of the other plant species (*B. buceras*, *O. ventosa*, *V. infausta* and *X. Kraussiana*), the active compounds were visible in varying extracts. *B. salicina* extracts had the highest antifungal activity against plant and animal pathogens. Amongst the six plant species used in this current study, all four extracts of *O. ventosa* were very active against the plant pathogens but when tested against animal pathogenic *A. fumigatus* they had moderate to low antifungal activity. Not all plant extracts active against plant pathogenic fungi are also active against animal pathogens. This aspect of the study was initiated since other researchers obtained good results using plant extracts against *A. fumigatus* to protect poultry against aspergillosis. The promising activity of plant extracts found in the present research study against plant pathogenic fungi prompted a continued investigation on their potential antifungal activity against *A. fumigatus*. Leaf extracts of *B. salicina* showed strong antifungal activity against *A. fumigatus* and the plant may therefore be a good candidate for further research into a treatment for systemic fungal infections. In the next two chapters, further targeted investigation of the antifungal nature of *B. salicina* leaf extracts and isolation of the antifungal compounds from the plant will be discussed.

CHAPTER 6

Antifungal activity of *Breonadia salicina* leaf extracts

6.1 Introduction

Breonadia salicina (Vahl) Hepper and J.R.I Wood belongs to the family Rubiaceae and is found in Limpopo, Mpumalanga and KwaZulu-Natal provinces (Furness and Breen 1980). The Rubiaceae family is one of the largest of the angiosperms with 10 700 species distributed in 637 genera. It is subdivided into four subfamilies, namely Cinchonoideae, Ixoroideae, Antirheoideae and Rubioideae (Mongrand et al. 2005, Robbrecht 1988, 1993b). Members of the Rubiaceae are mainly tropical woody plants and consist mostly of trees and shrubs, less often of perennial to annual herbs, as in the subfamily Rubioideae, which are found in temperate regions (Mongrand et al. 2005).

Breonadia salicina, commonly known as motumi (Sepedi), is a small to large tree up to 40 m in height. It usually grows in riverine fringes, forest, and usually near the banks or in the water of permanent streams and rivers. The bark is grey to grey-brown and rather rough, with longitudinal ridges. The leaves are usually in whorls of 4 and crowded at the ends of the branches (Palgrave 2002). Leaves are without hair; the veins are pale yellowish-green and the thickset petiole is up to 20 mm long. Flowers are small, pale mauve, sweetly scented, and are present in compact, round axillary heads up to 40 mm in diameter on long slender stalks up to 60 mm long, with 2 leaf-like bracts along their length. They are bisexual, all floral parts are in fives, widening into a funnel-shaped throat and 5-lobed cup-shaped disc. The stamens are inserted in the throat of the tube protruding from the mouth. The 2-chambered ovary with light yellow balls grows in the leaf origin from November to March. The fruit is a small, brown, 2-lobed capsule and is densely clustered into round heads which grow in the leaf origin, giving a rough, crusty, wart-like appearance. The diameter of the fruit is 2 - 3 mm and they are visible during January and February (Palgrave 2002).

Many plant species from the Rubiaceae family are used traditionally for the treatment of various diseases. In particular, *B. salicina* is used to treat wounds, ulcers, fevers, headaches, gastrointestinal illness, cancer, arthritis, diabetes, inflammation and bacterial and fungal

infections (Chang et al. 1989). 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).

Previous work indicates that anthraquinones have been isolated from species in the family Rubiaceae, and these compounds have *in vivo* activities such as antimicrobial, antifungal and antimalarial activity (Sittie et al. 1999, Rath et al. 1995). On the other hand, alkaloids, terpenes, quinonic acid glycosides, flavonoids, and coumarins, have also been isolated from the Rubiaceae (Heitzman et al. 2005). No chemical isolation and characterization of constituents of *B. salicina* have been reported in the literature surveyed.

(a) (b)

Figure 6-1 A photograph of (a) small and large tree and (b) leaves of *Breonadia salicina* taken from Lowveld National Botanical Garden in Nelspruit**.**

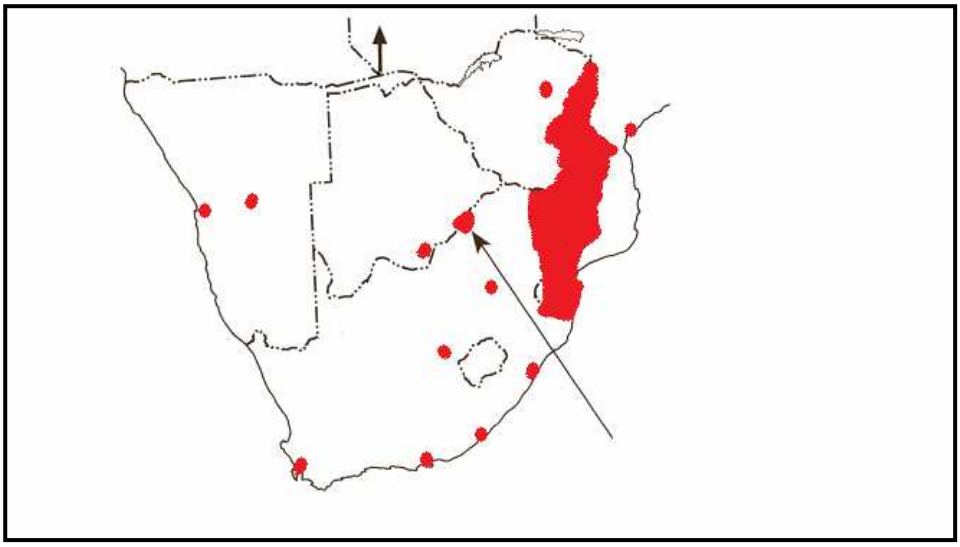

Figure 6-2 Map showing geographic distribution of *Breonadia salicina* (Palgrave 2002). The red shaded areas indicate the places where the plant species grows and the arrows show other places/countries where the plant species is found.

6.2 Materials and methods

6.2.1 Exhaustive sequential extraction

The plant material was collected in February 2007 and prepared as explained in section **2.2.3**. Finely ground leaf material (500 g) was serially extracted with 1500 ml of solvents of increasing polarities, namely hexane, chloroform, acetone and methanol. In each step, the solvent was allowed to extract the ground plant material for three hours on a Labotec Model 20.2 shaking apparatus. The extract was filtered through Whatman No.1 filter paper using a Büchner funnel. With each solvent, the plant material was extracted four times using fresh solvent (1500 ml) to exhaustively extract the material, and the process was repeated with chloroform, acetone, and methanol in sequence. The resulting filtrates were dried under reduced pressure at 40ºC in a rotavapor (Büchi rotary evaporator) and the reduced extracts were transferred into vials and allowed to dry. The masses of the extract yields were determined.

6.2.2 Solvent-solvent fractionation

The chloroform extract (10 g) was dissolved in 500 ml chloroform and transferred into a 1 L separatory funnel before being mixed with water (500 ml)). When separation of the two layers occurred, the bottom layer was collected to yield the chloroform fraction, and the process was repeated three times by extracting the water fraction with fresh chloroform. Following this, one litre of butanol was added to the water fraction and the top layer was collected, yielding the butanol fraction. The chloroform and butanol fractions were evaporated to dryness at 45ºC under reduced pressure using a Büchi Rotavapor R-114. The water fraction was evaporated using a Specht Scientific freeze dryer.

6.2.3 Microplate dilution assay

The fractions and extracts were tested for antifungal activity against seven plant pathogenic fungi. The method is described in section **3.3.2.** The total activity of each fraction was also calculated as described in section **3.2.2.1**.

6. 2.4 TLC fingerprinting

TLC fingerprinting was done on the fractions according to the method described in section **2.2.5**.

6.2.5 Bioautography assay

Bioautography was used to determine the number of active compounds in the fractions after each stage of serial extraction and solvent-solvent fractionation. The fractions were tested against seven plant pathogenic fungi. The method is described in section **3.2.2.2.**

6.3 Results and discussion

6.3.1 Serial extraction with different solvents

Almost a quarter of the plant material (a total of 112.4 g) was extracted from 500 g of *B. salicina* dried leaves with four different extractants, namely hexane, chloroform, acetone and methanol, as shown in Figure 6-3. Methanol extracted the largest quantity of plant material 12.3% (61.5g), followed by acetone 5.6% (27.8 g), hexane 2.6% (12.8 g) and chloroform 2.1% (10.3 g).

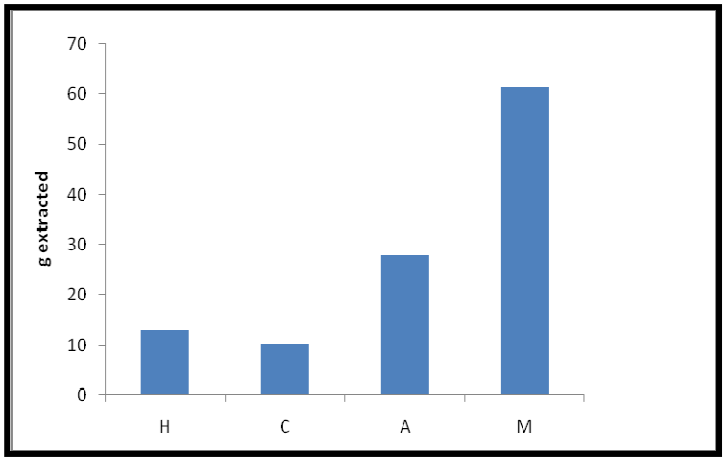

Figure 6-3 Quantity of plant material sequentially extracted from 500 g of *B. salicina*, with different extractants. **Lanes from left to right:** hexane (H), chloroform (C), acetone (A), and methanol (M).

The minimum inhibitory concentration (MIC) values and total activity of the four extracts from the serial extraction process against seven plant pathogenic fungi were determined in triplicate (Table 6.1). The standard deviation was zero for all experiments for all the tables. The highest total activity was observed in the methanol extract (141 ml/g) and the lowest was shown by the hexane extract (25.0 ml/g). The results confirm that where the average MIC value was low (0.54 mg/ml) the total activity was high (102.8 ml/g), consistent with the results in Chapter Two. The chloroform extract had good antifungal activity with an MIC value of 0.16 mg/ml against three fungi, namely: *P. expansum*, *P. janthinellum* and *F.*

oxysporum. The methanol extract also showed good activity with MIC = 0.16 mg/ml against *P. janthinellum*. However, the hexane and acetone extracts were not active against the tested microorganisms, with high MIC values ranging between 0.32 and 1.25 mg/ml. *Aspergillus parasiticus* was relatively resistant to the acetone, hexane, chloroform and methanol extracts with high MIC values between 1.25 and 2.5 mg/ml.

Table 6-1 Minimum inhibitory concentration (MIC) and total activity of four serial extracts against seven plant pathogenic fungi. The results show the average of three replicates and the standard deviation was 0 in all cases

Plant pathogens	Time (hr)	Extractants MIC (mg/ml)				
		Hexane	$CHCl_3$	Acetone	MeOH	AmpB
Aspergillus parasiticus	24	1.25	1.25	1.25	2.5	2.5
Aspergillus niger	48	0.63	0.63	0.32	1.25	1.25
Colletotrichum gloeosporioides	48	1.25	0.63	0.32	0.63	<0.02
Penicillium expansum	48	0.63	0.16	0.32	0.32	<0.02
Penicillium janthinellum	48	2.5	0.16	0.32	0.16	<0.02
Trichoderma harzianum	48	0.63	1.25	0.63	0.63	0.63
Fusarium oxysporum	48	0.32	0.16	0.63	0.63	2.5
Quantity of fraction in mg		12800	10300	27800	61500	112400
Average		1.03	0.61	0.54	0.87	-
Total activity (ml/fraction)		25	34	103	141	303
% of total activity		42.24	34.0	91.75	202.97	370.95

The total activity values of the acetone extract of *B. salicina* against seven plant pathogenic fungi are given in Table 6-2. The highest total activity was found in the acetone leaf extracts of *B. salicina* (174 ml/g) against *A. niger*, *C. gloeosporioides*, *P. expansum* and *P. janthinellum* whilst the lowest activity (45 ml/g) was observed against *A. parasiticus*. These values were in the same range of values found in the antibacterial activity of different *Combretum* spp (Eloff 1999).

Table 6-2 Total activity of the crude acetone extract of *B. salicina* leaves tested against seven plant pathogenic fungi

Plant pathogens	Total activity (ml/g)
Aspergillus parasiticus	45
Aspergillus niger	174
Colletotrichum gloeosporioides	174
Penicillium expansum	174
Penicillium janthinellum	174
Trichoderma harzianum	88
Fusarium oxysporum	88

The chloroform solvent-solvent fraction had reasonable antifungal activity against *A. niger*, *C. gloeosporioides*, *P. janthinellum* and *T. harzianum* with MIC values ranging between 0.16 and 1.25 mg/ml (Table 6-3). The aqueous fraction was less active with MIC values ranging between 0.32 and 2.5 mg/ml against A. *parasiticus*, *A. niger*, *C. gloeosporioides* and *P. janthinellum*. However, the butanol fraction had the lowest antifungal activity against *A. parasiticus*, *A. niger*, *T. harzianum* and *F. oxysporum*, with MIC values ranging between 1.25 and 2.5 mg/ml. The highest average MIC value (1.25 ml/g) was observed in the aqueous fraction, while the lowest average MIC value (0.43 ml/g) was obtained in the chloroform fraction. Furthermore, the lowest total activity of 7 ml/g was recorded for the butanol fraction, while the highest total activity (48.2 ml/g) was observed in the chloroform fraction.

The crude acetone extracts had the best activity against *P. janthinellum* with an MIC value of 0.08 mg/ml. It appears that serial extraction and solvent-solvent fractionation removed some of the compounds with synergism since the aqueous, butanol and chloroform fractions were relatively inactive against the tested plant pathogenic fungi.

Table 6-3 Minimum inhibitory concentration (MIC) and total activity of solvent-solvent fractions against plant pathogenic fungi. The results show the average of three replicates with standard deviation 0 in all cases

Plant pathogens	Time (hr)	Fractions MIC (mg/ml)			
		Aqueous	Butanol	$CHCl_3$	AmpB
Aspergillus parasiticus	24	2.5	1.25	0.63	2.5
Aspergillus niger	48	2.5	2.5	0.16	1.25
Colletotrichum gloeosporioides	48	0.32	0.32	0.16	<0.02
Penicillium expansum	48	0.63	0.63	0.32	<0.02
Penicillium janthinellum	48	0.32	0.63	0.16	<0.02
Trichoderma harzianum	48	1.25	1.25	1.25	0.63
Fusarium oxysporum	48	1.25	1.25	0.32	2.5
Average		1.25	1.12	0.43	-
Total activity (ml/g)		21	7	48	-

6.3.2 TLC analysis

6.3.2.1 Separation of compounds in the serial extraction fractions

The BEA solvent system separated more compounds from the serial extraction fractions than CEF and EMW, after chromatograms were sprayed with vanillin-sulphuric acid (Figure 6-3). With the BEA eluent, some separation of compounds was observed in the acetone and chloroform fractions, but separation of constituents was seen in the hexane and methanol fractions at the base of chromatograms. Addition of a more polar solvent can enhance the separation, moving the compounds further up the TLC chromatograms. In contrast to BEA, a different separation was observed in the CEF solvent system, since the relatively polar compounds moved to the top of the TLC chromatograms. Only one separated compound was visible in the acetone, hexane and $CHCl_3$ fractions, while no movement from the origin was observed in the MeOH fraction. However, using the EMW eluent, most of the compounds moved to just below the solvent front. Only one compound (R_f= 0.41) was visible under UV-

light in both the acetone and $CHCl_3$ fractions, and two compounds (R_f = 0.11 and 0.41) were also visible in methanol fraction (circled in pencil in Figure 6-3). Furthermore, no compounds were visible under UV-light in the hexane fraction.

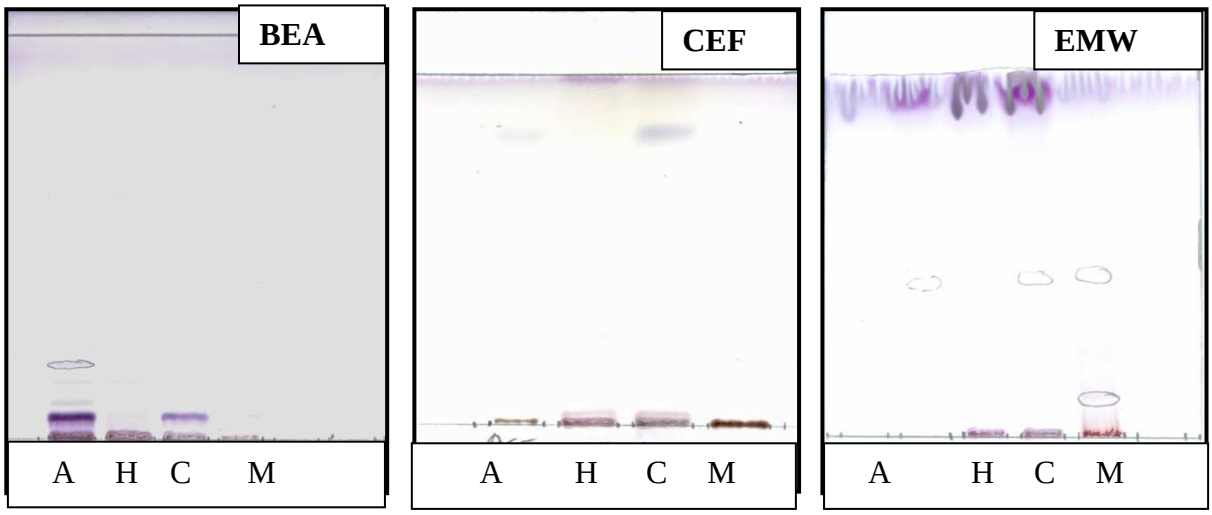

Figure 6-3 Chromatograms separated in BEA (left), CEF (centre) and EMW (right) solvent systems, sprayed with vanillin-sulphuric acid. **Lanes from left to right:** (A) = Acetone, (H) = Hexane, (C) = Chloroform and (M) = Methanol.

6.3.2.2 Separation of compounds in the solvent-solvent fractions

Figure 6-4 shows chromatograms of the fractions from solvent-solvent fractionation developed with BEA (left), CEF (centre) and EMW (right) solvent systems, sprayed with vanillin-sulphuric acid. With the BEA eluent, more compounds were separated in the $CHCl_3$ fraction at the base of the chromatograms, while no compounds were observed in the aqueous and butanol fractions. However, one compound in the aqueous and two compounds in the $CHCl_3$ fraction were visible in the CEF solvent system, indicating better separation with CEF than BEA. In EMW, the separation was no improvement in the aqueous fraction, since the compounds were observed near the base of the chromatograms under UV-light (R_f = 0.08, 0.15, 0.21 and 0.32). No compounds were visible in the butanol fractions using the three solvent systems.

