University of Venda

Bioactivity and chromatographic profiles of the selected medicinal plants against *Candida albicans.*

By

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Declaration

I, declare that the work described in this dissertation is original and where use has been made of the work of others, it has been acknowledged. It has never been submitted or presented for any other degree in any University or academic institution before. I, Takalani Millicent Mulaudzi declare that the above statement is true.

Signature:..... Date:24/04/2015

This work was done under the supervision of Dr N.A. Masevhe and Mr P.E.L. Mojapelo attached to the Department of Botany and Chemistry respectively.

Signature:..... Date:24/04/15

Signature:..... Date:24-04-2015.

Dedication

This work is dedicated to God who strengthens me every day. He has blessed me with a wonderful family, my mom, dad, my son, brothers, sister and my niece for the patience, understanding, love and the encouragement that you showed me when I was busy with my studies. I am motivated to work even beyond my imagined capabilities.

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I would like to thank the Holy Spirit which has guided me every day in everything I do.

Special thanks to my supervisor and mentor Dr. N.A. Masevhe, the assistance you gave me was beyond normal duties of a supervisor. I will always treasure the knowledge and skills that I have gained throughout my study. My co-supervisor Mr. P.E.L Mojapelo for the support, valuable discussions and guidance. I will always treasure the knowledge and skills I have gained from them during my study.

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Lastly I would like to thank National Research Foundation and the University of Venda for financial assistance.

List of Abbreviations

Amp B	Amphotecerin B
ATCC	American Type Culture Collection
BEA	Benzene: ethyl acetate: ammonia 90:10:1
CA	*Candida albicans*
CEF	Chloroform: ethyl acetate: formic acid 5:4:1
CN	*Cryptoccocus neoformans*
DPPH	1, 1- dipheny-2- picrylhydrozyl radical
EMW	Ethyl acetate: Methanol: water
INT	*p*-iodonitrotetrazolium violet
MIC	Minimum inhibitory concentration
R_f	Retardation factor
TLC	Thin layer chromatography

Abstract

Twelve medicinal plants were selected through ethnobotanical use and were screened for antifungal activity against candida albicans (ATCC 10231) and clinical isolates: *Aspegillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans*. The dried leaves of *Breonadia microcephala, Colophospermum mopane, Commiphora pyracanthoides, Diplorrhynchus condylocarpon, Elaeodendron transvaalense, Elephantorrhiza elephantine, Eugenia natalitia, Leucaena leucocephala, Vernonia corymbosa, Zanthoxylum humile, Ziziphus mucronata* and *Ornithogalum ornithogaloides were* extracted with acetone and water. The antifungal activity was determined using the micro-dilution and bioautography methods.The minimum inhibition concentration (MIC) of the acetone plant extracts ranged from 0.04 to 1.25 mg/ml while those of aqueous extracts had very weak antifungal activity against the test microorgaanisms ranging from 0.63 to more than 2.25 mg/ml. Acetone extracts of *C. mopane* and *D. condylocarpon* were the most active against *C.albicans* (ATCC 10231), *A. fumigatus*, *C. neoformans* with the MIC value of 0.04mg/ml. *C. mopane* had the highest total activity of 13375 ml/g followed by *D. condylocarpon* with 7617 mg/l against *C. albicans* respectively. *L. leucocephala* had the lowest activity of 504 mg/l against clinical isolates *C. albicans*, *C. neoformans* and *A. fumigatus* respectively. This therefore means that acetone extract from 1 g of the plant material could be diluted 13375 times and still kill the microorganism.

Acetone extracted the highest amount of the plant material than water in all plant species. The highest amount was extracted from *O. ornithogaloides* with 81.2 % and the least extracted was *Z. humile* with 21 %, with regard to water, the highest amount was also extracted in *O. ornithogaloides*. Acetone was selected an extractant in this study because it dissolves both hydrophilic and lipophilic components, has low toxicity and is useful in bioassays. Water was selected as an extractant because it is used as the main medium of extraction in the traditional medicine.

With regard to bioautography the acetone plant extracts has some bands of *B. microcephala*, *C. pyaracanthoides* and *E. natalitia* had less zones of inhibition against *C. neoformans* with an R_f value of 0.9. *C. mopane*, *E. elephantina* and *L. leucocephala* also had less zones of inhibition against *C. albicans* with the same R_f value of 0.9. Aqueous plant extracts had poor activity against both *C. albicans* and *C. neoformans* since no inhibition were observed.

In Phytochemical analysis, a number of different bands were observed on TLC plates showing the diversity of compounds present in plant extracts. Some compounds showed same colour and R_f values in the same solvent but different extracting solvents, this may suggest that compounds are of similar nature. BEA solvent system separated compounds acetone plant extracts more efficiently than CEF and EMW and more bands were observed in BEA than CEF and EMW solvent system. On the other hand water extracts separated poorly on the TLC plates.

Antioxidant activity of the twelve medicinal plants was investigated using qualitative assay 2, 2- diphenyl - picryl - hydrazyl (DPPH). TLC plates were developed in different mobile phases of varying polarities, namely, BEA (90:10:1), CEF (5:4:1), EMW (40:5:4:4) and FAWE 70: 20:3:2. The light yellow bands were observed on EMW, BEA and CEF solvent system of acetone plant extracts. The highest activity was observed in *E. transvaalense* with five bands present, followed by *B. microcephala*, *C. mopane* and *E. elephantina* with a total of four bands each, *O. ornithogaloides*, with only three bands and *D. condylocarpon* with only two bands. On the other hand, aqueous plant extracts hardly showed antioxidant activity.

In depth investigation was conducted using UPLC-MS in order to record the chemical profiling of the plant extracts and to identify some of the major compounds. UPLC-MS data indicated the prominent chromatograms representing different compounds. Flavonoids were identified as the major compounds in the leaves of all plant species. This study has shown that UPLC-MS is an excellent technique to evaluate the quality and content of pharmacologically important plant extracts.

Conference Presentation

This work was presented at SAAB 2015 hosted by the University of Venda from 11-15 January 2015

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CHAPTER ONE

Introduction

Background

The incidence of fungal infections has increased dramatically in recent decades as a consequence of chemotherapy, blood and marrow transplantation, solid-organ transplantation, HIV infection and the prolonged survival of seriously ill patients as a result of advances in intensive care medicine (Casalinuovo *et al*., 2004). These individuals are at heightened risk for many invasive fungal infections, including aspergillosis, candidiasis, cryptococcocis, pneumocystosis and zygomycosis. In developing countries the burden of these diseases is large and increasing (Warnock, 2007). The common pathogenic fungi responsible for these fungal infections included *Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans, Pneumocystis pneumonia* and *Scedosporium* species (Pfaller et al., 2006).

Although several antibiotics are available for treating fungal infections are present, their use is limited by factors such as low potency, poor solubility, emergence of resistant strains and toxicity (Portillo et al., 2001). It is therefore essential to search for more effective, new antifungal agents (Fenner et al., 2012). From time immemorial, medicinal plants have been used to get relief from various ailments and the widespread belief that green medicine is healthier than synthetic products has revived the interest in natural drugs (Bora, 2014). Plants produce a great deal of secondary metabolites, many of them with antimicrobial activities, well-known examples of these compounds include flavonoids, phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides and glucosinolates (Quiroga *et al*., 2001).

In the present study, some plants used by traditional practitioners to treat fungal infections were screened for their antifungal activities against three common fungal pathogens, namely: *Aspergillus fumigatus, Candida albicans* and *Cryptococcus neoformans* and plant extracts were also evaluated for their antioxidant activity. The chemical fingerprints of the plant extracts were determined using High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC).

Statement of the Problem

C. albicans is the most common cause of oral and vaginal candidosis, accounting for over 80% of infections (Shai *et al.*, 2008). Most of the currently used antifungal drugs display unwanted side effects and ineffective treatment of fungal infections by the antifungal agents usually result in rapid development of resistant strains of pathogens (Khan *et al.*, 2003). Thus, screening of plants used traditionally to treat fungal infections may be a viable option. The research question for this study is: do plants contain compounds that may be effective against *C. albicans* infections?

Hypothesis

Medicinal plants secondary metabolites possess anti-infectious properties against human pathogenic fungi. The most active plant extracts could be developed into potential drugs for use in treating and protecting humans against fungal infections.

Aim

The aim of this study is to provide scientific validation for the traditional uses of the selected plants against *Candida albicans.*

Objectives

- To investigate the chemical profiles of the selected plants using Thin Layer Chromatography.and bioautography.
- To evaluate the antifungal activity of the selected plants against *C. albicans* using microplate dilution technique.
- To determine the antioxidant activity of the plant extracts.

- To investigate the chemical composition of the selected plants and identification of the main metabolites using Ultra Performance Liquid Chromatography- Mass Spectrometry (UPLC-MS).

CHAPTER TWO

Literature review

2.1 Importance of fungal infections

Fungal infections are estimated to occur in over a billion people each year, and recent evidence suggests that the rate is increasing (Brown, 2012). Fungi are eukaryotic organisms that are more closely related to humans than bacteria at a cellular level (Khan *et al*., 2010). Fungi can infect almost any part of the body including skin, nails, respiratory tract, urogenital tract, alimentary tract, or can be systemic (Long, 2009). Anyone can acquire a fungal infection, but the elderly, critically ill, and individuals with weakened immunity, due to diseases such as HIV/AIDS or use of immunosuppressive medications, have a higher risk (Baddely, 2011). Fungi can cause significant number of human diseases represented by pathogens such as *Trichophyton* sp, *Epidermophyton* sp, *Histoplasma* sp, *Blastomyces* sp, *Sporothrix* sp, *Coccidiodes* sp, and *Paracoccidiodes* sp, capable of infecting healthy people or opportunistic invaders such as *Aspegillus* sp, *Candida* sp, *Cryptococcus* sp, *Fusarium* sp, and *Rhizopus* sp, which are normally virulent in healthy people but could be disseminated to deep tissue and cause fatal disease in unhealthy people (Khan *et al*., 2010).

2.1.1 Causative agents

The most well-known causes of opportunistic mycoses include *Candida albicans, Cryptococcus neoformans,* and *Aspergillus fumigatus*. The estimated annual incidence of invasive mycoses due to these pathogens is 72–228 infections per million population for *Candida* species, 30–66 infections per million population for *C. neoformans,* and 12–34 infections per million population for *Aspergillus* species (Pfaller *et al*., 2006).

Fungal diseases can be broadly classified as dermatophytosis, histoplasmosis, blastomycosis, coccidiomycosis, candidiasis, cryptococcosis, aspergillosis, hyalophyomycosis and zygomycosis as described by many authors (Sullivan *et al*., 2005). Candidiasis encopmpasses

secondary or opportunistic infections ranging from acute, sub-acute, and chronic to life-threatening mycoses. Infections are localized in the mouth, throat, skin, vagina, fingers, bronchi, lungs and gastrointestinal tract or sometimes become systemic as candidemia, endocardistis and meningitis. A number of Candida spp encountered in candidiasis are C. *albicans, C. glabrata, C. tropicalisis, C. krusei, C. dubliniensis, C. parapsilosis (*Khan *et al.*, 2010). *C. albicans* is a member of the commensal microflora of the intestine. It is pleomorphic and undergoes reversible morphogenic transitions between yeast, pseudohyphal, and hyphal growth forms. Healthy persons generally encounter superficial infections but in immunocompromised patients could also occur. Approximately 75 % of all women experience a clinically significant episode of vulvovaginal candidiasis (VVC) at least once during the reproductive period (Ventolini and Baggish, 2006).

Candida are the third most frequently seen organism (after coagulase negative *Staphylococcus* and *Staphylococcus aureus*), isolated in the onset sepsis in very low birth weight infants. The preterm infants are predisposed to more Candida infections because of immature immunity and invasive interventions. Candida may be transmitted by vertical i.e. from maternal vaginal infection or nosocomial i.e. from hospital acquired infection (Gurjeeth *et al.,* 2013).

2.1.2 Pathogenesis

Pathogenecity of a fungus depends on the ability to adapt to the tissue environment and to withstand the lytic activity of the host's cell defences. Filamentous forms of *C albicans* display increased adhesion to human epithelium. It is unclear if this is attributable to the larger surface area of the filament, with increased density of the various adhesins, or to factors intrinsic to the filament itself, since certain genes are expressed only in the hyphal phase (Bendel, 2003). Many human fungal pathogens are dimorphic (capable of reversible transitions between yeast and hyphal forms), and the morphogenetic transitions between these forms is often stimulated by growth forms in the host and correlated with host invasion (Khan *et al.*, 2010). It is clear that at present the frequency of fungal infection rate is increasing, up to 90% of patients with disseminated candidiasis, aspergilosis or cryptococosis. Therefore fungal pathogenesis is a multifactorial phenomenon (Kuleta *et al.*, 2009).

2.1.4 Treatment

Despite the increase in fungal infections, therapeutic options are very limited and are often unsatisfactory because of elevated toxicity and an inability to eradicate infections (Masoko *et al*., 2005). The pharmacologic treatment of invasive *Candida* infections has traditionally been limited to amphotericin B, flucytosine, and fluconazole; however, the development of newer generation azoles and echinocandins may ultimately expand the therapeutic options for single-agent and combination therapy for candidiasis (Chapman, 2007). However it has been found that the treatment with these drugs, especially for extended periods of time can lead to problems with toxicity to the patients (Motsei *et al*., 2013). There is also increasing evidence suggesting that Candida organisms are developing drug resistance (Njunda *et al*., 2012).Thus, there is a need for development of new antimicrobial agents.

2.1.5 Importance of herbal medicine

Despite the extensive use of antibiotics, antimicrobial infections continue to be a leading cause of morbidity and mortality worldwide (Casalinuovo *et al*., 2004). For centuries plants have provided mankind with useful, sometimes lifesaving drugs (Hostettmann *et al.,* 2000). In Africa and in many developing countries, medicinal plants are used in the treatment of various ailments and a large number of people depend on medicinal plants because they have no access to modern medicines (Runyoro *et al*., 2006).

The use of drugs derived from plants has been in practice for a very long time. Therefore such plants should be investigated to better understand their properties, safety and efficacy (Verma *et al*., 2013) Medicinal plants have attracted considerable research attention as new sources of antimicrobial agents (Van Wyk *et al*., 2009).

2.2. Plants used in the study

In an attempt to find practical solution to the problem, an ethnobotanical study was conducted in Vhembe district Municipality, Limpopo Province, South Africa, in order to identify plants used traditionally to treat fungal infections. The plant species were selected based on

frequency of use and the fact that literature search has shown that they have not been subjected previously to extensive pharmacological and phytochemical investigations. In order to have enough plant materials to work with, the availability of the plant species in their natural habitat was also taken into consideration and plant species that were found in abundance were preferred.

Author interviewing one of the traditional practitioner during an ethnobotanical survey.

2.3 Distribution and botanical description of plants selected for the study.

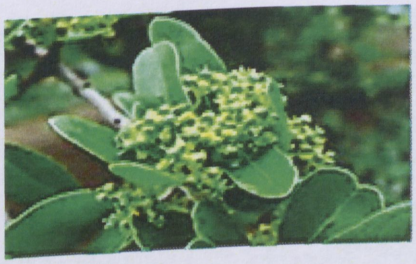
2.3.1 *Elephantorrhiza elephantina* (Burch.) Skeels

It is the most widespread and most commonly encountered from the southern parts of Angola, Namibia, Botswana, Zimbabwe, Mozambique and the South African provinces of Limpopo, Northwest, Gauteng, Mpumalanga, Free State, KwaZulu-Natal, Northern Cape and Eastern Cape as well as Swaziland and Lesotho. The plant has several unbranched, annual stems of nearly one metre in height, growing from an enormous underground rhizhome of up to eight metres long. The finely divided leaves have numerous, small narrow leaflets. Clusters of small, cream-coloured flowers are produced along the lower half of the aerial stem (Van Wyk *et al*., 2007).

Leaves of *E. elephantina*

2.3.2 *Elaeodendron transvaalense* (Burtt Davy) R.H.Archer

It grows from KwaZulu-Natal, Swaziland, Mpumalanga and through the northern parts of South Africa into Mozambique, Zimbabwe and Zambia; it also extends westwards to Botswana, Angola and Namibia. It favours soils rich in lime, grows in various soils and is found in forests, bushveld, scrub, thornveld and woodland, along streams and often on termite mounds. It is small to medium-sized tree that grows up to 6-8 m. In other areas the tree may reach 18 m. It has a conspicuously pale grey, smooth bark that is sometimes finely fissured horizontally. Its dwarf spur branchlets are characterized by a cluster of leaves at the tips. The leaves are often arranged in threes, but can alternate or arranged spiraly on longer stems (Van Wyk *et al*., 2007).



Leaves and flowers of *E. transvaalense*

2.3.3 *Zanthoxylum humile* (E.A.Bruce) P.G.Waterman

It is endemic to the north-eastern part of South Africa and South-eastern Zimbabwe. It is relatively abundant in the Pafuri and Skukuza areas of the Kruger National Park. It is dense impenetrable shrub to 3m; often prickly. The leaves are alternate, imparipinnate, glandular pinnate. It is often found in an open bushveld and mopane woodland (Van Wyk *et al* ., 2013)

Leaves of *Z. humile*

2.3.4 *Colophospermum mopane* (J.Kirk ex Benth.)

