Isolation and characterization of antifungal compounds from *Clerodendron glabrum* var *glabrum* (Verbenaceae) used traditionally to treat candidiasis in Venda, South Africa

By

Ndivhaleni Anox Masevhe (24275744)

MSc (University of Venda, SA)

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Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science.

Supervisor **Prof J.N. Eloff**

Co-supervisor **Dr L.J. McGaw**

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Declaration

I declare that this thesis which I hereby submit is my own investigation except where the work of others is acknowledged and it has not been submitted to any other tertiary institution.

Signed:----------

N. A. Masevhe

Dedication

This work is dedicated to the loving memory of my late mother (Masindi Rambani Masevhe) who passed on while I was busy with this study.

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I thank God, The Almighty, for giving me health and strength to complete my studies and also for protection in all my trips from Venda to Pretoria (Ps 103v1)

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Conference presentations

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List of abbreviations

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)
Amp B	Amphotericin 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	*Cryptococcus neoformans*
^{13}CNMR	Carbon 13 Nuclear magnetic resonance
DPPH	1, 1-diphenyl-2-picrylhydrazyl radical
EC	*Escherichia coli*
EC_{50}	Effective concentration 50
EF	*Enterococcus faecalis*
EMW	Ethyl acetate: methanol: water (40:5.4:4)
FRAP	Ferric reducing antioxidant power
H NMR	Proton Nuclear Magnetic Resonance
IC_{50}	Inhibitory concentration
INT	*p*-iodonitrotetrazolium violet
LC_{50}	Lethal concentration 50
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium
MS	Mass spectrometry
MH	Müller Hinton
PA	*Pseudomonas aeruginosa*
R_f	Retardation factor
SA	*Staphylococcus aureus*
SD	Sabouraud dextrose
STI	Sexually Transmitted Infections
TA	Total activity
TLC	Thin layer chromatography

Abstract

The aim of this study was to isolate and characterize antifungal compounds from the most active medicinal plant species that could be used to address secondary infection problems in immunocompromised patients.

An ethnobotanical study was conducted and 45 medicinal plant species used traditionally to treat candidiasis and related infections in HIV/AIDS patients were identified and documented. The most popular plant species used included *Acacia caffra*, *Clerodendrum glabrum*, *Croton gratissimus, Elaeodendron transvaalense*, *Faurea saligna, Hippocratea longipetiolata*, *Osyris lanceolata*, *Richardia brasiliensis*, *Schkuhria pinnata*, *Schotia brachypetala, Spilanthes acmella*, *Strychnos potatorum*, *Vangueria infausta subsp. infausta* and *Withania somnifera.* The plant parts used in the therapeutic preparations were roots (26.7%), bark (22.2%), and a combination of roots and bark (17.7%). Decoctions (44.4%), infusions (20%) and macerations (17.7%) were used. Most of the herbal remedies were administered orally.

Chemical profiles of the plant species were established by using thin layer chromatography. Leaf extracts of these plant species were tested for antimicrobial activity against two common pathogenic fungal species in humans (*Candida albicans* and *Cryptococcus neoformans*) and four nosocomial bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*) using a two-fold serial microdilution method and bioautography. All plant species investigated had some degree of antimicrobial activity against the test microorganisms. The hexane and the acetone extracts of *Clerodendrum glabrum, Hippocratea longipetiolata, Schkuhria pinnata* and *Withania somnifera* were the most active with MIC values ranging from 0.06 to 0.08 mg/ml. The most susceptible pathogen to the test samples was *C. neoformans* while *C. albicans* was resistant to most of the plant extracts. The water extracts of *Withania somnifera* and *Hippocratea longipetiolata* (14%) had MIC < 1 mg/ml against *C. albicans*. *C. neoformans* was susceptible to nine water plant extracts (64%) with MIC < 1 mg/ml and the promising activity was observed in *Hippocratea longipetiolata* and *Faurea saligna* extracts with MIC values of 0.16 and 0.31 mg/ml respectively. The hexane extract of *C. glabrum* was the most active against *C. albicans* with an MIC value of 0.06 mg/ml and total activity of 550 ml/g. In the bioautography, most plant extracts tested had few active compounds, others had no active components at all and this may be attributed to the disruption of synergism by the thin layer chromatography. *C. glabrum* had eight active antifungal compounds on bioautograms and most of these components were observed in the EMW solvent system. Based on this and its wide distribution in rural areas, *C. glabrum* was chosen for further study.

The antioxidant activity and possible immune boosting potential of the species were determined using 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), 2, 2' azinobis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) assays. In the DPPH qualitative assay, the aqueous plant extracts had several prominent antioxidant components than the organic plant extracts. The aqueous plant extracts which had the most prominent antioxidant activity were *F. saligna* with 8 compounds, followed by *E. transvaalense, H. longipetiolata O. lanceolata, R. brasiliensis* and *S.brachypetala*, with five compounds each and their R_f values ranged from 0;06 to 0.94. This appears to validate the ethnomedicinal use of the plant species to some extent because decoction is the most common method used in the preparation of the remedy by the traditional healers. With regard to the organic plant extracts, only one plant extract, *F. saligna* had two prominent antioxidant components at R_f values 0.81 and 0.88. A third of the plant species had a high level of free radical scavenging activities in the DPPH, ABTS and FRAP assays. However, all plant extracts had lower antioxidant activity than the positive control (Trolox) used.

The selected plant species were also evaluated for their *in vitro* toxicity against the Vero monkey kidney cell line using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. The acetone plant extracts of *O. lanceolata, S. acmella, S. pinnata* and *S. brachypetala* had high cytotoxic activity against Vero cells with IC_{50} values of 13.7±0, 19.9±0.001, 21.6±0.001 and 28.34±0.001 µg/ml respectively. However, their IC_{50} values were higher than that of the positive control, doxorubicin (IC_{50} = 9.9±0 µg/ml). The rest of the acetone plant extracts (64%) had moderate cytotoxic activity (30 < IC_{50}<100 µg/ml). The aqueous plant extracts were relatively non-toxic to the Vero cells with IC_{50} values ranging from 137 to > 500 µg/ml. This supports the use of aqueous extracts in the traditional medicine. However, their low selectivity index values ranging from 0.26 to 1.68 suggest that the plant extracts are probably suitable for external use only.

Fractionation of the hexane extract of the leaves of *C. glabrum* by chromatographic techniques yielded six fractions of which fractions C and D had significant antifungal activity (average MIC value = 0.1 mg/ml) against *C. albicans* and *C. neoformans.* From these fractions, one new triterpenoid, 3-(1-oxobutyl)-11*α*-hydroxytaraxast-20(30)-ene-24,28-dioic acid (clerodendrumic acid) (**1**) was isolated along with known heptadecanoic acid (**2**). *C. albicans* was relatively insensitive to clerodendrumic acid (**1**) (MIC value = 125 µg/mL) and was resistant to heptadecanoic acid (**2**) (MIC value = 188 µg/ml). Compounds **1** and **2** were non-toxic against monkey kidney Vero cells *in vitro* with IC_{50} values of 202.6 and 108.4 µg/ml respectively. Due to its low antifungal activity, the novel compound clerodendrumic acid (**1**) is not a viable candidate for drug development which could be used to combat candidiasis and related fungal infections. However, due to its relative safety, it may possibly be used as a lead compound to produce new chemically modified active derivatives or could be used together with known antibiotics to mitigate their undesirable side effects. To the best of our knowledge, the isolation of a novel, clerodendrumic acid (**1**) and a known heptadecanoic acid (**2**) compounds from leaf extracts of *C. glabrum* is reported herein for the first time.

The results obtained from this study generally substantiate the rationale behind the use of the selected plant species in the traditional medicine to treat candidiasis and related infections to some extent. This study showed the potential of studying traditional medicine in the search for effective plant extracts or new lead compounds that could be developed into drugs for combating microbial infections among the rural poor people.

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Chapter 1.

1.1. Introduction

HIV/AIDS is a syndrome comprising a dysfunction of the immune system, compounded by opportunistic infections of bacterial, fungal, protozoan or viral aetiology. These opportunistic infections in immunocompromised individuals are responsible for considerable morbidity and mortality (Mirowski et al., 1997). The infection with HIV/AIDS, creates a window for infections with other microbes, which were hitherto contained by the immune system. Another concern is that weakened human immune system also becomes a favourable environment for the adaptation of traditionally non-human infectious agents (Bessong and Obi, 2006). Studies all over the world show that 90% of all patients contract fungal, bacterial infections etc at some point during the primordial stage or after developing AIDS (Motsei *et al*., 2003).

A major predisposing factor of opportunistic infections in HIV-infected individuals is a decreased CD^{+4} T-cell counts (Hoepelman and Dupont, 1996). Common examples of opportunistic infections include cryptosporidiosis caused by *Cryptosporidium parvum* which is considered to be one of the opportunistic infections that complicates AIDS, by causing life-threatening diarrhoea (Tzipori and Honorine, 2002); cryptococcosis caused by *Cryptococcus neoformans*, candidiasis caused by *Candida albicans,* aspergillosis caused by an exposure to *Aspergillus fumigatus* (Dan and Levitz, 2006) and tuberculosis (TB) caused by *Mycobacterium tuberculosis* (McGaw et al., 2008). Among the different HIV/AIDS associated microbial infections, candidiasis caused by *C. albicans* is one of the earliest opportunistic infections in HIV infected individuals (Fennel *et al*., 2004).). *Candida* species are responsible for a wide range of systemic as well as superficial opportunistic infections (candidiasis) occurring most frequently in vaginal or oral mucosa (Van Wyk et al., 2009). On the other hand, Caston-Osorio et al. (2008) report that infections by *Cryptococcus* species are the second most common cause of opportunistic infections caused by yeasts in immunosuppressed patients.

Standard therapeutic regimen for candidiasis consists of common antifungal agents such as azoles, polyenes, echinocandins and flucytosine (Dan and Levitz, 2006). These antifungal agents play a vital role in controlling fungal species so that the patient may recover. Minimizing the opportunistic infections in AIDS patients improves the quality of life of an individual (Bessong and Obi , 2006).

Management of candidiasis is however complicated by the emergence of strains of *Candida* that are resistant to the currently used antifungal agents and these antifungal agents are limited in number, costly and are known to be toxic (van Wyk et al., 2009). According to Sanglard and Odds (2002) *C. albicans* and related species pathogenic to man become resistant to antifungal agents by the expression of efflux pumps that reduce drug accumulation, alteration of the structure or concentration of antifungal target proteins.

The above-mentioned factors prompt the need for the screening and investigation of plants as potential sources of new antimicrobial compounds for primary healthcare in rural areas (Shai et al., 2008). Medicinal plant species that are traditionally used by the traditional healers to treat fungal infections and related ailments can be a good source for new, safe, biogradable and renewable antifungal drugs (Hamza *et al*., 2006). Even the discovery of modern drugs such as reserpine from *Rauvolfia serpentine* for treating hypertension, vinscrine from *Catharanthus roseus* for treating leukemia, digoxin and digitoxin from *Digitalis purpurea* for artrial fibrillation, quinine from *Cinchona pubescens* for treating malaria, aspirin from *Filipendula ulmaria* for analgesic and inflammation, just to mention a few, have been discovered through ethnobotanical leads (Plotkin, 1988).and this signify the huge potential that still exists for the discovery of many more novel pharmaceuticals. To date however, no antifungal compounds from plants appears to have been commercialized.

In this study, fourteen plant species used traditionally to treat candidiasis in Venda were collected and evaluated for their antimicrobial activity using microdilution method and bioautography, in an attempt to validate their traditional use. The selected plant species were also evaluated for their antioxidant activity because the therapeutic benefit of medicinal plants is often attributed to their antioxidant properties (Ljubuncic et al., 2005). Plant species were also evaluated for their safety because based on the long history of medicinal plant use, users of traditional medicines just accept that they are safe for human consumption, yet there is no knowledge on the potential toxicity of many indigenous plants (Street et al., 2008), particularly in the long term. Antifungal compounds were isolated from the most active plant species using chromatographic methods in an endeavour to find a compound that could be used to treat candidiasis in immunocompromised individuals.

1.2. Statement of the problem

In South Africa where about 5.6 million patients are immunocompromised as a result of the HIV/AIDS pandemic (UNAIDS, 2011), opportunistic infection such as candidiasis caused mainly by *C. albicans* is responsible for considerable morbidity and mortality. Management of these infections is problematic because of toxicity and microbial resistance to the currently available drugs.

1.3. Hypothesis

Medicinal plants used to treat candidiasis in Venda contain metabolites with anti-infective activity against resistant and susceptible pathogens associated with opportunistic infections in immune-suppressed individuals

1.4. Aim

The aim of this study was to isolate and characterize antifungal compounds from the most active medicinal plant species that could be used to address secondary infection problems in immunocompromised patients.

The following objectives were identified in order to achieve the aim:

1. To determine which plants are being used traditionally to treat candidiasis in HIV/AIDS positive people in Venda and to select promising plant species based on a number of parameters
2. To determine the antifungal activity of the selected plant species against *C. albicans*
3. To determine the antioxidant activity that may be related to immune supporting capacity of plant extracts
4. To determine the cellular safety of plant extracts.
5. To isolate and characterize the antifungal compounds responsible for the activity from the plant species with the most promising characteristics.
6. To determine the antimicrobial activity and the cellular safety of the isolated compounds.

Two types of styles have been used in this work, namely, Chapter 1 and 2 have been written in the traditional style for the thesis and the rest of the chapters have been written in the form of manuscripts.

Chapter 2.

Literature review

2.1. Importance of fungal diseases

Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide (Duraipandiyan and Ignacimuthu, 2011). They are produced by fungi that are widely distributed in the environment and, therefore, very difficult to eradicate (Blanco and Garcia, 2008). There are more than 150,000 known fungal species, more than 100 species are pathogenic for humans, but only 10 to 15 are relatively common human pathogens that cause infections. Clinically, fungal infections rarely occur in individuals with intact immunity systems (Pirofski, 2001). The most frequent fungal infections are caused by *Candida* and *Aspergillus* species (Hoepelman and Dupont, 1996, Pfaller, 1995) and other fungi such as *Cryptococcus neoformans* and *Fusarium* species, dematiaceous molds such as *Pseudoallescheria boydii,* and *Zygomycete*s are increasingly being reported (Rodriguez and Patrick, 2001). People who are at risk of developing these fungal infections, include patients undergoing blood and bone marrow transplantation, solid-organ transplantation, patients with HIV/AIDS, neoplastic disease, advanced age; patients receiving immunosuppressive therapy, premature infants etc. (Epstein and Polsky, 1998). The estimated annual incidence of invasive mycoses due to *Aspergillus* species is 12–34 infections per million population, 30–66 infections per million population for *C. neoformans*, 72–228 infections per million population for *Candida* species and the cost of the latter in the United States only is more than 1 billion dollars per year (Pfaller et al., 2006, Berman and Sudbery, 2002).

2.1.1. Causative agents

Normally, *Candida albicans* is a commensal organism commonly found in the normal flora of the skin, mouth, intestinal tract and vagina. However, in immunocompromised settings, *C. albicans* infections lead to oral, and oropharyngeal, vulvovaginal and systemic candidiasis. Oral and oropharyngeal candidiasis is one of the first clinical signs of HIV infection and is diagnosed in up to 95% of HIV^+ patients before onset of full-blown AIDS (White et al., 1999, Pfaller, 1995). Other *Candida* species such as *Candida tropicalis, Candida krusei, Candida guilliermondii* and *Candida parapsilosis* have recently also emerged as causes of the infections (Pauw and Picazo, 2008).

Oral candidiasis encompasses infections of the hard and soft palate, tongue, buccal mucosa and floor of the mouth, and can present as reddened patches on the palate, dorsum of the tongue and is known as erythematous candidiasis, white curd-like lesions on the buccal mucosa, tongue and other oral mucosal surfaces that can be wiped away, leaving a red or bleeding underlying surface (pseudomembranous candidiasis), angular cheilitis appears as

cracking, peeling, fissuring at the corner of the mouth and candidal leukoplakia appears as white patches usually located at the sides of the tongue and cannot be scraped off. Patients may exhibit one or a combination of any of these presentations (Wong et al., 1998).

Vulvovaginal candidiasis involves infections of the vaginal lumen as well as the vulva and symptoms include burning, itching, soreness, an abnormal discharge and dyspareunia. It has been estimated that 75% of all adult women worldwide suffer from this condition at some time during their lives, and in many the disease is recurrent (Kaplan et al., 1997). Some of the predisposing factors that favour the onset of vaginal candidiasis include pregnancy, diabetes, luteal phase of the menstrual cycle, the use of antibiotics, oral contraceptives and hormone replacement therapy (Magliani et al., 2002).

Candida species are normal inhabitants of the gastrointestinal (GI) tract and persistence in the GI tract may lead to gastrointestinal candidiasis (infection of the stomach, small and large intestines). GI colonization and infection predispose patients to systemic candidiasis due to outgrowth of *Candida* species in the GI tract and the predisposing factors are immune deficiency and antibiotic therapy. *C. albicans* is capable of flourishing in low oxygen levels in the anaerobic environment of the gastrointestinal tract (Harriott and Noverr, 2011). Candidiasis of the trachea, bronchi and lungs likewise occur as an end stage of the disease event (Durden and Elewski, 1997).

2.1.2. Pathogenesis

Most fungal pathogens undergo morphological transformations during host invasion and have the ability to grow well at human body temperature. The yeast, *C. albicans* is an example of a highly successful opportunistic pathogen which has the ability to change from yeast morphology to various filamentous forms (true hyphae and pseudohyphae). Filamentous forms play an important role in the adhesion to human epithelium while yeasts and pseudohyphae are found in tissues once infection has been established (Bendel, 2003). During the invasion, *C. albicans* also secretes aspartic proteinase which is characterized as a major virulence factor because it plays the main role in both adhesion and penetration of the host cells and the yeast phase is thought to be important for dissemination during systemic infection (Bastert et al., 2001).

Some of the organisms are referred to as environmental pathogenic fungi and these include filamentous fungal molds (*Aspergillus fumigatus*), pathogenic yeasts (*Cryptococcus neoformans, Cryptococcus gattii*), and the dimorphic fungal pathogens (*Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis, Penicillium marneffei, Sporothrix schenckii*) and humans are exposed to these fungi by inhaling spores or small desiccated yeast cells, leading to an initial pulmonary infection in the lungs (Gow et al., 2002).

2.1.3. Resistance

Antifungal agents have greatly contributed to the improvement of public health. Nevertheless, antifungal resistant pathogens have increased during the past decade and are becoming a serious threat. According to Eloff (2000) no group of antibiotics has been introduced to which resistance had not been observed. *C. albicans* has been the most extensively studied pathogen in antifungal resistance because of the morbidity and mortality associated with infections in immunocompromised patients (Casalinuovo et al., 2004). A variety of antifungal classes is used to treat fungal infections and each of the antifungal classes utilizes a different means to kill or inhibit the growth of fungal pathogens (Pfaller, 2012). Mechanisms of antifungal resistance by *Candida* spp against antifungals agents is well documented in literature. Some of the most common mechanisms of resistance are discussed and illustrated in Figure 2.1:

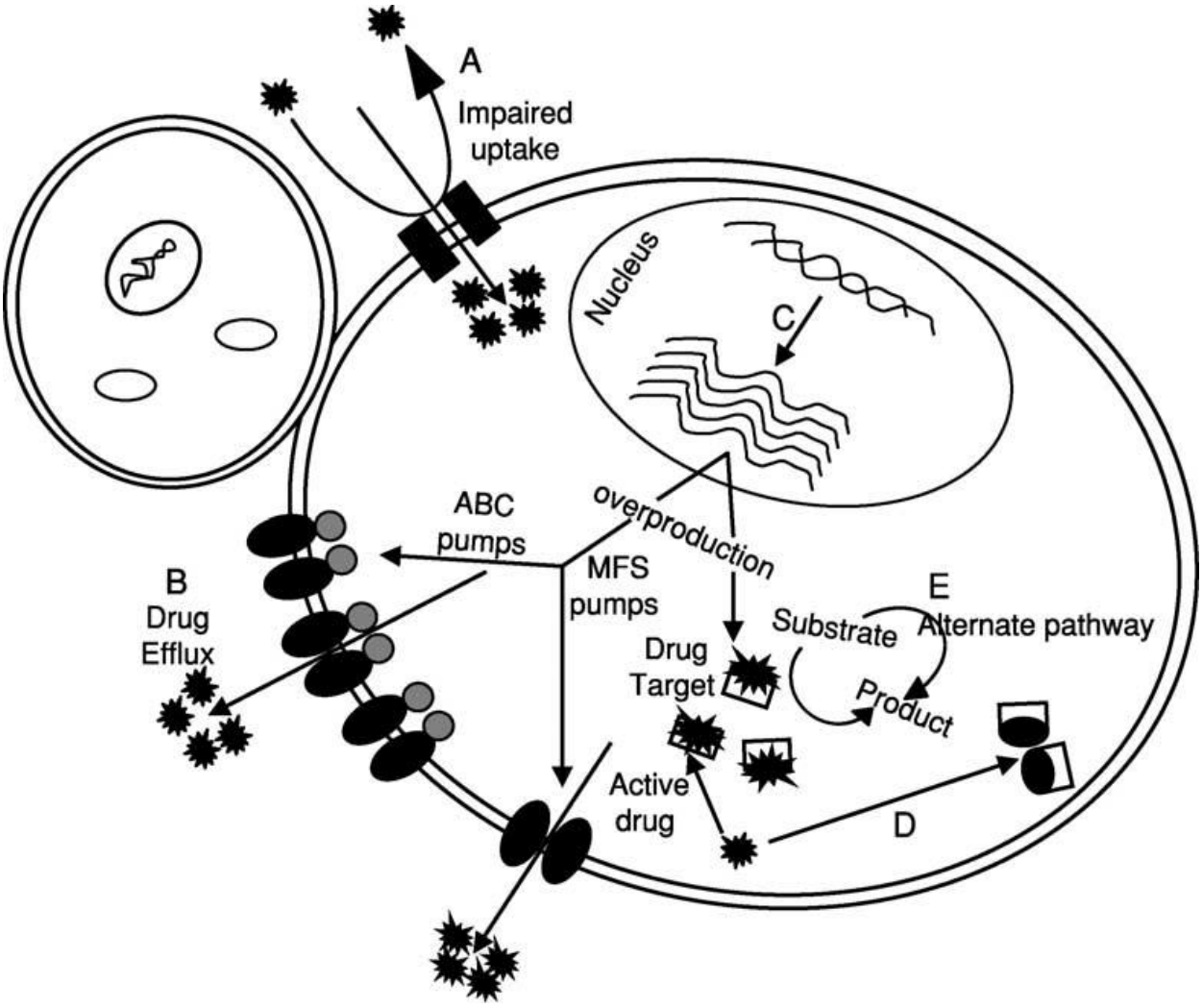

Figure 2. 1. Predominant drug resistance mechanisms of *Candida*. (A) Reduced drug uptake (import) due to compositional changes in the cell wall or plasma membrane. (B) Rapid efflux of drugs mediated by the ABC or MFS transporters. (C) Overexpression of the genes encoding drug target ERG11 and/or of efflux pumps, e.g., Cdr1, Cdr2, and CaMdr1. (D) Mutation in drug target Erg11p (P45014DM) leads to reduction or loss in drug binding. (E) Activation of alternate ergosterol pathways such as $\Delta^{5,6}$-Desaturase. (Source: Prasad and Kapoor, 2005)

Drug efflux mechanism.

The first mechanism associated with resistance of *Candida* species to azole antifungals is induction of efflux pumps that lead to decreased drug concentration at the enzyme target within the fungal cell. Upregulation of multidrug efflux transporter genes have been linked to this phenomenon, these transporters include the ABC proteins (ATP-binding cassette), namely, Cdr1 and Cdr2 (*Candida* Drug Resistance), and (MFS) major facilitator Superfamily, Mdr1. The ABC transporters are energy-dependent by ATP hydrolysis while the MFS transporters operate through a proton gradient. Azole-resistant isolates of *C. albicans* as well as other fungal pathogens like *Aspergillus fumigatus* and *Cryptococcus neoformans* display transcriptional activation of efflux pump encoding genes ABC (Cdr1p, Cdr2p) or

major facilitator Superfamily (CaMdr1p-*Candida albicans* Multidrug Resistance proteins). Cdr1p and Cdr2p are the two major drug extrusion pumps of *C. albicans*. These pump proteins not only efflux azoles and its derivatives but also transport a wide range of toxic drugs out of cells. Rapid efflux of incoming drug prevents cells from accumulating lethal concentration of azoles and enable the cells to survive (Casalinuovo et al., 2004; Sanglard and Odds, 2002).

Alterations in Erg11p.

Another common mechanism of resistance is the acquisition of point mutations in the gene (P45014DM) encoding for the target enzyme (ERG11p), resulting in an altered target with reduced affinity for binding to azoles leading to resistance (Lupetti et al., 2002).

$\Delta^{5,6}$-Desaturase (ERG3)

The enzyme, $\Delta^{5,6}$-Desaturase (ERG3) which is involved in ergosterol biosynthesis pathway, also contributes to azole's resistance. A defect in ERG3 leads to the accumulation of 14α-methylfecosterol instead of 14α - methylergosta-8,24-dien-3β,6α-diol. Accumulation of sufficient amounts of 14α-methylfecosterol compensates for ergosterol in the membranes and thus contributes to azole resistance in *C. albicans.* The decrease in ergosterol content due to a defect in $\Delta^{5,6}$-Desaturase in fluconazole-resistant clinical isolates of *C. albicans* also results in cross-resistance to amphotericin B (Prasad and Kapoor, 2005).

Membrane lipid composition

In addition to membrane ergosterol, which mainly provides rigidity, stability, and resistance to physical stresses in fungi, there are other membrane lipid components, which also affect drug susceptibilities of *Candida* cells. They exhibit altered membrane phospholipids as well as sterol composition (Sanglard, 2002).

Another potential mechanism of azole resistance in *Candida* species involves the development of bypass pathways, which negate the membrane disruptive effects of azole drugs that are associated with inhibited fungal growth. This has been linked with mutation of the *ERG3* gene in certain resistant strains of *Candida* (Pfaller, 2012).

Mechanisms of polyene resistance are less well studied. However, one explanation is the reduced ergosterol content in the fungal cell membrane. The ergosterol is replaced by other sterols that have reduced affinity for the polyene. Flucytosine resistance is most commonly due to mutational changes in cytosine deaminase or uracilphosphoribosyltransferase, which is involved in the pyrimidine salvage pathway. Although resistance to allylamine (terbinafine) appears to be rare in clinical yeast isolates, it has been shown that some azole resistant strains which over-express either Cdrl or Mdrl are cross-resistant to terbinafine (Rogers, 2002).

Echinocandin resistance has not been investigated in detail, because of insufficient clinical experience.

However, it has been reported that echinocandins inhibit 1,3-β-D-glucan synthase and thereby disrupt biosynthesis of 1,3- β -D-glucan, a key component of the fungal cell wall. This causes the formation of a defective cell wall associated with cellular instability and lysis in yeasts and aberrant hyphal growth in molds. Mutations in the gene encoding for elements of the 1,3-β-D-glucan synthase complex have been associated with *Candida* resistance to echinocandins (Pfaller, 2012). One of the strategy which is being investigated to overcome antifungal resistance is the development of new drugs with better antifungal activity from plant species used traditionally to treat fungal infections.

2.1.4. Treatment

Treatment strategies of fungal infections are often ineffective due to delays in treatment caused by lack of quick, reliable and robust diagnostic techniques, undesirable side effects, drug interactions or decreased drug absorption and an increasing incidence of antifungal resistance (Reedy et al., 2007). However, classes of antifungal agents available for systemic use against fungal pathogens, including *C. albicans,* a causative agent of candidiasis include: polyenes (amphotericin B and nystatin) which target ergosterol, a fungal cell membrane sterol to create membrane spanning channels or pores which in turn cause leakage of potassium and other cytoplasmic constituents resulting in the death of the cell, nystatin is restricted to topical administration because of its greater potential for toxicity while amphotericin B frequently causes nephrotoxicity, and this limits its role as an effective antifungal agent (Anderson, 2005).

Azoles (fluconazole, itraconazole, voriconazole, posaconazole) also target the plasma membrane, but mainly through the inhibition of ergosterol biosynthesis. The azoles inhibit cytochrome P-450-dependent 14-α demethylation of lanosterol, the precursor of ergosterol, a vital component of fungal cell membranes. The enzyme, 14α-sterol demethylase converts lanosterol to ergosterol removing the 14α-methyl group from lanosterol. The 14α-sterol demethylase is a cytochrome P450-dependent enzyme (P450-Erg11p or Cyp51p) which contains a heme moiety in its active site. The azoles bind to the heme iron through an unhindered nitrogen, thus inhibiting the enzymatic reaction (Rogers, 2002). The analysis of the safety profile of some azoles e.g. fluconazole shows adverse effects such as nausea, vomiting, abdominal pain and diarrhoea (Rodriguez and Patrick, 2001).

Echinocandins (caspofungin and micafungin) are lipopeptides that inhibit synthesis of β-1,3-glucan, an essential component of the cell wall of many fungi and the safety profile of echinocandins is excellent, with few reported adverse events such as abnormal liver function, phlebitis or histamine-like reactions (Mean et al., 2008), allylamines (terbinafine), inhibit ergosterol biosynthesis at the level of squalene epoxidase, resulting in ergosterol depletion and in the accumulation of toxic squalene.

This finally leads to inhibition of growth and cell death (Rodriguez and Patrick, 2001). Flucytosine, enters fungal cells through the action of a cytosine permease and is converted by cytosine deaminase into the active molecules that inhibit both DNA and RNA synthesis. It is active against both *Candida* spp and *Cryptococcus neoformans.* More recently, there has been interest in combining flucytosine with azoles to avoid nephrotoxicity. Flucytosine is rarely used alone because of the likelihood of development of resistance (Dan and Levitz, 2006). Fungal infections are difficult to prevent because there is a few varieties of vaccines available against them. Treatment is also problematic because compared to the antibacterials, the number of antifungal drugs available at present is very small, with much greater difficulty in production, with many side-effects, and with the appearance of resistance (Blanco and Garcia, 2008). Thus, screening of indigenous plants to find cheap alternative medicines with less side effects and little chance of eliciting resistance in the treatment of fungal infections is rational (Shai et al., 2008).

2.2. The human immune system in the fight against fungal infections

The integrated human immune system defends the body against microbes which cause infections. It consists of two branches, innate and adaptive immune systems which are the major defence mechanisms possessed by higher organisms. The innate immune system recognizes the presence of pathogens in the body and provides the first line of defense while adaptive immunity fight against any "foreign" substance that enters the body (Turvey and Broide, 2010). The immune response varies with respect to the fungal species encountered. The focus here, is on *C. albicans,* causative agent of candidiasis which is among the most common mycotic infections of immunocompromised patients. Human immunodeficiency virus (HIV) is one of the most important predisposing factors of candidiasis. Other risk factors include extreme old age, diabetes mellitus, nutritional deficiencies, excessive use of antibiotics, salivary abnormalities and destruction of mucosal barriers with radiotherapy or cytotoxic chemotherapy (Shoham and Levitz, 2005).

2.2.1. Innate immune system

The first innate defensive mechanism is the physical barriers that separate the organism from the environment: i.e. skin and the mucous membranes of the respiratory, gastrointestinal and genito-urinary tracts. The skin and mucous membranes are physical barriers, and they have antimicrobial substances on their surfaces, some of them synthesized by the epithelial and endothelial cells. They also have a commensal microflora of saprophytic microorganisms that impede colonization by pathogenic microorganisms (Blanco and Garcia, 2008).

Saliva secreted by salivary glands is also a highly important element of innate protection against the overgrowth of *C. albicans* in the oral cavity. Salivary flow provides an important physical force to flush microbes through swallowing, there by preventing pathogen adhesion to mucosal and dental surfaces. Saliva is also a major source of immunoglobulin A (IgA) antibodies, many of which recognize *C. albicans* and other potential pathogens. It is highly enriched with antimicrobial proteins such as lysozyme, lactoferrin, histatins, cathelicidins, calprotectins, beta-defensins and mucins which are key factors that help to control *C. albicans* growth and attachment to the oral epithelium. Healthy oral epithelial cells inhibit blastoconidia or hyphal growth of several *Candida* species (Fig 2.2). (Conti and Gaffen, 2010).

Walls of most pathogenic fungi consist of β-glucans which are probably important in stimulating the immune system to protect man from attack by pathogenic microbes and from harmful effects of environmental toxins and carcinogens. β-glucans activate phagocytic cells, macrophages, neutrophils, monocytes, dendritic cells, natural killer (NK) cells, and NK T cells to enhance the host's innate response to fungal infections. Additional effector cells, including neutrophils and monocytes, are recruited to sites of infection by the action of inflammatory signals, such as cytokines, chemokines and complement components. Yeast cells are killed or damaged by production or release of reactive oxygen intermediates and antimicrobial peptides. The latter, have a direct influence on the adaptive immune responses by activation of different immune factors such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1, and gamma-interferon (IFN-Ɣ) (Chen and Seviour, 2007).

To augment the above-mentioned cellular defences, innate immunity also has a humoral component that includes well-characterized components, such as complement proteins, LPS binding protein, C-reactive protein and other pentraxins, collectins, and also antimicrobial peptides. Circulating innate immune proteins are involved in both sensing of microbes and effector mechanisms to facilitate clearance of the infection. For example, mannose-binding lectin, a member of the collectin family of receptors, binds mannose-containing carbohydrates on microbes, triggering activation of the complement cascade, which enhances clearance of the pathogen (Turvey and Broide, 2010).

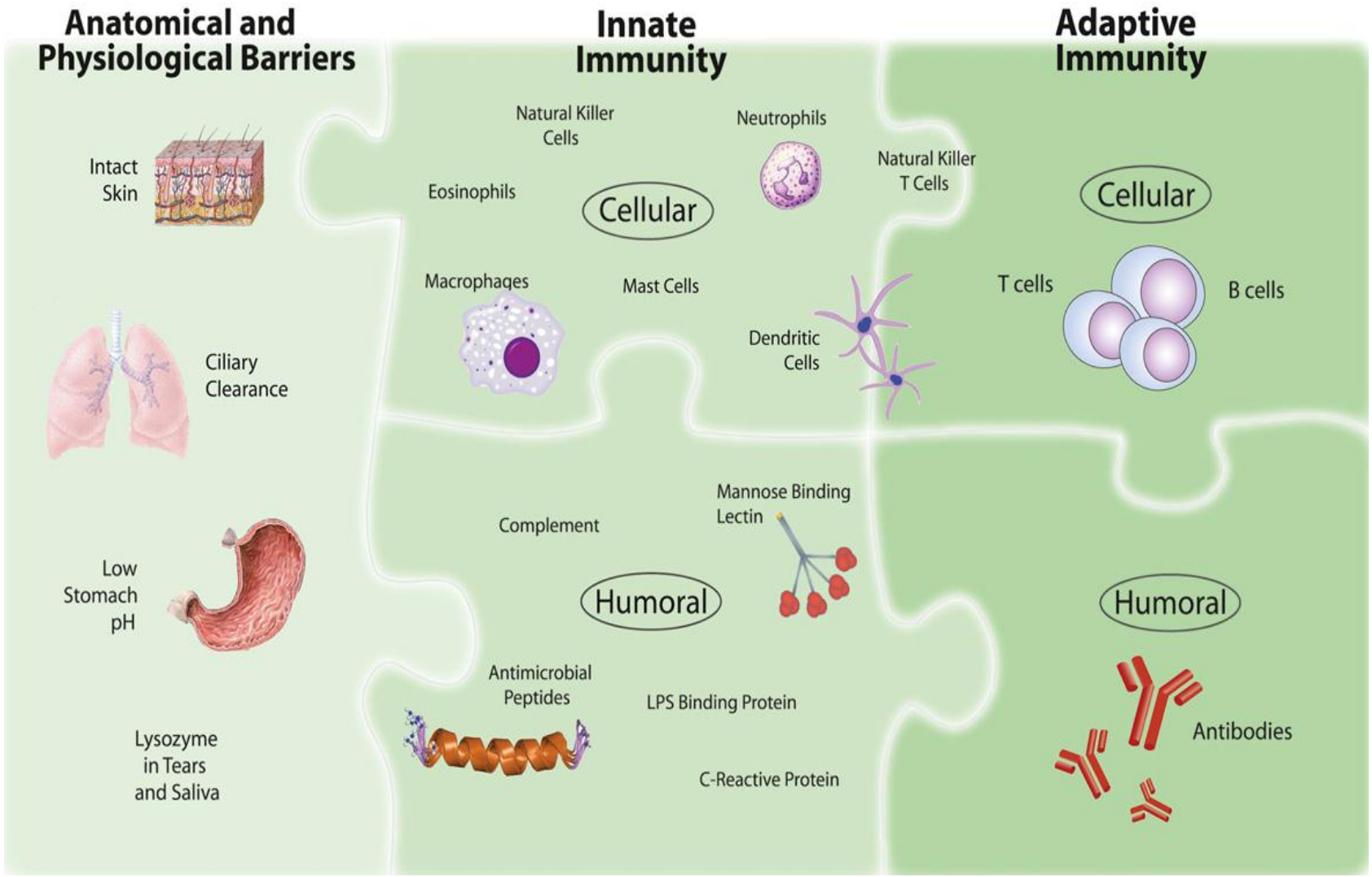

Figure 2.2..Human immune system. The human microbial defense system can be simplistically viewed as consisting of 3 levels: (1) anatomic and physiologic barriers; (2) innate immunity; and (3) adaptive immunity Source: (Turvey and Broide, 2010)

It has only recently been recognized that innate immune system actually guides and instructs how the adaptive immune system will respond to a particular type of microorganism. This is achieved by the innate immune system's ability to 'sample' microorganisms via pattern-recognition receptors (PRRs) on mammalian cells which recognize pathogen-associated molecular patterns (PAMPs). Different PRRs trigger distinct signaling pathways, leading to production of selected panels of cytokines and chemokines which then lead to activation of appropriate mechanisms of the adaptive immune response, ensuring that the strategy employed is the appropriate one for eradicating that particular microorganism. For example, the complete Toll-like receptors (TLRs) family allows the host to detect infection by most types of microbial pathogens (Lilic, 2009).

2.2.2. Adaptive immunity

The adaptive immune system responds to foreign antigens introduced in the body. It involves both B and T lymphocytes. The former produce antibodies to mediate humoral immunity whereas T cells induce cell-mediated immunity. Cytokines promote $CD4^+$ T cell differentiation to helper T cells 1 (Th1) and 2 (Th2), which mediate cell and humoral immunities respectively (Chen and Seviour, 2007). The adaptive immune response also involves dendritic cells, derived from monocytes, which play an important role in linking innate with adaptive immunity. Dendritic cells kill *C. albicans* yeasts and hyphae by a process that utilizes distinct receptors for each morphotypes. Dendritic cells that ingest the yeast, induce differentiation of $CD4^+$ T cells toward a Th1 pathway. Differentiation of $CD4^+$ T cells along a T-helper (Th) cell type 1 (Th1) or type 2 (Th2) pathway and the development of Th1 or Th2 responses is an important determinant of the host's ability to contain fungal infections. Development of Th1 responses is influenced by the intensive action of cytokines, such as interferon (INF)-ϒ, interleukin (IL)-6, tumour necrosis factor (TNF)-α, and IL-12, in the relative absence of Th2 cytokines, such as IL-4 and IL-10. The predominance of Th1 over Th2 type cytokines is associated with protection against invasive fungal infections (Fig.2.3) (Shoham and Levitz, 2005).

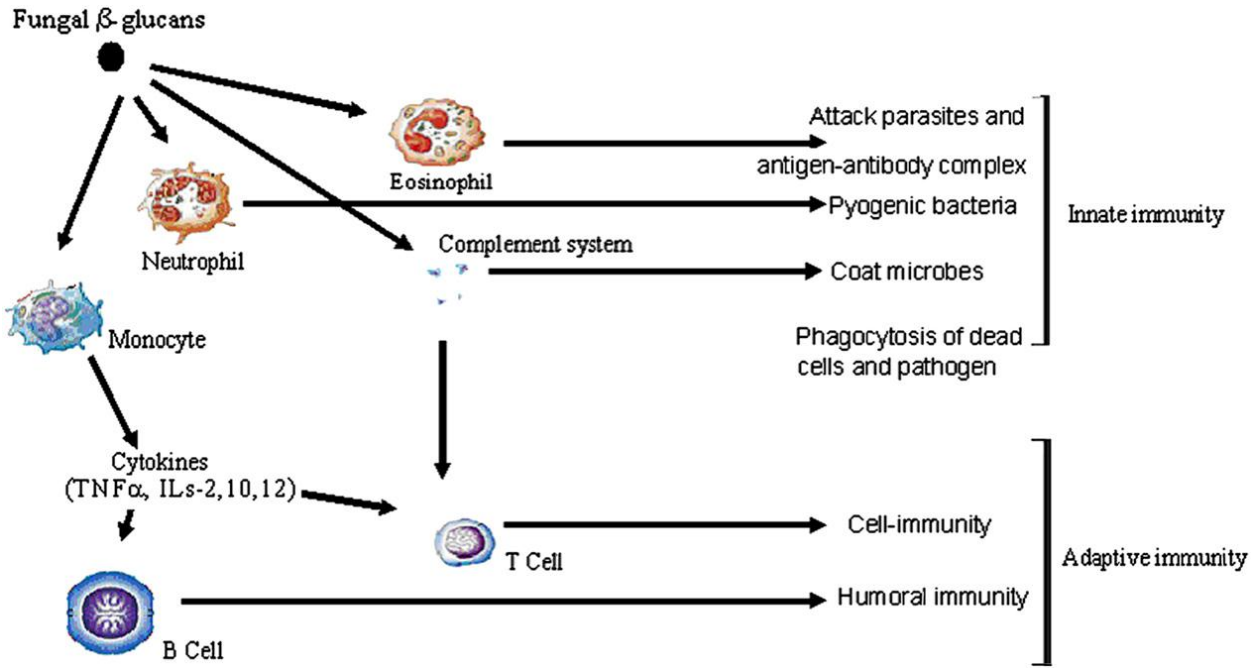

Figure 2.3. Immunostimulation by fungal β-glucans. Source: (Chen and Seviour, 2007**).**

2.3. Opportunistic organisms

2.3.1. Fungal pathogens

Fungi have emerged worldwide as an increasingly frequent cause of opportunistic infections (Mean et al., 2008). Opportunistic infections caused by fungal pathogens are defined as infections that occur primarily in immunodeficient individuals and rarely in immunocompetent individuals. They have emerged as important causes of morbidity and mortality in immunocompromised patients and remain a major challenge for infectious diseases clinicians (Canuto and Rodero, 2002). Among the myriad of opportunistic fungal pathogens, the three most important groups include *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (Pfaller et al., 2005). However, this study focuses on the first two pathogens.