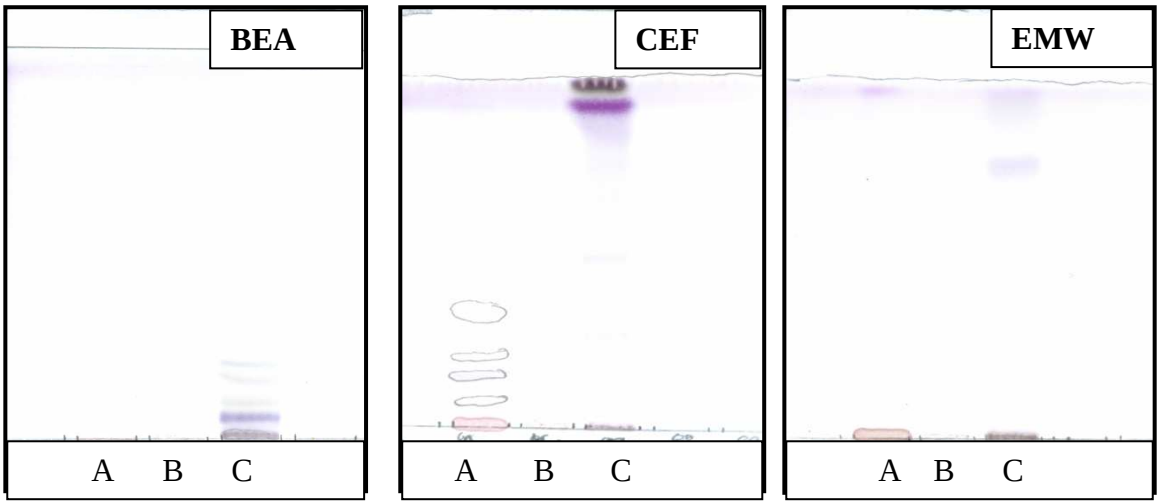

Figure 6-4 Chromatograms of *Breonadia salicina* fractions, developed in BEA (left), CEF (centre) and EMW (right), left to right: Aqueous (A), Butanol (B) and Chloroform (C) and sprayed with vanillin-sulphuric acid (0.1% in vanillin in sulphuric acid).

6.3.3 Bioautography assay

Figure 6-5 shows the chromatograms of the extracts developed in BEA and EMW and sprayed with *A. parasiticus*. The TLC chromatograms developed in the CEF solvent system showed no antifungal compounds and were not included in the Figure. In the BEA eluent system, one antifungal compound was visible in the acetone and chloroform fractions, with R_f value of 0.15. However, no compounds were observed in the hexane and methanol fractions. For extracts separated using EMW, only one compound ($R_f = 0.90$) was observed in the hexane and chloroform extracts. No antifungal compounds were observed in the fractions against the other six plant pathogenic fungi.

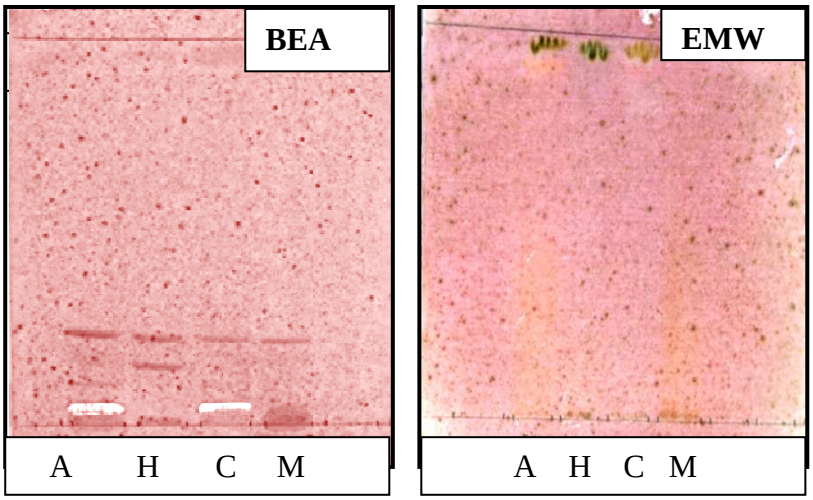

Figure 6-5 Bioautograms of *Breonadia salicina* extracts, serially extracted with A= Acetone (A), Hexane (H), Chloroform (C) and Methanol (M), developed in BEA, and EMW, and sprayed with *A. parasiticus*. White areas indicate inhibition of fungal growth.

6.4 Conclusion

The four serial extraction fractions were not very active against the tested plant pathogenic fungi since they had high MIC values. The methanol fraction had the lowest MIC value (0.16 mg/ml) against *P. expansum*, *P. janthinellum* and *F. oxysporum*. The average MIC values of the fractions varied, with the acetone fraction displaying the lowest average MIC value (0.54 mg/ml). The highest total activity was observed in methanol fraction (141 ml/g) while the hexane fraction had the lowest total activity (25 ml/g).

Of the three fractions resulting from solvent-solvent fractionation only the chloroform fraction had reasonable activity with an MIC of 0.43 mg/ml. This may suggest that there were some inactive compounds still present in the fractions that are associated with high MIC values or separation affected the antifungal activity by disrupting synergy. As could be expected based on the best MIC values, the highest total activity was observed in the chloroform fraction (48 ml/g), while the butanol fraction had the lowest total activity (7 ml/g).

The chloroform serial extraction fraction had highly visible compounds in the chromatograms prepared using BEA, CEF and EMW, and was used for solvent-solvent fractionation to yield aqueous, butanol and chloroform fractions (Figure 6-4). Only the chloroform fraction showed

visible compounds after spraying with vanillin sulphuric acid and no compounds were visible in the aqueous and butanol fractions. However, in the bioautography assay, no antifungal compounds were observed in the fractions (aqueous and butanol) in BEA, CEF and EMW solvent systems. This may suggest, firstly, that some of the compounds may have been volatile and evaporated during the drying period of the TLC chromatograms after developing using three solvent systems. Secondly, some of the residues of formic acid or ammonia following evaporation could have inhibited growth of the plant pathogenic fungi.

To summarise, in serial extraction procedure, the four fractions showed varying degrees of activity against seven plant pathogenic fungi. The chloroform fraction showed the lowest MIC values. After solvent-solvent fractionation of the chloroform fraction, the aqueous, butanol and chloroform fractions had the lowest activity against the tested microorganism. This may suggest that the antifungal activity of *B. salicina* may involve synergistic effects of several compounds. The crude acetone extract had the best antifungal activity when tested against *P. janthinellum* and *F. oxysporum* (MIC value of 0.08 mg/ml, Table 3-1). For further investigation, it therefore appears to be best to focus on the crude extract without preliminary serial extraction. For quality control purposes it is important to know the identity of the active compounds even if they have much lower activity than the crude extract. In the next chapter, isolation of antifungal compounds from leaves of *Breonadia salicina* and their activity against seven plant pathogenic fungi will be discussed.

CHAPTER 7

Isolation of antifungal compounds from leaves of *Breonadia salicina*

7.1. Introduction

In chapter 6 *Breonadia salicina* was selected as the best plant species for further phytochemical investigation and isolation of antifungal compounds.

Plant extracts may contain highly polar and/or highly non-polar substances which can interfere with the separation of pure compounds during isolation if they are present in a very high concentration. Commonly known examples of polar substances are carbohydrates, glycosides and amino acids, while non-polar substances include waxes, oils, sterols and chlorophylls (Klejdus et al. 1999). Preliminary removal of inactive highly polar or non-polar substances during isolation is useful since it increases extract purity and allows more accurate determination of antifungal activity and easier isolation of active compounds.

The polarity of solvents is important when extracting plant material, in terms of targeting specific compounds from crude extracts. Various solvents such as water, alcohols, acetone and ether are used to extract bioactive substances from natural products. Ether is used to extract low polarity ingredients, such as aromatic compounds. Methanol is frequently used to extract specific bioactive ingredients from various natural resources (Kim et al. 2007).

Isolation of antifungal compounds aims at targeting pure compounds from plant material that inhibit the fungi of interest. Isolation and purification of compounds from plant extracts is often demanding and time consuming. In order to yield pure compounds, several steps need to be followed and this includes: extraction, isolation, purification, separation, detection of the active compounds and quantitative data analyses (Abidi 2001). The major disadvantage is the time taken to isolate and to characterise the active components from the extracts. The purification process is necessary since it reduces or eliminates interference of other substances. Moreover, improving diversity, quality of sample source and screen suitability and by automating and standardising early isolation steps, the effectiveness of natural products research can be enhanced (Pieters and Vlietinck 2005).

Several methods have been used to acquire compounds for drug discovery, including isolation from plants and other natural sources, and synthetic chemistry (Balunas and Kinghorn 2005). This includes column chromatography (CC), high performance liquid chromatography (HPLC), gas chromatography (GC), planar chromatography (PC) and thin layer chromatography (TLC). HPLC with a variety of columns and solvents is commonly used for the separation and quantitation of secondary compounds in plant extracts and allows the recovery of pure compounds in the 1-100 mg range (Jagota and Cheatham 1992, Bouvier and Martin 1997). However, HPLC sometimes suffers from poor peak shape, insufficient selectivity and inadequate retention control for basic compounds (Kagan et al. 2008).

A well known isolation procedure is the solvent extraction of the plant sample followed by column chromatography on different sorbents (Štěrbová et al. 2004). Column chromatography and TLC techniques are most affordable procedures and are suitable for sample purification, qualitative assays and preliminary estimates of the compounds in plant extracts (Heftmann 1995). Planar chromatography (PC) requires small amounts of solvent and provides a method for the isolation and recovery of the heaviest fractions (Lazaro et al. 1999). In this study, we follow column chromatography for isolating antifungal compound since it can purify larger samples and also use normal phase systems, i.e. a polar stationary phase (silica) eluted with organic solvents of increasing polarities.

7.2. Materials and methods

The procedure for isolation of antifungal compounds from leaves of *Breonadia salicina* is explained in a schematic representation in Figure 7-1.

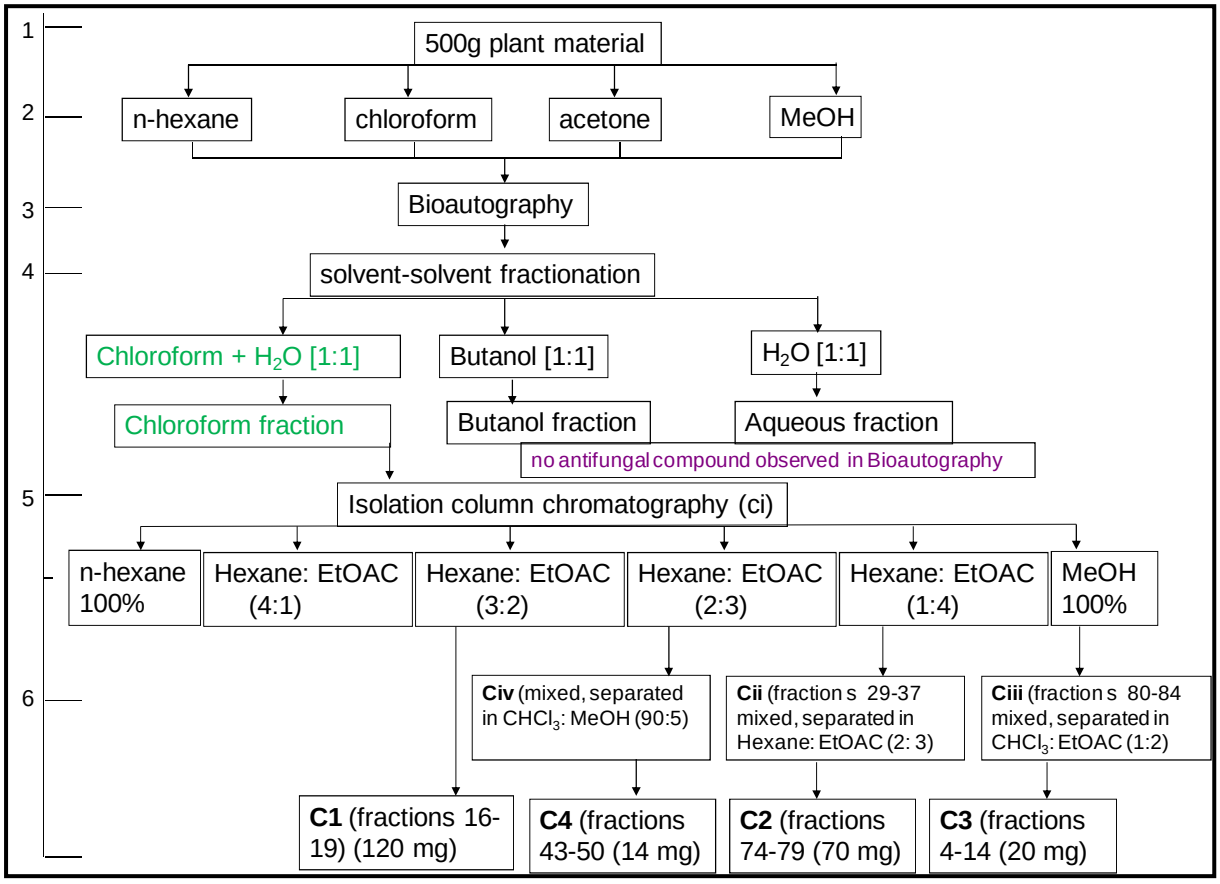

Figure 7-1 Schematic representation of bioassay-guided isolation of four antifungal compounds from the leaf extract of *B. salicina*. The isolation pathway include the following stages: (1) 500 g plant material was ground to fine powder, (2) Serial extraction was carried out using four extractants (Hexane, $CHCl_3$, Acetone and MeOH), (3) Bioautography assay was used to determine antifungal compounds, (4) Solvent-solvent fraction was carried out using chloroform fraction since antifungal compounds were present on bioautograms, (5) Isolation of antifungal compounds with column chromatography, (6) Six fractions were collected in the first column (Ci); Hexane: EtOAC (3:2) fraction yielded 120 mg of compound (C1), Further column chromatography Cii, Ciii and Civ yielded compounds C2 (70 mg), C3 (20 mg) and C4 (14 mg) respectively.

7.2.1 Isolation of antifungal compound(s)

7.2. 1.1 Column chromatography

The chloroform fraction from serial extraction was separated by solvent-solvent fractionation and the chloroform fraction was fractionated by column chromatography. Silica gel (200 g) was mixed with 500 ml hexane to form a slurry and packed to a glass column (denoted as column i) to a height of 30 cm and a diameter of 3 cm. The chloroform fraction (2 g) was dissolved in a small volume of $CHCl_3$ and mixed with 0.5 g silica gel and allowed to dry under a stream of cold air, then thinly spread on top of the column. The fraction was covered with cotton wool and a volume of 500 ml of 100% hexane was initially used to elute the column, followed by the same volume of each of the following solvent mixtures: hexane: ethyl acetate (4:1), (3:2), (2:3) and (1:4) and finally the column was eluted with 100% MeOH . Fractions of 500 ml each were collected. TLC chromatograms of the fractions were prepared in duplicate and developed in hexane: ethyl acetate (3:1). One set was sprayed with vanillin as the reference chromatograms for visualising compounds and the other was sprayed with *A. niger*, *A. parasiticus*, *C. gloeosporioides*, *T. harzianum*, *P. expansum*, *P. janthinellum* and *F. oxysporum* to locate the antifungal compounds present in the fractions.

7.2.1.1a Compound 1 (column i)

Fractions 15-19 from the first column (Ci) contained a pure compound C1. The pooled fraction was concentrated under vacuum at 45°C and transferred to a pre-weighed glass vial to dry completely.

7.2.1.1b Compound 2 (column ii)

Silica gel (13 g) was dissolved in $CHCl_3$: EtOAC (3:2) and used to pack the column ii (20 × 1.0 cm). Hexane: EtOAC (1:4) fractions (0.13 g) from column i were mixed with a small portion of silica gel and allowed to dry. The mixture was spread on top of the column and $CHCl_3$: EtOAC (2:3) was used as eluent solvent system. Fractions of 10 ml volume were collected. Fractions 75-80 contained a pure compound C2.

7.2.1.1c Compound 3 (column iii)

Silica gel (10 g) was mixed with $CHCl_3$: EtOAC (1:2) and packed in column iii (20.0 × 1.0 cm). Hexane: EtOAC (3:2) (110 mg) obtained from column ii was dissolved in $CHCl_3$, mixed with a small portion of silica gel 60, dried and loaded on the packed column. The column was eluted with 300 ml $CHCl_3$: EtOAC (1:2), (1:3) and (1:4). Fractions of 10 ml volume were collected. Fraction 4-12 contained a pure compound C3.

7.2.1.1d Compound 4

Silica gel (15 g) was mixed with $CHCl_3$: MeOH (90:5) and packed in column iv (20.0 × 1.0 cm). Hexane: EtOAC (4:1) (150 mg) obtained from column i was dissolved in $CHCl_3$, mixed with a small portion of silica gel 60, dried and loaded on the packed column. The column was eluted with 300 ml $CHCl_3$: MeOH (90:5). Fractions of 10 ml volume were collected. The fractions 43-50 containing only one compound based on TLC chromatograms were combined to yield compound 4.

7.3 Microplate dilution assay

The crude extracts and four isolated compounds were tested for antifungal activity against seven plant pathogenic fungi. The method is described in section **3.2.2.2.**

7.4 Bioautography assay

Bioautography was used to determine the number of active compounds in the crude extracts, as well as activity of the isolated compounds. The method is described in section **3.2.2.2.**

7.5 Results and discussion

Fractions 16-19 from column i contained a single spot on TLC chromatograms after spraying with vanillin and sulphuric acid and were pooled together and evaporated under reduced pressure to yield 120 mg of compound 1 (Figure 7-2). The TLC chromatograms of fractions 25-40 contained some impurities and were purified further.

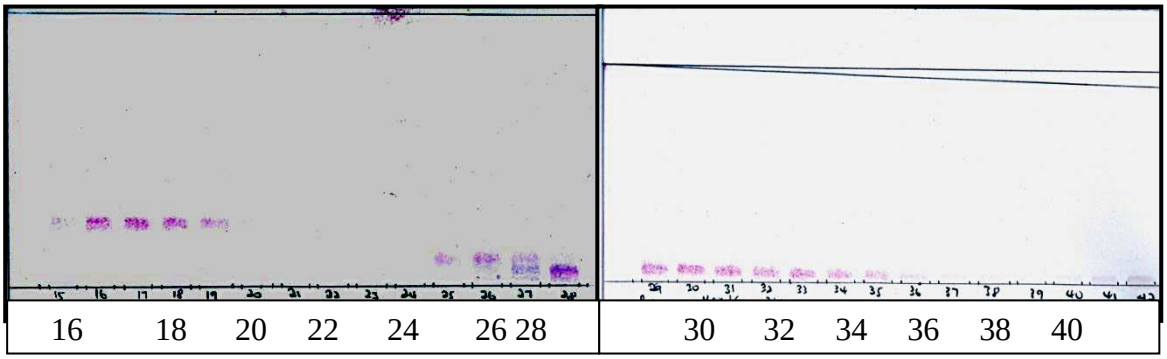

Figure 7-2 Fractions of column i developed in Hexane: EtOAC (3:1) and visualized using vanillin-sulphuric acid.

The pooled fractions were analyzed by bioautography against *F. oxysporum* (Figure 7-3). Clear zones of growth inhibition were observed on the bioautograms in fraction 15-20 and 26-40, and this indicates that the plant components inhibited the growth of fungi. The fractions were tested immediately to observe the presence of active compounds to avoid problems associated with decomposition or photo-oxidation. In bioautography, all seven plant pathogens showed sensitivity, but only the results for *F. oxysporum* are shown. Fractions 15-20 showed the presence of an active compound against *F. oxysporum* while antifungal compounds were observed against the other six plant pathogens from fractions 25-40. Bioautogram of *P. janthinellum* are shown in figure 7-4, showing the presence of active compounds from fraction 25-40.

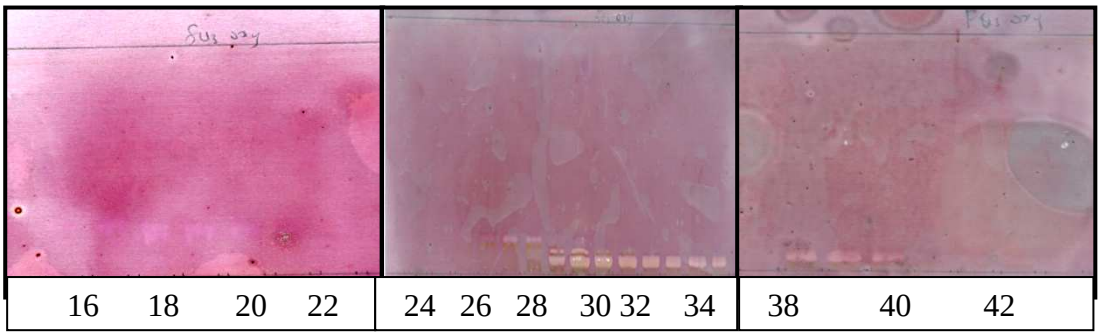

Figure 7-3 Bioautograms of fractions showing activity of fractions developed in hexane: EtOAC (3:1) and sprayed with *F. oxysporum.* White areas indicate inhibition of fungal growth on bioautograms.