It grows in hot, dry, low-lying areas, in far northern parts of South Africa, into Zimbabwe, Mozambique, Botswana, Zambia, Namibia, Angola and Malawi. It is found growing in alkaline (high lime content) soils which are shallow and not well drained.it is a shrub or tall tree up to 30 m in the northern part of its range, depending on soil condition and water availability. It has a tall narrow crown. The compound leaves are divided in two so that the leaflets resemble butterfly wings or a camel's foot. It is deciduous (sometimes semi-deciduous) tree with lovely spring and autumn colours. The greyish brown bark is very deeply fissured in vertical fissures. It has a very rough, ropy appearance and is very distinctive (Van Wyk *et al*., 2007).

Leaves of *C. mopane*

2.3.5 *Eugenia natalitia* Sond.

It is endemic to South Africa, discontinuously through the Eastern Cape, KwaZulu-Natal, with isolated populations in Mpumalanga, Limpopo Province and Swaziland. It is a shrub or small tree. The leaves are opposite, oblong-elliptic, glabrous, somewhat leathery, gland dotted, midrib prominent. Flowers singly or in small clusters. The fruits are edible round berries which are red to purple when ripe (Van Wyk *et al*., 1997).

Leaves of *E. natalitia*

2.3.6 *Diplorrhynchus condylocarpon* (Müll. Arg.) Pichon

The horn-pod tree is distributed in tropical Africa in Angola, Zambia, Zimbabwe, Malawi and Mozambique and in southern Africa in Namibia, Botswana and South Africa (Limpopo, North-West, Gauteng and Mpumalanga Provinces). It grows as a shrub or small, deciduous, multistemmed tree up to 8 m high, with a grey-brown to blackish bark. The trunk is covered with small knob-like outgrowths (lenticels) and becomes scaly with age, cracking into small segments. The leaves are light to dark green, leathery and smooth, 30-70 x 19-50 mm. All parts of the plant contain milky sap. The flowers are white to cream-coloured, very small and fragrant, and are borne in terminal sprays (Van wyk *et al*., 2013).

Leaves and flowers of *D. condylocarpon*

2.3.7 *Leucaena leucocephala* (Lam.) de Wit

It is found in parts of South America (outside its native range), Asia, Southern USA, Southern Europe, Australia, Africa and many oceanic islands with warm climates. It is a shrub or small tree usually growing 2-10 m tall, but occasionally reaching 15 m or more in height. Leaves are bipinnate with 6-8 pairs of pinnae bearing 11-23 pairs of leaflets 8-16 mm long. The inflorescence is a cream coloured globular shape which produces a cluster of flat brown pods 13-18 mm long containing 15-30 seeds (Orwa *et al*., 2009).

Leaves and flowers of *L. leucocephala*

2.3.8 *Commiphora pyracanthoides* Engl.

It occurs in Africa, Arabia, the Indian Ocean islands and India. There are two centres of diversity; one in northern Namibia and one in North-East Africa. In Southern Africa 38 species are found naturally (including subspecies). 25 species occur in Madagascar. It is a multi-stemmed shrub or tree with stems branching repeatedly at ground level, or trees with a single upright stem, often spiny, with smooth or papery bark. Leaves vary between simple and compound, 1- to 3-foliolate or pinnate. Small white inconspicuous flowers are produced

in axillary panicles or on dwarf lateral shoots. The fruits split into two sections when ripe, revealing the stone (seed) with a brightly coloured fleshy appendage (Coates palgrave, 2002).

Leaves of C. pyracanthoides

2.3.9 *Vernonia Corymbosa* Less.

It grows in clumps, sometimes in colonies, among rocks and on open grassy slopes from 5 up to 2200 m, widespread in the Eastern Cape, Free State, KwaZulu-Natal, Lesotho, Gauteng, Mpumalanga, Limpopo, North West and Swaziland and in Angola, Zambia, Zimbabwe and Malawi.

Leaves of *V. corymbosa*

2.3.10 *Breonadia microcephala* (Delile) Ridsdale

It is widely distributed in tropical Africa, from Côte d'Ivoire and Mali eastward to Ethiopia, and southward to Angola, Zimbabwe, Mozambique and Madagascar. It also occurs in South Africa, Swaziland and Yemen. This tree is found along the permanent rivers, occurring at low altitudes, and in riverine fringe forests. It is a single-trunked tree, which overhangs large, flowing rivers and both small and large trees are easily identifiable. They are medium to large trees, 10 - 40 m, with moderate density, and an irregular, narrow canopy of fresh, shiny, green leaves, which are thin and lancet-shaped, and are crowded at the end of the branches. The leaves tend to grow upwards, like the leaves of a pineapple. The bark is grey-brown and rough, with grooves that run lengthways (Coates Palgrave, 2002).

Leaves and flowers of *B. microcephala*

2.3.11 *Ziziphus mucronata* Willd.

It is distributed throughout the summer rainfall areas of sub-Saharan Africa extending from South Africa northwards to Ethiopia and Arabia. The buffalo-thorn is one of the most widely distributed of all South African trees. This widely used medicinal plant is a small to medium-sized tree up to 9m in height, occasionally taller, occurring in a wide variety of habitats, in open woodland, often in alluvial soils along the rivers, and frequently on termite mounds; it is said to indicate the presence of underground water. It has a wide, spreading crown and rough, greyish-brown bark. Sharp thorns are usually present on the twigs. The leaves are bright green and shiny above, slightly paler beneath, with three veins arising from the base with the margins toothed in the upper half. Small yellowish-green flowers are borne in clusters above each leaf. The fruits are small, rounded berries of about 10 mm in diameter, which become reddish-brown when mature (Coates Palgrave,1988).

Leaves and fruits of *Z. mucronata*

Table 2.2.1: Summary on the data and ethnomedicinal uses of the plants used in the study

Scientific names	Local names (Venda) (Tsonga)	Family	Parts used	Voucher number	Location	Reported ethnomedicinal uses
Breonadia microcephala (Delile) Ridsdale	Mutulume (V)	Rubiaceae	Leaves	T1	Tshikundamalema	Stomach complains (Hutchings *et al*., 1996).
Colophospemum mopane (J.Kirk ex Benth.)	Mupani (V)	Fabaceae	Leaves	T2	Nwanedi	Stomach ache and swollen legs (Cheikhyoussef *et al*., 2011)
Commiphora pyracinthoides Engl.	Mutalu (V)	Burseraceae	Leaves	T3	Nwanedi	Not available
Diplorhynchus condylocarpon (Müll. Arg.) Pichon	Tsowa (T)	Apocynaceae	Leaves	T4	Mhinga	Malaria (Ngarivhume *et al*., 2015)
Elaeodendron transvaalense (Burtt Davy) R.H.Archer	Mukuvhazwivhi (V)	Celastraceae	Leaves	T5	Ha-lambani	Sexually transmitted diseases (Tshikalange et al., 2005)
Elephantorrhiza elephantina (Burch.) Skeels	Gumululo (V)	Fabaceae	Leaves	T6	Tshiungani	Dysentery, diarrhea, food supplement (Msimanga et al 2013)
Eugenia natalitia Sond.	Museri(V)	Myrtaceae	Leaves	T7	Tshiungani	Not available
Leucaena leucocephala (Lam.) de Wit	Muchugulu (T)	Fabaceae	Leaves	T8	Mhinga	Stomach diseases, abortion (Aderibigbe *et al*., 2011)
Ornithogalum ornithogaloides (Kunth) Oberm.	Tshukelani(T)	Hyacinthaceae	Leaves	T12	Mhinga	Not available
Vernonia corymbosa Less.	Phathaphathane(V)	Asteraceae	Leaves	T9	Tshikundamalema	Stomach ache, hysteria (Hutchings *et al*.,1996).
Zanthoxylum humile (E.A.Bruce) P.G.Waterman	Tshinungwana(V)	Rutaceae	Leaves	T10	Nwanedi	HIV/AIDS (Semenya et al., 2013)
Ziziphus muronata Willd.	Mukhalu (V)	Rhamnaceae	Leaves	T11	Nwanedi	Boils, swollen glands, wounds and sores (Mthethwa *et al*., 2009).

Plant species selected for this study are claimed to be used by local traditional practitioners to treat fungal infections and related ailments. Literature search shows that the plant species are also used elsewhere to treat variety of human diseases (Table 1). In the next chapter, we investigate the phytochemicals present in the leaves of the selected plants and their activity using basic techniques.

CHAPTER THREE

Phytochemical analysis and bioautography of the selected plant species

Preface

In this chapter fingerprints of the selected plant species would be investigated using thin layer chromatography. The antifungal activity of the phytochemicals present in each plant extract would be determined using bioautography technique.

3.1 Introduction

Thin layer chromatography (TLC) is a common rapid and cost-efficient method used for finger printing plant extracts (Hajimehdipoor et al., 2009). It is a powerful method used for separating mixtures of different compounds of very different polarities (Encyclopaedia, 2005). It is frequently used for the analysis of herbal medicine, it also provide first characteristic fingerprints of herbs. Rather, TLC is used as an easier method of initial screening together with other chromatographic techniques (Amol., 2008). TLC has a wide application for profession to combine the best elements of traditional healing system and modern medical practices, to improve health care system, as it can be used for almost every chemical class of compounds, with the exception of the highly volatile constituents (Joshi, 2012).

On the other hand, TLC bioautography assay is the method of choice due to several advantages that include flexibility, simplicity and high throughput (Yue *et al*, 2012). Bioautography belongs to microbiological screening methods commonly used for the detection of antimicrobial activity. The screening can be defined as the first procedure, which is applied to an analysed sample, in order to establish the presence or absence of analytes (Motsei *et al*., 2013). It can quickly detect and separate the active compounds in a complicated plant extract, and has additional advantages such as convenience, being simple to run, cheap and requiring no specialized equipment (Gu *et al*., 2009). The aim of this chapter was to determine the chemical profiles of the selected plants using thin layer chromatography and to determine their antifungal activity using direct bioautography.

3.2.1 Plant Material

Plant materials were selected through ethnobotanical survey and literature reports. The plant leaves were collected in different areas (See Table 1) and were dried at room temperature. The materials were then ground to fine powders using a batch mill (IKA M20) and stored in airtight containers at room temperature.

Author collecting leaves from plant species in their natural habitat.

3.2.2 Extraction of plant material

Twenty five gram of plant material was extracted with 950ml of acetone and boiled distilled water respectively. The water extract was allowed to cool before the lid could be closed. The mixtures were left overnight in a mechanical shaker at 150rpm for 24h at room temperature and then filtered through Whatman No.1 paper using a Buchner funnel. The acetone plant extract were concentrated to dryness under reduced pressure at 40^0C using a Büchi rotary evaporator while the water were freeze dried using a Telstar LyoQuest bench top freeze dryer.

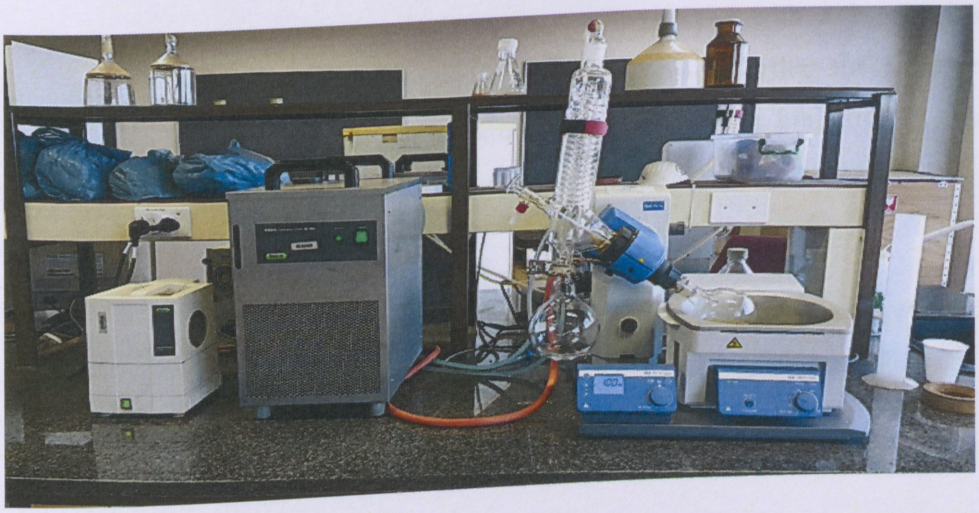


Figure 3.2.1: Büchi Rotavapor® RII (with distillation chiller and vacuum pump) used for evaporating organic extracts.

3.2.3 Phytochemical analysis

The chemical constituents of the plant extracts were analysed using thin layer chromatography (Silica gel 60 F254 plates, Merck). The plates were developed in different mobile phases of varying polarities, namely, Benzene/ethanol/ammonium hydroxide (BEA) (90:10:1), chloroform/ethyl acetate/formic acid (CEF) (5:4:1) and ethyl acetate/ methanol/ water (EMW) (40:5.4:5). Sample of (10 µl) was spotted on the TLC plate using a micropipette and developed immediately to minimise the possibility of photo-oxidation. The TLC plates were developed in closed glass tanks saturated with the mobile phase. In order to detect the separated constituents, vanillin-sulphuric acid (0.1 g vanillin: 28 methanol: 1 ml sulphuric acid) reagent was sprayed on the chromatograms and heated at 110°C to allow for optimal colour development.

3.2.4 Bioautography assay

Thin layer chromatography was used to separate and to identify the active constituents. TLC plates aluminium-backed, Silica gel 60 F254 (Merk) were loaded with 10 µl of the plant extracts and dried before developing in mobile phases of varying polarities, namely, ethyl acetate/methanol/water: 40:5.4:4 (EMW), chloroform/ethyl acetate/formic acid: 5:4:1 (CEF) and benzene/ethanol/ammonium hydroxide: 90:10:1 (BEA) (Kotze and Eloff, 2002). The chromatograms were dried for 2-3 days under a stream of moving air and sprayed with a concentrated suspension of actively growing cells of fungal cultures until they became moist, using a spray gun. The moist plates were incubated overnight at 37 ^{0}C in a closed chamber at

100 % relative humidity. The TLC plates were sprayed with a 2 mg/ml solution of p-Iodonitrotetrazolium violet, (Sigma) and were incubated further for 12 hours (Begue and Kline, 1972). Clear zones on the chromatograms indicate the inhibition of organisms by the separated plant constituents and the resultant red colour indicate the viability of cells.

3.3 Results and discussion

Chromatograms of 12 plant species extracted with acetone and developed in BEA, CEF and EMW solvents are shown in figure 3.3.1.

The R_f value was calculated using the following formula:

$$R_f = \frac{\text{distance travelled by the solute}}{\text{distance travelled by the solvent}}$$

The number of different bands observed on the TLC plates show the diversity of the compounds present in plant extracts. The chromatograms revealed complex mixture of compounds which exhibited different coloured reactions with vanillin, H_2SO_4 spray reagent. The classes of compounds in the acetone extracts included terpenoids (purple or bluish purple) (Taganna *et al*., 2011) and phenolics such as flavonoiods (yellow, pinkish or orange), stilbenes (bright red to dark pink colour) and proanthocyanidin (pink colour) (Wettasinghe et al., 2001).

Some of the compounds showed same colour and R_f values in the same solvent system but in different extracting solvents, this may suggest that the separated compounds are of similar nature. Comparing the three solvent systems (acetone extracts); BEA separated compounds in the plant extracts more efficiently than CEF and EMW. BEA solvent system showed more bands compared to CEF and EMW solvent systems (Table 3.3.2). Thus most of the separated compounds were non-polar. These results are consistent with what has been found by Masoko et al, (2008). On the other hand, water extracts separated poorly on the TLC plate, (Fig 3.3.2)

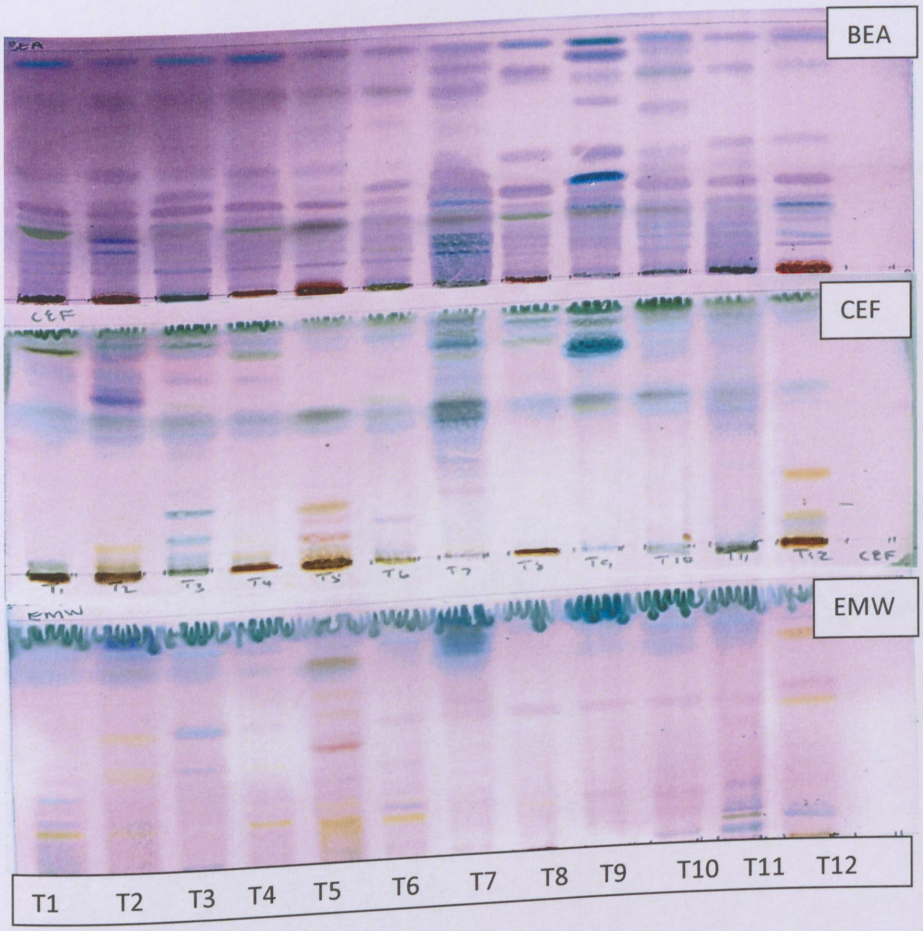


Figure 3.3.1: Chromatograms of plant species (T1 –T12) developed in BEA, EMW and CEF solvent systems and sprayed with vanillin sulphuric acid to show compounds extracted by acetone.