Candida albicans is a commensal microbe that resides in the human oropharynx and gastrointestinal tract. It becomes an opportunistic pathogen of mucosal surfaces, with breakdown of the integument or when immune defence mechanism has been weakened. Candidiasis caused by *C. albicans* is the most prevalent opportunistic infections in individuals infected with HIV. The most characteristic manifestation of oral candidiasis is plaque-like lesions that can become confluent throughout the oral cavity (Karkowska-Kuleta et al., 2009).

Cryptococcus neoformans is ubiquitous facultative, encapsulated non-mycelial, budding yeast found particularly in soil contaminated by bird droppings and rarely causes disease in individuals with normal immunity. In the case of HIV infection, *C' neoformans* is an opportunistic pathogen that causes cryptococcosis in individuals with profoundly decreased immune function. It is acquired from the environment via the respiratory route. Symptoms are non-specific and include fever, cough, dyspnea, sputum production and pleuritic chest pain (Pirofski, 2001).

Aspergillus species are also ubiquitous molds, found in soil and water, grow as filamentous fungi. The *Aspergillus fumigatus* rarely cause disease in immunocompetent individuals, but they have emerged as major causes of morbidity and mortality in immunocompromised patients. Aspergillosis is a common cause of life-threatening opportunistic infection in neutopenic patients. Clinical symptoms include chronic cough, sputum production, fever, weight loss and weakness (Franquet et al., 2004). *Pneumocystis carinii*, a unicellular organism actually classified as a fungus, is a common cause of life-threatening opportunistic infection, pneumonia in patients with AIDS. Clinical symptoms of *P. carinii* pneumonia include non-productive cough, shortness of breath, and hypoxia on room air (Franquet et al., 2004).

2.3.2. Bacterial pathogens

Bacterial species, namely *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), E*scherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were also investigated in this study, they are the major cause of nosocomial infections in hospitals and these strains are recommended for use by NCCLS (National Committee for Clinical Laboratory Standards) (Masoko et al., 2008).

Pseudomonas aeruginosa

A Gram-negative, free-living bacterium, commonly found in soil and water. It is an aerobic bacterium, which is an important cause of infection in patients with compromised host defence mechanisms. It causes urinary tract infections, pneumonia, bacteremia, bone and joint infections, a variety of systemic infections, particularly in patients with severe burns, and in cancer and AIDS patients who are immunosuppressed. It is notorious for its resistance to antibiotics, only a few antibiotics are effective against it e.g. fluoroquinolones, gentamicin and imipenem, even these antibiotics are not effective against all strains (Habbal et al., 2011;Todar, 2011)

Staphylococcus aureus

They are spherical, Gram-positive bacteria, facultative anaerobic bacteria that are found on nasal passages, skin and mucous membranes and are able to invade the host via the broken skin or mucous membrane. It causes skin lesions such as boils, furuncules and more serious infections such as pneumonia, phlebitis, osteomyelitis, septicemia,

meningitis and urinary tract infections (Seanego and Ndip, 2012). Strains of *S. aureus* are usually resistant to a variety of antibiotics but some can still be treated with vancomycin or with combination therapy using sulfa drugs and minocycline or rifampin (Todar, 2011).

E*scherichia coli*

They are facultatively anaerobic Gram-negative rods that are the inhabitant of the human intestinal tract. Pathogenic strains of *E. coli* are responsible for three types of infections in humans: urinary tract infections, intestinal diseases and neonatal meningitis. It is responsible for different outbreaks of diseases associated with bad diarrhoea and an insufficiency of the kidney, which in some cases proved to be lethal (Hostettmann et al. 2000)

Enterococcus faecalis

It is a Gram positive, a commensal inhabiting the harsh environment of the human and animal intestines. It is naturally and easily adaptable, quickly finding the way to turn into a true opportunistic pathogen in nosocomial infections. At the present time, it is frequently responsible for urinary tract infections, endocarditis, wound infections, intra-abdominal abscesses and food borne diseases. Risk factor for enterococcal infection is a long-term hospitalization with broad-spectrum antibiotic therapy (Arciola et al., 2007).

2.3.3. Parasitic pathogens

Human parasitic infections are a serious problem in tropical and subtropical developing countries, despite the discovery of new antiprotozoal and antibiotic medicines (Mesia et al., 2008). Free-living protozoans: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploid* have cosmopolitan distribution in soil and water, providing multiple opportunities for contacts with humans and animals, and they cause opportunistic and non-opportunistic infections in both humans and animals. The major factor in the wide distribution of these amoebae in water and soil is the presence of bacterial food supply except for *Balamuthia* which feeds on other amoebae (Schuster and Visvesvara, 2004a). Infections caused by the pathogenic and opportunistic free-living amoebas such as *Acanthamoeba, Balamuthi*a, and *Naegleria* have been recorded from all parts of the world. Because these infections occur most often in immunocompromised persons, greater numbers of cases would be expected in sub-Saharan Africa, South Asia, and other areas where the HIV epidemic is burgeoning (Visvesvara and Maguire, 2006). These amoebae are unusual pathogens, they have the ability to exist normally as free-living organisms, yet they are able to survive within the tissues of humans and other animals and live as parasites, because of this unique feature they are called amphizoic amoebae. Diseases they cause have received little attention even in developed countries because of lack of familiarity with them, resulting in a high mortality rate in developing countries (Visvesvara and Schuster, 2008a).

Several species of *Acanthamoeba* spp *(A. castellanii, A. culbertsoni, A. healyi, A.polyphaga, A. rhysodes*) cause an insidious and chronic disease, granulomatous amoebic encephalitis (GAE) as well as disseminated infections in lungs, kidneys, adrenals, cutaneous and nasopharyngeal infections, primarily in immunocompromised individuals due to HIV/AIDS, organ-transplant patients and persons in a debilitated state of health. *Balamuthia mandrillar* a close relative of *Acanthamoeba* also causes granulomatous amoebic encephalitis, cutaneous, nasopharyngeal, and central nervous system infections in immunocompromised or debilitated individuals (Visvesvara, 2010). Portals of entry by amoebae into the human body may either be through breaks in the skin in contact with contaminated soil or water or by wind-blowing cysts that might lodge in the nasal mucosa. Once in the body, amoebae can spread haematogenous to the central nervous system (CNS) and various organs (Schuster and Visvesvara, 2004b).

Naegleria fowleri causes primary amoebic meningoencephaltis in immunocompetent children and young adults. The infection is acquired by swimming or bathing in warm fresh waters, such as lakes, ponds, hot springs, and thermally polluted rivers or streams (Visvesvara and Schuster, 2008b). Multiple drugs, particularly those active against fungi, are used in treatment so that there may be synergistic effects, these include 5-fluorcytosine (flucytosine), fluconazole, pentamidine isethionate, a macrolide antibiotic (azithromycin or clarithromycin), sulfadiazine, and a phenothiazine compound (trifluorperazine or thiroridizine) (Schuster and Visvesvara, 2004a).

2.3.4. Viral organisms

Viruses are made up of infectious nucleic acids enclosed in a protein cover. They have membranes but they do not have cytoplasm or their own metabolism. Therefore, they penetrate the host cells to replicate their compounds and to form new viruses. Those that present the most problems for public health include HIV (Human Immunodeficiency Virus), Herpes simplex virus, hepatitis B virus, influenza virus, and avian influenza (Nester et al., 2012)

HIV (Human Immunodeficiency Virus)

It is currently the most significant infectious pathogen with devastating consequences. Since the description of HIV as the causative agent of Acquired Immune Deficiency Syndrome (AIDS), HIV has produced a worldwide pandemic (Bessong et al., 2006). Two distinct retroviruses, HIV-1 and HIV-2, cause HIV infection in humans. HIV-2 is less aggressive virus than HIV-1, both in terms of transmission and pathogenesis, geographically limited to West Africa (Balint, 2001). The vast majority of the HIV-1 strains responsible for the global AIDS pandemic belong to group M, which has evolved in humans to form at least 10 genetic subtypes, designated by letters from A to K.

HIV-1 subtype B predominates in industrialized countries as well as in Latin America and the Caribbean. Subtypes A and D are more common in Central Africa. Subtype C accounts for the majority of infections in India, eastern Africa and southern Africa. The latter remains the hardest-hit region in the world, with at least 25 million infected people, accounting for 60% of the people living with HIV/AIDS in the world. Mode of transmission is through sexual contact, injection of infected blood or blood-derived products, and from mother-to-child. Basically, HIV/AIDS is characterized by the progressive depletion of $CD4^+$ helper T-cells which are responsible for coordinating the immune response against the hostile world of antigens, pathogens and cancerous cells, resulting in an immunodeficiency syndrome that paves the way to opportunistic infections which are either of viral, bacterial, parasitic or fungal origin. However, the introduction of the Highly Active Antiretroviral Treatment (HAART) has transformed HIV/AIDS infection from a lethal disease to an effectively manageable chronic disease (Girard et al., 2011; Girard et al., 2006, Manavi et al., 2006).

Herpes simplex virus (HSV)

Herpes simplex virus (HSV) has two serotypes, Herpes simplex virus type 1 (HSV 1 and Herpes simplex virus type 2 (HSV-2), is one of the most common human pathogens, infecting 40–80% of people worldwide. HSV most commonly causes mucocutaneous infections, resulting in recurrent orolabial or genital lesions. HSV-1 is responsible mainly for oral infections and HSV-2 for genital infections but either virus can infect oral or genital sites (Mirowski et al., 1997). Primary infections of HSV occur in regions of the respiratory/oropharyngeal or genital mucosa, which are then further transmitted to peripheral sensory neurons where latent infections are established for the lifetime of the host. Reactivation leads to recurrent lesions in the vicinity of the primary infected area. HSV infection can also cause ocular herpes or encephalitis in adults while in neonates it can cause encephalitis and severe disseminated infection with neurological impairment and high mortality (Kelly et al., 2009)

Hepatitis B virus.

It is responsible for Hepatitis B. This virus can be acquired by contact with an infected person, by ingestion or through wounds. It can also pass from a mother's infected blood to her foetus through the placenta. Hepatitis B infection is the main cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Around 2 billion persons are infected by this virus (HBV) in the world and 350–400 millions of them are chronic carriers. This shows that Hepatitis B is still a significant health concern. Symptoms include fever, fatigue, jaundice and destruction of liver cells (Devesa and Pujol, 2007).

Influenza virus

It is a respiratory tract infection that is transmitted from one person to another through the inhalation of contaminated drops that are present in the air when somebody sneezes or coughs. It is an RNA virus with three known subtypes (A, B and C) and clinically it causes fever, chills, malaise, headache, muscle pain and non-productive cough that last for 3 or 4 days. There may be complications of different types, including particularly pneumonias and secondary bacterial infections, fundamentally in individuals with chronic respiratory disease (Diaz et al., 2011).

Avian influenza

Highly pathogenic avian influenza A (H5N1) virus is very contagious among birds and has been reported to be responsible for many human deaths since 1997. The primary target for H5N1 virus in humans is the alveolar epithelial cell in the lungs. Most patients present severe pneumonia, with rapid progression to acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury. The virus may also cause disseminated infection beyond respiratory tract. To date no effective regimen is available (Ma et al., 2011).

2.4. Plants as potential therapeutic agents

Plants have been used for centuries to treat infectious diseases and present an obvious source of new antimicrobial compounds (Eloff et al., 2005). Plants are complex chemical storehouses of undiscovered biodynamic compounds with unrealized potential for use in modern medicine. It has long been established that naturally occurring substances in plants have anti-bacterial and anti-fungal activities (Rangasamy et al, 2007).

2.4.1. Plants as antifungal and antibacterial agents

The increasing incidence of opportunistic infections associated with HIV/AIDS and the development of resistance by fungal and bacterial pathogens gives a fresh impetus to the search for novel antimicrobial agents from plants. Svetaz et al. (2010) state that plants provide unlimited opportunities for the isolation of new antimicrobial compounds because of the unmatched availability of chemical diversity. African plants are good candidates in the search for antimicrobial compounds because they have to resist difficult conditions and attack by all manners of parasites (Hostettmann et al., 2000). In our laboratory several plants presenting antifungal and antibacterial activities have been studied and several compounds with interesting antimicrobial properties have been isolated. Triterpenoids (betulinic acid, ursolic acid and 2α-hydroxyursolic acid) isolated from *Curtisia dentate* leaves have antifungal and antibacterial properties with MIC values ranging from 8 to 63 µg/mL (Shai et al., 2008),. *Markhamia obtusifolia* (Nchu

et al., 2010) yielded anti-*Candida albicans* compounds 3β-hydroxyurs-12-en-28-oic acid, ursolic acid,3β, 19α-dihydroxyurs-12-en-28-oic acid, pomolic acid and 2β, 3β, 19α -trihydroxy-urs-12-en-28-oic acid, 2-epi-tormentic acid; The triterpenes, asiatic acid and arjunolic acid isolated from *Combretum nelsonii* leaf extracts have antifungal activity against fungal animal pathogens (Masoko et al., 2008). *Combretum Paniculatum* yielded cholest-5-en-3-ol, 2-phyten-1-ol, isoquercitrin, *p*-coumaric acid, 2,3,8-tri-*O*-methylellagic acid, beta-sitosterol, gallocatechin, apigenin and apigenin-7-glucoside with good activity against bacteria as well as fungi (Eloff et al., 2008). The bioflavonoids, amentoflavone and 4"-methoxy amentoflavone isolated from leaf extract of *Garcinia livingstonei* have excellent activity with MICs of 6 and 8 µg/ml against *Enterococcus faecalis* and *Escherichia coli* respectively (Kaikabo et al., 2009). From *Dodonaea viscosa* var. *angustifolia* 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone and 3,4',5,7-tetrahydroxy flavone (kaempferol) had antibacterial properties (Teffo et al., 2010). A novel compound, oleanene-type triterpenoid glycoside, 1α, 23β-dihydroxy-12-oleanen-29-oic-acid-23β-O-α-4-acetylrhamnopyranoside isolated from *Combretum padoides* has antibacterial activity (Angeh et al., 2007). A stilbene, 2',3',4-trihydroxyl-3,5,4'-trimethoxybibenzyl is the main antibacterial compound isolated from the leaves of *Combretum woodii* has excellent antibacterial and antioxidant activity (Eloff et al., 2005). Martin et al. (2004) isolated several antibacterial flavonoids (apigenin; genkwanin; 5-hydroxy-7,4'-dimethoxyflavone, rhamnocitrin; kaempferol; quercetin-5,3'-dimethylether; rhamnazin) from *Combretum erythrophyllum.* In many cases the bioactive compound had much lower activity than could be expected from the activity of the crude extract even if there was only one main bioactive compound present in the crude extract and there was no possibility of isolating an artefact (Eloff et al. 2005).

2.4.2. Plants as antioxidants

Plants offer a wide range of natural antioxidants due to the structural diversities of their secondary metabolites (Teffo et al., 2010). This is not surprising because plants have some mechanism of protection against light energy being transformed to chemical energy by reduction of compounds yielding potentially damaging free radicals in the process. Various investigations conducted in our laboratory have shown that many compounds isolated from plant extracts possess antioxidant properties. For instance quercetrin and kaempferol isolated from *Combretum apiculatum* subsp. *apiculatum* has strong antioxidant activity with EC_{50} values of 11.81±0.85 and 47.36±0.03 µM (Eloff et al., 2008); from *Croton zambesicus* quercetin-3-*O*-β-6"(*p*-coumaroyl) glucopyranoside-3'-methyl ether, helichrysoside-3'-methyl ether, kaempferol-3-*O*-β-6"(*p*-coumaroyl) glucopyranoside, tiliroside and apigenin-6-C-glucoside, isovitexin had antioxidant properties (Aderogba et al., 2011); flavonoid glycosides, quercetin-3-O-galactopyranoside, myricetin-3-O-galactopyranoside and 2"-O-rhamnosylvitexin isolated from the leaves of *Bauhinia galpinii* had antioxidant properties (Aderogba et al.,2007); from *Dodonaea viscosa* Jacq. var. *angustifolia* 3, 5, 7-trihydroxy-4'-methoxyflavone and 3,4',5,7-tetrahydroxy flavone (kaempferol) had antioxidant properties (Teffo et al., 2010).

2.4.3. Plants as antivirals

Viral infections remain a major threat to humans and animals and there is a crucial need for new antiviral agents especially with the development of resistant viruses (Bagla et al., 2011). South Africa has a rich plant biodiversity and a long tradition of medicinal use of plants with approximately 3000 species of plants used as medicines and several of these plants may contain novel anti-HIV compounds (Klos et al., 2009). For example, gallotannin isolated from the stem-bark of *Peltophorum africanum* has strong activity against HIV-1 reverse transcriptase and integrase in an enzyme cell-free system (Bessong et al., 2005); galangin (3,5,7-trihydroxyflavone) isolated from the shoots of *Helichrysum aureonitens* possess a significant antiviral activity (Meyer et al., 1997); luteolin isolated from *Senna petersiana* also has antiviral activity (Tshikalange et al., 2005); *Podocarpus henkelii, Plumbago zeylanica and Carissa edulis,* plant species used traditionally to treat infectious diseases have potent antiviral properties (Bagla et al., 2011); extracts of *Terminalia sericea, Elaeodendron transvaalense* and *Zanthoxylum davyi,* plant species used traditionally to treat sexually transmitted diseases have also been shown to possess anti-HIV-1 properties (Tshikalange et al., 2008). These few examples indicate that plants can be excellent potential source of antioxidants, antiviral, antibacterial and antifungal agents. Runyoro et al. (2006) stressed that despite the availability of different approaches for discovery of medicines, plants still remain as one of the best reservoirs of new structural types.

2.4. Botanical description of plant species used in the study

Fourteen plant species were selected on the basis of their use in traditional medicine to treat candidiasis and for evaluation of the activity against *Candida albicans*, *Cryptococcus neoformans* and four nosocomial bacteria, namely *Staphylococcus aureus*, *Enterococcus faecalis*, E*scherichia coli* and *Pseudomonas aeruginosa.* The selected plant species were mainly trees i.e. *Acacia caffra* Thunb., *Clerodendrum glabrum* E. Mey var. *glabrum*, *Croton gratissimus* Burch var. *gratissimus, Elaeodendron transvaalense* (Burtt Davy) R.H. Archer, *Faurea saligna* Harv*., Hippocratea longipetiolata* Oliv., *Osyris lanceolata* Hochst. & Steud, *Richardia brasiliensis* Gomes, *Schkuhria pinnata* (Lam.) ex Thell, *Schotia brachypetala* Sond*, Spilanthes acmella* (L.) Murray, *Strychnos potatorum* L.f., *Vangueria infausta* Burch*. subsp. infausta* and *Withania somnifera* (L.) Dunal*.* A short description of each of these is presented next. A more exhaustive list of their traditional uses is presented in the next chapter (Table 3.2).

Acacia caffra (**common hook-thorn**), **Mimosaceae**

It is a shrub to medium-sized deciduous tree with a rounded crown and drooping foliage; occurring in bushveld, grassland and coastal scrub, often on rocky ridges. Thorns in pairs below the nodes, hooked, rather sparse. Leaves with 8-35 pairs of pinnae, fresh green and feathery; leaflets 13-50 pairs per pinna; petiolar gland usually present; glands on rachis variable. Flowers in elongated spikes, yellowish white. Pods flat, pale brown to reddish brown, straight dehiscent. In the Eastern Cape the wood is used for making tobacco pipes; hard wood makes good fence

posts; bark is used for tanning leather; long thin branches are pliable and are used for making baskets. Leaves and pods are browsed by stock. Various parts are used for medicinal purposes (Venter, 2007).

Figure 2.4. Leaves and flowers of *A. caffra* (Picture taken at Tshipise, October 2009).

Clerodedrum glabrum (**Tinderwood**), **Verbenaceae**

It is a shrub or small to medium-sized deciduous tree, crown often drooping; occurring in bushveld and along forest margins. Branchlets with small raised whitish dots. Leaves 3-6 whorled or opposite, often drooping, usually thin and soft, variable in shape and size, usually covered with short soft hairs below, with a pungently fouls smell when crushed. Flowers in dense, rounded, terminal heads, white to pinkish, foul or sweet scented. Fruit fleshy, round, yellowish white, surrounded by the weathered persistent cup like calyx. Poles made from the tree are used in the construction of huts and fish kraals. Wood white to pale brown, hard and used for utility carving and small pieces of furniture. Was once used as tinder-wood to start fires, hence the common name (van Wyk and van Wyk, 1997).

Figure 2.5. *C. glabrum* growing in its natural habitat (Picture taken at Tshipise, October 2009).

Croton gratissimus **Burch. var. *gratissimus* (lavender fever-berry), Euphorbiaceae**

It is a shrub or small tree of up to 10 m in height, with a rough, grey bark; occurring naturally over a large area in the north of South Africa, usually in bushveld and in wooded places in grassland, often on rocky ridges; leaves are dark green above but have a characteristic silver white lower surface, dotted with brown glands. The flowers are small, cream to yellow and inconspicuous; small yellowish brown fruit, three lobed capsules, dehiscent are produced. The plant is browsed by game and stock. Leaves aromatic, used by San people in dried and powdered form as a perfume. Leaves and bark are used medicinally (van Wyk et al., 2009).

Figure 2.6. *C. gratissimus* growing in its natural habitat (Picture taken at Nzhelele, September 2009)

***Richardia brasiliensis,* Rubiaceae**

Short lived perennial herb with trailing stems; covered with bristly hairs throughout. Leaves narrow to broad, egg shaped, inflorescence comprising stalkless flowers clustered at the ends of branches, surrounded by broad leaf like bracts. A weed from South America, naturalized in disturbed places (van Wyk and Malan, 1988).

Figure 2.7. *R. brasiliensis* growing in its natural habitat (Picture taken at Gundani, September 2009)

***Elaeodendron transvaalense* (Burtt Davy) R.H. Archer (Transvaal saffronwood), Celastraceae**

It is a shrub or small to medium-sized tree, multi-branched, with rigid arching stems. It is widely distributed in the north-eastern parts of South Africa (Mpumalanga, Gauteng and Limpopo province). The bark is generally smooth and has a characteristic pale, grey colour. Tufts of leaves are crowded on the ends of rigid side shoots. The leaves are oblong in shape, about 50 mm long and 20 mm wide, with firm texture and conspicuous venation on the upper and lower surfaces. The leaf margin is sometimes toothed. Small and inconspicuous greenish flowers are produced in summer, followed by oblong, yellow to dark orange, berry-like fruits which are edible. Wood pale reddish brown, hard, fine textured, used for carving household utensils (van Wyk et al., 2009).

Figure 2.8. Leaves and fruits of *E. transvaalense* (Picture taken at Tshaulu, September 2009)

***Faurea saligna* (Transvaal beech), Proteaceae**

It is a small to medium-sized semi-deciduous tree with an erect trunk and narrow crown with drooping foliage. It occurs in bushveld, often on sandy soils and on stony hillsides, sometimes along river banks. Colour of the bark vary from dark grey-brown to almost black and is deeply fissured longitudinally. Leaves are long, narrow elliptic, slightly sickle-shaped, drooping, almost hairless; margin is wavy; petiole is up to 20 mm long and is red in colour. Flowers are found in pendulous spikes up to 150 mm long, they are sessile, colour vary from greenish to cream white. Fruit is a small nutlet, with long hairs and has a slender persistent style. The wood is reddish brown, attractively figured and is suitable as a general purpose timber; makes beautiful furniture. The bark is used for turning leather (Palgrave, 2002).

Figure 2.9. Leaves and flowers of *Faurea saligna* (Picture taken at Thengwe, October 2009).

***Osyris lanceolata* Hochst. & Steud. (Transvaal sumach), Santalaceae**

It is an evergreen shrub or small slender tree with sparse blue-green foliage; widely distributed in bushveld and wooded grassland, often on rocky hill slopes. Bark grey, smooth leaves oval, up to 35 x 17-27 mm thick and leathery, blue green with a grey bloom, hairless, petiole about 2 mm long. Flowers in axillary clusters, small and yellowish green. Fruit a drupe, oval, crowned with the persistent calyx, ripening bright red to purplish black (van Wyk and Gericke, 2007).

Figure 2.10. *Osyris lanceolata* growing in its natural habitat (Picture taken at Hamashau, October 2009).

***Spilanthes acmella* (L.) Murray (toothache plant), Asteraceae**

A tender annual plant which grows up to 40 cm, with soft branching stems; leaves opposite, ovate-lanceolate, with wavy margins; flowers bright yellow in terminal heads. Flower process a tingling sensation while chewing. Seeds many, small compressed and brown coloured. The plant has been used as a local anaesthetic for gums and teeth. It has also many other properties as anti septic (van Wyk and Malan, 1988).

Figure 2.11. *Spilanthes acmella* growing in its natural habitat (Picture taken at Gundani, September 2009)

***Schotia brachypetala* (weeping boer bean), Fabaceae**

It is a medium-sized to large deciduous tree of up to 16 m in height, with a wide spreading crown and a rough brown bark. The leaves are divided into four to six pairs of small, glossy green leaflets, each about 30 mm long, with a distinctly asymmetric base. Large clusters of dark red flowers are borne on the old wood of the tree. They produce

copious amounts of nectar, which drips from the flowers, attracting several species of birds. The fruits are large woody pods with a characteristic persistent rim, containing large, pale brown seeds. Bark is used medicinally and for tanning. It is endemic to the north-eastern parts of southern Africa (Venter and Venter, 2007).

Figure 2.12. *Schotia brachypetala* growing in its natural habitat (Picture taken at Tshaulu, September 2009)

Strychnos potatorum (**black bitterberry**), **Loganiceae**

It is a small to medium-sized deciduous tree and the crown densely foliated. It occurs in open woodland, along dry water courses often on termites wounds and in riverine fringes. Branches are often dichotomously branched, with a ring-like scar left by the first pair of deciduous, reduced leaves on the new season's growth. Leaves are elliptic to ovate, thinly textured, glossy dark green above, paler green below, hairless, net-venation is distinct, apex narrowly tapering; base rounded; petiole is up to 7 mm long. Flowers are whitish to yellow green, in stalked heads near the base of the branchlets in axils of reduced leaves. Fruit is small, almost round, rind is soft, blue black when mature and has pale brown, single seed. Bark and roots are toxic and are used as a fish poison. The seeds are used to purify drinking water (Palgrave, 2002).

Figure 2.13. Leaves and fruits of *Strychnos potatorum* (Picture taken at Hamashau, September 2009)

Vanguria infausta **subsp.** ***Infausta*** **(Wild medlar), Rubiaceae**

It is a deciduous tree of up to 8 m tall; widely distributed in all types of woodland, especially on rocky ridges and hill slopes, stretching from Malawi in the north to the Eastern Cape in the south. Its wood is taboo for making fire. Leaf blade soft, covered with dense hairs, tips bluntly pointed with net-veining conspicuous on under surface and veins on upper side sunken, margin smooth. Flowers in clusters in axils of leaves, greenish white to cream coloured, with hairs inside the lower tube. Fruit roundish and up to 40 mm in diameter, with a glossy brown leathery skin (Venter and Venter, 2007).

Figure 2.14. Leaves and fruits of *Vanguria infausta* (Picture taken at Hamashau, September 2009)

***Withania somnifera* (L.) Dunal** (**Winter cherry**), **Solanaceae**

It is an erect, perennial shrublet with densely velvety stems and leaves. It is widely distributed and is indigenous to South Africa and has become a weed of disturbed places. The leaves are oblong, pale green and covered with short, dense hairs when young. Small white or yellowish flowers are produced in short clusters, followed by small round, orange-red berries of about 8 mm in diameter. The berries are completely enclosed in brown papery and bladder structures (van Wyk et al., 2009).

Figure 2.15. *Withania somnifera* growing in its natural habitat (Picture taken at Nzhelele, September 2009)

In conclusion, the work presented here has demonstrated that opportunistic infections that arise due to HIV/AIDS infection pose a serious threat to human health. The difficulties associated with the management of these opportunistic infections necessitated the search of new, safe and effective antifungal agents from indigenous plant species used in the traditional medicine in order to widen the spectrum of activity against *C. albicans* and to try to fight the resistant strains. Some workers elsewhere have reported that plants used in traditional medicine usually constitute an important source of new biologically active compounds (Portillo et al. 2001, Masoko et al., 2005). In addition, Webster et al., (2008) reports that the search for novel, antifungal agents relies in great part on ethnobotanical information and ethnopharmacologic exploration. Hence, an ethnobotanical survey was conducted to document plant species used by local traditional healers to treat candidiasis and related infections.

Chapter 3.

Ethnobotanical use of plants to manage candidiasis and related infections in Venda, South Africa.

Ndivhaleni A. Masevhe, Lyndy J. McGaw, Jacobus N. Eloff

Preface

An ethnobotanical survey was conducted in order to determine which plant species are used by Venda traditional healers to treat candidiasis and related infections. The following chapter describes which plants are used, what plant parts are used, how it is prepared as well as the route of administration and dosage.

Abstract

Aim of study: This paper presents findings of an ethnobotanical survey of medicinal plants used for the management of candidiasis and related fungal infections in the Venda area, South Africa.

Materials and Methods: Ethnobotanical data about the uses of plants was gathered from eleven rural traditional healers using semi-structured interviews.

Results: A total of 45 species belonging to 24 different families were identified, of which the dominant family was the Fabaceae with 13 species (28.9%) followed by the Asteraceae and Solanaceae with 3 plants each (6.7 %). A total of 27 of these plant species (60%) have either been scientifically evaluated for anticandidal activity or have been recorded for antifungal uses in the literature. Amongst the 45 species recorded, 51% were trees, 33% were shrubs, and 15.5% were herbs. The most widely used plant species included *Acacia caffra*, *Clerodendrum glabrum*, *Croton gratissimus, Elaeodendron transvaalense*, *Faurea saligna, Hippocratea longipetiolata*, *Osyris lanceolata*, *Richardia brasiliensis*, *Schkuhria pinnata*, *Schotia brachypetala, Spilanthes acmella*, *Strychnos potatorum*, *Vangueria infausta subsp. infausta* and *Withania somnifera.* The plant parts used in the therapeutic preparations were roots (26.7%), bark (22.2%), and a combination of roots and bark (17.7%). Decoctions (44.4%), infusions (20%) and macerations (17.7%) were used. Most of the herbal remedies were administered orally.

The main factors threatening the conservation status of these plants are unsustainable methods of harvesting, logging for firewood, building materials and crafts.

Conclusion: The Venda area is rich in plant diversity and local indigenous knowledge of medicinal plants can play an important role as a model for low cost primary health care. Further studies are in progress to validate the indigenous plants recorded as traditional remedies in this area.

Keywords: ethnobotanical survey, candidiasis, decoction, Venda, traditional healers

3. 1. Introduction

Since ancient times, plants have been used all over the world as unique sources of medicines and constitute the most common human use of biodiversity (Ribeiro *et al.,* 2010). In Africa and in many developing countries a large number of people depend on medicinal plants because they have no access to modern medicines (Runyoro et al., 2006). The dependence on medicinal plants and traditional healers may be attributed to the low proportion of medical doctors to patients in Africa (South Africa 1:1 639; Ethiopia 1:33 000; Kenya 1:7 142; Tanzania 1:33 000; Uganda 1:25 000, Malawi 1:50 000; Mozambique 1:50 000; Swaziland 1:10 000) (Bekalo et al., 2009). In these communities, traditional healers operate closer to the people, taking advantage of the biodiversity of plant species in such areas to treat various diseases and ailments (Kambizi and Afolayan, 2001).

Herbal medicines have been used to treat many diseases that are obstinate and incurable in other systems of medicine and they are gaining popularity because of several perceived advantages such as fewer side effects, better patient tolerance, relatively lower expense and more ready acceptance due to a long history of use (Vermani and Garg, 2002). However, indigenous knowledge on medicinal plants is being lost at a rapid rate with the increase of modern education, which has led the younger generation to underestimate its traditional values (Zerabruk and Yirga, 2012). This useful information about medicinal plants is also still passed on from one generation to another by oral communication, posing the danger of loss of valuable knowledge (Maregesi et al., 2007). In a study on the scientific validation of traditional medicinal plants, it was reported that there is still a lack of detailed documentation on the use of medicinal plants in South Africa (Taylor et al. 2001). It is becoming increasingly urgent to document the medicinal use of African plants because of the rapid loss of the natural habitat for some of these plants due to anthropogenic activities (Bisi-Johnson et al., 2010). Thus, there is a necessity to conduct research relating to the documentation of useful medicinal plants in southern Africa before this important knowledge vanishes.

Ethnobotanical studies are important in disclosing locally important plants used by communities in the management of a range of ailments affecting them. Previous ethnobotanical studies conducted in the Limpopo Province, South Africa, to document medicinal plants included those on diabetes mellitus (Semenya et al., 2012), the antifungal activity of plants used to treat fungal related ailments (Samie et al., 2010), diarrhoea (Mathabe et al., 2006), sexually transmitted diseases (Tshikalange et al., 2005) and plants used in traditional medicine in general (Arnold and Gulumiam, 1984). As far as our literature search could ascertain, this is one of the few studies on the medicinal plants used to treat candidiasis in Venda. Thus, the present study reports on information gathered from local traditional healers concerning plants used for the treatment of candidiasis and related infections. With the high incidence of HIV/AIDS, about 5.6 million infected people in South Africa (UNAIDS, 2011), candidiasis is a serious challenge to the public health system.

Opportunistic infections such as those caused by *Candida albicans*, the causal organism of candidiasis, are a significant cause of morbidity and mortality in AIDS patients (Wiliams and Lewis, 2011). Candidiasis is diagnosed in 9-90% of patients depending on the stage of HIV/AIDS disease (Reichart, 2003). The major concern with candidiasis is that it is associated with a mortality rate of 10–49% in immune compromised patients (Pfaller et al., 2006).

3. 2. Study site

The study took place in Venda, Limpopo Province, South Africa. In this area (Fig 3.1) traditional medicine still plays a significant role in the lives of local people, despite recent advances in Western medicine (Meyer et al., 2008). The region lies in the north-eastern corner of the Soutpansberg and is located between latitudes 22° 15" and 23° 45" S. and longitudes 29° 50" and 31° 30" E. (Lahiff, 1997). It has an estimated population of 1.1 million people (Bornman et al., 2012). Male and female roles are clearly defined, with the men responsible for keeping livestock, ploughing and the building of huts, while the women do most of the harvesting as well as all the domestic duties. Maximum temperatures vary from 25 to 40^{o}C in summer and from 22 to 26^{o}C in winter. Rainfall is seasonal with 80% occurring between October and March (Mzezewa et al., 2010). The Venda community is one of the most remote tribes in South Africa with their own language and a distinct culture and knowledge of medicinal plants. They depend on the natural environment for their health care and survival (Mulaudzi et al., 2012). It is an area dominated by a great diversity of plants and many of them are used in alleviating hunger, for shelters, fuel, artifacts and traditional medicine (Mabogo, 1990). It is important to preserve these plant species and the knowledge associated with their uses.

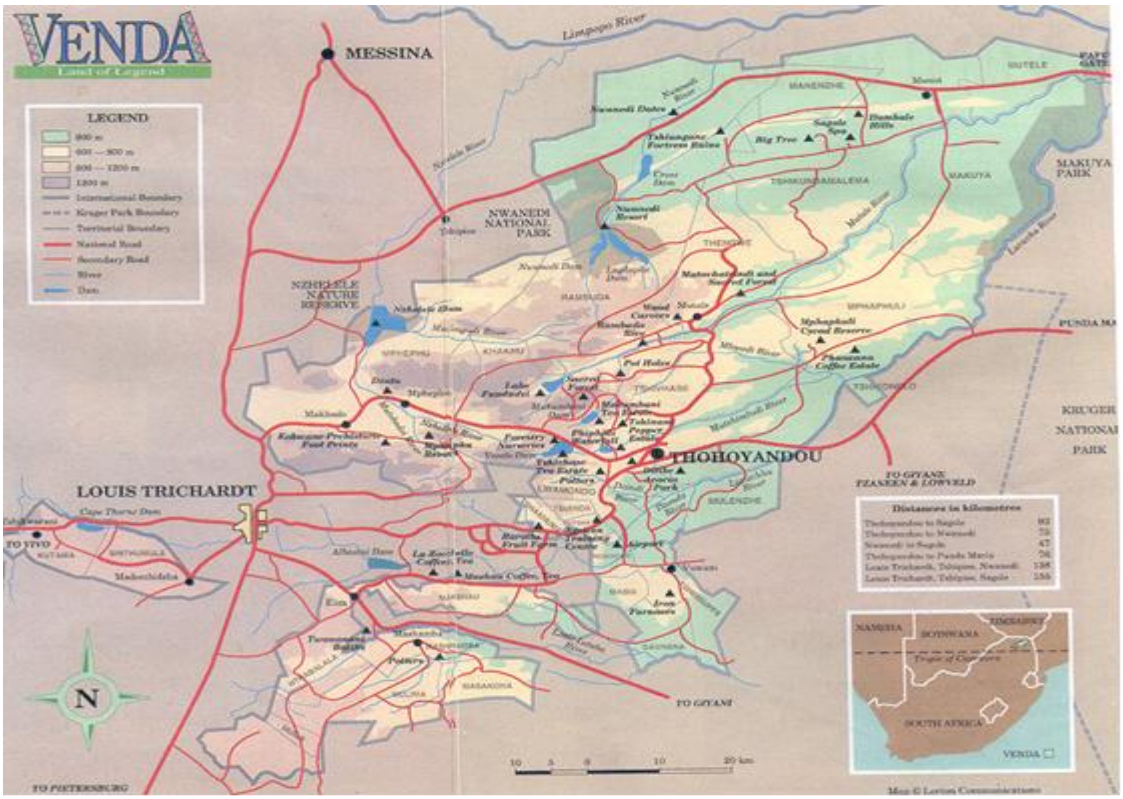

Figure 3. 1. Map of the Venda region (Lorton communications, 1993)

3. 3. Materials and Methods

Traditional healers were identified in every village and were visited with the help of a senior headman. The first author is a Venda, teaches ethnobotany at the University of Venda and because he grew up in the rural environment knows all the local customs. The aim of the study was explained to the traditional healers before the interview was conducted, and informed consent was obtained so that they could share their knowledge with us. Ethnobotanical data were collected using semi-structured interviews and were done using the local language (Tshivenda) and later translated into English. The informants were queried about the symptoms of candidiasis as well as information about the plants they use, including their local names, nature of the plant, parts used, methods of preparation, administration techniques and dosage form of the remedy. Personal information was also recorded (Appendix A). A monetary incentive was given to the traditional healers for their time. Plants were collected from the wild with the assistance of traditional healers, identified by two botanists from the University of Venda and were authenticated by SANBI (South African National Biodiversity Institute) in Pretoria. Voucher specimens were prepared and deposited at the University of Venda herbarium, South Africa.

Statistical analysis

Analysis of data was done using inferential and descriptive statistics such as percentages and frequencies.

3. 4. Results and Discussion

3.4.1. Interviews with traditional healers

Eleven traditional healers were interviewed, namely three males (27.3%) and eight females (72.7%), ranging in age from 36 to 69 years (Fig 3.2). Some of them (18%) received primary school education while 82% did not have any formal schooling. Most of these traditional healers (9) were registered with the Vhembe Traditional Healers Association and have certificates. The association is recognized by the government but they are not yet allowed to practice in public health facilities. However, 18% of the traditional healers indicated that occasionally they refer patients to the hospital for check-up after treatment.

According to the traditional healers candidiasis is known as “Makuma” a Venda word referring to ulcers either oral or genital. The ulcers begin in the stomach, move up the alimentary tract until they manifest externally either in the mouth or the genitals. With regard to the causes, there are two schools of thoughts, some believe that it is caused by germs and others believe that it is caused by the transmission of sexually transmitted diseases from one person to another. Traditional healers are aware of the fact that HIV/AIDS is infectious and consists of a variety of diseases which destroy “Phila” Venda word meaning the immunity system resulting in the failure of the body to fight off the opportunistic infections such as candidiasis.

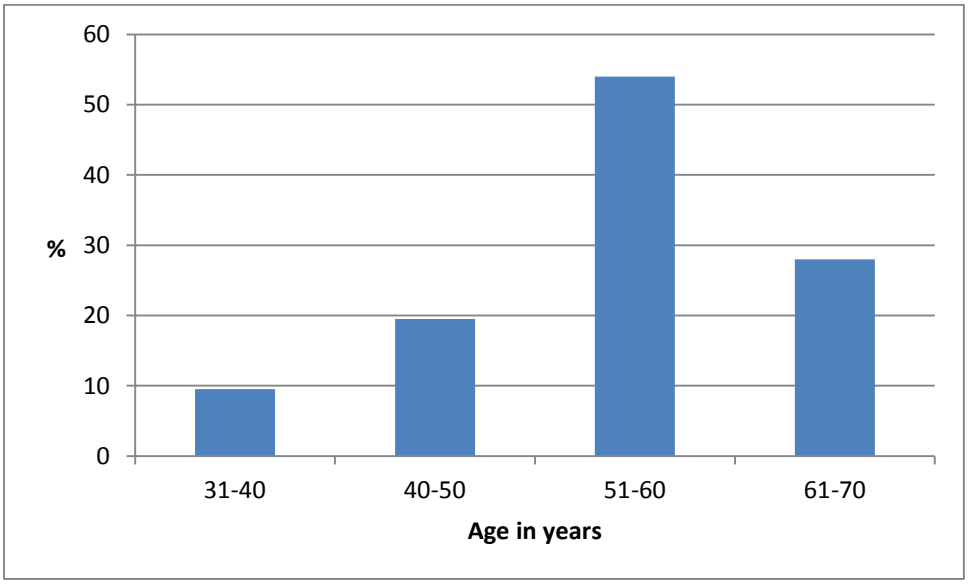

Figure 3. 2. Demographics of interviewed traditional healers.

Most males were generally unwilling to participate in the survey even though they were assured that the information was for research purposes only. They indicated that they could not give away their ancestral knowledge to the researchers.

That is why we ended up interviewing 27.3% male traditional healers only. This is a handful of traditional healers who gave us consent and took part in the survey. On the other hand females were open, participated freely and even wanted their pharmacopoeia to be tested for efficacy and toxicity.

The fact that 54% of traditional healers interviewed ranged in age from 51-60 years showed the wealth of indigenous knowledge gained in this survey as more experienced healers could be assumed to have greater knowledge than younger healers (Fig 3.2). On the other hand, this shows an urgent need for documenting more ethnobotanical data in the area because the future of indigenous knowledge is being endangered by remaining largely in the hands of older people. This is due to the fact that young people are commonly not interested in learning traditional medicine practices to ensure its continuity to the next generation.

3.4.2. Plant species and their families

The study collected data on 45 medicinal plant species used by local traditional healers to treat candidiasis and related infections. Table 3.1 shows the botanical name, family, common name, voucher number, frequency of citation, part used, method of preparation and route of administration of the herbal remedy. Plant species were distributed among 41 genera and 24 different botanical families.

