Ethnobotanical survey of medicinal plants with antifungal activities in Makhado Local Municipality, Limpopo Province, South Africa

by

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Botany has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Machaba TC (Ms) _____

Surname, Initials (title) Date

DEDICATION

I dedicate this work to my parents Selaelo Moses and Maria Hangwani Machaba, my daughter Tshilidzi, sisters Morwafe, Mokgadi and Kgomotso, my brothers Delton and Dennis Machaba. To my late grandmother Tshinakaho Machaba, for her support and making these research a success.

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ABSTRACT

The aim of the study was to investigate medicinal plants used for the treatment of various ailments by the traditional healers and local people and to determine antifungal activities against animal fungal pathogens. Ethnobotanical survey was conducted to identify medicinal plants used by local people and traditional healers to treat various ailments in Makhado Local Municipality, Vhembe District, Limpopo, South Africa. A questionnaire was designed to gather information on the local name of plants, plant parts used and the methods of preparation and administration by the traditional healers. In our findings, sixty-three medicinal plants belonging to thirty-three families were identified to be used for treatment of various diseases such as chest complaint, sexual transmitted infections, headache, swollen legs, hypertension, blood purification, asthma, and infertility. Specific parts of the plant used for medicinal purposes vary from species to species and from one traditional healer to another. The dominant families were Fabaceae, Celastraceae and Euphorbiaceae. Of the sixty-three plants species identified, trees were the most predominant plant form (53%), followed by shrubs (23%), herbs (14%), and climbers (10%). Root, fruit, bark, leaves, seeds and in some instances the whole plant are used for the preparation of medicine while decoction and infusion were the general methods of preparation. The mode of administration of medicine was mainly oral. The most frequently used plant species were *Warbugia salutaris* (Bertol.f.) Chiov*, Sclerocarya birrea* (A.Rich) Hochst and *Eleondron transvaalense* (Burtt Davy) R.H. Archer*.*

Eight plant species (*Asparagus buchananii* Bak., *Albuca seineri* (Engl. & K.Krause) J.C Manning & Goldblatt, *Elephantorrhiza elephantina* (Burch.) Skeels, *Indigofera circinnata* Benth*, Maerua juncea* Pax, *Pentarrhinum insipidum* E. Mey., *Senna italica* Mill. and *Schinus molle* L.) were selected based on the information given by the local people and the traditional healers for further phytochemical analysis and microbiological assays. Antifungal activities of the selected plant species were determined against three fungal pathogens namely, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Of the tested plant species, hexane leaf extracts of *M. juncea*, ethyl acetate leaf extracts of *S. italica*, *A. buchananii* and *E. elephantin*a were the most active against *Candida albicans*, *Cryptococcus*

neoformans and *Aspergillus fumigatus* with Minimum inhibitory concentration (MIC) values ranging between 0.02 mg/ml and 0.08 mg/ml.
Bioautography assay was used to determine the number of active compounds in the plant extracts. No active compounds were observed in some plant extracts against the tested animal fungal pathogens indicating possible synergism. The most promising plant species were: *A. buchananii*, *A. seineri* and *M. juncea*, all had shown good activity with 4 compounds against *A. fumigatus*. Acetone and methanol extracts had the same active compounds visible on bioautograms. Most of the active compounds were observed in TLC chromatograms developed Benzene: ethanol: ammonia hydroxide (BEA) eluent solvent system.

Based on excellent antifungal activity against the tested microorganisms, leaf extracts of *A. buchananii, A. seineri M. juncea, P. insipidum* and root extracts of *I. circinnata* were also tested for cytotoxicity against the Vero kidney cells. All plant extracts investigated were relatively not toxic against the cells with LC_{50} ranging between 0.131 mg/ml and $>$ 1 mg/ml. Water extracts of *A. buchananii, A. seineri* and *M. juncea* had $LC_{50} > 1$ mg/ml. The leaf aqueous extracts of *P. insipidum* were less toxic than root aqueous extracts *of I. circinnata* with LC_{50} of 0.65 mg/ml and 0.49 mg/ml against the Vero kidney cells respectively.

The results indicate that the local people and traditional healers in Makhado Local Municipality use medicinal plants and their indigenous knowledge on the treatment of fungal infections and related ailments.

ABBREVIATIONS

A	Acetone
A. F	*Aspergillus fumigatus*
AMP B	Amphotericin B
ATCC	American type culture collection
BEA	Benzene, ethanol ammonia (90:10:1)
C. A	*Candida albicans*
CEF	Chloroform, ethyl acetate, formic acid (5:4:1)
C. N	*Cryptococcus neoformans*
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EA	Ethyl acetate
EMW	Ethyl acetate methanol, water (40:5.4:4)
EtOH	Ethanol
H	Hexane
H_20	Water
INT	p-iodonitrotetrazolium violet
LC_{50}	Lethal Concentration for 50% of the cells
MeOH	Methanol
MIC	Minimum inhibitory concentration
MTT	(3-(4,5-dimethylthiazol)-2,5 diphenyltetrazolium bromide
NGOs	Non-government organisations
R_f	Retention factor
SDA	Sabouraud Dextrose Agar
STIs	Sexually transmitted infections
TLC	Thin layer chromatography
WHO	World health organisation

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CHAPTER 1

MEDICINAL PLANTS

1.1 Introduction

Medicinal plants are widely used as a primary source of prevention and control of various diseases in both animals and human. In developing countries, medicinal plants are utilised as alternatives to treat health related problems (Duarte et al., 2005). About 80% of people in developing countries use traditional medicines for their primary health care since these plants are easily accessible and cheaper as compared to western medicine (Ekor, 2014). In South Africa, over 30 000 species of higher plants were recorded and 3 000 of these species are used in traditional medicine (Drewes, 2012). Local people and traditional healers utilise indigenous, exotic or invasive plants as medicine. Almost 27 million local people in South Africa support the use of indigenous medicinal plants (Mander, 1998). On the other hand, local people also use these plants as source of fire wood, furniture, timber, fencing and protection of their homesteads and for commercial purposes.

Traditional medicines are more acceptable in developing countries and are part of the culture and religion amongst various ethnic groups. This is attributed to their accessibility and affordability (Steenkamp, 2003). South Africa has a huge diversity of tribes which is reflected in the systems of medicinal practises (Van Wyk et al., 1997). Some rural people prefer traditional medicines for cultural reasons cost effectiveness, acceptability and their accessibility (Yirga, 2010). People utilise medicinal plants based on their beliefs in traditional knowledge and due to relatively poor access to clinics or primary health care facilities (Upadhyay et al., 2007).

Indigenous knowledge rests with traditional healers and local people, and information is generally acquired from survey and interview. In most cases, traditional local knowledge is passed on orally from generation to generation (Togola et al., 2005). Local people prefer to consult traditional healers since they are easily accessible and their medication is not expensive. Traditional healers are recognized by the community to provide health care by using organic substances (plants and animals) based on the social, cultural and religious background. In South Africa, the

elderly people have more indigenous knowledge of the plants that are used for medicinal purposes.

In this dissertation, antifungal activities of selected medicinal plants in Makhado Local Municipality were investigated. This is important for detecting plant extracts that have the ability to inhibit growth of microorganisms. Furthermore, the toxicity effects of these plants are important in order to determine the safety of potential plant extracts. Plant extracts showing activities against tested microorganisms can provide lead to the discovery of novel antifungal agents. Thus, thorough ethnobotanical survey on various plant species has been conducted. Ethnobotanical surveys play an important role in gathering information about plant species used for medicinal purpose and also could lead into the development of new safer and cheaper potent drugs (Parveen et al., 2007). It is therefore important to document the indigenous knowledge of medicinal plants for future generations before it gets lost.

1.2 Rationale

Indigenous knowledge based on medicinal plants is lacking worldwide due to the ever increasing dependency on modern western medicine. Information on the medicinal uses rests with traditional healers and the information is not well documented for some parts of South Africa (Van Wyk et al., 1997 and Madikizela et al., 2012). Most of these plants have novel uses for combating infectious diseases on animals and humans, mainly caused by fungi (Senguttuvan et al., 2013).

Fungal pathogens causing diseases on plants, animals and humans are difficult to control due to their ability to be metabolised to many substances (Kavitha and Satish, 2016). The increasing resistance of fungal pathogens to currently available drugs such as Amphotericin B, Polyenes and Azoles is a major threat to public health. Furthermore, the currently available antifungal drugs are expensive, and some fungal species are resistant against them (Sanguinetti et al., 2015).

Infectious diseases account for a high proportion of the health problems in developing countries (Bansod and Rai, 2008). Moreover, it is difficult to control infectious diseases such as *Candida* infection (Aumeeruddy-Elalfi et al., 2016). Several antifungal agents have been developed and clinically used, but the cure

rates are still low, probably due to high resistance to current drugs (Singh et al., 2016).

Recently, research has focused more on medicinal plants for the development of new antimicrobial substance (Aumeeruddy-Elalfi et al., 2016). It has been reported that plants produce a variety of compounds with natural antimicrobial activities (Kavitha and Satish, 2016). In the current work, antifungal activity and cytotoxicity of plant extracts have been studied to identify plant extracts that are active against fungal pathogens namely, *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans*. Firstly, ethnobotanical survey was conducted in order to provide lead on screening of medicinal plants that may have antifungal activities. This is important for the discovery of novel antifungal agents that are more effective, and not toxic, to combat fungal infections in humans and animals.

1.3 Aim

The aim of the study was to investigate plant species used for the treatment of various ailments by traditional healers and local people using a semi-structured questionnaire, and to determine the antifungal activities and cytotoxicity of selected plant species against animal fungal pathogens.

1.4 Objectives

The objectives of the study were to:

i. Conduct an ethnobotanical survey on medicinal plants used to treat various ailments using a semi-structured questionnaire.
ii. Select and identify plant species with antifungal activities against animal fungal pathogens for further phytochemical analysis.
iii. Determine the antifungal activities of selected plants against *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*.
iv. Determine the cytotoxicity of the crude extracts against Vero monkey kidney cells.

1.5 Outline of study

Chapter 1 is concerned with general background on medicinal plants, traditional medicine and indigenous knowledge system. The rationale, aim and objectives were given.

Chapter 2 is concerned with the literature review based on medicinal plants and their uses. A brief review on various infectious diseases and fungal pathogens is also given.

Chapter 3 is concerned with the ethnobotanical survey of plant species in Makhado Local Municipality. The materials and methods used to select plant species are outlined. The results of the identified medicinal plants used by local people and traditional healers are discussed and finally the conclusion is given.

Chapter 4 is concerned with extraction and phytochemical analysis of plant species. The materials and methods used for extraction of plant materials and phytochemical analysis are also discussed. The results on extracted materials and chemical components of plant extracts are discussed.

Chapter 5 is concerned with antifungal activities and cytotoxicity of selected medicinal plants. The materials and methods such as serial dilution, bioautography assay are given. The results on antifungal activity, presence of antifungal compounds and toxic effects of plant extracts are discussed.

Chapter 6 gives summary and conclusion of the study. Lastly, the references used in the study are listed.

CHAPTER 2

LITERATURE REVIEW

2.1 Importance of medicinal plants

Medicinal plants are the major source of drugs in traditional system of medicine and thousands of plants species are used for the treatment of different infections (Predeep et al., 2014). Infectious diseases are the common cause of death and sickness in developing countries (Nabavi et al., 2015). However, it is difficult and more challenging to treat infectious diseases (Aumeeruddy-Elalfi et al., 2016). Eighty percent of the world's population use traditional medicines for primary health care (Predeep et al., 2014). In addition, plants extracts are recommended as primary source of treatment of infectious diseases by the local people and traditional healers.

Fungal pathogens are the common cause of morbidity and mortality in the world especially in an immune compromised population such as cancer, HIV and AIDS (Kavitha and Satish, 2016). Medicinal plants have been used to produce many modern medicines. However, most of the plants should be evaluated for toxicity, since most people believe that medicinal plants are safe, less toxic, have economic values and are primary source of natural drugs (Al-Daihan et al., 2013 and Akter et al., 2014). There is a need to search for new antifungal drugs since the production and availability of antifungal drugs in a market are very low (Kavitha and Satish, 2016).

2.2 Uses of medicinal plants

Plants are used as a source of medicine; almost 50% of all commercial drugs are derived directly from the plants (Drewes, 2012). Drug discovery from medicinal plants led to the isolation of early drugs such as, cocaine, codeine, digitoxin and quinine of which some are still in use (Samuelsson, 2004). In South Africa, there are drugs that were derived from plants such as fosbretabulin and platensimycin which are used as anti-cancer drugs (Davies-Colman, 2010). However, there are also other medicinal plant-derived drugs that are introduced in the market, for example, arteetheris a potent anti-malarial drug, Galantamine and nitisinone are used to treat

a rare inherited disease such as tyrosinaemia (Balunas and Kinghorn, 2005). Drugs such as aspirin, ephedrine, tubocurarine, atropine are produced from *Catharanthus roseus* worldwide (Gurib-Fakim, 2006). Though the use of medicinal plants is increasing and their importance in drug discovery, the future of these medicinal plants is being threatened and need to be conserved (Rout et al., 2009).

In South Africa, large parts of commonly daily used medicines are derived from plants (Taylor et al., 2001). Medicinal plants such as *Maerua angolensis* and *Rhus lancea* are used to treat stomach aches and diseases related to smallpox (Mabogo, 1990). *Maerua juncea* is used to treat tuberculosis (Chinsembu et al., 2015). The bark of *Ekebergia capensis* is used as a poultice to abscesses and boils (Grierson and Afolayan, 1999). *Ziziphus mucronata* is used to treat bilharzia, chronic coughs, boils, diarrhoea, and infertility in women, menorrhagia, venereal disease, oedema, pneumonia, toothache and wounds (Gelfand et al., 1985). *Withania somnifera* roots are used as an effective remedy for toning up the uterus of women who habitually miscarry and the leaves poultice is used on open cuts, abscesses and wounds (Grierson and Afolayan, 1999 and Parveen et al., 2007). The leaves and roots of *Sanseviera hyacinthoides* are widely used to treat ear infections, stomach disorders, toothache and ulcers (Nyenya and Stedje, 2011). The roots and bark of *Peltophorum africanum* are traditionally used in Southern Africa to treat diarrhoea, dysentery, infertility wounds and chronic pains (Watt and Breyer-Brandwijk, 1962). *Catharanthus roseus* is an example of important plant used by the local people in the treatment of leukaemia.

2.2.1 Conservation of endangered plant species

Almost 13% of South Africa‘s plant taxa are threatened with extinction (Raimondo, 2011). *Warbugia salutaris* is an example of highly used medicinal plant in Southern Africa and is regarded as endangered species (Hamilton, 2004). The increasing demand for herbal medicines encourages traditional healers and traders to destroy natural population of important medicinal plants; these can lead to loss of genetic diversity and natural habitat destruction (Netshiluvhi and Eloff, 2016).

Measures should be taken on the conservation, sustainable use of plants species and to pass the knowledge to the next generation (Thring and Weitz, 2006). These

can be achieved by teaching the local people and the traditional healers about the sustainable ways of harvesting medicinal plants. The education of people about the sustainable use and conservation can lay an important foundation for the conservation of natural habitats of medicinal plants (Hamilton, 2004). The elders and women should be considered in the transmission of the knowledge of medicinal plants usage. People should be encouraged to harvest plant parts such as leaves, seeds and fruits for herbal preparation because they could be less destructive. The process of using cultivated medicinal plants should also be encouraged. Hamilton, (2004) reported that total number of species of medicinal plants cultivated on any scale is few; most medicinal plants are collected from the wild. However, the importance of introduced medicinal plants prevents overuse of indigenous plant species (Begossi et al., 2002).

2.2.2 Economic importance of medicinal plants

Natural resources such as medicinal plants play a significant role in the socio-economic development of the African continent (Geldenhuys and Van Wyk, 2002). Medicinal plants are utilized by local people and traditional healers due to abundance and being less expensive than western medicine. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatments of various diseases (Azaizeh et al., 2003). For example, trading of medicinal plants in the markets, contributes to job creation for unemployed people in the community. Most women who are not employed are involved in trading of medicinal plants to reduce poverty in their homesteads, while most men are not involved since they have salary based jobs than women (Ndawonde et al., 2007). The trade of medicinal plants forms part of multi million rand income in Southern Africa (Cunningham, 1997). However, other medicinal plants are sold in large volume in local markets though they are not yet formally commercialized (Van Wyk, 2008).

Recognition and development of medicinal and economic benefits of medicinal plants are on the increase in both developing and developed countries (Sirajudeen et al., 2017). In South Africa, about 38 indigenous species that are regularly used as traditional medicine have been commercialized as processed materials in modern

packaging and in various dosage forms such as teas, tablets and ointments (Van Wyk, 2008).

2.3 Indigenous knowledge

Indigenous knowledge is the local knowledge that is unique to a culture or society and the knowledge is acquired over generations by communities. In some developing countries, there is a deterioration of indigenous knowledge on medicinal plants throughout the generations (Yineger et al., 2008 and Lulekal et al., 2013). Some researchers have indicated that younger generation usually consider the belief in plant remedies as a sort of superstition and less effective compared to modern medicine (Parveen et al., 2007). According to Maema et al. (2016), it is crucial for young people to learn about indigenous knowledge as this information will be preserved amongst future generations. More importantly, documentation of this knowledge is necessary for future generations before it disappears.

Western style health care services provided by government have contributed to the decline in traditional knowledge on medicine since people put more trust in western medicine than in traditional medicine (Bussmann et al., 2011). Necessary effort should be made to avoid erosion of this knowledge in South Africa, and also to conserve the information of useful plants (Grierson and Afolayan, 1999). Traditional medical knowledge of medicinal plants and their use by indigenous healers are not only useful for conservation of cultural traditions and biodiversity but also for community health care and drug development in the present and future (Pei, 2001). The knowledge on the use of medicinal plants is enormous but if this traditional knowledge is not rapidly researched and recorded, indications are that it will be lost with succeeding generations (Hostettmann et al., 2000).

2.4 Plants Secondary metabolites

Medicinal plants contain secondary metabolites which provide physiological action in human body and these bioactive substances include tannins, alkaloids, terpenoids, steroids, saponins and flavonoids (Yadav and Agarwala, 2011). Plants secondary metabolism is an important source of many fine chemicals such as drugs. Some medicinal plants contain bioactive compounds that have antimicrobial activities (Arif et al., 2009). It is important to note that bioactive compounds from plants secondary

metabolites exhibit a wide range of mechanisms for inhibiting microbial growth that causes diseases to humans (Potroz and Nam-Joon, 2015). Furthermore, some of the secondary metabolites are used as a source of medicine and could lead to the discovery of new pharmaceuticals (De Boer et al., 2005). Previous work indicated that some compounds are derived from plants secondary metabolites, and could be used to combat human ailments such as malaria, cancer, flu and others (Drewes, 2012).

2.5 Infectious diseases

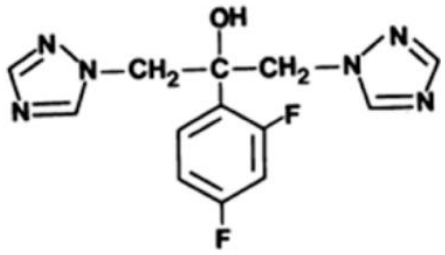
Infectious diseases are currently considered as one of the leading causes of global morbidity and mortality especially in developing countries (Aumeeruddy-Elalfi et al., 2016). Fungal infection is a creamy white, curd-like patch on the tongue or other mucousal surfaces which are removed by scraping (Motsei et al., 2003). Opportunistic fungus infections are increasing with the increase in number of immune-compromised patients in the health care systems in developing countries (Steenkamp et al., 2007). In South Africa, opportunistic fungi such as *Cryptococcus neoformans* cause cryptococcal meningitis. *Candida albicans* causes candidiasis in human. *Aspergillus fumigatus* causes aspergillosis in immune-compromised individuals (Liu et al., 2014).