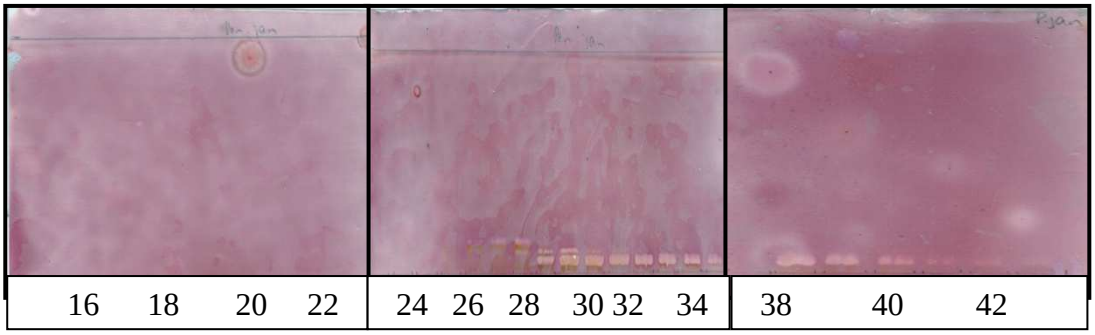

Figure 7-4 Bioautograms of fractions showing activity of fractions developed in hexane: EtOAC (3:1) and sprayed with *P. janthinellum*. White areas indicate inhibition of fungal growth on bioautograms.

Fractions 74-79 from column ii contained a single blue spot on chromatograms after spraying with vanillin spray reagent and were pooled together and evaporated to dryness to yield 70 mg of a white powder (Figure 7-5). Fractions 80-84 contained some minor impurities and were combined and evaporated under reduced pressure before being purified further.

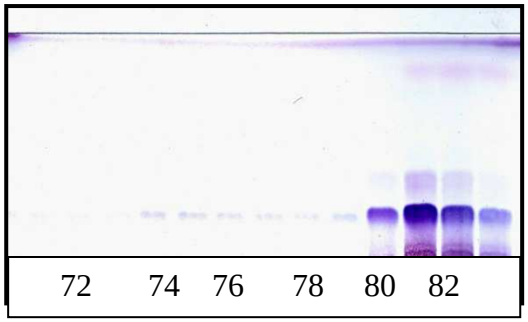

Figure 7-5 Fractions of column ii developed in hexane: EtOAC (3:2) and visualized using vanillin-sulphuric acid.

Fractions 4-14 contained a single blue compound after spraying with vanillin- sulphuric acid, and were combined and evaporated to dryness (Figure 7-6). The resultant pure compound, C3, yielded 20 mg and it was white powder.

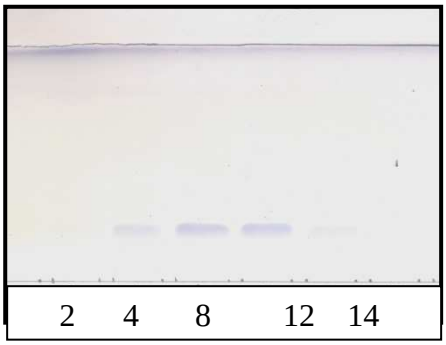

Figure 7-6 Fractions of column iii developed in $CHCl_3$: EtOAC (1:2) and visualized using vanillin-sulphuric acid.

Fractions 43-50 from column iv contained a single purple compound and were combined and evaporated to dryness (Figure 7-7). The resultant compound was a white powder, C4, and yielded 14 mg.

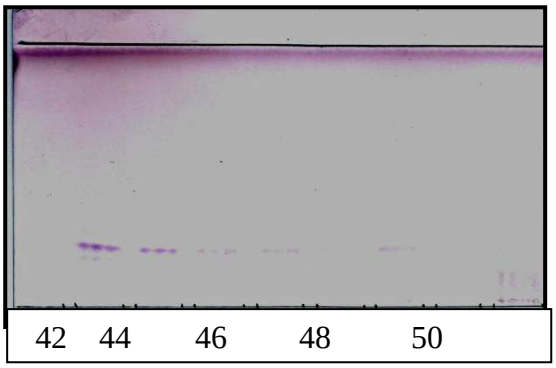

Figure 7-7 Fractions of column iv developed in $CHCl_3$: MeOH (90:5) and visualized using vanillin-sulphuric acid.

7.5.1 TLC analysis

Several compounds in the crude extract were visible after spraying the plates developed in three solvent systems with vanillin sulphuric acid (Figure 7-8 and Table 7-1). TLC chromatograms developed in BEA showed no compounds after spraying with vanillin-sulphuric acid for compounds 1 and 2. Some impurities were however visible in compounds 3

and 4 suggesting that these compound did not move in the solvent system used. Compound 1 had an R_f value of 0.82 in CEF. No visible compounds were observed in CEF with regard to compounds 2 and 4. However, compounds 1 and 3 had R_f values of 0.82 and 0.68, respectively. In TLC chromatograms developed in EMW, all compounds were observed just below the solvent front with the same R_f value of 0.92. Compound 1, C2 and C3 were not visible under UV-light at 254 and 362 nm. Visualising isolated compounds using different TLC systems helps to confirm that the compounds are sufficiently pure for structure elucidation.

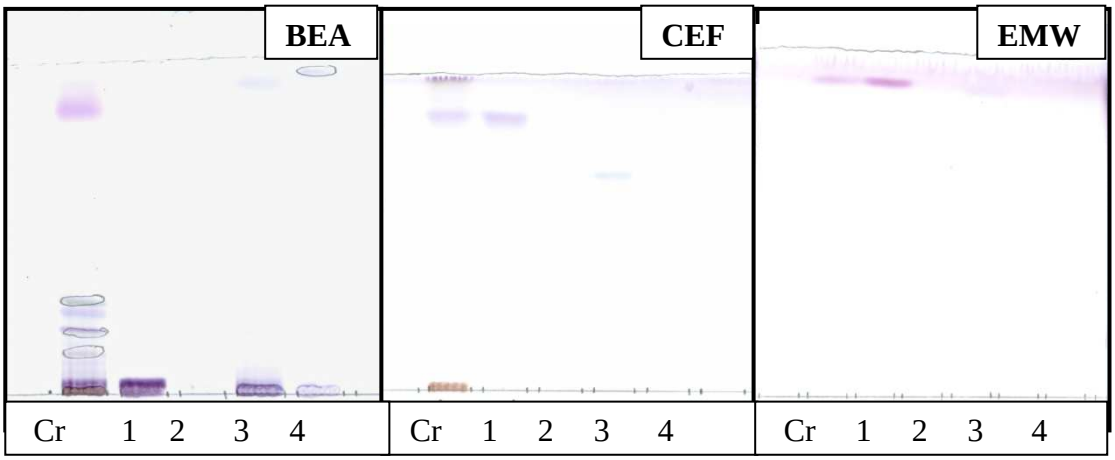

Figure 7-8 Chromatograms of 100 µg of isolated compounds developed with BEA, CEF and EMW and sprayed with vanillin-sulphuric acid. **Lanes from left to right:** Cr= Crude extract, 1= Compound 1, 2= Compound 2, 3= Compound 3 and 4= Compound 4.

Table 7-1 R_f values of compounds separated in BEA, CEF and EMW. The compounds were visualized using visible light, UV light at 254 or 365 nm, and sprayed with vanillin-sulphuric acid.

Solvent system	R_f of isolated compound (s)				
	Crude	1	2	3	4
BEA	0.12	-	-		
	0.18				
	0.29				
	0.88				
	0.90				
CEF	0.82	0.82	-	0.68	-
EMW	0.92	0.92	0. 92	0.92	-

7.6 Conclusion

Isolation of active compounds from leaves of *B. salicina* using column chromatography yielded 4 purified compounds. Compound 1 was isolated in the largest quantity (120 mg), followed by compound 2 (70 mg), compound 3 (20 mg) and compound 4 (14 mg). As expected, in the region of about 10% of plant extract was lost during isolation (packing column using silica gel and TLC analysis of the fractions). In the next chapter, the structure of the isolated compounds will be determined using NMR, EIMS and MS spectroscopy techniques.

CHAPTER 8

Structure elucidation of four isolated compounds

8.1 Introduction

Recently, more than 40% of newly registered drugs were derived from natural products (Humpf 2002, Skowroneck and Gawronski 2000). Compounds derived from natural products are mostly identified using techniques such as nuclear magnetic resonance (NMR) and mass spectroscopy (MS) that provides structural information leading to the complete structure determination of natural products.

Structural elucidation based on these techniques has been the most successful for determining both simple and complex structures (Conolly et al. 1991). Before undertaking NMR analysis of a complex mixture, separation of the individual compounds by chromatography is required (Silva-Elipe 2003). Nuclear magnetic resonance is the best method for complete structure elucidation of non-crystalline samples. When elucidating the structure of secondary natural products, ^{1}H NMR, ^{13}C NMR and 2D NMR spectroscopy are important since hydrogen and carbon are the most abundant atoms in natural products (Džeroski et al. 1998).

However, there are some difficulties encountered when using NMR because it has a very low sensitivity compared to MS and it therefore requires much larger samples for analysis. The machine can detect proton (^{1}H) sensitivity, high isotopic natural abundance and its ubiquitous presence in the organic compounds. When using NMR, all samples require signal averaging to reach an acceptable signal-to-noise level. The NMR analysis depends entirely on the size of the sample, and can range anywhere from several minutes to several days. For example, in the case of metabolites with a mass of 1-10 µg, an overnight experiment with a very powerful apparatus is required (Silva-Elipe 2003).

MS does not always provide conclusive structural information, especially when isomers of bioactive compounds are studied (Albert 2004). It can be used to determine the molecular weight and confirm the structure of the isolated compounds or natural products. In this chapter, we used NMR, MS and EIMS (electron impact mass spectrometry) to determine the

structure of four compounds isolated from leaves of *B. salicina*. The structures were elucidated from the spectroscopic data in collaboration with Dr X.K Peter of the CSIR**.**

8.2 Materials and methods

8.2.1 Structure elucidation

8.2.2.1 Nuclear Magnetic Resonance

An analytical Varian-NMR-vnmrs 600 instrument operating at proton frequency of 600 MHz was used for ^{1}H and ^{13}C. Four compounds isolated from leaves of *B. salicina* (Chapter 7) were weighed (10-30 mg) and dissolved in deuterated $CDCl_3$ since the compounds were soluble in $CHCl_3$. All of the samples were sent to the Council for Scientific and Industrial Research (CSIR) for NMR analysis. Each sample were dissolved in 0.7 ml $CHCl_3$ and transferred into NMR tubes (5 mm).

8.2.2.2 Mass Spectroscopy

An analytical THERMO electron DFS magnetic sector mass spectrometer at low resolution was used and the samples were ionized by electron impact ionization (EI). Approximately 2 mg of each isolated compound was dried, placed into a 2 ml glass vial and sent to the University of the Witwatersrand, Department of Chemistry for MS analysis.
Aliquots of the four isolated compounds were transferred into separate 1 ml HPLC vials. The samples (2 mg) were each dissolved in approximately 1 ml of DMSO (fraction 1) of which 2 µL was transferred to a direct probe crucible, and inserted into the MS. The MS source temperature was 250°C and the probe was heated from 50 to 250°C.

8.2.2.3 Electron impact mass spectrometry (EIMS)

Analytical EIMS was used to displace an electron from the organic molecule to form a radical cation known as the molecular ion**.** Compound 1 was ionized in a negative mode electron impact mass spectrometry (EIMS) with molecular ion $[M-H]^-$. This mass spectrum was used to confirm the accurate mass measurement of the isolated compound. Approximately 10 mg of compound 1 were weighed and sent to CSIR for EIMS. Before analyzing the sample, 1 mg of isolated compound was dissolved in 1 ml aceto nitrile (CH_3CN) and then direct infusion was applied.

8.3 Results and discussion

8.3.1 Structure elucidation

8.3.1.1 Compound 1

In the mass spectrum peaks were observed at *m/z*. 189.53, 207.58, 219.56 248.63 and 249.64 m/z (See p 142). The ^{13}C NMR showed the presence of 7 methyl groups at signals δ 14.1, 15.4, 15.5, 16.9, 17.0, 18.2 and 21.1 (See p 139). Furthermore, C12- C13 was identified as an olefinic group at signal δ 137.9 and 125.8 whilst an acidic group was observed at 206.9. The rest of the spectra were aliphatic CH_2 groups. The ^{1}H NMR had a signal at δ 5.19 for an olefinic proton at hydrogen 12, and hydrogen 3 was observed next to a hydroxyl group (OH) and was shifted down-field at signal δ 3.12 (See p 140). Furthermore, three hydroxyl groups were observed at signal δ 3.96. Based on ^{1}H and ^{13}C NMR spectra compound 1 was identified as the triterpenoid ursolic acid and the spectral data is in agreement with the literature (Moghaddam et al. 2006). However, our ^{13}C NMR spectrum at C28 had a peak at δ 206. 9 compared to that of Moghaddam et al. (2006) at δ 179.1. It is unlikely that the difference of the peaks could be due to the fact that we used $CDCl_3$ solvent while DMSO was used in Moghaddam et al. (2006) (Table 8-1). The structure was further confirmed by electron impact mass spectrometry (EI-MS), in a negative mode (See p 141). The spectrum displayed an accurate molecular ion peak at *m/z* 455.4 $[M-1]^+$ corresponding to the molecular formula of 456 of $C_{30}H_{48}O_3$. This was in good agreement with the ^{1}H NMR and ^{13}C NMR spectroscopic data. Previously, ursolic acid with a molecular ion peak at m/z $[M]^+$ 456 was reported (Moghaddam et al. 2006).

Ursolic acid has been isolated from *Satureja* species and also from the Lamiaceae and Oleaceae family (Escudero et al. 1985, Giannetto et al. 1979, Kontogianni et al. 2009). This compound has been previously isolated from the dichloromethane (DCM) extract of *Curtisia dentata* and stem bark of *Hyppocratea excels* (Shai et al. 2008, Cáceres-Castillo et al. 2008).

Table 8-1 ^{13}C NMR spectroscopic data for compound 1

Number of Carbon	Compound 1 ($CDCl_3$) δ ^{13}C (ppm)	Ursolic acid (DMSO) Moghaddam et al. (2006)
1	39.4	39.2
2	27.9	27.8
3	77.2	77.7
4	39.4	39.2
5	55.2	55.6
6	18.2	18.9
7	33.9	33.6
8	39.4	40.0
9	47.8	47.9
10	38.7	37.4
11	23.7	23.7
12	125.8	125.4
13	137.9	139.0
14	41.9	42.5
15	28.1	28.4
16	24.4	24.7
17	47.8	47.7
18	52.6	53.2
19	39.4	39.4
20	39.0	39.3
21	31.9	31.1
22	38.5	37.2
23	29.2	29.1
24	15.5	16.1
25	17.0	16.9
26	18.2	17.8
27	24.1	24.1
28	206.9	179.1
29	16.9	17.9
30	21.1	21.9

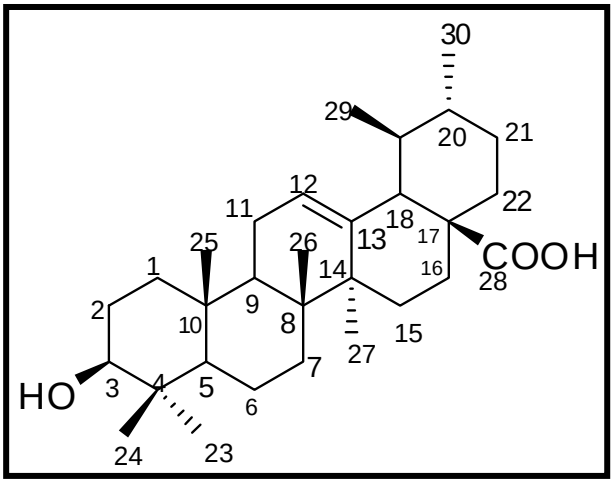

Figure 8-1 Structure of ursolic acid isolated from leaves of *Breonadia salicina*

8.3.1.2 Compound 2, 3 and 4

Compounds 2, 3 and 4 were isolated as white powders. The presence of long chain fatty acids was detected when these compounds were analysed in mass spectrometry. Despite a thorough use of different solvents to remove fatty acids from the compound, the MS still indicated the presence of long chain fatty acids among masses not explained by fatty acids. Based on the outcome of the mass spectra results and the low quantity of material available, the compound was not analysed further by NMR.

8.4 Conclusion

Four compounds were isolated from leaves of *B. salicina* and the structure of compound 1 was elucidated using NMR and MS technique as ursolic acid ($C_{30}H_{48}O_3$). With the other three isolated compounds (2, 3 and 4), only mass spectrometry was performed. To the best of our knowledge, no chemical isolation and characterization of bioactive constituents of *B. salicina* has been reported before. Ursolic acid has been previously isolated from leaves of *Curtisia dentata* (Shai et al. 2008) and stem bark of *Hippocratea excels* (Ca´ceres-Castillo et al. 2008).

Three compounds (2, 3 and 4) appeared to consist of long chain fatty acids or carboxylic acids as shown by MS. These were probably not pure and in all cases there was a significant loss of $(CH_2)_n$. In particular, there was no distinction between compound 2 and 3 from MS results.

CHAPTER 9

Antifungal and antibacterial activity and cytotoxicity of isolated compounds

9.1 Introduction

In the previous chapter, the structure of compound 1 was elucidated as the triterpenoid ursolic acid and the other three compounds consisted of long chain fatty acids. Triterpenoids form a large group of natural substances which includes steroids and consequently sterols. Steroids are one of the largest groups and a very small amount is present in bacteria but more are found in plants and animals (Connolly and Hill 1992). Various biological activities of the triterpenoid and fatty acids have been reported. Previously, an iridal triterpenoid isolated from *Iris germanica* L., has been reported to have antifungal activity against *Candida albicans* (Benoit-Vical et al. 2003). Bioassay guided fractionation led to the isolation of the active triterpenoid ergosterol-5,8-endoperoxide from *Ajuga remota* and it was active against *Mycobacterium tuberculosis* (MIC of 1 µg/ml) (Cantrell et al. 2001). Ursolic acid has been previously isolated from the dichloromethane extract of *Curtisia dentata* and was reported to have high antifungal activity against *Sporothrix schenckii* and *Microsporum canis* with MIC values of 12 and 32 µg/ml respectively (Shai et al. 2008).

Fatty acids, in particular 2-alkynoic fatty acids have been known to have antifungal activity. The activity of this compound depends on the fatty acid chain length and pH of the medium (Gershon and Shanks 1978). The optimal chain lengths of 8 and 16 carbons have been established for the 2-alkynoic fatty acid to exert maximum fungistatic effects. Another type of fatty acid, hexadecanoic acid has been reported to have antifungal, antimicrobial and cytotoxic properties (Konthikamee et al. 1982, Wood and Lee 1981).

Previously, a novel acetylenic fatty acid, known as 6-nonadecynoic acid was isolated from the ethanol extract of roots of *Pentagonia gigantifolia* (Li et al. 2003). The antifungal mechanism was due to interference of the compound with fungal sphingo-lipid biosynthesis. It was discovered to be fungitoxic to *Cryptococcus neoformans* but inactive towards *Candida albicans* (Li et al. 2008).

Crude extracts and pure compounds of medicinal plants are important in drug discovery; however their toxicity requires extensive attention since this can cause various side effects (biological implications) to human and animals. In general, cell type cytotoxic specificity of plant extracts is likely due to the presence of different classes of compounds (such as terpenes or terpenoids, and alkaloids) in the extracts. There are several types of cytotoxicity assays that can be used to determine the level of toxicity in the plant extracts, and this includes inferior organisms, biochemical assays, cell cultures and isolated organs. However, cytotoxicity with cell cultures is highly preferred because it is very common, rapid, inexpensive, and does not have ethical implications (Fernandes et al. 2005).