The Fabaceae was best represented with 12 plant species, followed by the Asteraceae and Solanaceae (3 plant species each), Apocynaceae, Ebenaceae, Celastraceae, Loganiaceae, Rubiaceae (2 plant species each), and the rest of the families were represented by one plant species each. Frequent use of members of the Fabaceae family has been documented by several surveys in Nigeria (Offiah et al, 2011); Ethiopia (Lulekal et al., 2008), Democratic Republic of Congo (Libman et al., 2006), South Africa (Bisi-Johnson et al., 2010) and India (Ayyanar and Ignacimuthu, 2011). This is not surprising because it is the third largest family of angiosperm plants with approximately 730 genera and over 19 400 species worldwide (Arabi et al., 2010).

Table 3.1. Plants and procedures used to treat candidal infections by local traditional healers.

Botanical name Family	Local name	Voucher no	Habit	Citation	Part used	Method of preparation	Route of administration
Acacia karoo Fabaceae	Muunga	Rn 34	Tree	1	roots, bark	Roots and stem bark are boiled together	Mouthwash or applied to genitals, 2x per day for a week
Acacia caffra Fabaceae	Murovhambado	Rn 40	Tree	5	leaves	Dried, burnt and mixed with animal fats	Applied directly on mouth ulcers or vaginal ulcers
Amaranthus spinosus Amaranthaceae	Tshithavhamisis	Rn 5	Herb	2	leaves	Put fresh leaves in hot water	Oral, ½ cup of the infusion is taken 3x a day
Burkea africana Fabaceae	Mufhulu	Rn 24	Tree	1	seeds roots	Dried roots, seeds are ground up and then boiled	Oral, taken 2x a day, overdose causes diarrhoea
Carica papaya Caricaceae	Mupapawe	Rn 9	Tree	4	roots	Grind roots of *Carica papaya* and *Psidium guava*, put them into hot water	Oral, cup is taken 3x a day or douching
Carpobrotus edulis Mesembryanthemaceae	Lutele	Rn 32	Shrub	1	bark	Grind dry bark into fine powder, boil for 20-30 min	Oral, as mouth wash, 3x per day for 1 week
Cissampelos torulosa Menispermaceae	Lukandulo	Rn 13	tree	3	bark	Grind dry bark into fine powder, boil in water	Oral, gargle or douche
Clerodendrum glabrum Verbenaceae	Munkhatingwe	Rn 46	shrub	7	roots, bark	Dried, pulverized and then boiled in water	¼ cup of decoction is taken orally 3x per day

Table 3.1 cont...

Botanical name Family	Local name	Voucher no	Habit	Citation	Part used	Method of preparation	Route of administration
Croton gratissimus Euphorbiaceae	Mafunyungule	Rn 38	Shrub	6	bark	Fresh bark is crushed, soaked in hot water or boiled in water for few minutes	Cupful infusion or decoction is drunk orally 2x per day after meals.
Dichrostachys cinerea Fabaceae	Murenzhe	Rn 23	Shrub	4	dried stem	Burned and liquid that oozes out at the other end is used	Topical application on mouth ulcers or vaginal ulcers
Diospyros whyteana Ebenaceae	Munyavhili	Rn 6	Tree	3	leaves	Dried leaves are put in hot water to make tea	Infusion is used as a douche
Diospyros mespiliforms Ebenaceae	Musuma	Rn 31	Tree	4	fruit	Crush raw fruit and add little water	Infusion is used as mouth wash or douche 3x per day
Dodonaea angustifolia Sapindaceae	Muthathavhana	Rn 26	Shrub	2	leaves	Fresh leaves are crushed and macerated in water. It is used together with the bark of *Dovyalis zeyheri*	Maceration is taken orally, ½ a cup 2x per day
Elaeodendron transvaalense Celastraceae	Mukuvhazwivhi	Rn 41	Tree	8	roots	Dried, powdered and boiled in water	½ cup of decoction is drunk 3x per day

Table 3.1 cont….

Botanical name Family	Local name	Voucher no	Habit	Citation	Part used	Method of preparation	Route of administration
Elephantorrhiza burkei Fabaceae	Tshisevhufa	Rn 17	shrub	2	bark	Grind dry bark into fine powder	Topical application, 2x per day
Erythrina lysistemon Fabaceae	Muvhale	Rn 27	Tree	4	roots, stem bark	Grind up roots and stem bark and boil for 1h	Mouth wash or douche, 2x per day for a week
Faurea saligna Proteaceae	Mutango	Rn 44	Tree	7	bark	Dried, powdered and soaked in warm water for at least 30 min	The infusion is used as a douche for vaginal ulcers
Ficus carica Moraceae	Muhuyu	Rn 3	Tree	3	fruit	Latex is collected from fig's raw fruit, crushed leaves and some water added	Mixture is used as mouth wash/douche 3x per day
Hippocratea longipetiolata Celastraceae	Mutshaliri	Rn 37	shrub	6	roots, bark	Used in combination with few leaves of *Olinia rochetiana*, boiled in water	A cupful decoction is drunk orally 3x per day.
Knowltonia bracteata Ranunculaceae	Thauyakhomba	Rn 18	shrub	2	roots	Roots are pulverized and soaked in water for at least two days.	Cupful of the maceration is taken 3x per day
Osyris lanceolata Santalaceae	Mpeta	Rn 45	shrub	7	bark	Grind bark into fine powder and then boil in water	Oral, cup of decoction is taken 2x per day

Table 3.1. cont….

Botanical name Family	Local name	Voucher no	Habit	Citation	Part used	Method of preparation	Route of administration
Ozoroa engleri Anacardiaceae	Mudumbula	Rn 8	Tree	3	roots	Grind dry roots and boil for 1h, poisonous when not boiled properly	Decoction is used as mouthwash or douche
Pappea capensis Sapindaceae	Murodolo	Rn 30	Tree	1	leaves	Fresh leaves are pounded in some water and filtered	Juice is used as douche on vaginal ulcers
Peltophorum africanum Fabaceae	Musese	Rn 15	Tree	4	bark	Dry bark is soaked in water for at least two days	Infusion is taken orally, ½ cup 3 times per day
Piper capense Piperaceae	Mulilwe	Rn 21	shrub	2	bark	Grind dry bark into fine powder and boil	Oral, 1 cup taken 3x per day
Pterocarpus rotundifolius Fabaceae	Muataha	Rn 29	Tree	1	stem bark	Grind bark into fine powder and pour into boiling water	Oral, quarter of a cup taken 2x per day
Richardia brasiliensis Rubiaceae	Mulegere	Rn 14	Herb	5	whole plant	Dried, burnt, pinch mixed with animal fats	Applied directly on mouth ulcers or vaginal ulcers
Rinorea angustitolia Violaceae	Mafambaborile	Rn 28	shrub	2	roots	Roots are boiled for at least1h, inappropriate preparation may cause stomach-ache	Oral, ½ a cup is taken 2x per day

Table 3.1. cont….

Botanical name Family	Local name	Voucher no	Habit	Citation	Part used	Method of preparation	Route of administration
Schotia brachypetala Fabaceae	Mununzu	Rn 39	Tree	6	roots, bark	Roots and barks are ground together, boiled in water	A cupful decoction is taken orally 3x per day after meals
Schkuhria pinnata Asteraceae	Luswielo	Rn 35	herb	8	whole plant	Dried, powdered and boiled in water	A cupful of decoction is drunk 3x per day. Also used to treat HIV/AIDS
Senna auriculata Fabaceae	Muduwish ango	Rn 12	Tree	2	fresh leaves	Fresh leaves are crushed, water is added and mixed with powdered shell of snail	Oral, ½ cup taken 3x per day after meals
Senna petersiana Fabaceae	Munembe nembe	Rn 2	shrub	3	seeds	Dry seeds ground into fine powder and boiled in water	Oral, ½ cup taken 2x per day after meals
Strychnos madagascarienses Loganiaceae	Mukwakwa	Rn 25	Tree	2	stem bark	Ground into fine powder and boiled in water	Oral, ½ cup is taken 3x per day for a week
Solanum nigrum Solanaceae	Muxe	Rn 7	herb	3	fruit	Green berries are pounded, water is added and is filtered	A cupful of the juice is taken orally 3x per day

Table 3.1. cont….

Botanical name Family	Local name	Voucher no	Habit	Citation	Part used	Method of preparation	Route of administration
Solanum panduriforme Solanaceae	Ndhulwani	Rn 19	shrub	2	fruit	Dry fruits are burnt, ashes mixed with animal fat.	Topical application on the female genitals 2x per day for a week.
Sophora *microphylla* Fabaceae	Muthanyi	Rn 10	shrub	1	roots	burned and mixed with water	Mixture is used as a gargle or douche
Spilanthes acmella Asteraceae	Tshishengel apofu	Rn 36	herb	8	hole plant`	Powdered, soaked in warm water for 30 min to 1h	½ a cup of infusion is taken orally 3x per day, also used to treat STI
Strychnos potatorum Loganiaceae	Mukongovhoti	Rn 34	tree	5	bark	Used together with the roots of *Annona senegalensis* and then soaked in cold water for 2 days	Maceration is used as mouth wash or douche 2x per day
Tabernaemontana elegans Apocynaceae	Muhatu	Rn 1	tree	4	roots	Dry roots are pulverized and boiled in water	Decoction is taken orally, a cupful 3x per day
Tagetes minuta Asteraceae	Mushashthuri	Rn 20	herb	3	lower leaves	Grind dry leaves and boil in water	Oral, 1 cup is taken while still hot once per day

Table 3.1. cont….

Botanical name Family	Local name	Voucher no	Habit	Citation	Part used	Method of preparation	Route of administration
Trimeria grandifolia Flacourtiaceae	Xidengani	Rn 4	Tree	1	leaves	Chewing of fresh leaves	Oral, 3x per day
Vangueria infausta Rubiaceae	Muzwilu	Rn 43	Tree	5	roots, bark	Chopped into small pieces and then boiled	Oral, cupful decoction is taken 3x per day, also used to treat diarrhoea
Warburgia salutaris Canellaceae	Mulanga	Rn 22	Tree	4	roots	Ground into powder, 1 spoonful is added into the soft porridge	Oral, twice per day, Overdose causes stomach ache
Withania somnifera Solanaceae	Musalamar ubini	Rn 33	shrub	7	roots	Used together with few leaves of *Ensete ventricosum* which are boiled together	A cupful of decoction is taken orally 3x per day
Wrightia natalensis Apocynaceae	Musunzi	Rn 11	Tree	3	roots, bark	Ground into fine powder and put into hot water	Infusion is used as gargle 2x per day

The most popular plants cited by traditional healers were *Hippocratea longipetiolata* Oliv., *Spilanthes acmella* (L.) Murray, *Schkuhria pinnata* (Lam.) Kuntze ex Thell, *Clerodendrum glabrum* var. *glabrum*, *Osyris lanceolata* Hochst. & Steud., *Faurea saligna* Harv., *Richardia brasiliensis* Gomes, *Withania somnifera* (L.) Dunal, *Elaeodendron transvaalense* (Burtt Davy), *Acacia caffra* Thunb. Wild., *Strychnos potatorum* L.f., *Schotia brachypetala* Sond., *Croton gratissimus* Burch. var. *gratissimus* and *Vangueria infausta* Burch. subsp. *infausta,* with their total citations ranging from 5 to 8 out of 11 (Table 3.1).

The fact that we obtained some duplication, that the same plants were shown to us by several traditional healers, and that most of the plants collected have been reported in the literature to be used as medicinal plants, indicated that traditional healers could be trusted for the information they gave us about the plants they use. Furthermore, the use of the plant species by different cultural groups may indicate their potential pharmacological effectiveness. It is worth noting that 27 species, representing 60% of those recorded, have either been scientifically shown to have anti-*Candida* activity or have been recorded for antifungal use elsewhere in the literature (Table 3.2). Only one plant species, *Solanum panduriforme*, has been reported to be inactive against *Candida albicans* (Table 3.2). However, this may be due to the fact that water was used as an extraction solvent while in the traditional preparation dried fruits are burnt and mixed with animal fat. This gives some credibility to the information which was collected from the informants. Based on our literature search, the following 12 plant species were recorded for the first time to be used to treat candidiasis: *Acacia caffra, Amaranthus spinosus, Cissampelos torulosa, Faurea saligna, Ozoroa engleri, Pterocarpus rotundifolius, Rinorea angustifolia, Schkuhria pinnata, Strychnos madagascarienses, Sophora microphylla, Trimeria grandifolia* and *Wrightia natalensis.* They have never been tested for anti-*Candida* activity and except for *Rinorea angustifolia,* has not been reported as medicinal plants to treat other ailments (Table 3.2).

Table 3.2. Traditional uses of the species reported in this study and their reported relevant antifungal activity.

Botanical name	Proven anti-*Candida* activity/active chemical constituents	Relevant reported ethnomedicinal uses	Previous report on ethnomedicinal uses
Acacia karoo	(Mulaudzi et al., 2011)	**No report**	Venereal diseases, diarrhoea (Mabogo, 1990)
Acacia caffra	**No report**	**No report**	Blood cleansing, abdominal disorder (Venter and Venter, 1996)
Amaranthus spinosus	**No report**	**No report**	Analgesic, laxative, piles (Kumar et al., 2011)
Burkea africana	(Steenkamp et al., 2007)	**No report**	Herpes simplex (Chisembu, 2010), dysentery (Grant and Thomas, 2000)
Carica papaya	(Runyoro et al., 2006)	Vaginal candidiasis (Runyoro et al., 2006)	Infectious diseases (Magassouba et al., 2007), cancer (Otsuki et al., 2010)
Carpobrotus edulis	(Motsei et al., 2003)	Thrush (Thring and Weitz, 2006)	Diarrhoea (Bisi-Johnson et al., 2010)
Cissampelos torulosa	**No report**	**No report**	Syphilis, toothache (De Wet and Van Wyk, 2008)
Clerodendrum glabrum	**No report**	Oral ulcers (Mabogo, 1990)	Diarrhoea (Bisi-Johnson et al., 2010), Coughs (McGaw et al., 2008)
Croton gratissimus	(van Vuuren and Naidoo, 2010)	**No report**	Syphilis, earache (van Vuuren and Viljoen, 2008), pneumonia (McGaw et al., 2008)

Table 3.2. cont…

Botanical name	Proven anti-*Candida* activity/active chemical constituents	Relevant reported ethnomedicinal uses	Previous report on ethnomedicinal uses
Diospyros whyteana	Isodiospyrin (Singh et al., 2012)	**No report**	Dysmenonrrhoea, rash (Steenkamp, 2003)
Diospyros mespiliforms	Isodiospyrin (Singh et al., 2012)	Fungal infections (Mabogo, 1990)	Malaria (Chinsembu, 2010), hypertension (Adamu et al., 2005)
Dodonaea angustifolia	(Motsei et al., 2003)	Oral candidiasis (Patel and Coogan, 2008)	Pneumonia, TB (van Wyk and Gericke, 2000) rhinitis, piles (Vermani and Garg, 2002)
Elaeodendron transvaalense	(Samie et al., 2010)	Candidiasis (Bessong et al., 2005)	Stomachache, fever (Drewes et al., 1991), dysmenorrhoea (Steenkamp, 2003)
Elephantorrhiza burkei	(Mulaudzi et al., 2011}	**No report**	Venereal diseases, aphrodisiac (Mabogo, 1990), abortifacient (Steenkamp, 2003)
Erythrina lysistemon	(Motsei et al., 2003)	**No report**	Wounds, arthritis (van Wyk and Gericke, 2000) toothache, abscesses (Hutchings et al., 1996)
Faurea saligna	**No report**	**No report**	Diarrhea (Hamill et al., 2000), epilepsy (Stafford et al., 2008)
Ficus carica	(Aref et al., 2010)	Sore throat (Jeong et al., 2009)	Eyesore, diabetes (Aref et al., 2010)
Hippocratea longipetiolata	**No report**	**No report**	Invocation of ancestors (Mabogo,1990)
Knowltonia bracteata	(Buwa and van Staden, 2006)	**No report**	Sexually transmitted diseases (Buwa and van Staden, 2006)

Table 3.2. cont…

Botanical name	Proven anti- *Candida* activity/active chemical constituents	Relevant reported ethnomedicinal uses	Previous report on ethnomedicinal uses
Osyris lanceolata	(Mulaudzi et al., 2011)	**No report**	Ringworm (Muthee et al.,2011)
Ozoroa engleri	**No report**	**No report**	Sexually transmitted infections (de Wet et al., 2012)
Pappea capensis	(Mulaudzi et al., 2011)	**No report**	Aphrodisiac, venereal diseases (Hutchings et al., 1996)
Peltophorum africanum	(Steenkamp et al., 2007)	Sore throat (Bessong et al., 2005)	Diarrhea (McGaw et al., 2008), Venereal diseases (de Wet et al., 2012)
Piper capense	(Steenkamp et al., 2007)	Sore throat (Mabogo, 1990)	Diarrhoea, cough (Chahal et al., 2011), Ulcers, fever (Obi et al., 2002))
Pterocarpus rotundifolius	**No report**	**No report**	Sore eyes (Venter and Venter, 1996)
Richardia brasiliensis	(Adekunle, 2000)	**No report**	Diabetes, anti-emetic (Pinto et al., 2008)
Rinorea angustifolia	**No report**	**No report**	No report
Schotia brachypetala	(Samie *et al*., 2010)	**No report**	Dysentery, diarrhoea (McGaw et al.,2000), Ulcers (Venter and Venter, 1996)
Schkuhria pinnata	**No report**	**No report**	Abortifacient (van Wyk and Gericke, 2000)
Senna auriculata	(Muthukumaran et al., 2011)	**No report**	Ulcers, leprosy, diabetes (Subhadradevi et al., 2011)

Table 3.2. cont…

Botanical name	Proven anti-*Candida* activity/active chemical constituents	Relevant reported ethnomedicinal uses	Previous report on ethnomedicinal uses
Senna petersiana	Samie et al., 2010	**No report**	Venereal diseases (Tshikalange et al., 2005); infertility (Steenkamp, 2003)
Strychnos madagascarienses	**No report**	**No report**	Fever (Ribeiro et al., 2010), diarrhoea (de Wet et al., 2010)
Strychnos potatorum	**No report**	(Hamza et al., 2006)	Leucoderma, diabetes (Ekambaram et al., 2010)
Solanum nigrum	(Mehjabeen et al., 2011)	**No report**	Ringworm (Jain et al., 2011), Antiflammatory (Ravi et al., 2009)
Solanum panduriforme	No activity (Steenkamp et al., 2007)	**No report**	Diarrhoea (McGaw et al., 2008)
Spilanthes acmella	**No report**	Sore throat (Pfoze et al., 2012)	Cancer (Graham *et al.*, 2000), toothache (Mbeunkui et al., 2011)
Tabernaemontana elegans	(Steenkamp et al., 2007)	**No report**	Sexually transmitted infections (De Wet et al., 2012)
Tagetes minuta	(Motsei et al., 2003)	Oral candidiasis (Hamza et al., 2006)	Smallpox, earache, fevers (Shahzadi et al., 2010)

Table.3.2..cont…

Botanical name	Proven anti- *Candida* activity/active chemical constituents	Relevant reported ethnomedicinal uses	Previous report on ethnomedicinal uses
Trimeria grandifolia	**No report**	**No report**	Heart burns (Okello et al., 2010)
Vangueria infausta	(de Boer et al., 2005)	Oral candidiasis (Chinsembu and Hedimbi, 2010)	Coughs (McGaw et al., 2008) malaria, pneumonia, (Venter and Venter, 1996)
Warburgia salutaris	(Motsie et al., 2003).	Thrush (van Wyk, 2011)	Malaria (Bussmann et al., 2006), HIV/AIDS (Larmorde et al., 2010),
Withania somnifera	(Jain and Varshney, 2011)	**No report**	Cancer, fever (Maregesi et al., 2007), Diarrhoea (McGaw et al., 2008)
Wrightia natalensis	**No report**	**No report**	Gonorrhoea, aphrodisiac (Obi et al., 2002)

3.4.3. Growth form and plant parts used

Growth form analysis showed that trees made up the highest proportion, being represented by 23 species (51.1%), followed by shrubs (15 species, 33.3%) and herbs only 15.5%;. During interviews and discussions with the traditional healers, they indicated that they prefer to use trees and shrubs because these growth forms are available throughout the year and are not affected by any seasonal variations. From the chemical point of view, some studies elsewhere investigated the relationship between species habit and classes of chemical compounds such as phenols, tannins, alkaloids, triterpenes and quinones, and found that trees usually bear greater quantities of such compounds than do shrubs and herbaceous species (Cartaxo et al., 2010), and this may also provide a reason why trees are preferred to other growth forms by the traditional healers.

Frequently used plant parts were roots, bark, combination of roots and bark, leaves and fruit, while the whole plant was the least used. The most widely used plant parts in the therapeutic preparations of remedies were roots (26.7%) and bark (22.2%), followed by roots and bark together (17.7%) (Fig 3.3). Several indigenous communities elsewhere also utilize mostly roots for the preparation of herbal remedies (Okello et al., 2010, Steenkamp, 2003; Oyedemi et al., 2009**;** Appidi et al., 2008; Gessler et al., 1995). Some traditional healers prefer to use roots and bark because they believe that they have more healing powers than other plant parts. Studies elsewhere have shown that roots and other underground parts have high concentrations of bioactive compounds (Maroyi, 2011). Worthy of note is that plant species in the area under study are regularly exposed to long periods of drought and they shed their leaves, so this may be an additional reason why roots and bark are preferred. According to the informants, plant materials are mainly collected in the winter season and time of collection is early in the morning. They believe that plants absorb plenty of water during the summer rainfall which may dilute the active principles, resulting in the loss of healing power of the remedy. Scientific literature disagrees with this because Buwa and Van Staden (2007) reported that the highest antimicrobial activity of aqueous extracts has been detected with plant material collected both during winter (June 2003) and summer (December 2003) but this may vary according to the plant species. In a study of the variation in antibacterial activity of *Schotia brachypetala* ethanol and water leaf extracts, McGaw et al. (2002) reported that monthly variation in activity against a panel of bacterial species was not marked, although there appeared to be a tendency towards slightly higher activity in the summer months.

Frequent harvesting of roots and bark has a negative influence on the survival of plants, and is therefore discouraged. Some literature studies (van Vuuren and Naidoo, 2010), (Shai et al., 2009), (Lewu et al., 2006) and (Eloff, 2001) have shown that plant leaves can be used as biologically active alternatives in traditional medicine. Thus, to foster sustainability, traditional healers should be encouraged to use plant leaves. However, this might only be valid if the chemistry of the roots or bark is similar to that of the leaves.

There are several communities in Africa and elsewhere who are mostly using leaves to treat a variety of ailments, in Burkina Faso (Nadembega et al., 2011), Cameroon (Telefo et al., 2011), Pakistan (Abbasi et al., 2010), Ethiopia (Bekalo et al., 2009), Uganda (Ssegawa and Kasenene, 2007), India (Namsa et al., 2009) and Portugal (Camejo-Rodrigues et al., 2003).

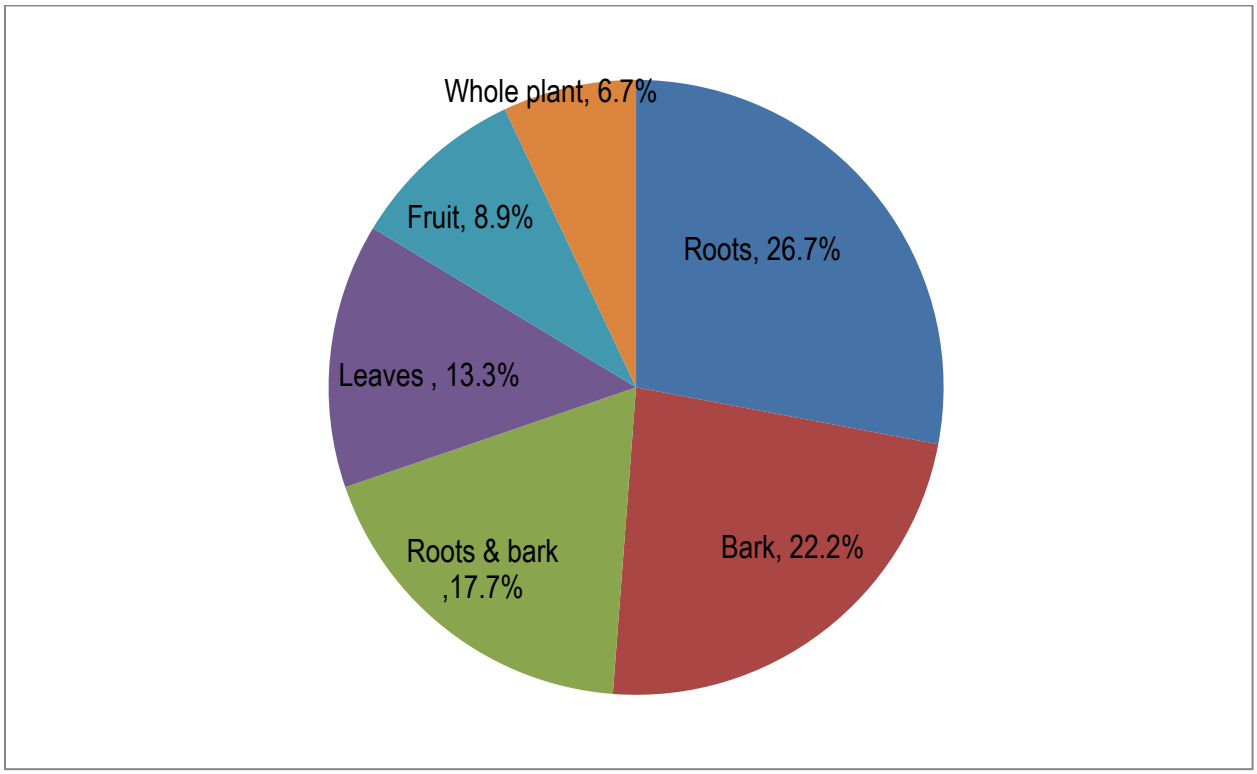

Figure 3.3 Plant parts used for preparing remedies

3.4.4. Preparation, dosage and route of administration of the remedy.

The most frequently used methods in the preparation of the herbal remedies (Fig 3.4) were decoctions (44.4%), infusions (20%), macerations (17.7%) and burning (11.1%). The use of decoctions and infusions as the methods of choice is supported by several other studies (Keter and Mutiso, 2012, Namukobe et al., 2011, Ajibesin et al., 2008). A decoction is obtained by boiling plant parts in water while an infusion is prepared by soaking plant parts in water for a few hours or days. According to Okello et al. (2010), the advantage of using the latter method is that active principles are extracted with almost no alteration of their chemical structure, thus preserving almost all their properties, and this may enhance the efficacy of the plant extracts. With regard to burning, plant materials such as roots and bark are burnt to a certain degree, and then the fire is doused so that they do not become ash lest they lose their healing properties.

In some plant species such as *Dichrostachys cinerea*, dry splinters of wood are burnt at one end and the liquid that oozes out is collected and used as a remedy for treating fungal infections.

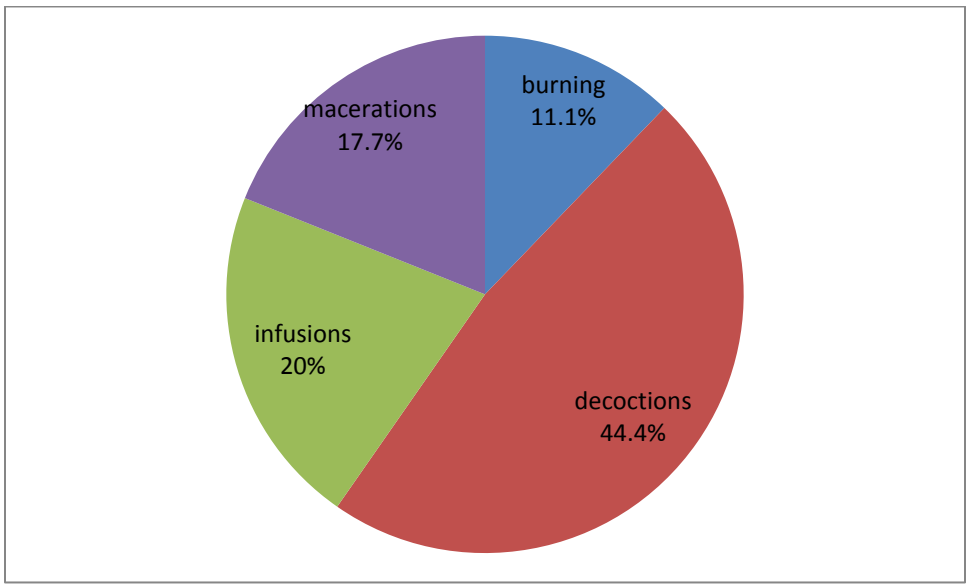

Figure 3.4. Reported methods of preparing the remedies

The majority of these preparations were constituted by single plant species (84%) and 16% was constituted from multiple plant species; similar findings were reported by other researchers (Packer et al., 2012, Ranganathan et al., 2012, Rosas-Pinon et al., 2012, Giday et al., 2003). In the present study most traditional healers reported that multiple preparations of the remedy are normally administered to the patient if the disease is severe to increase its healing power. There was a consensus among the traditional healers that some plants enhance the action of others, powdered roots of *Elaeodendron transvaalense* were reported to be added to most of the herbal preparations. For example, in one of the preparations, it is mixed with the powdered roots of *Ozoroa paniculosa, Ehretia rigida*, *Ziziphus mucronata* and boiled in the clay pot and a cup of the decoction is taken three times per day. The plant species is also reported as having antiviral and anticandida properties (Samie et al., 2010 and Bessong et al., 2005).

During interviews and discussions with the traditional healers, we found that dosage is estimated using either lids, spoons, cups, pinches or handfuls. It was difficult to get the information on how much plant material was used to make the extracts. In most cases dosage is determined according to the severity of the disease and age of the patient i.e. a greater amount of remedy is normally given to adults than children to treat the same disease. However, there is still lack of precision in the determination of the dosage to be taken by the patient and this is one of the disadvantages of the use of traditional remedies.

McGaw et al. (2007) also reported that drawbacks of traditional medicinal plant remedies include uncertain dosages and lack of standardization.

Traditional healers reported that the prepared therapeutic remedies may be stored in powder form until needed, especially for scarce medicinal plants, and liquid remedies (decoctions or infusions) are prepared and administered to patients on a daily basis when needed. The latter could also be stored for up to 3-5 days, but thereafter could not be used because they believed the remedy would have lost its healing powers. The therapeutic preparations are normally stored in dried horns and skins of domestic and wild animals, calabashes, clay pots and wooden bottles. In the workshop there was agreement that the use of tins, plastics or glass bottles is discouraged because they absorb heat which may destroy the healing properties of the remedy. In most instances, the storage containers were not labelled and this might be attributed to illiteracy. However, the informants claimed that they were able to identify their remedies by colour, smell, etc. Unprocessed plant material such as roots, fruit and stem bark are stored in dried form for future use in the traditional surgery which has a thatched roof.

With regard to the administration routes of the remedies, four main routes were reported, namely douche, oral, mouthwash and topical (Fig 3.5). However, oral application was the most commonly used route of administration. This is in agreement with the results of various ethnobotanical studies elsewhere (Mukazayire et al., 2011; Philander et al., 2011, Wambugu et al., 2011; Idowu et al., 2010;). The use of the oral route of administration did not come as a surprise because this is in line with orthodox medicine where the preferred route is frequently oral.

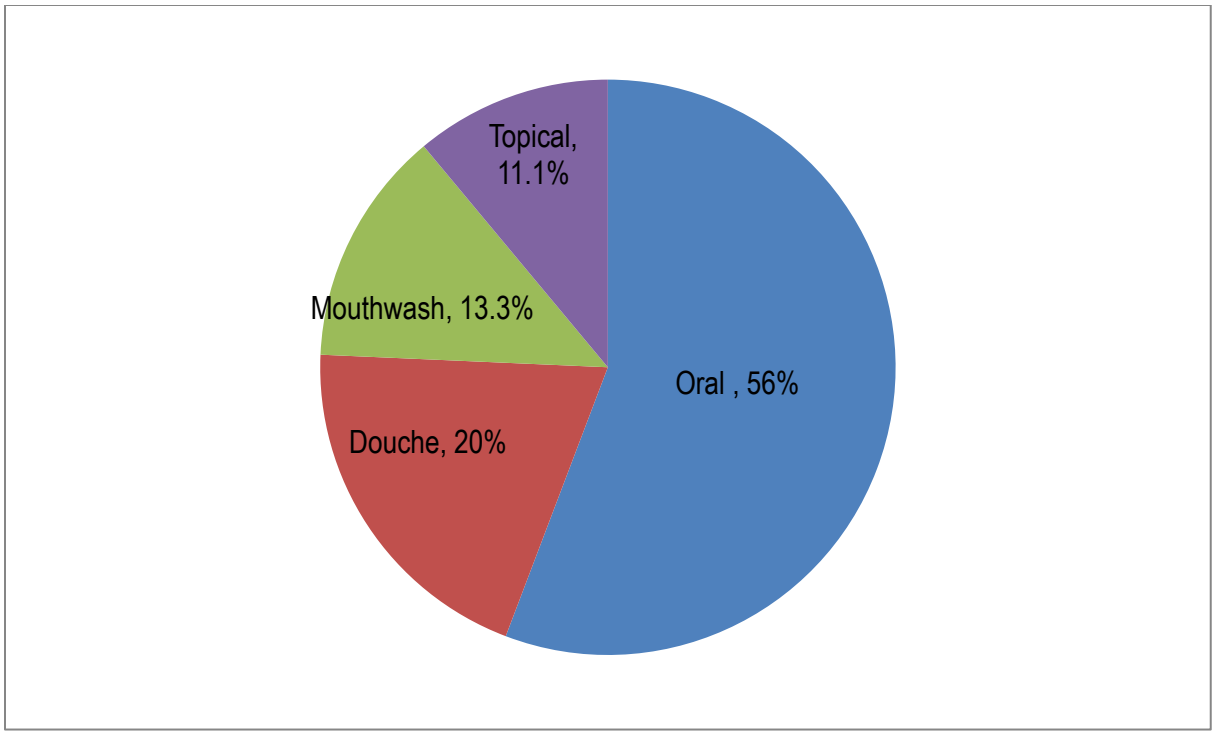

Figure 3. 5. Reported routes of administration

This may explain why traditional healers prefer to use this route of administering the remedy to their patients. Most of these remedies were taken with some food twice or three times per day and some additives such as fats were also used, especially for topical application.

3.4.5. Conservation status of medicinal plants

The main factors threatening the conservation status of medicinal plants recorded during the interviews were unsustainable methods of harvesting, logging for firewood, use of plants for building materials, crafts and current agricultural trends. However, the informants stressed that they practice sustainable methods of harvesting the medicinal plants from the wild because they know that these plants are their primary source of income. In some of the instances, before the collection, they conduct some rituals such as sprinkling of snuff, bowing, invoking of their ancestors etc. The informants blamed herbalists for the destruction of the medicinal plants. Some of the harvesting techniques employed by the traditional healers include stripping of bark from the western and eastern sides of the tree to avoid ring barking of the stem; when collecting the whole plant, they ensure that some individuals remain behind; some leafy twigs are collected using teeth only; when collecting roots, side roots are collected, leaving the main ones for the plant to survive etc. The informants believed that if the plant from which they collected the plant material dies, then the patient would not be cured by the remedy. This is another way of instilling sustainable methods of harvesting plant material, resulting in the conservation of the medicinal plants.

Most traditional healers (60%) stated that to avoid trekking to the wild to collect the medicinal plants frequently, they have planted some common medicinal plant species in their yards. The present inventory represents a contribution of the natural flora of this area to a global approach in controlling candidiasis and related infections.

3.5. Conclusion

A total of 45 medicinal plant species used by local traditional healers in the Venda region, to manage candidiasis and related fungal infections were documented. The results of this study have shown that traditional medicine still plays an important role in meeting the primary health care needs of rural people in this area. We reported for the first time 12 medicinal plants used to treat candidiasis, and local traditional healers' current knowledge in the management of candidiasis and related fungal infections.

Post script

The medicinal plant species recorded here create a base for ethnopharmacological evaluation which could lead to the discovery of biologically active compounds that can be used as starting materials in the development of new drugs. Preliminary screening of the plant species about this was undertaken in our laboratory. In the next chapter we investigate the chemical profiles of the selected plants using thin layer chromatography and their antimicrobial activity using the bioautography.

Chapter 4.

Phytochemical and bioautographic investigation of selected South African medicinal plant species

Ndivhaleni A. Masevhe, Lyndy J. McGaw, Jacobus N. Eloff

Preface

Plant species recorded in the previous chapter as the most widely used to treat candidiasis were screened to determine the number of antimicrobial compounds. Thin layer chromatography was employed to fingerprint the complex plant extracts while bioautography was used to detect the activity of the separated compounds in the plant extracts. These methods were used in order to get an idea of the diversity of compounds present in the plant extracts and to determine the number of active compounds in each plant extract with a view of selecting the promising plant species for further investigation. This chapter has been prepared for submission to the African Journal of Traditional, Complementary and Alternative Medicines for publication

Abstract

Leaf extracts of *Acacia caffra*, *Clerodedrum glabrum*, *Croton gratissimus, Elaeodendron transvaalense*, *Faurea saligna, Hippocratea longipetiolata*, *Osyris lanceolata*, *Richardia brasiliensis*, *Schkuhria pinnata*, *Schotia brachypetala, Spilanthes acmella*, *Strychnos potatorum*, *Vangueria infausta* and *Withania somnifera* were separated by thin layer chromatography to detect the number of active compounds against *Candida albicans* of clinical origin, and *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia. coli* (ATCC 27853). Leaves were extracted using hexane, acetone, methanol and water. Thin layer chromatography (TLC) developed in benzene/ethanol/ammonium solution (BEA), chloroform/ethyl acetate/formic acid (CEF), ethyl acetate/methanol/water (EMW) solvent systems, ethylacetate/water/formic acid/acetic acid (FAWE) and vanillin-sulphuric acid spray reagent was used to determine the chemical composition of extracts. Bioautography was used to determine the number of antimicrobial compounds in the plant extracts separated by TLC. The BEA solvent system separated the highest number of compounds in the plant extracts, this indicates that most compounds were relatively non-polar. In the bioautography method, only 10% of all the compounds separated by BEA, CEF, EMW and FAWE solvent systems were active against *C. albicans* and most of the antifungal compounds were separated by the EMW solvent system. Plant species which had the highest number of active compounds against *C. albicans* were *C. glabrum, F.saligna* and *R. brasiliensis* (21%). In the bacteria, the Gram-positive, *S. aureus* was inhibited by the largest number of compounds separated on the TLC plates by the solvent systems. Gram negative bacteria (*P. aeruginosa, E. coli*) were sensitive to fewer separated compounds. Some active compounds separated on the bioautography using the same solvent system had the same R_f values,

suggesting that the same compounds may be present in different species. Based on our experience it does not mean that extracts that contained no separated compounds inhibiting growth are not active. This may be attributed to the disruption of synergism between compounds that are not active once separated by the thin layer chromatography. The results obtained from this investigation justify the use of some of the plant species in traditional medicine to some extent. This has to be confirmed by determining the quantitative antimicrobial activity.

Keywords: Thin layer chromatography, bioautography, *Candida albicans*, medicinal plants

4.1. Introduction

In South Africa and other developing countries many patients are immune-compromised as a result of the HIV/AIDS pandemic, cancer, organ transplantation and opportunistic infections such as candidiasis caused by *C. albicans* and other pathogens are therefore common (Nagaraja et al., 2011). Management of candidiasis and other microbial infections is facing a number of problems such as resistance to antimicrobial agents, drug toxicity and high costs of antimicrobial agents (Hamza, et al., 2006). Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from various sources such as medicinal plants (Masoko et al., 2008). Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value (Nostro et al., 2000). Plant extracts are regarded by ancient civilizations to be significant for the treatment of various human ailments (Soberon et al., 2006). The substances that can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered candidates for developing new antimicrobial drugs (Ahmad and Beg, 2001).

Now, in an effort to discover new lead compounds, many research groups screen plant extracts to detect secondary metabolites with relevant biological activities (Suleiman et al., 2010). Currently, the available screening methods for the detection of antimicrobial activity of natural products are diffusion, bioautographic and dilution methods (Valgas et al., 2007). Diffusion and bioautographic methods are qualitative, they only give an idea of the presence or absence of substances with antimicrobial activity while dilution methods are quantitative in that they determine the minimal inhibitory concentration of the plant extracts or compounds. Compared to other methods, thin layer chromatography (TLC) bioautography method , herein will be used in this investigation because it is considered as the most efficacious assay for the separation and detection of the number of antimicrobial compounds in a plant extract (Shahverdi et al., 2007). 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). This method permits the localization of antimicrobial active compounds that have been separated by TLC (Suleiman et al., 2010).

In the search for new bioactive compounds, an ethnobotanical survey was undertaken looking for plants used by local traditional healers to treat candidiasis and related infections. The selected plants were: *Acacia caffra*, *Clerodedrum glabrum*, *Croton gratissimus, Elaeodendron transvaalense*, *Faurea saligna, Hippocratea longipetiolata*, *Osyris lanceolata*, *Richardia brasiliensis*, *Schkuhria pinnata*, *Schotia brachypetala, Spilanthes acmella*, *Strychnos potatorum*, *Vangueria infausta* and *Withania somnifera.* Extracts of these species were fractionated by thin layer chromatography (TLC) and active compounds detected by bioautography. Some studies report that there is a high correlation between the traditional use of plants and the presence of active compounds within the plant extracts (McRae et al., 2007).

4.2. Materials and Methods

4.2.1. Plant collection

The plant species were collected from Nzhelele, Tshipise, Tshaulu and Hamashau in Venda, Limpopo province, South Africa. The plant species were collected in their natural habitats during summer between September and November 2009. They were identified by a botanist from the University of Venda and were further authenticated by SANBI (South African National Biodiversity Institute) in Pretoria. Voucher specimens were deposited in the herbarium of the Department of Botany at the University of Venda

4.2.2. Plant drying and storage

For the conservation of plant species, only leaves were harvested for investigation in this study, although traditional healers preferred to use either stem bark or roots. Leaf materials were dried at room temperature. Dried leaf materials were ground into a fine powder using a Macsalabmill (Model 200 LAB), Eriez®, Bramley. They were stored at room temperature in airtight containers in the dark until required.

4.2.3. Extraction of plant materials

The ground leaf materials (1.0 g) were separately extracted with10 ml of hexane, acetone and methanol (technical grade Merck) in a shaking machine (Labotec model 20.2) at a moderate speed. The plant extracts and residual plant materials were centrifuged at 3500 rpm for10 min, then filtered using filter paper (Whatman No.1) into pre-weighed labelled containers. The process was repeated three times on the same plant material to exhaustively extract the phytochemicals and the plant extracts were then combined. The plant extracts were dried under a stream of air in a fume hood at room temperature.