2.5.1 Resistance of fungal pathogens

The resistance that pathogenic infections develop against antibiotics has increased a great interest in the search of new antimicrobial drugs (Al-Fatimi et al., 2007). The treatment of bacterial and fungal pathogens that are drug resistant is even more complicated especially in acquired immune deficiency syndrome (AIDS) patients (Sanguinetti et al., 2015). However, antimicrobial properties have been reported more frequently in a wide range of plant extracts and natural products in an attempt to discover new chemical classes of antifungal drugs that could resolve this problem (Kavitha and Satish, 2016). Herbal medicines used in traditional folk remedies may help to overcome the increasing resistance to antifungal drugs and their related toxic effects (Uddandapu et al., 2016).

Failure of drug treatment in fungal infections is harmful to human health. An improvement of performance and standardization of anti-fungal susceptibility testing

is being attempted to address the problem of anti-fungal resistance and its underlying mechanisms (Schmourlo et al., 2005). Examples of drugs that are resistance to fungal pathogens include itraconazole (Denning, 1998). The other drugs are shown by their chemical structure in figure 2.1.



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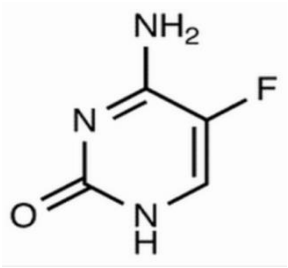

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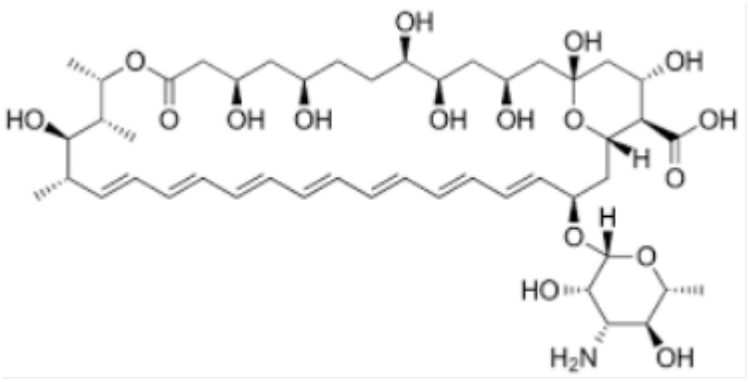


(c)

Figure 2.1 Chemical structures of (a) fluconazole, (b) flucytosine and (c) Amphotericin B (Vandeputte et al., 2012)

2.5.2 Plant as antifungal agents

Antimicrobial agents occurring naturally have been derived from plants (Coker et al., 2015). Plants are a source of antimicrobial drugs and new anti-effective agents. Furthermore, plant derived compounds play a major role in fighting against pathogens (Singariya et al., 2012). Secondary metabolites such as alkaloids, tannins, phenolic compounds derived from plants possess antibacterial properties and can be used as medicinal agents (Pradeep et al., 2014). Recently, many modern medicinal compounds are derived from plants (Sirajudeen et al., 2017).

Phytochemicals have two categories; primary and secondary constituents. Primary constituents contain chlorophyll, proteins sugar and amino acids while secondary constituents contain terpenoids and alkaloids (Wadood et al., 2013). Phytochemicals found in plants play an important role in the treatment of various ailments and can serve as a good source of useful drugs (Yadav et al., 2014). However, the beneficial medicinal effects of plant materials such as, antifungal, antibacterial and anti-inflammation activities usually result from the combination of secondary metabolites present in the plant (Kapoor et al., 2015).

2.6 Fungal pathogens

2.6.1 *Aspergillus fumigatus*

Aspergillus fumigatus is commonly found in poultry and causes localized infection of lungs, sinuses in non–immunocompromised individuals (Suleiman et al., 2010 and Morace et al., 2014). Inhalation of spores from the environment gives rise to invasive infection of the lungs or sinuses and dissemination to other organs in immunocompromised persons. Aspergillosis is frequently seen in patients with leukemia, induced bone marrow suppression and patients with the late-stage of AIDS (Patterson et al., 2000).

Treatment failure is common in patients with aspergillosis since the correlation of resistance to antifungal treatment is difficult to establish (Perea et al., 2002). For example, Amphotericin B treatment failure against *Aspergillus* is very common (Moore et al., 2000). The development of azole resistance observed in some *A. fumigatus* strains should urge a thorough study of molecular mechanism resistance. Previously, it was reported that Aspergillus species are resistant to the current

available drugs such as voriconazole, posaconazole and ravunazole (Sheehan et al., 1999).

2.6.2 *Candida albicans*

Candida albicans is an opportunistic pathogen that can cause local and systemic infections in predisposed persons, commonly affecting immunocompromised patients and those undergoing prolonged antibiotic treatment (Correia et al., 2014). The management of candida infections is facing a number of challenges such as limited and unavailability of antifungal drugs, and also resistance of *Candida* to commonly used drugs (Liu et al., 2014). Furthermore, there are difficulties associated with the management of *Candida* infections which necessitate the discovery of new anti-fungal agents (Shokri et al., 2012). It was also reported that *Candida* is resistant to currently available azoles drugs such as fluconazole, triazoles and intraconazoles (Garcia-Rubio et al., 2017).

2.6.3 *Cryptococcus neoformans*

Cryptococcus neoformans is one of the most harmful opportunistic fungal pathogen that cause life threatening to the central nervous system in immunocompromised individuals (Gago et al., 2016), mainly HIV-infected patients and those receiving immunosuppressive treatment for cancer, organ transplantation, and other critical medical conditions (Lai et al., 2016). In addition, the resistance to therapy against *Cryptococcus neoformans* can be caused by a variety of factors, such as hydrocephalus, drug intolerance, poor compliance with therapy, and pharmacokinetic factors. The lack of development either primary or secondary drug resistance may also enhance resistance (Morace et al., 2014 and Nyazika et al., 2016).

2.7 Cytotoxicity of medicinal plants

Cytotoxic screening of plants is the preliminary method to identify toxic effect of plant extracts (Sahranavard et al., 2009). However, the assessment of cytotoxicity on plant extracts is important for conducting and monitoring if there are any harmful effects on plants especially those commonly used by traditional healers to treat various ailments. Most medicinal plants used are potentially toxic and toxicity studies help to ensure that the biological activities of the plant’s extract is not due to a general metabolic toxic effect. More importantly, toxicological evaluations of all medicinal

plants are important in order to ascertain their safety (Asare et al., 2012 and Dzoyem et al., 2016).

Nearly 4000 medicinal plants taxa are used in South African traditional healthcare and relatively few are considered likely to give rise to serious toxicity (Fennell et al., 2004). Okem et al. (2012) reported that evaluating natural products for their efficacy and toxic potential before applying them as therapeutic agents is becoming increasing important.

2.8 Botanical description of selected medicinal plants used in the study

Eight plant species have been selected based on their medicinal uses for the current study. All pictures of the selected plant species were taken from Muduluni village in Makhado Local Municipality.

Elephantorrhiza elephantina (Figure 2.2) is a shrub from the family Fabaceae. The plant has several unbranched, annual stems of nearly one metre in height, growing from an underground rhizome. The finely divided leaves have numerous small, narrow leaflets. Clusters of small, cream-coloured flowers are produced along the lower half of the aerial stem, giving rise to the seed pods (Hutchings et al., 1996). *Elephantorrhiza elephantina* often occurs gregariously in hot, dry areas with grassland and open scrub (Van Wyk and Gericke, 2000).

Figure 2.2 *Elephantorrhiza elephantina*

Senna italica is a herb from the family Fabaceae and is shown in Figure 2.3. Many species of the genus *Senna* are used traditionally to treat a number of ailments such

as intestinal complications, haemomorphoids, circulatory system problems, calculi in the urinary system and sexually transmitted diseases.

Figure 2.3 *Senna italica*

Schinus molle also known as pepper tree, belonging to the family Anacardiaceae is an evergreen tree that grows to 15 meters (Figure 2.4). It has a thin, long leaves and it is often used in subtropical climates for landscaping (Deveci et al., 2010).

Figure 2.4 *Schinus molle*

Asparagus buchananii is a woody climber or shrub from the family Asparagaceae, with yellowish brown to grey stems with zigzag branches, spines 6-20 mm long flattened towards the base. Occurs in forest margins and drier bush land, the plant is shown in Figure 2.5.

Figure 2.5 *Asparagus buchananii*

Albuca seineri is a perennial herb growing from the bulbs, belonging to the family Asparagaceae. It is generally fleshy with mucilaginous juice (Manning, 2008). The flowers may be stiff and the fruits are rounded with three lobed capsule containing shiny black seeds (Figure 2.6).

Figure 2.6 *Albuca seineri*

Indigofera circinnata is a small shrub belonging to Fabaceae family (Figure 2.7), usually less than 1 m, with orange spines, flowers with gaping lips, bicoloured, white and fleshy pink and the pods are coiled (Shrire, 2012).

Figure 2.7 *Indigofera circinnata*

Maerua juncea is a woody climber or scrambling shrub in the canopy of other trees or shrubs, belonging to the family Capparaceae (Figure 2.8). Leaves are alternate, grey-green. The fruits ellipsoid with a smooth surface, and they are green to orange when ripe.

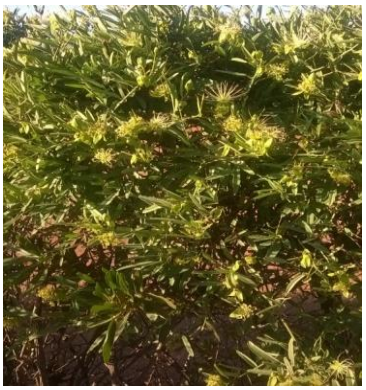


Figure 2.8 *Maerua juncea*

Pentarrhinum insipidum from the family Apocynaceae commonly known as phulule (Venda) is a wild vegetable, slender fast growing. The plant is endemic to South Africa and other African countries. It is widespread in Savanna and shrubland. The plant is shown in Figure 2.9.

Figure 2.9 *Pentarrhinum insipidum*

CHAPTER 3

ETHNOMEDICINAL USE OF PLANT SPECIES

3.1 Introduction

Medicinal plants are an important source of indigenous medical systems in South Africa and across the globe. About 60-80% of South African population relies on traditional medicine for the treatment of human ailments (Shai et al., 2008). The use of traditional medicines by the traditional healers plays an important role in the health care of millions of people (Sigidi et al., 2016). However, practices of usage may differ from country to country for the treatment of human ailments such as ulcers, headaches, burns colds and malaria (Masango and Nyasse, 2015 and Saibu et al., 2015). In South Africa, *Ziziphus mucronata* is used medicinally to treat measles, chest complain and swelling (Sigidi et al., 2016).

South Africa has more than 200 000 traditional healers and they conduct healing practices of body and mind for more than 27 million people (McFarlane, 2015). Most people still rely on the traditional healers for the treatment of various ailments because they are found within the community, and the consultation is more affordable than western health care. Moreover, it is possible for traditional healers to treat human ailments since they are well conversant with the culture and tradition of the people.

Ethnobotanical surveys and documentation of medicinal plants are crucial for gaining information and preserve knowledge about medicinal plants and their uses (Seid and Tsegay, 2011). Medicinal plants are subjected to screening and such a process could provide a lead in the discovery of novel antifungal agents. Majority of traditional medicinal plants have not been intensively studied in African countries (Edori and Dibofori-Orji, 2016). Furthermore, indigenous knowledge is important in conservation of cultural traditions, community healthcare and for future drug development. In this chapter, a detailed investigation on ethnomedicinal use of plant species will be discussed based on ethnobotanical survey and indigenous knowledge system.

3.2 MATERIALS AND METHODS

3.2.1 Location and demographics of study area

The study was conducted in five selected Kutama villages (Muduluni, Tshikwarani, Ha-Madodonga, Maebane and Ha-Manavhela) situated in Makhado Local Municipality in Vhembe District, Limpopo Province. Makhado Local Municipality lies between 23.0000°S and 29.7500°E dissection. Almost 70% of the municipality is rural and Makhado is the administrative headquarters (Moyo et al., 2012). The area is found in the western side of Louis Trichardt, and comprised of three ethnic groups: Vhavenda, Pedi, and Tsonga.

3.2.2 Climate

Makhado Local Municipality experiences rainfall mostly in mid-summer. Rainfall ranges between 185 mm and 495 mm per year. Winter usually lasts from June to August. Summers experience warm and often humid temperatures with the occasional afternoon thunderstorms. The average temperature for summer is around 30°C whilst the winter temperature varies between 20°C to 25°C. Higher temperatures are experienced in the west and north of the mountain range.

3.2.3 Vegetation

Makhado Local Municipality is located in the lowveld and consists of savannah. The fauna and flora range from savannah plains to Mopani and thorn bushveld towards the south and west of Makhado and north of the mountain. Sub-tropical vegetation and even rainforests and lakes are found towards the east.

3.3 Data collection

3.3.1 Ethnobotanical survey

The ethnobotanical survey was conducted in Makhado Local Municipality, Vhembe District, Limpopo Province (Figure 3.1). Permission to conduct the research was obtained from Local authorities, in order to access the communities. Before conducting the survey, the informants were given a consent form to complete before providing the information on the medicinal plants. Twenty informants (15 traditional healers and 5 the local people) from different areas were selected using snowball method. Data was collected using a semi-structured questionnaire (Appendix A) and

guided field work with traditional healers. A questionnaire was designed to gather information on the names of plants used for the treatment of human ailments, the source of these plants, the part/s of plants used, and methods of preparation of medications, diagnosis of different ailments and other information.

3.3.2 Plant collection and identification

Plants were collected from their natural habitat from Soutpansberg West Mountain and five local villages in Makhado Municipality, South Africa during March-May 2016 with the help of traditional healers. Collected plants were identified using the literature and Larry Leach herbarium (UNIN). Voucher specimens were collected and deposited to the University of Limpopo Herbarium. Plant parts such as leaves and roots were collected and plant materials were separated according to the different parts used. Plant materials collected were allowed to dry at room temperature (25° C) before grinding. The dried leaf and roots material were ground to fine powder and stored in the dark in sealed container until extraction.

3.4 Data analysis

Data were analysed using descriptive and inferential statistics such as percentages and frequencies. Frequency index of each plant species were calculated using the formula: FI= FC/N x 100, where FC is the number of traditional healers who mentioned the use of the plant and N is the total number of informants.

3.5 Ethical considerations

The current study was conducted in Makhado Local Municipality, and no samples were collected from animals or human beings. Each traditional healer was requested to sign a consent form approved the University of Limpopo. The proposal was submitted to Turfloop Research Ethics Committee (TREC) after it was approved by the Faculty Higher Degrees Committee (FHDC).

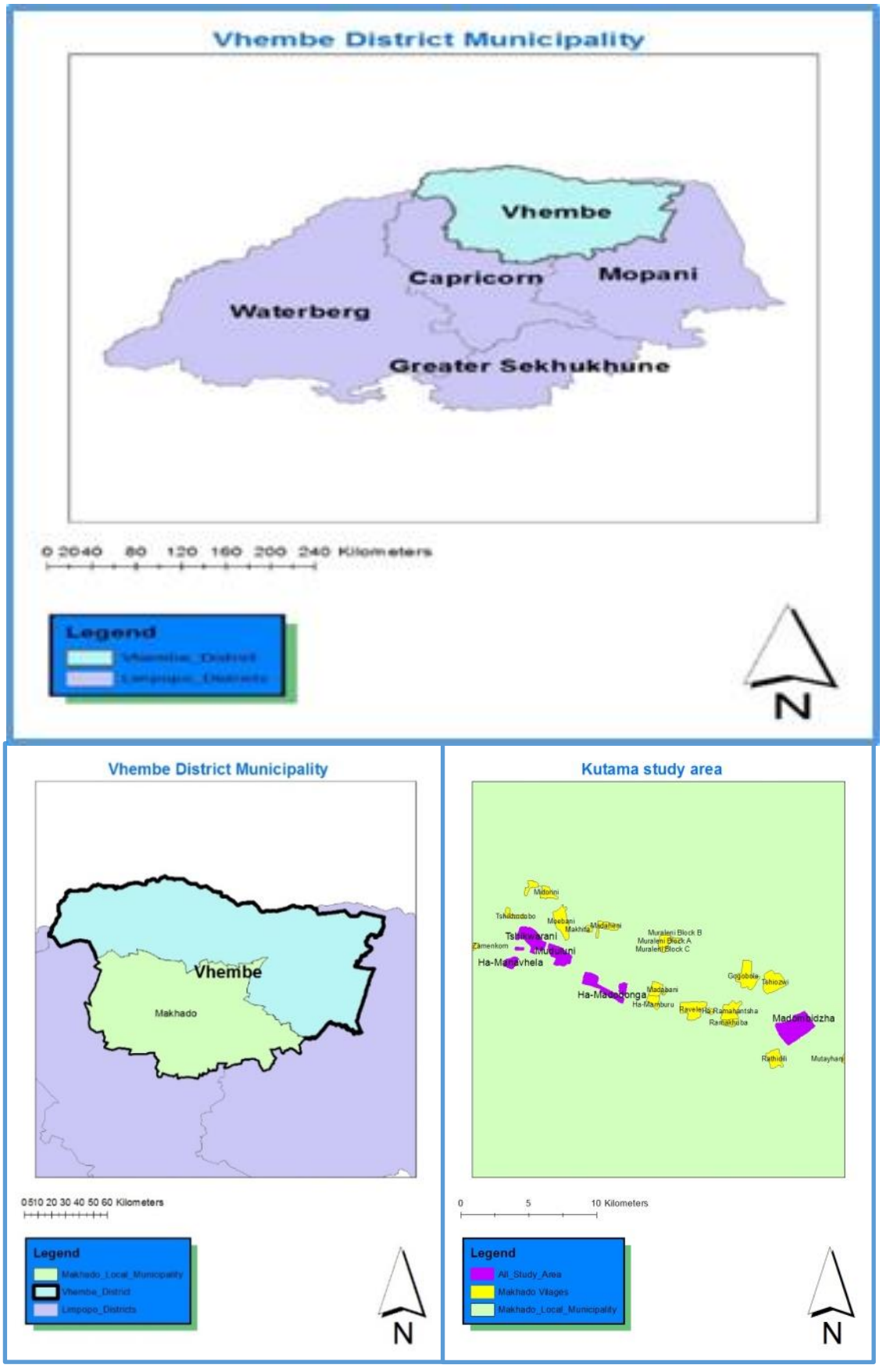


Figure 3.1 Map of the study area showing Makhado Local Municipality.

3.6 Results and Discussion

3.6.1 Demographic information

3.6.1.1 Age and gender of informants

The informants were both male and female. It was found that female traditional healers were dominant in the interview with 85% more than male 15%. Noticeably, majority of healers were women and pensioners and most of them were using traditional healing practice through the ancestral calling. This is true despite a strong traditional belief that women should perform their duties at home, taking care of their families including children and elderly people. Previously, it was indicated that women have more knowledge about the indigenous knowledge on the use of medicinal plants than men (Parveen et al., 2007). However, other findings reported that men were found to have knowledge on medicinal plants than women (Giday et al., 2010 and Belayneh and Bussa, 2014).