Key: T1, *B. microcephala)* T2, *C. mopane* T3, *C. pyracantoides* T4, *D. condylocaporn T5, E. transvaalense T6, E. elephantina T7, E. natalitia T8, L. leucocephala T9,V. corymbosa T10, Z. humile T11, Z. mucronata* and T12, *O. ornithogaloides.*

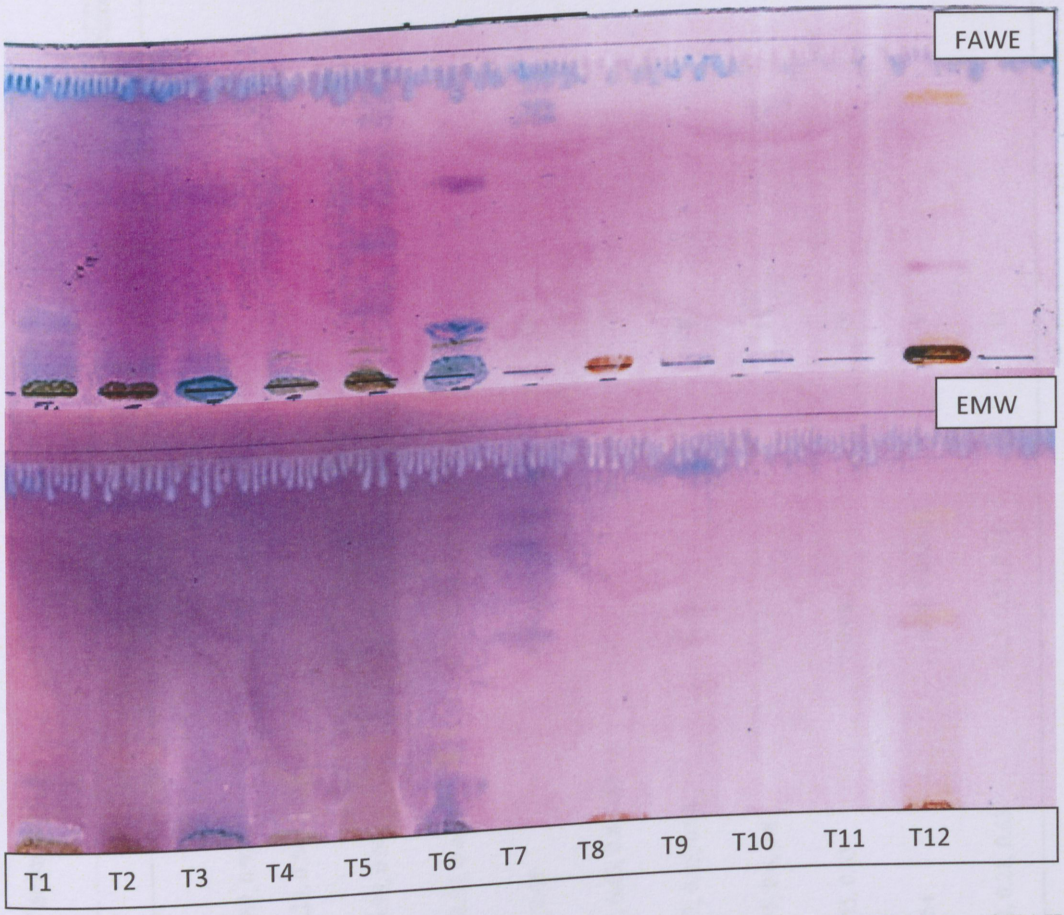


Figure 3.3.2: Chromatograms of plant species (T1 –T12) developed in EMW and FAWE solvent systems and sprayed with vanillin sulphuric acid to show compounds extracted by water.

Key: T1, *B. microcephala* T2, *C. mopane* T3, *C. pyracantoides* T4, *D. condylocaporn T5, E. transvaalense T6, E. elephantina T7, E. natalitia T8, L. leucocephala T9,V. corymbosa T10, Z. humile T11, Z. mucronata* and T12, *O. ornithogaloides.*

Table 3.3.2: Results of TLC finger printing of acetone plant extracts.

Plant samples	Solvent system (R_f Values) BEA	CEA	EMW	Total number of components separated
B. microcephala	0.13, 0.17, 0.2, 0.29, 0.36, 0.93	0.91	0.22, 0.18, 0.29	10
C. mopane	0.11, 0.2, 0.24, 0.29, 0.37, 0.47, 0.93	0.09, 0.69, 0.923	0.18, 0.38, 0.54, 0.82	14
C. pyracanthoides	0.1, 0.16, 0.23, 0.29,0.34, 0.39, 0.93	0.12, 0.23, 0.54, 0.62, 0.66	0.04, 0.40, 0.56, 0.87	16
D. condylocarpon	0.09, 0.26, 0.34, 0.49, 0.93	0.06, 0.58, 0.68	0.18	9
E. transvaalense	0.1, 0.27, 0.33, 0.91	0.11, 0.25, 0.46	0.19, 0.44, 0.59, 0.66, 0.9	12
E. elephantina	0.1, 0.14, 0.27, 0.31,0.77, 0.91	0.54, 0.65	0.18, 0.21	10
E. natalitia	0.11, 0.19, 0.25, 0.28, 0.31, 0.77, 0.91	0.57, 0.63, 0.8, 0.87	-	11
L. leucocephala	0.24, 0.34, 0.49, 0.83, 0.93	0.62, 0.85, 0.94	0.18	9
V. corymbosa	0.24, 0.26, 0.34, 0.37, 0.5, 0.7,0.87, 0.93	0.55, 0.6, 0.8	-	11
Z. humile	0.1, 0.14, 0.2, 0.27, 0.36, 0.81,0.93	0.55, 0.63	0.03	10
Z. mucronata	0.08, 0.23, 0.34, 0.48, 0.93	0.54	0.04, 0.08,0.11	9
O. ornithogaloides	0.08, 0.13, 0.2, 0.26, 0.93	0.18, 0.28, 0.63	0.25, 0.53, 0.78	11

The results of bioautography of acetone plant extracts against *C. albicans* and *C. neoformans* are shown in Fig 3.3.3 and 3.3.4. The experiment was conducted several times but the bioautograms results were poor. Other researchers also noted that the bioautography technique is more difficult with fungi because they grow more slowly and contamination can also be problematic (Suleiman et al., 2010). However, some bands in acetone extracts of *B. microcephala* (T1), *C. pyracantoides* (T4) and *E. natalitia* (T8) had less visible zones of inhibition against *C. neoformans* with an R_f value, 0.9 (Fig 3.3.3). *C. mopane* (T2), *E. elephantina* (T7) and *L. leucocephala* (T9) also had less intense zones of inhibition against *C. albicans* at the same R_f value, 0.9 (Fig 3.3.4). This might be an indication of antifungal compounds present in low concetrations or they do not react with p- Iodonitrotrazolium violet (INT) (Eloff *et al.*, 2005). Aqueous plants had poor activity against both C. *albicans* and *C. neoformans* since no inhibition were observed (results not shown) and this is in line with the previous findings (Eloff *et al.*, 2005). This shows that water did not extract inhibiting compounds from the dried ground leaves, possibly because the inhibiting compounds were made unavailable by soluble membranes (Eloff *et al.*, 2008).

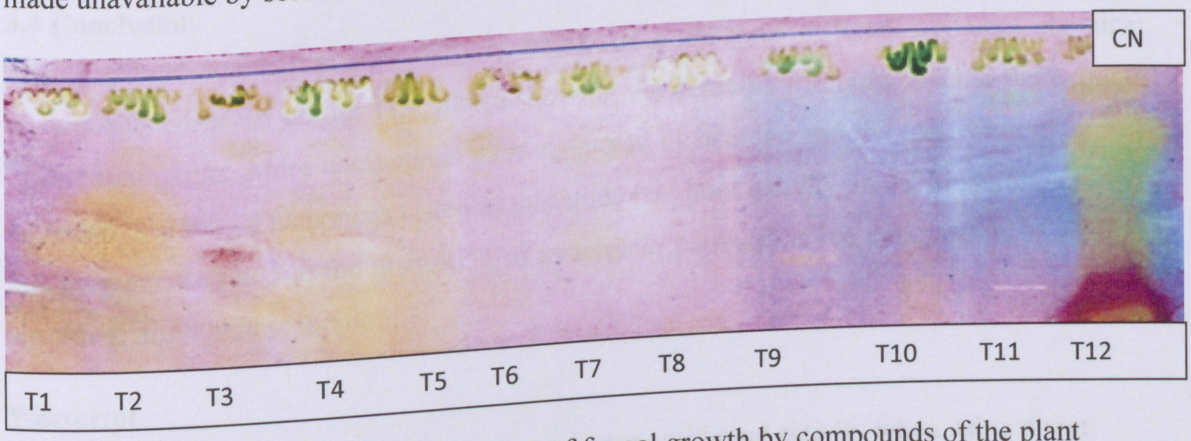


Figure 3.3.3 TLC plates showing inhibition of fungal growth by compounds of the plant extracts after 24 hrs of incubation at 37 °C.

Key: T1, *B. microcephala)* T2, *C. mopane* T3, *C. pyracantoides* T4, *D. condylocaporn T5, E. transvaalense T6, E. elephantina T7, E. natalitia T8, L. leucocephala T9,V. corymbosa T10, Z. humile T11, Z. mucronata* and T12, *O. ornithogaloides. CA, C. albicans, CN, C. neoformans*

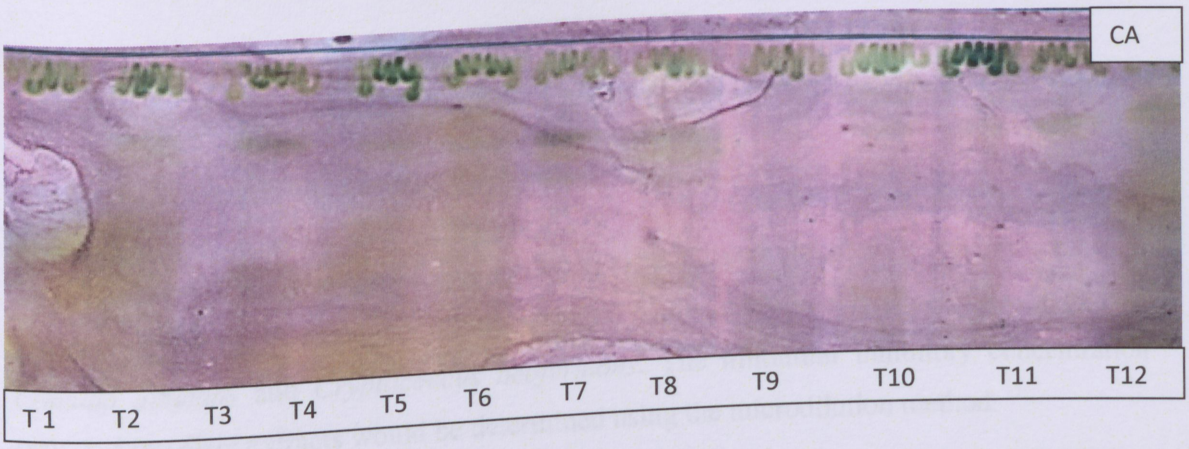


Figure 3.3.4 TLC plates showing inhibition of fungal growth by compounds of the plant extracts after 24 hrs of incubation at 37 °C.

Key: T1, *B. microcephala)* T2, *C. mopane* T3, *C. pyracantoides* T4, *D. condylocaporn T5, E. transvaalense T6, E. elephantina T7, E. natalitia T8, L. leucocephala T9,V. corymbosa T10, Z. humile T11, Z. mucronata* and T12, *O. ornithogaloides. CA, C. albicans, CN, C. neoformans*

3.4 Conclusion

Phytochemical analysis in this study revealed the number of different coloured chemical components observed on the TLC plates showing the diversity of compounds in the extracts of different plants. More compounds were extracted by acetone and the least were extracted by water. The fungal pathogens tested in this study had poor activity on the bioautograms and this may be attributed to the disruption of synergism between active components caused by the chromatographic separation.

Postscript

Plant species investigated in this study showed poor antifungal activity when using direct bioautography. In the next chapter, they will be tested against the common fungal pathogens using the microdilution method in order to quantify their activity.

CHAPTER FOUR

Antifungal activity of the selected plant species against *Candida albicans*

Preface

The plant species which were selected in the previous chapter would be investigated for their efficacy against the three common human fungal pathogens, namely: *Aspergillus fumigatus, Candida albicans* and *Cryptococcus neoformans*. The minimum inhibitory concentration (MIC) of the plant extracts would be determined using the microdilution method.

4.1 Introduction

In South Africa 60–80% of the population relies solely or partially on traditional herbal medicines to treat a variety of animal and human diseases (Shai *et al*., 2008). Traditional medicine is used by many people because it is more accessible, more acceptable to them and is cheaper than orthodox medicine (Sofowora, 1996). Many traditionally used medicinal plants in South Africa are sold in market places due to increased demands for cheap medicines, high unemployment rate and increase in HIV infections (Fyhrquist *et al*., 2002). This reliance on plants as sources of medicines warrants scientific validation of their safety, efficacy, quality and the appropriate dosage of the plant material used (Masika and Afolayan, 2002). Plants need to be screened and investigated as potential sources of new antimicrobial compounds for primary healthcare in rural areas (Shai *et al*., 2008). Antimicrobials of plant origin may be effective in the treatment of infectious diseases while simultaneously mitigating the side effects that are often associated with synthetic antimicrobials (Kokoska *et al*., 2002).

World Health Organisation is also encouraging research and development of new antimicrobial agents in order to combat the threat of drug resistance (WHO, 2001). The aim of this chapter was to evaluate the antifungal activity of the selected plants against C. *albicans* using microplate dilution technique.

4.2 Materials and Methods

4.2.1 Plant Material

Plant materials were selected according to ethnobotanical and literature reports. The plant leaves were collected in different areas (See Table 1) and then left to dry at room temperature. The material was then ground to fine powders and stored in containers at room temperatures.

4.2.2 Extraction of plant material

Twenty five gram of plant material was extracted with 950 ml of acetone and boiled distilled water respectively. The water extract was allowed to cool before the lid could be closed. The mixtures were left overnight in a mechanical shaker at 150rpm for 24h at room temperature and then filtered through Whatman No.1 paper using a Buchner funnel. The acetone plant extract were concentrated to dryness under reduced pressure at 40^0C using a Büchi rotary evaporator while the water were freeze dried.

4.2.3 Test organisms

Clinical isolates: *C. albicans, A. fumigatus and C. neoformans* and *C. albicans* (ATCC 10231) were used. The test organisms were obtained from Microbiology Department, University of Venda. These fungi are the most common and important disease-causing fungi in humans. Commercial antimicrobial drug, Amphotericin B was used as positive control.

4.3 Antifungal assay

The microplate method developed by Ellof (1998) was used to determine the minimal inhibitory concentration (MIC) which was recorded as the lowest concentration of the plant extract that inhibits growth. Two-fold serial dilutions were dispensed into 96-well microplates; distilled water (100 µl) was placed in each well using micropipette and 100 µl of a 10 mg/ml plant extract was placed in each of the first wells of the relevant series of dilution, and thereby diluted the extracts to 50%. Hundred microlitres was removed from the wells and placed into the next well. The process was repeated all the way to the bottom of the plates with 100 µl from the last rows being discarded to ensure that all wells contained the same amount of extract. Exactly 100 µl of the microorganisms was added in each well. *P*-iodonitrotetrazolium (0.2 mg/ml in water was added) 40 µl in each well. The plates were incubated for 18 hours at 37 °C in a humidified atmosphere. The minimal inhibitory

concentration was then recorded as the lowest concentration of the extract that inhibits growth. The reduction of INT in respective red formazan indicated fungal growth.

4.4 Results and discussions

Yield of the plant extracts

Fig.4.1.1 shows the percentage of dried plant materials extracted by acetone and water from the plant species used in this study. Acetone extracted the highest amount of the plant material than water in all the plant species. The highest amount was extracted from *O. ornithogaloides* (T12) representing 81.2% and the least was extracted from *Z. humile* (T10) representing 21%. With regard to water, the highest amount was also extracted from *O. ornithogaloides* (T12).