The ground leaf materials (1.0 g) were also suspended in 10 ml of distilled water, boiled for 20 min and then cooled down. The extracts were filtered, put in the freezer overnight at -40 ^{0}C and then freeze dried.

4.2.4. Test microorganisms

Fungal cultures of *Candida albicans* were obtained from the Microbiology Laboratory (Department of Veterinary Tropical Diseases, University of Pretoria). *C. albicans* was isolated from a Gouldian finch and its density was approximately 3 ×10^6. The cultures were maintained in Sabouraud Dextrose (SD) agar at 4 ^{0}C (Oxoid, Basingstoke, UK) and were inoculated in SD broth (Oxoid, Basingstoke, UK) and incubated at 37 ^{0}C prior to conducting bioautography assay. Bacterial test organisms used were two Gram-positive bacteria, *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212), and two Gram-negative bacteria, *P.aeruginosa* (ATCC 25922) and *E. coli* (ATCC 27853), and their densities were approximately 3 ×10^{12}, 2 ×10^{10}, 5 ×10^{13} and 3 ×10^{11} cfu/ml respectively (Suleiman et al., 2010). They were maintained on Mueller Hinton (MH) agar (Oxoid, Basingstoke, UK) at 4 ^{0}C and cultured in MH broth (Fluka, Switzerland) at 37 ^{0}C before use.

4.2.5. TLC fingerprinting

Chemical constituents of the plant extracts were analyzed by thin layer chromatography using Silica gel 60 F_{254} plates (Merck). The TLC plates were developed in four eluent systems of varying polarities, namely: ethyl acetate/methanol/water: 40:5.4:4 (EMW), chloroform/ethyl acetate/formic acid: 5:4:1 (CEF), benzene/ethanol/ammonium hydroxide: 90:10:1 (BEA) and ethylacetate/water/formic acid/acetic acid: 70: 20:3:2 (FAWE) (Kotze and Eloff, 2002). TLC plates (10 x 20 cm, aluminium-backed, Merk, Silica gel 60 F_{254}) were loaded with 10 µl of the plant extracts and were developed in a closed tank in which the atmosphere has been saturated with the eluent vapour. The separated components were visualized under UV light (254 nm and 360 nm, Camac Universal lamp TL-600). In order to detect the separated constituents, vanillin-sulphuric acid (0.1g vanillin: 28 methanol: 1ml sulphuric acid) reagent was sprayed on the chromatograms and heated at 110 °C to allow optimal colour development (Masoko et al., 2005). The retention factor (R_f) of the separated components was calculated using the formula, R_f = distance moved by the compound/distance moved by solvent front.

4.2.6. Bioautography

Thin layer chromatography as described by Begue and Kline (1972) was used with some slight modification to separate and identify the active plant constituents (Masoko and Eloff, 2005). TLC plates (10 x 20 cm, aluminium-backed, Merk, Silica gel 60 F_{254}) were loaded with 10 µl of the plant extracts (10 mg/ml) 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), benzene/ethanol/ammonium hydroxide: 90:10:1 (BEA), ethylacetate/water/formic acid/acetic acid: 70: 20:3:2 (FAWE) (Kotze and Eloff, 2002). The TLC plates were dried under a stream of air in a fume hood at room temperature and sprayed with a concentrated suspension of actively growing cells of test organisms until they become completely moist using a spray gun. The moist plates were incubated at 37 ^{0}C overnight in a closed chamber at 100 % relative humidity. The TLC plates were sprayed with a 2 mg/ml solution of p-Iodonitrotetrazolium violet (INT) (Sigma) and were incubated further for 2 hours. Clear zones on the chromatograms indicated the inhibition of the test organism by the separated plant constituents and the resultant red colour indicated the viability of cells i.e. ability of cells to reduce INT to its red formazan (Begue and Kline, 1972).

4.3. Results and discussion

4.3.1. TLC fingerprinting analysis

Chromatograms of 14 plant species extracted with solvents of different polarities hexane, acetone, methanol and water, and developed in BEA, CEF, EMW and FAWE solvent systems are shown in Fig.4.1.The number of different coloured bands observed on the TLC plates show the diversity of the compounds present in the plant extracts. Pascaline and co-workers report that the different colours of the fluorescence rings are due to different atoms present in the compound having different wavelengths. When atoms are excited to a higher energy level, they may fall back to their original position using the same or a different wavelength resulting to the emission of different colours (Pascaline et al., 2011). 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.

Only 582 compounds were separated by the four solvent systems because most of the plant extracts did not separate well, they remained at the baseline of the TLC plate, this might be attributed to too much plant extracts applied to the baseline or compounds in the plant extracts were too polar to be eluted because normal phase silica of TLC plate retains polar components (Fried and Serma, 1999). Comparing the four solvent systems, BEA separated compounds in the plant extracts more efficiently than CEF, EMW and FAWE, representing 38% of the separated compounds, followed by EMW with 29%, CEF with 26% and the least was FAWE with only 7%. Our group has also observed the separation of more bands in the BEA solvent system than in CEF and EMW solvent systems (Masoko et al., 2008, Eloff et al., 2005). This indicated that most of the separated compounds were non-polar.

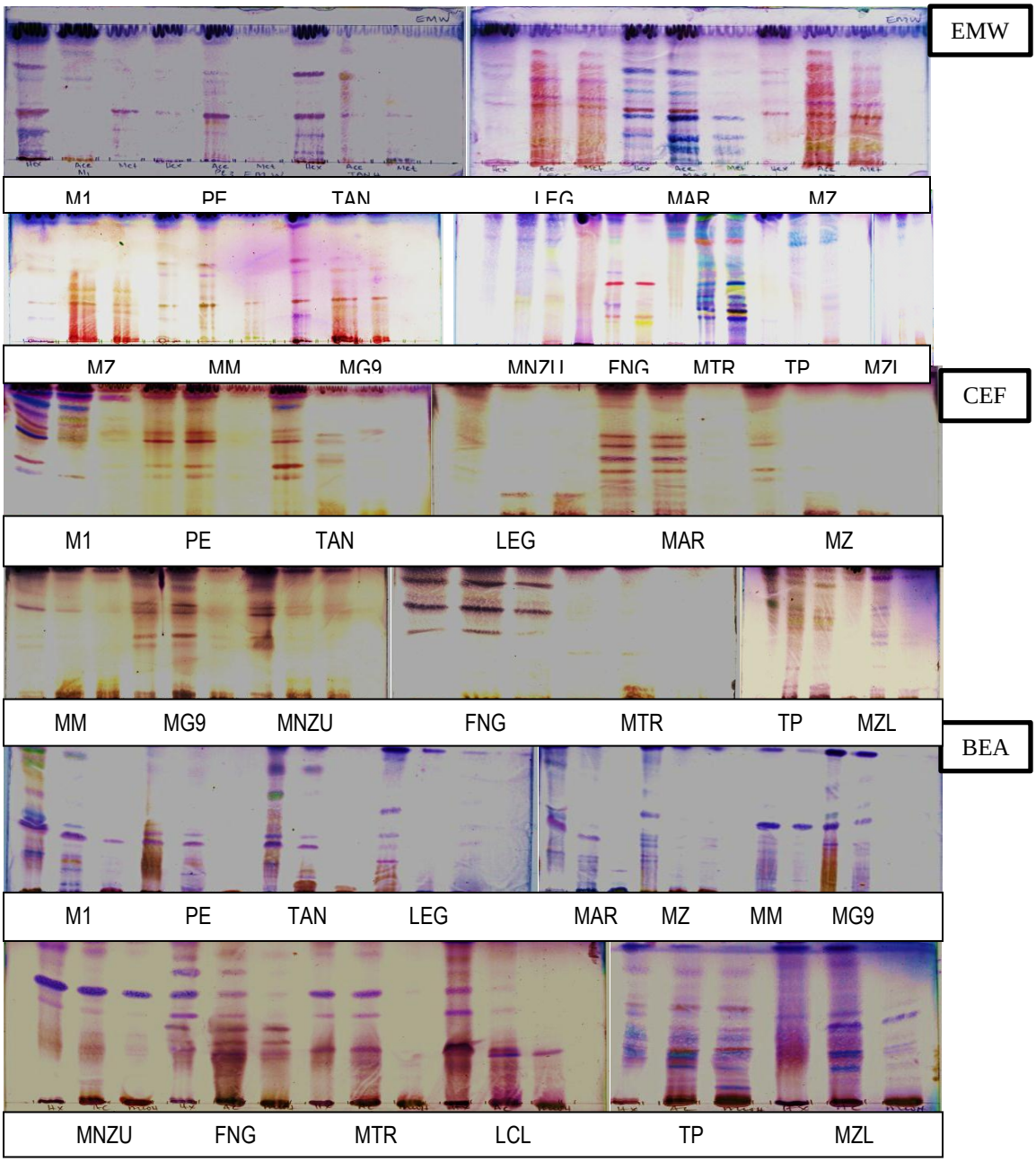

Figure 4.4. Chromatograms of M1-*C. glabrum*, PE-*O. lanceolata,* TAN-*F. saligna,* LEG-*R. brasiliensis,* MAR-*W. somnifera,* MZ-*E. transvaalense,* MM-*A. caffra,* MG9-*S. potatorum,* MNZU-*S. brachypetala,* FNG-*C. gratissmus,* MTR- *H. longipetiolata,* LCL-*S. pinnata,* TP-*S. acmella,* MZL-*V. infausta* species developed in EMW, BEA and CEF solvent systems and sprayed with vanillin–sulphuric acid to show compounds extracted with hexane, acetone and methanol in lanes from left to right for each group.

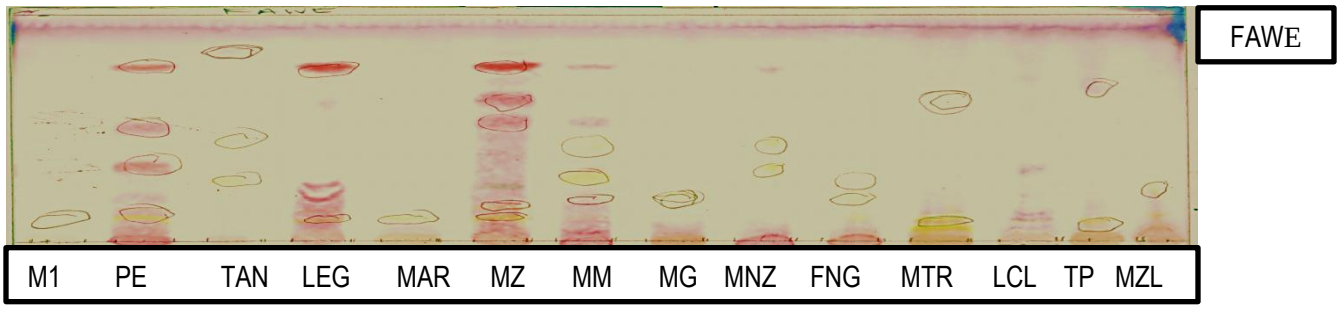

Figure 4.2. Chromatograms of M1-*C. glabrum*, PE-*O. lanceolata,* TAN-*F. saligna,* LEG-*R. brasiliensis,* MAR-*W. somnifera,* MZ-*E. transvaalense,* MM-*A. caffra,* MG9-*S. potatorum,* MNZ-*S. brachypetala,* FNG-*C. gratissmus,* MTR-*H. longipetiolata,* LCL-*S. pinnata,* TP-*S. acmella,* MZL-*V. infausta* species developed in FAWE solvent system and sprayed with vanillin–sulphuric acid to show components extracted with water in lanes from left to right for each group.

4.3.2. TLC bioautography of fungal species

The results of bioautography of plant extracts against *C. albicans* are shown in Fig.4.3 and their corresponding R_f values are shown in Table 4.1. Both BEA, CEF and EMW solvent systems had few and less visible clear bands of inhibition against *C. albicans.* This might be an indication of antifungal compounds present in low concentrations or that they do not react with the p-Iodonitrotetrazolium violet (INT) (Eloff et al., 2005). Aqueous plant extracts developed in FAWE solvent system had poor activity against *C. albicans* since no bands of inhibition were observed (results no 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 lipid soluble membranes (Eloff et al., 2008). Our group has acknowledged the difficulties of getting good bioautograms against fungi (Suleiman et al., 2010). However, number of active compounds against *C. albicans* was 53 representing only 9 % of all the components separated by BEA, CEF, EMW and FAWE solvent systems. Amongst the 53 active compounds, 72% (38) were relatively polar compounds separated by EMW solvent system with R_f values ranging from 0.16 to 0.82, followed by 21% (11) components having intermediate polarity separated by CEF solvent system with R_f values ranging from 0.23 to 0.94 and the least 8% (4) non-polar components separated by BEA solvent system and their R_f values ranged from 0.11 to 0.34. Out of the fourteen plant species tested. *F.saligna* and *R. brasiliensis* had 8 active compounds each while *O. lanceolata, A. caffra* and *C. gratissimus* had 1 active component each. *C. glabrum* had 8 active compounds against *C. albicans,* 5 of them were observed in the hexane extract, 1 compound was observed in acetone extract, 2 components observed in methanol extract had the same R_f values as those observed in the hexane extract, 0.16 and 0.17 and this is indicative of similar compounds. Therefore, hexane extract of *C. glabrum* was targeted for the isolation of antifungal compounds and even its MIC value was 0.06 mg/ml in the previous experiments. The experience in our group has shown that most of the antimicrobial activity is found highly in non-polar compounds (Eloff, 2001).

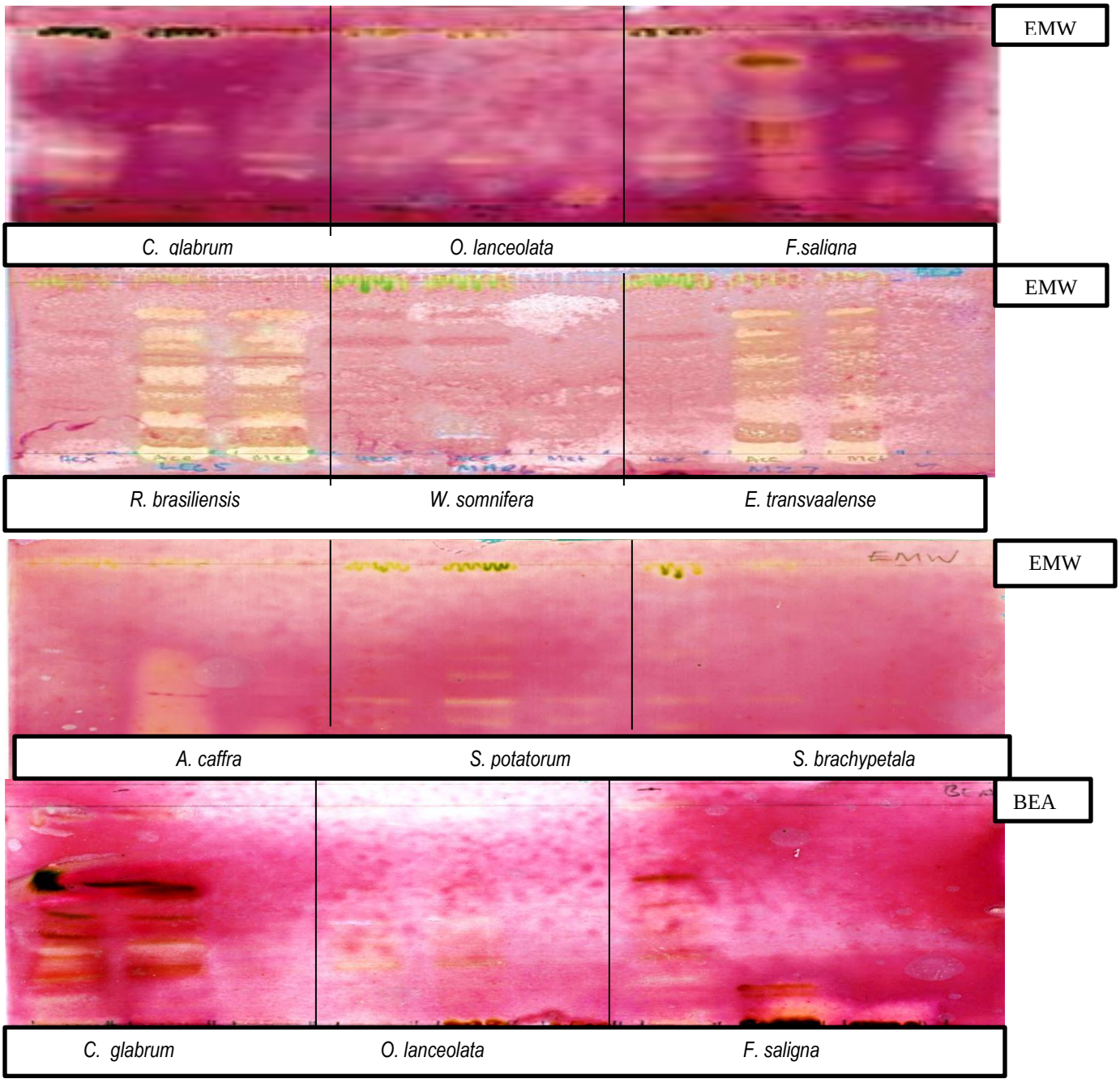

Figure 4.5. Bioautography of M1-*C. glabrum*, PE-*O. lanceolata,* TAN-*F. saligna,* LEG-*R. brasiliensis,* MAR-*W. somnifera,* MZ-*E. transvaalense,* MM-*A. caffra,* MG9-*S. potatorum,* MNZU-*S. brachypetala* , FNG-*C. gratissimus,* MTR-*H. longipetiolata,* LCL-*S. pinnata,* TP-*S. acmella,* MZL-*V. infausta* species extracted with hexane, acetone and methanol, separated by BEA, CEF, EMW and sprayed with *C alibicans* and 24 hrs later by INT. White spots indicate zones of inhibition by compounds of the plant extract after 24 hrs of incubation at 37 ^{0}C.

Table 4.1. Number of active antifungal bands and R_f values of the selected plant species against *C. albicans*

Plant	Extract	Solvent system (R_f values)											No of active components	Total
		BEA			CEF			EMW						
C. glabrum	Hex	0.11	0.29	0.34	-	-	-	0.16	0.17	_	_	_	5	8
	Ace	-	-	-	-	-	-	0.42	-	-	-	-	1	
	Met	-	-	-	-	-	-	0.16	0.17	-	-	-	2	
O. lanceolata	Hex	-	-	-	-	-	-		-	-	-			1
	Ace	-	-	-	-	-	-	0.17	-	-	-	-	1	
	Met	-	-	-	-	-	-	-	-	-	-	-		
F. saligna	Hex	0.21	-	-	-	-	-	0.17	0.26	-	-	-	3	10
	Ace	-	-	-	0.71	0.83	0.92	0.81	-	-	-	-	4	
	Met	-	-	-	0.84	0.94	-	0.81	-	-	-	-	3	
S. potatorum	Hex	-	-	-	-	-	-	0.29	-	-	-	-	1	6
	Ace	-	-	-	-	-	-	0.17	0.29	0.43	-	-	3	
	Met	-	-	-	-	-	-	0.17	0.29	-	-	-	2	
S. brachypetala	Hex	-	-	-	-	-	-	0.16	0.29	-	-	-	2	2
	Ace	-	-	-	-	-	-	-	-	-	-	-	-	
	Met	-	-	-	-	-	-	-	-	-	-		-	

Table 4.1. cont...

Plant	Extract	Solvent system (R_f values)											No of active components	Total
		BEA			CEF			EMW						
Acacia caffra	Hex	-	-	-	-	-	-	-	-	-	-	-	-	1
	Ace	-	-	-	-	-	-	0.62	-	-	-		1	
	Met	-	-	-	-	-	-	-	-	-	-	-	-	
C. gratissimus	Hex	-	-	-	-	-	-	-	-	-	-	-		1
	Ace	-	-	-	-	-	-	-	-	-	-	-		
	Met	-	-	-	0.23	-	-	-	-	-	-	-	1	
H. longipetiolata	Hex	-	-	-	0.23	-	-	-	-	-	-	-	1	5
	Ace	-	-	-	0.27	0.44	0.53	-	-	-	-	-	3	
	Met	-	-	-	0.28	-	-	-	-	-	-	-	1	
R. brasiliensis	Hex	-	-	-	-	-	-	-	-	-	-	-		10
	Ace	-	-	-	-	-	-	0.19	0.45	0.63	0.71	0.82	5	
	Met	-	-	-	-	-	-	0.19	0.45	0.63	0.71	0.82	5	
V. infausta	Hex	-	-	-	-	-	-							6
	Ace	-	-	-	-	-	-	0.26	0.54	0.71	0.82		4	
	Met	-	-	-	-	-	-	0.26	0.72	-	-	-	2	
Grand Total		4			11			35						50

4.3.3. TLC bioautography of bacterial species

In the bioautography of bacterial species only acetone plant extracts of the selected plant species were tested because acetone is known to extract compounds with a wide variety of polarity from plants, easy removal of solvent after extraction and safety to microorganisms in bioassays (Eloff, 1998). The results of bioautograms of *S. aureus* are shown in Fig.4.4 and their corresponding R_f values are shown in Table 4.2. The bioautograms of *E.faecalis* were not shown because they didn't come out well after several attempts. Bands of inhibition of active compounds against *S. aureus* were prominent in both BEA, CEF and EMW solvent systems. These findings are supported by the results obtained by Martin and Eloff (1998) and Suleimann et al. (2010) indicating that *S. aureus* has the highest number of inhibition bands. In the BEA solvent system clear bands of inhibition were near the base line, suggesting that the active compounds were relatively polar and EMW solvent system showed most inhibition bands near the solvent front, suggesting that the active components were relatively non-polar. Mdee et al. (2009) also observed the same trend in the investigation of the activity of extracts of seven common invasive plant species on fungal phytopathogens.

Total number of active compounds against *S. aureus* was 103, 36 compounds were separated by BEA solvent system, followed by CEF solvent system with 35 compounds and the least was EMW solvent system with 32 compounds. The use of solvent systems which differ in polarity, BEA (non-polar), CEF (intermediate) and EMW (polar) has managed to separate an average of 34 active compounds which were non-polar, intermediate and polar. This is supported by Eloff (1998), when he asserted that the larger the variety of compounds that are extracted by the extractant, the better the chance that biologically active components will also be extracted if a specific class of chemical component is not targeted.

Amongst the plant species tested against *S. aureus, W. somnifera* had the highest number of active compounds (14) and R_f values ranged from 0.26 to 0.97, followed by *F. saligna* with 10 active compounds, R_f values ranged from 0.17 to 0.95. Plant species which had fewer active components were *S. acmella* and *V.infausta* with 3 components each, R_f values of the former ranged from 0.51 to 0.84 and of the latter, R_f values ranged from 0.82 to 0,89. As shown by bioautograms of the three solvent systems (Fig.4.4), plant extracts had a good activity against *S. aureus* which is known to be resistant to a number of antibiotics (Ejikeme et al., 2010). However, in CEF solvent system, traces of formic acid are suspected to be also involved in inhibiting the growth of the *S.aureus* (Masoko and Eloff, 2005).

The results of bioautograms of *P. aeruginosa* are shown in Fig.4.5 and their corresponding R_f values are shown in Table 4. 3. Both BEA, CEF and EMW solvent systems had less intense clear zones of bands on bioautograms and total number of active compounds were very few (27), representing only 5% of the separated compounds. Similar trend of activity was displayed by *E. coli* with a total of 28 active compounds (Table 4.4) (Bioautograms results not shown). In general, most plant extracts had at least one or two activecompounds against either *P. aeruginosa* or *E. coli.* These results have shown that Gram negative bacteria (*P.aureginosa, E. coli*) were resistant to the diversity of compounds of plant extracts separated on the TLC plates than Gram positive bacteria. This may be attributed to the fact that cell wall in Gram positive bacteria consists of a single layer, whereas, gram negative cell wall is a multilayered structure bounded by an outer cell membrane (Grosvenor et al., 1995). In contrast to the investigation done by Shai et al. 2008, it is reported that the plant species screened had activity against both Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative (*P. aeruginosa* and *E. coli*) bacterial species.

Compounds of plant extracts tested here had few antimicrobial compounds because some showed no bands of inhibition, some had less visible clear bands of inhibition and large clear bands of inhibition were not observed. The non-activity or low activity of these plant extracts in bioautography could be explained by volatilization or oxidation of antifungal compounds during the extended removal of the TLC eluents or the very low concentration of the active compounds in the crude extract under the tested conditions or by the disruption of synergism between active constituents caused by TLC separation (Mdee et al., 2009, Masoko and Eloff, 2006).

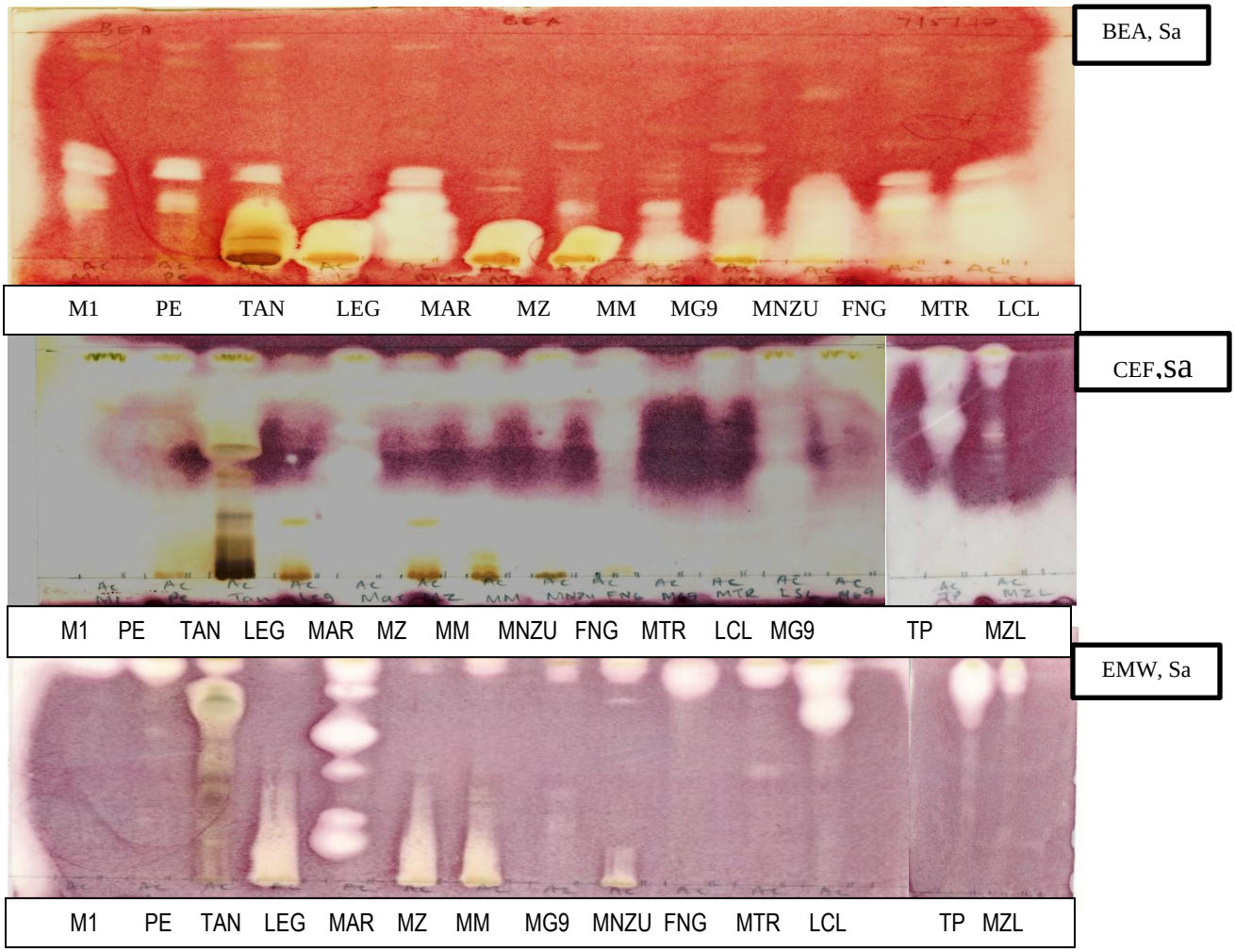

Figure 4.4. Bioautography of M1-*C. glabrum*, PE-*O. lanceolata,* TAN-*F.saligna,* LEG-*R. brasiliensis,* MAR-*W. somnifera,* MZ-*E. transvaalense,* MM-*A. caffra,* MG9-*S. potatorum,* MNZU-*S. brachypetala,* FNG-*C. gratissimus,* MTR-*H. longipetiolata,* LCL-*S. pinnata,* TP-*S. acmella,* MZL-*V.infausta* species extracted with acetone, separated by BEA (top), CEF (middle) and EMW (bottom) and sprayed with *S. aureus* and 24 hrs later by INT. White spots indicate zones of inhibition by components of the plant extract after 60 mins of incubation at 37 ^{o}C.

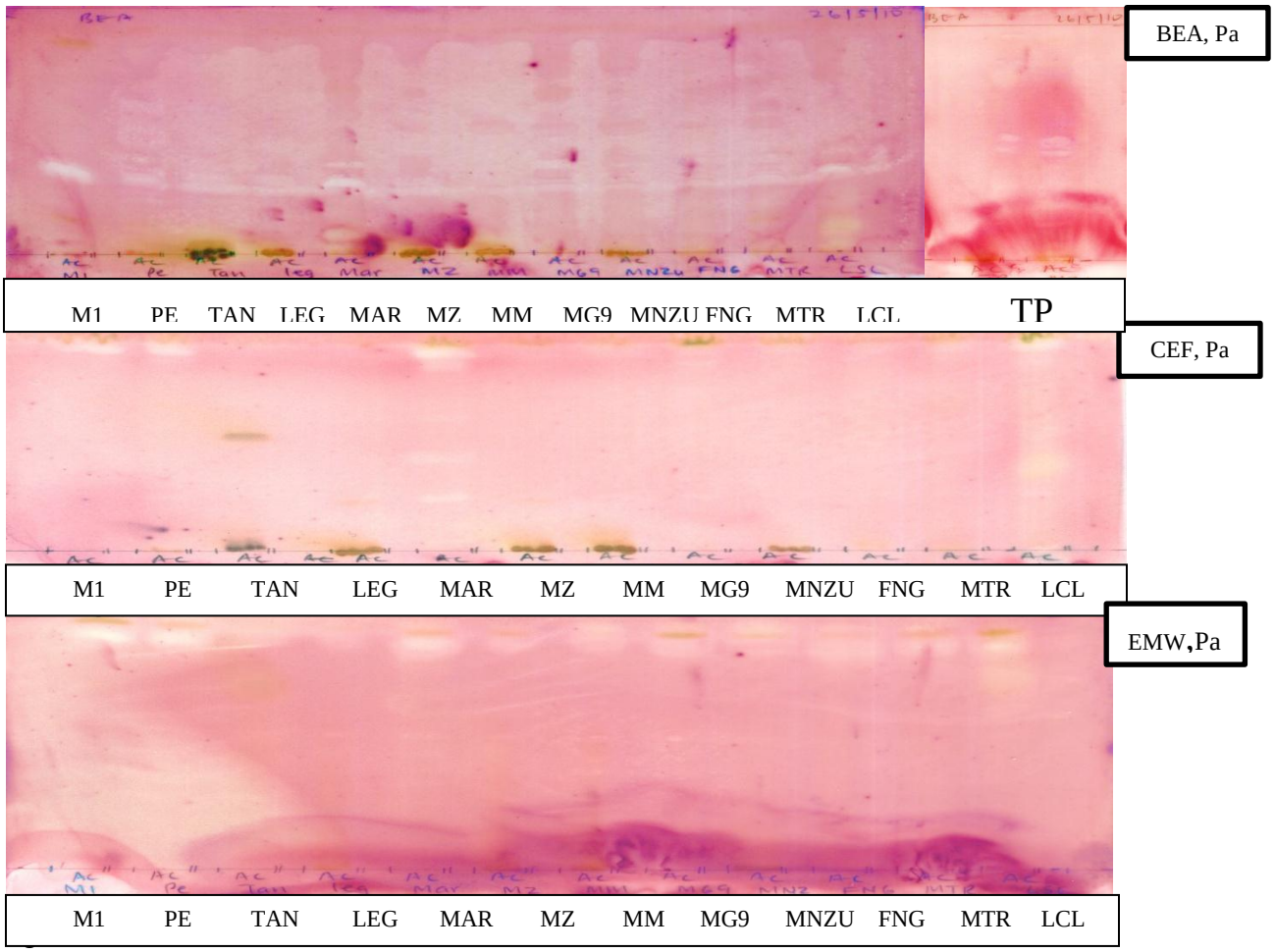

Figure 4.6. Bioautography of M1-*C. glabrum*, PE-*O. lanceolata,* TAN-*F. saligna,* LEG-*R. brasiliensis,* MAR-*W. somnifera,* MZ-*E. transvaalense,* MM-*A. caffra,* MG9-*S. potatorum,* MNZU-*S. brachypetala,* FNG-*C. gratissimus,* MTR-*H. longipetiolata,* LCL-*S. pinnata,* TP-*S. acmella,* MZL-*V. infausta* species extracted with acetone, separated by BEA (top), CEF (middle) and EMW (bottom) and sprayed with *P. aeruginosa* and 24 hrs later by INT. White spots indicate zones of inhibition by components of the plant extract after 60 min of incubation at 37 °C.

Table 4.2. Number of active antibacterial bands and R_f values of the selected plant species against *S. aureus*

Acetone extract	Solvent system (R_f values)														No of active components
	BEA				CEF				EMW						
C. glabrum	0.34	0.45	0.51	-	0.85	-	-	-	0.95	-	-	-	-	-	5
O. lanceolata	0.31	0.45	0.89	0.97	0.63	0.91	-	-	0.95	-	-	-	-		7
F. saligna	0.17	0.28	0.45	0.95	0.4	0.59	0.74	0.91	0.79	0.95	-	-	-	-	10
R. brasiliensis	0.17	-	-	-	0.31	0.85	-	-	0.1	0.21	0.95	-	-		6
W. somnifera	0.28	0.34	0.45	0.97	0.51	0.63	0.8	0.91	0.26	0.35	0.53	0.68	0.84	0.95	14
E.transvaalense	0.17	0.36	0.45	-	0.8	0.91	-	-	0.1	0.21	0.95	-	-	-	8
A. caffra	0.17	0.28	0.57	-	0.65	0.8	0.91	-	0.1	0.21	0.95	-	-	-	9
S. potatorum	0.17	0.27	0.34	-	0.91	-	-	-	0.32	0.42	0.95	-	-	-	7
S. brachypetala	0.17	0.28	0.57	-	0.63	0.8	0.91	-	0.21	0.74	0.95	-	-	-	9
C. gratissimus	0.23	0.34	0.74	-	0.63	0.8	0.91	-	0.89	-	-	-	-	-	7
H. longipetiolata	0.23	0.34	0.45	-	0.8	0.9	-	-	0.56	0.95	-	-	-	-	7
S. pinnata	0.34	0.51		-	0.45	0.6	0.74	0.91	0.79	0.95	-	-	-	-	8
S. acmella	-	-	-	-	0.51	0.72	-	-	0.84	-	-	-	-	-	3
V. infausta	-	-	-	-	0.59	0.82	-	-	0.89	-	-	-	-	-	3
Total	12	11	10	36	14	12	6	35	14	9	6	1	1	32	103

Table 4.3. Number of active antibacterial bands and R_f values of the selected plant species against *P. aeruginosa*

Acetone extract	Solvent system (R_f values)													No of active components
	BEA				CEF				EMW					
C. glabrum	0.45	-	-	-	-	-	-	-	0.95	-	-	-	-	2
O. lanceolata	0.4	-	-	-	-	-	-	-	0.95	-	-	-	-	2
F. saligna	0.34	-	-	-	-	-	-	-	0.95	-	-	-	-	2
R. brasiliensis	0.34	-	-	-	-	-	-	-	0.95	-	-	-	-	2
W. somnifera	0.17	0.4	0.49		-	-	-	-	0.9	0.95	-	-	-	5
E.transvaalense	0.34	-	-	-	-	-	-	-	0.95	-	-	-	-	2
A. caffra	0.34	-	-	-	-	-	-	-	0.95	-	-	-	-	2
S. potatorum	0.38	-	-	-	-	-	-	-	0.95	-	-	-	-	2
S. brachypetala	0.38	-	-	-	-	-	-	-	0.95	-	-	-	-	2
C. gratissimus	0.4	-	-	-	-	-	-	-	0.95	-	-	-	-	2
H. longipetiolata	0.4	-	-	-	-	-	-	-	0.95	-	-	-	-	2
S. pinnata	0.45	-	-	-	-	-	-	-	0.9	-	-	-	-	2
S. acmella	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. infausta	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	12	1	1						12	1				27

Table 4.4. Number of active antibacterial bands and R_f values of the selected plant species against *E.coli*

Acetone extract	Solvent system (R_f values)												No of active components
	BEA				CEF				EMW				
C. glabrum	0.33	-	-	-	0.95	-	-	-	0.9	-	-	-	3
O. lanceolata	0.33	-	-	-	0.95	-	-	-	0.9	-	-	-	3
F. saligna	-	-	-	-	0.95	-	-	-		-	-	-	1
R. brasiliensis	-	-	-	-	-	-	-	-		-	-	-	
W. somnifera	-	-	-	-	-	-	-	-	0.9	-	-	-	1
E. transvaalense	-	-	-	-	-	-	-	-		-	-	-	
A. caffra	0.53	-	-	-	-	-	-	-	0.9	-	-	-	2
S. potatorum	-	-	-	-	-	-	-	-	0.9	-	-	-	1
S. brachypetala	0.56	-	-	-	-	-	-	-	0.9	-	-	-	2
C. gratissimus	0.33	-	-	-	-	-	-	-	0.9	-	-	-	2
H. longipetiolata	0.33	-	-	-	-	-	-	-		-	-		1
S. pinnata	0.28	-	-	-	0.95	-	-	-	0.9	-	-	-	3
S. acmella	0.6	-	-	-	0.9		-	-	0.68	0.9	-	-	4
V. infausta	0.56	-	-	-	0.84	0.9	-	-	0.9	0.95	-	-	5
Total	9				6	1			10	2			28

4.4. Conclusion:

The results obtained in this study indicated that compounds separated from different plant extracts had varying degree of effectiveness in inhibiting the growth of the tested pathogens. Even though our group has found that plants used traditionally to treat infections have low antimicrobial activity in *in vitro* assays (Makhafola and Eloff, 2012), these results justify the use of the plant species in the traditional medicine to some extent. Further study on these plants is important in order to isolate, identify, characterize and elucidate the structure of the bioactive compounds.

Postscript

Plant extracts had few active compounds against the tested fungal and bacterial pathogens. However, some plant extracts had prominent active compounds which could be targeted for isolation through bioassay-guided fractionation. The next step is to determine the minimum inhibitory concentration (MIC) of the plant extracts with a view of getting a total picture about the efficacy of the plant extracts.

Chapter 5.

Antifungal and antibacterial activity of some South African medicinal plants used traditionally to treat candidiasis.

N A Masevhe, M.M. Suleiman, L.J. McGaw, J.N. Eloff

Preface

The following section describes the determination of minimum inhibitory concentration (MIC) and the total activity (TA) of the plant species against the fungal pathogens that cause opportunistic infections. Nosocomial bacteria were also included as test organisms. This was done with a view of selecting the most active plant species for further investigation. This chapter has been prepared for submission to the African Journal of Traditional, Complementary and Alternative Medicines for publication.

Abstract

According to an ethnobotanical study carried out as part of this research, 14 plants used traditionally to treat candidiasis were collected. They were tested against fungal species (*Candida albicans* and *Cryptococcus neoformans*) and bacterial species (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*) using a two-fold serial microdilution method. Twenty four of 42 plant extracts, representing 57%, exhibited moderate to good microbial growth inhibition with minimum inhibitory concentrations (MICs) values ranging from 0.06 to 0.31 mg/ml. The hexane and the acetone extracts of *Withania somnifera* (L) Dunal, *Hippocratea longipetiolata* Oliv., *Clerodendrum glabrum* var *glabrum* and *Schkuhria pinnata* (Lam.) Thell. were the most active with MIC values ranging from 0.06 to 0.08 mg/ml. The most susceptible pathogen to the test samples was *C. neoformans* while *C. albicans* was resistant to most of the plant extracts. Average MIC values of the plant extracts against the tested fungi ranged from 0.06 to 1.88 mg/ml while the average total activity ranged from 22 to 684 ml/g. Only two water extracts, *Withania somnifera* and *Hippocratea longipetiolata* (14%) had MIC < 1 mg/ml against *C. albicans*. *C. neoformans* was susceptible to nine water plant extracts (64%) with MIC < 1 mg/ml and promising activity was observed in *Hippocratea longipetiolata* and *Faurea saligna* extracts with MIC values 0.16 and 0.31 mg/ml respectively.

In the antibacterial assays, plant extracts were more active against Gram-positive bacteria than Gram-negative bacteria. The hexane and the acetone extracts of *Withania somnifera*, *Croton gratissimus, Clerodendrum glabrum* and *Schkuhria pinnata* had good activity against Gram-positive *S.aureus* and *E. faecalis* with MIC value 0.08 mg/ml. In contrast, MIC values against Gram-negative *E. coli* and *P. aeruginosa* ranged from 0.16 to 1.25 mg/ml. Total activity of the plant extracts against the tested bacteria ranged from 66 to 1982 ml/g. Gram-positive bacteria (*E.faecalis* and *S.aureus*) were sensitive to 12 water plant extracts (86%) with an MIC <1 mg/ml while Gram-negative bacteria (*E. coli* and *P.aeruginosa*) were sensitive to eight water extracts only (57%) with the lowest MIC value 0.31 mg/ml. The investigated plants can be potential sources of anti-infective agents, thus providing some scientific rationale for the ethnomedicinal use of the plant species, although the non-polar extracts were more active than the aqueous extracts.

Key words: traditional medicine, plant extracts, antimicrobial, *Candida albicans, Cryptococcus neoformans*

5.1. Introduction

There has been an increasing incidence of fungal infections due to increased numbers of immunocompromised patients such as organ transplant recipients, as well as cancer and HIV/AIDS patients (Garcıa *et al.,* 2003). Development of opportunistic and superficial mycoses, such as candidiasis, cryptococcosis and aspergillosis caused by *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* respectively has increased dramatically in recent years (Portillo *et al*., 200). The most frequent fungal infections are oropharyngeal candidiasis (Sanglard *et al*., 1995), oral candidiasis (Hamza *et al*., 2006) and vaginal thrush (Shai *et al*., 2008). Cryptococcal meningitis caused by *Cryptococcus neoformans* remains a leading cause of death in HIV-infected patients in Africa and Asia (Harrison and Brouwer, 2009). *Cryptococcus* is the fourth leading cause of all opportunistic infections in HIV-infected individuals (Burden and Elewski, 1997) and its treatment failure rate is reported to be in the region of 10 to 25% (Paugam *et al*., 1994).