In this chapter I will investigate the antifungal activity of the isolated compounds against seven plant pathogens as well as against three bacteria including the Gram-positive *Staphyloccocus aureus* (ATCC 29213) and the Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aureus* (ATCC 27853). I will also determine the cytotoxicity against Vero monkey kidney cells to evaluate the safety of the isolated compounds.

9.2 Materials and methods

9.2.1 TLC fingerprint

Ten milligrams of pure compounds were separately resuspended in 1 ml acetone to a known concentration (10 mg/ml) and were separated on TLC plates. The method is described in section **2.2.5.**

9.2.2 Bioassays for antifungal activity

9.2.2.1 Microdilution method

The crude acetone extracts and four isolated compounds were tested for antifungal activity against seven plant pathogenic fungi. The method is described in section **3.2.2.2.**

9.2.2.2 Bioautography assay

Bioautography was used to determine the number of active compounds in the crude extracts, as well as activity of the isolated compounds. The method is described in section **3.2.2.2.**

9.2.3 Antibacterial activity

Bioautography was used to determine the number of active compounds in the crude extracts, as well as activity of the isolated compounds. The assay was conducted as described by Eloff (1998c). The method is basically the same as the one outlined in section **3.2.2.2** the only difference is that INT (0.2 mg/ml) was added following overnight incubation of compounds with bacteria. Overnight cultures of the bacteria were diluted 1:100 with fresh Mueller Hinton (MH) broth prior to use in the assay

9.2.4 Cytotoxicity assay

9.2.4.1 Tetrazolium-based colorimetric assay (MTT)

The method described by Mosmann (1983) and slightly modified by McGaw et al. (2007) was used to determine the cytotoxicity of the crude extracts and four isolated compounds. The plant extracts and compounds were tested for cytotoxicity against Vero monkey kidney cells obtained from the Department of Veterinary Tropical Diseases (University of Pretoria). The cells were maintained in minimal essential medium (MEM, Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5×10^3 cells into each well of a 96-well microtitre plate. Plates were incubated overnight at 37ºC in a 5% CO_2 incubator and the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the plant extracts (200 mg/ml) and isolated compounds (20 mg/ml) were prepared by dissolving them in DMSO. Serial 10-fold dilutions of each extract and isolated compounds were prepared in growth medium and added to the cells. The viable cell growth after 120 hours incubation with plant extracts and isolated compounds was determined using the tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT), Sigma) described by Mosmann (1983). Briefly, after incubation, 30 µl of MTT (5 mg/ml in phosphate buffered solution,

PBS) was added to each well and the plates were incubated for a further 4 hours. The medium was aspirated from the wells and 50 µl DMSO added to each well to solubilize the formazan produced by mitochondrial activity. The absorbance was measured on a Versamax microplate reader at 570 nm. Berberine chloride (Sigma) was used as a positive control. The intensity of colour was directly proportional to the number of surviving cells. Tests were carried out in quadruplicate and each experiment was repeated three times.

9.3 Results and discussion

9.3.1 Biological activity of the isolated compounds

9.3.1.1 Bioautography assay

Figure 9-1 shows bioautograms developed in BEA, CEF, and EMW and sprayed with *T. harzianum* (left), *A. parasiticus*, *P. janthinellum* centre and *F. oxysporum*. In TLC chromatograms developed in BEA, clear zones were observed with ursolic acid against *T. harzianum* and *A. parasiticus*, with R_f values of 0.07 and 0.15 respectively. The antifungal compounds 2 and 3 were visible at the origin ($R_f = 0$). Ursolic acid was visible at $R_f = 0.87$, and compounds 2 and 3 had the same $R_f = 0.66$ for the TLC chromatogram developed in CEF against *P. janthinellum*. For the chromatograms developed in EMW, ursolic acid, 2 and 3 showed clear inhibition zones indicating the presence of antifungal compounds against *F. oxysporum* ($R_f = 0.94$). In general, ursolic acid had a distinct active band than the other compounds. No clear visual growth inhibition was found with compound 4 against the tested microorganisms.

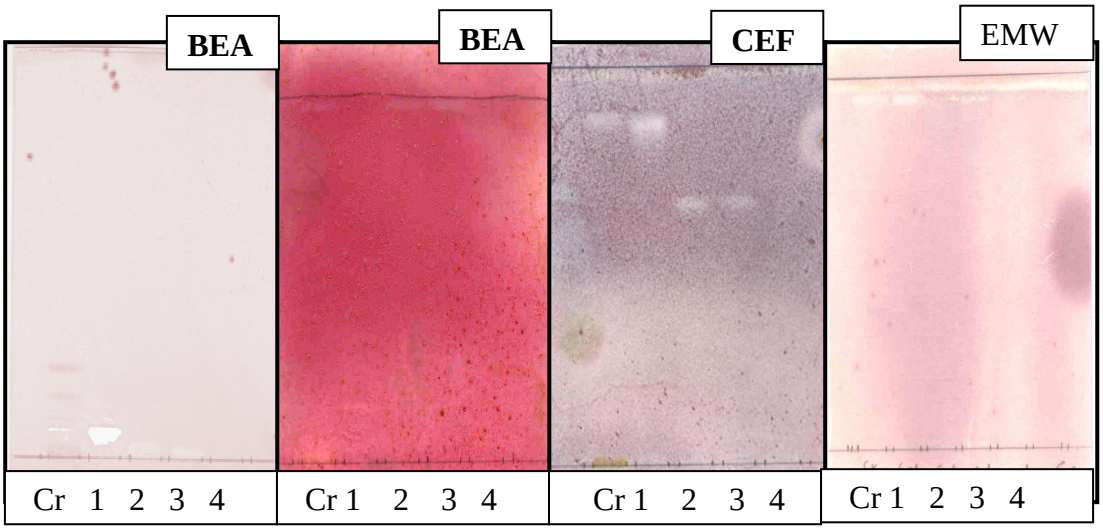

Figure 9-1 Bioautograms of 100 µg of isolated compounds, chromatograms developed with BEA, CEF and EMW and sprayed with *Trichoderma harzianum*, *Aspergillus parasiticus* (left), *Penicillium janthinellum* (centre) and *Fusarium oxysporum* (right). White areas indicate inhibition of fungal growth. **Lanes from left to right:** Cr = Crude extract, 1 = Ursolic acid, 2 = Compound 2, 3 = Compound 3 and 4 = Compound 4

Figure 9-2 shows bioautograms of ursolic acid, 2, 3, and 4 and crude extracts, with TLC chromatograms developed in BEA and EMW sprayed with *E. coli*, *P. aeruginosa*, and *S. aureus*. Ursolic acid, 2 and 3 showed active compounds against *E. coli*, *S. aureus* and *P. aeruginosa*. The results showed that the crude extract and ursolic acid had the same antibacterial compound in TLC chromatograms developed with BEA. The crude extract showed the presence of compound 1 by revealing an active band at the same R_f value as that of ursolic acid. (R_f = 0.05 against *E. coli* and *S. aureus*). Similarly, the same band in the crude extract and ursolic acid showed antibacterial compound with R_f value of 0.92 against *S. aureus* in TLC chromatograms developed in EMW. In TLC chromatograms developed in CEF, the compounds were visible below the solvent front with the same R_f value of 0.86 against *S. aureus*.

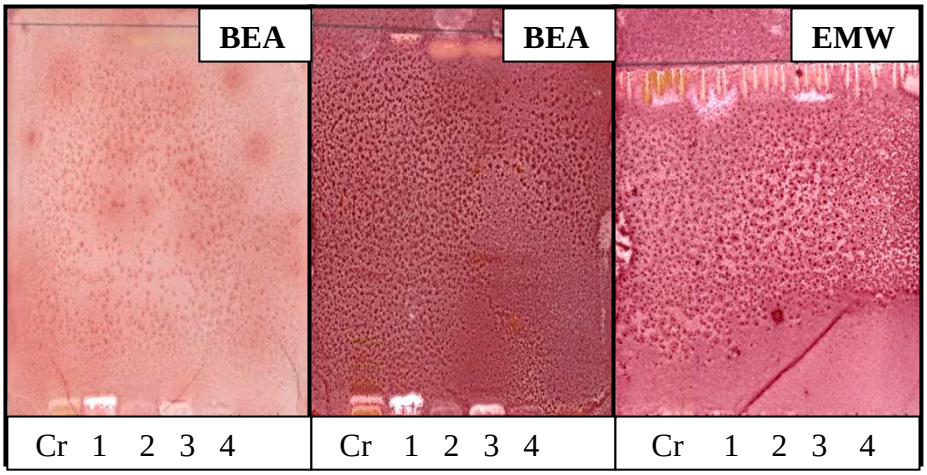

Figure 9-2 Bioautograms of compound 1, 2, 3, 4 and crude extracts, chromatograms developed in BEA and EMW sprayed with *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. White areas indicate inhibition of fungal growth. **Lanes from left to right:** Cr = crude extract, 1 = Ursolic acid, 2 = Compound 2, 3 = Compound 3 and 4 = Compound 4.

9.3.1.2 Microplate dilution assay

The crude extracts and four compounds were tested for antifungal activity against the plant pathogens. Compound 3 and 4 had good antifungal activity against *A. parasiticus* and *P. janthinellum* with MIC value of 10 and 16 µg/ml. Ursolic acid and C2 had activity with MIC values ranging between 20 and 250 µg/ml (Table 9-1). These results suggest that during isolation, 80% (crude extract= 2.5 mg/ml and compound 3 MIC = 10 µg/ml) of other impurities were removed since the compounds had low MIC values.

Table 9-1 Minimum inhibitory concentration (MIC) of four isolated compounds against seven plant pathogenic fungi. Standard deviations were 0 in all cases.

Micro-organisms	Time (hrs)	MIC (µg/ml)	MIC (µg/ml)				
		Crude extract	Ursolic acid	2	3	4	AmpB
Aspergillus parasiticus	24	630	20	20	10	30	6.4
Aspergillus niger	48	2500	120	120	120	120	1.6
Colletotrichum gloeosporioides	48	1250	30	60	20	60	3.2
Fusarium oxysporum	48	3200	50	50	50	50	3.2
Penicillium expansum	48	1250	125	32	32	125	3.2
Penicillium janthinellum	48	80	125	16	25	16	3.2
Trichoderma harzianum	48	630	125	250	125	250	3.2

9.3.2 Cytotoxicity assay

The cytotoxicity of four compounds was determined against Vero cells using the MTT assay. Berberine was used as a positive control and it was toxic with an LC_{50} of 13 µg/ml (Figure 9-3). The crude extract was less toxic than ursolic acid with LC_{50} of 82 µg/ml (Figure 9-9). Compounds 2 and 3 were not toxic at the highest concentration tested (200 µg/ml) (Figure 9-6 and 9-7) towards the Vero cells. However, C4 (compound 4) was more toxic to the cells with an LC_{50} of 35 µg/ml (Figure 9-8).

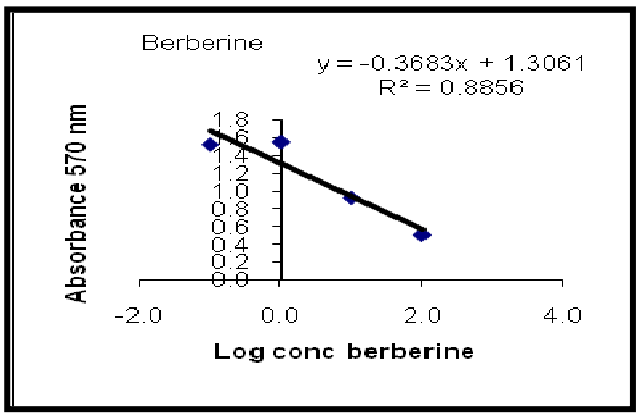

Figure 9-3 Cytotoxicity of berberine with LC_{50} = 13 µg/ml against Vero cells

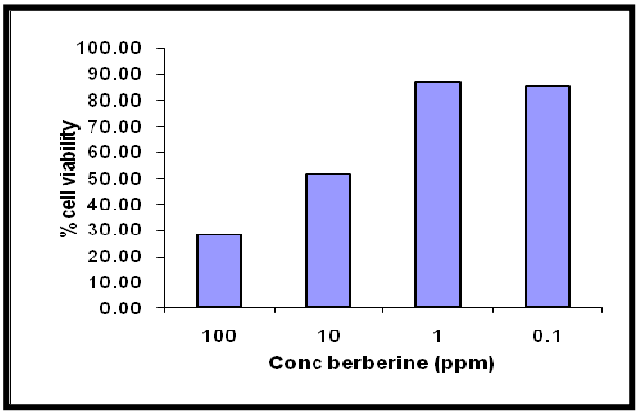

Figure 9-4 Percentage (%) cell viability of berberine

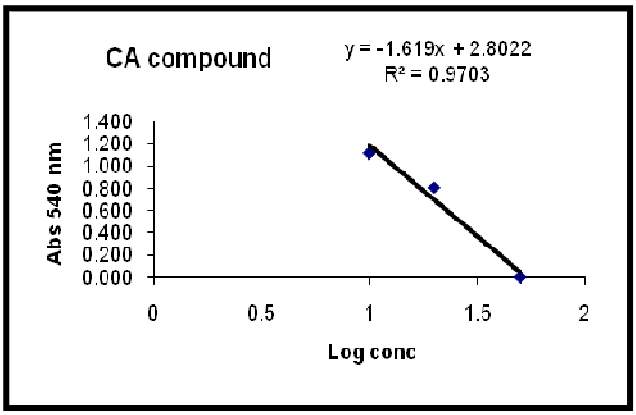

Figure 9-5 Cytotoxicity of ursolic acid with LC_{50}= 25 µg/ml against Vero cells

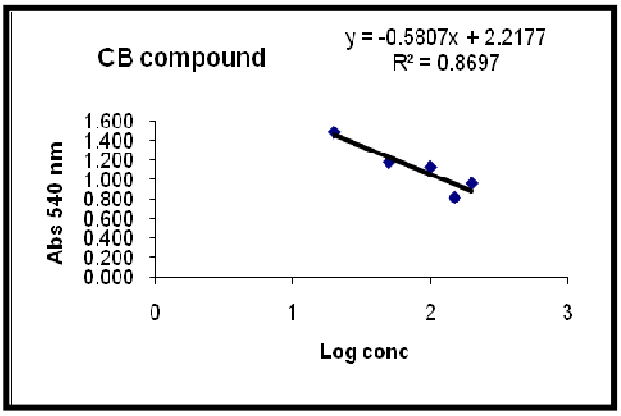

Figure 9-6 Cytotoxicity of C2 with LC_{50} = 525 µg/ml against Vero cells

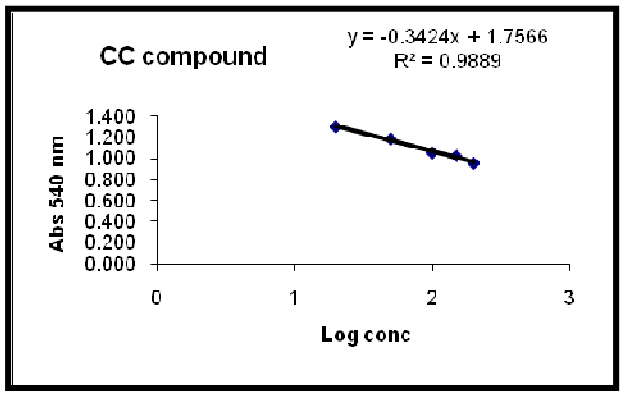

Figure 9-7 Cytotoxicity of C3 with LC_{50} = 1849µg/ml against Vero cells

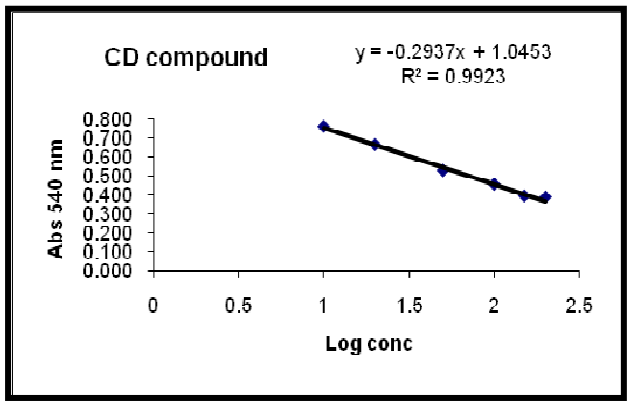

Figure 9-8 Cytotoxicity of C4 LC_{50} = 35 µg/ml against Vero cells

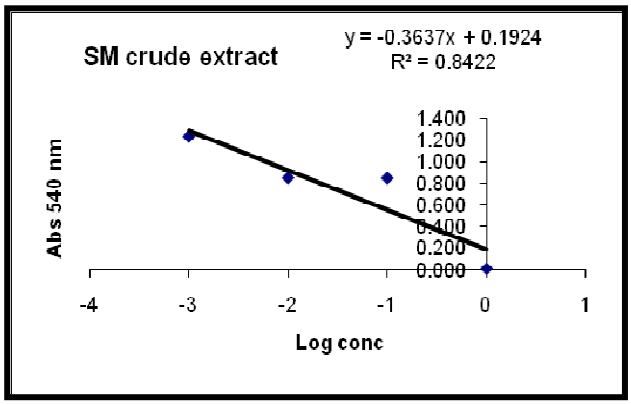

Figure 9-9 Cytotoxicity of crude extract with LC_{50} = 82 µg/ml against Vero cells

9.3.2.1 Therapeutic index of the crude extract and isolated compounds

The therapeutic index for the four antifungal compounds was calculated using the cytotoxic concentrations of the compounds.

The therapeutic index for each fungus was calculated as follows:

Therapeutic index (TI) = LC_{50} against Vero cells in mg/ml divided by the MIC in mg/ml

Table 9-3 shows the therapeutic index of four isolated compounds against different plant pathogens. Amongst the four compounds, the highest therapeutic index was observed in C3 with TI = 185 against *A. parasiticus* and the lowest was found in ursolic acid with 0.2 against *Penicillium* species and *T. harzianum*. The higher the therapeutic index the better the compounds can be considered for use in drug discovery.

Table 9-2 Cellular toxicity and minimum inhibitory concentration of crude extract and four isolated compounds against seven plant pathogenic fungi.

Micro-organisms	Time (hrs)	MIC (mg/ml)	MIC (µg/ml)				
		Crude Extract	Ursolic acid	2	3	4	AmpB
Aspergillus parasiticus	24	0.63 (0)	20(0)	20(0)	10(0)	30(0)	6.4
Aspergillus niger	48	2.50 (0)	120(0)	120(0)	120(0)	120(0)	1.6
Colletotrichum gloeosporioides	48	1.25(0)	30(0)	60(0)	20(0)	60(0)	3.2
Fusarium oxysporum	48	0.32 (0)	50(0)	50(0)	50(0)	50(0)	3.2
Penicillium expansum	48	1.25 (0)	125(0)	32(0)	32(0)	125(0)	3.2
Penicillium janthinellum	48	0.08 (0)	125(0)	16(0)	25(0)	16(0)	3.2
Trichoderma harzianum	48	0.63 (0)	125(0)	250(0)	125(0)	250(0)	3.2
Cytotoxicity (µg/ml)			25	525	1849	35	

Table 9-3 The Therapeutic Index (TI) of four isolated compounds against seven plant pathogenic fungi.