Acetone was selected as an extractant in this study because it dissolves both hydrophilic and lipophilic components, has low toxicity to fungi, and hence is very useful in bioassays (Mdee *et al.*, 2009). On the other hand, plant species used in this study are used by local traditional practitioners for treating fungal infections and related ailments. Hence, water was selected as an extractant because it is used as the main medium of extraction in the traditional medicine (Luseba *et al.*, 2007).

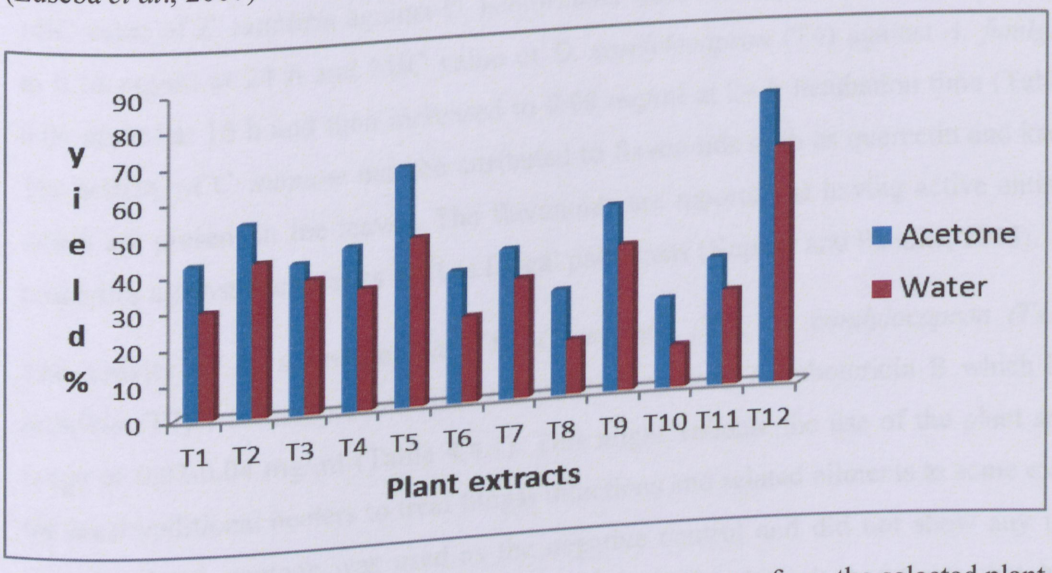


Figure 4.1. 1. Percentage yield extracted by acetone and water from the selected plant species

B. microcephala (T1), *C. mopane* (T2), *C. pyracantoides* (T3), *D. condylocaporn* (T4), *E. transvaalense* (T5), E. elephantine(T6), *E. natalitia* (T7), *L. leucocephala* (T8), *V. corymbosa* (T9), *Z. humile* (T10), *Z. mucronata* (T11) and *O. ornithogaloides* (T12).

Minimum inhibitory concentration (MIC) of organic plant extracts

MIC results of the acetone plant extracts are shown in Table 4.4.1. Due to the fact that crude plant extracts were investigated in this study, antifungal activity has been graded as follows: good antifungal activity = MIC less than 0.1 mg/ml; moderate antifungal activity = MIC of 0.1 to 0.5 mg/ml; weak antifungal activity = MIC of 0.5 to1 mg/ml; MIC of greaterthan1 mg/ml was considered inactive (Suleimanet *et al.*, 2010).

The majority of the plant extracts (61%) had moderate antifungal activity against the test organisms. Plant extracts (14%) had a good antifungal activity, namely *B. microcephala* (T1), *C. mopane* (T2), *D. condylocapron* (T4) and *E. natalitia* (T7) with minimum inhibitory concentration values as low as 0.04 and 0.08 mg/ml (Table 4.4.1). These plant extracts were strong inhibitors of the growth of standard *C. albicans* strain, and clinical isolates *C. neoformans* and *A. fumigatus*. Generally, the activity of the active compounds present in the plant extracts was fungistatic, for example MIC value of *C. mopane* (T2) against the standard *C. albicans* strain was 0.04 mg/ml at 16 h then rose to 0.08 mg/ml at 24 h incubation time, MIC value of *E. natalitia* against *C. neoformans* was 0.08 mg/ml at 16 h and then increased to 0.16 mg/ml at 24 h and MIC value of *D. condylocapron* (T4) against *A. fumigatus* was 0.04 mg/ml at 16 h and then increased to 0.08 mg/ml at 24 h incubation time (Table 4.4.1). The activity of *C. mopane* may be attributed to flavonoids such as quercetin and kaempferol which are present in the leaves. The flavonoids are reported as having active antimicrobial properties against bacterial as well as fungal pathogens (Kapoor and Pandita, 2013).

The activity of *B. microcephala (T1), C. mopane (T2), D. condylocapron (T4)* and *E. natalitia* (T7) was comparable with the positive control, Amphotericin B which had MIC range of 0.02-0.04 mg/ml (Table 4.4.1). This might validate the use of the plant species by the local traditional healers to treat fungal infections and related ailments to some extent. On the other hand, acetone was used as the negative control and did not show any inhibition against the pathogenic fungi. The results also showed that plant extracts that had weak antifungal activity against the test organisms were 13% while those that had no activity at all were 11% only. *C albicans* clinical isolate was relatively resistant to the plant extracts

because most of them had MIC value greater than 0.1 mg/m except one plant extract of *E. natalitia* which had MIC value of 0.08 mg/ml after 16 h incubation (Table 4.4.1)

Table 4.4.1: Minimum inhibitory concentration (MIC) of acetone extracts (mg/ml) against *Candida albicans* (ATCC) and clinical isolates: *C. albicans*, *C. neoformans* and *A. fumigatus*

	16Hrs	24Hrs	16Hrs	24Hrs	16Hrs	24Hrs	16Hrs	24Hrs
Plant samples	*C. albicans* (ATCC)		*C. albicans*		*C. neoformans*		*A. fumigatus*	
B. microcephala (T1)	0.08	0.16	0.16	0.63	0.08	0.16	0.16	0.31
C. mopane (T2)	0.04	0.08	0.16	0.31	0.04	0.16	0.04	0.08
C. pyracinthoides (T3)	0.31	0.31	0.16	0.16	0.16	0.63	0.16	0.31
D. condylocapron (T4)	0.04	0.08	0.16	0.31	0.16	1.25	0.04	0.08
E. transvaalense (T5)	0.31	0.31	0.63	1.25	0.63	2.5	0.31	1.25
E. elephantine (T6)	0.63	1.25	0.63	0.63	0.31	0.31	0.63	0.63
E. natalitia (T7)	0.16	0.16	0.08	0.16	0.08	0.16	0.16	0.16
L. leucocephala (T8)	0.16	0.31	0.31	1.25	0.31	1.25	0.31	1.25
V. corymbosa (T9)	0.16	0.31	0.16	0.16	0.16	0.16	0.31	0.31
Z. humile (T10)	0.63	0.63	0.16	0.31	0.16	0.31	0.16	0.63
Z. mucronata T11	0.31	0.31	0.16	0.31	0.16	0.31	0.31	0.31
O. ornithogaloides (T12)	0.16	0.31	0.31	1.25	0.63	1.25	0.31	0.31
Amphoterim B	0.04	0.04	0.04	0.04	0.02	0.02	0.02	0.02

ATCC, American Type Culture Collection

Minimum inhibitory concentration (MIC) of aqueous plant extracts

Only two plant species, *D. condylocapron (T4)* and *O. ornithogaloides (T12)* had moderate antifungal activity against *C. neoformans* and *A. fumigatus* with minimum inhibitory concentration (MIC) value of 0.31 mg/ml respectively. The antifungal activity of the plant extracts may be attributed to the presence of water soluble tannins which are well known to possess antimicrobial properties (Mathabe et al., 2006). A total of 16% of the plant extracts had very weak activity against *C. neoformans* and *A. fumigatus* with minimum inhibitory concentration (MIC) value of 0.63 mg/ml. This included plant species such as *B. microcephala (T1), C. mopane (T2)* and *C. pyracinthoides (T3)*. The majority of the plant extracts (78%) did not show antifungal activity against the tested organisms, especially against the Candida species (Table 4.4.2). Thus, the standard *C. albicans* strain and *C. albicans* clinical isolate were relatively resistant to the plant extracts. Makhafola and Eloff, (2012) report that the low activity exhibited by the plant extracts may be related to the

extractant used, in this case water and according to their report aqueous plant extracts usually have very low antimicrobial activity. This is also supported by the work done by Luseba *et al.*, (2007). However, the fact that a biological activity has not been detected or is very low does not mean that the plant is useless. It may contain natural products with other activities or useful lead compounds which can be modified to provide interesting therapeuticals (Hostettmann *et al.*, 2000).

Table 4.4.2: Minimum inhibitory concentration (MIC) of aqueous extracts against ATCC strain of *Candida albicans* and Clinical isolates *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*

	16Hrs	24Hrs	16Hrs	24Hrs	16Hrs	24Hrs	16Hrs	24Hrs
Plant samples	*C. albicans* (ATCC)		*C. albicans*		*C. neoformans*		*A. fumigatus*	
B. microcephala (T1)	1.25	1.25	1.25	1.25	0.63	0.63	0.63	1.25
C. mopane (T2)	1.25	1.25	1.25	1.25	0.63	0.63	0.63	0.63
C. pyracinthoides (T3)	2.5	2.5	>2.5	>2.5	0.63	0.63	0.63	1.25
D. condylocapron (T4)	1.25	1.25	2.5	2.5	0.31	0.63	0.31	0.63
E. transvaalense (T5)	1.25	1.25	1.25	1.25	1.25	1.25	0.63	0.63
E. elephantine (T6)	2.5	>2.5	2.5	>2.5	>2.5	>2.5	>2.5	>2.5
E. natalitia (T7)	2.5	>2.5	2.5	>2.5	>2.5	>2.5	>2.5	>2.5
L. leucocephala (T8)	2.5	>2.5	2.5	>2.5	1.25	2.5	0.63	0.63
V. corymbosa (T9)	1.25	1.25	1.25	1.25	1.25	2.5	1.25	2.5
Z. humile (T10)	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
Z. mucronata (T11)	2.5	2.5	2.5	2.5	1.25	1.25	2.5	2.5
O. ornithogaloides (T12)	1.25	1.25	>2.5	>2.5	0.31	0.31	0.31	0.31
Amphoterim B	0.04	0.04	0.04	0.04	0.02	0.02	0.02	0.02

ATCC, American Type Culture Collection

It was of worthy to note that the activity of both organic and aqueous plant extracts was fungistatic because the minimum inhibitory concentration (MIC) values of the plant extracts increased with the incubation time. This might be attributed to the decomposition of the active compounds in the plant extracts allowing the tested organisms to grow or the pathogenic fungi were able to overcome the initial inhibitory effects of the active compounds (Masoko *et al.*, 2005),

Total activity

In order to compare the activity of the plant species under the investigation and to consider the plant extract worth for further studying, not only the MIC value is important, but also the total activity should be considered. The latter indicates the degree to which the active compounds in one g of plant material can be diluted and still inhibit the growth of the tested microorganism (Eloff, 2004).

It was calculated as follows:

Total activity = quantity of the material extracted from 1 g of the plant material divided by MIC value of the plant extract

i.e. TA = $\frac{\text{mass in mg extracted from 1 g}}{\text{MIC (mg/ml)}}$

Plant species that had high total activity were *C. mopane* (T2), 13375 ml/g and *D. condylocarpon* (T4), 7617 ml/g against the standard *C. albicans* strain respectively, *E. natalitia* (T7), 3483 ml/g against clinical isolates *C albicans* and *C. neoformans* respectively, *V. corymbosa* (T9), 3244 ml/g against clinical isolates *C albicans* and *C. neoformans* respectively, *O. ornithogaloides* (T12), 3123 ml/g against the standard *C. albicans* strain, *C. pyracinthoides* (T3), 2625 ml/g against the clinical isolates *C albicans* and *L. leucocephala* (T8) had the lowest total activity of 504 ml/g against the clinical isolates *C albicans* , *C. neoformans* and *A. fumigatus* respectively (Table 4.4.3).The order of total activity of the four plant species against the tested fungal pathogens was as follows: *C. mopane* > *D. condylocapron* > *E. natalitia* > *V. corymbosa*. According to Makhafola and Eloff (2012), the higher the total activity, the greater is the potential for application of the specific plant extract. Suleiman *et al.* (2010) also report that higher value of total activity indicates increased usefulness and potential economic value of the plant to the rural poor people.

Table 4.4.3: Total activity of acetone plant extracts (ml/g)

Plant samples	C.albicans (ATCC)	C.albicans	C.neoformans	A.fumigatus	Average
B. microcephala (T1)	3217	965	585	2413	1795
C. mopane (T2)	13375	2229	5350	8917	7468
C. pyracinthoides (T3)	1355	2625	1050	1750	1695
D. condylocapron (T4)	7617	1904	644	7617	4445
E. transvaalense (T5)	2155	711	418	2386	1417
E. elephantine (T6)	455	679	1381	679	799
E. natalitia (T7)	2613	3483	3483	2613	3048
L. leucocephala (T8)	1512	504	504	504	756
V. corymbosa (T9)	1996	3244	3244	1674	2539
Z. humile (T10)	397	1042	1042	625	776
Z. mucronata T11	861	1113	1113	861	987
O. ornithogaloides (T12)	3123	1041	864	2619	1912

ATCC, American Type Culture Collection

In conclusion, plant extracts investigated here showed varying degree of antifungal activity against the test organisms. However, acetone leaf extracts of *B. microcephala, C. mopane, D. condylocapron* and *E. natalitia* had the most antifungal activity against the tested pathogens. Thus, they serve as promising candidates for antifungal agents.

Postscript

Seeing that the plant species investigated in this study had promising antifungal activity, we decided to subject them to a further research work. In the next chapter, we will investigate the antioxidant activity of the plant extracts.

CHAPTER FIVE

Antioxidant activity of the selected plants

Preface

The following text describes the antioxidant activity of the plant extracts. This is important in order to determine whether the selected medicinal plants have got the ability to boost the immune system in order to fight off antifungal infections.

5.1 Introduction

Antioxidants are effective free radical scavengers which tend to retard or prevent the oxidation of other molecules by capturing free radicals (Mahlo et al., 2013). Free radicals are highly reactive and are generated in the body through normal cellular function. Free radicals are believed to cause lipid oxidation leading to cellular membrane damage (Khaza *et al.*, 2011). It is now well established that a series of oxygen-centred free radicals and other reactive oxygen species (ROS) contribute to the pathology of many disorders including atherogenesis, neurodegeneration, chronic inflammation, cancer and physiological senescence. Therefore, antioxidants are considered important nutraceuticals on account of many health benefits that are widely used in food industry as potential inhibitors of lipid perocidation (Deng *et al.*, 2011). Free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, pollution, pepticides, etc. Normally, there is a balance between the quantity of free radicals generated in the body and the antioxidant defence systems which scavenge/ quench these free radicals preventing them from causing deterious effects in the body. The antioxidant defence systems in the body can only protect the body when the quality of free radicals is within the normal physiological level (Masoko *et al.*, 2007).

Cells use different types of defence mechanisms against reactive oxygen species, one of which is low in molecular weight antioxidants (most often in edible plant) (Chevion et al., 2000). Therefore antioxidants are widely used in medicines and foodstuffs and medicines because of their benefits to life (Barros *et al.*, 2011).

5.2 Materials and Methods

5.4.1 Plant Material

This was done as described in section 4 2.1.

5.4.2 Extraction of plant material

This was done as described in section 4.2.2.

5.3 Antioxidant activity

Plant extracts were evaluated for their antioxidant activity as an indicator of their ability to boost the immune system of the host in order to fight microbial infections. The qualitative assay was conducted using 2, 2- diphenyl-1-picryl hydrazyl (DPPH) according to the method described by Takao *et al* (1994). Thin layer chromatograms were developed in different mobile phases of varying polarities, namely, Benzene/ethanol/ammonium hydroxide (BEA) (90:10:1), chloroform/ethyl acetate/formic acid (CEF) (5:4:1) and ethyl acetate/ methanol/ water (EMW) (40:5:4:4) (Kotze and Eloff, 2002).

The development of the TLC chromatograms was done in a closed tank in which the atmosphere has been saturated with the eluent vapour and was sprayed with 0.2% DPPH in methanol. Antioxidant activities were assessed by visually observing colour changes. A change of colour from the purple background to yellow indicated the presence of antioxidants.

5.4 Results and discussion

Antioxidants react with DPPH, a stable nitrogen-centred free radical, and convert it to α,α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the scavenging potential of the antioxidant extracts (Kumar *et al*., 2012). Any molecule that can donate an electron or hydrogen to a mixture will react with and bleach DPPH. And the latter is reduced from a purple compound to a light yellow compound by electrons from oxidant compounds (Masoko *et al*., 2007). The light yellow bands were observed on EMW, BEA and CEF solvent system of acetone plant extracts (Fig 5.4.1). However, the activity was not so intense since the yellow bands were not crystal clear. Most yellow bands were only visible in six plant samples out of the twelve tested. The highest activity was observed in *E. transvaalense* with five bands present, followed by *B. microcephala*, *C. mopane* and *E. elephantina* with a total of

four bands each, *O. ornithogaloides*, with three bands and *D. condylocarpon* with two bands. When comparing the three solvent systems, the order of activity was as follows: EMW > CEF >BEA. In CEF and EMW solvent systems, intense activity of antioxidants was observed at the baseline i.e the mobile phase failed to separate them well (Fig 5.4.1). This may be attributed to overloading during spotting on the TLC plate.