Most informants in this study were pensioners above the age of 65 and very few were young people between the ages of 35 and 45. These support the findings by Yineger et al. (2008) that older traditional healers had greater knowledge of traditional medicine (Mowobi et al., 2016). Most recently, it has been a concern that the indigenous knowledge on the use of medicinal plants is declining amongst the younger generation, which could be attributed to low interest of younger generation to inherit and use ethnomedicinal knowledge. Therefore, the transfer of indigenous knowledge is liable to erosion as it could vanish when knowledgeable elders pass on before the knowledge is transferred to individuals (Addis et al., 2002)

3.6.1.2 Educational background

A total number of 20 traditional healers participated in this study. Among 20 informants, 55% of the informants did not have formal education, 30% have acquired primary education while 15% received secondary education. The highest percentage of traditional healers received the primary education and few were illiterates in the study conducted in Eastern Cape Province of South Africa (Lawal et al., 2014). Other studies conducted in Ethiopia found that the majority of the informants were illiterate with 53% and those that could read and write were 33% while 13% attended grade one to four (Yineger et al.,2008 and Asnake et al., 2016).

3.6.1.3 Experience

In our findings, most traditional healers (65%) fall between 21-30 years of healing practice. Of the 20 traditional healers consulted in this study, 35% have less than 10 years in healing practice. Lawal et al. (2014) found that in Eastern Cape Province of South Africa most informants had about 16-20 of experience in traditional practice.

3.6.1.4 Consultation

Most traditional healers have less than 15 consultations per month. The patients' returns if they happen not to be healed and traditional healers do not have the same patients throughout; new patients also come for consultation. People consult traditional healers because they provide personalized health care that is customized to the needs, expectations of patients and also paying special respect of social and spiritual matters (Homsy et al., 2004).

3.6.1.5 Legislation

Of the twenty traditional healers interviewed in Makhado Local Municipality, 75 % were not registered with the Traditional Association for Healers. Only 25 % traditional healers were registered with the Association, the reason being that the Association has strict rules and not seeing the importance of being registered.

3.7 Methods of plant collection

3.7.1 Plant collection

Most traditional healers (60%) collect their plants from the wild while other healers cultivate some plants in their home gardens. Traditional healers believe that cultivated plants have less healing powers than those found in the wild and also that the cultivated plants are contaminated with human behaviours and evil spirits. Previous studies indicated that most medicinal plants were harvested from veld, and only few plant species were harvested from cultivated areas (Kose et al., 2015 and Asnake et al., 2016). In the current study, some traditional healers are too old to collect their plant materials in the field and they prefer to buy their medicinal plants from herbalist. In some instances, they rather hire someone to collect plants from the wild. Tabuti et al. (2003) found that traditional healers hire plant collectors.

Plant species in the study area are mostly collected during winter. Traditional healers collect their plants during winter because they also believe that during summer plants parts such as leaves have not reached maturity stage to contain the healing activities, and also that the roots and bark will have more water content that will reduce the healing power. Early in winter traditional healers collect the leaves before they are shed off from the mother plant. Mabogo, (1990) reported that plant materials must be collected at the appropriate season as the active compounds of some plants vary from season to another.

Collected plants are dried in the sun, ground and put into the bottles for future use, while some plants material such as roots are dried without grounding. It was noted that traditional healers store plant material in an open room on the floor for preservation. In some instances, they store plant material in the sun for an hour to avoid microbial growth. In contrast, Tabuti et al. (2003) reported that sun drying makes medicinal plants to be potentially harmful as the fungi and bacteria may grow on the plant tissue, and due to that the process is done unhygienically on bare ground.

3.8 Traditional healing practice

3.8.1 Plant part(s) used

The roots were the mostly used plant part (49.3%), followed by the bark (23.3%), leaves (19.2%), bulbs and the seeds (2.7%) and the least were the stem and fruits (1.4%) (Figure 3.2). Similar results were found in other studies where the roots were frequently used plant part (Appidi et al., 2008 and Cheikhyouseff et al., 2011). In contrast, other studies reported that the leaves were the most frequently used plant parts (Belayneh and Bussa, 2014). However, leaves do not cause any significant threat to the survival of individual plants as compared to other plants parts such as the roots, stem, bark and whole plant (Giday and Teklehaymanot, 2013).

Herbalists and traditional healers use underground plants part such as the roots and bark since they were reported to contain the highest concentration of potent healing agents (Masevhe et al., 2015). The roots and other underground plant parts have high concentration of bioactive compounds. However, harvesting the roots of herbaceous plants for medicinal purpose is not sustainable and this might have a

negative impact on the survival and continuity of medicinal plants (Lulekal et al., 2008). The fact that the usage of roots account for the highest percentage of all plant parts used in this study, may lead to extinction of some medicinal plants.

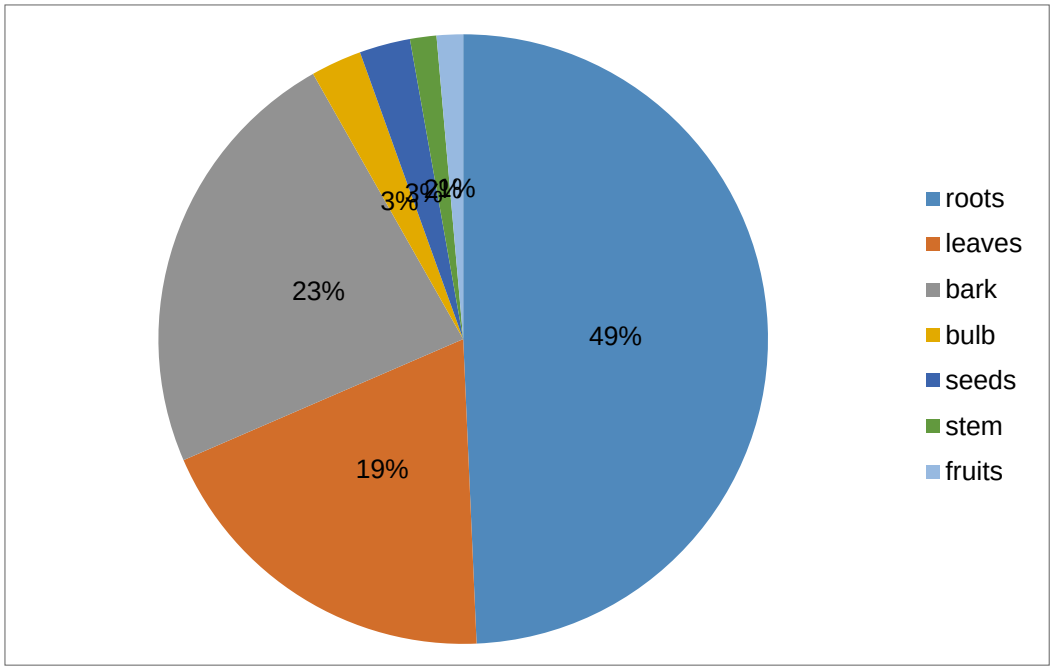


Figure 3.2 Plant parts used in medicine

3.8.2 Diagnosis of different diseases

In order for the traditional healers to diagnose the ailments, 75% of them rely on both ancestors and patients, while 25% rely on patients alone. In general, most patients believe and trust their traditional healers and they are familiar with the patient's culture and environment (Rennie, 2001). More importantly, traditional healing involves rituals and spiritual aspects, rather than the use of plants (Leffers, 2003). In some cases, traditional healers refer their patients to the health care service especially in the cases of chronic diseases such as HIV/AIDS, heart diseases, diabetes, stroke and respiratory problems.

3.8.3 Preparation and treatment

The most common methods of preparation used by the traditional healers are decoction (65%) and infusion (35%). Decoction is a method of choice when extracting tougher and more fibrous bark and roots because they have more water soluble chemicals (Mowobi et al., 2016). Other studies reported similar results

indicating that decoction and infusion were the most frequently used methods of preparation of medicinal plants to treat various ailments (Appidi et al., 2008 and Otang et al., 2012). Of all the modes of administration, the most frequently used was orally (36.9%), followed by external application 20%, bathing and mixing with soft porridge (10.8%), inhalation (9.2%), chewing (4.6%), gargling (3.1%), steaming, brushing and lotion or smear all with the lowest percentage (1.5%) (Figure 3.3). The results also revealed that some medicinal plants were used in more than one mode of administration to treat various ailments, for example, a decoction of roots of *Ziziphus mucronata* is taken orally to treat diarrhoea, whereas the leaves are chewed and swallowed to relieve the pains. Seeds of *Ximenia caffra* are heated then ground to smear the wound and the decoction of roots is taken orally to treat sores.

Traditional healers mostly prepare their medicinal plants in some form of aqueous extraction (Togola et al., 2005). In the current study, traditional healers prefer water rather than lipophilic solvents. Lipophilic solvents have the ability to enable non-polar compounds to dissolve in fats and non-polar solvents. However, researchers use lipophilic solvents such as ethanol, hexane and others for plant extraction because water does not extract the antimicrobial compounds that usually have an intermediate or non-polar character (Kelmanson et al., 2004 and Eloff et al., 2005).

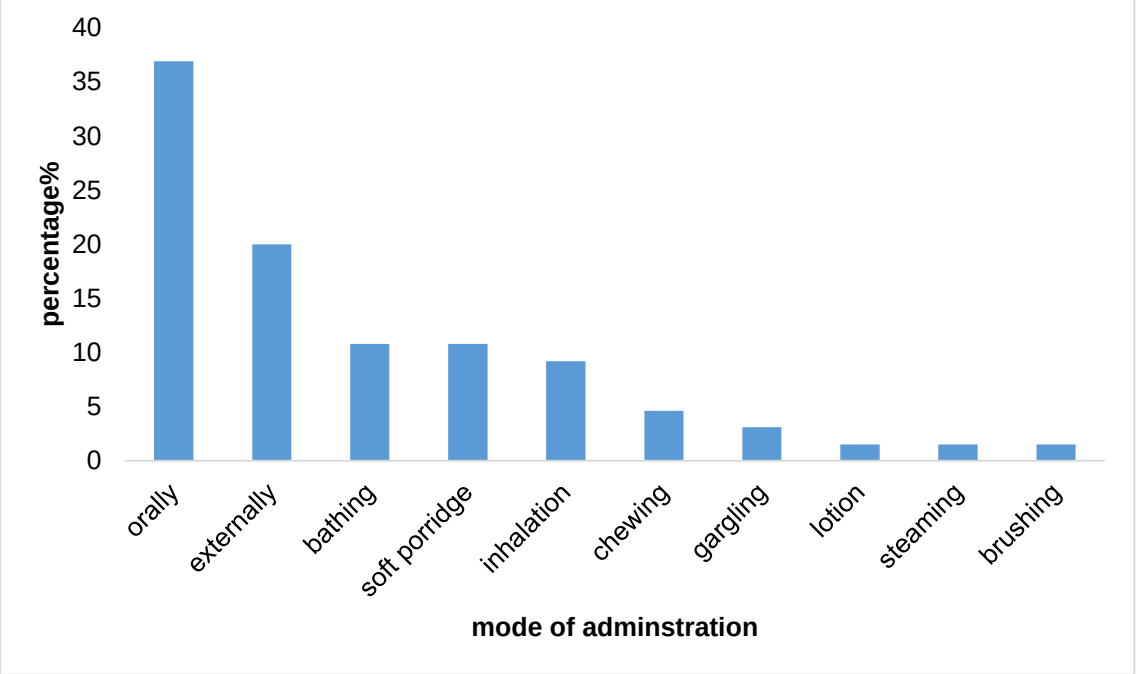


Figure 3.3 Mode of administration of medicinal plants in percentage

3.8.4 Identified medicinal plants

In the current study, sixty-three plants species belonging to thirty-three families were identified as being used for treatment of various ailments. The common names of plants, family names, scientific names, plant forms, plant parts used, method of preparation and administration are represented in Table 3.2. The most dominating families were Fabaceae 33%, followed by Celastraceae 15%, Capparaceae and Euphorbiaceae have the same percentage 12%, Anacardiaceae and Rutaceae 9%, the rest of the families have the lowest percentage of 3%. Similar results were found from other studies were Fabaceae and Euphorbiaceae were among the dominant families (Cheikhyouseff et al., 2011 and Belayneh and Bussa, 2014).

The most commonly used plants were *Warburgia salutaris* used for the treatment of sores. *Sclerocarya birrea* is used to treat ulcers. *Senna italica* is used to relieve backaches and to induce diarrhoea; *Elaeodendron transvaalensis* is used to remove the evil spirits in people. Based on literature, *E. transvaalense* was reported to be used to treat fever, diarrhoea, cramps and as a stomach cleanser (Ndawonde et al., 2007). *Asparagus buchananii* is used to stop a person from vomiting. In general, most medicinal plants in the current study were used for the treatment of stomach aches, diarrhoea, wounds, sore throat, ulcers, vomiting, toothaches and infertility.

3.9 Plant forms

Of the sixty-three plants species found in this study, trees were most predominant plant form (53%), followed by shrubs (23%), herbs (14%), and climbers (10%) (Figure 3.4). Most frequently used plants for medicinal purposes are tree species (Van Wyk et al., 1997). Other researchers found that trees were mostly used in Limpopo Province, and the use of trees and shrubs most frequently might be due to their availability throughout the year and they are relatively drought resistant (Masevhe et al., 2015).

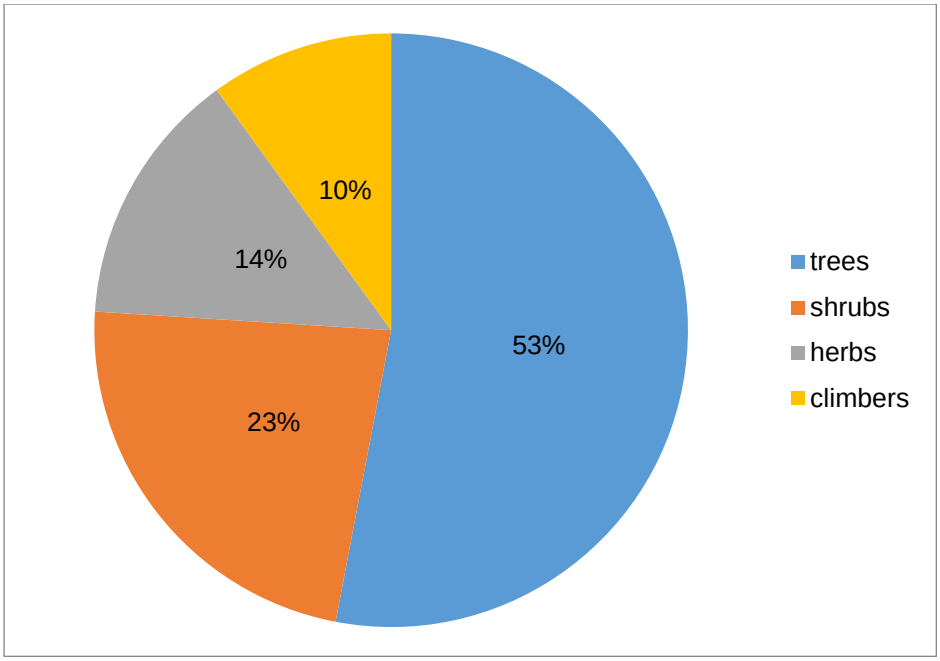


Figure 3.4 Plant forms used in medicine

3.10 Frequency index

Frequency index of each plant species were calculated (Figure 3.5) using the formula described in section 3.4. The frequency index was directly proportional to the number of informants. These suggest that, the higher the number of informants who mentioned a particular plant, the higher the frequency index. In this study, *Warburgia salutaris* was found to be the most frequently used species and is used in the treatment of sores, with a frequency index of 64.7%. *Warburgia salutaris* is also used as a remedy for influenza, coughs, sinus (Rabe and Van Staden, 2000), skin complaints, aphrodisiac, backache, chest complaints, colds, malaria (Mabogo, 1990), diarrhoea, indigestion, fever, snake bites, pneumonia (Mukamuri and Kozanayi, 1999), venereal diseases, stomach ulcers (Van Wyk et al., 1997).

Sclerocarya birrea was the second most frequently used species with a frequency of 58.8%, followed by *Elaedendron transvaalensis*. Based on literature, *S. birrea* is used to treat wounds, ulcer infertility (Mabogo, 1990). Previously, it was reported that *E. transvaalense* is used to remove evil spirits in humans, and in literature is used to treat dysmenorrhoea (Van Wyk, 1972). *Senna italica* is used to induce diarrhoea and also relieve the backaches. *Asparagus buchananii* is used to prevent vomiting, all with 52.9% frequency index. It was noted that *Withania somnifera, Adonsonia*

digitata, Peltophorum africanum, Capparis sepiaria and *Ziziphus mucronata* had the same frequency index (47.1%). *Withania somnifera* gave higher frequency of index in the study conducted by Belayneh and Bussa, (2014). Previous work indicated that *W. somnifera* is also used for toning up the uterus of women who habitually miscarry (Parveen et al., 2007). The higher frequency of index of these species indicates their importance for local communities (Belayneh and Bussa, 2014). The lowest frequency index (5.9%) were observed with *Tabernaemontana elegans, Bolusanthus speciosus, Cassine eucleiformis, Garcinia livingstonei, Crassula ovata, Heteropyxis natalensis, Ekebergia capensis, Acacia albida, Ochna holstii, Zanthoxylem leprieurii* and *Osyris lanceolata*.

Previous work reported that *Ekebergia capensis* is used to treat headaches and wounds (Grieson and Afolayan, 1999), for regulation of menstruation, and for treating venereal diseases, chronic cough, backache and skin disease (Mulaudzi et al., 2011). However, in our findings *Clausena anisata* was the least plant used while in literature it was found to be the species with highest fidelity level and used by traditional healers in Ethiopia to treat rheumatism (Yineger et al., 2008). Previously, it was reported that *C. anisata* has antimicrobial and hypoglycaemic activities (Gundiza et al., 1994 and Hamza et al., 2006)

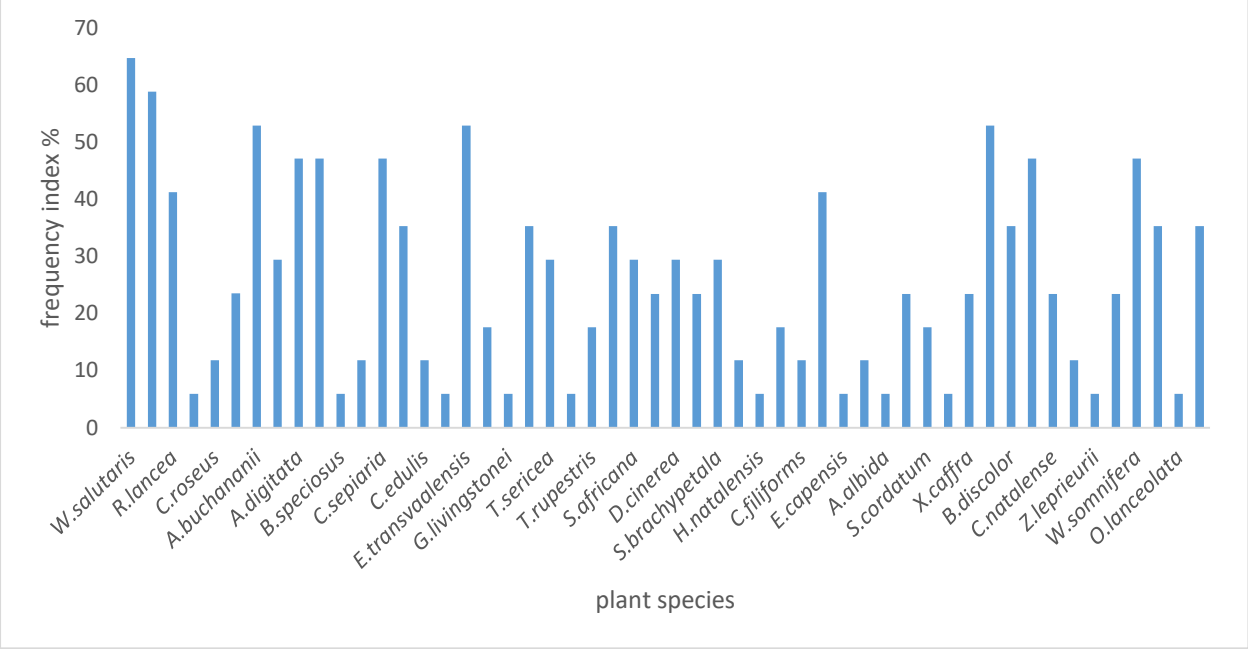


Figure 3.5 Frequency index of plant species used.