Plant pathogens				
	ursolic acid	2	3	4
Aspergillus parasiticus	1.25	26.3	185	1.2
Aspergillus niger	0.21	4.4	15.4	0.3
Colletotrichum gloeosporioides	0.83	8.8	93	0.6
Fusarium oxysporum	0.5	10.5	37	0.7
Penicillium expansum	0.2	16.4	15	0.29
Penicillium janthinellum	0.2	33.0	116	2.3
Trichoderma harzianum	0.2	4.2	7.4	0.29

9.4 Conclusion

Various compounds are present in crude extracts and this may be the reason why the MIC value was lower (0.08 mg/ml) against *P. janthinellum* than the isolated compounds. Ursolic acid had good antifungal activity against *A. parasiticus*, *C. gloeosporioides* and *F. oxysporum*. Compounds 2 and 4 had good antifungal activity against *P. janthinellum* (MIC 16 µg/ml) while compound 3 inhibited the fungus at the lowest concentration of 10 µg/ml. The inactive constituents were removed during isolation, and as a result the MIC values for all four compounds are lower compared to the crude extract as expected. The initial crude extract loaded on the first column was 2 g (20%) and ursolic acid yielded 6% followed by C2 (3.5%), C3 (1%) and C4 (0.7%). The four compounds may act additively or synergistically as the activity of the individual compounds was not as high as expected.

Amongst the four isolated compounds, only three (1, 2 and 3) had antifungal activity against the tested microorganisms. Moreover, compound 1 was most active compared to the other compounds against the plant fungal pathogens and also against the bacteria. In bioautography assay, the crude extract and compound 1 showed an active compound at the same R_f value against *A. parasiticus*, *T. harzianum* and *P. janthinellum*. This indicates that compound 1 was not an artefact of the isolation procedure.

In the cytotoxicity assay three compounds were very toxic at the concentration tested and the crude extract was less toxic than the isolated compounds. Of the four compounds tested, the highest therapeutic index was observed in C3 with 185 against *A. parasiticus* and the lowest was found in ursolic acid with a ratio of 0.2 against *Penicillium* species and *T. harzianum*. The higher the therapeutic index the safer the compounds can be considered to be in drug discovery.

From the efficacy and safety of the three unidentified compounds it may mean that the crude extract could have higher potential than the isolated compounds. It is a pity that the structure of compound 3 was not able to be elucidated because this compound had good activity and a low toxicity. In the next chapter the *in vivo* efficacy of a crude acetone extract containing a mixture of the isolated compounds and ursolic acid will be tested on fungi infecting oranges.

CHAPTER 10

In vivo experiment: Plant extracts active against *Penicillium* species

10.1 Introduction

Fruit forms an important part of the human diet because it supplies essential nutrients such as vitamins and minerals, which are necessary for prevention of harmful diseases in humans. Citrus (*Citrus sinensis* L. Osbeck, belonging to the family Rutaceae) is well known and is grown in over 100 countries on six continents, with a worldwide crop of about 70 billion kg in 2004 (Anon 2005), and its production exceeds that of any other fruit. However, there are serious problems encountered in citrus production, in particular, postharvest losses which are frequently caused by plant pathogenic fungi. Up to 25% of the total production of harvested fruit is subject to fungal attack in both industrialized and developing countries, and damage is often higher, exceeding 50% (Spadaro and Gullino 2004). Infection occurs through injury during the picking or handling of fruit and results in decay during storage or marketing.

It is essential to control postharvest diseases in order to maintain the quality and improve the shelf life of citrus fruit. However, there are some implications in the market place where transport from producer to consumer may take several weeks to deliver the fruit and storage in packing houses can exceed the maximum period. These factors all influence fruit decay.

Penicillium expansum is one of the most common fruit pathogens, causing a condition known as “blue rot” on nectarines and peaches (Karabulut and Baykal 2002, Karabulut et al. 2002, Vero et al. 2002). *Penicillium expansum* is also harmful to humans since it produces patulin, a mycotoxin known to cause immunological, neurological, and gastrointestinal toxic effects in animals (Pitt 1997). Exposure to high levels of patulin results in vomiting, salivation, anorexia, polypnea, weight loss and leukocytosis.

Green mold caused by *Penicillium digitatum* (Pers.Fr.) Sacc., is generally the most serious postharvest disease of citrus and this results in significant economic losses to the fruit industry. The fungus infects fruit through injuries where moisture and nutrients are available to stimulate spore germination. Infection can occur though very minor injuries that involve damage to individual oil glands of the fruit exo-and mesocarp (flavedo), and through more

extensive puncture injuries encompassing oil glands that extend deeper into the mesocarp (albedo) (Kavanagh and Wood 1971).

Postharvest decay is the major factor limiting the extension of storage life of many fresh harvested commodities. All fresh fruits and vegetables for domestic or export markets should be free of dirt, dust, pathogens and chemicals before they are packed. Chemical fungicides are used widely to prevent the spreading of plant diseases in fruit and also to avoid losses in the commercial fruit industry. There are some difficulties involved, for example residues in fruit which can cause serious complications in human health when the fruit is consumed (Cabras et al. 1999). The susceptibility of freshly harvested products to postharvest diseases increases during prolonged storage as a result of physiological changes that enable pathogens to develop in the fruit (Eckert and Ogawa 1988).

Penicillium digitatum is relatively resistant to the currently available antifungals, for example imazalil, thiabendazole and sodium *o*-phenylphenate (Harding 1972, Holmes and Eckert 1999, Kuramoto 1976). However, the efficacy of these fungicides is compromised due to the emergence and dominance of resistant fungal populations (Holmes and Eckert 1999). Azoxystrobin, fludioxonil and pyrimethanil are recently registered new fungicides for postharvest citrus disease control (Kanetis et al. 2007). Azoxystrobin is very effective in controlling a broad range of plant diseases including green mold of citrus. However, because of their site specific modes of action, quinone outside inhibitor fungicides are at high risk of resistance development in the targeted phytopathogenic fungal populations (Uesugi 1998, Gullino et al. 2000).

There is a need to introduce a new fungicide with a different mode of action that can be used to combat plant fungal pathogens. An alternative way to combat plant fungal pathogens would be to consider natural plant defence mechanisms. Many plant species have excellent activity against fungi (Masoko et al. 2007). Phenolic compounds can play a vital role as phytoalexins in some citrus species (Arcas et al. 2000). The peel of citrus fruit is a rich source of flavonones and many polymethoxylated flavones, which are rare in other species (Horowitz 1961). Naringin and hesperidin are the principal flavones in *Citrus paradise* (grape fruit) and *Citrus sinensis* (oranges).

Because acetone leaf extracts of *Breonadia salicina* had excellent activity *in vitro* against *P. janthinellum* (Chapter 3), the potential use of these extracts and isolated compounds from leaves of *B. salicina* on *Citrus sinensis* artificially infected with *Penicillum* species was examined.

10.2 Materials and methods

10.2.1 Microplate dilution assay

Dried acetone leaf extracts (10 mg) of *B. salicina* were dissolved in acetone (1 ml) and tested for antifungal activity against *Penicillium expansum*, *P. digitatum* and *P. janthinellum* using the serial dilution assay described in section **3.3.2.**

10.2.2 Isolate and culturing of fungi

To obtain a naturally occurring *Penicillium digitatum*, a sterile loop was rubbed carefully on the skin of infected oranges purchased from the market (Fruit and Vegetable) and then streaked on Potato Dextrose agar (PDA) plates. The plates were then incubated overnight at 24ºC. The growth of fungi was observed after 24 hours and the agar plates were removed from the incubator and stored in the refrigerator until further used. The fungal culture was rubbed carefully with a sterile cotton swab and transferred to a sterile tube with fresh Potato Dextrose broth (50 ml).

Penicillium expansum and *P. janthinellum* from Department of Microbiology and Plant Pathology at the University of Pretoria were subcultured from their original fungal strains on Potato Dextrose (PD) agar plates and incubated overnight. Fungal cultures were subcultured (1% inoculum) in PD broth before being used in the experiment.

The identity of the fungus isolated from the infected orange was confirmed as *Penicillium digitatum* by Ms Candice Johnston from the Department of Microbiology and Plant Pathology at the University of Pretoria. This is a well known vigorous citrus pathogen.

10.2.3 Quantification of fungal inoculum

For quantification of the fungal cultures, the haemocytometer cell-counting method described by Aberkane et al. (2002) with some modifications was used. The method is described in section **3.3.1.**

10.3. In vivo experiment

10.3.1 Fruit Decay test

A modification of the method described by Muňoz et al. (2007) was used for the fruit decay test using orange fruit (*Citrus sinensis* L. Osbeck*)*. Experiments were carried out on freshly harvested navel orange fruits (*Citrus sinensis* L. Osbeck) purchased at a greengrocer. Approximately 10 ml of Sunlight liquid dishwashing soap were added into a container containing five litres of water and freshly harvested fruits were soaked for five minutes. This was done to remove the wax layer on the oranges, which were then rinsed repeatedly with tap water and allowed to air dry. The oranges were marked on the outer skin into eight sections labelled N (acetone negative control), H (highest concentration of 10 mg/ml of extract), L (lower concentrations 0.16, 0.08 and 1.25 mg/ml of the extract and (P) the positive control, which was amphotericin B (0.16 mg/ml based on the MIC previously determined), in one experiment. Each of the 8 sections on the orange had a duplicate treatment leading to a total of sixteen treatments and four replicates per treatment on each orange. Four oranges per replicate were prepared for each treatment. The oranges were then wounded with a sterile needle by making punctures approximately 5 mm in depth and 0.6 mm in diameter at appropriate places. Acetone extracts was used in this experiment since the solvent is not toxic to the fungi at the concentration tested (Eloff et al. 2007). The extraction procedure is described in section **2.2.4.1** (Chapter 2).

We completed three experiments to test the efficacy of the isolated compound and plant extract. In the first experiment, 1.25, 0.16 and 0.08 mg of crude extract were dissolved in 1 ml acetone and then 100 µl of the acetone extracts were applied on the wounds. These concentrations were chosen on the basis of the MICs of the extracts on the growth of *P. expansum*, *P. digitatum* and *P. janthinellum* respectively when tested for antifungal activity in section **10.2.1**. The oranges were allowed to dry completely for 20 minutes to remove traces of acetone. Ten µl inocula contained 1.0×10^6 cells/ml of *P. expansum*, *P. digitatum* and *P. janthinellum* of the culture was applied to each puncture wound to simulate infections after oranges were treated. This equated to about 10 000 fungal cells on the wound. Because four oranges were used for each fungus there were 16 replicates per treatment. After treatment the fruits were placed in a container with the following dimensions: 27cm wide, 27 cm long and 14.5 cm high. The container had a tightly closing lid sealed with a tape. The growth chamber

was maintained at 20°C and 100% relative humidity by putting moistened cotton wool at the bottom of the container. The zone of growth on the infected area was visible after 4 days and minor changes were observed on the fifth and sixth days. Measurements were then recorded after 4 and 7 days as the number of infected wounds per replicate, and mean diameter in mm ±SD (standard deviation).

In the second experiment we wanted to test the activity of the isolated ursolic acid and to confirm that acetone did not have a negative indirect effect by e.g. interacting with the surface of the orange. One mg of ursolic acid was dissolved in 1 ml acetone and then 100 µl was applied on the wound as described above. In this experiment each orange was marked on the outer of the skin into four sections and each section had two duplicates to make eight replicates per orange fruit. Acetone was used as the negative control and amphotericin B (0.16 mg/ml) was the positive control.

In order to test the dose response of the extract and to determine the LC_{50} in the third experiment, different concentrations of the extract were tested 0.0, 1.0, 2.0, 4.0 and 8.0 mg/ml. In the case of amphotericin B, it was difficult to determine the low dose effect in experiments mentioned above (1 and 2) since we tested the highest concentration of 0.16 mg/ml. The concentration of the positive control was decreased to 0.04 and 0.08 mg/ml.

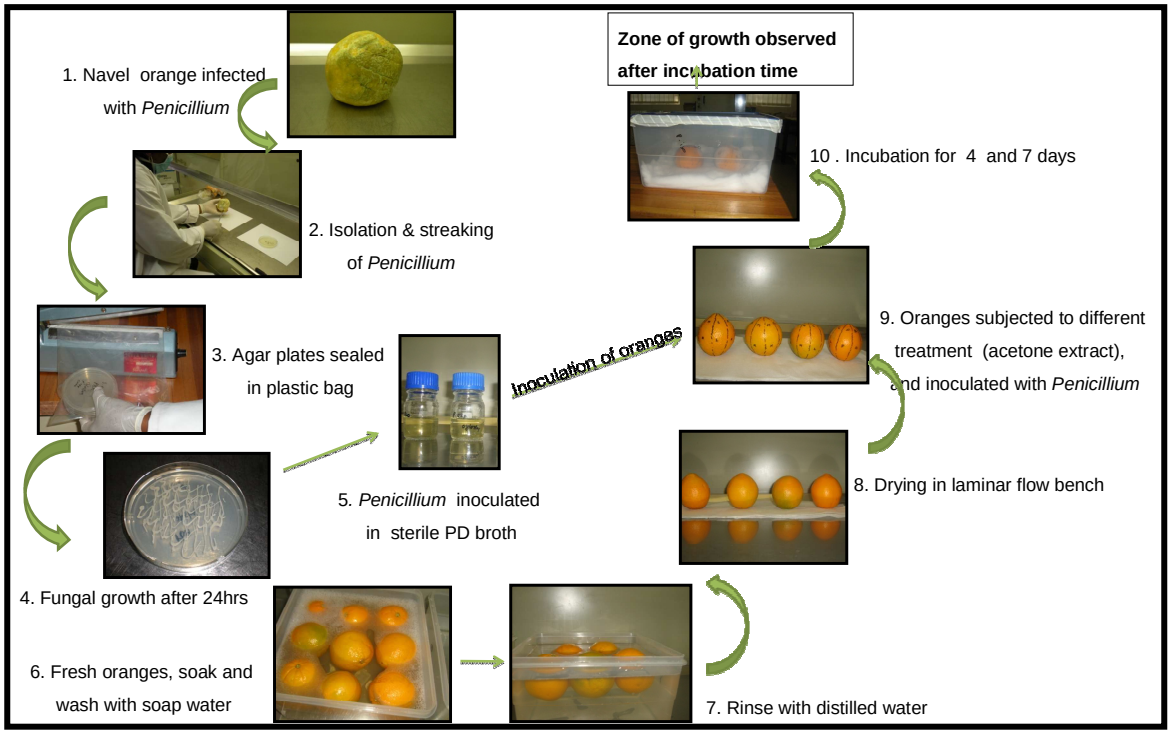

Figure 10-1 Schematic representation of orange infection experiment using plant pathogen isolated from rotten orange and *P. expansum* and *P. janthinellum*. (2) Sterile loop was used to streak *P. digitatum* on agar plate and (3) incubate overnight. (4) Observation of fungal growth after 24 hrs. (5) The fungal culture was adjusted to approximately 1.0×10^{6} cells/ml (6-8) Navel oranges were soaked for 5 minutes and then washed with soap and allowed to dry. (9) Each orange was divided into eight sections with two duplicates in each section to make sixteen treatments. The oranges were punctured and then the extracts were applied into the wound at different concentrations and allowed to dry for 20 minutes. Acetone and amphotericin B were used as negative and positive controls. The fungal culture was applied to the wound and (10) the oranges were placed in a container and incubated until the zone of growth was visible.

10.4 Results and discussion

10.4.1 Microplate dilution assay

The minimum inhibitory concentration (MIC) values of acetone extracts against three plant pathogenic fungi were determined (Table 10-1). Acetone leaf extracts had good antifungal activity against *P. janthinellum* with MIC value of 0.08 mg/ml. The *Penicillium digitatum* and *P. expansum* were more resistant, both with MIC values of 1.25 mg/ml. Ursolic acid also had antifungal activity against *P. expansum*, *P. janthinellum* and *P. digitatum* with MIC value of 0.13 and 0.25 mg/ml. The *Penicillium digitatum* was 25 times more resistant to amphotericin B than the two pure cultures, possibly indicating why it was not inhibited by the treatment the oranges received prior to marketing.

Table 10-1 Minimum inhibitory concentration (MIC) after 48 h of crude extract, ursolic acid and amphotericin B against three *Penicillium* species. The results show the average of three replicates with a standard deviation of 0.

Plant pathogens	MIC (mg/ml)		
	Extract	Ursolic acid	AmpB
Penicillium expansum	1.25	0.13	3.2
Penicillium janthinellum	0.08	0.13	3.2
Penicillium digitatum	0.16	0.25	80

10.4.2 First experiment to evaluate the procedure

The experiments were designed to evaluate the potential use of the extract or isolated compound(s) against a plant fungal pathogen. In the first experiment, we determined the potential toxicity of the solvent and the crude extract and amphotericin B. After four days incubation time, the acetone treatment gave the highest growth with a diameter of 5.7 to 6.0 mm in all cases and probably did not inhibit the growth of fungi against three *Penicillium* species. This suggests that the solvent was not harmful to the fungi or had evaporated to such an extent that the residues left had no effect (Figure 10-2). The observations were repeated after seven days but with the exception of some growth of *P. expansum* dosed with 10 mg/ml (acetone extract) there were no striking differences between the values between 4 and 7 days.

From the low standard deviation within the different treatments is appears that the method gives reproducible results. The method used was further validated by the good correlation between the MIC obtained with the serial dilution method and the growth rate on the oranges. The results provided confidence to continue with the next steps.

Table 10-2 Growth of different *Penicillium* isolates in mm treated with acetone (0), acetone extracts and amphotericin B after 7 days incubation

	Penicillium expansum			*Penicillium digitatum*			*Penicillium janthinellum*		
	0.0	10	1.25	0.0	10	0.16	0.0	10	0.08
Diameter (mm)	6.2	1.32	5.17	5.89	0.0	4.73	6.77	2.01	6.7
AmpB	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

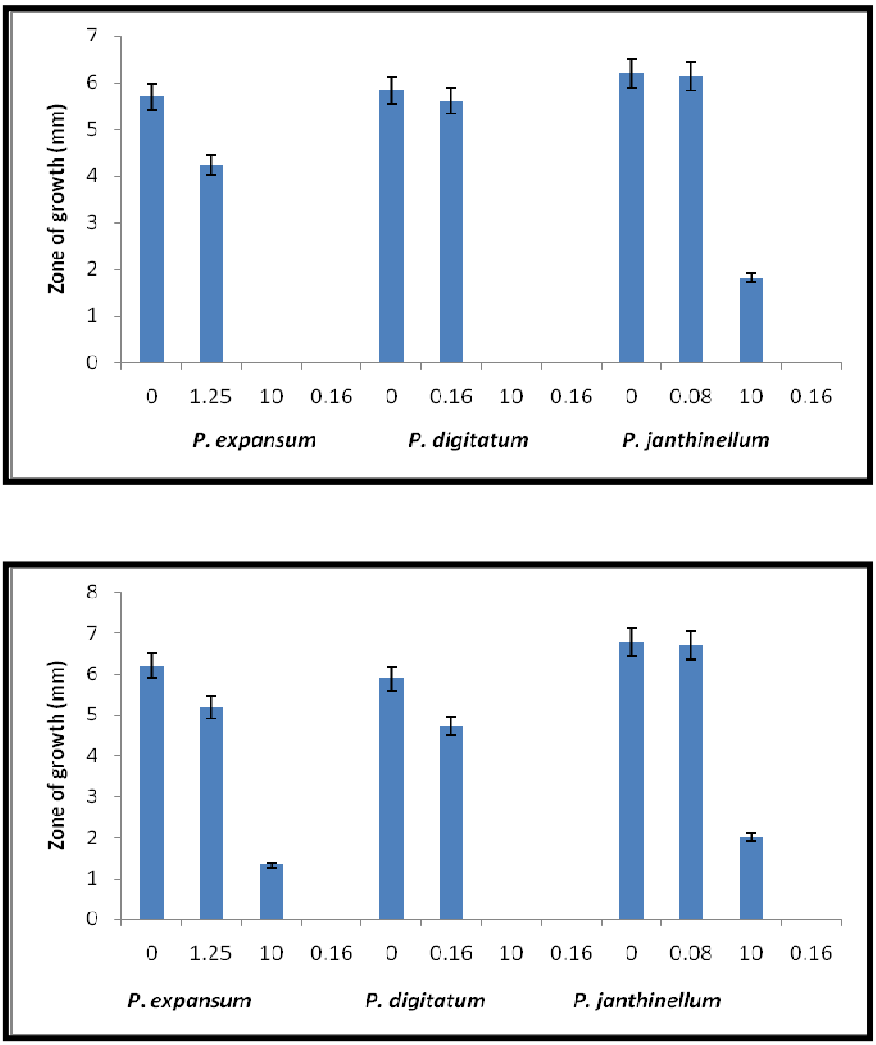

Figure 10-2 Orange fruits were inoculated with about 10 000 cells of *P. expansum*, *P. digitatum* and *P. janthinellum* on each wound and subjected to different concentrations of the crude extract. Diameters of growth (mm) were measured after 4 (top) and 7 (bottom) days incubation time. Results are shown as the average diameter of the infected wounds. Error bars show standard deviation. **Lanes from left to right:** Acetone extracts at different concentrations (0.0, 1.25 and 10 mg/ml), Amphotericin B (0.16 mg/ml).