Fungal infections are usually treated with antifungal agents such as nystatin, flucytosine, azoles, echinocandins and amphotericin B (Eggimann *et al*., 2003). The management of fungal infections faces a number of problems including a limited number of effective, non-toxic and low cost antifungal drugs (Beringer and Kriengkauykiat, 2003; Naeini *et al*,. 2009). The development and spread of resistant microbial strains is also a major challenge in the treatment of infectious diseases with many available drugs. These factors prompt the need for development of new antifungal agents in order to widen the spectrum of activities against pathogenic fungi and combat strains expressing resistance to the available antifungal agents (Runyoro, 2006).

Medicinal plants have a long history of use in traditional healthcare worldwide for the treatment of different diseases. In some African countries, up to 90% of the population relies exclusively on medicinal plants as a source of medicines (Hostettmann *et al*., 2000). In South Africa, traditional healing is widely practiced by approximately 80% of the black population and forms the backbone of rural healthcare (York *et al*., 2011). Traditional medicine is used by many people because it is more accessible, more acceptable to them and is cheaper than orthodox medicine (Sofowora, 1996). Hamza *et al.* (2006) reported that plants used traditionally in the treatment of fungal infections and related ailments may contain phytochemicals which could be a good source for new, safe and renewable antifungal drugs. Antimicrobials of plant origin may be effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Kokoska *et al*., 2002). The World Health Organization is also encouraging research and development of new antimicrobial agents in order to combat the threat of drug resistance (WHO, 2001). Substances that can either inhibit the growth of pathogens or kill them and have little or no toxicity to host cells are considered good candidates for developing new antimicrobial drugs (Masoko *et al.,* 2005).

Although medicinal plants are used widely in South Africa and other developing countries, there is a very limited scientific support for their use in the treatment of microbial infections. A major emphasis on ethnopharmacological research in South Africa has been establishing the scientific rationale for traditional medicine and validating their use. This is largely due to the current trend which is moving towards the integration of traditional herbal medicine with primary healthcare (Light *et al*., 2005).

Fourteen medicinal plants used in South African traditional medicine were selected on the basis of recommendations by the traditional healers. Symptoms of thrush such as sore, painful mouth ulcers, burning tongue, white patches that appear as discrete lesions on the surfaces of the buccal mucosa, pain when swallowing food, vaginal milk-like discharges, and lesions around the vulva (Khosravi *et al*., 2007, Vazquez, 2000) were described to traditional healers so that they could give the appropriate plants they use to treat them. The plant species were tested against two important fungal pathogens, namely *Candida albicans* and *Crytococcus neoformans*; and were further tested against four important nosocomial bacteria: *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa.* These bacteria cause nosocomial infections such as diarrhoea and pneumonia, and have high fatality rates (Tenover, 2006, Mathabe *et al.,* 2006).

The main focus of the study was to test plant extracts against the fungal pathogen, *Candida albicans* that commonly afflicts immunocompromised individuals, in order to obtain the plant species with the most promising antifungal activity so that its active compounds could be isolated. This is important as a result of the increasing occurrence of opportunistic mycotic infections associated with AIDS (Eloff *et al*., 2008).

Table 5.1. Summary of ethnobotanical data of the selected plants

Botanical name	Local name	Voucher no.	Other ethnomedicinal uses
Acacia caffra Thunb. (Mimosaceae)	Murovhambado	RN 640	abdominal disorder in infants, infusion used for blood cleansing (Venter, 2007)
*Clerodendrum glabrum var. glabrum (*verbenaceae)	Mukhatshilonwe	RN 646	stomach troubles, chest pains, colds, sore throats (Hutchings *et al*. ,1996)
Croton gratissimus Burch. var. gratissimus(Euphorbiaceae)	Mafunyungule	RN 638	flu, fevers, aromatic, used as perfume, rheumatism, blue tongue (van Wyk, 1997)
Elaeodendron transvaalense (Burtt Davy) R.H. Archer (Celastraceae)	Mukuvhazwivhi	RN 641	sore throat, haemorrhoids, veneral diseases, coughs and diarrhoea (van Wyk *et al.*, 2000)
Faurea saligna Harv. (Proteaceae)	Mutango	RN 644	pneumonia, venereal diseases, rheumatism; bilharzia , diarrhoea (Gelfand *et al*., 1985)
Hippocratea longipetiolata Oliv.(Celastraceae)	Mutshaliri	RN 637	baby food mixture, invocation of ancestors (Mabogo,1990)
Osyris lanceolata Hochst. & Steud. (Santalaceae)	Mpeta	RN 645	gynaecological complaints, wounds, veneral diseases (Steenkamp, 2003
Richardia brasiliensis Gomes (Rubiaceae)	Mulegere	RN 642	Anti-emetic and diabetes treatment (Pinto *et al*.,2008)
Schkuhria pinnata (Lam.) ex Thell (Asteraceae)	Luswielo	RN 635	malaria, rheumatism, diabetes, wounds, influenza (Watt and Breyer-Brandwijk ,1962)
Schotia brachypetala Sond. (Fabaceae)	Mununzu	RN 639	diarrhoea, heart diseases, stop bleeding nose, ulcers (Venter, 2007)
Spilanthes acmella (L.) Murray (Asteraceae)	Tshingelaphofu	RN 636	toothache; diarrhoea; fungal infections, arthritis; ulcers (Saraf and Dixit, 2002)
Strychnos potatorum L.f. (Loganiaceae);	Mukongovhoti	RN 634	aphrodisiac, gonorrhoea, diarrhoea, diabetes, wounds (van Wyk *et al.,* 1990)
Vangueria infausta Burch. subsp. Infausta (Rubiceae)	Muzwilu	RN 643	fever, aphrodisiac, malaria, pneumonia, sores (Venter, 2007, de Boer *et al*., 2005)
Withania somnifera (L.) Dunal (Solanaceae)	Musalamarubini	RN 633	wounds, asthma, rheumatism, tuberculosis (van Wyk *et al*., 2009, Arora *et al*., 2004)

5.2. Materials and methods

5.2.1. Plant materials

Medicinal plants listed in Table 5.1 cited by the traditional healers during interviews were collected with their assistance from four different locations in Venda, Limpopo province, South Africa. Plant species were collected in their natural habitats during summer between August and October 2009. They were identified by a botanist from the University of Venda and were further authenticated by SANBI (South African National Biodiversity Institute) in Pretoria. Voucher specimens were deposited at the herbarium of the Department of Botany, University of Venda and voucher numbers are quoted in Table 5.1.

5.2.2. Plant drying and storage

For the conservation of plant species, only leaves were harvested for investigation in this study, although traditional medical practitioners preferred to use stem bark and roots. The leaves were dried at room temperature. Dried leaves were ground into a fine powder using a Macsalabmill (Model 200 LAB), Eriez®, Bramley. They were stored at room temperature in airtight containers in the dark until required.

5.2.3. Extraction of plant materials

The ground leaf materials (1.0 g) were separately extracted with 10 ml of n-hexane, acetone and methanol (technical grade, Merck) in a shaking machine (Labotec model 20.2) at a moderate speed. The ground leaf materials (1.0 g) were also suspended in10 ml of distilled water and boiled for 20 min and then cooled down. Although some authors reported that water extracts have limited antimicrobial activity (Kotze and Eloff, 2002), it is important to use it because the remedy for treating the infections is normally prepared as either a decoction or infusion (personal communication Ramugondo).

The extracts and residual plant materials were centrifuged at 3 500 rpm for 10 min, then filtered using filter paper (Whatman No. 1) into pre-weighed labelled containers. The process was repeated three times on the same plant material to exhaustively extract the phytochemicals and the plant extracts were then combined. The extracts were dried under a stream of air in a fume hood at room temperature to quantify the extraction process. Water plant extracts were put in the freezer overnight at -40 ^{0}C and then freeze dried.

5.2.4. Test microorganisms

Fungal cultures of *Candida albicans* and *Cryptococcus neoformans* were obtained from the Microbiology Laboratory (Department of Veterinary Tropical Diseases, University of Pretoria). *C. albicans* was isolated from a Gouldian finch and *C. neoformans* was isolated from a cheetah. The cultures were maintained in Sabouraud Dextrose (SD) agar at 4 ^{0}C (Oxoid, Basingstoke, UK) and were inoculated in SD broth (Oxoid, Basingstoke, UK) and incubated at 37 ^{0}C prior to conducting microdilution assays. Bacterial test organisms used were two Gram-positive bacteria, *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212), and two Gram-negative bacteria, *P.aeruginosa* (ATCC 25922) and *E. coli* (ATCC 27853). They were maintained on Mueller Hinton (MH) agar (Oxoid, Basingstoke, UK) at 4 ^{0}C and cultured in MH broth (Fluka, Switzerland) at 37 ^{0}C before use.

5.2.5. Antimicrobial tests

A two-fold serial microdilution method was used to determine the minimum inhibitory concentration (MIC) values for plant extracts against bacteria (Eloff 1998a) and fungi (Eloff, 1998a), as modified by Masoko *et al*., (2005).

5.2.6 Antifungal assay

The hexane, acetone and methanol plant extracts were dissolved in acetone to a concentration of 10 mg/ml. Acetone dissolves hydrophilic and lipophilic components easily and has low toxicity to microorganisms (Eloff, 1998b). Aqueous plant extracts were dissolved in distilled water to a concentration of 10 mg/ml. Fungal cultures were inoculated in fresh SD broth and incubated at 37 ^{0}C for three days prior to conducting the assay. Densities of fungal cultures used were approximately: 2.5 × 10^4 cfu/ml for *C. albicans* and 2.6 × 10^4 cfu/ml for *C. neoformans* (Suleiman *et al*., 2010). Plant extracts (100 µl) in triplicate were serially diluted two-fold with sterile distilled water in 96-well microtitre plates. Thereafter, 100 µl of the fungal culture was added to each well. Amphotericin B was used as positive control and acetone was used as a negative control.

To indicate growth of microorganisms, 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium violet (INT, Sigma) was added to each well. The covered microplates were then incubated for two days at 37 ^{o}C in 100% relative humidity. The minimum inhibitory concentration (MIC) was taken as the lowest concentration of the extract that inhibited fungal growth after 24 and 48 h. The experiment was repeated twice in order to verify the results.

5.2.7. Antibacterial assay

The two-fold serial microdilution method was used to determine the minimal inhibitory concentration (MIC) values for plant extracts (Eloff, 1998a). Aliquots (100 µl) of plant extracts in triplicate were serially diluted two-fold with sterile distilled water in 96-well microtitre plates and 100 µl of freshly prepared bacterial culture in MH broth was added to each well. Densities of bacterial cultures used were approximately: 2.6×10^{12} cfu/ml for *S. aureus*; 1.5×10^{10} cfu/ml for *E. faecalis*; 5.2×10^{13} cfu/ml for *P. aeruginosa* and 3.0×10^{11} cfu/ml, *E. coli* (Suleiman *et al*., 2010). Acetone was used as negative control and gentamicin was used as a positive control. The covered microtitre plates were incubated overnight at 37 ^{o}C. Thereafter, 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium violet (INT) was added to each well to indicate microbial growth. The microtitre plates were further incubated at 37 ^{o}C and minimal inhibitory concentration (MIC) was determined 1 and 2 h after the addition of *p*-iodonitrotetrazolium violet (INT). MIC was determined as the lowest concentration of plant extract inhibiting microbial growth, indicated by a decrease in the intensity of the red colour of the formazan (Eloff, 1998a; Shai *et al*., 2008).

5.3. Results and discussion

Scientific names, families, voucher specimen numbers, local names in Tshivenda and other ethnomedicinal uses of the plant species used by the traditional medical practitioners to treat candidiasis and related ailments are presented in Table 5.1.

5.3.1. Mass extracted

Methanol extracted the highest quantity of plant material in almost all the plant species, acetone was intermediate and hexane extracted the least. The greatest amount of plant material was extracted with methanol from *F. saligna*, 396 mg (41%), while the least was extracted with hexane from *A. caffra* 4 mg (1%) (Fig.5.1). These results are comparable with data reported by Suleiman *et al*., (2010) where methanol extracted the highest quantity of plant material from *L. alata,* 218 mg and the lowest amount of extract was obtained from the hexane extraction of *K. wilmsii*, 20 mg of extractable material. Methanol was also quantitatively reported as the best extractant by Masoko *et al*., (2007).

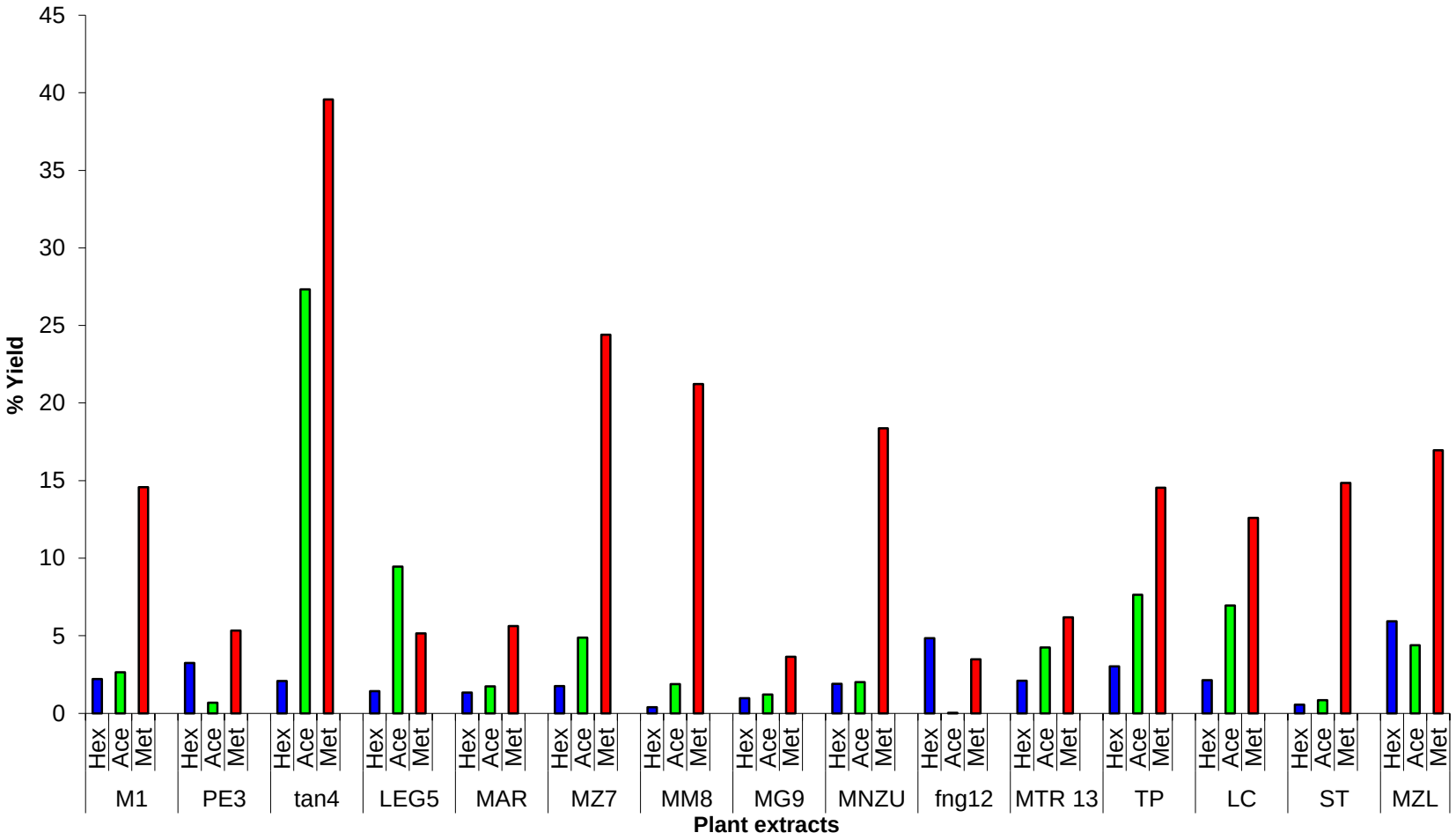

Figure 5.1. %yield extracted by hexane, acetone and methanol solvents from leaves of the selected plants.

Hex–hexane, Met–methanol, Ace–acetone, M1-*C. glabrum,* PE-*O. lanceolata,* TAN-*F.saligna,* LEG-*R. brasiliensis,* MAR-*W.somnifera,* MZ-*E. transvaalense,* MM–*A.caffra,* MG-*S. potatorum,* MNZ-*S. brachypetala,* FNG - *C.gratissimus,* MTR -*H .longipetiolata,* TP –*S. acmella,* LC –*S. pinnata,* MZL-*V.infausta*

5. 3.2. Minimum inhibitory concentration (MIC)

5.3.2.1. Fungal species

The results for the screening for antifungal activity are presented in Table 5.2. Cos *et al.* (2006) and Rís and Recio (2005) recommended that the criterion for the activity of all anti-infective bioassays should be MIC values below 0.1 mg/ml for plant extracts. However, MIC values of plant extracts in the present study were classified as follows: MIC ≤ 0.08 mg/ml: good antimicrobial activity, 0.1 ≤ MIC ≤ 0.31 mg/ml: moderate antimicrobial activity, MIC ≥ 0.4 mg/ml: poor antimicrobial activity.

A total of 42 plant extracts from 14 ethnobotanically selected plants were investigated. Plant species that had a good antifungal activity against either *C. albicans* or *C. neoformans* were *W. somnifera*, *H. longipetiolata, C. glabrum* and *S. pinnata* (29%). The hexane and the acetone extracts of *C. glabrum* were strong inhibitors of both *C. albicans* and *C. neoformans.* The hexane and the acetone extracts of *W. somnifera* were strong inhibitors of *C. neoformans* only, while the hexane extracts of *H. longipetiolata* and *S. pinnata* had a good antifungal activity against *C. neoformans* only. MIC values of the above-mentioned plant extracts ranged from 0.06 to 0.08 mg/ml (Table 5.2). In contrast to the investigation done in our laboratory by Shai et al. (2008), some plant extracts had the highest activity against fungal test organisms (*C. albicans*, *C. neoformans* etc) with MIC values as low as 0.02 mg/ml.

Literature reports on the antifungal activity of *C. glabrum* are scarce, however, other biological activity such as anthelmintic activity (McGaw *et al.,* 2000) and weak inhibition of α-*glucosidase* (Tshikalange *et al*., 2008) have been reported. It is generally accepted that when active compounds are found in one species, more species of the same genus are likely to contain active compounds of a similar nature (Lindsey *et al*., 1999). The hexane leaf extracts of *C. inerme* and *C. phlomidis* inhibited the growth of human pathogenic fungi such as *E. floccosum, T. rubrum and T. tonsurans*, and plant pathogens such as *A. niger, A. flavus* and *F. oxysporum.* The active constituents isolated from *C. inerme* were inerminoside-A1, inerminoside-D, 3-iridoid glycosides, 22E-trien-3β-ol, sterols and megastigmane glycosides (Anitha and Kannan, 2006). Thus, it is also possible that the activity of *C. glabrum* reported in this investigation could be attributed to similar or related compounds found in *C. inerme.*

A previous study by Kambizi and Afolayan (2008) reports that the methanol root extract of *W. somnifera* was effective against *C. albicans* at a concentration of 20 mg/ml. In this study the methanol leaf extract had some antifungal activity at 0.94 mg/ml; this was quite low compared to 20 mg/ml. The methanolic root extract of the same plant was also active against *C. albicans* using the agar well diffusion method (Jain and Varshney, 2011).

Antifungal activity of *W. somnifera* against phytopathogenic fungi is also recorded (Singh *et al*., 2010; Parkash *et al.,* 2005) and antiviral activity (Vermani and Garg, 2002). In the study done by Quiroga *et al*. (2001), the ethanolic extract of *S. pinnata* did not inhibit the growth of filamentous fungi such as *L. elegans, S. commune*, *F. oxysporum*, *P. notatum*, *A. niger* and *Trichoderma* spp. but in this investigation the hexane leaf extract of *S. pinnata* had a notable activity against *C. neoformans* with MIC value of 0.06 mg/ml. The activity of the latter may be explained in terms of tannins present in the plant (Fernandes *et al*., 2008). The disparity in the activity of this plant species may be attributed to the type of organic solvent used. In spite of an extensive search, it was not possible to find literature reports on the antifungal activity of *H. longipetiolata* against *C. albicans* or C. *neoformans.* However, the methanol root bark extract of its close relative, *H. indica,* was evaluated for its bioactivity and was inactive against *C. albicans* (Ogbole *et al*., 2007).

The acetone and the hexane extracts of *F. saligna, E. transvaalense, R. brasiliensis, S. potatorum*, *C. gratissimus, S. acmella* and *V.infausta* had moderate antifungal activity against either *C. albicans* or *C. neoformans* with MIC values ranging from 0.12 to 0.31 mg/ml. This represented 50% of the plant species which were investigated. Previous studies reported that the methanol bark extract of *E. transvaalense* had some activity against clinical *C. albicans* using the plate-hole diffusion assay (Steenkamp *et al.,* 2007). The hexane bark extract of the same plant had strong activity against *C. albicans* isolates (MIC = 0.46 mg/ml) while the acetone extract did not show any activity (Samie *et al*., 2010). In our case the hexane and the acetone leaf extracts of the same species had antifungal activity with MIC values 0.12 mg/ml and 0.4 mg/ml respectively under our assay conditions. Anti-HIV property of the same plant has also been reported (Tshikalange *et al*., 2008; Bessong *et al*., 2005). *E. transvaalense* bark contains tannins (McGaw *et al*., 2000) and high tannin content has been reported to inhibit growth of different micro-organisms (Fabry *et al*., 1998). It is also possible that the antifungal activity of the acetone and the hexane extracts of *E. transvaalense* observed in this study might be attributed to tannins. A study done by de Boer *et al*. (2005) reports that the ethyl acetate root extract of *V. infausta* had slight activity against *C. albicans* while the methanol extract had no activity against the same pathogen. The activity of *V.infausta* against plant pathogenic fungal species was also recorded (Mahlo *et al.,* 2010). Other biological activity reported included anthelmintic and antiplasmodial activity (Clarkson *et al.,* 2004; de Boer *et al*., 2005).

The activity of *R. brasiliensis* may be attributed to alkaloids, tannins, saponins, steroids, terpenes and flavonoids known to be present in Rubiaceae family (Pinto *et al*., 2008). *S. acmella* had some activity against plant fungi using the well diffusion method (Arora *et al.,* 2011). The dichloromethane and the methanol leaf, bark and root extracts of *C. gratissimus* had some activity against *C. albicans* and *C. neoformans* with MIC values ranging from 2-6 mg/ml (Van Vuuren and Naidoo, 2010). In this study, the acetone leaf extract of *C. gratissimus* had a higher activity with MIC values 0.31 and 0.12 mg/ml for *C. albicans* and *C. neoformans* respectively. Not much is known regarding

antifungal activity of *S. potatorum,* however, a recent study conducted on its close relative (Mallikharjuna *et al*., 2010) showed that extracts of *S.wallichiana* seeds and leaves had significant activity against *A. niger* and *Mucor* sp. using the agar well diffusion method.

Plant species that had poor antifungal activity were *A. caffra*, *S. brachypetala* and *O. lanceolata*; their MIC values were ≥ 0.4 mg/ml. The methanol extracts of almost all plant species tested had a poor antifungal activity against the tested pathogens (Table 5.2). This suggests that the active compounds of the plant extracts may either be among the non-polar or intermediately polar compounds as they occur in both hexane and acetone plant extracts. Previous studies report that the hexane bark extract of *S. brachypetala* had some activity against *C. albicans* and C. *neoformans* with MIC values of 3.75 and >7.5 mg/ml respectively (Samie *et al*., 2010). In our study the hexane leaf extracts of the same plant species had MIC values 0.94 and 0.24 mg/ml against *C. albicans* and *C. neoformans* respectively. The difference in the antifungal activity may be attributed to the different plant parts used because it is reported that the level of bioactivity and biologically active compounds can vary in different plant parts at different seasons (Ncube *et al.,* 2010). Insignificant activity of the *O. lanceolata* extract against *C. albicans* has been reported in a recent study (Mulaudzi *et al*., 2011), MIC values ranged from 1.56 – 6.25 mg/ml; in this study an MIC value f 0.31 mg/ml has been recorded for the acetone leaf extract. Not much is reported regarding the activity of *A.caffra*, however, in the study conducted by Hamza *et* al. (2006), its close relative *A. robusta* exhibited strong activity against *C. neoformans* but no activity has been detected against *C. albicans.*

The most sensitive pathogen was *C. neoformans* and the number of extracts with good activity against the organism was six with MIC values ranging from 0.06 to 0.08 mg/ml, this is in agreement with what has been reported by Shai et al. (2008). *C. albicans* had a high resistance to the plant extracts, it was sensitive to two plant extracts only, with MIC values ranging from 0.06 to 0.08 mg/ml (Table 5.2). Average MIC values of the plant extracts against the tested fungi ranged from 0.06 to 1.88 mg/ml. The positive control, amphotericin B, recorded low MIC values for *C. albicans* and *C. neoformans* with average values of 0.04 mg/ml and 0.02 mg/ml respectively. This confirms the superiority of amphotericin B over the plant extracts investigated as an antifungal agent. The negative control, acetone, did not inhibit the growth of the pathogens at the concentrations tested. The activity of the plant extracts was fungistatic because the test pathogens appeared to overcome the inhibition ability of the plant extracts and started growing again after 48 h of incubation. This might be attributed to the decomposition of active compounds necessitated by the extended period of incubation or growth was possibly promoted by contamination (Masoko *et al*., 2007).

In this study it was found that 39 out of 42 plant extracts prepared with organic solvents had average MIC values <1 mg/ml except the methanol extracts of *S. potatorum*, *C. gratissimus* and the hexane extract of *E. transvaalense* had average MIC values ranging from 1.1 to 1.9 mg/ml. The hexane and acetone extracts of *C. glabrum*; hexane extracts of *S. pinnata* and *S. potatorum* had the lowest average MIC values against *C. albicans* and *C. neoformans* with values of 0.06, 0.1, 0.11 and 0.12 mg/ml respectively.

The aqueous plant extracts had poor activity against both *C. albicans* and *C. neoformans*. Only three plant extracts *W. somnifera, F. saligna* and *H. longipetiolata* (21%) had some moderate activity against the tested pathogens with MIC values ranging from 0.16 to 0.31 mg/ml and the rest of the plant extracts (86 %) had MIC of > 1 mg/ml. Lack of antifungal activity by aqueous plant extracts may be due to boiling of plant extracts during extraction. This is consistent with what has been reported by Motsei *et al.* (2003) where a boiled extract of *A. sativum* had a total loss of activity whilst an extract of *P. myrtifolia* showed a decrease in activity after boiling. Fawole *et al.* (2009) also did not detect any antimicrobial activity in aqueous plant extracts which were tested. In this study *C. neoformans* was susceptible to nine aqueous plant extracts (64%) with MIC values < 1 mg/ml while *C. albicans* had some resistance to the aqueous plant extracts because it was susceptible to two plant extracts only (14%). Other researchers have also noted a high resistance of *C. albicans* to plant extracts (de Boer *et al*., 2005; Hamza *et al*., 2006; Kambizi and Afolayan, 2008). The fungal pathogens inhibited by plant extracts in this investigation are associated with candidiasis and cryptococcal meningitis occurring mostly in immunocompromised individuals. The local traditional healers treat the aforementioned infections with the tested medicinal plants and this provides some scientific rationale for the ethnomedicinal use of these plant species even though the traditional mode of preparing plant extracts was not followed.

Table 5.2. Minimum inhibitory concentrations (MIC) of plant species using four different extractants against *C. albicans and C. neoformans*

Organism	Time (h)	MIC (mg/ml)																			
		C. glabrum				*O. lanceolata*				*F. saligna*				*R..brasiliensis*				*W.* somnifera			
		H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W
Ca	24	**0.06**	**0.08**	0.47	1.25	0.63	0.31	0.94	1.25	0.12	0.63	0.94	1.25	0.31	0.63	0.63	1.25	0.31	0.24	0.94	0.31
	48	**0.06**	0.16	0.47	1.25	0.63	0.31	0.94	1.25	0.12	0.63	0.94	1.25	0.31	0.63	0.63	1.25	0.31	0.24	0.94	0.31
Cn	24	**0.06**	**0.08**	0.47	0.63	0.47	0.24	0.63	1.25	0.16	0.31	0.94	0.31	0.47	0.16	0.47	0.63	**0.08**	**0.08**	0.63	0.63
	48	**0.06**	**0.08**	0.47	0.63	0.47	0.24	0.63	1.25	0.16	0.31	0.94	0.31	0.47	0.31	0.47	0.63	0.16	**0.08**	0.63	0.63
Average		**0.06**	0.1	0.47	0.94	0.55	0.28	0.79	1.25	0.14	0.47	0.94	0.78	0.39	0.43	0.55	0.94	0.22	0.16	0.79	0.55

Table 5.2. Minimum inhibitory concentrations (MIC) of plant species using four different extractants against C. albicans and C. neoformans (Continued)

Organism	Time (h)	MIC (mg/ml)																			
		E. transvaalense				*A. caffra*				*S. potatorum*				*S. brachypetala*				*C. gratissimus*			
		H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W
Ca	24	0.12	0.4	0.47	1.25	0.94	1.25	0.63	1.25	0.12	0.24	1.25	2.5	0.94	0.31	0.47	1.25	0.94	0.31	1.25	1.25
	48	0.12	0.4	0.47	1.25	1.88	1.25	0.63	1.25	0.12	0.24	1.25	2.5	0.94	0.31	0.47	1.25	1.25	0.31	1.25	1.25
Cn	24	2.5	0.16	0.47	0.63	0.47	0.24	0.47	0.63	0.12	0.12	2.5	1.25	0.24	0.24	0.63	0.63	0.31	0.12	0.63	1.25
	48	2.5	0.16	0.47	0.63	0.47	0.24	0.47	0.63	0.12	0.12	2.5	1.25	0.24	0.24	0.63	1.25	0.31	0.12	1.25	1.25
Average		1.31	0.28	0.47	0.94	0.94	0.75	0.55	0.94	0.12	0.18	1.88	1.88	0.59	0.28	0.55	1.1	0.7	0.22	1.1	1.25

Table 5.2. . Minimum inhibitory concentrations (MIC) of plant species using four different extractants against C. albicans and C. neoformans (Continued)

Organism	Time (h)	MIC (mg/ml)															
		H. longipetiolata				*S. acmela*				*S. pinnata*				*V. infausta*			
		H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W
Ca	24	0.24	0.16	0.63	0.63	0.31	0.31	0.63	1.25	0.16	0.31	0.94	1.25	0.24	0.31	0.94	1.25
	48	0.24	0.16	0.63	0.63	0.31	0.31	0.63	1.25	0.16	0.31	0.63	1.25	0.24	0.31	0.94	2.5
Cn	24	**0.08**	0.24	0.47	0.16	0.12	0.24	0.63	0.63	**0.06**	0.16	0.47	1.25	0.12	0.12	0.24	1.25
	48	**0.08**	0.24	0.47	0.16	0.12	0.24	0.63	0.63	**0.06**	0.16	0.47	1.25	0.12	0.12	0.24	1.25
Average		0.16	0.2	0.55	0.4	0.22	0.28	0.63	0.94	0.11	0.24	0.63	1.25	0.18	0.22	0.59	1.56

Average MIC for positive control amphotericin B was 0.04 mg/ml for *C. albicans* and 0.02 mg/ml for *C.neoformans*,

H-hexane; A-acetone, M-methanol, W-Water, Ca – C.albicans, Cn – C. neoformans

5.3.2.2. Bacterial species

The results of the antibacterial activity studies of the plant species are given in Table 5.3. All plant species investigated had some degree of antibacterial activity against the test microorganisms. The hexane and the acetone extracts of *W. somnifera*, *C. gratissimus, C. glabrum*, and *S. pinnata* had good antibacterial activity against at least one of the Gram-positive bacteria with MIC values as low as 0.08 mg/ml (Table 5.3). The activity of *W. somnifera* and *S. pinnata* confirms the findings by Arora *et al*. (2004) and the activity of the former has been attributed to the presence of withaferin A and withanolide D (Arora *et al*., 2004). The ethanolic extract (aerial parts) of *W. somnifera* had some activity against *M. smegmatis* with MIC value of 1 mg/ml (Gautam *et al*., 2007). In the study for antibacterial efficacy of *W. somnifera* by Owais *et al*. (2005), the methanol as well as the aqueous extracts had strong activity while the hexane fraction was not effective at all against the bacteria tested using agar well diffusion method. However, in this study the hexane leaf extract had good activity against Gram-positive bacteria, *S. aureus* with an MIC value as low as 0.08 mg/ml. Reports on the activity of the methanol leaf extract of *C. gratissimus* against *S. aureus, E. faecalis, P. aeruginosa and E. coli* with MIC ranging from 1-6 mg/ml (van Vuuren and Viljoen, 2008) support the present findings but MIC values recorded in this study for the activity of the methanolic leaf extract against pathogens tested ranged from 0.47 -0.94 mg/ml.

The hexane and the acetone extracts of *O. lanceolata*, *E. transvaalense*, *A. caffra*, *S. potatorum*, *S. brachypetala, F. saligna, H. longipetiolata*, *S. acmella* and *V. infausta*, had moderate antibacterial activity against at least one of the Gram-positive test bacteria with MIC values ranging from 0.12 to 0.24 mg/ml (Table 5.3). Previous work indicated that the ethanol leaf extract of *S. brachypetala* had activity against *S.aureus* (MIC = 0.195 mg/ml), ethanol bark extract of *E. transvaalense* had strong activity against *S. aureus* with MIC 0.098 mg/ml (McGaw *et al*., 2000). In this study the hexane and the acetone leaf extracts of *S. brachypetala* had activity with MIC value of 0.16 mg/ml respectively; the hexane and the acetone leaf extracts of *E. transvaalense* had some activity against *S.aureus* and *E. faecalis* with MIC of 0.12 mg/ml and also recorded MIC values of 0.16 and 0.63 mg/ml against *P.aeruginosa* and *E. coli* respectively. Antibacterial activity of both *S. brachypetala and E. transvaalense* might be attributed to tannins present in the bark (McGaw *et al*., 2000), which might also be present in the leaves. In a recent investigation, the ethanol leaf extract of *F. saligna* had some activity against *B. subtilis* with MIC value of 0.5 mg/ml and there was no activity detected against *S. aureus* (Chitemerere and Mukanganyama, 2011). In this study the methanol extract of the same plant had some activity with MIC value 0.31 mg/ml. Little is known about the antibacterial activity of *H. longipetiolata* and *S. potatorum,* however, the methanol root bark extract of a close relative of the former, *H. indica,* is reported to have pronounced activity against *S. aureus* and *Pseudomonas* (Ogbole *et al*., 2007). In another recent study, a close relative of *S. potatorum, S. wallichiana* is reported to have activity against *E. coli* and *S. aureus* at 2 mg/ml and 4 mg/ml concentrations using the agar well diffusion method and the activity is attributed to the presence

of alkaloids, phenols, glycosides, steroids, sterols owing to their synergistic action (Mallikharjuna *et al*., 2010). It is likely that the activity of *H. longipetiolata* and *S. potatorum* observed in this investigation might be attributed to similar or related compounds found in *H. indica* and *S. wallichiana* respectively. Mulaudzi *et al*. (2011) has reported the activity of *O. lanceolata* extract against *S. aureus* and *E. coli* with MIC values ranging from 0.78 – 6.25 mg/ml. In this study the antibacterial MIC values ranged from 0.12 – 0.63 mg/ml.

The majority of the plant extracts investigated had a poor antibacterial activity against Gram-negative test bacteria, *E. coli* and *P.aeruginosa,* except hexane and acetone extracts of *C. glabrum* and *F. saligna* which had moderate activity with MIC values ranging from 0.16 to 0.2 mg/ml (Table 5.3). Thus, the results in this study indicated that the antibacterial activity of plant extracts was more pronounced against Gram-positive than against Gram-negative bacteria. These findings correlate with the observations of previous screenings of medicinal plants for antibacterial activity (Palombo and Semple, 2001, Kokoska *et al*., 2002, Tshikalange *et al*., 2005). This is mainly attributed to differences in their cell walls (Ali-Shtayeh *et al*., 1998). In the negative control, acetone, no inhibition of growth was observed, confirming no toxicity in bioassays (Eloff, 1998). The positive control gentamicin had the highest activity when compared to the plant extracts tested; the lowest MIC recorded was 0.2 µg/ml, and this shows the superiority of the antibiotic as an antibacterial agent although it is difficult to compare the activity of a purified compound with that of an extract.

No significant antibacterial activity was observed in the methanol extracts of most plant species against the tested bacteria except in methanol extracts of O. *lanceolata* and *S. brachypetala* which had activity against *S.aureus* with MIC values of 0.12 mg/ml and 0.16 mg/ml respectively. Average MIC values of the plant extracts against the tested bacteria ranged from 0.13 mg/ml to 0.63 mg/ml. Generally, the aqueous plant extracts had less antibacterial activity than organic plant extracts. This may be due to the fact that active substances might be in low concentrations in the aqueous plant extracts or active substances were soluble in organic solvents and therefore not present in high concentrations in aqueous plant extracts (Bhattacharjee *et al.,* 2011). Poor antibacterial activity of aqueous plant extracts was also reported by Vuuren and Naidoo (2010) and Ncube *et al.,* (2010).

Table 5.3. Minimum inhibitory concentrations (average of triplicate determinations) of four different plant extracts from the selected plant species tested against bacteria.

Organism	Time (h)	MIC (mg/ml)																		
		C. glabrum				*O. lanceolata*				*F. saligna*				*R. brasiliensis*				*W. somnifera*		
		H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W
Ec	1	0.16	0.16	0.47	1.25	0.47	0.24	0.63	1.25	0.16	0.16	0.33	0.31	0.31	0.31	0.63	0.31	0.31	0.24	0.63	1.25
	2	0.16	0.16	0.47	1.25	0.47	0.24	0.63	1.25	0.16	0.16	0.33	0.31	0.31	0.31	0.63	0.31	0.31	0.24	0.63	1.25
Pa	1	0.16	0.16	0.31	0.63	0.47	0.24	0.63	0.63	0.2	0.2	0.47	0.31	0.47	0.31	0.47	0.31	0.24	0.24	0.24	1.25
	2	0.16	0.16	0.31	1.25	0.47	0.24	0.63	0.63	0.2	0.2	0.47	0.31	0.47	0.31	0.47	0.31	0.24	0.24	0.24	1.25
Ef	1	**0.08**	**0.08**	0.31	0.63	0.16	0.12	0.63	0.63	0.12	0.12	0.63	0.63	0.24	0.24	0.31	0.63	0.16	0.16	0.24	0.63
	2	0.16	0.16	0.31	0.63	0.16	0.12	0.63	0.63	0.12	0.12	0.63	0.63	0.24	0.24	0.31	0.63	0.16	0.16	0.24	0.63
Sa	1	**0.08**	0.12	0.31	0.63	0.12	0.12	0.12	0.63	0.12	0.12	0.31	0.31	0.12	0.12	0.47	0.63	**0.08**	**0.08**	0.31	0.63
	2	**0.08**	0.12	0.31	0.63	0.12	0.12	0.12	0.63	0.12	0.12	0.31	0.31	0.16	0.12	0.47	0.63	**0.08**	**0.08**	0.31	0.63
Average		0.13	0.14	0.35	0.86	0.31	0.18	0.5	0.79	0.15	0.15	0.44	0.39	0.29	0.25	0.47	0.47	0.2	0.18	0.36	0.94

Table 5.3. Minimum inhibitory concentrations (average of triplicate determinations) of four different plant extracts from the selected plant species tested against bacteria (continued)

Organism	Time (h)	MIC (mg/ml)																		
		E. transvaalense				*A. caffra*				*S. potatorum*				*S. brachypetala*				*C. gratissimus*			
		H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W
Ec	1	0.63	0.24	0.63	0.31	0.24	0.47	0.63	0.63	0.31	0.24	0.94	0.63	0.47	0.31	0.47	0.63	0.63	0.24	0.47	1.25
	2	0.63	0.24	0.63	0.31	0.24	0.47	0.63	0.63	0.31	0.24	0.94	0.63	0.47	0.31	0.47	0.63	1.25	0.24	0.47	1.25
Pa	1	0.16	0.2	0.63	0.31	0.24	0.24	0.33	1.25	0.31	0.31	0.63	0.63	0.31	0.31	0.31	0.63	0.31	0.24	0.94	1.25
	2	0.16	0.2	0.63	0.31	0.33	0.33	0.33	1.25	0.31	0.31	0.63	0.63	0.31	0.31	0.31	0.63	0.31	0.24	0.94	1.25
Ef	1	0.12	0.12	0.24	0.31	0.24	0.24	0.24	0.63	0.12	0.16	0.24	0.63	0.24	0.24	0.24	0.63	0.16	0.16	0.47	1.25
	2	0.12	0.12	0.24	0.31	0.24	0.24	0.24	0.63	0.12	0.16	0.24	0.63	0.24	0.24	0.24	0.63	0.16	0.16	0.47	1.25
Sa	1	0.12	0.12	0.47	0.16	0.16	0.12	0.24	0.63	0.16	0.16	0.24	0.31	0.16	0.16	0.16	0.63	**0.08**	**0.08**	0.47	0.63
	2	0.12	0.12	0.47	0.16	0.16	0.12	0.24	0.63	0.16	0.16	0.24	0.31	0.16	0.16	0.16	0.63	0.16	0.16	0.47	0.63
Average		0.26	0.17	0.49	0.27	0.23	0.28	0.36	0.79	0.23	0.22	0.51	0.55	0.3	0.26	0.3	0.63	0.38	0.19	0.59	1.1

Table 5.3. Minimum inhibitory concentrations (average of triplicate determinations) of four different plant extracts from the selected plant species tested against bacteria (continued)

Organism	Time (h)	MIC (mg/ml)																
		H. ongipetiolata				*S. acmella*				*S. pinnata*				*V. infausta*				gent
		H	A	M	W	H	A	M	W	H	A	M	W	H	A	M	W	(ug/ml)
Ec	1	0.24	0.31	0.63	0.31	0.24	0.24	0.47	1.25	0.47	0.47	0.94	0.63	0.31	0.24	0.94	1.25	6.0
	2	0.24	0.31	0.63	0.31	0.24	0.24	0.47	1.25	0.47	0.47	0.94	0.63	0.31	0.24	0.94	1.25	8.0
Pa	1	0.31	0.31	0.63	0.31	0.24	0.24	0.47	1.25	0.24	0.24	0.63	1.25	0.47	0.24	0.63	1.25	0.3
	2	0.31	0.31	0.63	0.31	0.24	0.24	0.47	1.25	0.24	0.24	0.63	1.25	0.47	0.24	0.63	1.25	0.3
Ef	1	0.24	0.24	0.47	0.31	0.16	0.16	0.31	0.63	**0.08**	0.16	0.47	0.63	0.16	0.12	0.47	0.63	1.6
	2	0.24	0.24	0.47	0.63	0.16	0.16	0.31	0.63	0.16	0.16	0.47	0.63	0.16	0.12	0.47	0.63	1.6
Sa	1	0.16	0.16	0.47	0.31	0.12	0.12	0.31	1.25	0.16	**0.08**	0.47	0.63	0.12	0.12	0.47	1.25	0.2
	2	0.16	0.16	0.47	0.63	0.12	0.12	0.31	1.25	0.16	**0.08**	0.47	1.25	0.12	0.12	0.47	1.25	0.2
Average		0.24	0.26	0.55	0.39	0.19	0.2	0.39	1.1	0.25	0.24	0.63	0.86	0.27	0.18	0.63	1.1	

Ec - Escherichia. coli , Pa – *Pseudomonas aeruginosa, Ef - Enterococcus faecalis, Sa - Staphylococcus aureus,* gent - gentamicin

5.3.2.3. Total activity

In order to compare the activity of different plant species, not only the MIC should be considered but also the quantity extracted from the plant should be brought into the equation (Eloff, 2004). Total activity indicates the degree to which the active compounds in 1 g of plant material can be diluted and still inhibit the growth of the tested microorganism and the unit is ml/g (Eloff, 2000). In bacterial species, the highest total activity was observed in the acetone extract of *F. saligna* against *S. aureus* and *E faecalis* with a value of 2367 ml/g respectively. This was followed by the acetone extract of the same plant species with a value of 1775 ml/g against *E. coli* and the lowest total activity was observed in the hexane extract of *A. caffra* against *P.aeruginosa* (45 ml/g). Average total activity of different plant extracts against the tested bacteria ranged from 66 to 1982 ml/g (Table 5.5).