3.11 Ranking of ailments

The total numbers of nineteen diseases were ranked in Makhado Local Municipality; the ailments were ranked according to frequency mentioned by traditional healers to treat a particular ailment (Figure 3.6). The highest ranked ailments were diarrhoea with 47.4%, stomach ache 42.1%, throat sores 52.6% as shown in Table 3.1. Stomach disorders and infertility were ranked among the top ten treated ailments (Kose et al., 2015). Based on literature, stomach ache/problems, diarrhoea and wounds were mostly treated ailments with plant medicines (Bussmann et al., 2011). However, Kose et al. (2015) reported that most plants were mentioned to cure infectious diseases such as diarrhoea, blood impurities and skin sores and *Elephantorrhiza elephantina* was among the highest ranked plant mentioned to treat various ailments.

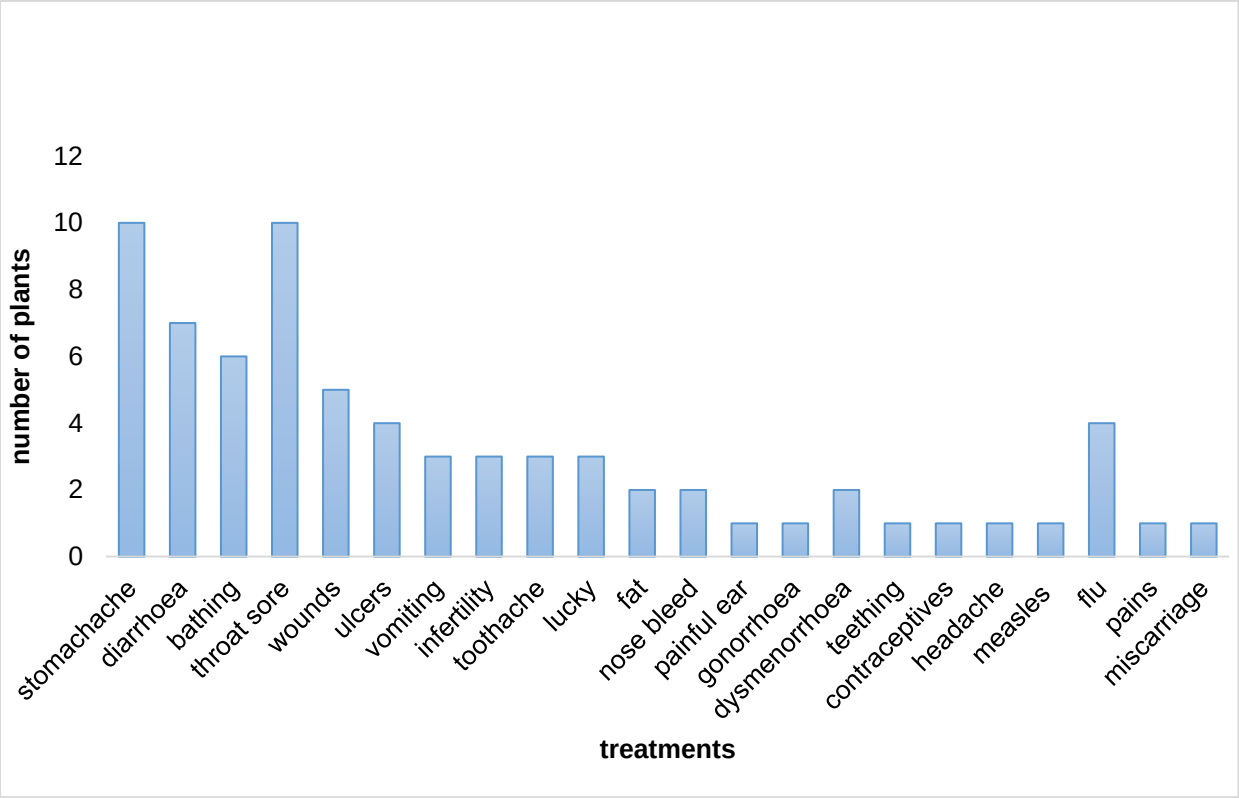


Figure 3.6 Number of plants used for treating various diseases.

Table 3.1. Ranking of ailments that are normally treated in Makhado Local Municipality

Ailments	Number of respondents	Ranking %	Ailment recognition	Reason for Occurrence
Toothache	3	15.8%	Painful and bleeding teeth	Infection
stomach ache	8	42.1%	Indigestion , bloating , painful stomach, constipation	Ingestion of contaminated food
Diarrhoea	9	47.4%	Running stomach	Eating contaminated food, *nyongwe*
Miscarriage	3	15.8%	Vaginal bleeding during pregnancy	Natural causes and too much termination of pregnancies
Throat sore	10	52.6%	Difficulties when swallowing and loss of appetite.	Caused by flu, and infection
Wounds (*tshifula*)	5	26.3%	Wounds on the leg or hand, and also cuts	Caused by witchcraft
Ulcers (*tshiliso*)	3	15.8%	Painful stomach more especially when eating	Caused by witchcraft
Vomiting	6	31.6%	Vomiting	Eating contaminated food
Infertility	3	15.8%	Not bearing children	Natural cause, witchcraft, frequency termination of pregnancy
Nose bleed	3	15.8%	Blood through the nose	Too much exposure to the sun
Earache	1	5.3%	Painful ear and sometimes reddish inside	Infection and bathing water.
Gonorrhoea	1	5.3%		Multiple sexual partners
Teething	2	10.5%	Whitish on the gums and rubbing of the gums	Part of growing up
Dysmenorrhoea	3	15.8%	Abdominal pains and cramps	Sharing clothes with a person who experience period's pains.
Measles	2	10.5%	High fever and reddish rushes all over.	Infection
Flu	7	36.8%	High fever and coughing	Infection
Backache	3	15.8%	Painful back	Working with heavy things
Hypertension	2	10.5%	Sweating too much	Too much blood

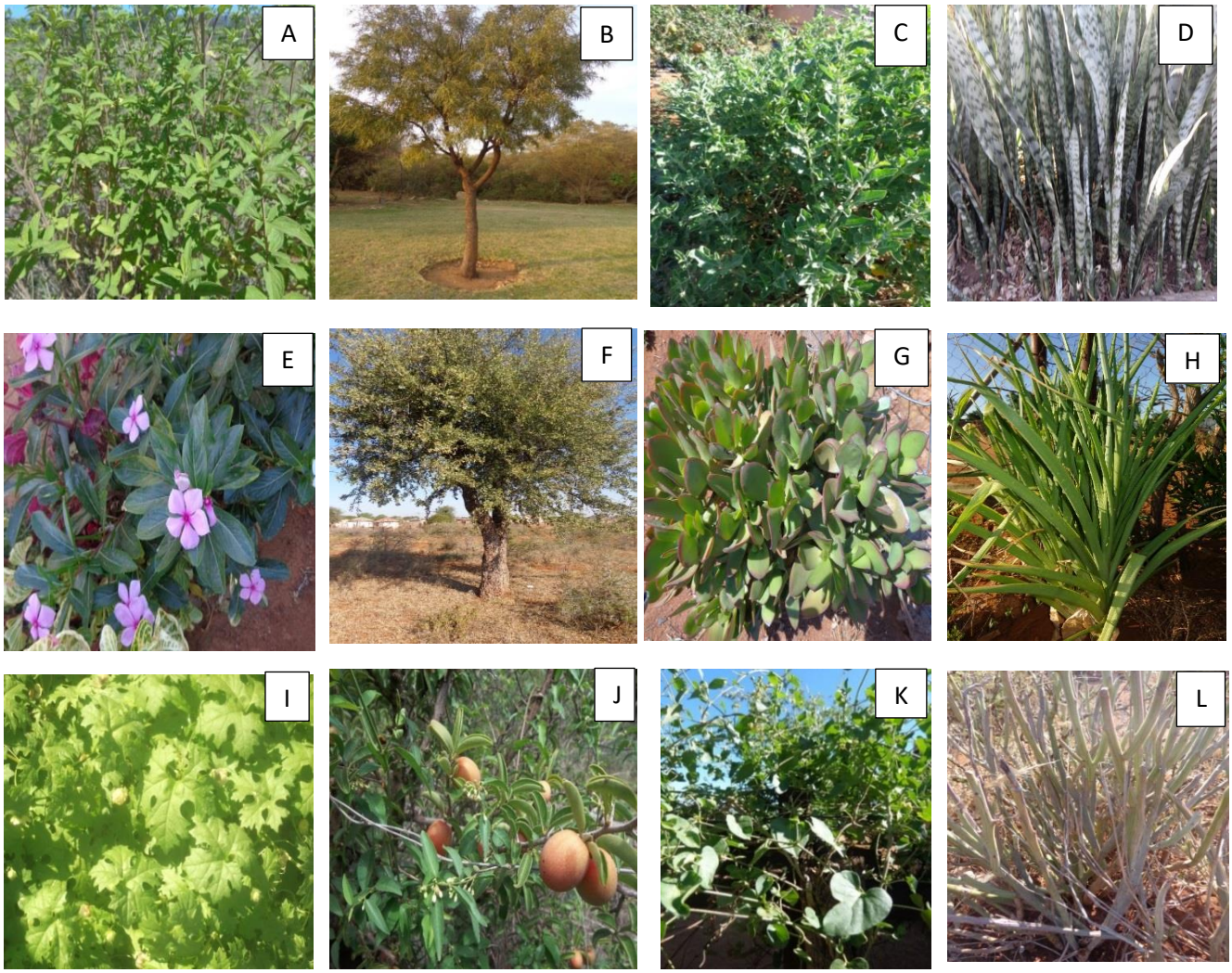


Figure 3.7. Medicinal plants used in Makhado Local Municipality, Vhembe district. A: *Lippia javanica*, B: *Rhus lancea*, C: *Withania somnifera*, D: *sansevieria hyacinthoides* E: *Catharanthus roseus*, F: *Combretum imberbe*, G: *Crassula ovata*, H: *Albuca seineri*. I: *Momordica balsamina*, J: *Ximenia caffra,* K: *Pentarrhinum insipidum*, L: *Kleinia longiflora*.

Table 3.2 Medicinal plants used in Makhado Local Municipality for the treatment of various ailments.

Family	Scientific name	Common name	Voucher number	Plant part(s) used	Uses	Mode of preparation	Plant form	Other medicinal uses in literature	References
Anacardiaceae	*Sclerocarya birrea* (A.Rich) Hochst	Mufula	TC1	Bark	Wound, infertility ulcers	The bark infusion is taken orally to treat ulcers	Tree	STIs, female infertility, sore eyes, diarrhoea, oral candidiasis	Mabogo, 1990; Chinsembu et al., 2015
	Rhus lancea L. fil	Mushakaladza	TC2	Leaves	Measles and flu	The leaves are boiled and decoction is used to bath a baby having measles, and also to steam a person having flu.	Tree	STIs	
	Schinus molle L.	Mubibiri	TC3	Leaves	Flu and sore throat	The decoction of the leaves is used to steam a person having flu covered with a blanket. A small amount of the decoction is taken orally to treat sore throat.	Tree	Malaria, jaundice, diarrheal, bloating, tonsillitis	Giday et al., 2007; Teklay et al., 2013
Annonaceae	*Tabernaemontana elegans* Stapf.	Muhatu	TC4	Roots	Bath	A decoction of roots is used for bathing to prevent various diseases.	Tree	Menorrhagia ,sores	Arnold & Gulumian, 1984; De Wet et al., 2013
Apocynaceae	*Catharanthus roseus* (L.)G. Don.	Unknown	TC5	Roots	Toothache and ulcer	A cold infusion is taken orally with a spoon to treat ulcers, and the infusion of roots is gargled to treat toothaches.	Herb	Stomach problems, breast cancer, hypertension	De Wet et al., 2016
Asclepladaceae	*Pentarrhinum insipidum* E. Mey.	Phulule	TC6	Roots	Fat	A decoction of roots is used to bath a baby to grow stronger and fat.	Climber		
Asparagaceae	*Asparugus buchananii* Bak.	Mufhaladzamakole	TC7	Roots	Vomiting	A decoction of the roots is drunk using a cup to prevent vomiting.	Shrub	Amenorrhoea	Arnold & Gulumian, 1984; Steenkamp, 2003
	Albuca seineri (Engl. & K.Krause) J.C Manning & Goldblatt	Kgofakgofane	TC8	Bulb and leaves	Wounds (*tshifula*)	The decoction of the bulb is used externally on the wound	Herb		

Asteraceae	*Kleinia longiflora DC.*	Muvhale	TC9	Stem	Wounds and pains	The stem is ground and boiled and allowed to cool then applied *(u kanda*) on the wounds and painful part of the body.	Shrub	Menstrual disorder, mental illnesses	Cheikhyoussef et al., 2011
Aizoaceae	*Carpobrotus edulis* (L.) L. Bolus	Unknown	TC10	Leaves	Toothache	The fleshy leaves are chewed and spit off to relief the toothache	Herb	Tuberculosis , candidiasis	Lawal et al., 2014; Masevhe et al.,2015
Bombacaceae	*Adansonia digitata* L.	Muvhuyu	TC11	Roots	Fat	The roots infusion is used to bath a baby to be fat.	Tree	Dysentery, diarrhoea	Chinsembu et al., 2015
Canellaceae	*Warburgia salutaris* (Bertol.f.) Chiov.	Mulanga	TC12	Bark	Sores	A decoction of bark is taken orally to treat sores.	Tree	Malaria, Venereal disease, sinus, respiratory complaints, stomach pain, skin complaints, stomach ulcers, skin sores	Mabogo, 1990; Rabe and Van Staden, 2000
Capparaceae	*Maerua angolensis* DC.	Mutambamme	TC13	Bark	Stomach pains	An infusion of the bark is taken orally to treat stomach aches.	Tree	Fever, pains, skin rashes, sores, womb cleansing, STIs	Mothana et al., 2009; Okatch et al., 2012
	Maerua edulis (Gilg & Gilg-Ben.) Dewolf.	Mutshalimela	TC14	Roots and bark	Venereal diseases	The decoction of both roots and bark is taken orally to treat venereal diseases.	Shrub	Ticks	Nyahangare et al., 2015
	Maerua juncea Pax	Mukundulela	TC15	Roots	Flu, respiratory problem	The roots decoction is taken orally treat flu.	Climber/ shrub	Tuberculosis	Chinsembu et al., 2015
	Capparis sepiaria Lam.	Muobadali	TC16	Roots and bark	Homestead protection, infertility	Infusion of bark and roots is used to treat infertility.	Shrub	Infertility, lice, bleeding after delivery	Giday et al., 2007
Celastraceae	*Maytenus heterophylla* (Eckl. & Zeyh.) Robson	Tshipandwa	TC17	Leaves and roots	Stomach pains *(tshilala)*	A decoction of both leaves and roots is given orally to a baby to treat stomach aches.	Shrub	Epilepsy	Kokwaro, 1976
	Catha edulis (Vahl.) Endl.	Luthadzi	TC18	Roots and leaves	Sore throat, *Tshiunza*	The infusion from the roots is used to cook soft porridge for a baby. Leaves are chewed to treat sore throat	Tree	Thrush, mouth ulcers, tuberculosis, stomach trouble, impotence	Thring et al., 2006
	Cassine eucleiformis (Eckl. & Zeyh.)	Munamu	TC19	Roots	Diarrhoea and vomiting	Roots infusion is taken orally for children to stop diarrhoea and vomiting.	Tree		

	Elaeodendron transvaalense(Burtt Davy) R.H. Archer	Mukuvhazwivhi	TC20	Bark	Body cleansing	A bark decoction is used for bathing to remove evil spirits.	Tree	Female infertility dysmenorrhoea	Van Wyk, 1972
	Salacia rehmannii Schinz	Dira a di bonwi	TC21	Roots and bark	Lucky	Roots and bark are grounded and burnt, the smoke is inhaled to get lucky in things that one needs, e.g. jobs	Shrub	Magical powers	Mabogo, 1990
Clusiaceae	*Garcinia livingstonei* T. Anderson	Muphiphi	TC22	Roots	Stomach pains	An infusion of the roots is used to treat stomach pains.	Tree	Contraceptives	Mabogo, 1990
Combretaceae	*Combretum imberbe* Wawra	Mudzwiri	TC23	Roots and leaves	Sores and *tshiunza*	Decoction of the leaves is used to treat sores and infusion of the roots is used to prepare soft porridge for the baby to prevent stomach problems.	Tree	Male dysfunction, gonorrhoea, impotent, ticks	Cheikhyoussef et al., 2011; Nyahangare et al., 2015
	Terminalia sericea Burch.ex DC.	Mususu	TC24	Roots and bark	Sores	An infusion of roots and bark is used to treat sores.	Tree	Infertility , leg pains diarrhoea, meningitis, gonorrhoea, syphilis	Arnold et al., 1984; Cheikhyoussef et al., 2011; Chinsembu et al., 2015
Crassulaceae	*Crassula ovata* (Mill.) Druce	Mubulomu	TC25	Fleshy leaves	Toothache	The fleshy leaves are chewed and spit off to treat toothaches.	Shrub	Diarrhoea, disinfecting wounds, warts, diabetes	Van Wyk, 2008, Muiruri and Mwangi, 2016
Cucurbitaceae	*Momordica balsamina* L.	Tshibavhe	TC26	Leaves	Hypertension	The decoction of the leaves is taken orally to lower the blood level	Climber	Epilepsy	Stafford et al., 2008
Euphorbiaceae	*Tragia rupestris* Sond.	Tshitondovhe	TC27	Roots	Teething	Ground roots are used to brush the gums of a baby to enhance teething.	Herb		
	Euphorbia inaequilatera Sond.	Maswi	TC28	Roots	Toothache	The root decoction is gargled to treat the painful and bleeding teeth	Herb	Malaria	Muregi et al ., 2003
	Bridelia micrantha (Hochst.) Baill.	Munzere	TC29	Roots and bark	Body cleansing	The roots decoction is used for bathing.	Tree	Gonorrhoea	Mabogo, 1990).
	Spirostachys Africana Sond.	Muonze	TC30	Bark	Nose bleed	The bark is burnt and the smoke is inhaled to treat nose bleeding.	Tree	blood purification, kidneys	Mabogo, 1990