10.4.3 Determining the dose related effect of the treatments

To determine the activity of ursolic acid the second experiment was repeated for four days using acetone, 1 mg/ml ursolic acid and 0.16 mg/ml amphotericin B on the *P. digitatum*. The

values for the acetone control (0 mg/ml) were in the same order as in the previous experiments and the value for 1 mg/ml ursolic acid was 2.89 mm (figure 10-3).

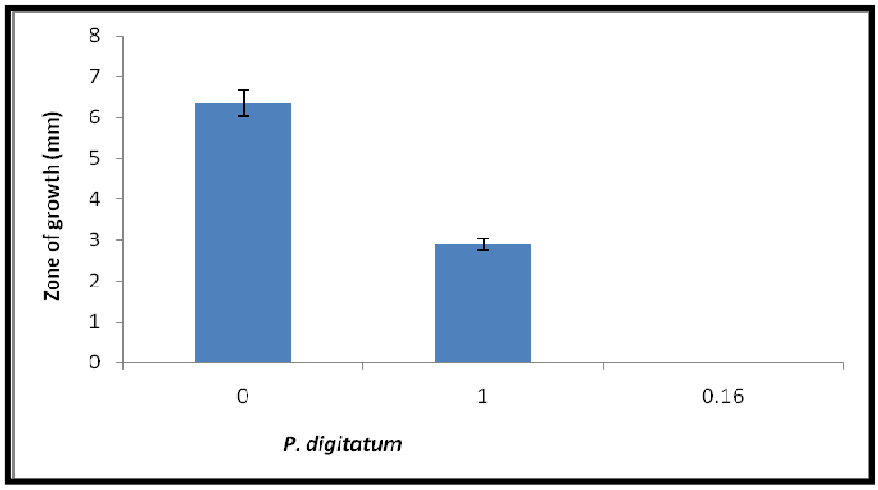

Figure 10-3 Orange fruits were inoculated with about 10 000 cells of *P. digitatum* on each wound and subjected to 1 mg/ml concentration of ursolic acid. Diameters of growth (mm) were measured after 4 days incubation time. Results are shown as the average diameter of the infected wounds. Error bars shows standard deviation. **Lanes from left to right:** Acetone (0.0), ursolic acid (1 mg/ml) and Amphotericin B (0.16 mg/ml).

Figure 10-4 shows the average diameter of the zone of growth tested on oranges against three plant pathogenic fungi, *P. expansum*, *P. digitatum* and *P. janthinellum*. In the first experiment, the lowest dose of amphotericin B was too high to determine the $LC_{50.}$ In the third experiment different concentrations were investigated: 1.0, 2.0, 4.0 and 8.0 mg/ml, amphotericin B (0.04 and 0.08 mg/ml). The highest zone of growth (2.7 mm) were observed in the negative control (acetone) while at 1 mg/ml the average diameter was reduced (2.2 mm) when acetone extracts were used as treatment against *P. expansum*. There was a good dose related inhibition by the crude acetone extract. Noticeably, when amphotericin B was used, there was no zone of growth observed on the fruit even at the lowest concentration of 0.04 mg/ml (Fig 10-4, Table 10-3).

Acetone (the negative control) had the highest zone of growth when oranges were treated against *P. digitatum* with average diameter of 2.9 mm and was reduced to 2.4 mm at 1 mg/ml.

There were slight differences in the average diameter at acetone extract concentration of 4 and 8 mg/ml (with average diameters of 1.1 and 0.9 mm, respectively). In contrast to *P. expansum* where there was no growth with amphotericin B at a concentration of 0.04 mg/ml, the *P. digitatum* was much more resistant with an average growth diameter of 2.9 mm (0.08 mg/ml) and 2.1 (0.04 mg/ml).

The zone of growth in the acetone negative control tested against *P. janthinellum* was 5.2 mm and this was reduced to 3.2 mm at an extract concentration of 1 mg/ml. In general, the zone of growth on the fruit was reduced when the concentration of the acetone extracts increased, for example, 1 mg/ml (average diameter 3.2 mm) and 2 mg/ml (2.7 mm), 4 mg/ml (1.7 mm) and 8 mg/ml (0mm). In contrast to *P. expansum* some growth of *P. janthinellum* took place at 0.04 mg/ml amphotericin B.

10.4.4 Determining the effect of dose and time of exposure

When the growth was determined after 4 and 7 days on the same oranges, there were not many changes in the dose related response. There were minor differences between measurements taken after 4 and 7 days with *P. expansum*. After 7 days there was also some growth at a dose of 8 mg/ml (Figure 10-4). With the *P. digitatum* there was a doubling of the area of fungal growth between day 4 and 7 without much change in the dose related response. After ten days however the whole orange was covered when infected with *P. digitatum* (Figure 10-6). It is possible that in the case of *P. digitatum* that the growth form changed from a yeast-like single cell to a mycelial growth form. With *P. janthinellum* more or less the same effect was found as with *P. expansum*, with not much growth between days 4 and 7.

P. expansum was sensitive to amphotericin B at the lowest concentration tested after both periods. *P. janthinellum* growth was inhibited by amphotericin B up to 4 days, but after 7 days the inhibition was overcome. In the case of the isolate it was resistant to the highest concentrations and between days 4 and 7 it grew even better than the negative control. This can probably be explained by changing from a yeast to a mycelial growth form.

Table 10-3 Growth of different *Penicillium* species in mm treated with different concentrations of the crude extract and two concentrations of amphotericin B after 7 days incubation

Pathogen						AmpB	
	0.0	1	2	4	8	0.04	0.08
Penicillium expansum	3.2	2.7	2.2	1.7	1.2	0.0	0.0
Penicillium digitatum	6.2	4.7	3.6	2.7	2.5	10.6	11.5
Penicillium janthinellum	6.7	4.7	3.3	2.2	2.8	6.7	0.0

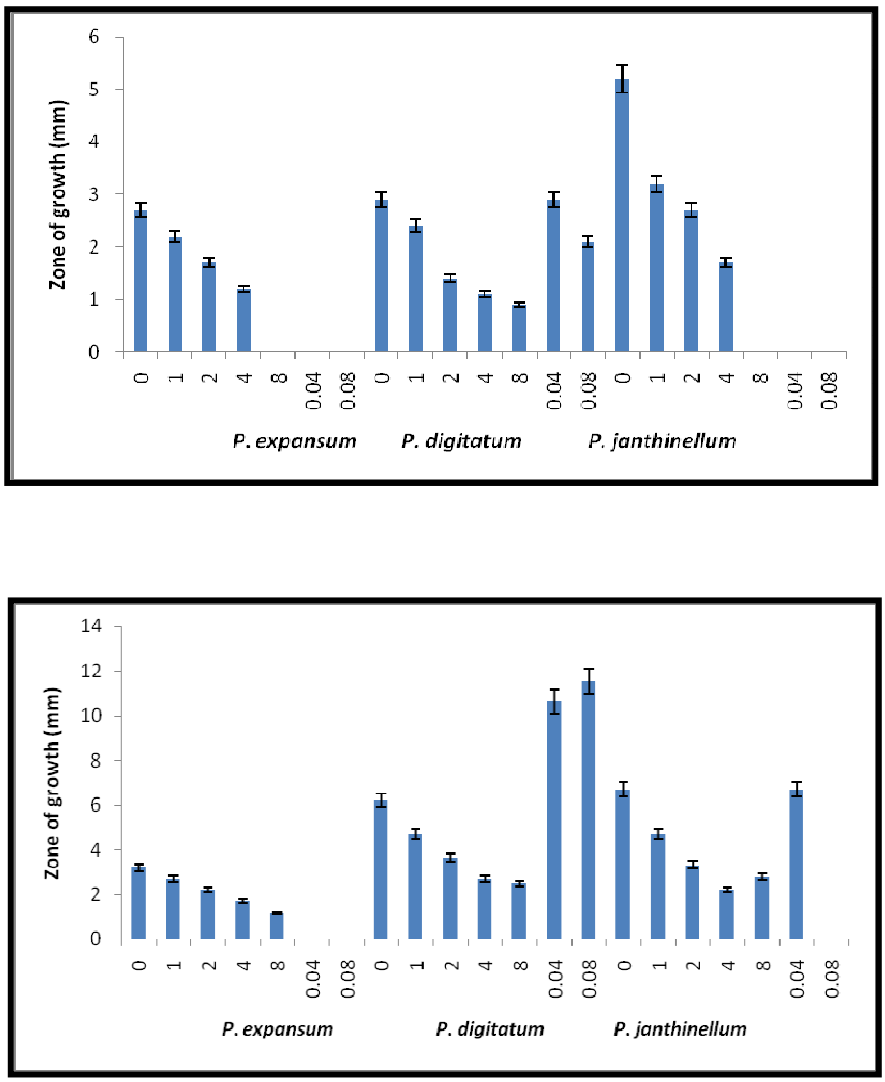

Figure 10-4 Orange fruits were inoculated with about 10 000 cells of *P. expansum*, *P. digitatum* and *P. janthinellum* on each wound and subjected to different concentrations of the crude extract. Diameters of growth (mm) were measured after 4 (top) and 7 (bottom) days incubation time. Results are shown as the average diameter of the infected wounds. Error bars shows standard deviation. **Lanes from left to right:** Acetone extracts at different concentrations (0.0), 1.25 and 10 mg/ml, Amphotericin B (0.16 mg/ml).

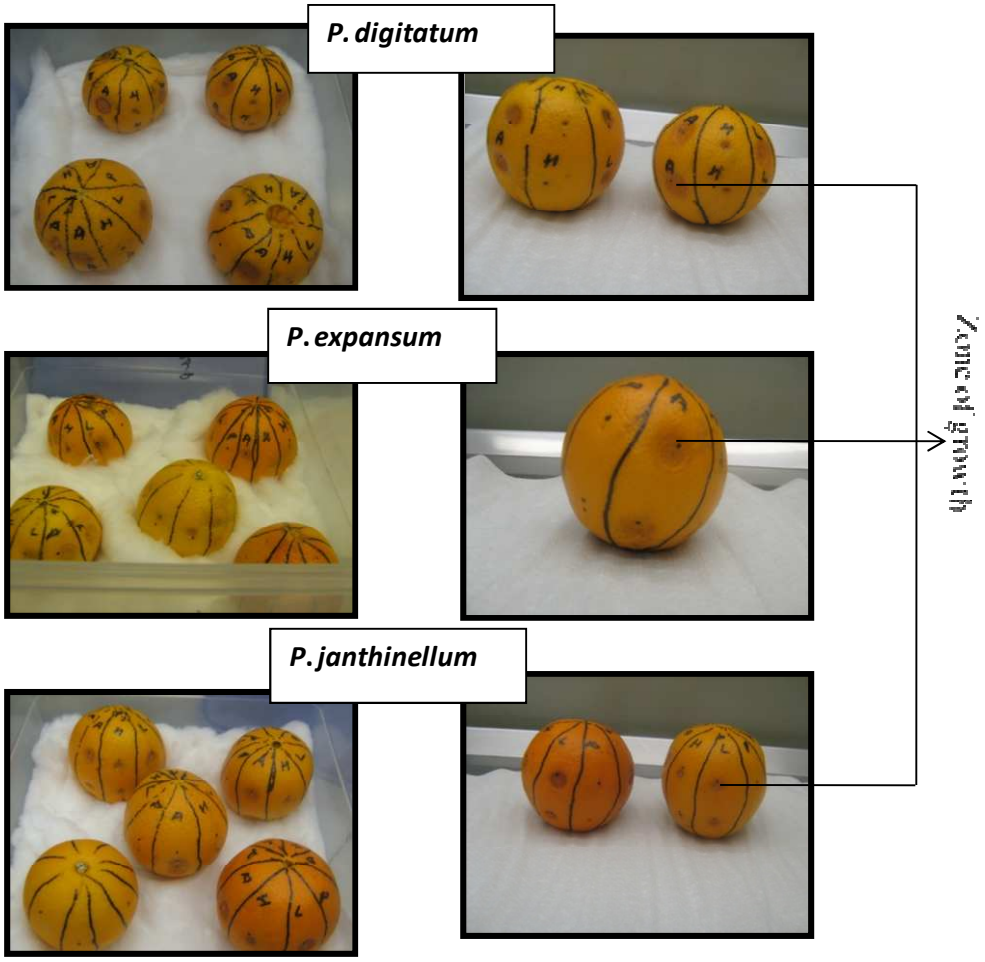

Figure 10-5 Orange fruits inoculated with 1.0×10^{6} cells/ml of *P. digitatum*, *P. expansum* and *P. janthinellum*, subjected to different treatment (A) acetone, (L) lower concentration (H) higher concentration and (P) Amphotericin B and then incubated for 4 days. Zone of growth (mm) of infected wounds were observed on the skin of fruit.

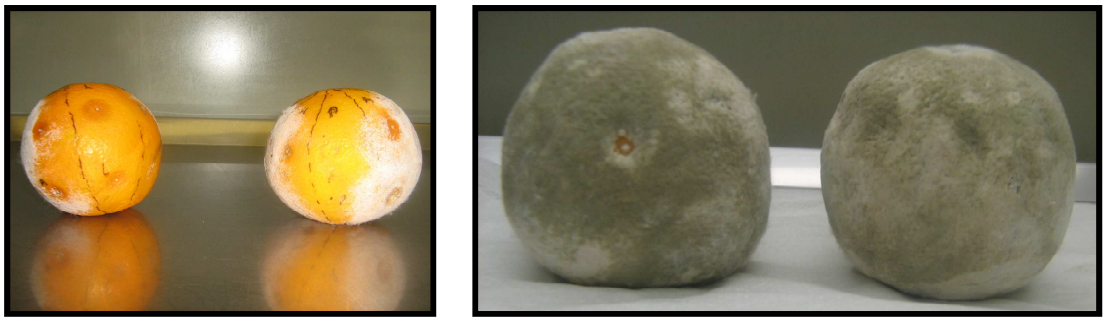

Figure 10-6 Oranges inoculated with 100 000 cells of *P. digitatum* and subjected to different treatments. **Left:** Oranges half infected with *Penicillium* after 8 days and **right:** oranges were covered with fungi after 10 days probably reflecting a change from a yeast to a mycelial growth form.

10.4.5 Determining the LC_{50} of the crude extract

In the third experiment we wanted to determine the dose related response of the crude extract in order to compare the activity relative to ursolic acid as well as to compare the ratio of toxicity of mammalian cells to activity against the fungal pathogens. To calculate the IC_{50} it would be more correct to calculate the growth area rather the growth diameter growth rate. The data presented in bar charts in Figure 10-4 were presented in a scatter diagram to enable calculation of LC_{50} values in Figure 10-7. To overcome the change of a yeast growth form to a mycelial growth form, experiments were only carried out for 4 days.

There was an excellent dose related response in all cases (Figures 10-7 and 10-8). The smooth lines again attest to the validity of the model we have developed. In Figure 10-7, the graph shows that at a zero concentration, the area of growth of *P. expansum* was 32.2 mm^2 and therefore the LC_{50} (dose that kills 50% of the cells) was 1.8 mg/ml, because this would have led to a 50% growth of 16.1 mm^2. In the case of the *P. digitatum*, the IC_{50} was 1.6 mg/ml and with *P. janthinellum* it was 1 mg/ml. The above analysis revealed that when the area of growth was very high, then the IC_{50} (inhibition concentration of fungi) was reduced.

There was a slight difference in IC_{50} when comparing data between 4 and 7 days of incubation (Figure 10-8). With *P expansum* the IC_{50} increased from 1.8 mg/ml to 1.9 mg/ml

with the longer incubation. With the *P. digitatum*, the value changed from 1.6 to 1.7 mg/ml and for *P. janthinellum* there was no difference in IC_{50} of 1 mg/ml with a longer period of incubation.

It is remarkable that in this experiment the growth area for *P. janthinellum* was substantially lower than that for the two other isolates whereas the diameter of growth was very similar in the first experiment. This may be related to changing from a yeast to a mycelial growth form by the two other isolates.

The effect of 0.04 and 0.08 mg/ml amphotericin B on the three isolates was also determined in the same experiment as shown in Table 10-4. The resistance of the *P. digitatum* in comparison to the other *Penicillium* species to amphotericin B may explain why it was isolated from commercially available oranges. It has been reported that *P. digitatum* isolate from citrus fruit were relatively sensitive to the three fungicides, sodium *o*-phenylphenate (*o*-phenylphenol), imazalil, and thiabendazole with mean EC_{50} values of 0.026, 0.1, and 6.3 µg/ml. However, in the case of packing house fruit *P. digitatum* was more resistant to imazalil with an EC_{50} ranging between 0.87 and 0.92 µg/ml (Holmes and Eckert 1999).

Table 10-4 Growth of different *Penicillium* isolates in mm^2 treated with two concentrations of amphotericin B after 7 days incubation

Pathogen	0.04 mg/ml	0.08 mg/ml
Penicillium expansum	0	0
Penicillium digitatum	89	105
Penicillium janthinellum	35	0

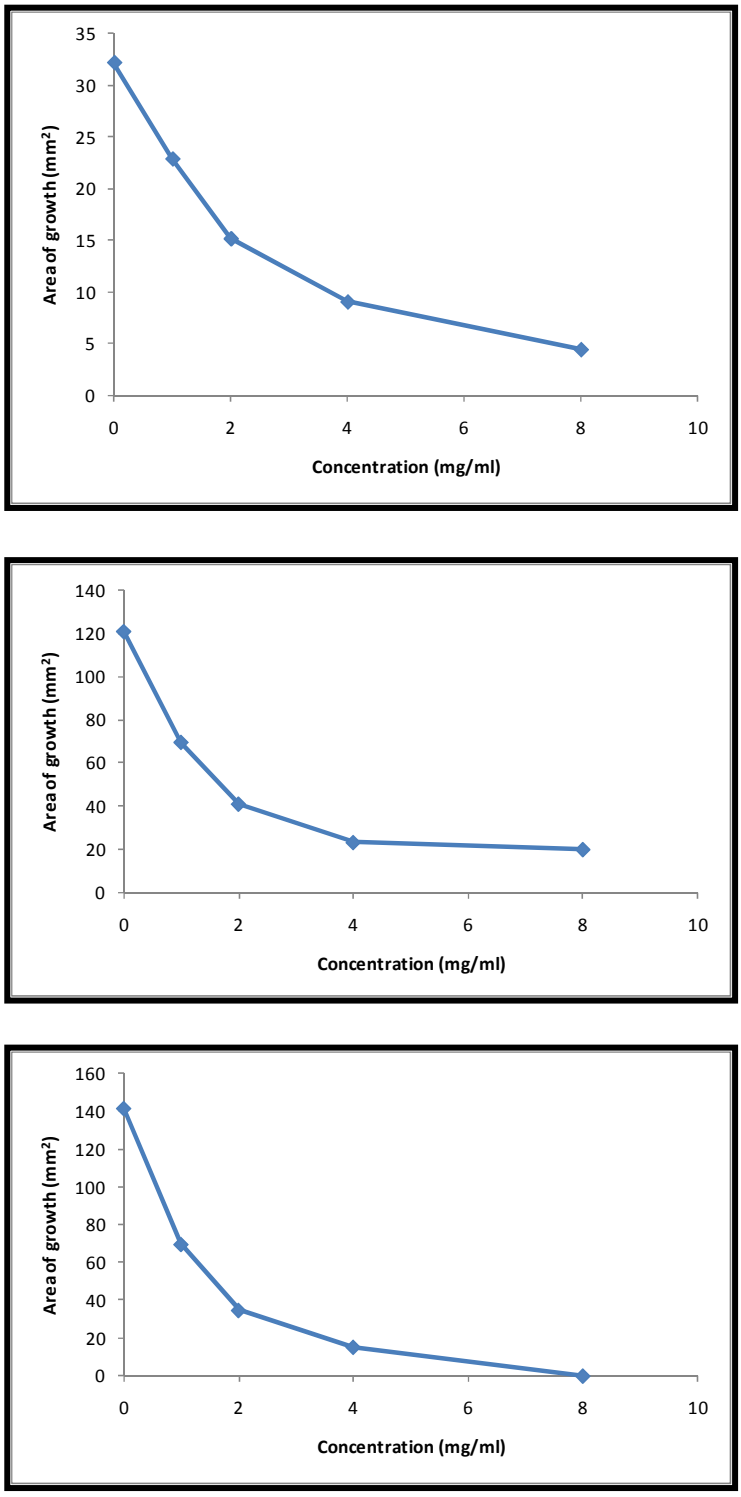

Figure 10-7: Oranges inoculated with 100 000 cells of *P. expansum* (top) *P. digitatum* (middle) and *P. janthinellum* subjected to different concentrations of the crude acetone leaf extract of *Breonadia salicina*. The area of growth in mm^2 was calculated and results are shown as the average infected area after 4 days of incubation time.