In the fungal species, the highest total activity against C. *albicans* was observed in the hexane extract of *C. glabrum* (550 ml/g) and the lowest was found in *A. caffra* (8 ml/g) over a 48 h incubation period (Table 5.4). The highest total activity against *C. neoformans* was found in acetone extract of *F. salgna* (917 ml/g) while the lowest was observed in hexane leaf extract of *E. transvaalense* (11 ml/g). This means that 1 g of *F. salgna* acetone leaf extract can be diluted with 917 ml of acetone and still inhibit the growth of *C. neoformans*. The average total activity of different plant extracts against the tested fungi ranged from 22 to 684 ml/g.

The good antimicrobial activity of *C. glabrum* may be attributed to the presence of chemical constituents like polyphenols, glycosides, sapponins and steroids in the leaves. Polyphenols are reported to be responsible for the antimicrobial properties of some medicinal plants (Amusan, 2007). The antimicrobial activity of *C. glabrum* was not surprising because other members of the genus *Clerodedrum* have been reported to possess some activity. Gbedema *et al*. (2010) reported that the methanol extract of the aerial parts of *C. splenders* had activity against C. *albicans* with MIC ranging between 64 and 512 µg/ml. Extracts of roots, leaves and stem of *C. viscosum* were effective against some bacteria and fungi including C. *albicans* (Oly *et al*., 2011). The observed low antimicrobial activity against the tested pathogens might be attributed to the season at which plants were harvested or the conditions in which they grew. Soil composition and seasonal variations in temperature, light and water availability can greatly influence the biochemical composition of plants (Romero *et al*., 2005).

Table 5.4. Total activity (ml/g) of selected plants screened for antifungal activity

Organism	Time(h)	Total activity (ml/g)																	
		C. glabrum			*O. lanceolata*			*F. saligna*			*R. brasiliensis*			*W. somnifera*			*E. transvaalense*		
		H	A	M	H	A	M	H	A	M	H	A	M	H	A	M	H	A	M
Ca	24	550	469	334	69	57	68	265	451	433	81	167	99	80	118	72	238	149	543
	48	550	234	334	69	57	68	265	451	433	81	167	99	80	118	72	238	149	543
Cn	24	550	467	334	93	74	102	198	917	432	54	659	133	309	353	107	11	373	543
	48	550	467	334	93	74	02	198	917	432	54	659	133	155	353	107	11	373	543
Average		550	409	334	81	66	85	232	684	433	68	413	116	156	236	90	125	261	543

Table 5.4. Total activity (ml/g) of selected plants screened for antifungal activity (Continued)

Organism	Time (h)	Total activity (ml/g)														
		A. caffra			*S. potatorum*			*S. brachypetala*			*C. gratissimus*			*H.longipetiolata*		
		H	A	M	H	A	M	H	A	M	H	A	M	H	A	M
Ca	24	16	24	354	173	96	38	32	100	414	63	35	37	133	334	115
	48	8	24	354	173	96	38	32	100	414	48	35	37	133	334	115
Cn	24	32	124	475	173	192	19	125	129	309	18	92	73	400	223	155
	48	32	124	475	173	192	19	125	129	309	18	92	37	400	223	155
Average		22	74	415	173	144	29	79	115	362	37	64	46	267	279	135

Table 5.4. Total activity (ml/g) of selected plants screened for antifungal activity (Continued)

Organism	Time(h)	Total activity (ml/g)								
		S.acmella			*S. pinnata*			*V. infausta*		
		H	A	M	H	A	M	H	A	M
Ca	24	133	278	248	202	335	442	293	177	192
	48	133	278	248	202	335	217	293	177	192
Cn	24	344	359	248	538	503	291	585	456	752
	48	344	359	248	538	503	291	585	456	752
Average		239	319	248	370	419	310	439	317	472

Ca – C.albicans, Cn – C. neoformans

Figure 5.5. Total activity (ml/g) of the selected plant species screened for antibacterial activity

Organism	Time (h)	Total activity (ml/g)														
		C. glabrum			*O. anceolata*			*F. saligna*			*R. .brasiliensis*			*W. somnifera*		
		H	A	M	H	A	M	H	A	M	H	A	M	H	A	M
Ec	1	206	316	334	94	90	102	200	1775	1233	81	342	100	81	117	106
	2	206	238	334	94	90	102	200	1775	1233	81	342	100	81	117	106
Pa.	1	206	238	506	94	90	102	160	1420	866	53	342	134	104	117	279
	2	206	238	506	94	90	102	160	1420	866	53	342	134	104	117	279
Ef.	1	413	475	506	275	180	102	266	2367	646	104	442	203	156	176	279
	2	206	238	506	275	180	102	266	2367	646	104	442	203	156	176	279
Sa.	1	413	317	506	367	180	533	266	2367	1312	208	883	134	208	350	216
	2	413	317	506	367	180	533	266	2367	1312	156	883	134	208	350	216
Average		284	287	463	208	135	210	223	1982	1014	105	502	143	137	190	220

Table 5.5. Total activity (ml/g) of the selected plant species screened for antibacterial activity

Organisms	Time(h)	MIC (mg/ml)														
		A. caffra			*S.potatorum*			*S. brachypetala*			*C. gratissimus*			*H. longipetiolata*		
		H	A	M	H	A	M	H	A	M	H	A	M	H	A	M
Ec	1	63	64	354	68	96	51	64	100	415	95	46	98	133	174	116
	2	63	64	354	68	96	51	64	100	415	48	46	98	133	174	116
Pa	1	63	125	676	68	74	76	97	100	629	194	46	49	103	174	116
	2	45	91	676	68	74	76	97	100	629	194	46	49	103	174	116
Ef	1	63	125	929	175	144	200	125	129	813	375	69	98	133	225	155
	2	63	63	125	929	175	144	200	125	129	375	69	98	133	225	155
Sa	1	94	94	250	929	131	144	200	188	194	750	138	98	200	338	155
	2	94	94	250	929	131	144	200	188	194	375	69	98	200	338	155
Average		69	69	141	722	111	103	132	119	131	301	66	86	142	228	136

Table 5.5. Total activity (ml/g) of the selected plant species screened for antibacterial activity

Organisms	Time(h)	Total activity (ml/g)											
		S. acmella			*S. pinnata*			*V. infausta*			*E. Transvaalense*		
		H	A	M	H	A	M	H	A	M	H	A	M
Ec	1	171	358	334	68	172	146	226	229	193	46	250	405
	2	171	358	334	68	172	146	226	229	193	46	250	405
Pa	1	171	358	334	133	338	217	148	229	287	181	300	405
	2	171	358	334	133	338	217	148	229	287	181	300	405
Ef	1	256	538	506	400	506	291	438	458	385	241	53	1063
	2	256	538	506	200	506	291	438	458	385	241	500	1063
Sa	1	342	717	506	200	1013	291	583	458	385	242	500	543
	2	342	717	506	200	1013	291	583	458	385	242	500	543
Average		171	358	334	175	507.	236	349	344	313	178	388	604

Ec - Escherichia. coli , Pa – *Pseudomonas aeruginosa, Ef - Enterococcus faecalis, Sa - Staphylococcus aureus*

5.4. Conclusion

The findings in this study suggest that plant species investigated have potential antimicrobial activity which can be exploited for the isolation and development of new broad spectrum antimicrobial compounds. Of the 14 plant species investigated, six representing 43 % had significant antimicrobial activity against the tested pathogens, namely: *F. saligna, W. somnifera, C. gratissimus, C. glabrum, S.pinnata* and *H. longipetiolata*. The results lend some support to the traditional use of these plant species. The challenge for the rural communities is that it is unlikely that they could extract those compounds which are responsible for the activity in the hexane and the acetone extracts because water is generally the only solvent available to them and our findings showed that water extracts had poor activity against the tested pathogens. Compared to other plant species tested, *C. glabrum* was chosen for further studies in order to isolate and characterize the active antifungal compounds because it exhibited the lowest average MIC values of 0.06 mg/ml in the hexane extract and the highest total activity of 550 ml/g against *C. albicans*.

Postscript

Ethnomedicinal use of the plant species was authenticated to some degree because most plant species had some antimicrobial potency against the tested pathogens. It is also possible that a plant extract with low antimicrobial activity could be useful if it could enhance the immune system of the patient. The next step was therefore to determine the anti-oxidant activity that may be related to the immune boosting capacity of the plant species and to verify whether the antimicrobial activity observed here was due to toxicity of the plant extracts or not. The antimicrobial results obtained in this chapter would be communicated back to the traditional healers in a duly constituted meeting of the author and the traditional healers.

Chapter 6.

Evaluating cytotoxicity and antioxidant activity of the selected South African medicinal plant species

N.A. Masevhe, S.A. Ahmed, L.J. McGaw and J.N. Eloff

Preface

Seeing that the selected plant species are used widely to treat candidiasis and related infections on daily basis, it was deemed necessary to test for their toxicity. This is also an important preliminary step in the isolation of target compounds from the plant. Plant species were also evaluated for their antioxidant activity to determine their capacity to boost the immune system. This chapter has been prepared for submission to the Journal of Pharmaceutical Biology for publication.

Abstract

Fourteen plant species ethnobotanically selected on the basis of their use in traditional medicine to treat candidiasis were evaluated for their *in vitro* toxicity against the Vero monkey kidney cell line using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. They were also evaluated for their antioxidant potential using 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), 2, 2' azinobis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP). The acetone plant extracts (29%): *Spilanthes acmella* (L.) Murray*, Schkuhria pinnata* (Lam.) Thell*, Osyris lanceolata* Hochst. & Steud and *Schotia brachypetala* Sond. exhibited high cytotoxic activity with IC_{50} values of 13.7, 19.8, 21.6 and 28.3 µg/ml respectively. However, their IC_{50} values were higher than that of the positive control, doxorubicin, which was 9.87 µg/ml. The acetone plant extract that was relatively non toxic was *Faurea saligna* Harv with an IC_{50} value of 201 µg/ml. The rest of the acetone plant extracts (64%) had moderate cytotoxicity activity ($30 < IC_{50} < 100$ µg/ml). However, aqueous plant extracts did not show cytotoxicity against the Vero cells, with IC_{50} values ranging from 137 to > 500 µg/ml.

Plant species that showed a high level of antioxidant activity in the DPPH, ABTS and FRAP assays were *O. lanceolata, F. saligna*, *A. caffra* and *R. brasiliensis*. The aqueous leaf extracts of these plants had a considerable antioxidant activity than the organic leaf extracts in the qualitative assays. .However, the plant extracts had less antioxidant activity than the positive control used. The results obtained in this study revealed that a high proportion of the acetone plant extracts (93%) contained cytotoxic compounds in their crude plant extracts while aqueous plant extracts were generally non-toxic to the Vero cells. On the other hand, medicinal plant species tested in this study

showed some free radical scavenging activities using three different methods and can be considered as promising sources of natural antioxidants for medicinal uses.

Key words: antioxidant activity; ABTS; DPPH; FRAP; cytotoxicity; Vero cells

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6.1. Introduction

It has been known for a long time that before the advent of orthodox medicine, African people relied on herbs growing in and around them to take care of their health problems and in some cases, as a simultaneous source of food (Awodele et al., 2012. Traditional medicine is commonly used as it is an accessible and affordable treatment to the rural communities (Bussmann et al., 2011). In South Africa 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 the rural area (Elgorashi et al., 2003). The reliance on herbal medicine demands information on the toxicity of various plant preparations used traditionally to treat variety ailments (Awah et al., 2011).

Based on the long history of medicinal plant use, users of traditional medicines just accept that they are safe for human consumption, yet there is no knowledge on the potential toxicity of these indigenous plants (Street et al., 2008, Ljubuncic et al., 2005), particularly in the long term. Scientific research shows that some plants used in traditional medicine are potentially toxic, mutagenic and carcinogenic (Verschaeve and van Staden, 2008; Akinboro and Bakare, 2007; Taylor et al., 2001). Cases of poisoning due to traditional medicines are not uncommon in South Africa, many of which have resulted in significant morbidity and mortality; due to insufficient data, estimates of mortality vary widely from 8000 to 20 000 per annum (Popat et al., 2001).

In the present study the cytotoxicity of the ethnobotanically selected medicinal plants (Table 6.1) was evaluated in cell culture using the Vero monkey kidney cell line because it is widely available, easy to culture and has been adopted by many laboratories as a model for screening plant extracts and compounds for cytotoxicity (Nchu et al., 2011). This has been done against the background that there is an increasing trend worldwide to integrate traditional medicine with primary health care and the amount of information about the relative safety of herbal remedies is limited (Fennel et al., 2004). *In vitro* cytotoxicity is necessary to define basal cytotoxicity such as the intrinsic ability of the plant extract or compound to cause cell death as a result of damage to several cellular functions (Tshikalange and Husein, 2010). Furthermore, to establish the safe use of medicinal plants, toxicological bioassays are necessary to scientifically recommend non-toxic plants for human consumption and for drug discovery (Serpeloni et al., 2011). The selected medicinal plants (Table 6.1) were also evaluated for their antioxidant activity because the therapeutic benefit of medicinal plants is often attributed to their antioxidant properties (Ljubuncic et al., 2005).

Antioxidants have been reported to prevent oxidative damage caused by free radicals; they interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers (Patel et al., 2010). The use of natural antioxidants for the treatment and prophylaxis of free radical induced pathologies such as cancer, diabetes, arthritis, neurodegenerative diseases etc has certain advantages because most of these agents produce no side effects, possess low toxicity and effectively act upon the main factors damaging the vascular system (Korotkova et al., 2003, Wong et al., 2006). There is an increasing interest of researchers in searching for new sources of natural antioxidants from plants to replace the synthetic ones, for example butylated hydroxyanisole and butylated hydroxytoluene have been harmful to health due to their potential toxicity and carcinogenicity (Gan et al., 2010). Another point worth noting is that little information is available on the antioxidant properties of South African herbal medicinal plants (Steenkamp et al., 2005). The selected plant species were evaluated for their antioxidant activity using three different methods:1,1-diphenyl-2-picrylhydrazine radical scavenging assay (DPPH) , 2,2'azinobis-(3-ethylbenzthiazoline- 6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) because many authors assert that total antioxidant activity of plant extracts cannot be evaluated by using a single method, due to the complex composition of phytochemicals as well as of oxidative processes (Wangensteen et al., 2004, McGaw et al., 2007).

Table 6. 1. Summary of ethnobotanical data of the selected medicinal plants from literature.

Botanical name	Family	Local name	Voucher number	Ethnomedicinal uses
Acacia caffra Thunb.	Mimosaceae	Murovhambado	Rn 40	abdominal disorder (Venter, 2007)
Clerodendrum glabrum var. *glabrum*	Verbenaceae	Munukhatshilongwe	Rn 46	chest pains, colds, sore throats, coughs (Hutchings et al. ,1996, McGaw et al., 2008)
Croton gratissimus Burch. *var.* gratissimus	Euphorbiaceae	Mafunyungule	Rn 38	coughs, fever, syphilis, ear ache, pneumonia (van Vuuren and Viljoen, 2008, McGaw et al., 2008),
Elaeodendron transvaalense (Burtt Davy) R.H. Archer	Celastraceae	Mukuvhazwivhi	Rn 41	sore throat, haemorrhoids, venereal diseases, coughs, diarrhoea, Steenkamp, 2003)
Faurea saligna Harv.	Proteaceae	Mutango	Rn 44	stomach ache, diarrhoea (Muthee et al., 2011, Hamill et al., 2000)
Hippocratea longipetiolata Oliv.	Celastraceae	Mutshaliri	Rn 37	baby food mixture, invocation of ancestors (Mabogo,1990)
Osyris lanceolata Hochst. & Steud.	Santalaceae	Mpeta	Rn 45	infertility, wounds, venereal diseases (Steenkamp, 2003)
Richardia brasiliensis Gomes	Rubiaceae	Mulegere	Rn 42	anti-emetic and diabetes treatment (Pinto et al.,2008)
Schkuhria pinnata (Lam.) Thell	Asteraceae	Luswielo	Rn 35	eye infections, pneumonia, diarrhoea (Luseba et al., 2007)
Schotia brachypetala Sond.	.Fabaceae	Mununzu	Rn 39	dysentery, diarrhoea (McGaw et al.,2000)
Spilanthes acmella (L.) Murray	Asteraceae	Tshishengela phofu	Rn 36	cancer, toothache, HIV/AIDS (Wongsawatkul et al.,2008; Graham et al., 2000)
Strychnos potatorum L.f.	Loganiaceae	Mukongovhoti	Rn 34	fungal infections, inflammation, diabetes (Ekambaram et al., 2010; Moshi et al., 2007),
Vangueria infausta Burch.subsp. infausta	Rubiceae	Muzwilu	Rn 43	pneumonia, sores, abdominal pains, parasitic worms (de Boer et al., 2005)
Withania somnifera (L.) Dunal	Solanaceae	Musalamarubini	Rn 33	breast cancer, skin diseases, fever, diarrhoea (Maregesi et al., 2007; McGaw et al.,2008)

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6.2. Materials and methods

6.2.1. Plant materials

Medicinal plant species listed in Table 6.1 were selected on the basis of their traditional use to treat candidiasis and availability. They were collected with the assistance of local traditional healers from four different locations in Venda, South Africa. They were collected in their natural habitats during summer between September and November in 2009. In this study only leaves of the plant species were collected due to economic and conservation reasons. They were identified by a botanist from the University of Venda and were further authenticated by SANBI (South African National Biodiversity Institute) in Pretoria. Voucher specimens were deposited at the herbarium of the Department of Botany, University of Venda and are quoted in Table 6.1.

6.2.2. Plant drying and storage

Only leaves were harvested for investigation in this study in order to promote sustainable use of the plants, although traditional healers preferred to use stem bark and roots in the preparation of their remedy. The leaves were dried at room temperature. Dried leaves were ground into a fine powder using a Macsalabmill (Model 200 LAB), Eriez®, Bramley. They were stored at room temperature in airtight containers in the dark in the laboratory until needed.

6.2.3. Extraction of plant materials

The ground leaves (1.0 g) were separately extracted with 10 ml of acetone and methanol (technical grade-Merck) for 2 h in a shaking machine (Labotec model 20.2) at a moderate speed. The ground leaves (1.0 g) were also suspended in10 ml of distilled water and boiled for 20 min and then cooled down. The plant extracts and residual plant materials were centrifuged at 3 500 rpm for 10 min, then filtered using filter paper (Whatman No. 1) into pre-weighed labelled containers. The process was repeated three times to exhaustively extract the phytochemicals and plant extracts were then combined. Plant extracts were dried under a stream of air in a fume hood at room temperature. Water plant extracts were put in the freezer overnight at -40 ^{0}C and then put in the freeze drier for 24 h.

6.2.4. Evaluation of the cytotoxicity of plant extracts

The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reduction assay is widely used for measuring cell proliferation and cytotoxicity. MTT (yellow) is reduced into a formazan (purple) by viable cells. The colour intensity of the formazan produced, which is directly proportional to the number of viable cells, is measured using a spectrophotometer. Viable cell growth after incubation with known concentration of plant extract was determined using the tetrazolium-based colorimetric assay (MTT assay) as described by Mosmann (1983). Monkey Vero cells of a subconfluent culture were harvested and centrifuged at 200 x g for 5 min, and resuspended in growth medium to a density of 2 x 10^3 cells/ml. Minimal Essential Medium (MEM, Sigma) supplemented with 0.1% gentamicin and 10% foetal calf serum (Sigma) were used. A total of 200 µl of cell suspension was added into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Growth medium (200 µl) was added into wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37 ^oC in a 5% CO_2 incubator, until the cells were in the exponential phase growth. The medium was then removed from wells using a hypodermic needle and immediately replaced with 200 µl of test plant extract or doxorubicin hydrochloride (Pfizer Laboratories) (positive control) at various known concentrations (quadruplicate dilutions prepared in growth medium). The microtitre plates containing treated and untreated cells were incubated at 37 ^oC in a 5% CO_2 incubator for a defined contact period with various treatments.

After incubation, 30 µl MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates were incubated for a further 4h at 37 ^0C. The medium in each well was carefully removed without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µl DMSO to each well, followed by gentle shaking of the MTT solution. The amount of MTT reduction was measured immediately by detecting absorbance at 570 nm using a microplate reader (Versamax). The wells in column 1, containing only medium and MTT but no cells, were used to blank the reader. The LC_{50} values were calculated as the concentration of plant extract resulting in a 50% reduction of absorbance compared to untreated cells. Selective activity of the plant extracts was calculated as follows: Selectivity index (SI) = LC_{50}/MIC.

LC_{50} = lethal concentration that kills 50% of the Vero cells, MIC = minimum inhibition concentration values against *C. albicans* and *C. neoformans*. MIC values used to calculate selectivity Index (SI) of plant extracts were obtained from the previous investigation.

6.2.5. Evaluation of antioxidant activity

6.2.5.1. Qualitative 1,1-diphenyl-2-picrylhydrazine (DPPH) radical-scavenging assay

Qualitative screening for antioxidant activity was done using the DPPH radical according to the method described by Takao et al. (1994) with some slight modification. Briefly, thin-layer chromatography (TLC) plates (10 x 20 cm, aluminium-backed, Merk, Silica gel 60 F_{254}) were loaded with 10 µl of the plant extracts and dried before developing in the mobile phase of ethyl acetate/methanol/water: 40:5.4:4 (EMW) (Kotze and Eloff, 2002). The DPPH radical test was performed directly on thin-layer chromatography (TLC) plates by spraying with DPPH (0.2% (w/v) in methanol to reveal the antioxidant activity of the plant extract. A change of colour from the purple DPPH background to yellow indicates the presence of an antioxidant (Bors et al., 1992).

6.2.5.2. Quantitative DPPH free radical-scavenging method

The 2, 2-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay was conducted as described by Mensor et al. (2001) with some slight modification. Different concentrations of the plant extracts were prepared in methanol at concentrations ranging from19 -2000 µg/ml and 40 µl methanol solutions of the plant extracts and control, trolox at concentrations between 19 -250 µg/ml were added to 160 µl of DPPH dissolved in 25 µg/ml of methanol in a 96 well-microtitre plates and were allowed to react in the dark for 30 minutes. Thereafter, absorbance was taken at 518 nm for each plant extract concentration against a blank at 1, 10, 20 and 30 min with microtitre plate reader (Versamax). Values obtained were converted to percentage antioxidant activity (AOX A %) using the formula:

$$AOX\ A\% = 100 - \{[(AbS_{sample} - AbS_{blank}) \times 100] / AbS_{control}\}$$

Where: AbS_{sample} = absorbance of the sample, AbS_{blank} = absorbance of the blank, $AbS_{control}$ = absorbance of the control

Antioxidant activity was expressed as effective concentration fifty (EC_{50}) values. The lower the EC_{50} value, the more effective is the antioxidant activity (Maw et al., 2011). All determinations were performed in triplicate.

6.2.5.3. ABTS free radical-scavenging method

This assay is based on measuring the scavenging of 2, 2'-azinobis (3-methyl-benzothiazoline-6-sulfonic acid) (ABTS) radical into a colourless product, recording the absorbance at 734 nm and was conducted as described by Re et al. (1999) with some modifications. A solution of ABTS was prepared by dissolving 7.68 $\times 10^4$ µg of potassium persulphate ($K_2S_2O_4$) in 10 ml of distilled water and 1.32$\times$ 10^4 µg of ABTS in 10 ml of 50% methanol solution, and made up to 200 ml with 50% methanol, and was put in the dark at room temperature for 12 hours. ABTS radical

solution was diluted with 50% methanol solution to an absorbance of 0.7- 0.8 at 734 nm. Plant extract (40 µl) was serially diluted between the concentration range of 19 -2000 µg/ml in 96 well-microtitre plate and 160 µl ABTS solution was added to each well. The absorbance was taken after 6 min of reaction time (A_{t2}) and blank absorbance (A_{b2}) was prepared using the plant extracts without ABTS radical. Inhibition % of ABTS radical was calculated using the following formula:

%Inhibition = $\{[A_{o2} – (A_{t2} - A_{b2})]/ A_{o2}\} \times 100$

Log concentrations of the plant extracts in the reaction medium were plotted against the percentage inhibition. The EC_{50} value was obtained by interpolation from sigmoidal dose-response best fit curve using Graphpad. All determinations were performed in triplicate

6.2.5.4. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing capacity of the plant extracts was determined by direct reduction of potassium ferric cyanide ($K_3Fe_3(CN)_6$) to potassium ferrocyanide ($K_3Fe_2(CN)_6$). The increase in absorbance following the addition of excess ferric ion was measured as described by Benzie and Strain (1996) with some modification. The reaction medium (200µl) containing 40 µl of the plant extracts and positive control, trolox at concentration range between 19 -2000 µg/ml, 100 µl of 1.0M hydrochloric acid, 20 µl of 1% (w/v) of SDS, 30 µl of 1% (w/v) of potassium ferric cyanide was incubated for 20 min at 50 ^{O}C, cooled down to room temperature and finally 20 µl of 0.1% (w/v) of ferric chloride was added. The absorbance was taken at 750 nm and blank absorbance was taken by preparing the reaction medium the same way except the addition of ferric chloride. The reducing activity of plant extracts were taken as slope obtained from the line of best fit of the absorbance against concentration using the linear regression curve. High slope values showed that components present in the plant extracts were more effective in donating electrons.

Statistical Analysis

The data were presented as mean±standard deviation (SD) for at least two independent determinations in triplicate for each experiment. Statistical software, Graphpad was used to analyze the differences between the controls and the plant extracts and $p < 0.05$ was considered the limit for significance.

6.3.-Results and Discussion

Table 6.1 contains a list of the ethnobotanically selected medicinal plants that were investigated for cytotoxic and antioxidant properties and some of their traditional uses reported from literature are given.

6.3.1. Cytotoxicity of plant extracts

In this study we investigated the effects of crude plant extracts on the Vero monkey kidney cell line using the MTT assay, and the results are shown in Tables 6.2 and 6.3. The principle of this assay is based on the reduction of a soluble tetrazolium salt by mitochondrial dehydrogenase activity of viable cells into a soluble colored formazan product that can be measured spectrophotometrically after dissolution (Ariffin et al., 2009). The LC_{50} value was used as a parameter for cytotoxicity. It is expressed as a concentration required for inhibiting cell growth by 50%. The criterion for the cytotoxicity for the crude plant extracts, as established by the American National Cancer Institute (NCI), is an $LC_{50} < 30$ µg/ml after an exposure time of 72 h (Caamal-Fuentes et al., 2011, Talib and Mahasneh, 2010, Itharat et al., 2004). Thus, results in this study were interpreted as follows: highly toxic: $LC_{50} < 30$ µg/ml, moderately toxic: $30 < LC_{50} \leq 100$ µg/ml and non-toxic: $LC_{50} > 100$ µg/ml.

The acetone plant extracts that had the highest cytotoxicity to the monkey Vero cell line were 4 out of 14 (29%), these included *S. acmella, S. pinnata, O. lanceolata* and *S. brachypetala* and had LC_{50} values of 13.7±0, 19.88±0.001, 21.62±0.007 and 28.34±0.001µg/ml respectively. These values are within the cut off endpoint of the NCI criteria for cytotoxicity in the screening of crude plant extracts. McGaw et al. (2007) reported that the hexane bark, methanol bark and methanol leaf extracts of *S. brachypetala* had no toxic effect on brine shrimp larvae whereas the hexane leaf extract had some lethal effect with LC_{50} value of 3.3 mg/ml. In the present study the acetone leaf extract of the same plant species had a high level of toxicity against the Vero cell line with $LC_{50} < 30$ µg/ml. Polyhydroxystilbenes isolated from the heartwood of *S. brachypetala* which may also be present in the leaves might account for the observed cytotoxicity (Ajaiyeoba et al., 2006; McGaw et al., 2000). The discrepancy in the results may be due to the fact that the brine shrimp assay was used earlier instead of the cell line cytotoxicity assay used in the present study and toxicity to a crustacean model may differ quite significantly from that observed against a mammalian cell line. Furthermore, this might also be due to differences in extraction procedures and the natural variability in plants (Steenkamp and Gouws, 2006).

From the chemical point of view, eucannabinolide was isolated as the active constituent from the stem of *S. pinnata* and had the highest toxicity against the melanoma UACC62 cell line with $LC_{50} < 6.25$ ug/ml (Fouche et al., 2008). Thus, the cytotoxicity activity of *S. pinnata* observed in the present study might be attributed to eucannabinolide which may also be present in its aerial parts. Cytotoxicity of *S. acmella* is due to ferulic acid, scopoletin and 3-acetylaleuritolicacid which are reported as having a strong cytotoxic effect against human lung carcinoma A549 cells (Prachayasittikul et al., 2009). Previous studies on the cytotoxicity of *O. lanceolata* are ill-defined, however, triterpenoids reported to be present in the root bark (Yeboah et al., 2010) and pyrrolizidine alkaloids present in the family Santalaceae might account for the observed cytotoxic activity (Thoppil and Bishayee, 2011, Roeder, 1999). High levels of cytotoxicity observed on *S. acmella, S. pinnata, O. lanceolata* and *S. brachypetala* suggest that the

four plant species have the likelihood of yielding anticancer compounds. Their study against cancer cell lines needs to be undertaken because cancer is a major health problem worldwide which claims more than six million lives per year (Akhir et al., 2011). However, the LC_{50} values of these plant extracts were higher than that of the positive control, doxorubicin (9.87±0 µg/ml). Thus, the plant extracts were less toxic than doxorubicin.

Plant extracts that had moderate cytotoxicity activity against the Vero cell line were 9 out of 14 (64%). These included *V. infausta, H. longipetiolata, C. gratissimus, S. potatorum, A. caffra, E. transvaalense, W. somnifera, R. brasiliensis and C. glabrum* (Table 6.2) and the plant extract that was relatively non-toxic was *F. saligna* with LC_{50} value 201.17±0.004 µg/ml. This seems to support the traditional use of this plant in the treatment of fungal infections and other pathogenic conditions. The leaf extract of *W. somnifera* had the strongest cytotoxic activity against human ECV304 cells with LC_{50} values of 2.1 µg/ml (PE/EtOAc) and 1.1 µg/ml (MeOH), respectively (Al-Fatimi et al., 2005). However, in this study the acetone leaf extract of *W. somnifera* had moderate cytotoxic activity against Vero cells with LC_{50} value 42.5±0.003 µg/ml. The difference in the results may be attributed to the different solvents, cell lines used and the difference in the sensitivity of the plant extract to the cell lines. The methanol leaf extract of *S. potatorum* had some toxicity against brine shrimp with LC_{50} 87.6 µg/ml (Moshi et al., 2007) whereas in the present study, the acetone leaf extract was toxic against the Vero cell line with LC_{50} value 46.2±0.006 µg/ml. The toxicity of *S. potatorum* is most likely due to the alkaloid strychnine which is known to be present in the genus and the alkaloid diaboline present in the same plant species but with less toxicity than strychnine (Phillipe et al., 2004). Terpenoids and scopoletin are some of the secondary metabolites that account for the cytotoxicity of *R. brasiliensis* (Thoppil and Bishayee, 2011; Pinto et al., 2008). The ethanol stem bark extract of *E. transvaalense* had no toxicity against Vero cells with LC_{50} value greater than 100 µg/ml (Tshikalange and Hussein, 2010), but in the present investigation the acetone leaf extract of the same plant was mildly toxic with LC_{50} value 54.9±0.011µg/ml, Quinonoid triterpenes might account for the toxicity of this plant (Drewes and Mashimbye, 1993). Inconsistency of the results observed here may be ascribed to extraction method and the type of solvent used. However, cytotoxic components such as tingenin B, cardiac glycoside and elaeodendroside A are also reported to be present in the genus *Elaeodendron* (Maregesi et al., 2010; Anjaneyulu and Rao, 1980) while terpenes and alkaloids are reported to be present in the family Celastraceae (Browning and Wagner, 1978). The activity of *C. gratissimus* may be accredited to diterpenoids, triterpenoids, kaurane and isoquinoline alkaloids reported to be present in the genus and cembranolides from the stem bark of the same plant which had moderate activity against ovarian cancer cells (Mulholland et al., 2010; Block et al., 2004). Chemical constituents such as alkaloids, cardenolides and terpenoids reported to be present in the bark of *C. gratissimus* might account for the observed activity (Steenkamp et al., 2005). A recent report shows that related plant species such as *Croton cajucara, Croton regelianus* and *Croton caracasanus* contained compounds which exhibited cytotoxic effects on human cancer cell lines (Alonso-Castro et al., 2011).

Little information is available with respect to the cytotoxicity *V. infausta,* however, the ethanol leaf extract of this plant has been shown to be non toxic to brine shrimp with LC_{50} value 144.7 µg/ml (Moshi et al., 2010). The related species, *V. tomentosa*, was non-toxic to the HT29 cell line but mildly toxic to HeLa cells (Kamuhabwa et al., 2000). These results are not surprising because fruits of *V. infausta* are eaten by both people and wild animals while different parts of this plant are used traditionally for the treatment of malaria, wounds, menstrual and uterine problems, and genital swellings among others (Mbukwa et al., 2007). In contrast, in the present study the acetone leaf extract *V. infausta* was toxic with LC_{50} value 36.9±0 µg/ml. Alkaloids isolated from its bark which may also be present in the leaves might account for the activity observed here (Lindsey et al., 1999). Literature data on the cytotoxicity of *H. longipetiolata* is limited, however, root bark of its close relative *H. excels* contained triterpenoid quinone methides and hippocrateine I which showed cytotoxic activity in the brine shrimp lethality test, and the alkaloid emarginatine A had a significant cytotoxicity against KB cells (Navarrete et al., 2002). With regard to the plant species *C. glabrum*, reports in the literature about its cytotoxic activity are rare, however, diterpenes, triterpenes, sesquiterpenes, cyanogenic glycosides and pheophorbides reported to be present in the genus might account for its toxicity in the present study (Shrivastava and Patel, 2007). Anti-inflammatory activity which is a biological activity related to cytotoxicity has been documented for this plant (Jager et al., 1996; Steenkamp and Gouws, 2006). A recent study of a related plant species, *C. viscosum* showed that all crude extracts (leaf, stem and root) were lethal to brine shrimp (Oly et al., 2011). It is also interesting to learn that the DCM and MeOH leaf extracts of another closely related plant species, *C. myricoides* had strong antimutagenic properties at doses as low as 0.05 mg/ml (Verschaeve and van Staden, 2008). Plants that show antimutagenic properties should be considered as potentially safe for human consumption and may be able to ameliorate cancer risks from mutagens. Cytotoxicity of *A. caffra* is ill-defined in literature, however, a number of secondary metabolites: alkaloids, cyanogenic glycosides, terpenes and tannins reported to be present in the genus might account for the activity observed here (Thoppil and Bishayee, 2011; Seigler, 2003). *Acacia* species are also reported as having strong antimutagenic properties (Krishnaiah et al., 2011)

Although most acetone plant extracts proved to be toxic, it is worthy to note that cytotoxicity of plant extracts in *in vitro* does not necessarily translate into *in vivo* activity. This situation can be explained by the fact that the active principles of the plant extract could be metabolized or detoxified in the animal body cells, and in consequence lose the possible antineoplastic effects (Alonso-Castro et al., 2011).
However, the aqueous plant extracts prepared following the traditional approach had very low cytotoxicity against Vero cells with LC_{50} values ranging from 137 to > 500 µg/ml (Table 6.3) and can be categorized as being practically non-toxic. This may be attributed to the fact that in traditional medicine, most remedies are prepared as simple water extracts, thus avoiding potential toxic effects.

This seems to be in agreement with what has been reported by Fouche et al. (2008) in their study for the i*n vitro* anticancer screening of South African plants wherein they screened a total of 7500 plant extracts against a panel of three cell lines (breast MCF7, renal TK10 and melanoma UACC62) and found that the majority of aqueous plant extracts had no cytotoxicity activity against the cell lines tested. Similarly, Bessong et al. (2005) observed no cytotoxicity on 17 aqueous plant extracts tested against HeLaP4 cell line. A recent study by Akhir et al. (2011) reported that natural herbal medicines usually offer less undesirable side effects, more efficiency and less toxicity to consumers. This suggests that medicinal plants used in the present study are safe but should also be tested further against other cell lines.

Comparatively, 93% of organic plant extracts were toxic to Vero cells while 100% of aqueous plant extracts were non-toxic. This may suggest that cytotoxic components were present in higher concentrations in organic plant extracts than in aqueous plant extracts, or water as a solvent was incapable of extracting large amounts of cytotoxic components from plant material. Thus, toxicity results from an animal model will be important as a way to conclusively evaluate the safety of the plants used in the present study. It is worth noting that although some plants selected for this study have previously been shown to have some cytotoxic effects, they were not tested against monkey Vero cells with the exception of one plant species, *E. transvaalense.*

6.3.2. The Selectivity Index

The Selectivity Index (SI) of each plant extract was calculated using the following formula:

Selectivity Index (SI) = LC_{50}/MIC

LC_{50} = lethal concentration that kills 50% of the Vero cells, MIC = minimum inhibition concentration values against *C. albicans* and *C. neoformans*. The Selectivity Index (SI) is defined as the ratio of the cytotoxic LC_{50} value and the minimum inhibition concentration (MIC) value (Lusakibanza et al., 2010). It is generally considered that the biological efficacy of the plant extract or compound is not due to cytotoxicity when SI ≥ 10, it is the cut off point ensuring that overdose does not put the life of the patient in danger (Caamal-Fuentes et al., 2011). Worthy of note is that if the plant extract is too toxic, the results of the biological activity are not valid because they will have a low therapeutic value (Rizwana et al., 2010). The selectivity ratios of the organic plant extracts were poor because they ranged from 0.051 to 0.484 (Table 6.2) while those of aqueous plant extracts ranged from 0.26 to 1.68 (Table 6.3). These values suggest that plant extracts are suitable for external use only and caution should be exercised in the use of these plants by traditional healers to treat a variety of ailments of fungal and bacterial origin. However, McGaw et al. (2007) reported that as toxicity can be associated with pharmacological activity in lower doses, plants containing toxic constituents may have useful biological activities

Table 6. 2. Cytotoxicity of organic plant extracts

Plant extracts	Cytotoxicity	SI		
	LC_{50} ug/ml SD	Ca	Cn	Average
C. glabrum	58.17±0.015	0.485	0.364	0.425
O. lanceolata	**21.62±0.007**	0.07	0.09	0.08
F. saligna	201.17±0.004	0.319	0.649	0.484
R. brasiliensis	74.62±0.023	0.118	0.311	0.215
W. somnifera	42.48±0.003	0.177	0.531	0.354
E. transvaalense	54.99±0.011	0.137	0.344	0.241
A. caffra	60.89±0.017	0.049	0.254	0.152
S. potatorum	46.23±0.006	0.193	0.385	0.289
S. brachypetala	**28.34±0.001**	0.091	0.118	0.105
C. gratissimus	46.52±0.003	0.15	0.388	0.269
H. longipetiolata	32.49±0.002	0.203	0.135	0.169
S. acmella	**13.7±0**	0.044	0.057	0.051
S. pinnata.	**19.88±0.001**	0.064	0.124	0.094
V. infausta	**36.98±0**	0.119	0.308	0.214
Doxorubicin	**9.87±0**			

LC_{50}: concentration of extract that produced a 50% decrease of cell growth, SD: standard deviation, SI: Selectivity Index Ca: *Candida albicans*, Cn: *Cryptococcus neoformans.* Plant extracts in bold are very toxic.