These results are in consistent with what has been found by Masoko et al., (2007) and Mahlo et al., (2013). On the other hand, aqueous plant extracts hardly showed antioxidant activity. However, in some of the plant extracts the same trend of having some slight yellow bands at the base line was also observed (results not shown)

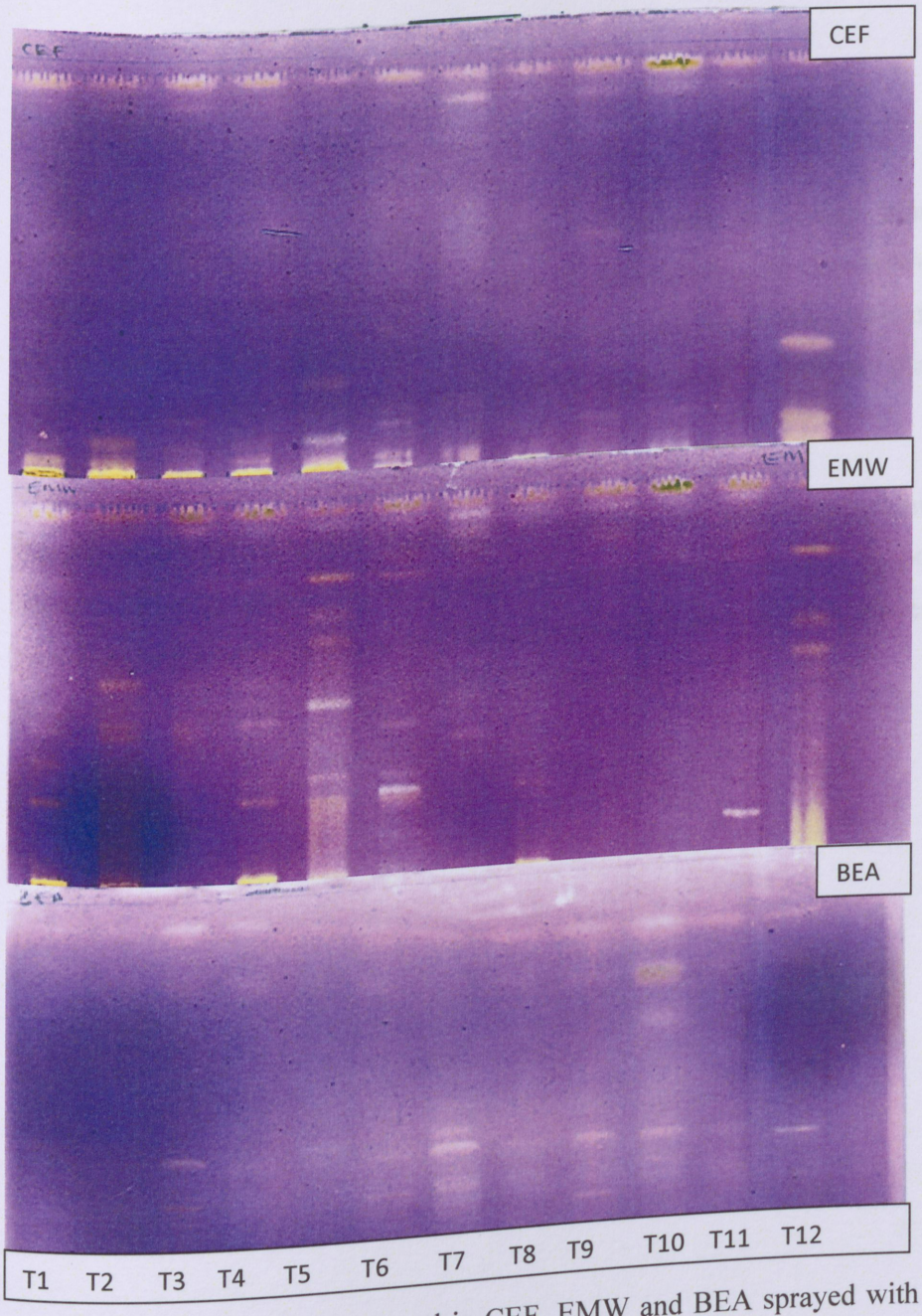


Figure 5.4.1: TLC plate developed in CEF, EMW and BEA sprayed with 0.2% DPPH, the different acetone plant extracts (T1-T12).

Key: T1, *B. microcephala)* T2, *C. mopane* T3, *C. pyracantoides* T4, *D. condylocaporn T5, E. transvaalense T6, E. elephantina T7, E. natalitia T8, L. leucocephala T9,V. corymbosa T10, Z. humile T11, Z. mucronata* and T12, *O. ornithogaloides.*

5.5 Conclusion

From the twelve acetone plant extracts investigated only five plant species of *E. transvaalense*, *B. microcephala*, *C. mopane*, *E. elephantina* and *O. ornithogaloides* had good antioxidant properties while the aqueous extracts hardly showed an antioxidant activity. This is the first report on the qualitative antioxidant properties of the selected plant species.

Postscript

Plant species selected for this study showed varying degree of antifungal and antioxidant activity. This shows that they are of value in the traditional medicine. In the next chapter, we would investigate the chemical profiling of compounds present in the leaves of the selected plant species using high definition UPLC-MS technique.

CHAPTER SIX

Chromatographic profiling and identification of flavonoids in the selected plants using UPLC-MS

Preface

In this chapter plant extracts would be analysed by Ultra high-performance liquid chromatography -mass spectrometry in order to reveal their chemical compositions. Knowledge of chemical profiling plays an important role in the quality control of plant products and also in the identification of plant species.

6.1 Introduction

Plants have a long history of use for the treatment of different diseases and complaints. In the developing countries, up to 80% of the population relies exclusively on plants as a source of medicines (Hostettmann *et al.*, 2000). Compounds with antimicrobial activity have been investigated and the searches for new anti-infections agents showed that medicinal plant extracts, essential oils or isolated compounds such as alkaloids, flavonoids, diterpenes, triterpenes and sesquiterpene lactones are potential sources of bioactive compounds (Boligon *et al.*, 2015).

The analysis of the compounds in plants is a challenging task because plant metabolites are structurally diverse, forming a highly complex spectrum of compounds of different size, solubility, volatility, polarity, quantity and stability (Summer *et al.*, 2003). The metabolic fingerprinting of species and, consequently, their bioactivity can vary depending on various factors such as age of the plant, time of harvest, soil conditions, weather conditions, part of the plant used, etc. (Wang *et al.*, 2013). Consequently there is no single method that is capable to meet the ultimate goal of quantitation of all compounds. Depending on the goal of the study different approaches can be applied (Verpoorte *et al.*, 2005)

Techniques such as nuclear magnetic resonance (NMR), gas chromatography (GC), high performance liquid chromatography (HPLC), Ultra high-performance liquid chromatography -mass spectrometry (UHPLC-MS), etc, have been used for metabolite fingerprinting and metabolic profiling (Hendriks *et al.*, 2005). Each method has advantages and disadvantages, and none of them can be able to analyse all the phytochemicals in the plant. According to Hostettmann *et al.*, (2000), HPLC is a suited technique for an efficient separation of crude

plant extracts and can be coupled with different spectroscopic detection methods e.g. HPLC coupled with UV photodiode array detection (LC/UV) has been used for more than a decade by phytochemists in the screening of plant extracts. However, in this study Ultra high-performance liquid chromatography-mass spectrometry (Waters Corporation, USA) was used in the analysis of the botanical metabolites. The UHPLC offers numerous advantages over the traditional HPLC, of which the most important includes higher resolution and peak capacity at the same analysis time and the ability to allow shorter chromatographic runs with similar but best separation than those attained by the traditional HPLC (Madala *et al*., 2013). The aim of this study was to produce chemical fingerprinting of the selected plants using UHPLC-MS and to identify some of the targeted constituents using multivariate data analysis (MVDA) and principal component analysis (PCA) methods

6.2 Materials and Methods

6.2.1 Plant collection, preparation and storage

The leaves of the selected plant species were collected from their natural habitats in different areas of Vhembe District Municipality (Table 2.2.1). Voucher specimens were prepared and stored at the Botany Department Herbarium, University of Venda. The plant materials were dried at room temperature and ground into fine powder using electric grinder. They were stored in closed containers at room temperature until needed.

6.2.2 Preparation of plant extracts

Dried, powdered plant materials were extracted with acetone as it has previously been shown to be the best solvent for extracting active compounds from plants (Eloff, 1998a). The solvent was used in the ratio 1:10 of the plant material: solvent to prepare the plant extracts. The plant extracts were concentrated to dryness under reduced pressure at 40 °C using a Büchi rotary evaporator. Aqueous extract were obtained by decoction in distilled water over 30 min and then cooled down. They were put in the freezer overnight at -40 ^{0}C and then freeze dried. The yields from different plant extracts was weighed and then used for quantitative analysis. The plant extracts were filtered through a 0.22 μm filter before injected into UPLC-MS.

6.3 UPLC–MS chromatographic conditions

The method developed by Madala *et al*, (2013) was used for data acquisition with some slight modification. High definition mass spectrometry was performed on SYNAPT G1 Q-TOF system (Waters) in V-optics and operated in electrospray negative mode to enable detection of flavonoid compounds. Leucine enkephalin (50 pg/mL) was used as reference lock mass calibrant to obtain typical mass accuracies between 1 and 5 mDa.

The optimal conditions of analysis were as follows:

- capillary voltage of 2.5 kV,
- the sampling cone at 30 V,
- the extraction cone at 4 V.
- The scan time was 0.1 seconds covering the 100 to 1000 Dalton mass range.
- The source temperature 120°C
- The desolvation temperature was set at 450°C.
- Nitrogen was used as the nebulisation gas at a flow rate of 700 L/h.

Data processing:
UPLC-MS raw data was exported and analysed using Markerlynx XS software (Waters, MA, USA).

6.4 Results and Discussions

More than 4000 phytochemicals have been discovered to date and the latter have shown great potential in treating human ailments and diseases such as cancer, coronary heart diseases, diabetes and infectious diseases (Tripathi *et al*., 2012). Therefore, it was important to analyze the secondary metabolites present in the selected medicinal plants using modern techniques such as UPLC-MS. The latter is commonly used for fingerprinting as it can successfully separate out the different metabolites of the plant extract and can provide both qualitative and quantitative data (Loescher *et al*., 2015). The UPLC distinctive fingerprints of the selected medicinal plants are shown in Fig 1-12 respectively. The high resolution of the chromatograms shown in Fig 1-12 was achieved by optimizing the extraction, separation and analytical conditions and by using an effective ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS). The UPLC analysis of *B. microcephala* acetone extract shows the presence of ten main metabolites as indicated by the chromatograms obtained at 0.96, 1.05, 3.82, 3.93, 4.76, 4.85, 5.00, 7.42, 11.44, 18.27

retention times with mass of 341, 191, 353, 389, 403, 609, 463, 793, 617, 197 respectively. These are the main metabolites in the leaves of *B. microcephala* at run time of 20 min. The peak of interest eluted at 4.76 retention time with a mass of 403 (Fig 7.1). Acetone extract of *C. mopane* shows seven prominent peaks at 4.93, 5.00, 5.46, 9.63, 11.31, 12.12 retention times with mass of 463, 463, 447, 333, 337, 379 respectively. The peaks represent the main metabolites present in *C. mopane* leaves. The peak of interest eluted at 5.46 retention time with a mass of 447.0947 represents the metabolite occurring in abundance in the leaves (Fig 7.2). The acetone extract of *C. pyracantoides* showed the various metabolites at 1.05, 4.05, 4.85, 4.91, 5.70, 7.75, 10.87, 12.70, 14.12 18.27 retention times with mass of 191, 431, 609, 495, 509, 327, 293, 555, 355, 197 respectively. The five compounds having 1.05, 4.85, 4.91, 10.87, 18.27 retention times are found in abundance in the leaves of *C. pyracantoides* (Fig 7.3). There are three main compounds in the acetone leaf extract of *D. condylocaporn* represented by the chromatograms eluted at 4.78, 4.85, 5.46 retention times (Fig 7.4). The chromatograms of *E. transvaalense* and *E. elephantine* showed that there are five main constituents at 4.19, 5.22, 3.83, 6.57, 7.73 and three main constituents at 2.75, 4.85, 5.22 retention times respectively (Fig 7.5). The leaves of *L. leucocephala*, *V. corymbosa* and *Z. humile* are composed of two main metabolites as represented by distinctive chromatograms at (1.05, 5.00), (5.50, 6.57) and (0.98, 4.63) retention times respectively (Fig 7.8-7.10). Leaves of *Z. mucronata* contain up to eleven main compounds whereas *E. natalitia* has got one main compound at 7.92 retention time.

It is interesting to note that the chemical fingerprinting of all the plant species investigated showed that they contain the same compound at 18.27 retention time. The other common compound occurred in six plant species (*B. microcephala, C. mopane, D. condylocaporn, E. transvaalense, E. elephantine, O. ornithogaloides*) at 0.96 retention time. The plant species (*B. microcephala, C. mopane, L. leucocephala, O. ornithogaloides*) contain the same compound at 5.00 retention time and plant species (*B. microcephala, C. pyracantoides, D. condylocaporn, L. leucocephala*) also contain the same compound at 1.05 retention time.

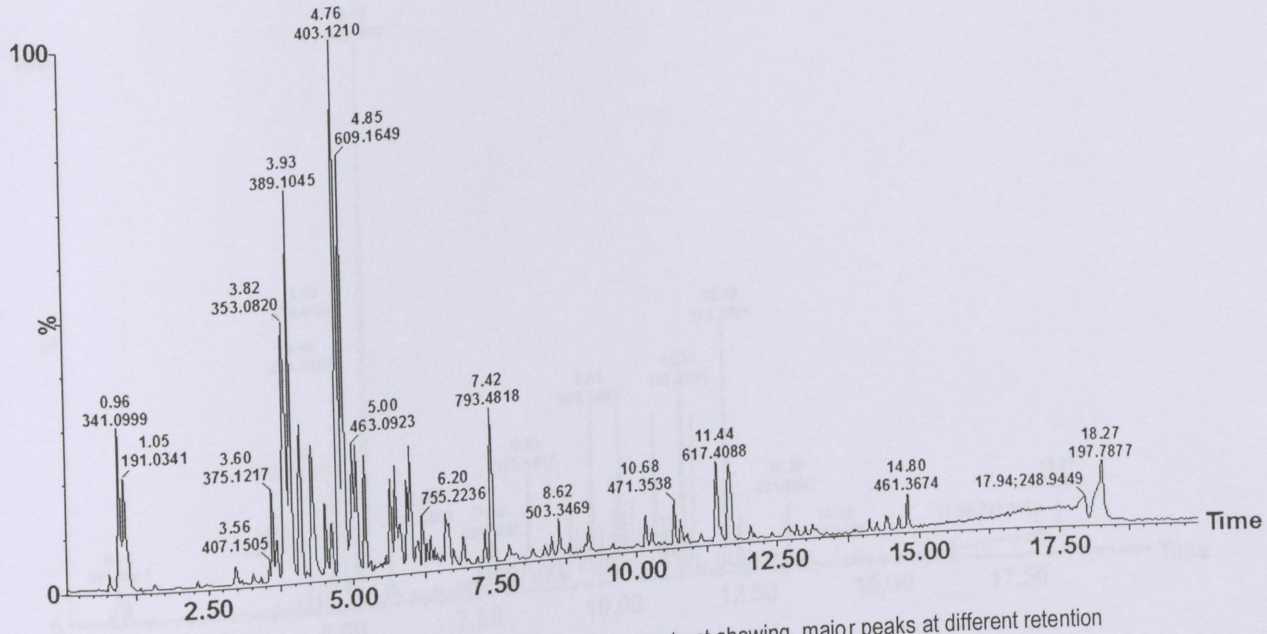


Fig. 7.1 .The UPLC chromatograms of *B. microcephala* (T1) acetone extract showing major peaks at different retention times (min) and molecular mass at a wavelength of 266 nm.