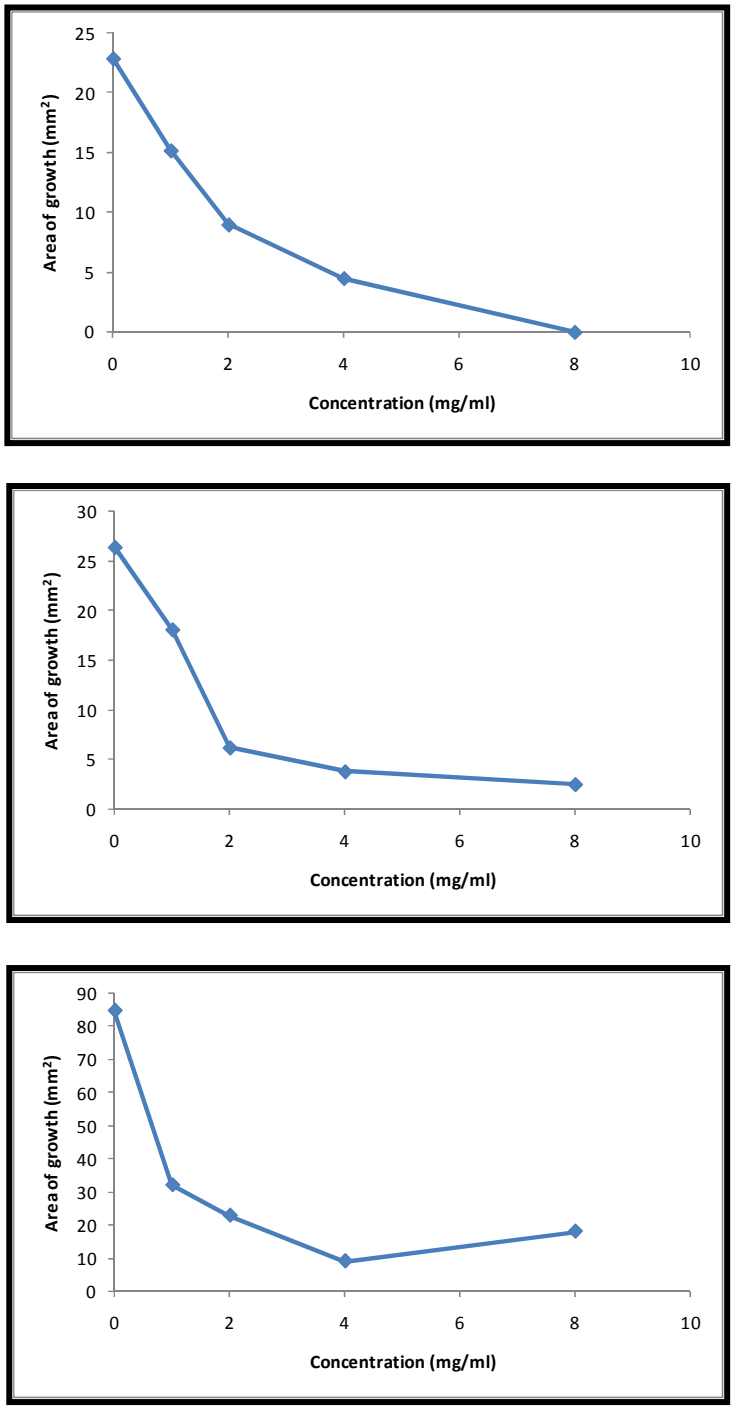

Figure 10-8: Oranges inoculated with 100 000 cells of *P. expansum* (top) *P. digitatum* (middle) and *P. janthinellum* (bottom) subjected to different concentrations of the crude acetone leaf extract of *Breonadia salicina*. Area of growth in mm^2 was calculated and results are shown as the average infected area of the after 7 days of incubation time.

Table 10-5 LC_{50} and area of growth after 4 and 7 days incubation time against three *Penicillium* species.

Plant pathogens	Time (days)	LC_{50} (mg/ml)	Area of growth (mm^2)
P. expansum	4	1.8	16.1
P. digitatum	4	1.6	60.4
P. janthinellum	4	1.0	70.5
P. expansum	7	1.9	11.45
P. digitatum	7	1.7	13.2
P. janthinellum	7	1.0	42.4

It is interesting that the LC_{50} of ursolic acid was in the same order as that of the crude extract. This indicates that the other antifungal compounds present in the crude extract probably play a role in the total antifungal activity.

10.5 Therapeutic Index

10.5.1 Therapeutic index of the crude extract and isolated compounds

The therapeutic index for the acetone extracts (treatment) was calculated using the cytotoxicity concentrations of the crude extracts (Chapter 9).

The therapeutic index for each fungus was calculated by dividing the concentration that would kill the pathogen by the concentration that would kill animal cells. The higher the value the safer the extract would be.

Because LD_{50} or LC_{50} are inversely related to activity the therapeutic index can be calculated by dividing the LC_{50} for animal cells (Vero) by the LC_{50} for the pathogen determined in section **10.5.5**.

Table 10-6 The Therapeutic Index (TI) of acetone extracts against three plant pathogenic fungi calculated by dividing LC_{50} with MIC using values after 4 days.

Fungi	LC_{50} in mg/ml for fungus	LC_{50} Vero cells mg/ml	Therapeutic Index
Penicillium expansum	1.8	0.082	0.05
Penicillium digitatum	1.6	0.082	0.05
Penicillium janthinellum	1.0	0.082	0.08

The therapeutic index of 0.05 to 0.08 means that the acetone leaf extract of *B. salicina* may be much too toxic to become a useful product to control *Penicillium* infections in oranges. Many fungicides are very toxic to mammal cells and it may be interesting to compare the safety of the fungicides commonly used to protect plant products with the extract we used.

One cannot accept that cellular toxicity equates to mammalian toxicity without any animal experiments. If toxic components are not taken up from the digestive system or if toxins are quickly metabolized to inactive compounds by liver enzymes, the inherent toxicity may be much lower than the value reflected by the cellular assay.

10.6 Conclusion

The method that was used to evaluate the protection by crude leaf extracts of *B. salicina* gave reproducible results. A crude leaf extract at a concentration of less than 1 mg/ml gave the same level of protection as 1 mg/ml ursolic acid (2.89 mm diameter, Figure 10-3), the main antifungal compound in the extract. The crude extract therefore gave practically the same protection as the isolated compound. This indicates that a low cost product could be developed from leaves of *B. salicina.* This LC_{50} was however two to three orders of magnitude higher than that of the commercially used antifungals. In practical terms all antifungal compounds have some toxicity, unfortunately our extract was more toxic to Vero monkey cells than to the fungi. It may be possible to remove the toxic component from the extract without major changes.

These results point to the feasibility of using plant products as biopesticides. This degree of success was attained with a plant species that had relatively low activity compared to the antifungal activity of other plant extracts investigated in the Phytomedicine Programme. It is likely that wide screening against *Penicillium* species may be a viable option to develop useful antifungal preparations for post harvest protection of plant products.

CHAPTER 11

Summary and conclusion

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

To attain this aim the following objectives were identified:

1. 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.
2. To determine the antifungal activity of leaf extracts of the selected plant species against *Aspergillus fumigatus* due to its potential use in protecting production animals against this pathogen.
3. 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.
4. To isolate antifungal compounds from the selected plant species and to determine the structure of these compounds.
5. To determine the biological activity of the crude extract and the isolated compounds in antimicrobial and cytotoxicity assays.
6. To evaluate the potential use of the extract or isolated compound(s) against a plant fungal pathogen.

Objective 1. To select plant species to be tested for activity against plant fungal pathogens

The species selected for evaluation were: *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana*. Acetone, hexane and methanol extracts of *B. buceras* had the best antifungal activity against *Penicillium expansum*, *P. janthinellum*, *Trichoderma harzianum* and *Fusarium oxysporum* with MICs ranging between 0.02 and 0.08 mg/ml. All the extracts of *B. salicina* had the best activity against *P. janthinellum* with MIC values of 0.08 mg/ml. Acetone extracted a large quantity of plant

material (108.3 mg) and MeOH was the second best extractant, extracting 100.4 mg. Some of the plant extracts had strong antifungal activity, but individually separated compounds were not active based on the bioautography assay. All extracts of *O. ventosa* had the highest activity against *T. harzianum* with MIC value of 0.04 mg/ml. Although *Olinia ventosa* extracts gave very promising results, the plant was not readily available for collection of test material. *Breonadia salicina* was selected for further phytochemical investigation based on the good antifungal activity and bioautography results.

Objective 2. To determine the antifungal activity of plant extracts against *Aspergillus fumigatus*

Based on the antifungal activity against plant pathogenic fungi, further investigation was carried out to test the activity of the plant extracts against an animal fungal pathogen with serious economical implications in poultry production. Leaf extracts of the six plant species had antifungal activity against *A. fumigatus*. All extracts of *B. salicina* had a strong antifungal activity against *A. fumigatus* with MIC values as low as 0.08 mg/ml. In general, extracts were not as active against animal fungal pathogens although all of the plant extracts were active against both plant and animal pathogenic fungi. In the bioautography assay, the same antifungal compounds were apparently present in extracts of *B. salicina* against plant and animal pathogenic fungi. Since the extracts had good antifungal activity against *A. fumigatus*, a clinical study could in future be carried out to test the efficacy of the extracts using chickens as done by Suleiman (2009) with other extracts.

Objective 3. To screen leaf extracts of plant species for qualitative antioxidant activity

There are two ways in which a plant extract can protect the host against infections. In the first place it could directly inhibit the growth of pathogens. It is however also possible that the extract could stimulate the immune system of the host so that it is able to withstand the infection. Antioxidant activity was determined qualitatively by spraying TLC plates with 0.2% DPPH. Antioxidant activity was observed in the methanol extract of *V. infausta* and *X. kraussina* with the yellow band. However, the activity was not strong since the yellow band was not very clear. The acetone, hexane and DCM extracts were less effective than the

methanol extract. No plant extract had strong antioxidant activity and quantitative analysis was not investigated. It appears that the effect of extracts of *B. salicina* used traditionally may therefore be due to direct inhibition of microbial growth.

Objective 4. To isolate antifungal compounds from the selected plant species

Preliminary fractionation of *B. salicina* was carried out using serial extraction with solvents of increasing polarities. Acetone and chloroform were the most promising fractions of the serial extraction procedure. Solvent-solvent fraction was performed on the chloroform fraction and led to the separation of three fractions (n-butanol, chloroform and aqueous). Amongst the three fractions, the chloroform fraction of the solvent-solvent fractionation was the most active and showed several antifungal compounds in the bioautography assay.

Column chromatography using silica gel was used for gradient elution of extracts, leading to isolation of four compounds. Nuclear Magnetic Resonance (NMR) spectroscopy and MS were used for identification of isolated compounds. Only one compound was identified as the triterpenoid ursolic acid while MS revealed that the other three compounds appeared to be mixtures of fatty acids and the structures were not elucidated.

Objective 5. To determine the biological activity of the crude extract and the isolated compounds in antimicrobial and cytotoxicity assays

Biological activities of all isolated compounds were determined. Although many compounds have been isolated, frequently the biological activity has not been determined. All the isolated compounds had antifungal activity against the tested organisms, with MIC values ranging between 10-125 µg/ml. In the bioautography assay, antifungal compound was visible in the bands represented by ursolic acid, C2 and C3, but not in compound C4. Based on the bioautography assay, the four isolated compounds had both antibacterial (against *E. coli*, *P. aeruginosa* and *S. aureus*) and antifungal activity.

It is important to determine cytotoxicity to know whether the compounds are potentially harmful to humans and animals. The cytotoxicity of the crude acetone extract and isolated

compounds were investigated against Vero monkey kidney cells using the MTT assay. The crude acetone extract had an LC50 of 82 µg/ml. Ursolic acid and C4 were toxic towards Vero cells at an LC_{50} of 25 and 36 µg/ml respectively. Compound 2 and C3 were not toxic toward Vero cells with LC_{50} greater than 200 µg/ml. Ursolic acid was very toxic despite showing good antibacterial and antifungal activity against the tested microorganisms. The crude acetone extract, ursolic acid and compound 4 were too toxic to investigate further to develop drugs that can be used to combat fungal infectious diseases in plants, animals and humans. If the structures of compounds 2 and 3 can be determined there may be some possibility for further use.

The crude extract of *B. salicina* was active against the animal fungal pathogen *Aspergillus fumigatus*, with an MIC of 80 µg/ml, but was also too toxic to consider for further use. In all cases, changes of the basic chemical structure may increase the activity and decrease the toxicity of the compound to animal cells, but this was beyond the scope of this study.

Objective 6. To evaluate the potential use of the extract or isolated compound(s) against a plant fungal pathogen

In *in vivo* studies, the potential use of the extract or isolated compound(s) against three plant fungal pathogens (*Penicillium expansum*, *P. janthinellum* and *P. digitatum* isolated from infected oranges) were investigated after treating the oranges with the crude acetone extract and ursolic acid. The method used in the experiment gave good, reproducible results. The crude leaf extracts and ursolic acid inhibited the growth of fungi at 8 and 1 mg/ml. The LC_{50} for the three fungi were *P. janthinellum* 1 mg/ml, *P. digitatum* 1.6 mg/ml and *P. expansum* was 1.8 mg/ml. These values are three orders or magnitude lower than the LC_{50} values of commercial fungicides such as sodium *o*-phenylphenate (*o*-phenylphenol), imazalil, and thiabendazole. *Penicillium digitatum* was more resistant to amphotericin B in comparison to other *Penicillium* species. These results nevertheless suggest that a low cost product could be produced from leaves of *B. salicna* to protect fruit from plant pathogenic fungi. The therapeutic index for each fungus ranged between 0.05 and 0.08. This means that the acetone leaf extract of *B. salicina* may be much too toxic to be a useful product to control *Penicillium* infections in oranges.

The results did however illustrate the feasibility of developing a biopesticide from a plant product. The low cost of developing such a product may make it economically feasible if there were no safety concerns. Using a plant based fungicide may also find acceptance in the organic growth market.