Table 6 3. Cytotoxicity of aqueous plant extracts

Plant extracts	Cytotoxicity	SI		
	LC_{50} ug/ml SD	Ca	Cn	Average
C. glabrum	>500	0.4	0.79	0.59
O. lanceolata	>500±0.096	0.4	0.4	0.4
F. saligna	240±0.028	0.19	0.77	0.48
R. brasiliensis	137±0.009	0.22	0.29	0.26
W. somnifera	291±0.021	0.94	0.46	0.7
E. transvaalense	424.3±0.153	0.34	0.67	0.51
A. caffra	>500	0.4	0.79	0.59
S. potatorum	450.4±0.109	0.36	0.18	0.27
S. brachypetala	>500	0.4	0.79	0.59
C. gratissimus	>500	0.4	0.4	0.4
H. longipetiolata	>500	0.22	3.13	1.68
S. acmella	288±0.009	0.46	0.46	0.46
S. pinnata.	>500	0.4	0.4	0.4
V. infausta	>500	0.4	0.4	0.4
Doxorubicin	4.06±0.966			

LC_{50}: concentration of extract that produced a 50% decrease of cell growth, SD: standard deviation, SI: Selectivity Index Ca: *Candida albicans*, Cn: *Cryptococcus neoformans*

6.3.3.1. Qualitative DPPH radical-scavenging assay

A total of 14 plant species evaluated exhibited a variation of antioxidant activity in the aqueous and methanol plant extracts as indicated by the difference in the number of yellow bands observed. .The aqueous plant extracts which had prominent antioxidant activity included *F. saligna* with 8 compounds and R_f values ranged from 0.13 to 0.88, followed by *O. lanceolata, R. brasiliensis*, *S.brachypetala*, *E. transvaalense, H. longipetiolata* with five compounds each and their R_f values ranged from 0;06 to 0.94 respectively (Fig.6.1). With regard to methanol plant extracts, only one plant extract, namely *F. saligna* had two prominent antioxidant components at R_f values 0.81 and 0.88 (results not shown). These results are in agreement with what has been reported by McGaw, 2007 and co-workers who detected eight antioxidant components in the water extracts of the *Athrixia* species and five antioxidant components in the ethanol extracts of the same plant species. The aqueous plant extracts had several prominent antioxidant compounds than the organic plant extracts. It is also worthwhile to note that all aqueous plant extracts had active antioxidant constituents at the base line implying that they were very polar because they did not move upwards (Katerere and Eloff, 2008). These findings have revealed the potential of the aqueous plant extracts as sources for natural antioxidants. Various investigations have shown that these antioxidant compounds usually possess other activities such as anti-inflammatory, antitumour, antimutagenic, anticarcinogenic, antibacterial or antiviral activities (Eloff et al., 2008). This appears to validate the ethnomedicinal use of the plant species because infusion or decoction is the most common method used in the preparation of the remedy by the local people

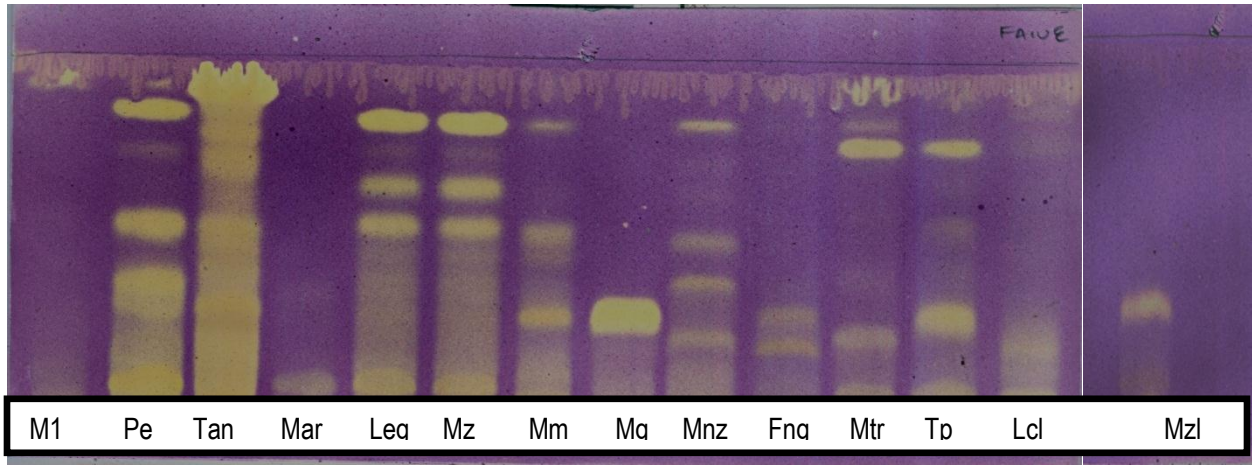


Figure 6.1: Chromatograms of Chromatograms of M1-*C. glabrum*, PE-*O. lanceolata,* TAN-*F. saligna,* LEG-*R. brasiliensis,* MAR-*W. somnifera,* MZ-*E. transvaalense,* MM-*A. caffra,* MG9-*S. potatorum,* MNZU-*S.brachypetala,* FNG-*C. gratissmus,* MTR-*H. longipetiolata,* LCL-*S. pinnata,* TP-*S.acmella,* MZL- *V. infausta* species developed in FAWE solvent system and sprayed with 0.2% DPPH in methanol. Yellow zones indicate antioxidant activity of compounds extracted with water.

6.3.3. 2. DPPH free radical-scavenging method

The DPPH, a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of plant extracts. As antioxidants in the plant extracts donate protons to these radicals, the absorbance decreases. The decrease in absorbance is taken as a measure of the extent of radical scavenging activity (Turkoglu et al., 2007). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. EC_{50} value was used as a parameter for antioxidant activity. This is defined as the amount of antioxidant necessary to decrease the absorbance of DPPH by 50% of the initial absorbance (Yang et al., 2010). Free radical scavenging capacity of the plant extracts, measured by DPPH assay, is shown in Table 6.4. Plant extracts showed a concentration-dependent scavenging activity with DPPH radical scavenging capacity varying from 0.015 to 2.201 µg/ml. Potent DPPH free radical scavenging activity was observed in *H. longipetiolata* (0.015±0.001), *A. caffra* (0.019±0.00), *F. saligna* (0.024± 0.01), *S. brachypetala* (0.034±0.02), *R. brasiliensis* (0.034 ±0.003) and *O. lanceolata* (0.036±0.004). This means that the plant extracts contain compounds with some radical scavenging capacity and the antioxidant properties of these compounds may be the ones which are responsible for beneficial effects during the treatment of fungal infections and other diseases by traditional healers, particularly as aqueous extracts are generally used in traditional remedies. However, all plant extracts tested in this study had less potent DPPH free radical scavenging activity than the control, trolox (0.002±0.001). In general, the DPPH radical scavenging efficiency of the plant extracts from strongest to weakest antioxidant activity was in the following order:*H. longipetiolata* > *A. caffra* > *F. saligna* >*S. brachypetala* > *R. brasiliensis* > *O. lanceolata* > *C. gratissimus* > *V. infausta* > *S. pinnata* > *E. transvaalense* > *W. somnifera* > *S. acmella* > *C. glabrum* > *S. potatorum.* Weak DPPH scavenging activity by some of the plant species may be attributed to lack of electron or hydrogen-donating capacity by constituents present in the plant extracts.

6.3.4. ABTS free radical-scavenging method

This assay reflects the ability of hydrogen or electron-donating antioxidants to scavenge the ABTS•+ radical cation compared with that of the control, trolox, and is an excellent tool for evaluating the total antioxidant potential of the plant extracts (Aruoma , 2003). A high level of antioxidant activity was observed in the leaves of *H. longipetiolata* (0.015±0.002), *A. caffra* (0.019±0.004), *F. saligna (*0.024 ±0.01), *R. brasiliensis* (0.034 ±0.002) and *O. lanceolata* (0.036 ±0.002). Overall, antioxidant activity was observed in the following order: *H. longipetiolata* > *A. caffra* > *F. saligna* > *R. brasiliensis* > *S. brachypetala* > *O. lanceolata* > *C. gratissimus* > *V. infausta* > *S. pinnata* > *E. transvaalense* > *W. somnifera* > *S. acmella* > *C. glabrum* > *S. potatorum.* Most plant extracts tested (71 %) had the capacity to scavenge ABTS+ radical cation although their antioxidant activity was relatively low as compared to the control, trolox (Table 6.4).

6.3.5. FRAP assay

This assay is based on the ability of the plant extracts to reduce ferric ions (Fe^{3+}). An antioxidant in the plant extract capable of donating a single electron to the ferric iron (Fe^{3+}) complex would cause the reduction of this complex into the blue ferrous iron (Fe^{2+}) complex which absorbs strongly at 593 nm (Wong et al., 2006). The antioxidant power of the plant extracts is denoted by the slope calculated from the linear regression best fit curve. High slope values show that components present in the plant extracts had strong antioxidant capacity i.e. were more effective in donating the electrons. As shown in Table 6.4, potent ferric ion reducing power activity was observed in *A. caffra* (0.716 ± 0.06), *F. saligna* (0.671 ± 0.06), *E. transvaalense* (*0*.619 ± 0.05), *O. lanceolata* (0.582± 0.05), *S. brachypetala* (0.526 ± 0.04) and *R. brasiliensis* (0.457 ± 0.04). However, the control, trolox had the strongest antioxidant capacity of all the plant extracts tested. The ferric ion reducing power capacity of the plant extracts from strongest to weakest antioxidant activity was in the following order: *A. caffra* > *F. saligna* > *E. transvaalense* >*O. lanceolata* > *S. brachypetala* > *R. brasiliensis* > *C. gratissimus* > *S. acmella* > *H. longipetiolata* > *W. somnifera* > *V. infausta* > *S. potatorum* > *C. glabrum* > *S. pinnata.*

Comparing the three methods used, the results showed that 29% of plant species had a high level of antioxidant activity in the DPPH, ABTS and FRAP methods. The plant species were *O. lanceolata, F. saligna, A. caffra and R. brasiliensis*. This suggests that antioxidants in the plant extracts were capable of scavenging the free radicals DPPH and $ABTS^{+}$ and reducing ferric ions. Plant species that had potent antioxidant activity in the two most extensively used antioxidant assays for plant samples, namely DPPH and ABTS (Krishnaiah et al., 2011), included *O. lanceolata, F. saligna, A. caffra, R. brasiliensis* and *H. longipetiolata.* The pattern for ferric ion reducing activity of the 14 plants did not differ markedly from their DPPH free radical scavenging activities, when a comparison was made (Table 6.4). Similar to the results obtained for the DPPH free radical scavenging assay, *O. lanceolata, F. saligna, A. caffra, R. brasiliensis* and *S. brachypetala* also had relatively potent ferric ion reducing activity. This may suggest that compounds present in the plant extracts were capable of reducing both DPPH radicals and ferric ions.

Data from published literature indicate that *R. brasiliensis* possesses antifungal properties and phytochemical analysis showed the presence of tannins, anthraquinones, flavonoids, phlobatannins, saponins and steroids (Adekunle, 2000). Isorhamnetin-3-*O*-rutinoside, oleanolic acid, coumarin, scopoletin, *p*-hydroxy-benzoic and *m*-methoxy-*p*-hydroxybenzoic acids are some of the components isolated from the same plant (Pinto et al., 2008). Some of these compounds are known antioxidant components (Abalaka et al., 2011), and this explains why antioxidant activity was observed in the plant extract.

F. saligna has been shown to possess some antibacterial activity (Chitemerere and Mukanganyama, 2011) and to date no antioxidant components are reported to have been isolated. Some compounds isolated from the root bark of *O. lanceolata* such as dihydro-beta-agarofuran polyesters, 1 alpha, 9 beta-difuranoyloxy-2-oxodihydro-beta-agarofuran and pentacylic triterpenoids had antimicrobial activity but did not scavenge the DPPH radical (Yeboah et al., 2010). However, antioxidant activity of *O. lanceolata* observed in this study may be attributed to phenolic compounds reported to be present in its leaves (Mulaudzi et al., 2011). Phytochemical analysis of *E. transvaalense* showed the presence of alkaloids, flavonoids, glycosides, polyphenols, steroids and tannins (Amusan et al., 2007). Some of these compounds are known as antioxidant components (Korotkov et al., 2003) and this explains the antioxidant activity observed in this species. Antioxidant activity of *A. caffra* can be attributed to proanthocyanidins which are reported to be present in the stem bark and might also be present in the leaves (Xie and Dixon, 2005). A related species, *Acacia auriculiformis* is also reported as having strong antioxidant activity (Krishnaiah et al., 2011).

Previous studies have attempted to shed light on the antioxidant activities of some plants tested in the present study. Antioxidant properties of S. *brachypetala* and *C. gratissimus* (Steenkamp et al., 2005), *W. somnifera* (Auddy et al., 2003, Scartezzini and Speroni, 2000), *S. potatorum* (Sanmugapriya and Venkataraman, 2006), *V. infausta* (Mbukwa et al., 2007; Abeer, 2011), *S. acmella* (Wongsawatkul et al., 2008), *S. pinnata (*León et al., 2009), confirmed those reported in the literature. Bouayed et al. (2007) reported that medicinal plants contain a wide variety of natural antioxidants, such as phenolic acids, flavonoids and tannins, which possess more antioxidant activity than dietary plants. As far as our literature survey could ascertain, this is the first report on the antioxidant activity of *A. caffra, H. longipetiolata, E. transvaalense, O. lanceolata,* and *F. saligna.* Even though the plant species had less antioxidant activity than the control used, they can still serve as valuable sources of natural antioxidants and further isolation and purification of the antioxidant components is required.

Table 6.4. DPPH, ABTS and FRAP radical scavenging activities of plant extracts expressed as EC_{50} (µg/ml)

Test samples	DPPH	ABTS	FRAP
C. glabrum	1.696 ±0.31	1.76 ±0.19	0.045 ± 0.01
O. lanceolata	0.036±0.004	0.036 ±0.002	0.582 ± 0.05
F. saligna	0.024± 0.01	0.024 ±0.01	0.671 ± 0.06
R. brasiliensis	0.034 ±0.003	0.034 ±0.002	0.457 ± 0.04
W. somnifera	0.64± 0.09	0.64±0.07	0.121 ± 0.01
E. transvaalense	0.35 ±0.09	0.63 ±0.03	0.619± 0.05
A. caffra	0.019±0.00	0.019±0.004	0.716 ± 0.06
S. potatorum	2.201±0.72	2.11 ±0.11	0.063 ± 0.01
S. brachypetala	0.034±0.02	0.035±0.001	0.526 ± 0.04
C. gratissimus	0.182±0.84	0.18±0.001	0.208± 0.02
H. longipetiolata	0.015±0.001	0.015±0.002	0.151 ± 0.01
S.acmella	0.65±0.06	0.65±0.06	0.168±0.02
S. pinnata.	0.289±0.09	0.29±0.022	0.025± 0.01
V. infausta	0.260±0.02	0.26±5.23	0.078 ±0.02
Control (*Trolox*)	0.002±0.001	0.001±0.04	13.142±0.0

All values are the means of three measurements.

6.4. Conclusion

Cytotoxicity of organic crude plant extracts revealed that 13 out of 14 plant species investigated had a moderate to very active cytotoxicity and only one plant species, *F. saligna,* was relatively non-toxic. Plant extracts that had pronounced cytotoxic activity such as *S. camellia, S. pinnata, O.lanceolata* and *S. brachypetala* should be investigated further for anticancer activity and isolation of active components. On the other hand, the aqueous plant extracts were non-toxic to Vero cells, indicating their safety but should be used with caution by traditional healers because of their low selectiivity indexes. They also need to be tested further for mutagenicity and genotoxicity in order to verify their lack of toxicity.

Plant extracts tested in this study had varying degrees of free radical scavenging activities using the DPPH, ABTS and FRAP methods. The use of the three methods when evaluating the antioxidant activity of the plant extracts gave a better estimate of comparative antioxidant potential of the plant extracts, *O. lanceolata, F. saligna, R. brasiliensis* and *A. caffra* had potent antioxidant properties in both assays. These plant species can be considered as promising sources of natural antioxidants for medicinal uses because they are easily available in rural areas, but further isolation and purification of the antioxidant components is needed.

Postscript

Plant species had variable potency of toxicity and antioxidant properties. The low toxicity of the aqueous plant extracts support their use in the traditional medicine to some extent. Due to the low antioxidant activity observed, the activity of *C. glabrum* extract may be more related to the killing of pathogens directly rather than boosting the immune system to combat the infection. Since the hexane leaf extract of *C. glabrum* had good activity against *C. albicans* in the previous investigation, the next chapter discusses the isolation of antifungal compounds from the hexane extract of leaves of this plant.

Chapter 7.

Clerodendrumic acid, a new triterpenoid from *Clerodendrum glabrum* (Verbenaceae) and antimicrobial activity of fractions and constituents

Ndivhaleni A. Masevhe, Maurice D. Awouafack, Aroke S. Ahmed, Lyndy J. McGaw, Jacobus N. Eloff

Preface

Hexane leaf extract of *C. glabrum* var. *glabrum* had good activity and contained several antifungal compounds. The next step was therefore to isolate and characterise the antifungal compounds and to determine their biological activities. This chapter has been accepted for publication in Helvetica Chimica Acta.

Abstract

One new triterpenoid, 19*b*-3-(1-oxobutyl)-11*α*-hydroxytaraxast-20(30)-ene-24,28-dioic acid (clerodendrumic acid) (**1**) was isolated from the hexane extract of the leaves of *Clerodendrum glabrum* var. *glabrum* along with heptadecanoic acid (**2**). The structure of the new compound was elucidated by interpretation of its NMR (1 and 2D), MS and IR data. Combined fractions C and D from the column chromatography of the hexane extract had significant antifungal activity (average MIC of 0.10 mg/ml) against *Candida albicans* and *Cryptococcus neoformans*. *C. albicans* was relatively resistant to clerodendrumic acid (**1**) (MIC value = 125 µg/ml) and was resistant to heptadecanoic acid (**2**) (MIC of 188 µg/ml). Both compounds had low antibacterial activity against two Gram-positive and two Gram-negative bacteria with average MICs of 157 and 172 µg/ml, respectively. Compounds were relatively non-toxic against Vero monkey kidney cells *in vitro* with IC_{50} values of 202.6 and 108.4 µg/ml.

Keywords: *Clerodendrum glabrum*; Verbenaceae; Clerodendrumic acid; Antimicrobial activity; Cytotoxicity

7.1. Introduction

The genus *Clerodendrum* in the Verbenaceae family which consists of about 580 species is widely distributed in Asia, Australia, America and Africa (Shrivastava and Patel, 2007). *Clerodendrum glabrum* E. Mey var. *glabrum*, known as white cat's whiskers or tinder wood and called "Munukhatshilongwe" in Venda, is a small to medium-sized deciduous tree, with the crown often drooping, which is widely distributed in Bushveld and along forest margins (Van Wyk and Van Wyk, 1997). The leaves are used for treating round-worms and threadworm infections, as a wound dressing, as purgatives in domestic animals, for treatment of stomach troubles, coughs and fevers, colds, mouth ulcers, sore throats and intestinal worms in humans, while their roots are used for arthritis and fractured bones (Thomas et al., 2004). Their leaves have an unpleasant odour and are used as an insect repellent. Pounded leaves are placed in the armpit and neck to induce sleep and to provide a remedy for convulsions in children (Watt and Breyer-Brandwijk, 1962). Previous phytochemical investigations on some species of the genus *Clerodendrum* have reported the isolation of phenols, flavonoids, terpenoids, steroids and cyanogenic glycosides (Shrivastava and Patel, 2007; Choudhury et al., 2009; Liu et al., 2009).

Various local uses of the leaves of this species in addition to the search for bioactive chemical constituents from South African medicinal plants motivated our efforts to commence with the bioguided fractionation of *C. glabrum* var. *glabrum*. To the best of our knowledge, no phytochemical and biological work has been reported on the leaves of this species. We report herein the isolation and characterization of one new triterpenoid, clerodendrumic acid (**1**), along with a known compound (**2**) from the leaves of *C. glabrum* var. *glabrum* as well as the antimicrobial activity of fractions and constituents, and the cytotoxicity of the isolated compounds.

7.2. Results and Discussion

The n-hexane crude extract of the leaves of *C. glabrum* was subjected to repeated silica gel column chromatography and Sephadex LH-20 to yield clerodendrumic acid (**1**) along with a known heptadecanoic acid (**2**) (Fig.7.1). The antimicrobial and cytotoxicity of fractions and isolated compounds (**1**-**2**) were determined.

Clerodendrumic acid (**1**) was obtained as a white powder and gave a positive result with the Liebermann Burchard test characteristic for triterpenes. Its HR-ESI-MS had a pseudo-molecular ion ($[M+H]^+$) peak at *m/z* 573.4290 corresponding to the molecular formula $C_{34}H_{53}O_7$ in conjunction with the NMR data. The bands observed on its IR spectrum at 3300 and 1709 cm^{-1} were indicative of the presence of hydroxyl and carbonyl groups. The ^{1}H-NMR data (Table 7.1) of **1** had four singlets assignable to four methyl groups (δ(H) 0.63, 1.00, 1.12 and 1.13), one doublet methyl group (δ(H) 1.96, J = 6.0 Hz), two oxygenated methine protons [δ(H) 4.51 (*dd*, J = 7.1, 3.4 Hz) and 4.57 (*m*)] and two exomethylene protons [δ(H) 4.75 (br.*d*, J = 1.3 Hz) and 4.82 (br.*d*, J = 1.2 Hz)].

The ^{13}C-NMR data (Table 7.1), in combination with DEPT and HSQC revealed the presence of a total number of 34 carbons including nine quaternary carbons, seven methines, twelve methylenes and six methyl groups. Signals due to two carboxylic and one ester carbonyl groups were observed on the quaternary carbons at δ(C) 178.8 C(28), 178.7 C(24) and 169.5 C(1') while signals owed to oxygenated carbons were found on the methines carbons at δ(C) 79.1 C(3) and 77.7 C(11). The conclusion for the exomethylene group to be situated on **1** was substantiated by the signals at δ(C) 103.2 C(30) and 154.5 C(20) on its ^{13}C-NMR spectrum. Important HMBC correlations (Fig. 7.1) between protons at δ(H) 1.12 Me(23), 2.30 H(5) and carbon at δ(C) 178.7 C(24), and between protons at δ(H) 1.77 H(22), 2.22 H(18) and carbon at δ(C) 178.8 C(28) were relevant for the carboxylic groups to be sited at positions C(24) and C(28), respectively. Further HMBC correlations (Fig.7.1) between the proton at δ(H) 4.51 H(11) and carbons at δ(C) 43.9 C(10) and 48.6 C(13) were indicative for the hydroxyl group to be attached at position C(11). Signals observed on the ^{13}C-NMR spectrum at δ(C) 38.0 C(2'), 32.2 C(3') and 15.9 C(4') were assignable to the n-propyl group that was established from the HMBC correlations (Fig.7.1) [between proton at δ(H) 1.24 H(2'a) and carbons at δ(C) 169.5 C(1') and 32.2 C(3'), and between proton at δ(H) 4.57 H(3) and carbon at δ(C) 169.5 C(1')] to be attached at position C(1'), which in turn linked to C(3). Evidently, the presence of this group was materialized on the ESI-MS spectrum by the ion fragment peak $[M+H\text{-}CH_3CH_2CH_2COO]^+$ at *m/z* 486 (Fig 7.2). The α- and β-configurations of the hydroxyl group at position C(11) and the methyl group at position C(19) were confirmed by the $^3J_{H,H}$ couplings (J = 7.1, 3.4 Hz and J = 10.9 Hz) of axial protons H(11) and H(19), respectively (Petrović et al., 1999). The ESI-MS fragmentation pattern of **1** (Fig 7.2) revealed ion fragments in agreement with the proposed structure for clerodendrumic acid (**1**). From the above spectroscopic data and by comparison with previously reported literature for pentacyclic taraxastane triterpenoids (Petrović et al., 1999; Mahato and Kundu, 1994; Atta-ur-Rahman et al., 2008), the structure of clerodendrumic acid (**1**), isolated and characterized herein for the first time, was assigned as 19β-3-(1-oxobutyl)-11α-hydroxytaraxast-20(30)-ene-24,28-dioic acid.

Minimum inhibition concentration (MIC) values of fractions and isolated compounds were determined against fungal and bacterial pathogens. Many authors consider antimicrobial activity of extracts to be significant if the MIC value is 100 µg/ml or lower (Kuete et al., 2011; Eloff, 2004). Based on this criterion, fractions C and D had significant activity against *C. albicans* and *C. neoformans* with average MIC values of 0.10 mg/ml (Table 7.2). This correlated with the results of bioautography showing clear zones of inhibition against *C. albicans* (Fig 7.3). The highest total activity was found in fractions C and E with an average value of 15750 ml/g (Table 7.3). This value shows the volume to which the active constituent present in one milligram of the fraction can be diluted and still kills the test organism (Eloff, 2004).

According to Rios and Recio (2005) stringent endpoint criteria for pure compounds isolated from plants to be considered as having noteworthy activity is an MIC value of ≤ 10 µg/ml. MIC values of the isolated compounds (**1** and **2**) against the test fungi and bacteria ranged from 125 to 188 µg/ml (Table 7.2) and clerodendrumic acid (**1**) had moderate activity against *C. albicans*, *C. neoformans* and *A. niger* (MIC value of 125 µg/ml and total activity of 8 ml/mg). The three fungi were more resistant to compound **2** with an average MIC of 167 µg/ml and total activity of 6.2 ml/mg (Tables 7.2 and 7.3). The positive control, amphotericin B, had a very low average MIC value of 0.65 µg/ml against the tested fungi and this confirms its superiority over the isolated compounds as an antifungal agent.

The antibacterial activity of the compounds (**1** and **2**) recorded here can be regarded as being weak against the tested microorganisms because MIC was >100 µg/ml (Kuete, 2010). The bacteria *P. aeruginosa* and *S. aureus* were more resistant to the compounds than the tested fungi with an MIC value of 188 µg/ml and total activity of 5.3 ml/mg (Tables7. 2 and 7.3). This trend of activity seems not to be related with the structure of the cell wall of the tested bacteria as *P. aeruginosa* is Gram-negative while *S. aureus* is Gram-positive. This is in agreement with the results of other workers elsewhere who reported that the compounds (terpenoids) inhibited the test fungi (*A. niger*, *C. albicans*, etc.) but not the bacteria (*S. aureus, S. epidermidis* and *Bacillus subtilis*) (Duraipandiyan et al., 2012). However, it was also found that different compounds in the tested fractions were active against bacterial species (*S. aureus, E. coli, P. aeruginosa* and *E. faecalis)* on bioautography.

Antifungal activity of fractions C and D from which the compounds were isolated, was higher than that of the isolated compounds (Table 7.2). This suggests the presence of synergistic interactions of different compounds in the fractions. Weak potency by the compounds may be due to some decomposition which might have occurred during the isolation process (Odebode, 2003). In this case the isolated compounds had the same R_f value as the active compounds in the fractions which makes it unlikely that an artefact has been isolated. However, further investigation could be done especially on the novel compound, clerodendrumic acid (**1**), to potentiate its biological activity. For instance, it can either be used together with another compound in order to increase its activity or due to its likely safety it can be used together with known antibiotics to boost their potency and to mitigate their undesirable side effects (Ríos and Recio, 2005).

The isolated compounds did not show appreciable activity against the tested pathogens. Our group has found that in some cases crude extract or fractions have an excellent potential to use in treating microbial infections and may even have as good or higher activity than commercially used antimicrobials in field (Eloff, 2007) or clinical (Eloff et al., 2010) trials. Major or minor constituents in the plant extract interacting in a synergistic manner may not be highly active when they are not part of a mixture with synergistic compounds.

To the best of our knowledge, the biological activity of the fractions and isolated compounds from the leaves of this plant against the selected bacteria and fungi is being reported for the first time.

Compounds **1** and **2** were evaluated for their cytotoxic activity against monkey kidney Vero cells *in vitro* by means of the MTT (3-(4,5-dimethylythiazol-2-yl)-2,5-diphenyl-2H-tetrazolium hydrobromide) assay and were relatively inactive in inhibiting the proliferation of Vero cells with IC_{50} values > 100 µg/ml (Table 7.4). According to the American National Cancer Institute (NCI), the criterion of cytotoxic activity for pure compounds is $IC_{50} \leq 4$ µg/ml (Tanamatayarat et al., 2003). Thus, compounds **1** and **2** were relatively non toxic because they had IC_{50} values of 202.6 and 108.4 µg/ml respectively compared to the positive control doxorubicin (IC_{50} value of 9.9 µg/ml). The higher IC_{50} values of compounds **1** and **2** indicate their probable safety in terms of cytotoxicity, but more cell lines could be included in the comprehensive screen to confirm these results. Selectivity index (SI) values of these compounds were poor because their SI values ranged from 0.58 to 1.62. It is generally considered that the ratio for a good therapeutic index for a remedy or drug should be ≥ 10, which is a cut-off point ensuring that overdose does not put the life of the patient in danger (Caamal-Fuentes et al., 2011). To the best of our knowledge, there are no other reports on the cytotoxicity of these isolated compounds against Vero cells.

7.3. Conclusion

A novel compound clerodendrumic acid (**1**) and a known compound, heptadecanoic acid (**2**) were isolated for the first time from the leaves of *C. glabrum*. *C. albicans* was relatively resistant to clerodendrumic acid (**1**) with an MIC value of 125 µg/ml and was resistant to heptadecanoic acid (**2**) with an MIC of 188 µg/ml. The low antifungal activity of clerodendrumic acid (**1**), disqualifies it from being a feasible antifungal compound. However, due to its relative safety, clerodendrumic acid (**1**) could either be used as a lead compound to produce new chemically modified active derivatives to fight microbial infections or could be used together with known antibiotics to mitigate their undesirable side effects. Other active compounds visualized in bioautography remain to be isolated and identified, and these may possibly contribute more to the activity of the crude extract and fractions than the compounds isolated in this study.

7.4. General experimental procedures

General. IR spectra were recorded on a Bruker Alpha FT-IR spectrometer (Optik GmbH, Germany). ESI-MS were measured on a Waters Synapt HDMS spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded with a Varian spectrometer at 600 MHz, and chemical shifts (δ) are quoted in ppm with TMS as internal standard. Column chromatography was performed on MN silica gel 60 (0.063-0.2 mm / 70-230) mesh.

Precoated plates of TLC silica gel 60 F_{254} (Merck, Germany) were used for monitoring fractions and spots were detected with UV light (254 and 365 nm) and then sprayed with vanillin-sulfuric acid spray reagent followed by heating up 110°C for 3 min.

7.4.1. Plant material.

The leaves of *Clerodendrum glabrum* var. *glabrum* were collected in October at Tshipise (Limpopo province of South Africa). The plant was identified by MP Tshisikhawe, a botanist at the University of Venda (South Africa) where the specimen was deposited in the herbarium of the Department of Botany under the voucher number Rn 46.

7.4.2. Extraction and isolation.

The dried and powdered leaves of *C. glabrum* (2.85 kg) were extracted with 5 l of n-hexane for 5 h under shaking and at room temperature to afford a crude extract (34.59 g) after filtration and concentration *in vacuo*. A part of the crude extract (10 g) was subjected to silica gel column chromatography (45 cm × 100 mm, 938 g) and eluted with n-hexane and EtOAc in increasing polarity (0 to 100%) to give 84 fractions of 300 ml each that were combined using comparative TLC into 6 main fractions: A (1-14, 1.5 g), B (15-17, 1.2 g), C (18-31, 1.44 g), D (32-38, 1.78 g), E (39-63, 1.44 g), F (64-84, 0.09 g). Fractions A, B, E and F did not have as many and as active antimicrobial compounds separated by TLC in bioautography as fractions C and D, and were not further investigated. Fraction D (1.78 g) was subjected to a purification silica gel column chromatography (50 cm x 2 cm, 70 g) and eluted with hexane and EtOAc in increasing polarity to give 87 fractions of 50 ml each that were monitored with co-TLC to 9 sub-fractions. Subfraction D_4 (0.34 g) was subjected to further silica gel CC using hexane and EtOAc (from 96:4 to 80:20) to afford 41 fractions of 30 ml each. Sub-fractions $D4_{13\text{-}18}$ from D_4 gave mainly **1** (19.4 mg). Fraction C (1.44 g) was similarly subjected to a purification silica gel CC as described for fraction D to afford **2** (24.7 mg).

19β-3-(1-Oxobutyl)-11α-hydroxytaraxast-20(30)-ene-24,28-dioic acid or *clerodendrumic acid* (**1**): White powder; IR ν_{max}: 3300, 2900, 1709, 1442, 982 cm^{-1}; ^1H- (600 MHz) and ^{13}C-NMR (150 MHz) in acetone-d_6: Table 7.1; ESI-MS: *m/z* (rel. Int.) 573 $[M+H]^+$ (42), 555 (8), 529 (40), 486 (13), 485 (25), 441 (16), 413 (35), 368 (37), 273 (26), 229 (100). HR-ESI-MS: *m/z* 573.4290 ($[M+H]^+$), $C_{34}H_{53}O_7$ calcd. for 573.4308.

7.4.3. Antimicrobial Assay.

The two-fold serial microdilution method was used to determine the minimum inhibitory concentration (MIC) values for fractions and isolated compounds against bacteria (Eloff, 1998) and for fungi a modification of (Eloff, 1998) by Masoko et al. (2005). The MIC of the samples were evaluated using two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212), two Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922); and the pathogenic fungi *Candida albicans*, *Cryptococcus neoformans* (clinical isolates) and *Aspergillus niger*.

In the antibacterial tests, 100 µl of fractions (10 mg/ml) or compounds (4 mg/ml) in triplicate dissolved in acetone were serially diluted two-fold with sterile distilled water in 96-well microtitre plates and 100 µl of freshly prepared overnight bacterial culture in Mueller Hinton broth (Fluka, Switzerland) was added to each well. Densities of bacterial cultures used were approximately: 2.6×10^{12} cfu/ml, *S. aureus*; 1.5×10^{10} cfu/ml, *E. faecalis*; 5.2×10^{13} cfu/ml, *P. aeruginosa* and 3.0×10^{11} cfu/ml, *E. coli* (Suleiman et al., 2010). Acetone and gentamicin were used as negative and positive controls, respectively. The microtitre plates were sealed in air-tight polythene plastic bags and were incubated overnight at 37 °C. Thereafter, 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium violet (INT) was added to each well to indicate microbial growth. The microtitre plates were further incubated at 37 °C and minimal inhibitory concentration (MIC) was determined 1 and 2 h after the addition of INT. MIC was determined as the lowest concentration inhibiting microbial growth, indicated by a decrease in the intensity of the red colour of the formazan (Shai et al., 2008).

For the antifungal assay, fractions and isolated compounds were dissolved in acetone to a concentration of 10 mg/ml and 4 mg/ml respectively. Fungal cultures were taken from agar culture plates and inoculated in fresh Sabouraud Dextrose broth and incubated at 37 °C for three days prior to conducting the assay. Densities of fungal cultures used were approximately: 2.5×10^4 cfu/ml, *C. albicans*; 2.6×10^4 cfu/ml, *C. neoformans*; and 1×10^6 cfu/ml (Suleiman et al., 2010). The fractions or compounds (100 µl) in triplicate were serially diluted two-fold with sterile distilled water in 96-well microtitre plates. Thereafter, 100 µl of the fungal culture was added to each well. Amphotericin B and acetone were used as positive and negative controls, respectively. To indicate growth of microorganisms, 40 µl of 0.2 mg/ml of INT was added to each well. The microplates were put in air-tight plastic bags and then incubated for two days at 37 °C in 100% relative humidity. MIC was taken as the lowest concentration of the extract that inhibited fungal growth after 24 and 48 h. The experiment was conducted twice in order to verify the results.

7.4.4. Cytotoxicity assay.

The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reduction assay is widely used for measuring cell proliferation and cytotoxicity. MTT (yellow) is reduced into a formazan (purple) by viable cells. The colour intensity of the formazan produced, which is directly proportional to the number of viable cells, is measured using a spectrophotometer. Viable cell growth after incubation with known concentration of either fractions of plant extract or isolated compound was determined using the colorimetric MTT assay as described by Mosmann (1983) . Monkey Vero cells of a subconfluent culture were harvested and centrifuged at 200 × g for 5 min, and resuspended in growth medium to a density of 0.1 x 10^6 cells/ml. Minimal Essential Medium (MEM, Sigma) supplemented with 0.1% gentamicin and 5% foetal calf serum (Sigma) was used. A total of 100 µl of cell suspension was added into each well of columns 2 to 11 of a sterile 96-well microtitre plates. Growth medium (200 µl) was added into wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37 °C in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. Test samples or doxorubicin (Sigma) (as the positive control prepared in growth medium (100 µl) at various known concentrations were added to each well (in quadruplicate, except for column 2 where only growth medium was added). The microtitre plates containing treated and untreated cells were incubated at 37 °C in a 5% CO_2 incubator for 2 days with the various treatments. After incubation, the medium was removed from each well, and each well was rinsed with PBS before fresh growth medium (200 µl) was added. Following this washing step, 30 µl MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates were incubated for a further 4 h at 37 °C. The medium in each well was carefully removed without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µl DMSO to each well, followed by gentle shaking of the MTT solution. The amount of MTT reduction was measured immediately by detecting absorbance at 570 nm using a microplate reader (Versamax). The wells in column 1, containing only medium and MTT but no cells, were used to blank the reader. The IC_{50} values were calculated as the concentration of plant extract or test compound resulting in a 50% reduction of absorbance compared to untreated cells. Selective activity of the isolated compounds was calculated as follows: selective index (SI) = IC_{50}/MIC.

	***δ* (C)**	***δ* (H)**
CH_2(1)	38.3	0.91 (*m*), 1.56 (*m*)
CH_2(2)	27.1	1.62 (*m*), 1.73 (*m*)
H-C(3)	79.1	4.57 (*m*)
C(4)	46.5	
H-C(5)	41.8	2.30 (*m*)
CH_2(6)	17.6	
CH_2(7)	26.9	1.39 (*m*), 1.46 (*m*)
C(8)	22.6	
H-C(9)	51.7	1.55 (*m*)
C(10)	43.9	
H-C(11)	77.7	4.51 (*dd*, 3.4, 7.1)
CH_2(12)	45.0	1.29 (*m*), 1.55 (*m*)
H-C(13)	48.6	1.61 (*m*)
C(14)	46.1	
CH_2(15)	39.4	0.94 (*m*), 1.88 (*m*)
CH_2(16)	33.2	1.49 (*m*), 1.74 (*m*)
C(17)	46.9	
H-C(18)	42.4	2.22 (*m*)
H-C(19)	43.5	2.06 (*d*, 10.9)
C(20)	154.5	
CH_2 (21)	45.2	2.05 (*m*), 2.25 (*m*)
CH_2(22)	36.7	1.77 (*m*), 1.61 (*m*)
Me(23)	17.0	1.12 (*s*)
C(24)	178.7	
Me(25)	15.8	1.13 (*s*)
Me(26)	19.7	0.63 (*s*)
Me(27)	14.2	1.00 (*s*)
C(28)	178.8	
Me(29)	20.2	1.96 (*d*, 6.0)
CH_2(30)	103.2	4.75 (br.*d*, 1.3), 4.82 (br.*d*, 1.2)
C(1')	169.5	
CH_2(2')	38.0	1.24 (*m*), 1.96 (*m*)
CH_2(3')	32.2	1.30 (*m*), 2.05 (*m*)
Me(4')	15.9	1.09 (br.*s*)

Table 7.1. ^{1}H- and ^{13}C-NMR [600 and 150 MHz in $(CD_3)_2CO$] data for clerodendrumic acid (1), *δ* in ppm and *J* in Hz

Table 7.2: Minimum inhibitory concentration (MIC) of fractions (**A**-**F**) in mg/ml and compounds (**1** and **2**) in µg/ml from *C. glabrum* against fungi (**C.a**, **C.n**, **A.n**) and bacteria (**S.a**, **P.a**, **E.f**, **E.c**). The results are the means of three replicates and the standard deviation was zero.

Samples	Fungi (MIC values)							Bacteria (MIC values)								
	C. a		C. n		A. n		Average	S.a		P.a		E.f		E.c		Average
	24 h	48 h	24 h	48 h	24 h	48 h		1 h	2 h	1 h	2 h	1 h	2 h	1 h	2 h	
Fractions																
A	0.31	0.63	0.08	0.16	NT[a])	NT[a])	0.30	0.63	0.63	0.63	1.25	0.63	0.63	1.25	1.25	0.86
B	0.08	0.16	0.08	0.16	NT[a])	NT[a])	0.12	0.63	0.63	1.25	1.25	0.63	1.25	1.25	1.25	1.02
C	0.08	0.16	0.08	0.08	NT[a])	NT[a])	0.10	0.31	0.31	0.31	0.31	0.16	0.16	0.31	0.31	0.27
D	0.08	0.16	0.08	0.08	NT[a])	NT[a])	0.10	0.16	0.31	0.31	0.31	0.08	0.16	0.31	0.63	0.28
E	0.08	0.16	0.08	0.16	NT[a])	NT[a])	0.12	0.16	0.16	0.31	0.63	0.31	0.31	0.31	0.63	0.35
F	0.31	0.63	0.63	0.08	NT[a])	NT[a])	0.55	0.63	0.63	0.63	1.25	0.63	1.25	1.25	1.25	0.94
Compounds																
1	125	125	125	125	125	125	125	188	188	188	188	125	125	125	125	157
2	188	188	125	125	188	188	167	188	188	188	188	125	125	188	188	172
Controls																
Amp B	0.02[b]) and 0.78[c])	0.04[b]) and 0.78[c])	0.04[b]) and 0.78[c])	0.08[b]) and 0.78[c])	NT[a]) and 0.39[c])	NT[a]) and 0.39[c])	0.05[b]) and 0.65[c])	-	-	-	-	-	-	-	-	
Gen	-	-	-	-	-	-	-	0.39[b]) and 0.19[c])	0.39[b]) and 0.19[c])	0.39[b]) and 0.78[c])	0.78[b]) and 0.78[c])	0.78[b]) and 0.39[c])	0.16[b]) and 0.39[c])	0.78[b]) and 0.59[c])	0.78[b]) and 0.59[c])	0.56[b]) and 0.49[c])

C.a: *Candida albicans*, C.n: *Cryptococcus neoformans,* A.n: *Aspergillus niger,* E.c: *Escherichia coli,* E.f: *Enterococcus faecalis,* S.a: *Staphylococcus aureus,* P.a: *Pseudomonas aeruginosa,* Amp B: Amphotericin B, Gen: *Gentamicin*, [a]): not tested, [b]):MIC values for reference standard when testing fractions, [c]): MIC values for reference standard when testing compounds.

Table 7.3: Total activity in ml of fractions (**A** - **F**) from *C. glabrum* calculated by dividing mass in mg of fraction with the MIC of the fraction [13]. The inverse of MIC in ml/mg was calculated for compounds (**1** and **2**). This indicated to what volume one mg of the compound can be diluted and still kills the relevant microorganism.

Samples	Fungi							Bacteria								
	C. a		C. n		A. n		Average	S.a		P.a		E.f		E.c		Average
	24 h	48 h	24 h	48 h	24 h	48 h		1 h	2 h	1 h	2 h	1 h	2 h	1 h	2 h	
Fractions																
A	4839	2381	18750	9375	NT [a])	NT [a])	8836	7730	7730	7730	3896	7730	7730	3896	3896	6292
B	15000	7500	15000	7500	NT [a])	NT [a])	11250	1905	1905	960	960	1905	960	960	960	1314
C	18000	9000	18000	18000	NT [a])	NT [a])	15750	4645	4645	4645	4645	9000	9000	4645	4645	5734
D	17750	8875	17750	8875	NT [a])	NT [a])	13313	8875	4581	4581	4581	17750	4581	4581	2254	6473
E	18000	9000	18000	18000	NT [a])	NT [a])	15750	9000	9000	4645	2285	4645	4645	4645	2285	4858
F	290	143	143	143	NT [a])	NT [a])	180	290	143	143	72	290	143	72	72	153
Compounds																
1	8	8	8	8	8	8	8	5.3	5.3	5.3	5.3	8	8	8	8	6.7
2	5.3	5.3	8	8	5.3	5.3	6.2	5.3	5.3	5.3	5.3	8	8	5.3	5.3	6

C.a: *Candida albicans,* C.n: *Cryptococcus neoformans,* A.n: *Aspergillus niger,* E.c: *Escherichia coli,* E.f: *Enterococcus faecalis,* S.a: *Staphylococcus aureus,* P.a: *Pseudomonas aeruginosa,* [a]): not tested.