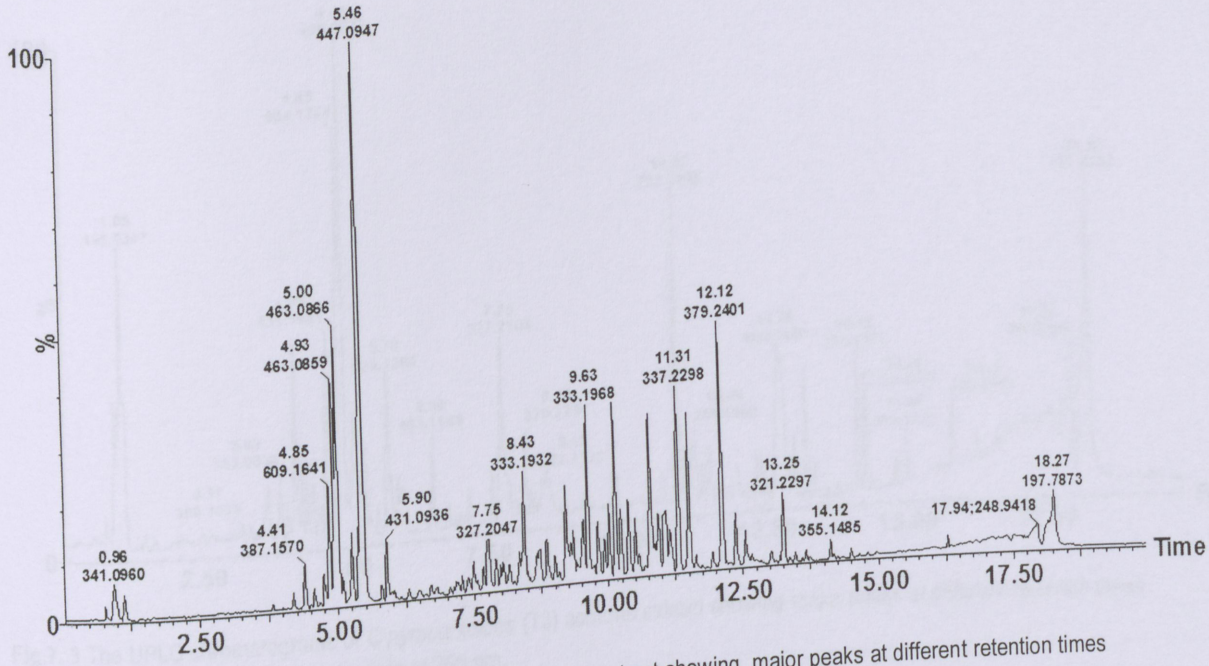


Fig. 7.2 The UPLC chromatograms of *C. mopane* (T2) acetone extract showing major peaks at different retention times (min) and molecular mass at a wavelength of 266 nm.

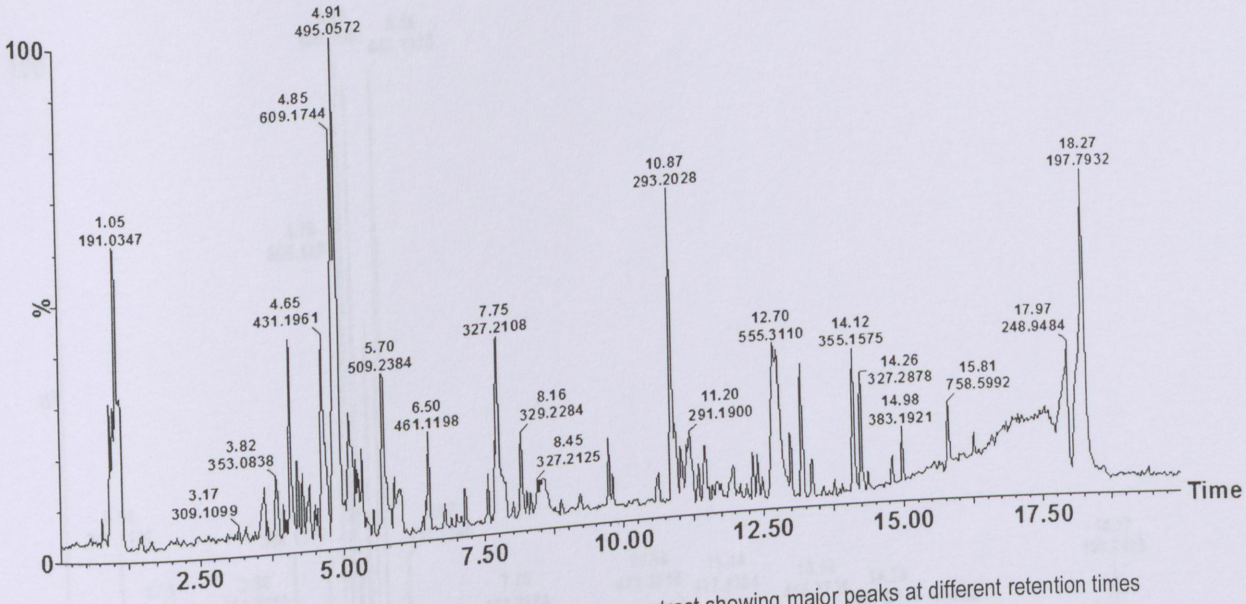


Fig.7. 3 The UPLC chromatograms of *C pyracantoides* (T3) acetone extract showing major peaks at different retention times (min) and molecular mass at a wavelength of 266 nm.

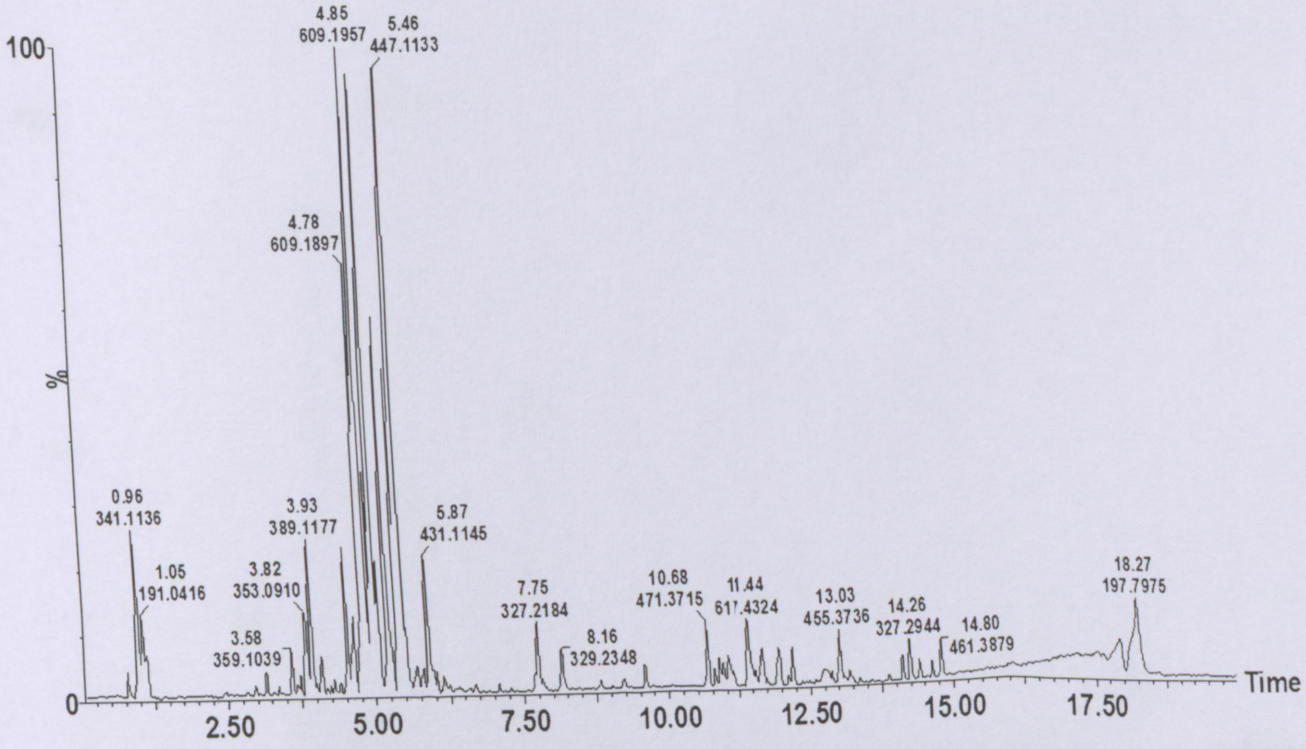


Fig.7. 4 The UPLC chromatograms of *D. condylocaporn* (T4) acetone extract showing major peaks at different retention times (min)and molecular mass at a wavelength of 266 nm.

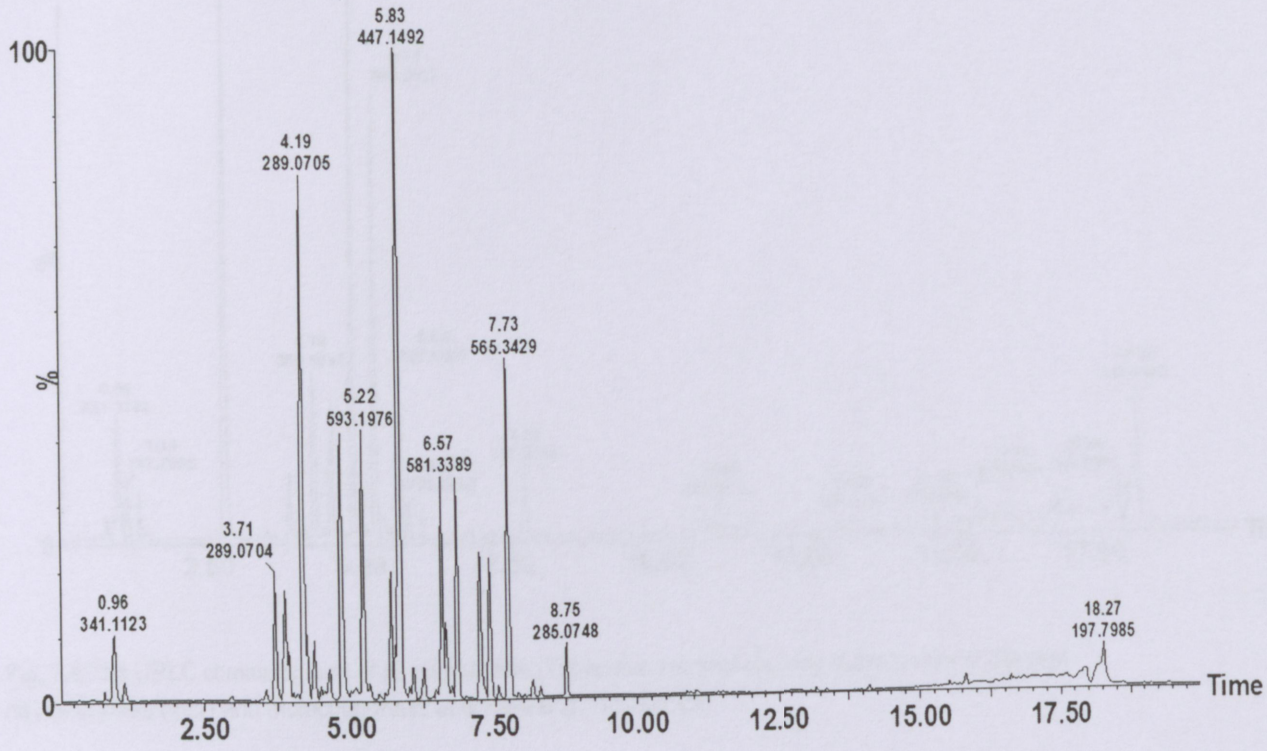


Fig 7.5. The UPLC chromatograms of *E. transvaalense* (T5) acetone extract showing major peaks at different retention times (min) and molecular mass at a wavelength of 266 nm.

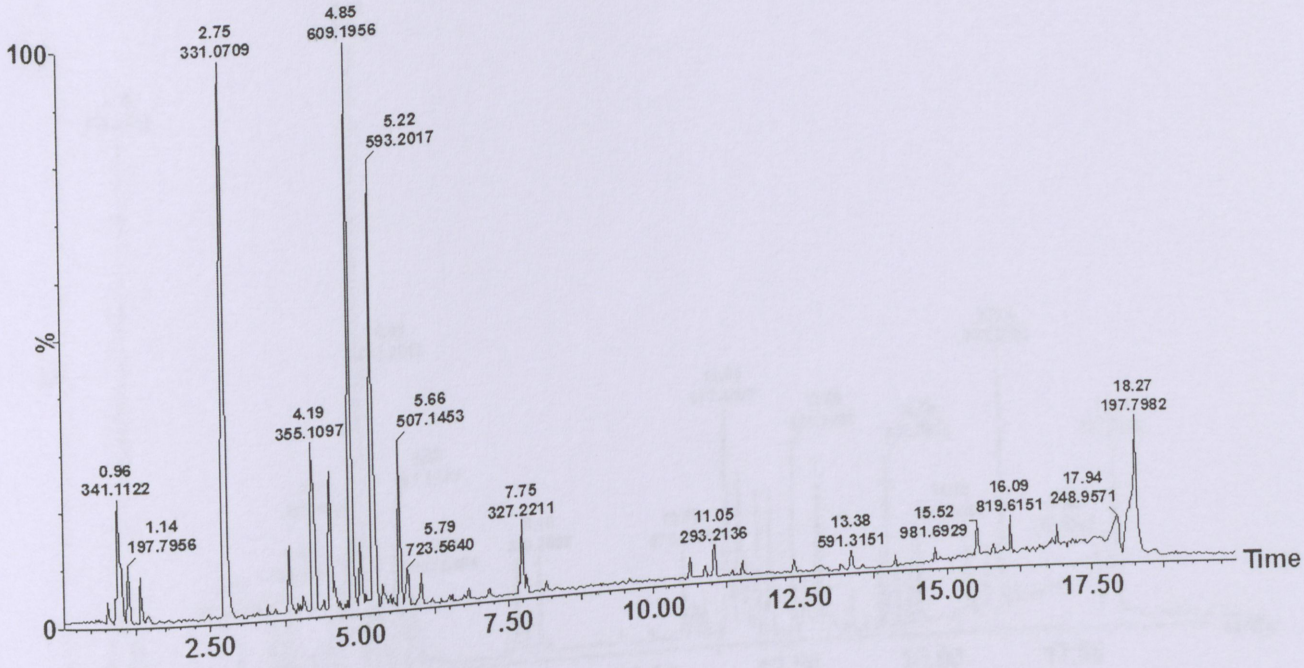


Fig. 7.6 The UPLC chromatograms of *E. elephantine* (T6) acetone extract showing major peaks at different retention times (min) and molecular mass at a wavelength of 266 nm.

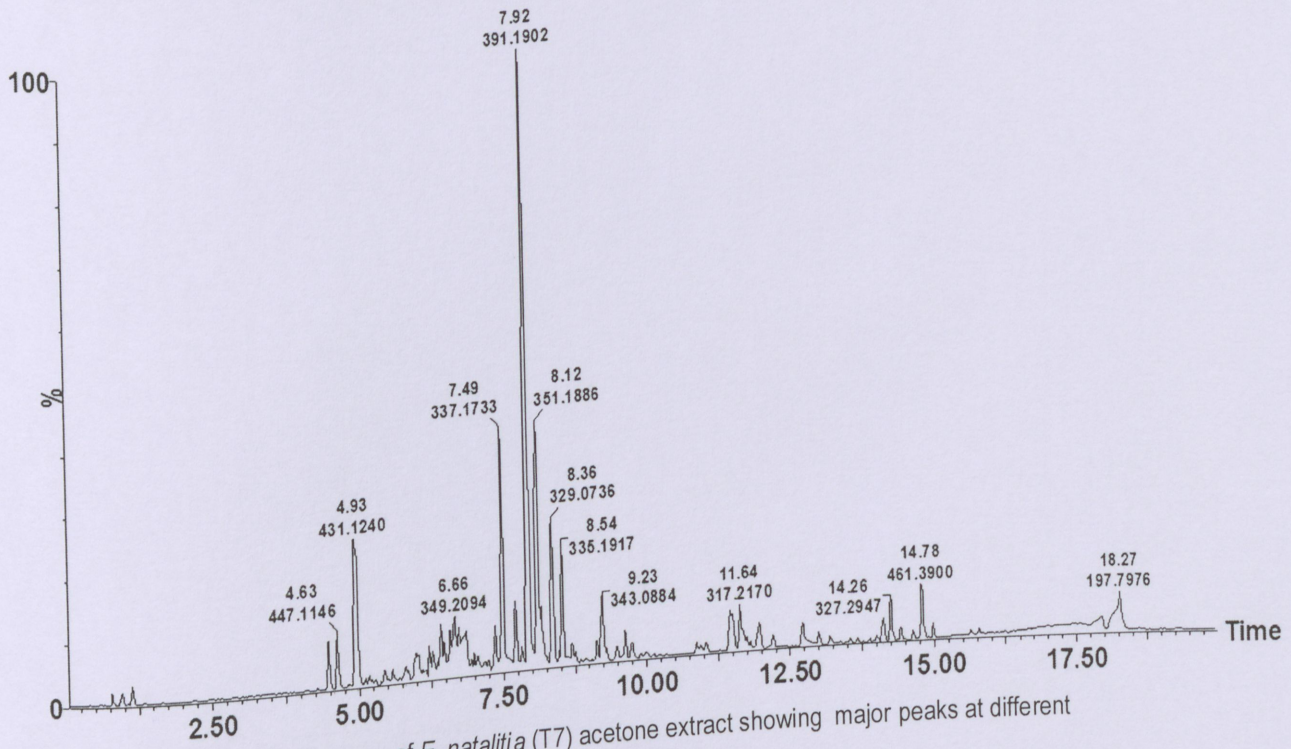


Fig 7. 7 The UHPLC chromatograms of *E. natalitia* (T7) acetone extract showing major peaks at different retention times (min.)and molecular mass at a wavelength of 266 nm.