CHAPTER 12

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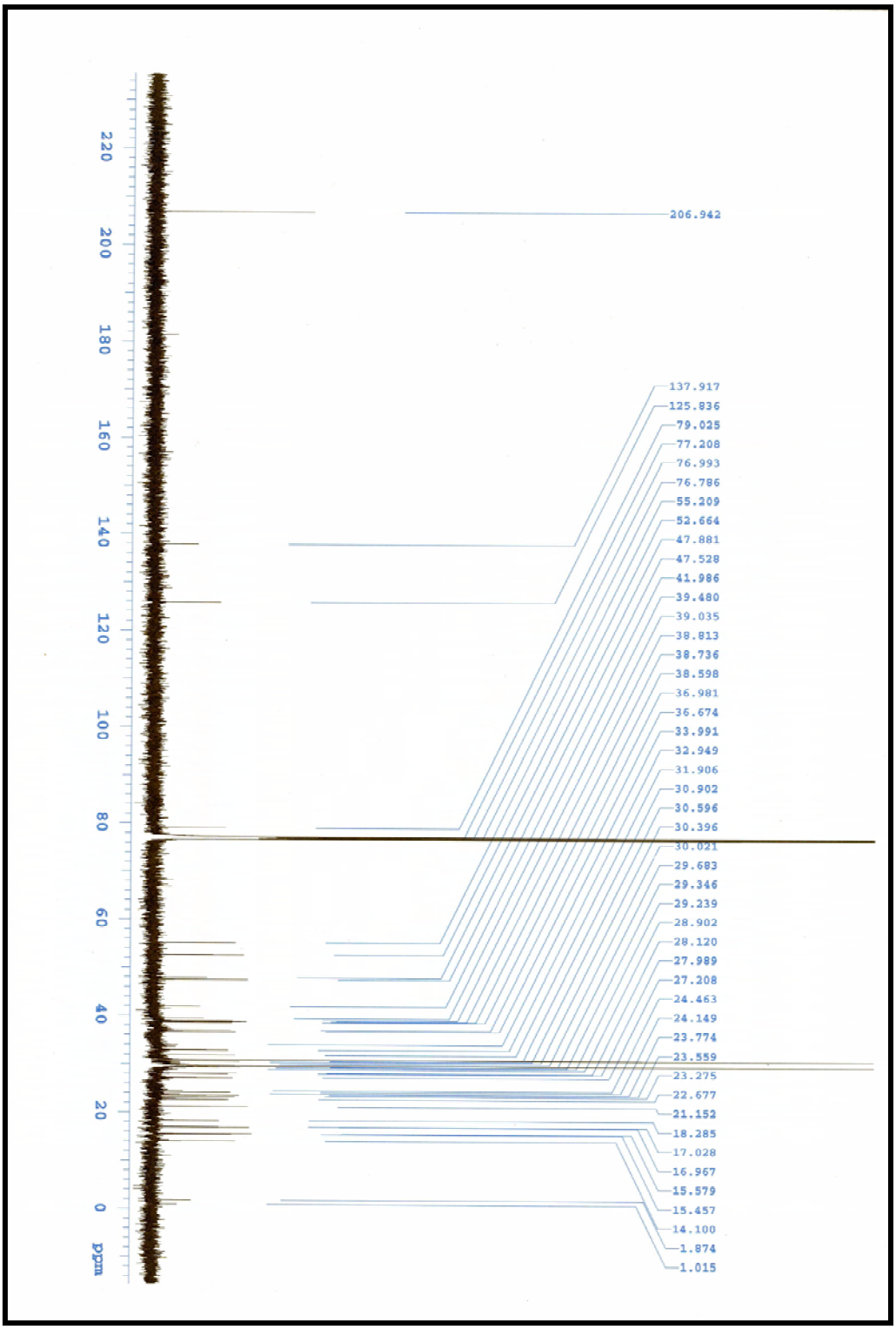
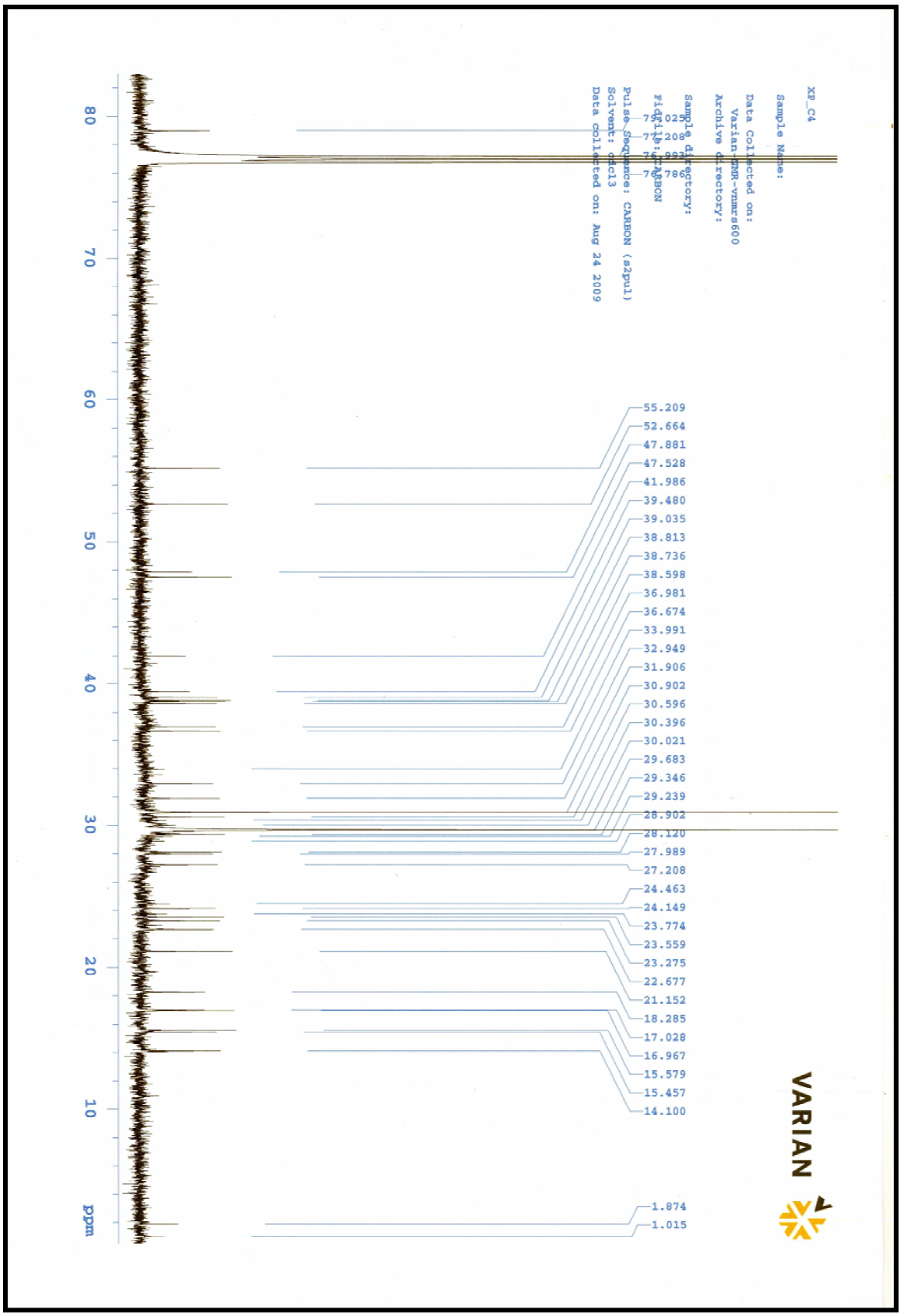
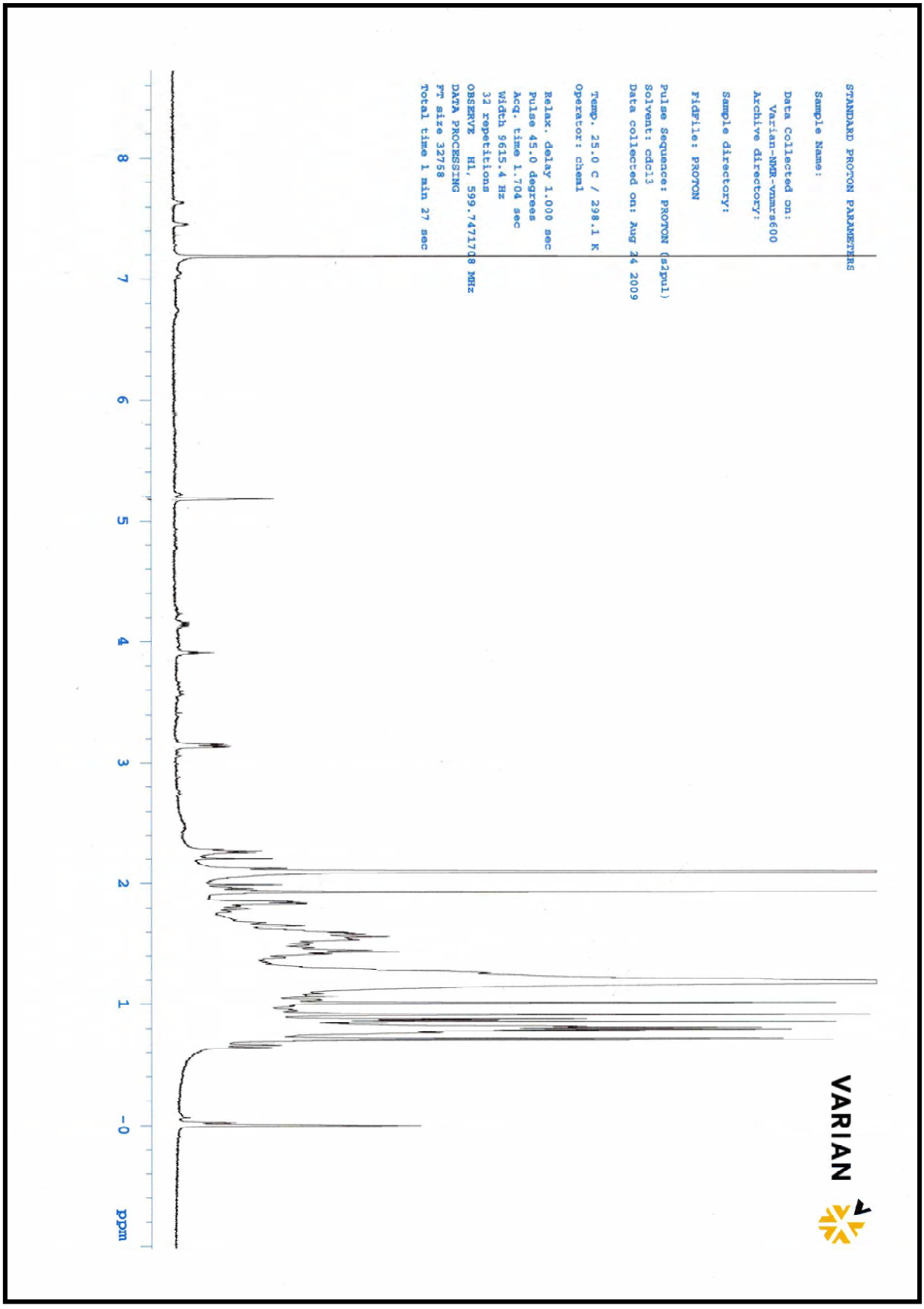
Appendix



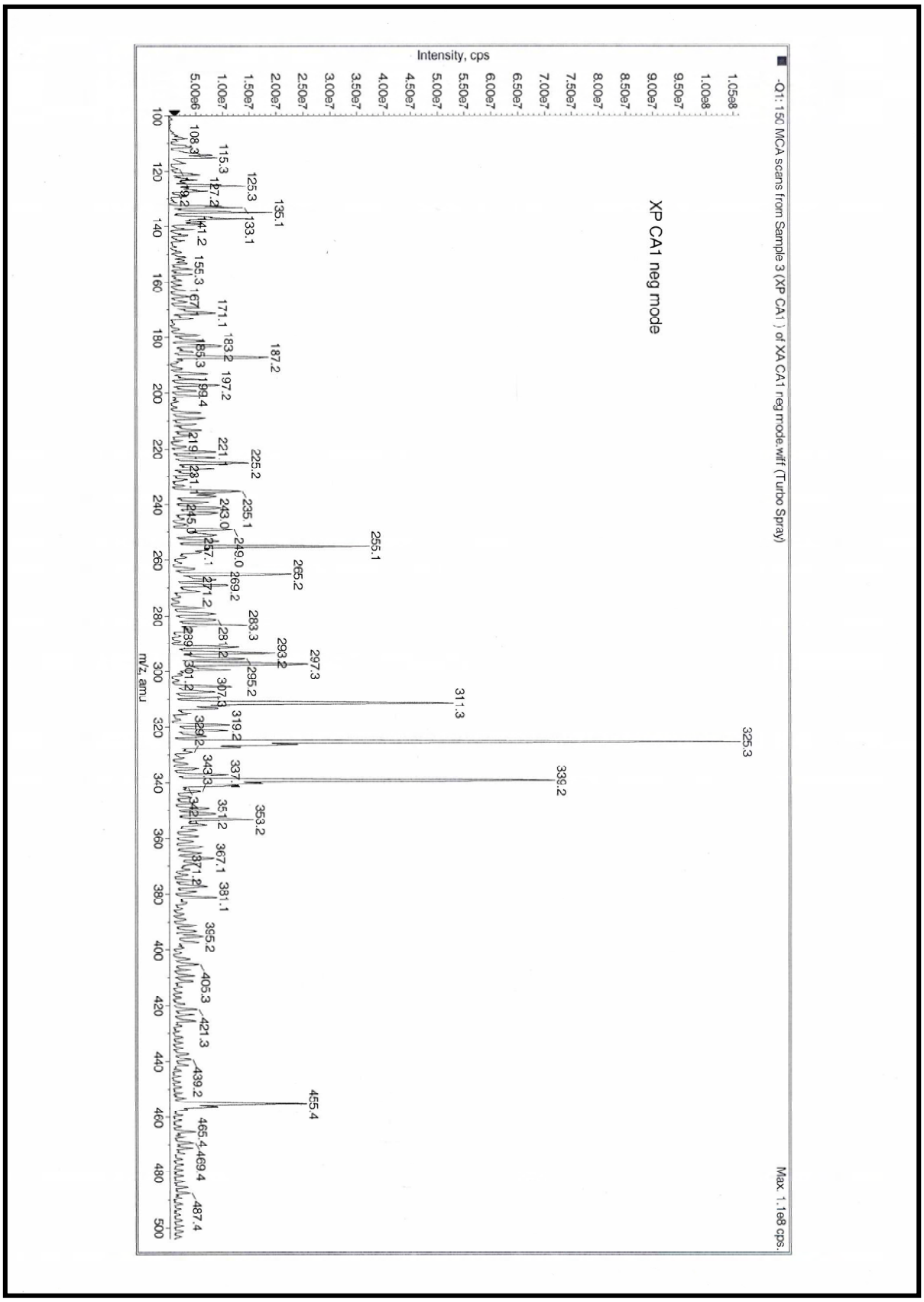
^{13}C NMR spectrum of ursolic acid



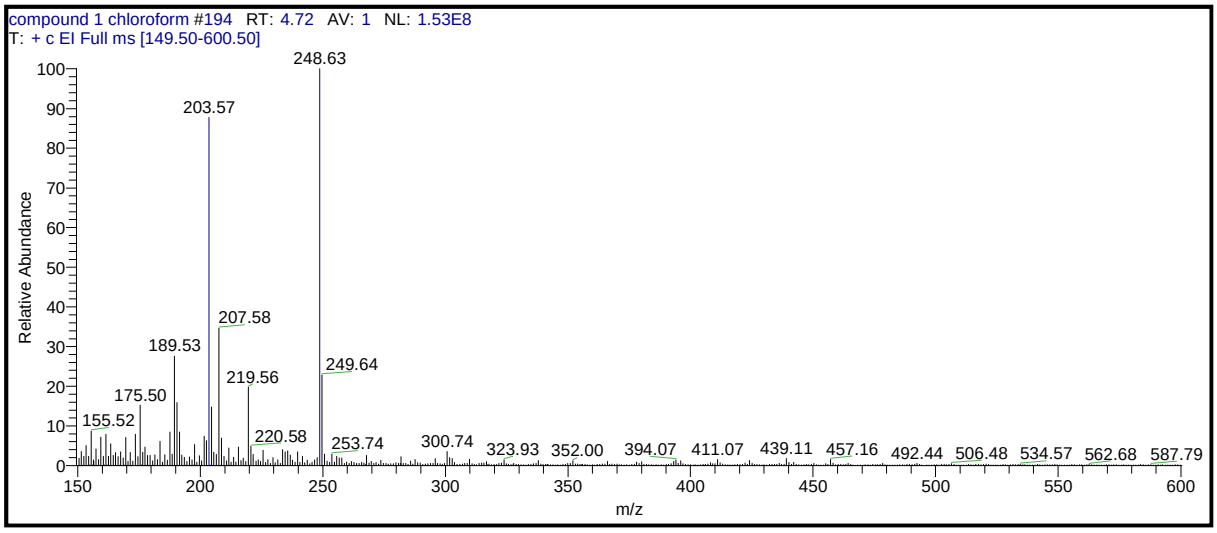
^{13}C NMR of ursolic acid, enlarge/ focused from 0 to 80 ppm



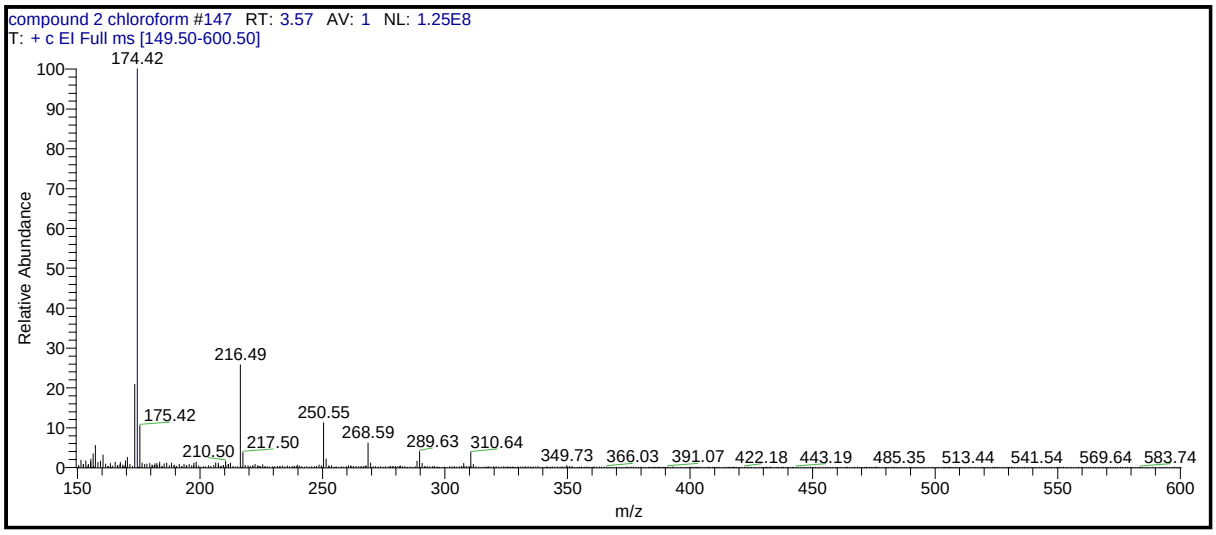
^{1}H NMR spectrum of compound 1 (ursolic acid)



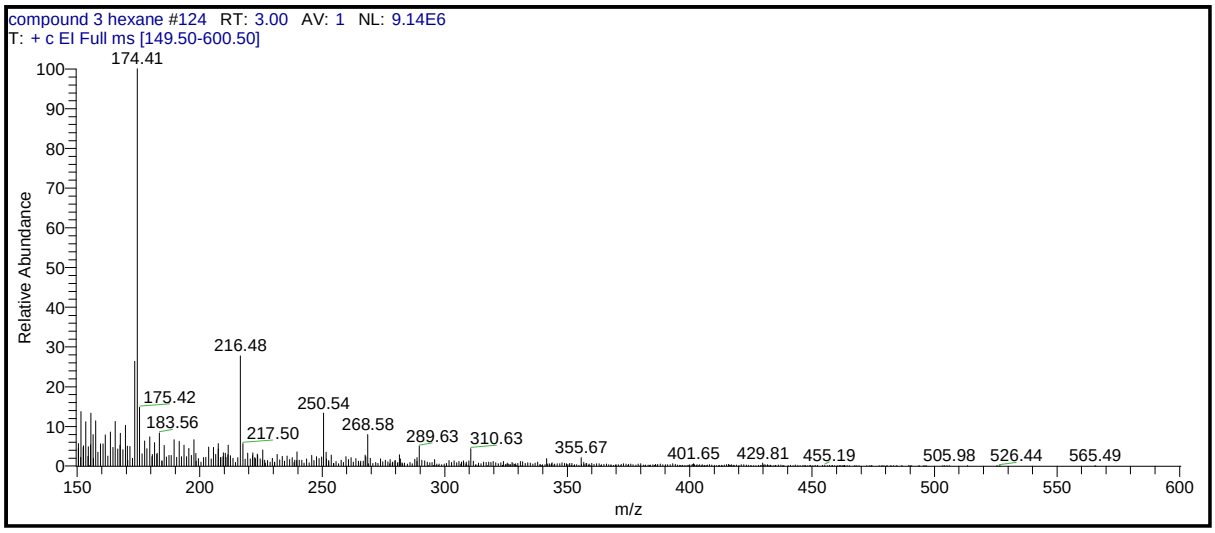
Electron ion impact mass spectrometry (EIMS) of ursolic acid



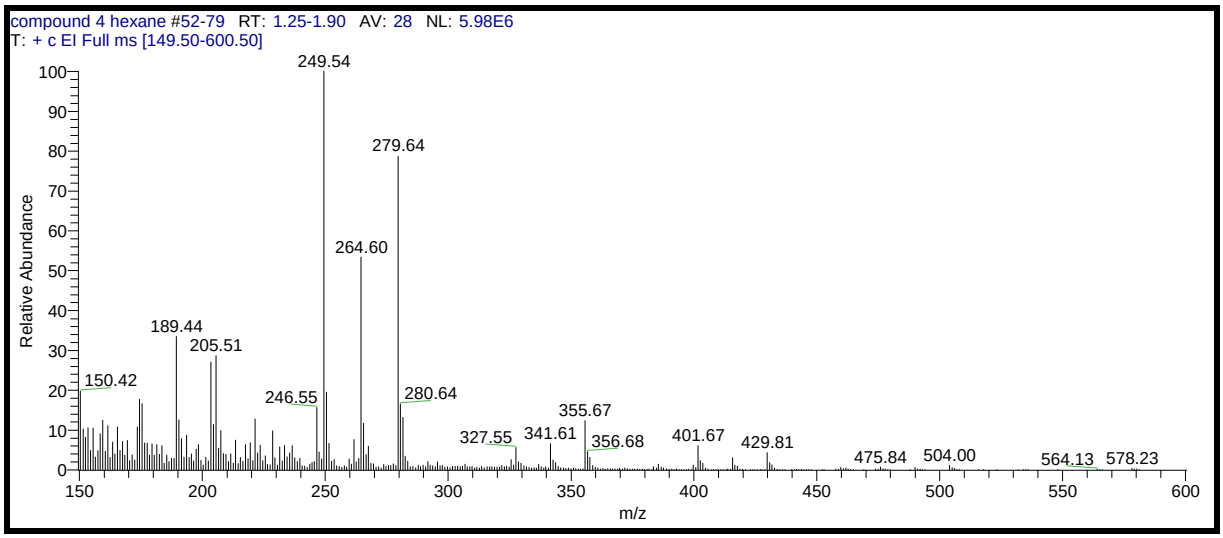
Mass spectrum of ursolic acid.



Mass spectrum of compound 2.



Mass spectrum of compound 3.



Mass spectrum of compound 4.