Fabaceae	*Senna petersiana* (Bolle) Lock	Munembenembe	TC31	Roots	Vomiting and stomach ache	Roots infusion is taken orally to treat stomach aches and vomiting.	Tree	Infertility	Mabogo, 1990; Mahwasane et al., 2013
	Dichrostachys cinerea (L.)Wright &Arn	Murenzhe	TC32	Seeds and roots	Wounds and stomach problems	The powered seeds are used to treat the wounds and a decoction of roots is prepared with soft porridge to treat stomach problems.	Tree	Oral candidiasis, stomach ache, dysentery, malaria, sores	Chinsembu et al., 2015; De Wet et al., 2013
	Albizia tanganyicensis Baker.	Mulelu	TC33	Roots	Body cleansing	An infusion of roots is used externally for bathing.	Tree		
	Senna italica Mill.	Murundelatshotshi	TC34	Roots	Back pain and diarrhoea	A decoction of roots is used externally (*u kanda*) to relief the back pains, and decoction taken orally to induce diarrhoea.	Herb		
	Schotia brachypetala Sond.	Mulubi	TC35	Roots and bark	Relief pain	A decoction of roots and bark is used externally (*u kanda*) to relief the pains.	Tree	Heartburns, dysentery, sores	Mabogo, 1990; De Wet et al., 2013
	Bolusanthus speciosus (Bolus) Harms	Mukamba	TC36	Roots	Miscarriage	Roots infusion is taken orally to prevent miscarriage and if overdose is taken a person might become drunk.	Tree	Venereal diseases, epilepsy	Mulaudzi et al., 2013; Stafford et al.,2008
	Elephantorrhiza burkei Benth.	Gumululo	TC37	Roots	Stomach disorder	An infusion of ground roots is used to prepare a soft porridge to babies to treat stomach disorders. The ground roots are used for adults (*nowa i tshi kuma*)	Shrub	Venereal diseases, amenorrhoea, candidiasis	Mulaudzi et al., 2013; Masevhe et al., 2015
	Elephantorrhiza elephantina (Burch.) Skeels	Tshisesana	TC38	Roots	Body cleansing, stomach problems	Infusion of roots is used for bathing, and taken orally to treat stomach problems.	Shrub	Diarrhoea, impotence, shingles	Madikizela et al., 2012; De Wet et al., 2013
	Peltophorum africanum Sond.	Musese	TC39	Bark	sore throat and diarrhoea	A decoction of bark is taken orally to treat sore throat and stops diarrhoea.	Tree	Female infertility, STIs, leg pains, sore eyes, toothache, tuberculosis, coughs, dysentery	Cheikhyoussef et al., 2011; Chinsembu et al., 2015

	Lonchocarpus capassa Rolfe	Mufhanda	TC40	Leaves , bark and roots	Diarrhoea, stomach problems	The decoction of both roots and bark is taken orally to treat stomach problems, and the decoction of the leaves is taken orally to treat diarrhoea.	Tree	Hallucination, epilepsy	Amusan et al., 2002; Moshi et al., 2005
	Indigofera circinnata Benth	Mutahala	TC41	Roots	Throat and mouth sores, flu, measles.	The decoction of roots is gargled to treat sores, for the treatment of flu the decoction is taken orally, and for the measles treatment, a kid is bathed with the decoction.	Shrub		
Heteropyxidaceae	*Heteropyxis natalensis* Harv.	Mudedede	TC42	Roots	Stomach disorder	The infusion of the roots is taken orally to treat stomach disorders.	Tree	Menorrhagia	Arnold et al., 1984
Icaoraceae	*Pyrenacantha grandiflora* Baill.	Bwere	TC43	Roots	Lucky	Roots are grounded and burnt, the smoke is inhaled to get a lucky in things that one needs, e.g. jobs	Climber	Magical powers, malaria	Mabogo, 1990; Bapela et al., 2014
Lauraceae	*Cassytha filiforms* L.	Luangalala	TC44	Leaves and bulb	Relief pain	A decoction of leaves and bulb is taken orally to relief the pain	Climber	Pregnancy, diseases of the eyes	Catarino et al., 2016
	Persea americana Mill.	Avocado	TC45	Seed and leaves	Hypertensio n and ulcers	The decoction of the leaves is taken orally to treat hypertension, the decoction of the ground seed is taken orally to treat ulcers.	Tree	Kidney	Tribess et al., 2015
Liliaceae	*Sansevieria hyacinthoides* (L.) Druce	Savha	TC46	Bulb, leaf	Painful ear and *tshiunza*	Infusion of the bulb is used to make soft porridge for babies, and a squeezed fluid from the leaf is used to treat the painful ear.	Herb	Diarrhoea, ticks	Mabogo, 1990; Nyahangare et al.,2015
Meliaceae	*Ekebergia capensis* Sparrm.	Mutobvuma	TC47	Bark	Headaches	The bark is burnt and the smoke is inhaled to treat headache.	Tree	Boils, wounds	Grierson et al., 1999

Menispermaceae	*Cissampelos torulosa* E. Mey. ex Harv	Lukandululo	TC48	Leaves	Sores	A decoction of leaves is taken orally to treat the sores.	Climber	Candidiasis	Masevhe et al., 2015
Mimosaceae	*Acacia albida* Del.	Muhoto	TC49	Roots and bark	Venereal diseases and stomach problems	Decoction of the bark is used to treat venereal disease through bathing and roots infusion is used to prepare a soft porridge for babies to prevent stomach problems.	Tree	Skin infections	Aliyu et al ., 2008
Myrtaceae	*Syzygium cordatum* Hochst. ex Sond.	Mutu	TC50	Leaves	Stomach pains	An infusion of leaves is taken orally to treat stomach aches.	Tree	Tuberculosis, burns	De Wet et al., 2013; Lawal et al., 2014
Ochnaceae	*Ochna holstii* Engl.	Tshipfure	TC51	Leaves	Wounds	Infusion of leaves is used externally to treat the wounds.	Tree		
	Ximenia caffra Sond.	Mutshili	TC52	Leaves, Seeds and roots	infertility, Wounds, sores	The seeds are heated, ground and mixed with soil found were the road crosses each other, and smeared on the wound, a decoction of roots is taken to treat sores.	Shrub	Culture bound syndrome, gonorrhoea, backache, diarrhoea, venereal diseases, wounds, tuberculosis, skin rashes	Cheikhyoussef et al., 2011; Chinsembu et al., 2015
Rhamnaceae	*Berchemia discolor* (Klotzsch) Hemsl.	Munie	TC53	Roots	Stomach problems *(Tshiunza)*	An infusion of roots is used to prepare soft porridge for babies to prevent stomach problems	Tree	Abdominal pains, coughs, vomiting, amenorrhoea	Chinsembu et al., 2015; Steenkamp, 2003
	Ziziphus mucronata Willd.	Mukhalu	TC54	Leaves and roots	dysmenorrhoea	A decoction of roots is taken to relieve period pains; the leaves are chewed and swallowed to relief other pains.	Tree	Diarrhoea, infertility, wounds, boils, abdominal pains, STIs, diarrhoea, dysentery	Cheikhyoussef et al.,2011; Madikizela et al 2012, Chinsembu et al., 2015
Rubiaceae	*Conostomium natalense* (Hochst.) Bremek.	Ndilele	TC55	Roots and bark	Lucky	Roots and bark are grounded and burnt, the smoke is inhaled to get a lucky in things that one needs, e.g. jobs	Herb		

Rutaceae	*Clausena anisata* (Willd.) Hook.f.	Murandela	TC56	Roots	Ulcers	An infusion of roots is taken orally to treat ulcers.	Tree	Tuberculosis, epilepsy	Lawal et al., 2014; Stafford et al., 2008
	Zanthoxylum leprieurii Guill. et Perr.	Mununguu	TC57	Roots and bark	Diarrhoea and sores	The decoction of roots and bark is taken orally to treat diarrhoea and sores.	Tree	Wounds, syphilitic sores, leprous ulcers, toothache, rheumatic pain, skin and urinary tract infections, dysentery, intestinal worm infection	Agyare et al ., 2009; Agyare et al., 2014
Rutaceae	*Zanthoxylum humile* (E.A.Bruce) P.G. Waterman	Lunungwane	TC58	Roots and bark	Stomach problems	The decoction of the roots and bark are taken orally to treat sores	Shrub		
Sapindaceae	*Pappea capensis* Eckl. & Zeyh	Muvundambado	TC59	Roots and bark	Relief pain	A decoction of both roots and bark is taken orally to relieve the pains.	Tree	Candidiasis, venereal diseases	Masevhe et al., 2015; mulaudzi et al 2011
Solanaceae	*Withania somnifera* Dunal (Ashgandh)	Musalamarubini	TC60	Leaves and roots	Relief pains	A decoction of roots is used externally (*u kanda*) to relief the pains.	Shrub	open cuts, wounds, stomach problems, malaria, tuberculosis	Bussmann et al., 2011; Asnake et al., 2016; Lawal et al., 2014
	Solanum incanum L.	Mututulwa	TC61	Fruits, roots	Toothache	The fruits are burned and inhaled smoke taken by opening the mouth is used to treat the toothache and the decoction of roots is gargled in the mouth then spit out to treat toothache.	Herb	Stomach problems, scabies, skin infections, skin cancer, malaria	Bussmann et al., 2011; Chinsembu et al., 2015; Asnake et al., 2015
Sontalaceae	*Osyris lanceolata* Hochst. & Steudel	Mpeta	TC62	Roots and bark	Diarrhoea	A decoction of both root and bark are taken orally to treat diarrhoea.	Shrub	Candidiasis	Masevhe et al.,2015
Verbenaceae	*Lippia javanica* Spreng. (Burm.f)	Musudzungwane	TC63	Leaves	Nose bleed	The leaves are burned and the smoke is inhaled through the nose to treat nose bleeding.	Shrub	Stomach pains, sores, hypertension	De Wet et al., 2013; De Wet et al., 2016

4. CONCLUSION

Local people and traditional healers in Vhembe District still rely on medicinal plants as a source of primary health care. The survey revealed that more than twenty medicinal plants used to combat various diseases have not been documented in Makhado Local Municipality. It was noted that traditional healers use bark and roots to prepare the remedies. This could lead to extinction of some of plants due to overexploitation and deforestation. Therefore, the sustainable way of plant collection should be taught to our traditional healers and local people, more especially to conserve plants that are indigenous to South Africa. They should also be encouraged to cultivate their own traditional medicinal plants in their home gardens. The indigenous knowledge on medicinal plants should also be passed to new generation, because it was found that the young generation is less knowledgeable about the medicinal plants. The valuable knowledge regarding the folk medicinal uses of plants should be recorded before it is lost completely.

In the next chapter, we will discuss methods of extraction of eight selected medicinal plants and also investigate phytochemical analysis of the crude extracts.

CHAPTER 4

EXTRACTION AND PHYTOCHEMICAL ANALYSIS OF EIGHT MEDICINAL PLANT SPECIES

4.1 Introduction

Plants are natural source of medicine and produce a wide range of bioactive chemical constituents with a wide spectrum of activities such as antifungal activity (Quiroga et al., 2001). Most natural plant products have not been intensively investigated in the past even though they are known to contain structurally diverse molecules, many of which are unknown (Ngula et al., 2016). However, some plant secondary metabolites are toxic, which can induce adverse effects leading to mutation and degenerative disease (Popat et al., 2001).

Extraction procedure is important in the analysis of medicinal plants especially or conducting phytochemical analysis as well as antifungal activity. This process requires extracting the desired chemical components from the plants for further analysis. The extraction solvent depends on the specific nature of biologically active compound being targeted from the plant materials (Sasidharan et al., 2011). The solvents in general should not interfere with the bioassay and also non-toxic against the tested microorganisms (Ncube et al., 2008). Traditional healers use water to extract the plant material by decoction and infusion. More importantly, the water extracts is preferred since water are readily available and is part of their cultural tradition (Lekganyane et al., 2012).

There are several solvents that are mainly used, such as alcohol, acetone, hexane and others. For example, alcohol extracts provide a more complete extraction including less polar compounds, and many alcohol extracts were found to have antifungal activities against *Candida* species (Webster et al., 2008). Acetone and hexane are two solvent system of opposite polarities, and these are important for determining the activity of the compounds with different polarities (Samie et al., 2010). The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate while lipophilic compounds use dichloromethane or mixture with methane (Sasidharan et al., 2011). The aqueous extract did not show

the activity while the alcohol showed the activity; this suggests that the less polar compounds are the active components (Webster et al., 2008). This observation will be crucial for the recent study and will assist in the evaluation of the current selected plant species. Another critical phenomenon of evaluating medicinal plants is to check their toxicity.

4.2 Materials and methods

4.2.1 Plant selection

Eight plant species documented in the previous chapter used to treat fungal infections were selected for further phytochemical analysis and biological assay.

4.2.2 Plant collection

Plants were collected from Soutpansberg West Mountain and five local villages in Makhado Municipality, with the help of traditional healers. Collected plants were identified using the literature and Larry Leach Herbarium at the University of Limpopo and voucher specimens that were collected and were deposited in the University of Limpopo herbarium.

4.2.3 Plant extraction

The plant materials such as leaves, bark and roots were dried at room temperature (25 ºC) for three weeks. The dried plant materials were ground to fine powder using a laboratory grinding mill and stored in airtight bottles. Each finely ground plant material (4g) was extracted with 40 ml hexane, dichloromethane, acetone, ethanol, ethyl acetate, methanol and water in polyester plastic tubes, while shaking vigorously for 3-5 minutes on a shaking machine at high speed of 3500 rpm. The plants were filtered after centrufuging using Whatman No.1 filter paper to separate the supernatant and the sediment. The supernatants were decanted into labelled vials. The process was repeated three times and the extracts were combined. The solvents were removed under a stream of cold air at room temperature. Aqueous extracts were frozen in a deep freezer. The crude extracts were re-dissolved in acetone prior to biological assay.

4.2.4 Phytochemical analysis

Chemical components of the extracts were analysed using aluminium-backed Thin Layer Chromatography (TLC) plates and developed using three different eluent systems: Ethyl acetate: methanol: water: 40:5.4:4 [EMW], Chloroform: ethyl acetate: formic acid: 5:4:1 [CEF] and Benzene: ethanol: ammonia hydroxide: 90:10:1 [BEA] (Kotze and Eloff, 2002). Ten microliters of each sample were loaded on TLC plates. Chemical components were visualized under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600). For chemical compounds that were not visible under UV light, vanillin-sulphuric acid spray reagent (Stahl, 1969) was used for detection.

4.3 Results and discussion

4.3.1 Plant extraction using various solvents

Methanol extracted the highest amount of the plant material from all plant species (13%), followed by ethanol (7%), acetone (5.3%) and the least was hexane (3.8%) (Figure 4.1). Previously, Masevhe (2013) found that methanol extracted large quantity of plant material from *Faurea saligna* Harv (41%) and the least was hexane (1%).

The highest amount of plant material was extracted from methanol leaves of *E. elaphantina* with 21%, followed by methanol leaf extracts of *S. molle* 16.5%. Similar results were obtained in other studies with *L. alata* extracting large quantity 21.8% followed by methanol (Suleiman et al., 2010). Ethanol, acetone and dichloromethane extracted large quantity of plant material respectively. Ethyl acetate and hexane was the least effective solvent extracting 0.75% from *S. italica* leaves. Some researchers found that hexane extracted the lowest plant material (Shai et al., 2008). In contract, (Shai et al., 2013) reported that acetone extracted more plant material than other solvents. Acetone was used as an extractant because it has the ability to extract a combination of polar and non-polar compounds and it was found to be less toxic to microorganisms than other solvents (Eloff, 1998).

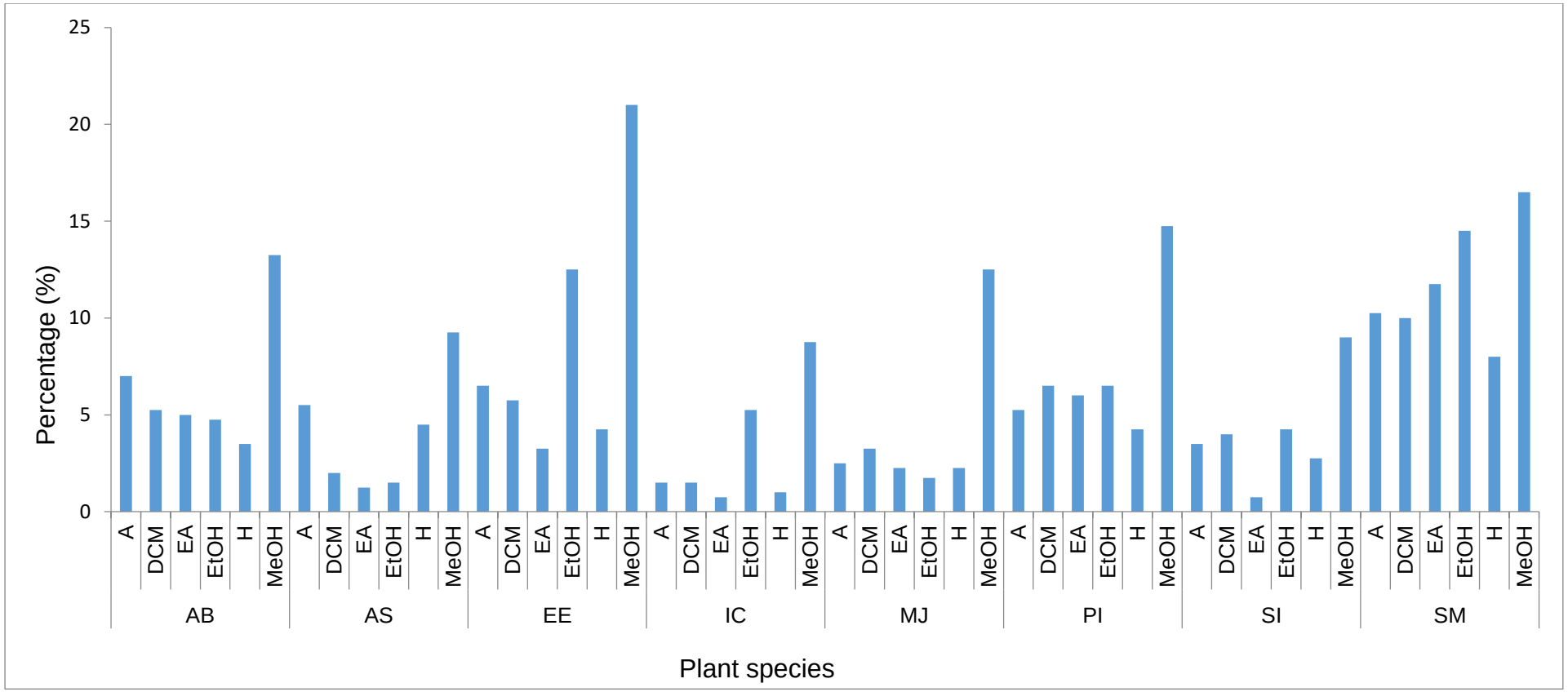


Figure 4.1 Percentage of plant extracted from 4g of (AB) *Asparagus buchananii,* (AS) *Albuca seineri,* (EE) *Elephantorrhiza elephantina,* (IC) *Indigofera ciricinnata,* (MJ) *Maerua juncea,* (PI) *Pentarrhinum insipidum,* (SI) *Senna italica* and (SM) *Schinus molle* with different extractants.