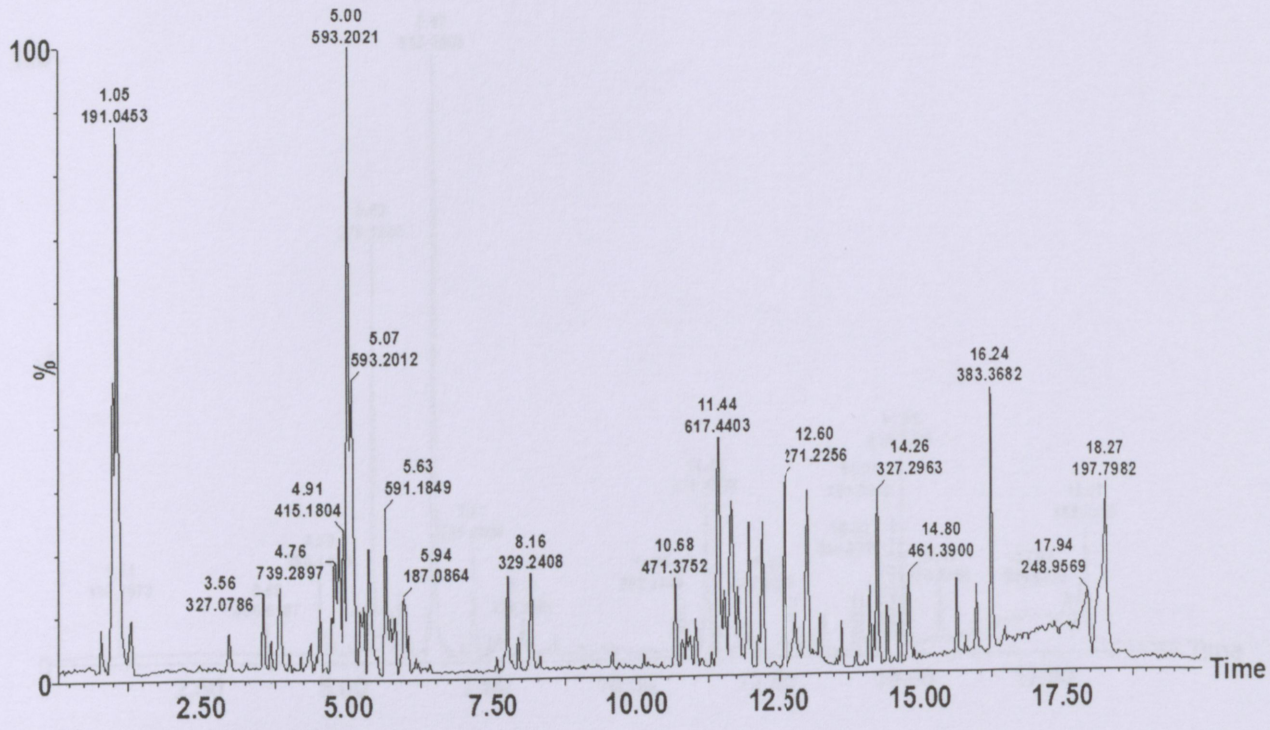


Fig. 7.8 The UHPLC chromatograms of *L. leucocephala* (T8) acetone extract showing major peaks at different retention times (min) and molecular mass at a wavelength of 266 nm.

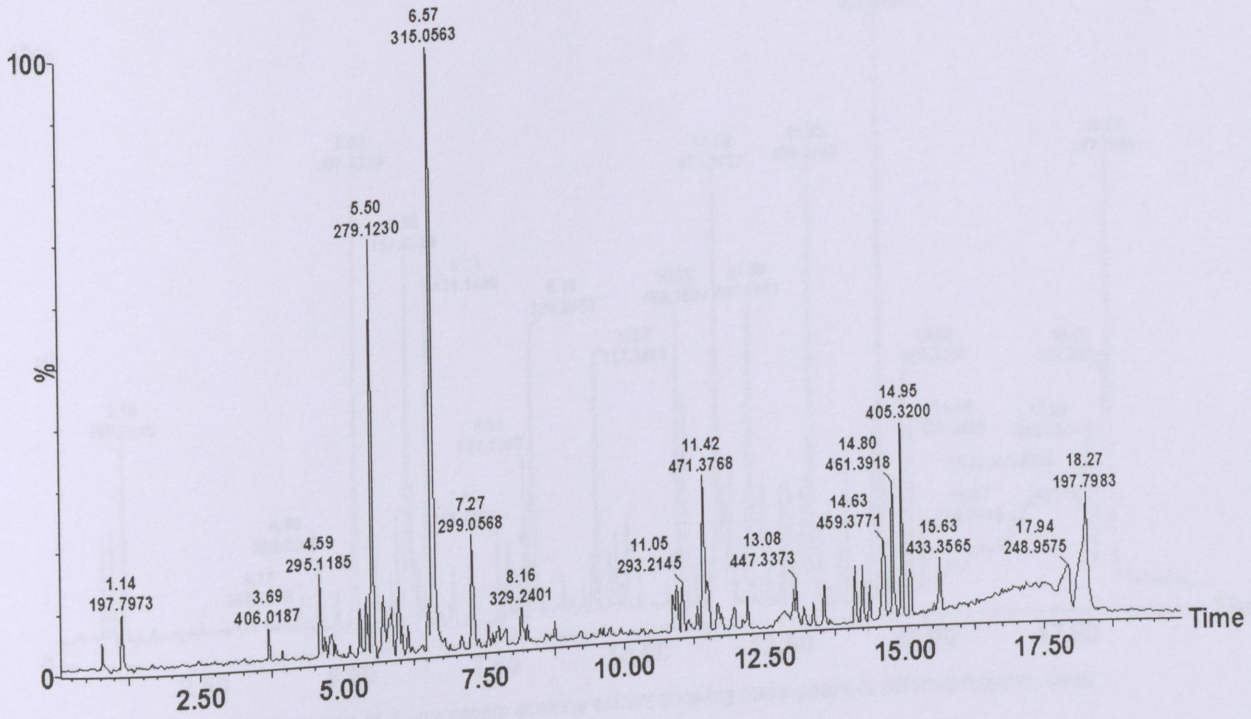


Fig. 7.9. The UHPLC chromatograms of *V. corymbosa* (T9) acetone extract of showing major peaks at different retention times (min) and molecular mass at a wavelength of 266 nm.

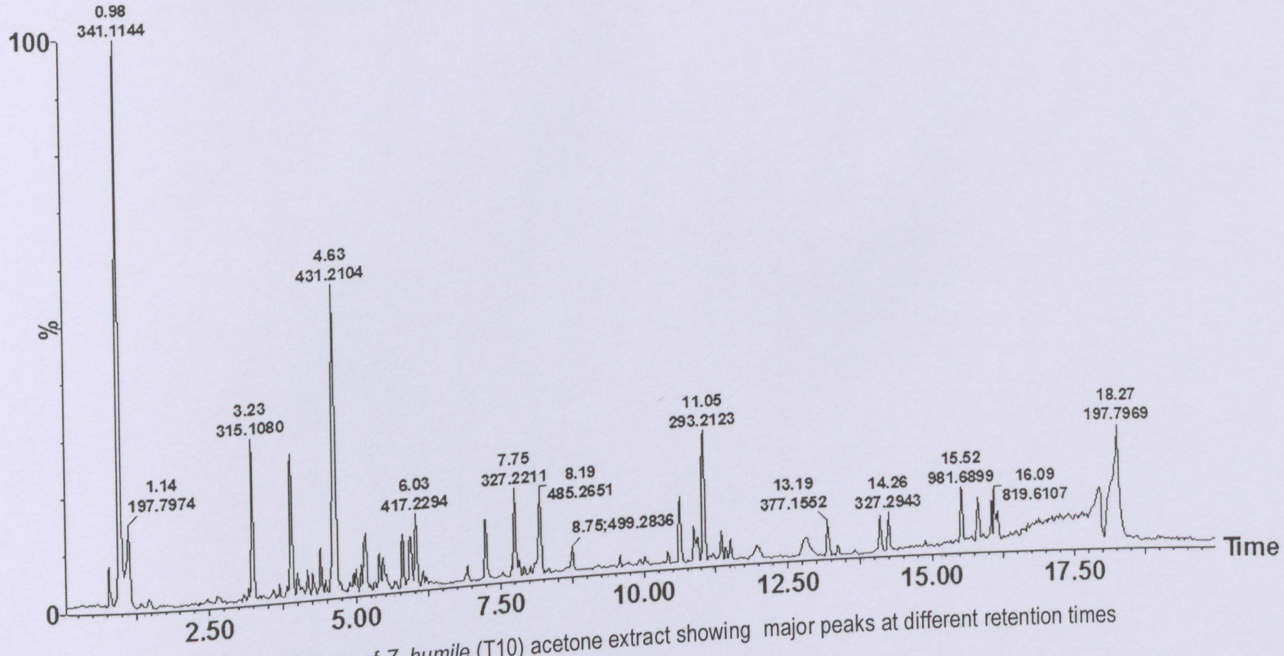


Fig.7.10 The UHPLC chromatograms of *Z. humile* (T10) acetone extract showing major peaks at different retention times (min) and molecular mass at a wavelength of 266 nm.

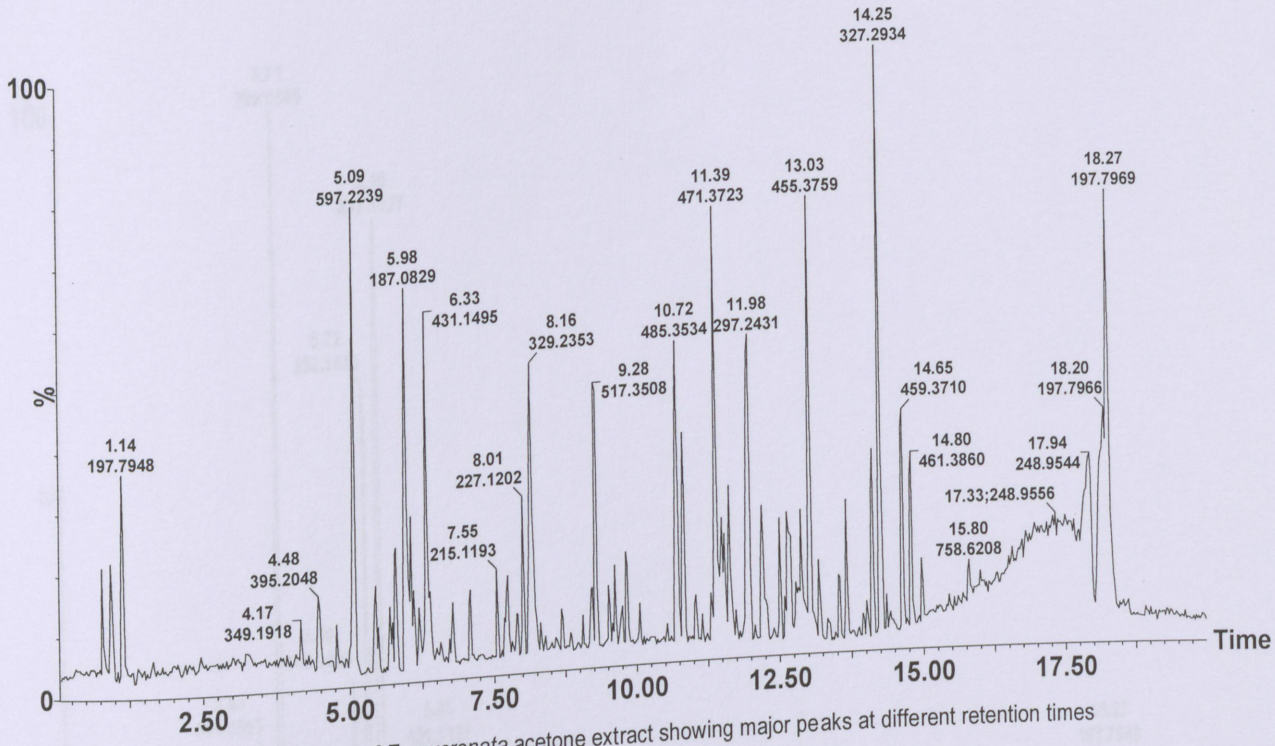


Fig 7.11. The UHPLC chromatograms of *Z. mucronata* acetone extract showing major peaks at different retention times (min) and molecular mass at a wavelength of 266 nm.

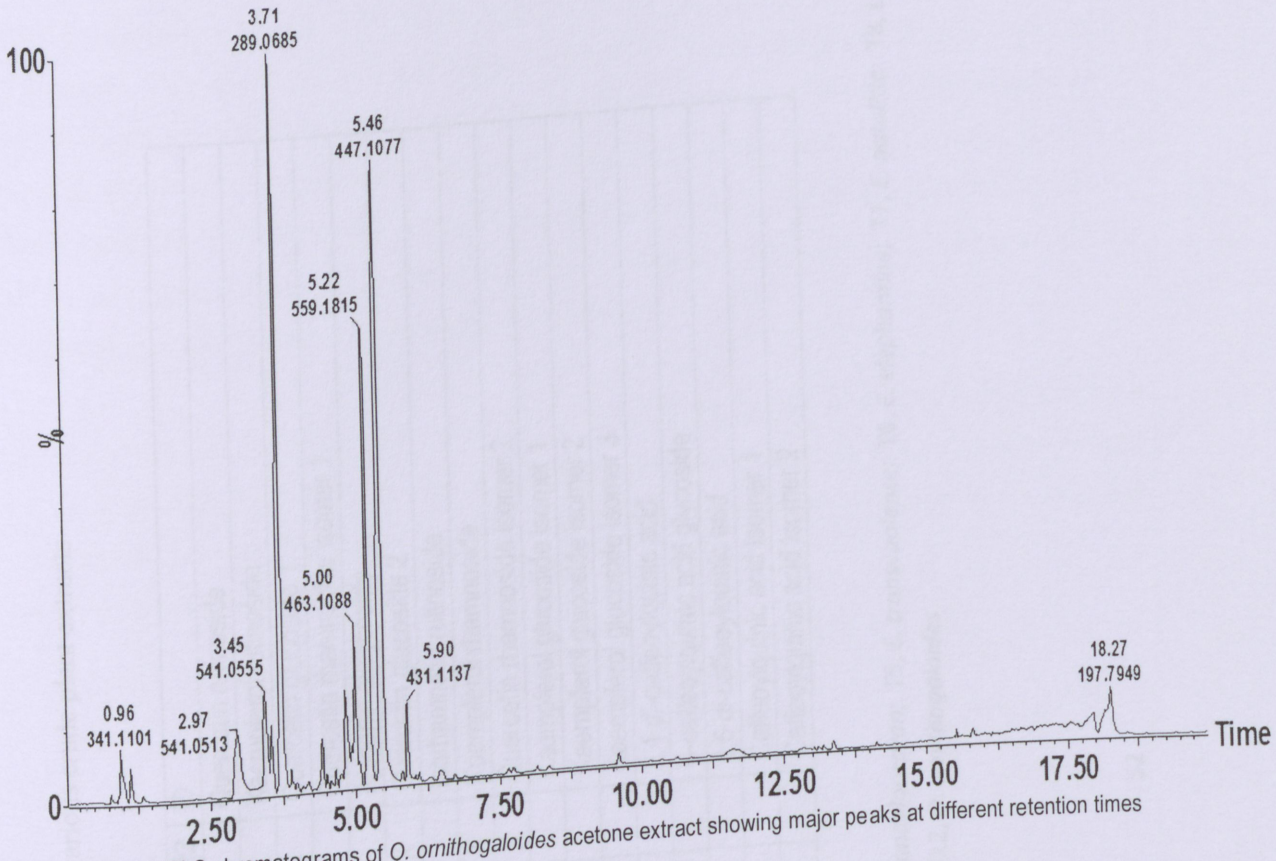


Fig 7.12 The UHPLC chromatograms of *O. ornithogaloides* acetone extract showing major peaks at different retention times (min.)and molecular mass at a wavelength of 266 nm.