Table 7.4: Cytotoxicity for compounds (**1** and **2**) from *C. glabrum* and their selectivity index against three fungal and four bacterial species

Compounds	Cytotoxicty		Selectivity Index						
	IC_{50} µg/ml	SD	C.a	C.n	A.n	E.c	P.a	E.f	S.a
1	202.64	±3.61	1.62	1.62	1.62	1.62	1.08	1.62	1.08
2	108.37	±3.69	0.87	0.87	0.87	0.58	0.58	1.62	0.58
Dox	9.87	±0.10							

C.a: *Candida albicans,* C.n: *Cryptococcus neoformans,* A.n: *Aspergillus niger,* E.c: *Escherichia coli*, E.f: *Enterococcus faecalis,* S.a: *Staphylococcus aureus,* P.a: *Pseudomonas aeruginosa,* Dox: Doxorubicin.

Figures

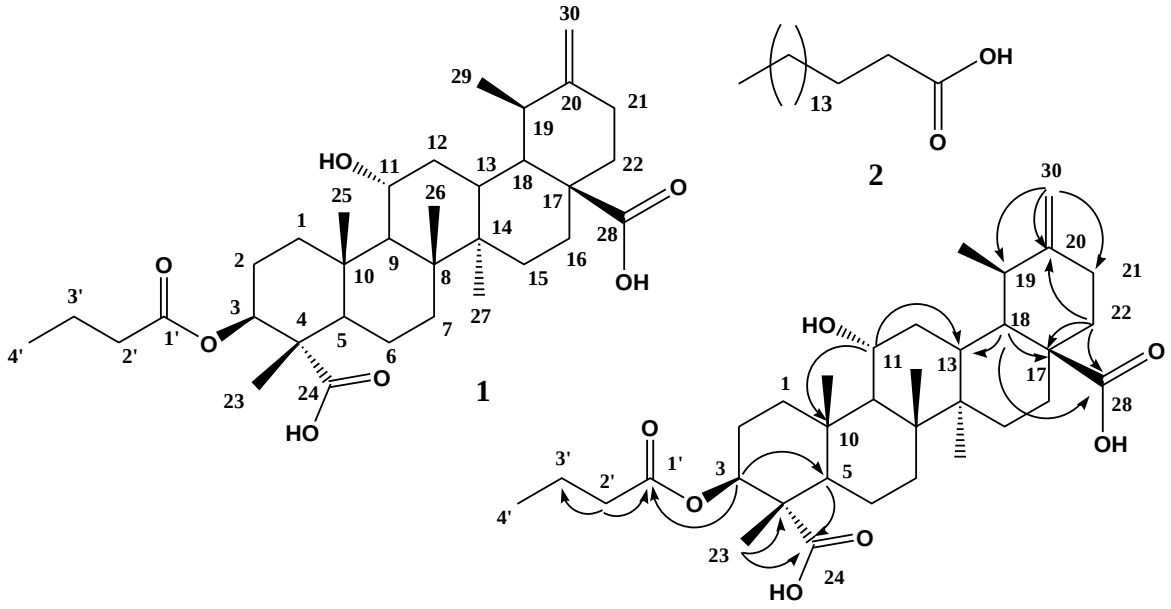


Figure 7. 1.. Compounds (1 and 2) from *C. glabrum* and key HMBC correlations of clerodendrumic acid (1)

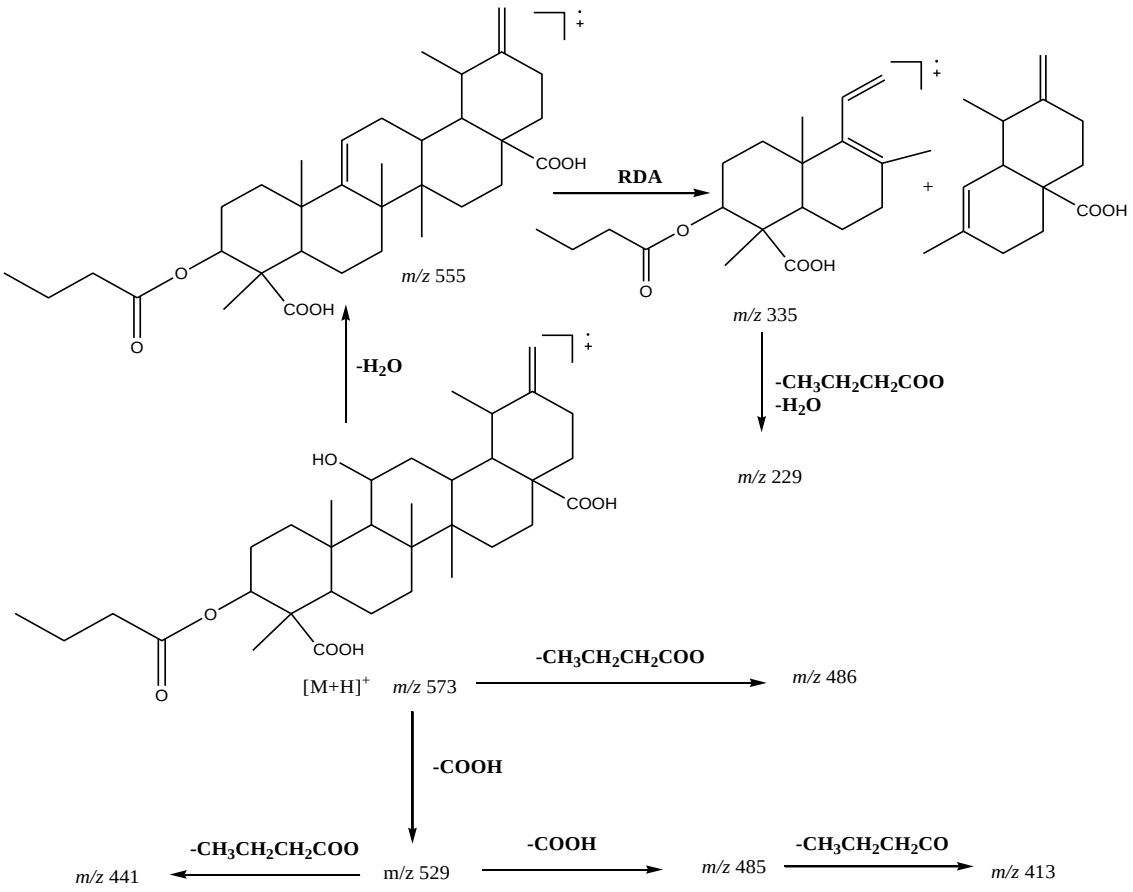
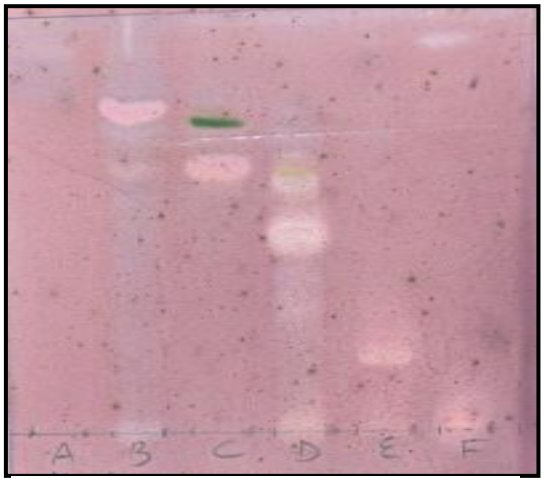


Figure 7. 2. Important ESI-MS fragmentation pattern of clerodendrumic acid (1)



A B C D E F

Figure 7.3: Anti-*Candida* activity of fractions (**A**-**F**) from the extract of *C. glabrum* using bioautography technique on TLC plate and sprayed with *Candida albicans* culture Heptadecanoic acid (**2**) was the main active compound in fraction C and clerodendrumic acid (**1**) was the main active compound in fraction D.

Postscript

The optimistic expectation of isolating at least one high activity antifungal compound which could possibly be developed into a safe and affordable antifungal drug by rural people was not attained because the activity of the isolated compounds was poor even though the compounds isolated were relatively safe against cell cultures. It is however, exciting that the compound isolated has not been found in nature previously. In the next chapter, an overview of the whole work is briefly discussed in terms of the identified objectives.

Chapter 8.

Summary and conclusions

There are many reports in the literature that opportunistic fungal infections are commonly encountered in immunocompromised individuals and *C. albicans* is one of the earliest opportunistic infections in HIV/AIDS patient populations. Considerable morbidity and mortality is attributed to these opportunistic infections. Plants are being used by traditional healers to combat these opportunistic infections. Hence, the aim of this study was to isolate and characterize antifungal compounds from the most active plant species that have been used to address secondary infection problems in immunocompromised patients. The following objectives were identified in order to achieve the aim. However, some of the objectives were not achieved to our satisfaction as discussed below.

8.1. Objectives

1. To determine which plants are being used traditionally to treat candidiasis in HIV/AIDS positive people.
2. To evaluate the antifungal activity of the selected plant species and select promising plant species to study further for its activity against *C. albicans*
3. To determine the antioxidant activity that may be related to immune supporting capacity of plant extracts.
4. To determine the cellular safety of plant extracts.
5. To isolate, characterize the antifungal compounds and to determine their antimicrobial activity and cellular safety.

8.1.1. Objective 1. To determine which plants are being used traditionally to treat candidiasis in HIV/AIDS positive people.

In an endeavour to search for the new, cheap, safer, effective antifungal molecules from plants which could be used to overcome the disadvantages such as fungal resistance, high cost of antifungal agents and their toxicity, an ethnobotanical study was undertaken. This kind of study is important in disclosing locally important plants used by communities to treat a range of ailments affecting them. A total of 45 plant species belonging to 24 different families were identified and recorded. It was interesting to note that 27 plant species representing 60% of those recorded, have either been scientifically shown to have anti-*Candida* activity or have been recorded for antifungal use in the literature. This indicated the value of local traditional healers knowledge that they shared with us. Several drugs which are being used today in the conventional medicine have been discovered by following ethnobotanical leads e.g. aspirin, reserpine, quinine, digoxin, artimisinin just to mention a few examples.

It is of worthy to note that 12 plant species were recorded for the first time as medicinal plant species used to treat candidiasis: *Acacia caffra, Amaranthus spinosus, Cissampelos torulosa, Faurea saligna, Ozoroa engleri, Pterocarpus rotundifolius, Rinorea angustifolia, Schkuhria pinnata, Strychnos madagascarienses, Sophora microphylla, Trimeria grandifolia* and *Wrightia natalensis.* This is a valuable contribution of new knowledge to the ever-increasing database of information relating to the potential medicinal value of the South African flora. Only 14 plant species were recorded as the most widely used by the traditional healers and these were *Acacia caffra*, *Clerodendrum glabrum*, *Croton gratissimus, Elaeodendron transvaalense*, *Faurea saligna, Hippocratea longipetiolata*, *Osyris lanceolata*, *Richardia brasiliensis*, *Schkuhria pinnata*, *Schotia brachypetala, Spilanthes acmella*, *Strychnos potatorum*, *Vangueria infausta subsp. infausta* and *Withania somnifera.* Considering the fact that knowledge of traditional medicine is largely oral, the information documented here is a permanent record which can be used as a starting point for future ethnopharmacological studies.

It was disheartening to learn that the most widely used plant parts in the therapeutic preparations of remedies were roots and barks. Some studies from our group report that in many cases where roots or barks are used traditionally leaf extracts are as effective. This study therefore focussed on leaf extracts of trees as a sustainable resource. It is highly recommended that traditional healers should be encouraged to use plant leaves instead of roots and bark in the preparation of their remedies because it has been shown that plant leaves can be used as biologically active alternatives in traditional medicine (Shai et al., 2009; Eloff, 2001).

8.1.2. Objective 2. Evaluation of antifungal activity of the selected plant species.

A total of 14 plant species were tested against fungal species (*C. albicans* and *C. neoformans*) and bacterial species (*S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa*) using a two-fold serial microdilution method and a TLC bioautography method. The hexane and the acetone extracts of *W. somnifera*, *H. longipetiolata, C. glabrum, C. gratissimus* and *S. pinnata* were the most active against at least one or two of the tested pathogens with MIC values ranging from 0.06 to 0.08 mg/ml. It was observed that 92% plant extracts prepared with organic solvents had average MIC values <1 mg/ml and this provides some scientific credibility to the documented traditional uses of the plant species. However, aqueous plant extracts had poor activity against *C. albicans* . This is a concern because water is the only solvent freely available to rural people and cannot afford to buy organic solvents in order to have an access of the active plant extracts (Eloff et al., 2008). The degree to which this objective has been achieved is satisfactory because the selection of *C. albicans* as a test organisms relate directly to the traditional uses of the plant species.

Among the tested plant extracts, the hexane extract of *C. glabrum* had a good antifungal activity against *C. albicans* with an MIC value as low as 0.06 mg/ml and the highest total activity of 550 ml/g. This value shows that 1 g of *C. glabrum* hexane leaf extract can be diluted with 550 ml and still inhibit the growth of *C. albicans* (Eloff, 2000). On the TLC bioautography, the same extract had 8 active components with R_f values ranging from 0.11 to 0.46. These factors coupled with a wider distribution of this plant species in the rural area led us to select it for further investigation.

8.1.3. Objective 3. To evaluate the potential immune supporting capacity of the plant extracts.

The organic and the aqueous leaf extracts were evaluated for their immune boosting capacity because the therapeutic benefit of medicinal plants is often attributed to their antioxidant properties (Ljubuncic et al., 2005). The antioxidant activity of plant extracts was determined using DPPH, ABTS and FRAP assays. Plant species that had potent free radical scavenging activity were *O. lanceolata, F. saligna, A. caffra, R. brasiliensis, A. caffra* and *H. longipetiolata* representing 43% of the tested plant extracts. The aqueous leaf extracts of these plants had considerably higher antioxidant activity than the organic leaf extracts. This means that they contain antioxidants that may boost the immune system so that the body could fight off microbial infections. This provides some rationale for the traditional use of these plants in the treatment of candidiasis and related infections. However, all plant extracts tested in this study had less potent radical scavenging activity than the control, Trolox used. Worthy of note is that, this is the first report on the antioxidant activity of *A. caffra, H. longipetiolata, E. transvaalense, O. lanceolata,* and *F. saligna.* The effect of the extract of *C. glabrum* appears to be the direct inhibition of microbial growth because it had low antioxidant activity. This study provides a better understanding of the antioxidant properties of these plant species because three different methods were employed. This was done to gain a fully comprehensive indication of the antioxidant efficacy of the plant extracts (McGaw et al., 2007). Plant species that had potent antioxidant activity could serve as valuable sources of natural antioxidants and further isolation and purification of the antioxidant components is important.

8.1.4. Objective 4. To determine the cellular safety of plant extracts

In order to establish the safe use of medicinal plants by our people and to scientifically recommend non-toxic plants for human consumption and for drug discovery, cytotoxicity of the selected plant species was determined against the Vero monkey kidney cell line using MTT assay. The acetone plant extracts of *S. acmella, S. pinnata , O. lanceolata* and *S.brachypetala* were highly toxic with IC_{50} value < 30 µg/ml. This suggests that plant species have the potential of yielding anticancer compounds. Only one acetone plant extract of *F. saligna* was relatively non-toxic and the rest of the plant species had moderate activity against the Vero cell line. On the other hand, the aqueous plant extracts were non-toxic and this supports their use in the traditional medicine.

However, their low selectivity Index values suggest that plant extracts are suitable for external use only and caution should be exercised in the use of these plant species by traditional healers to treat a variety of ailments of fungal and bacterial origin. Literature reports that the use of medicinal plants in their crude form, without scientific evaluation of their safety could be harmful (Light et al., 2005). Thus, this section has made some valuable contribution to the knowledge base of traditional medicine safety because this is an area where there is a great paucity of the available information. However, other cell lines need to be tested and the genotoxic effects of the plant species should also be investigated to confirm the results.

8.1.5. Objective 5. To isolate, characterize the antifungal compounds and to determine their antimicrobial activity and cellular safety.

The hexane crude extract of *C. glabrum* (10 g) was subjected to silica gel column chromatography and eluted with n-hexane and EtOAc in increasing polarity to give several fractions that were combined using comparative TLC into 6 main fractions. Fractions A, B, E and F did not have as many and as active anti-candida compounds based on bioautography as fractions C and D, and were not further investigated. Fraction C and D were subjected to a purification silica gel CC to afford compounds **2** and **1** respectively. A novel triterpenoid, 19β-3-(1-oxobutyl)-11α-hydroxytaraxast-20(30)-ene-24,28-dioic acid (clerodendrumic acid) (**1**) along with known heptadecanoic acid (**2**) were identified using NMR, MS and IR data. To the best of our knowledge, isolation of clerodendrumic acid (**1**) and heptadecanoic acid (**2**) from the leaves of *C. glabrum* is reported here for the first time.

The MIC values of fractions and isolated compounds were determined against fungal and bacterial pathogens. Fractions C and D had significant activity against *C. albicans* and *C. neoformans* with average MIC values of 0.10 mg/ml respectively. Compound (**1**) had moderate activity against *C. albicans*, *C. neoformans* and *A. niger* (MIC value = 125 µg/ml and total activity = 8 ml/mg). In contrast, Shai et al. (2008) reports that triterpenoids (betulinic acid, ursolic acid and hydroxyursolic acid) isolated from the leaves of *C. dentata* had appreciable inhibition of fungal growth (*C. neoformans*, *C. albicans* etc) with MIC values ranging from 8 to 63 µg/mL. The three fungi (*C. albicans*, *C. neoformans* and *A. niger*) were more resistant to compound **2** with an average MIC of 167 µg/ml and total activity of 6.2 ml/mg. The antibacterial activity of the compounds (**1** and **2**) was also weak against the tested microorganisms because MIC was >100 µg/ml. Antifungal activity of fractions C and D from which the compounds were isolated, was higher than that of the isolated compounds. This suggests the presence of synergistic interactions of different compounds in the fractions. Weak potency by the compounds may also be due to some decomposition of other antifungal compounds which might have occurred during the isolation process. The isolated compounds had the same R_f value as the active compounds in the fractions which makes it unlikely that an artefact has been isolated. To the best of our knowledge, the antimicrobial activity of the fractions and isolated compounds from the leaves of this plant against the tested pathogens is reported for the first time.

Cytotoxic activity of compounds **1** and 2 against the Vero cell line was determined using MTT assay, both of them were non-toxic to Vero cells with IC_{50} values > 100 µg/ml but more cell lines could be tested to confirm this results. Selectivity index values of the compounds were poor because a good therapeutic index for the compound which can be developed into a safe drug should be ≥ 10 (Caamal-Fuentes et al., 2011). To the best of our knowledge, there are no other reports on the cytotoxicity of the isolated compounds against Vero cells.

The hope of isolating a compound with a high antifungal activity which could be developed into an effective antifungal agent was not attained because the activity the of novel compound, clerodendrumic acid (**1**) was inadequately low. Some studies elsewhere also report that the identification of a single active chemical entity that is responsible for the antimicrobial activity of a plant is becoming more and more improbable (van Vuuren, 2008). However, due to its relative safety, clerodendrumic acid (**1**) could be used as a lead compound to produce new chemically modified active derivatives to fight candidiasis and related fungal infections in the immunocompromised patients.

This study provides some scientific rationale for the ethnomedicinal use of the selected plant species in terms of their efficacy, immune boosting capacity and safety. The study showed the potential of studying traditional medicine in the search for effective plant extracts or new lead compounds that could be developed into low cost drugs for combating candidiasis and related infections among the rural poor people.

Further work to be done should focus on testing the efficacy of the plant part combinations because this is how traditional remedy is prepared in most cases. Plant species which were highly toxic could also be investigated further for their anti-tumour properties.

Chapter 9. References

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Appendix A

Ethnobotanical study of plants used traditionally for the management of candidiasis and related infections in Venda, Limpopo Province, South Africa.

Questionnaire

1. Information about the traditional healers

Age:
Gender:
Physical address and contact no.:
Education:

2. Information about the disease

Do you treat people with the following symptoms?

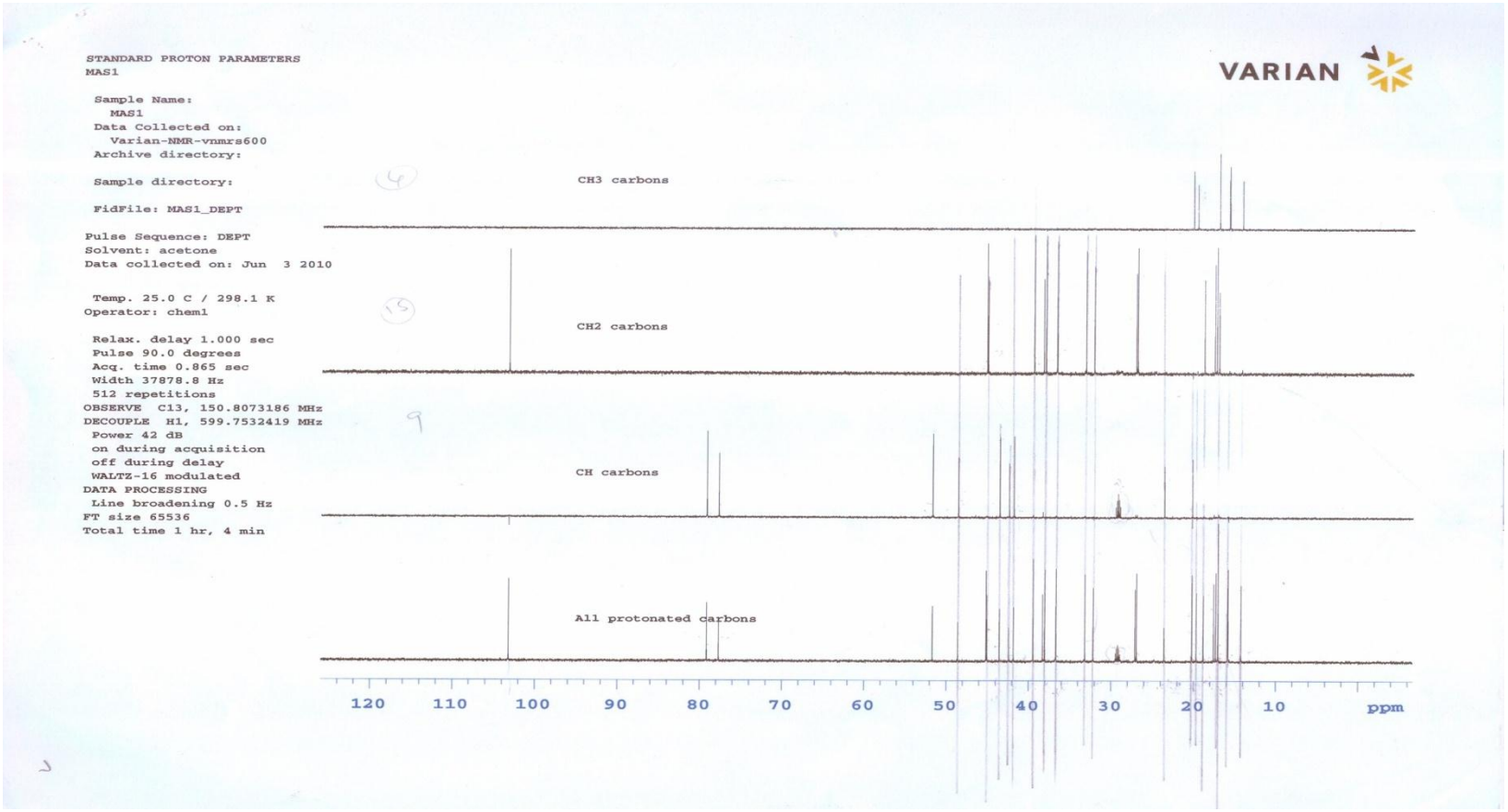
- mouth ulcers
- genital ulcers
- sore throat
- itching of genitals
- white discharge

3. Information about the medicinal plants

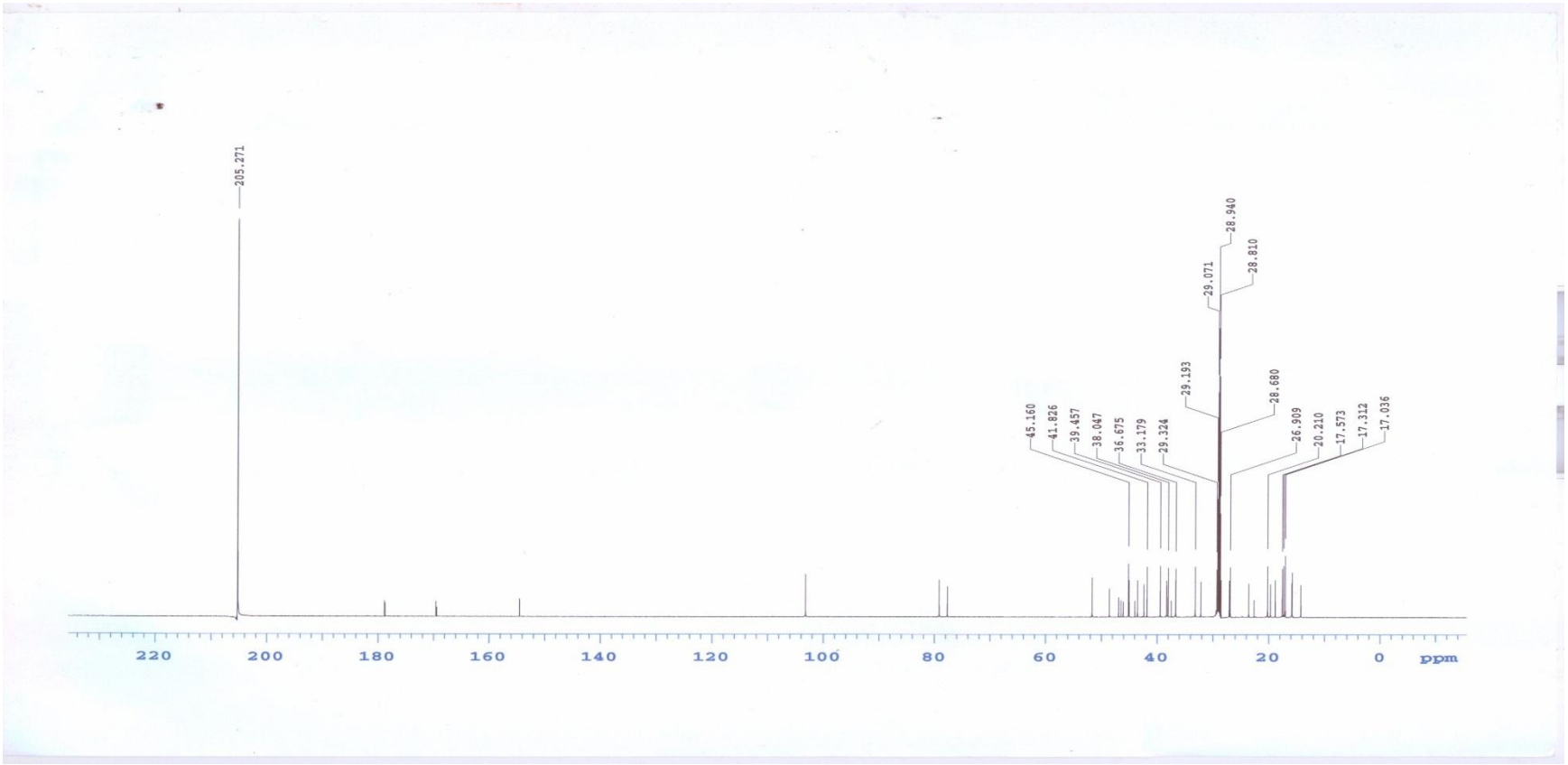
- where do you collect your plants?
- Do you conduct some rituals before the collection?
- Can you share with us some of your harvesting techniques?
- How do you store your remedy?

Local name	Habit	Citation	Part used	Method of preparation	Route of administration	Dosage

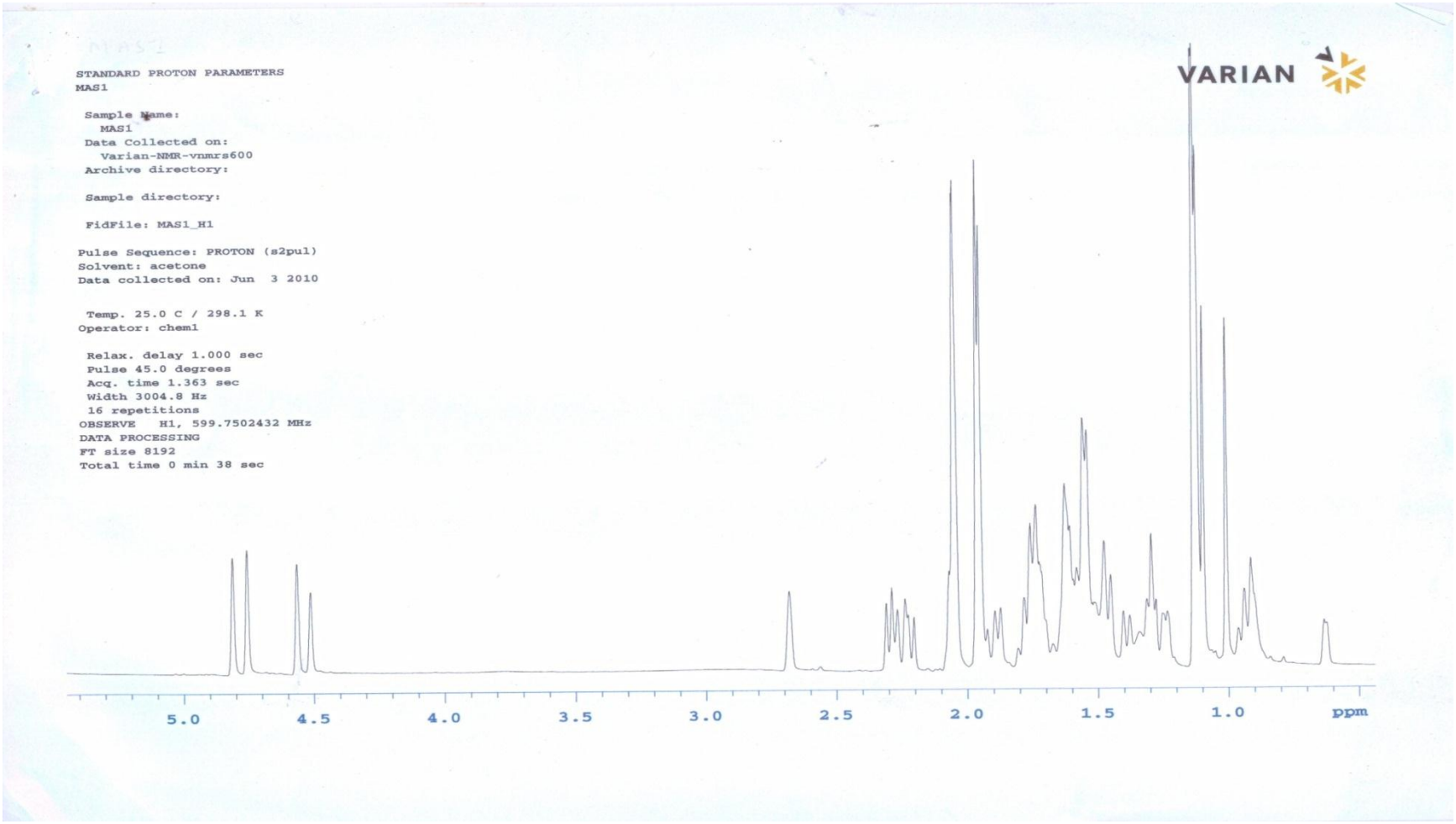
Appendix B.



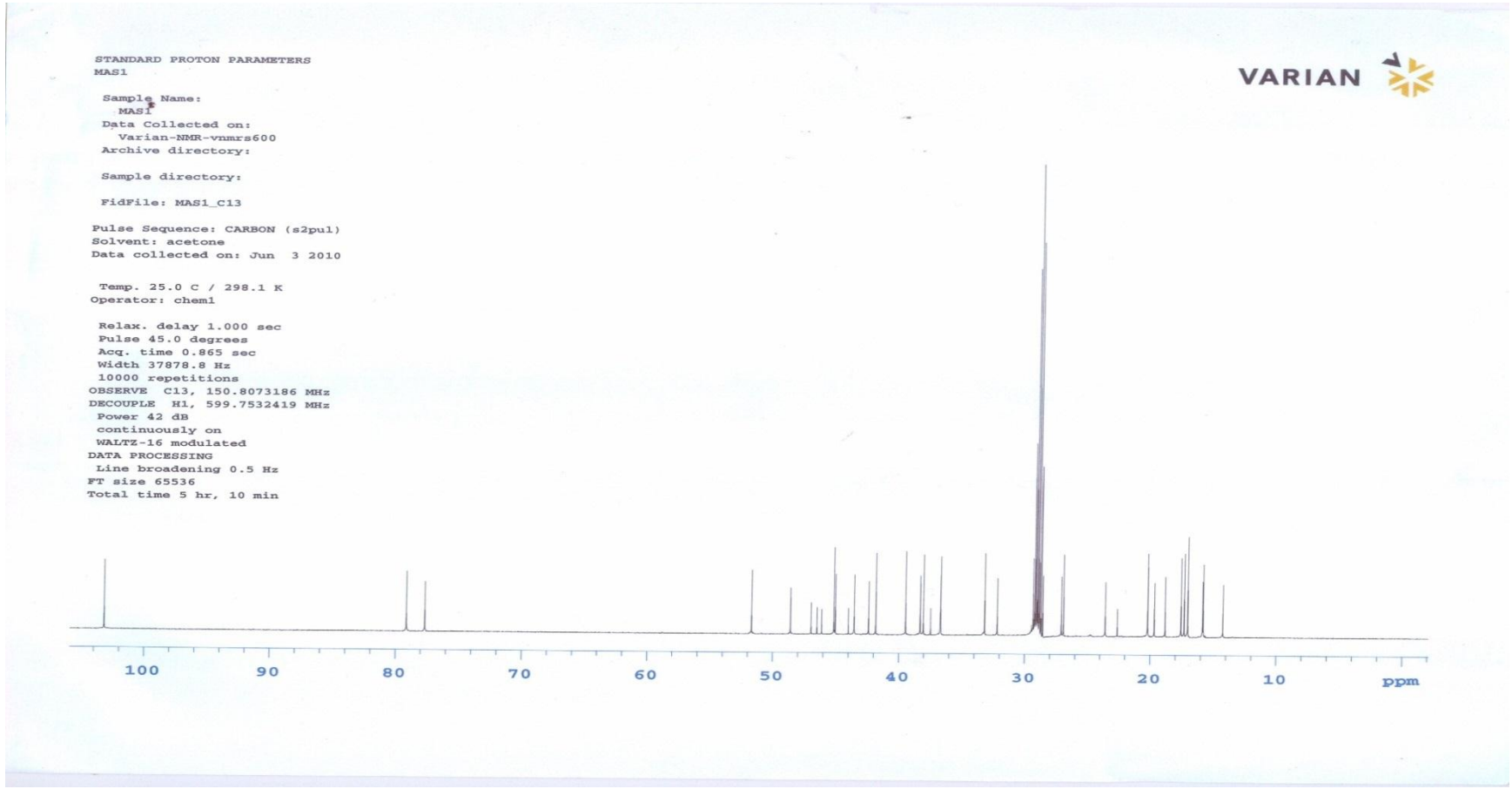
The ^{13}C –DEPT-NMR Spectroscopy of Clerodendrumic acid



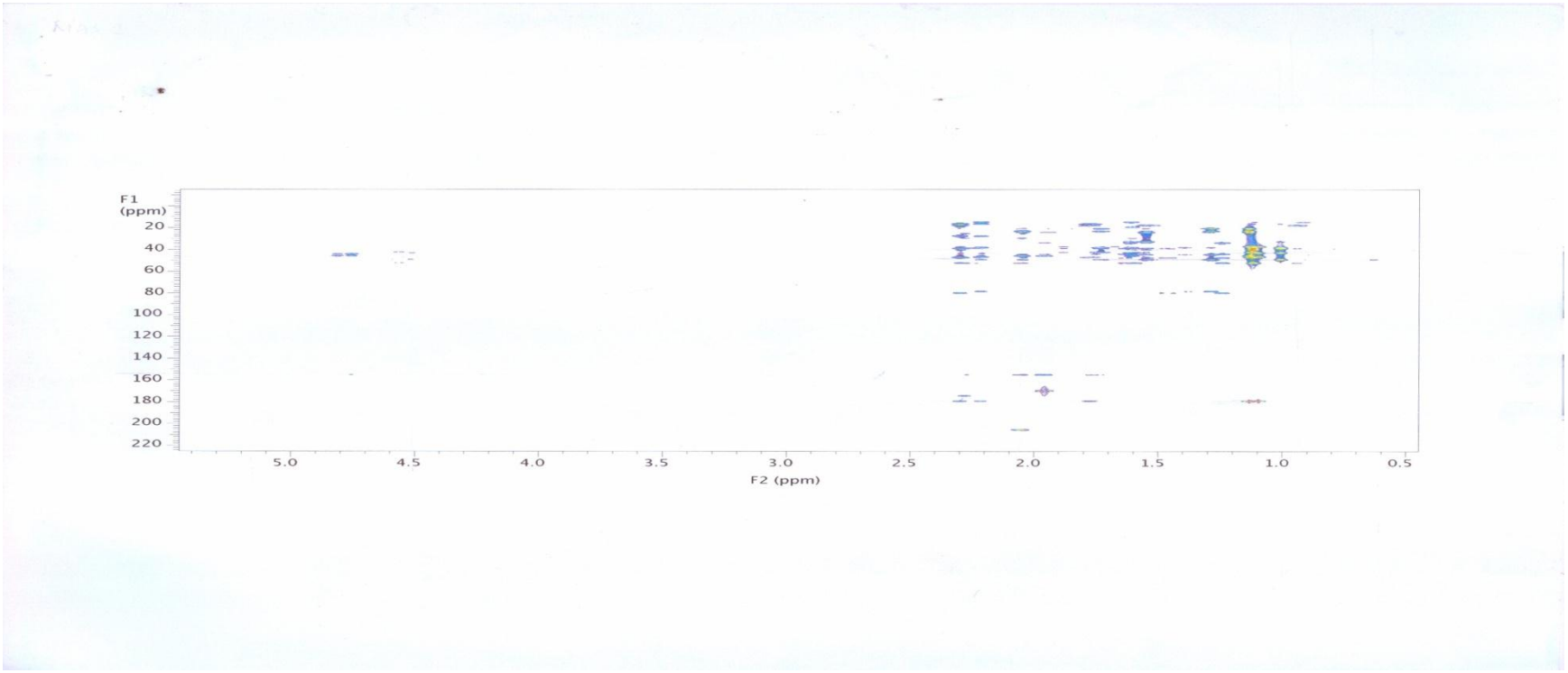
The ^{13}C NMR spectrum of of Clerodendrumic acid isolated from the leaves of *C. glabrum*



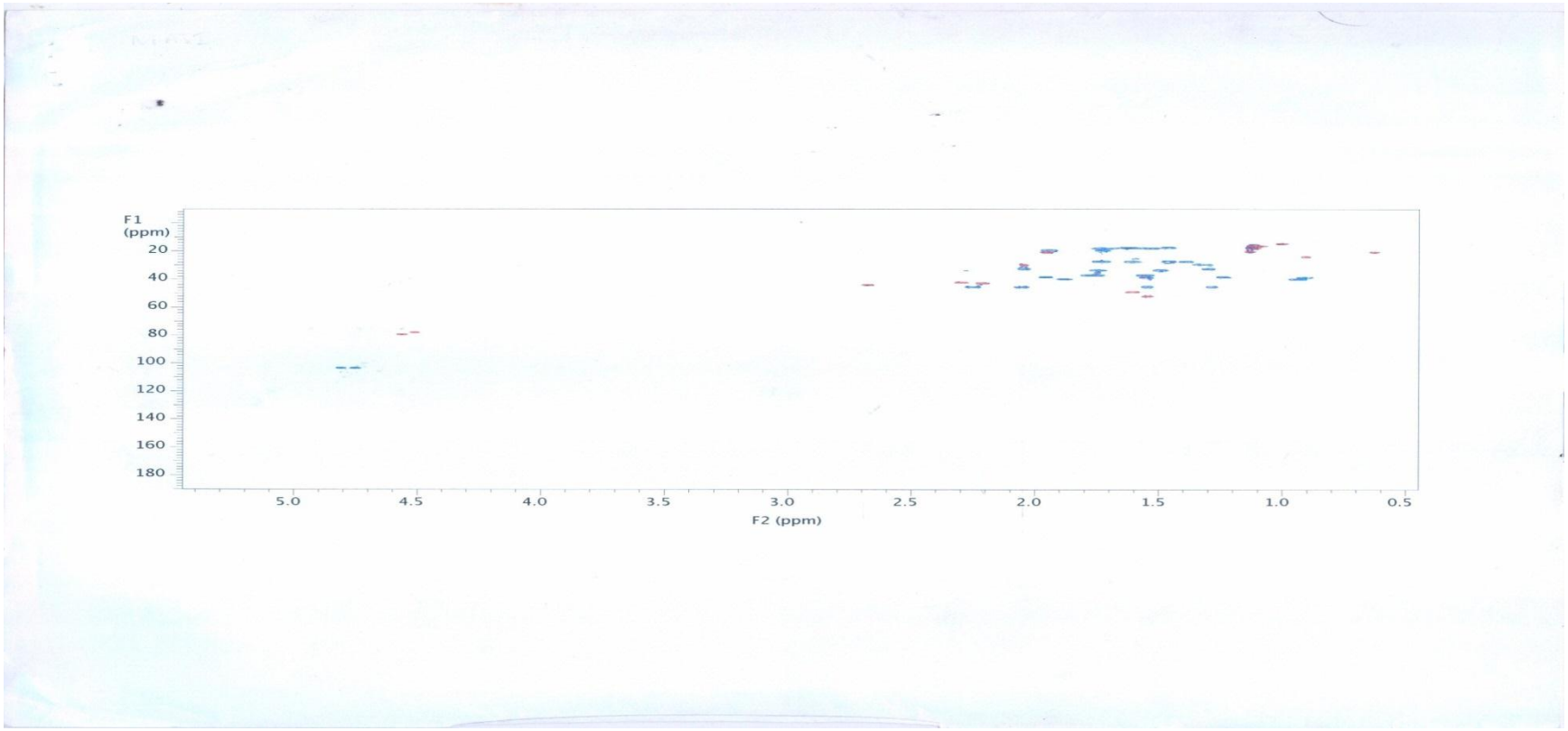
The ^{1}H NMR spectrum of Clerodendrumic acid isolated from the leaves of *C. glabrum.*



The ^{13}C NMR spectrum of Clerodendrumic acid isolated from the leaves of *C. glabrum*.



HMQC-NMR Spectroscopy of Clerodendrumic acid



HMBC-NMR Spectroscopy of Clerodendrumic acid