4.3.2 TLC fingerprint analysis

The TLC chromatograms of the eight plant species are shown in Figure 4.2. In TLC chromatograms separated with EMW and CEF, two compounds were observed in DCM extracts of *Maerua juncea* with R_f values of 0.14 and 0.79 (Table 4.1). BEA was the best solvent system by separating more compounds; this indicates that the active compounds were relatively non-polar. Similarly, Lekganyane et al. (2012) found that BEA was effective followed by CEF and EMW. In contrast, CEF eluent system separated many compounds when sprayed with vanillin sulphuric acid reagent spray (McGaw et al., 2013).

The water extracts of *Senna italica* had shown a chemical component under UV light with R_f value of 0.07. The R_f value of less than 0.5 indicates that the compound was less effective (McGaw et al., 2013). The polar eluent system was able to separate the compounds from the water extracts of some plants such as *S. italica*, *E. elephantina*, and *I. circinnata* with R_f values of 0.08, 0.45 and 0.65 respectively.

Table 4.1 R_f values of compounds separated in BEA, CEF and EMW extracted with (A) acetone, (DCM) dichloromethane, (H) hexane, (EA) ethyl acetate, (E) ethanol, (M) methanol and (H_2O) water.

Plant species	R_f value	Extractants							Total
		A	DCM	H	E	EA	M	H_2O	
Solvent system BEA									
Asparagus buchananii	0.14	2	2		1				5
	0.83								
Elephantorrhiza elephantine	0.31	2	1	1		1			5
	0.86								
Indigofera circinnata	0.19	3	3	1	2	3	2		14
	0.29								
	0.79								
Maerua juncea	0.14	2	2	2	2	2			10
	0.93								
Pentarrhinum insipidum	0.60	1	1		3	2	3		10
	0.81								
	0.91								
Senna italic	0.37	3	2		1	1	1		8
	0.79								
	0.87								
Schinus molle	0.71	2	1		3	3			9
	0.79								
	0.87								
CEF									
Albuca seineri	0.79	1	1						2
Elephantorrhiza elephantine	0.45	2						1	3
	0.81								

Table 4.1 continued. R_f values of compounds separated in BEA, CEF and EMW extracted with (A) acetone, (DCM) dichloromethane, (H) hexane, (EA) ethyl acetate, (E) ethanol, (M) methanol and (H2O) water.

Plant species	R_f value	Extractants							Total
		A	DCM	H	E	EA	M	H_2O	
Solvent system BEA									
	0.75								
Senna italic	0.08	3	1		3		1	1	9
	0.47								
	0.81								
Schinus molle	0.76						1		1
EMW									
Asparagus buchananii	0.46	3	2		2	2	1		10
	0.68								
	0.80								
Elephantorrhiza elephantine	0.42	3	1		2	2	2	2	13
	0.56								
	0.67								
Indigofera circinnata	0.65	1	1			1		1	4
	0.08								
Maerua juncea	0.65	2	2			2			6
	0.79								
Pentarrhinum insipidum	0.65	2	1		2	2	2		9
	0.79								
Senna italica	0.13	3	1		2	1			7
	0.46								
	0.77								
Schinus molle	0.77	1	1						2

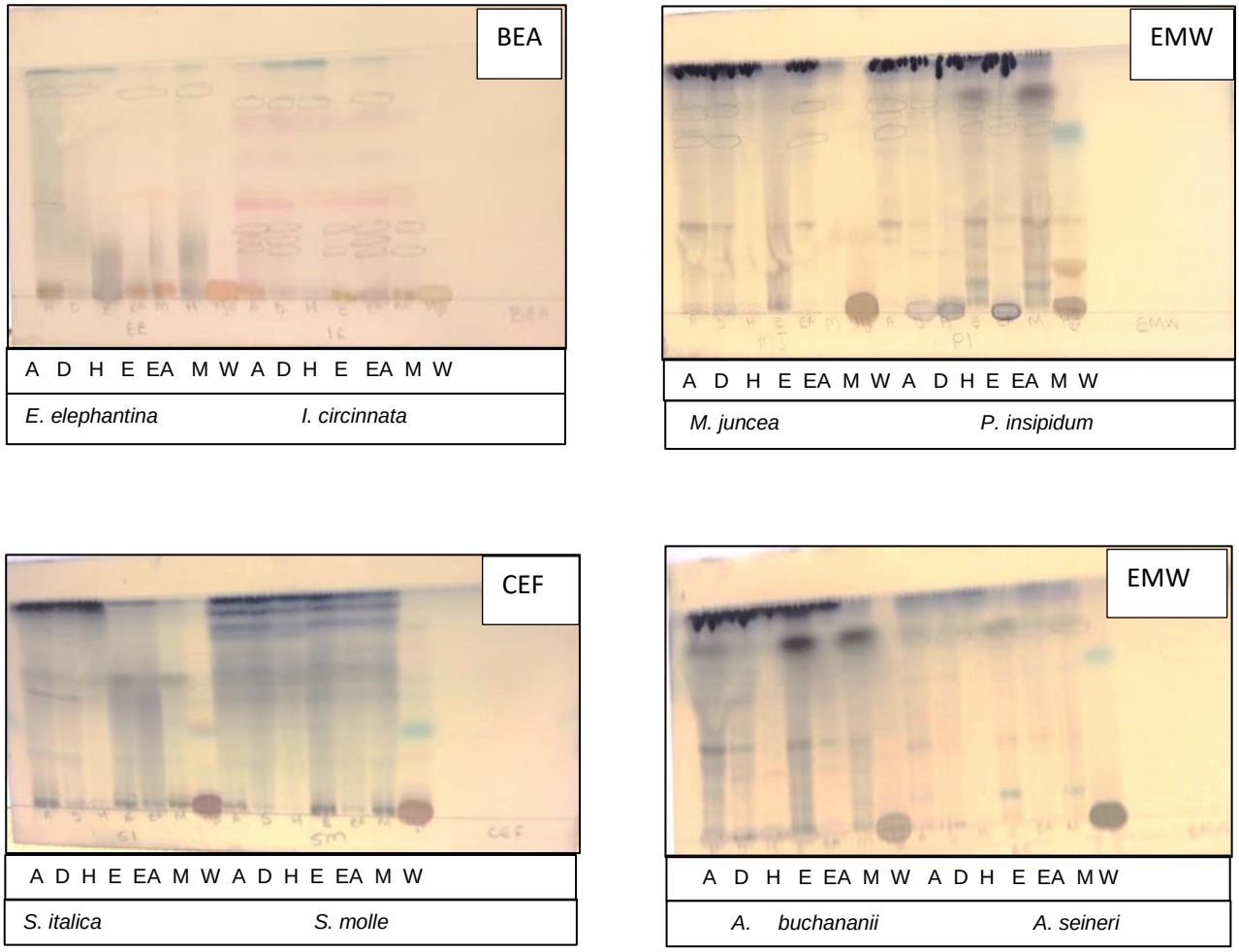


Figure 4.2 TLC chromatograms *of E. elephantina, I. circinnata, S. italica*, S*. molle, A. buchananii, A. seineri, Maerua juncea* and *Pentarrhinum insipidum* developed in BEA, CEF and EMW sprayed with vanillin-sulphuric acid. Lanes from left to right: acetone=A, dichloromethane=D, hexane=H, ethanol=E, ethyl acetate=EA, methanol=M and aqueous extract

4.4 Conclusion

Methanol extracted more quantity of plant material compared to other solvents. Dichloromethane root extracts of the eight plant species, had shown excellent antifungal activity against *C. albicans, C. neoformans* and *A. fumigatus. Candida albicans* and *C. neoformans* were more sensitive to the plant extracts while *A. fumigatus* was resistant to the plant extracts.

BEA was the best eluent solvent system since it separated more compounds from plant extracts under UV light. This indicates that the active compounds were relatively non-polar. BEA had 61 chemical constituents, followed by EMW with (51) and then CEF (19)

In the next chapter, antifungal activity of the eight selected medicinal plants against animal fungal pathogens will be discussed.

CHAPTER 5

ANTIFUNGAL ACTIVITY OF EIGHT SELECTED PLANT SPECIES

5.1 Introduction

Plants are the major source of antimicrobial agents and have been tested previously against variety of microorganisms (Edori and Dibofori-Orji, 2016). Different plant species are being studied to treat various ailments and for pharmaceutical uses (Suman-Kumar et al., 2013). However, there is a need to development more effective and safe antifungal drugs since the pathogenic infections are developing resistance against the currently used drugs (Mohammadi et al., 2014 and Seneviratne and Rosa, 2016).

Bioautography assay is an effective and inexpensive technique used to identify bioactive compounds (Dewanjee et al., 2015). The technique is used to determine the bioactive compounds from plant extracts and focuses on the antifungal activities of an extract on the chromatogram (Sasidharan et al., 2011). Furthermore, it disables the challenge of isolating active compounds from crude extracts with complex chemical components (Ncube et al., 2008).

Serial dilution is a fundamental procedure that is used for creating a range of test solution and highly diluted samples in the laboratory (Ahrar et al., 2014). However, it requires relatively large initial sample volume and overnight incubation is also required (Jiang et al., 2003). In the current study, microdilution assay is a method of choice for determining the antifungal activities of three fungal pathogens namely, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. The method allows the calculation of MIC values for active plant extracts against microbes and is time saving and less labour intensive. Agar diffusion assay involves the transfer of antimicrobial agent by diffusion from the chromatograms to an agar plate (Ncube et al., 2008). The method is inaccurate and misleading, time consuming and labour intensive (Eloff and McGaw, 2014 and Elisha et al., 2016).

Plants commonly used in the treatment of various diseases are considered toxic (Elisha et al., 2016). However, cytotoxicity assay plays an important role of validating the safety of medicinal plant extracts for human use (Ghuman et al., 2016).

Ethnobotanical survey described in chapter 3 was conducted to identify medicinal plants used for the treatment of fungal infections and related ailments. Eight selected plant species namely; *A. buchananii*, *A. seineri*, *E. elephantina*, *I. circinnata*, *M. juncea*, *P. insipidum*, *S. italica*, and *S. molle* were extracted with solvents of various polarities and screened for antifungal activity against the three animal fungal pathogens (*C*. *albicans* and *C. neoformans*) and moulds (*A. fumigatus*).

5.2 Materials and methods

Plant selection, collection and extraction were described in detail in chapter 4.

5.3 Determining antifungal activity

5.3.1 Fungal strains and inoculum quantification

Candida albicans (ATCC 10231), and clinical isolates such as *Cryptococcus neoformans* and *Aspergillus fumigatus* were obtained from the Department of Veterinary Tropical Diseases at the University of Pretoria. For quantification of fungi, the haemocytometer cell-counting method described by Aberkane et al. (2002) was used for counting the number of cells for each fungal culture. Filamentous fungal colonies were enumerated by using a haemocytometer. The inoculum of each isolate was prepared by growing the fungus on sabouraud dextrose (SD) agar slants for seven days at 35°C. The slants were rubbed with sterile loop and transferred to a sterile tube with fresh SD broth (50 ml). The sterile tubes were shaken for 5 min and appropriate dilutions were made in order to determine the number of cells by microscopic enumeration using a haemocytometer (Neubauer chamber; Merck S.A). The final inoculum concentrations were adjusted to approximately 1.0×10^{6} cells/ml.

5.3.2 Micro-dilution assay

The antifungal activity of plant extracts were determined using microplate method (Eloff, 1998a). The plant extracts were tested in triplicate in each assay, and the assays were repeated three times to confirm the results. Residues of different extracts were dissolved in acetone to a specific concentration. The plant extracts

(100 µl) were serially diluted 50% with water in 96 well microtiter plates (Eloff, 1998b), and 100µl of fungal culture were added to each well. Amphotericin B was used as the reference antibiotic and 100% acetone as the negative control. As an indicator of growth, 40µl of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) dissolved in water were added to the microplate wells. The microplates were covered and incubated for three to five days at 35ºC at 100% relative humidity after sealing in plastic a bag to minimize fungal contamination in the laboratory. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth.

5.3.3 Bioautography

The presence of active compounds in plant extracts were determined using bioautography assay. TLC plates (10x10 cm) were loaded with 10 µl of each of each plant extracts. The prepared plates were developed using different mobile systems of varying polarity: CEF, BEA and EMW. The chromatograms were dried at room temperature under a stream of air over night to remove the remaining solvent. Fungal cultures were grown on sabouraud dextrose (SD) agar for 3-5 days. Cultures were transferred into a SD broth from agar with sterile swabs. The plates were sprayed with fungal cultures and were incubated at 37 °C in a clean chamber at humidified chamber for overnight and further sprayed with a solution of p-iodonitrotetrazolium (INT) violet and incubated for 2-6 hours for fungal growth. White areas indicated where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the fungi. The plates were sealed in plastic to prevent the spreading of the fungi into the environment and to retain the humidity and then scanned to produce a record of the results.

5.3.4 Cytotoxicity assay

The cytotoxicity of plant extracts was determined using the (3-(4,5-dimethylthiazol) -2,5-diphenyltetrazoliumbromide (MTT) assay described by Mosmann, (1983) and (McGaw et al., 2000). The plant extracts were tested for cytotoxicity against African green monkey Vero kidney cells obtained from the Department of Veterinary Tropical Diseases (University of Pretoria). Minimal Essential Medium (MEM, Whitehead Scientific) was used to maintain the cells, supplemented with 0.1% gentamycin (Virbac) and 5% fetal calf serum (Highveld Biological). Two hundred µl of the cell suspension was pipetted into each sterile 96-well microtitre plates. The plates were

incubated for 24 h at 37ºC in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. The MEM was aspirated from the cells which were then washed with 150 µl phosphate buffered saline (PBS, Whitehead Scientific) and replaced with 200 µl of test compound at differing concentrations in quadruplicate. The serial dilutions of the test extracts and compounds were prepared in MEM. The microtitre plates were incubated at 37ºC in a 5% CO_2 incubator for 48 h with test compound or extract. Untreated cells and a positive control (doxorubicin chloride, Pfizer Laboratories) were included. After incubation of 4h in 30 µl of 5mg/ml MTT solution, the MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Biotek Synergy) at a wavelength of 570 nm. The LC_{50} values were calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

5.4 Results and discussion

5.4.1 Antifungal activity

5.4.1.1 Micro-dilution method

The antifungal activities of the plant extracts were tested against *Candida albicans*, *Cryptococcus neoformans*, *and Aspergillus fumigatus*. The minimum inhibitory concentrations (MIC) values are shown in Table 5.1. The leaf extracts of *S. italica* had excellent activity against *C. albicans* with the lowest MIC value of 0.02 mg/ml. Acetone, dichloromethane, hexane and ethanol extracts of *E. elephantina* had good activity against *C. albicans, C. neoformans* and *A. fumigatus* with MIC value ranging between 0.02 mg/ml and 0.04 mg/ml. Previously, it was reported that *E. elephantina* had shown antimicrobial activity against the tested microorganisms with MIC value ranging between 0.05 mg/ml and 1 mg/ml (Mabona et al., 2013). Hexane leaf extracts of *M. juncea* had shown good activity against *C. albicans, A*. *fumigatus* and *C. neoformans* with MIC value of 0.02 mg/ml. Previously, *M. juncea* did not possess strong anti-mycobacterial activity against screened strains (Lua et al., 2010).

Acetone, methanol and DCM leaf extracts of *A. seineri* had shown strong activity against *C. albicans* and *C. neoformans* with MIC value of 0.02 mg/ml. Previously, it was reported that most methanol extracts from seven South African plants were relatively inactive against the tested pathogens (Suleiman et al., 2010). Ethanol

extracts of *S. molle* had good activity against all tested fungal pathogen with the MIC value ranging between 0.02 mg/ml to 0.04 mg/ml. Some studies have reported antifungal activity of essential oil, ethanol and aqueous extracts of *S. molle* (Schmourlo et al., 2005). *Schinus molle* showed weak antifungal activity against *C. abicans* and other tested bacteria (Deveci et al., 2010). Hexane, methanol and aqueous leaf extracts of *A. buchananii* had shown excellent activity against *C. albicans* and *C. neoformans* with MIC values between 0.02 mg/ml and 0.04 mg/ml. Other studies found that hexane and chloroform fractions had low MIC value against C. *albicans* strains (Ramadwa et al., 2017). Furthermore, ethanol extracts of plants from the genus *Asparagus* has shown good antimicrobial activity (Olivier et al., 2017).

Plants from genus *Asparagus* are regarded as medicine and some are used in the treatment of epilepsy (Olivier et al., 2017). In some instances, other plant species are used in ethnoveterinary medicine (McGaw and Eloff, 2008 and Van Wyk et al., 2009). Dichloromethane, hexane, ethyl acetate, methanol and aqueous leaf extracts of *P. insipidum* had good activity against *C. albicans* and *C. neoformans* with MIC value ranging between 0.02 mg/ml and 0.04 mg/ml. Acetone extracts were moderately active against the fungal pathogens while *A. fumigatus* was relatively resistant against plant extracts.

In the current study, *C. albicans* and *C. neoformans* were relatively sensitive to all plant extracts. The plant extracts screened against these microorganisms might have the secondary metabolites that enabled the inhibition of the fungal growth. The presence of secondary metabolites such as alkaloids, terpenoids and proteins may be responsible for the antibacterial properties of plant extracts (Olivier et al., 2017). In our findings, *A. fumigatus* was relatively resistant to most plant extracts. In contrast, thirteen plant extracts showed high inhibition against *A. fumigatus* than other tested organisms (Zhang et al., 2013). Furthermore, some aqueous extracts of *S. molle*, *S. italica, I. circinnata* and *P. insipidum* had shown excellent activity against *C*. *albicans* with the lowest MIC value of 0.02 mg/ml. This supports the study reported that water extracts from other plants showed good antibacterial activities against the tested organisms (Madikizela et al., 2012). This indicates the usefulness of the plant species used by traditional healers using water as extracting solvent

(Shai et al., 2013). However, Parekh et al. (2005) found that organic solvents had better antimicrobial activity than the aqueous extracts. Moreover, it was found that water does not extract the antimicrobial compounds that have intermediate and non-polar characters (Eloff et al., 2005).

The acetone, DCM and methanol leaf extracts of *A. seineri* have excellent fungal activity against *C. albicans* and *C. neoformans* with lowest MIC of 0.02 mg/ml. Previuosly. It was reported that plant from the genus *Albuca* revealed the presence of saponins indicating a good potential as antifungal agent (Odeyemi et al., 2015). The antimicrobial activity of the plant extracts is considered significant when the MIC value is below 0.1 mg/ml and moderate between 0.1 and 0.625 mg/ml and low when more than 0.625 mg/ml (Olivier et al., 2017).