Table 6.1 Metabolites detected and identified from the MS spectra of various crude plant extracts

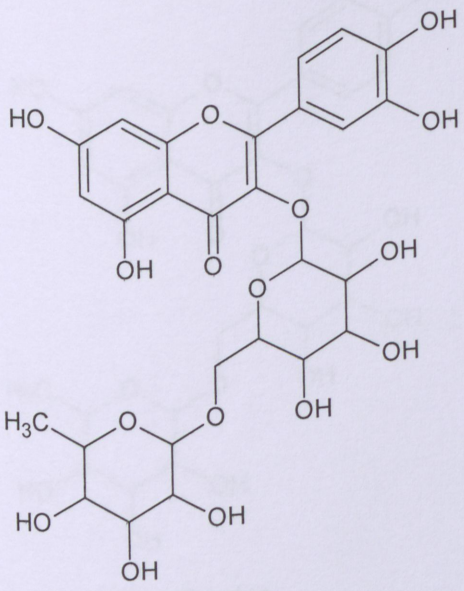
RT	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	ID
4.85	X	X	X	X	X	X		X	X			X	Quercetin rutinoside
5.20	X	X		X	X	X		X					Kaempferol rutinoside
5.00	X	X	X	X	X	X			X			X	Quercetin glucoside 1
5.45	X	X		X						X			Quercetin rhamnoside isomer 1
5.07	X	X		X				X					Apigenin diglycoside
4.90		X		X				X	X		X	X	Quercetin glucoside 2
5.28									X				Isorhamnetin rutinoside
5.90	X	X	X	X								X	Kaempferol rhamnoside
5.46	X		X						X		X	X	Quercetin rhamnoside isomer 2
5.30	X		X		X	X	X						Kaempferol glucoside isomer 1
5.80	X		X										Kaempferol glucoside isomer 2
6.80			X										Kaempferol glucoside isomer 3
5.66	X								X				3,4 *di*-caffeoylquinic acid
6.14	X							X					4-caffeoylquinic acid glycoside
5.40									X				3,5 *di*-caffeoylquinic acid
3.85	X	X	X	X									Caffeoylquinic acid Isomer 1
4.30	X												Caffeoylquinic acid Isomer 2

Key: T1, *B. microcephala;* T2, *C. mopane;* T3, *C. pyracinthoides;* T4, *D. condylocapron;* T5, *E. transvaalense;* T6, *E. elephantine;* T7, *E. natalitia* T8, *L. leucocephala;* T9, *V. corymbosa;* T10, *Z. humile ;* T11, *Z. mucronata;* T12, *O. ornithogaloides*

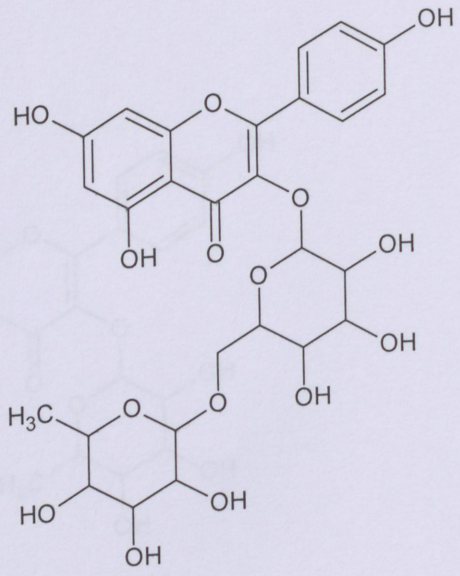
Different types of flavonoids were tantively identified in all plant species (Table 6.1) using masses, retention times, UV/visible spectra, MS/MS fragmentation and searching the databases. Two plant species, namely *C. mopane* (T2) and *had* high antifungal properties which might be attributed to the flavonoids. By visual inspection of the chromatogram of *C. mopane* (T2) the most dominant peaks represented flavonoids at different retention times e.g. Rutin Quercetin rutinoside at 4.8 min, Quercetin glucoside at 4.9 min, Quercetin glucoside at4.99 min, Kaempferol rutinoside at 5.2 min, Kaempferol rhamnoside at 5.88 min and the dominant one was Quercetin rhamnoside at 5.45 min .

D. condylocapron (T4) also showed different types of flavonoids at different retention times e.g. - Quercetin rutinoside at 4.7 min, Quercetin rhamnoside at 4.9 min, Kaempferol rutinoside at 5.2 min, Kaempferol glucoside at 5.36 min, Quercetin glucoside at 5.4 min and Kaempferol rhamnoside at 5.5 min.

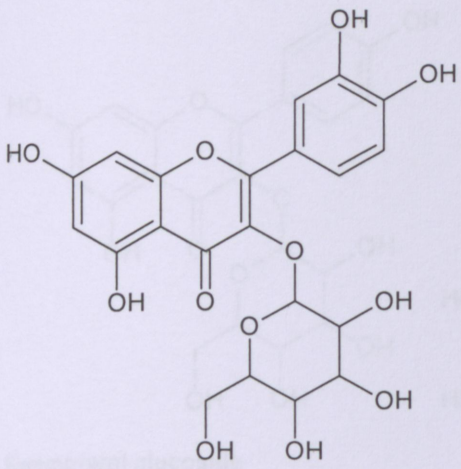
Chemical structures of different classes of flavonoids are shown in Fig 7.1.- 7.4.



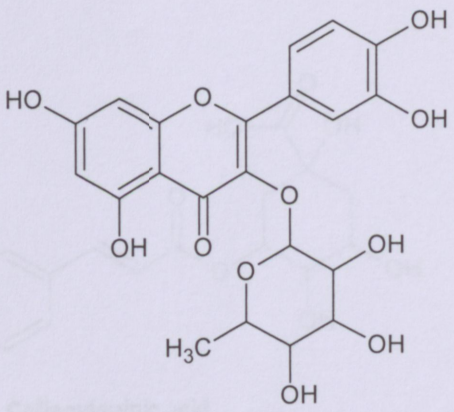
Quercetin rutinoside



Kaempferol rutinoside

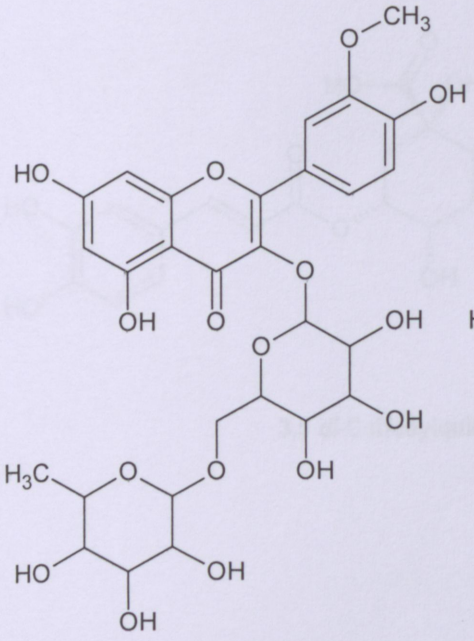


Quercetin glucoside

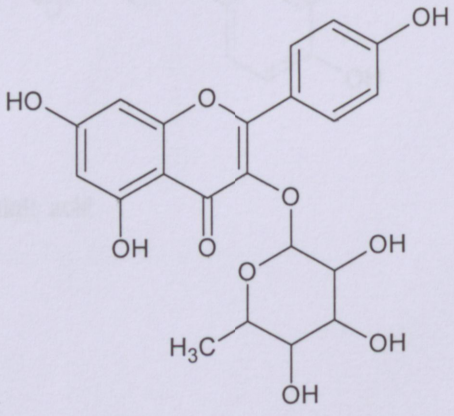


Quercetin rhamnoside

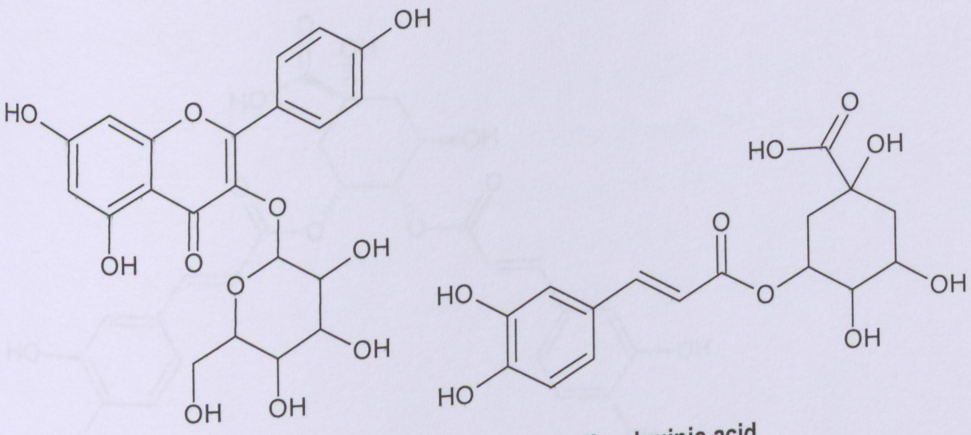
Fig 7.1 Chemical structures of the identified metabolites



Isorhamnetin rutinoside



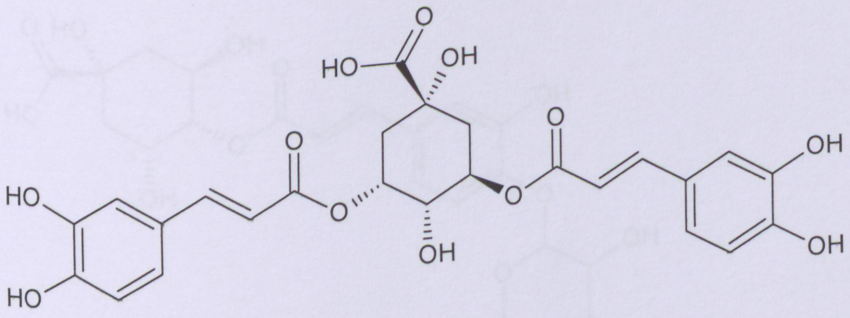
Kaempferol rhamnoside



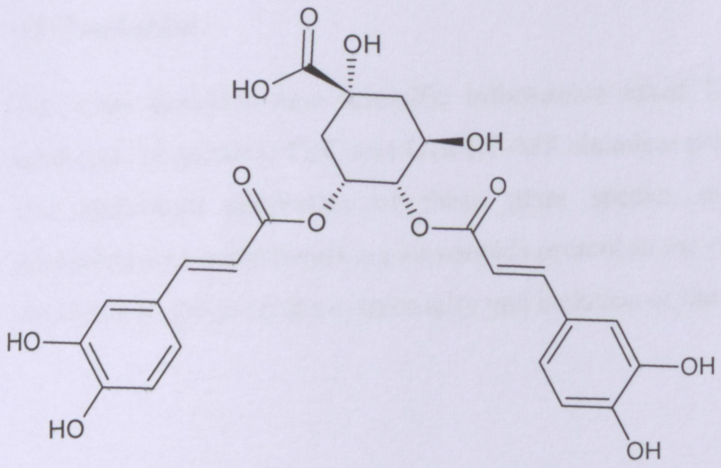
Kaempferol glucoside

Caffeoylquinic acid

Fig 7.2. Chemical structures of the identified metabolites

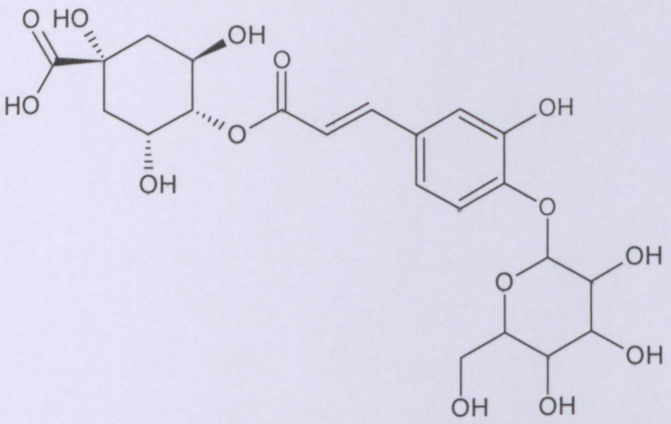


3,5 *di*-Caffeoylquinic acid

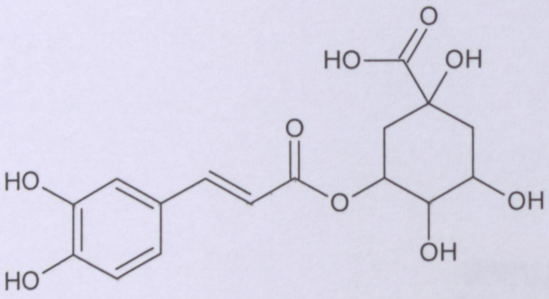


3,4 *di*-Caffeoylquinic acid

Fig 7.3. Chemical structures of the identified metabolites



4-Caffeoylquinic acid glycoside



Caffeoylquinic acid

Fig 7.4 Chemical structures of the identified metabolites

6.5 Conclusion

The study provides new scientific information about 12 medicinal plants, based on their antifungal properties, TLC and UHPLC-MS chemical profiling that has never been reported. The antifungal properties of these plant species may be attributed to the various phytochemical constituents e.g flavonoids present in the crude plant extracts. Further research work should focus on the cytotoxicity and isolation of the active components

CHAPTER SEVEN

General discussions and conclusion

In Africa and other developing countries around the world where many patients are imunocompromised as a result of the AIDS pandemic, opportunistic infections such as candidiasis caused mainly *by C. albicans* are common (Shai *et al*., 2008). *C. albicans* is the most common fungal pathogen and it is responsible for the majority of localized fungal infections in humans (Martin, 1999). The major concern with invasive candidiasis is that it is associated with an excess attributable mortality rate of 10%–49% (Pfaller *et al*., 2006). Another problem is the development of drug resistance by Candida species against the available antifungal agents. This problem can be circumvented by plants which are being used in the traditional medicine. Thus, the aim of this study was to provide scientific validation for the traditional uses of the selected plants against *C. albicans*.

Objectives

7.1. Objective 1. To investigate the chemical profiles of the selected plants using Thin Layer Chromatography and bioautography.

Phytochemical analysis of the plant extracts revealed a complex mixture of compounds separated by EMW, CEF and BEA solvent systems. These phytochemicals also act as a natural defense system for host plants and provide colour, aroma and flavour. More than 4000 of these compounds have been discovered to date and it is expected that scientists will discover many more (Tripathi *et al*., 2012).

The antifungal activity of the separated compounds against the common pathogens such as *C. albicans* and *C. neoformans* using the direct bioautography method was not encouraging. Acetone extracts of three plant species, namely *C. mopane, E. elephantina* and *L. leucocephala* showed some slight activity against the tested pathogens. Aqueous plant extracts hardly showed activity against the tested fungal pathogens. The poor activity of the plant extracts on the bioautography is generally attributed to the disruption of synergistic activities between the separated active components (Masoko *et al*., 2008).

7.2. Objective 2. To evaluate the antifungal activity of the selected plants against C. albicans using microplate dilution technique.

All the plant species investigated in this study showed varying degree of antifungal properties. Worthy of note is the acetone extracts of *C. mopane, D. condylocapron, B. microcephala* and *E. natalitia* which were the most active against the tested fungal pathogens: *C. albicans, A. fumigatus* and *C. neoformans* with minimum inhibition concentration (MIC) as low as 0.04 mg/ml. The activity of these plant species was comparable with the standard, Amphotericin B which had MIC range of 0.02-0.04 mg/ml. Thus, these plant species can serve as good candidates for further work in order to develop new antifungal agents. Hamza *et al*, (2006) report that plants that are used by the traditional practitioners in the treatment of a variety of ailments can be a good source of new, safe, biogradable and more effective antifungal drugs (Hamza *et al*., 2006).

On the other hand, the activity of aqueous plant extracts against the tested pathogens was disappointingly low. This is a serious challenge because our local poor people cannot afford to buy expensive organic solvents for extracting the active compounds from plants which are freely available to them. However, this study has revealed that some of the selected plant species are of value in the traditional medicine.

7.3. Objective 3. To determine the antioxidant activity of the plant extracts.

The high incidence of candidiasis in HIV/AIDS patients has made candidiasis a leading fungal infection in the immune-suppressed population (Runyoro et al., 2006). In view of this, plant species selected for this study were investigated for their antioxidant properties in order to assess their ability to boost the immunity system of the body. The analysis of total antioxidant activity of plant species was done using the quick, reliable, cost effective, qualitative DPPH method. The overall results showed that organic plant extracts had high antioxidant activity than aqueous plant extracts. The order of their activity from the highest to the lowest was as follows: *E. transvaalense* > *B. microcephala* > *C. mopane* > *E. elephantina* > *O. ornithogaloides*, > *D. condylocarpon* . The low activity of aqueous plant extracts might be attributed to the fact that water extracted the least concentration of the active components from the leaves or possibly the volatility of the compounds. The results of this study showed that some of the plant species might be good sources of antioxidants which help the body to fight off microbial infections.

7.4. Objective 4. To investigate the chemical composition of the selected plants and identification of the main metabolites using Ultra Performance Liquid Chromatography- Mass Spectrometry (UPLC-MS).

Acetone was used in the extraction process because it is the best extractant capable of extracting both polar and none polar metabolites (Eloff, 1998a). Chemical fingerprinting of the plant extracts is important in the quality control of plant products. It can also be used for chemotaxonomic analysis of plant species or any other organisms e.g microbes, worms etc. Madala *et al*., 2013 report that metabolic fingerprinting is increasingly becoming a valuable tool for rapid sample classification; e.g. disease diagnostics, monitoring wholecell biotransformations, plant taxonomy, plant-based medicines, etc. In this study a high definition UPLC-MS was used to analyse the chemical profiling of the selected plant species. Data analysis was done using Markerlynx XS software (Waters, USA). The UPLC analysis of plant extracts revealed a very complex mixture of metabolites eluted at different retention times. Major compounds which were tentatively indentified in the leaves of all plant species were flavonoids. Most of these fllavonoids were also identified even by visual inspection of the chromatograms of *C. mopane* and *D. condylocaporn* plant species. This may explain the reason why they had the highest antifungal activitiy against the tested fungal pathogens. Thus, UPLC-MS is an excellent tool for evaluating the quality and content of pharmacologically important plant extracts.

Conclusion

Generally, plant species showed varying degree of antifungal properties against the selected pathogens. Plant species that showed strong antifungal activity should be investigated further in order to isolate and identify the active compounds with the aim of developing new antifungal agents. The use of UPLC-MS has disclosed that the selected plant species contain diverse metabolites in their leaves which need to be investigated further for their pharmacological activities. The UPLC chemical profiles of the plant extracts were recorded and will serve as basic reference for all future investigations of the plant species. This is the first report on the chemical profiling of the selected plant species using UPLC-MS technique.

CHAPTER EIGHT

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