Table 5.1 Minimum inhibitory concentration (MIC) of eight plant species tested against three animal fungal pathogens

Fungi		MIC (mg/ml)														
		Asparagus buchananii							*Albuca seineri*							AMP B
	Time(h)	Extractants														
		A	D	H	E	EA	M	H_2O	A	D	H	E	EA	M	H_2O	
	24	0.04	0.04	0.08	1.25	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.08	0.02	2.5	<0.02
	48	1.25	0.32	0.02	1.25	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.62	<0.02
C. a	72	1.25	0.32	0.02	1.25	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.32	<0.02
	24	1.25	1.25	0.02	1.25	0.32	0.02	0.02	0.02	0.62	0.32	0.04	0.02	0.02	0.32	<0.02
	48	0.32	0.32	0.02	1.25	0.04	0.02	0.02	0.02	0.02	0.08	0.04	0.04	0.02	0.32	<0.02
C. n	72	0.32	0.32	0.02	1.25	0.08	0.02	0.02	0.02	0.02	0.08	0.08	0.04	0.02	0.32	<0.02
	24	0.02	0.02	1.25	0.62	0.04	2.5	2.5	2.5	0.08	0.02	0.04	0.16	0.08	0.08	<0.02
A. f	48	1.25	1.25	0.32	0.32	0.32	0.32	2.5	0.08	0.08	0.08	0.08	0.62	0.08	0.04	<0.02
Average		0.71	0.48	0.22	1.05	0.11	0.39	0.65	0.34	0.11	0.08	0.04	0.12	0.04	0.56	<0.02

Fungi		MIC (mg/ml)														
		Elephantorrhiza elephantine							*Indigofera circinnata*							AMP B
	Time(h)	Extractants														
		A	D	H	E	EA	M	H_2O	A	D	H	E	EA	M	H_2O	
C. a	24	0.02	0.02	0.02	0.02	2.5	2.5	2.5	0.02	0.02	0.02	0.02	0.04	0.02	0.02	<0.02
	48	0.02	0.02	0.02	0.02	2.5	2.5	2.5	0.02	0.02	0.02	0.02	0.08	0.02	0.02	<0.02
	72	0.02	0.02	0.02	0.02	2.5	2.5	2.5	0.02	0.02	0.02	0.02	0.08	0.02	0.02	<0.02
C. n	24	0.16	0.02	0.04	0.08	0.08	0.02	0.62	0.04	0.02	2.5	0.04	1.25	0.02	0.02	<0.02
	48	0.16	0.02	0.04	0.08	0.08	0.02	0.02	0.04	0.02	2.5	0.04	1.25	0.02	0.02	<0.02
	72	0.08	0.02	0.04	0.08	0.08	0.02	0.02	0.04	0.02	0.16	0.04	1.25	0.02	0.02	<0.02
A. f	24	0.16	0.04	0.02	0.32	0.02	2.5	2.5	0.16	0.02	0.02	0.16	0.04	0.16	0.32	<0.02
	48	0.62	1.25	0.62	2.5	0.62	2.5	0.62	0.16	1.25	0.04	1.25	0.08	0.08	1.25	<0.02
Average		0.15	0.18	0.10	0.34	1.05	1.57	1.41	0.06	0.17	0.67	0.19	0.51	0.04	0.21	<0.02

Abbreviations: C. a- *Candida albicans*, C. n- *Cryptococcus neoformans*, A. f- *Aspergillus fumigatus*, A- acetone, D- dichloromethane, H- hexane, E- ethanol, EA-ethyl acetate, M- methanol and H_2O- water.

Table 5.1 continued. Minimum inhibitory concentration (MIC) of eight plant species tested against three animal fungal pathogens.

Fungi		MIC (mg/ml)														
		Maerua juncea							*Pentarrhinum insipidum*							AMP B
	Time(h)	Extractants														
		A	D	H	E	EA	M	H_2O	A	D	H	E	EA	M	H_2O	
	24	0.04	0.04	0.02	0.02	0.08	0.02	2.5	0.02	0.02	0.02	0.02	0.04	0.04	0.02	<0.02
	48	0.04	0.04	0.02	0.02	2.5	0.02	2.5	0.02	0.02	0.02	0.02	0.04	0.04	0.02	<0.02
C. a	72	0.04	0.04	0.02	0.02	2.5	0.02	0.02	2.5	0.02	0.02	0.02	0.02	0.02	0.02	<0.02
	24	0.04	0.32	0.02	0.16	0.04	0.02	0.02	0.32	2.5	0.32	2.5	2.5	0.32	0.62	<0.02
	48	0.04	0.02	0.02	0.16	0.04	0.02	0.02	0.16	1.25	0.02	2.5	0.16	0.62	0.62	<0.02
C. n	72	0.04	0.02	0.02	0.16	0.04	0.02	0.02	0.16	1.25	0.02	0.08	0.08	0.62	0.62	<0.02
	24	0.62	0.32	0.02	0.02	1.25	0.62	2.5	0.16	0.16	1.25	0.04	0.04	0.04	0.04	<0.02
A. f	48	0.08	0.32	0.02	1.25	0.02	1.25	0.32	0.16	0.16	0.08	0.32	0.04	0.32	0.04	<0.02
Average		0.12	0.14	0.02	0.23	0.81	0.24	0.98	0.44	0.67	0.23	0.69	0.36	0.25	0.25	<0.02
Fungi		MIC (mg/ml)														
		Senna italica							*Schinus molle*							AMP B
	Time(h)	Extractants														
		A	D	H	E	EA	M	H_2O	A	D	H	E	EA	M	H_2O	
C. a	24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.62	0.62	0.62	0.02	2.5	1.25	1.25	<0.02
	48	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.62	0.62	0.62	0.02	2.5	0.02	0.02	<0.02
	72	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.04	0.04	0.04	0.02	2.5	0.02	0.02	<0.02
C. n	24	1.25	0.02	1.25	0.62	0.02	0.04	1.25	0.02	0.08	0.16	0.08	0.04	0.32	0.32	<0.02
	48	1.25	0.02	0.16	0.02	0.02	0.02	1.25	0.02	0.16	0.04	0.08	0.04	0.02	0.02	<0.02
	72	0.16	0.02	0.16	0.02	0.02	0.02	0.02	0.02	0.04	0.04	0.08	0.04	0.02	0.02	<0.02
A. f	24	0.32	0.08	0.02	0.02	0.02	1.25	0.02	0.04	0.02	0.04	0.04	0.08	2.5	2.5	<0.02
	48	0.16	0.16	0.16	0.16	0.02	0.62	0.02	0.32	0.08	0.02	0.04	0.08	0.08	0.08	<0.02
Average		0.40	0.04	0.23	0.11	0.02	0.25	0.33	0.21	0.21	0.19	0.05	0.97	0.52	0.52	<0.02

Abbreviations: C. a- *Candida albicans*, C. n- *Cryptococcus neoformans*, A. f- *Aspergillus fumigatus*, A- acetone, D- dichloromethane, H- hexane, E- ethanol, EA-ethyl acetate, M- methanol and H_2O- water.

5.4.1.2 Average MIC value of extractants.

Aqueous extract of *S. molle* and methanol extract of *E. elephantina* had the highest average MIC value of 1.57 mg/ml, followed by aqueous (1.41 mg/ml) and ethanol extracts (1.05 mg/ml) of *E. elephantina* as shown in Figure 5.1. The highest average MIC value shows that the water extracts was less active against the tested microorganisms. In the previous study, *E. elephantina* in combination with other plant species had average MIC of 0.5 mg/ml in dichloromethane and methanol extracts (Mabona et al., 2013).

Hexane leaf extracts of *M. juncea* and ethyl acetate extracts of *S. italica* had lowest average MIC of 0.02 mg/ml against all the test organisms, followed by methanol and ethanol of *A. seineri* methanol of *I. circinnata*, dichloromethane extracts of *S. italica* all with (0.04 mg/ml) and ethanol of *S. molle* (0.05 mg/ml). In contrast, dichloromethane and acetone extracts had the lowest average MIC against some microorganisms (Mahlo et al., 2013). Ethyl acetate and acetone extracts showed good average MIC values 0.30 mg/ml and 0.32 mg/ml while methanol had lowest average MIC of 0.16 mg/ml against *C. albicans* and *C. neoformans*. However, *C. albicans* and *C. neoformans* were sensitive to most of the plant extract.

Methanol and ethanol extracts of *Albuca seineri* had shown excellent average MIC value of 0.04 mg/ml against the tested fungal pathogens. Other plant species from the genus *Albuca* have been reported to be used by the traditional healers in Eastern Cape to treat diabetes mellitus and wounds (Van Huyssteen et al., 2011). It was found that methanol extracts of *Albuca* genus has shown higher anti-diabetic activity than the aqueous extracts (Odeyemi et al., 2015).

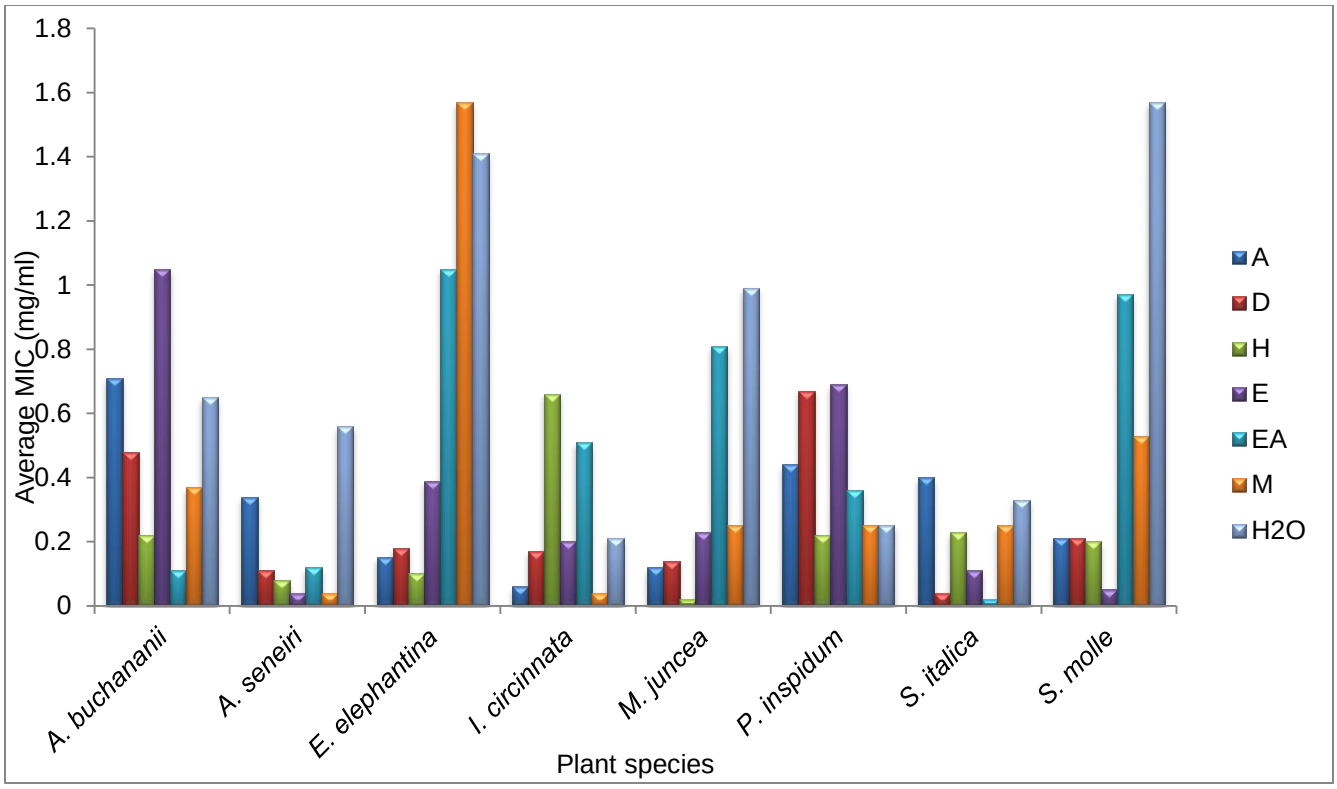


Figure 5.1 Average MIC value prepared with different extractants

5.4.1.3 Total activity

The total activities of plant extracts are shown in Table 5.2. The total activity was calculated by dividing the quantity extracted in milligrams from 1g leaves by the MIC value in mg/ml (Eloff, 2000). The total activity is used to determine the exact volume an extract from 1 g of plant material can be diluted and still inhibit the growth of the test organism (Eloff, 1999).

The highest total activity was observed in methanol extracts of *E. elephantina* (10500 ml/g) against *C. neoformans*, high total activity of *E. elephatina* was due to high extractability of the plant material with methanol. The lowest total activity was observed in hexane extracts of *I. circinnata* (4 ml/g) against *C. neoformans*. It was suggested (Ramadwa et al., 2017) that high total activity shows that relatively non-polar compounds play an important role in antimicrobial activity of the plant extracts.

Table 5.2 Total activity in ml/g of eight plant species extracted with acetone (A), dichloromethane (d), hexane (H), ethanol (E), ethyl acetate (EA) and methanol (M).

Fungi		Total activity (ml/g)																	
		Asparagus buchananii						*Albuca seineri*						*Elephantorrhiza elephantina*					
	Time(h)	Extractants																	
		A	D	H	E	EA	M	A	D	H	E	EA	M	A	D	H	E	EA	M
	24	1795	1346	449	38	2500	6625	2750	1000	2250	750	160	4625	3250	2875	2125	6250	13	84
	48	56	168	1750	38	2500	6625	2750	1000	2250	750	625	4625	3250	2875	2125	6250	13	84
C. a	72	56	168	1750	38	2500	6625	2750	1000	2250	750	625	4625	3250	2875	2125	6250	13	84
	24	56	42	1750	38	160	6625	2750	32	144	385	625	4625	417	2875	1090	1603	417	10500
	48	224	168	1750	38	1282	6625	2750	1000	577	385	321	4625	417	2875	1090	1603	417	10500
C. n	72	224	168	1750	38	641	6625	2750	1000	577	192	321	4625	833	2875	1090	1603	417	10500
	24	3500	2625	28	77	1282	53	22	257	2250	385	80	1186	417	1474	2125	399	1625	84
A. f	48	56	42	112	152	160	423	705	257	577	192	20	1186	105	46	69	50	53	84
Average		746	591	1167	57	1378	5028	2153	693	1359	474	347	3765	1492	2346	1480	3001	371	3990
	Time(h)	*Indigofera circinnata*						*Mearua juncea*						*Pentarrhinum insipidum*					
		Extractants																	
		A	D	H	E	EA	M	A	D	H	E	EA	M	A	D	H	E	EA	M
	24	750	750	500	2625	192	4375	641	833	1125	875	289	6250	2625	3250	2125	3250	1539	3782
	48	750	750	500	2625	96	4375	641	833	1125	875	9	6250	2625	3250	2125	3250	1539	3782
C. a	72	750	750	500	2625	96	4375	641	833	1125	875	9	6250	21	3250	2125	3250	3000	7375
	24	385	750	4	1346	6	4375	641	104	1125	112	577	6250	168	26	136	26	24	471
	48	385	750	4	1346	6	4375	641	1625	1125	112	577	6250	337	52	2125	26	385	238
C. n	72	385	750	64	1346	6	4375	641	1625	1125	112	577	6250	337	52	2125	833	769	238
	24	96	750	500	337	192	561	40	104	1125	875	18	202	337	417	34	1667	1539	3782
A. f	48	96	12	257	42	96	1122	321	104	1125	14	1125	100	337	417	545	208	1539	471
Average		450	658	291	1537	86	3492	526	758	1125	481	398	4725	848	1339	1418	1564	1292	2517

Table 5.2 continued. Total activity in ml/g of eight plant species extracted with acetone (A), dichloromethane (D), hexane (H), ethanol (E), ethyl acetate (EA) and methanol (M) the tested fungal pathogens

Fungi		Total activity (ml/g)											
		Senna italica						*Schinus molle*					
	Time (h)	Extractants											
		A	D	H	E	EA	M	A	D	H	E	EA	M
C. a	24	1750	2000	1375	2125	375	4500	165	161	129	7250	47	132
	48	1750	2000	1375	2125	375	4500	165	161	129	7250	47	8250
	72	1750	2000	1375	2125	375	4500	2628	2564	2051	7250	47	8250
C. n	24	28	2000	22	69	375	2308	5125	1282	513	1859	3013	527
	48	28	2000	176	2125	375	4500	5125	164	2051	1859	3013	8250
	72	224	2000	176	2125	375	4500	5125	2564	2051	1859	3013	8250
A. f	24	112	513	1375	2125	375	72	2628	5000	2051	3718	1507	66
	48	224	257	176	273	375	145	328	1282	4000	3718	1507	2116
Average		733	1596	756	1637	375	3128	2661	1647	1622	4345	1524	4480

C. a- Candida albicans, *C. n- Cryptococcus neoformans*, *A. f- Aspergillus fumigatus,* A- acetone, D- dichloromethane, H- hexane, E- ethanol, EA- ethyl acetate, M-methanol,

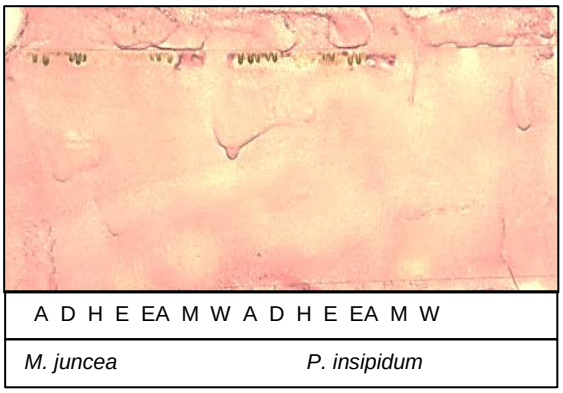
5.5. TLC bioautography of fungal species

5.5.1 TLC bioautogram of *Candida albicans*

Bioautography assay was used to determine the number of active compounds in different plant extracts and the results are shown in Figure 5.2 and R_f values are shown in Table 5.3. TLC chromatograms developed in BEA separated non-polar compounds with 87 (59%), followed by CEF 38 (25%) separate intermediate polarity and the least was EMW 23 (16%). This shows that most antifungal compounds were non-polar. Similar results were observed in other studies; BEA was the best eluent solvent system that separated more bands than other eluent systems (Adamu et al., 2012). All plant extracts have at least one compound when separated with BEA against the test fungal organisms. However, no active compounds were observed in chromatograms developed in BEA eluent system against the tested microorganisms (Mahlo et al., 2016). The results of chromatograms for *A. buchananii, A. seineri, E. elephantorrhiza, I. circinnata, M. juncea, P. insipidum, S. italica* and *S. molle* plant extracts separated in BEA and EMW were not shown since the chromatograms were not clearly visible after repeating several times. More active compounds were observed in DCM extracts of most plant species compared to other plant extracts.

In TLC chromatograms separated with BEA, the same antifungal compound was observed in water extracts of *A. buchananii* and *A*. *seineri* with similar R_f value of 0.06 against *C*. *neoformans* (Table 5.3). This indicates that the compounds were more non-polar to be separated with polar eluent system. In contrast, the antifungal compounds with water extracts against *C. albicans* separated with polar (CEF) eluent system were observed (McGaw et al., 2013). The active compounds were visible in acetone, dichloromethane and ethyl acetate extracts of *A*. *seineri* and had similar R_f value of 0.76 against *C*. *albicans*. Other studies found that other plants species from the genus *Albuca* had antidiabetic compounds (Odeyemi et al., 2015). More interesting, other active compounds with similar R_f value of 0.25 were also observed in acetone and dichloromethane extracts of *M*. *juncea* and *P*. *insipidum* against *C*. *neoformans*. Methanol extracts of the tested plant species had shown the zone of inhibition against *C*. *albicans* (Dube et al., 2017).

The aqueous extracts developed in BEA, EMW and CEF had poor antifungal activity against *C*. *albicans*, *A. fumigatus* and *C*. *neoformans.* Antifungal compounds were observed in dichloromethane extracts of *A. seineri*, *I. circinnata*, *A. buchananii* and *M. juncea* against *C. albicans* in EMW bioautograms with R_f values of 0.79, 0.96, 0.82 and 0.77 respectively (Table 5.3).



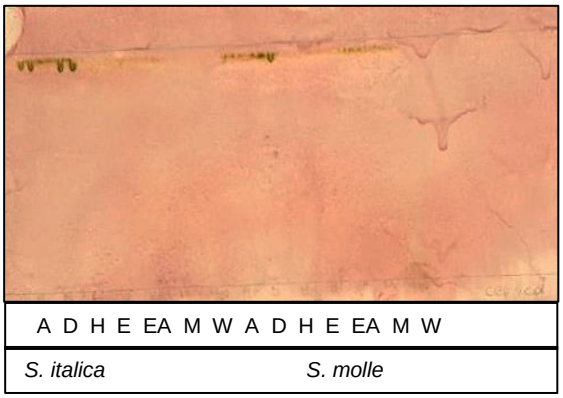


Figure 5.2 Bioautograms extracts *of S. italica*, S*. molle, Maerua juncea* and *Pentarrhinum insipidum*. TLC plates developed in CEF were sprayed with *Candida albicans*. White areas indicate inhibition of fungal growth. Lanes from left to right: acetone=A, dichloromethane=D, hexane=H, ethanol=E, ethyl acetate=EA, methanol=M and aqueous extract.

Table 5.3 R_f values of antifungal compounds separated with BEA, CEF and EMW eluent systems, extracted with acetone, dichloromethane, hexane, ethanol, ethyl acetate, methanol and water

Eluent system	R_f value	Extractants	Microorganisms			Total
			A. f	*C. a*	*C. n*	
BEA	*Asparagus buchananii*					
	0.36 0.76	A	1	1	1	3
	0.14 0.26 0.36 0.76	DCM	2	2	1	5
	0.36	H	1	1		2
	0.36	E	1			1
	0.11 0.36 0.76	EA	1	1	1	3
	0.06	H_2O			1	1
	Albuca seineri					
	0.11 0.31 0.36	A	2	1		3
	0.14 0.36	DCM	3	2		5
	0.14 0.31 0.36	H	3			3
	0.14 0.36 0.50	EA	3	2		5
	0.06	H_2O			1	1
	Elephantorrhiza elephantina					
	0.18 0.35 0.39	A	3			3
	0.18 0.35 0.42	DCM	3			3
	0.18 0.39	H	3			3
	0.35	EA	1			1

Table 5.3 Continued. R_f values of antifungal compounds separated with BEA, CEF and EMW eluent systems, extracted with acetone, dichloromethane, hexane, ethanol, ethyl acetate, methanol and water

Eluent system	Rf value	Extractants	Microorganisms			Total
			A. f	*C. a*	*C. n*	
	Indigofera circinnata					
BEA	0.18 0.35	DCM	2			2
	0.35	H	1			1
	0.18 0.35	EA	2			2
	Maerua juncea					
	0.25 0.35	A	1		1	2
	0.20 0.35	DCM	2	1	1	4
	0.20 0.35	H	2			2
	0.31	EA	1			1
	Pentarrhinum insipidum					
	0.25 0.49 0.56	A	2		2	4
	0.25 0.40 0.49	DCM	1		2	3
	0.25 0.49	E	1		2	3
	0.4	EA	1			1
	0.4	M	1			1
	Senna italica					
	0.33	A	1			1
	0.14 0.33	DCM	2			2
	0.33	H	1			1
	Schinus molle					
	0.23 0.29 0.40	A	3			3
	0.14 0.23 0.29 0.40	DCM	4			4

Table 5.3 Continued. R_f values of antifungal compounds separated with BEA, CEF and EMW eluent systems, extracted with acetone, dichloromethane, hexane, ethanol, ethyl acetate, methanol and water

Eluent system	Rf value	Extractants	Microorganisms			Total
			A. f	*C. a*	*C. n*	
BEA	*Schinus molle*					
	0.23 0.29	H	2			2
	0.23 0.29	E	2			2
	0.23 0.29	EA	2			2
	0.23 0.29 0.40	M	3			3
	Total					87
CEF	*Asparagus buchananii*					
	0.5 0.60	DCM	2	1	2	5
	Albuca seineri					
	0.96	A	1			1
	0.60 0.64 0.96	DCM	3	2		5
	0.96	H	1			1
	0.96	EA	1			1
	Elephantorrhiza elephantina					
	0.59 0.60	DCM	2			2
	Indigofera circinnata					
	0.52 0.60 0.90	DCM	3	1	1	5
	Maerua juncea					
	0.51 0.60 0.66	DCM		2	1	3
	0.66	EA		1		1
	Pentarrhinum insipidum					
	0.60 0.66	DCM		2		2
	0.66	H		1		1
	0.66	EA		1		1

Table 5.3 Continued. R_f values of antifungal compounds separated with BEA, CEF and EMW eluent systems, extracted with acetone, dichloromethane, hexane, ethanol, ethyl acetate, methanol and water

Eluent system	Rf value	Extractants	Microorganisms			Total
			A. f	*C. a*	*C. n*	
CEF	*Senna italica*					
	0.58 0.60	DCM	1	2		3
	Schinus molle					
	0.86	A	1			1
	0.83 0.86	DCM	1	1		2
	0.86	M	1			1
	Total					38
EMW	*Asparagus buchananii*					
	0.75 0.81	DCM	1	1		2
	0.93	H		1		1
	Albuca seineri					
	0.75 0.79	DCM	1	1		2
	Elephantorrhiza elephantina					
	0.74	DCM	1			1
	0.27 0.40 0.50	EA			3	3
	Indigofera circinnata					
	0.69 0.83 0.89 0.96	DCM	3	1		4
	Maerua juncea					
	0.77 0.82	DCM		1	1	2
	Pentarrhinum insipidum					
	0.74	DCM	1		1	2
	Senna italica					
	0.78	DCM	1			1
	Schinus molle					
	0.79	DCM			1	1
	0.69	M		1		1
	Total					23

5.5.2 TLC bioautogram of *Cryptococcus neoformans*

In TLC chromatograms separated with BEA, no active compounds were observed in all plant extracts of *E. elephantina*, *I. circinnata* and *S. molle* against *C. neoformans*. Active compounds with similar R_f value of 0.25 were visible in acetone and dichloromethane extracts of *M. juncea* and *P. insipidum* against *C. neoformans* (Table 5.3). More importantly, more compounds were observed against *C. neoformans*. The clear zones of inhibition may be due to the nature of the separated compounds on the TLC plate, some may have lost the activity because they were acting synergistically with the crude extracts (Kabonga-Kayoka et al., 2016). Other studies found that the antifungal compounds were visible in the CEF eluent system against *Cryptococcus neoformans* (McGaw et al., 2013).

DCM extracts of *Indigofera circinnata* has shown zone of inhibition against *C. neoformans* (Figure 5.3). However, no active compounds were observed in some plants from the genus *Indigofera* (Njeru et al., 2016). In the current study, more active compounds were visible against *A. fumigatus* as compared to *C*. *neoformans.* In summary, the results obtained have shown that there were more active compounds separated by CEF (total of 38) than by EMW (total of 23).

In CEF, acetone, dichloromethane, hexane, ethanol, ethyl acetate, methanol and water extracts of *A. seneiri*, *E*. *elephantina*, *P*. *insipidum*, *S. italica*, *and S*. *molle* had shown no active compounds against *C*. *neoformans* (Figure 5.3). These might be due to the presence of formic acid that inhibited the growth of microorganisms. Acetone, dichloromethane, hexane, ethanol, ethyl acetate, methanol and water extracts *A*. *buchananii*, *A*. *seneiri*, *I*. *circinnata* and *S. italica* developed in EMW had poor activity against *C*. *neoformans.* No active compounds were observed when separated with EMW eluent system. The lack of good activities among some of the extracts could be that the bioactive compounds are present in small amount (Okem et al., 2012). However, all these plants had shown some antifungal activity on microdilution method. These might be due to the synergistic effects between the active compounds or low concentration of compound present in the plant extracts (Mahlo et al., 2013).

The antifungal compounds separated well in CEF bioautograms than other eluent solvent systems. DCM extracts of *A*. *buchananii*, *I*. *circinnata* and *M*. *juncea* had shown the active compounds against *C. neoformans* with similar R_f values of 0.60 (Table 5.3). Acetone and DCM extracts of some plants eluted in CEF showed the zone of inhibition against the tested organisms (Mahlo et al., 2016)

Dichloromethane extracts of *M. juncea* and *P. insipidum* had similar active compounds with R_f value of 0.82 against *C. neoformans* (Table 5.3*)*. No antifungal compounds were observed in plant extracts of *E. elephantina* against *C. albicans*, since it had shown low MIC value against *C. albicans.* No active compounds were observed with the polar EMW eluent system and the R_f values were relatively high indicating the compound are probably of medium polarity (Netshiluvhi and Eloff, 2016).

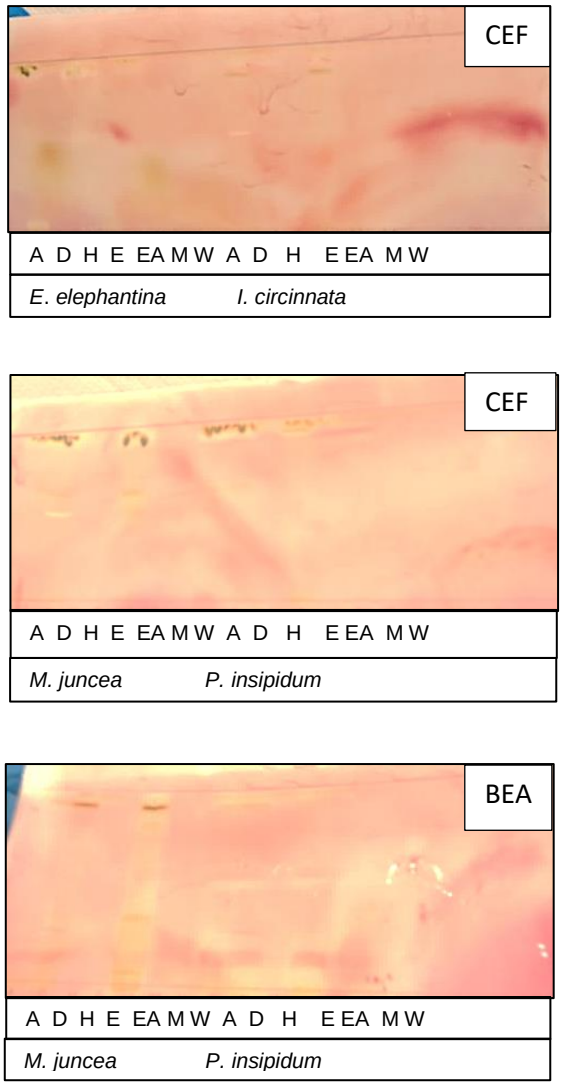


Figure 5.3 Bioautograms of extracts *of E. elephantina, I. circinnata, Maerua juncea* and *Pentarrhinum insipidum*. TLC plates developed in CEF and BEA sprayed with *C. neoformans*. White areas indicate inhibition of fungal growth. Lanes from left to right: acetone=A, dichloromethane=D, hexane=H, ethanol=E, ethyl acetate=EA, methanol=M and aqueous extracts=W.

5.5.3 TLC bioautogram of *Aspergillus fumigatus*

In BEA solvent system, two compounds were visible in dichloromethane, hexane, acetone and ethanol extracts of *S. italica* and *S. molle* with R_f values of 0.14 and 0.40 (Table 5.3). However, *S. italica* extracts had more compounds when separated with BEA eluent system (Lekganyane et al., 2012). Leaf extracts of *S. molle* had shown clear zone of inhibition against *A. fumigatus* (Figure 5.4) and had no activity when separated with BEA and CEF against *C. neoformans* and *C. albicans*. This

suggests that different plant extracts may have selective targets compounds (Zhang et al., 2013).

Acetone, dichloromethane, hexane, ethanol and ethyl acetate extracts of *A. buchananii* and *A. seineri* had shown active compounds in BEA bioautograms with similar R_f value of 0.36 against *A. fumigatus* (Figure 5.4). Some plant species from the genus *Albuca* possess highest phenolic contents (Odeyemi, 2015). Previously it was reported that *A. fumigatus* was more resistant to the plant extracts (Eloff et al., 2008). Active compounds were observed in DCM extracts in most plant species. Similarly Lekganyane et al. (2012) found that DCM showed more zones of inhibition against the tested organisms. In contrast, Eloff et al. (2008) found that acetone extracted most antifungal compounds from some Southern African *Combretum* species. Furthermore, clearer bands were observed on chromatograms developed in BEA eluent system followed by CEF. This shows that the active compounds were mostly non-polar.

In general, more bands of compounds were observed in chromatograms developed with BEA as compared to other eluent solvent systems. This may suggest that active compounds visible on chromatograms were non-polar. Adamu et al. (2012) reported that more active compounds were observed in TLC bioautograms developed in BEA eluent system against *C. albicans*.

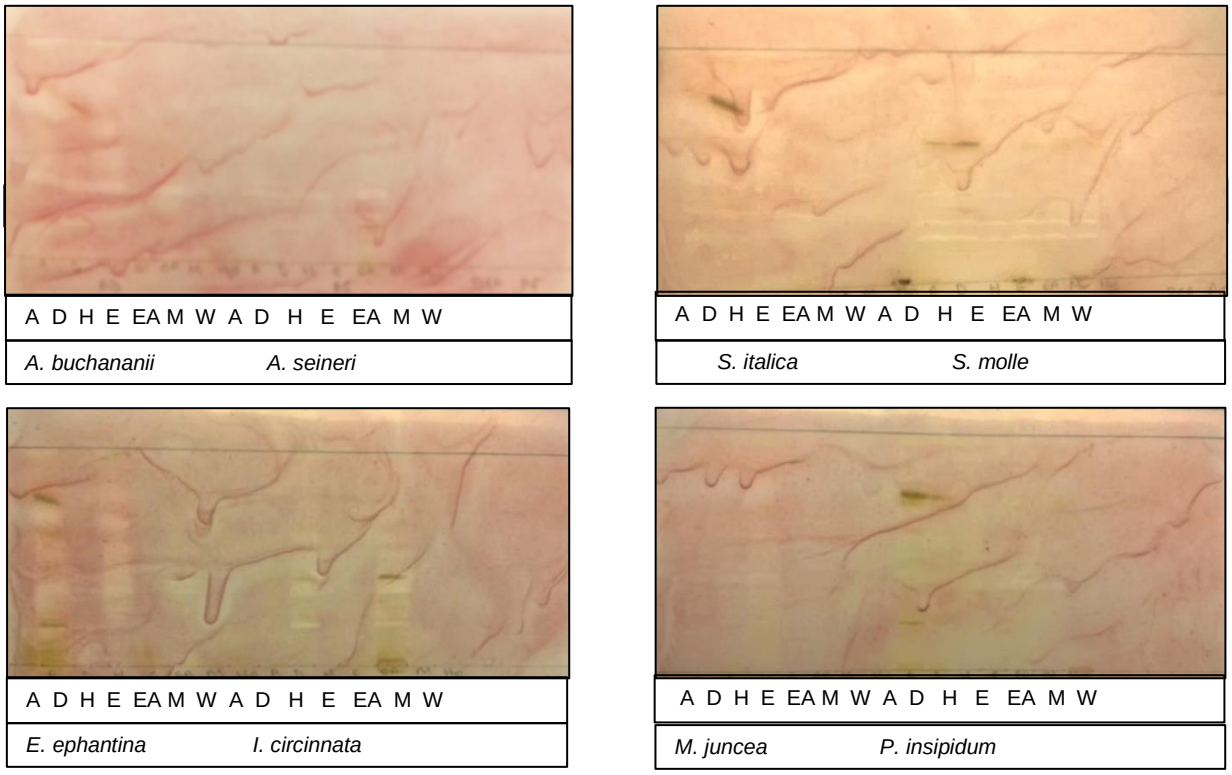


Figure 5.4 Bioautograms of extracts *of E. elephantina, I. circinnata, S. italica*, S*. molle, Maerua juncea* and *Pentarrhinum insipidum*. TLC plates developed in BEA sprayed with *A. fumigatus*. White areas indicate inhibition of fungal growth. Lanes from left to right: acetone=A, dichloromethane=D, hexane=H, ethanol=E, ethyl acetate=EA, methanol=M and aqueous=W

5.6 Cytotoxicity of plant extracts

Cytotoxicity assay was used to determine the toxicity effects of five selected plant species against African green monkey Vero cells. These plant species were selected based on excellent activity against the animal pathogens as described in section 5.5.1. Cytotoxicity assay was described in chapter 5 section 5.3.4. Plant extracts that had shown good activity against the tested microorganisms should be tested for cytotoxic effects, to ensure that the biological activity of the plant extract is not due to general metabolic toxic effect (Dzoyem et al., 2016), since the metabolic effect might be toxic to humans (Shai et al., 2013). Plant extracts with good biological activity against the test organisms and found to be toxic against the cells will not be of any value (Elisha et al., 2017). However, the plant extracts with prominent antifungal activity were selected for cytotoxicity testing.

Cytotoxicity of five plant extracts was evaluated using MTT assay against the Vero cells and the results are shown in Figure 5.6- 5.10. The extracts had varying degrees of cytotoxicity against the Vero kidney monkey cells with values ranged between 0.13 mg/ml and >1 mg/ml as summarised in (Table 5.4). Doxorubicin was used as a positive control in (Figure 5.6) and was toxic at LC_{50} = 4.34 µM. Hexane extracts of *A. buchananii* was less toxic with LC_{50} of 0.35 mg/ml and ethanol extracts of *P. insipidum* was also less toxic with LC_{50} of 0.28 mg/ml against African Vero cells. Hexane extracts of *M. juncea* was highly toxic with LC_{50} of 0.13 mg/ml. However, crude extract is considered to have cytotoxic effect if LC_{50} value is <0.5 mg/ml and highly toxic if LC_{50} is 0.1mg/ml (Ghuman et al., 2016) while other researchers (Elisha et al., 2017) considered plant extracts with LC_{50} >0.2 mg/ml as relatively non-toxic. However, plant extracts showing sensitivity of cell lines with LC_{50} >0.1 mg/ml are considered not cytotoxic to anticancer compounds (Kuete et al., 2010).

The water extracts of *A. buchananii*, *A. seineri* and *M*. *juncea* had LC_{50} of >1 mg/ml, suggesting that the extracts were non-toxic against the Vero cells. Similarly, Odeyemi et al. (2015) reported that water extracts of *Albuca* species were not toxic against the Chang liver cells, and methanolic extracts indicate little toxicity. Furthermore, Tarkany et al. (2012) found similar results where aqueous extracts were less toxic than ethanol extracts. Aqueous extracts of selected plants used as anthelmintic in Kenya were not toxic using Brine Shrimp Lethality test (Muthee et al., 2016). These confirm the safety usage of the water extracts by traditional healers in the treatment of fungal infections. Furthermore, other researchers also found that traditional medicine are mostly prepared in the form of decoction and infusion which uses water as a solvent (Mongalo et al., 2016). Moreover, this suggests that the antifungal activity of these plants is not due to their cytotoxic effects (Ali et al., 2001). Previous studies, found that all plants extracts tested against green monkey cells were not toxic with LC_{50} values of >1 mg/ml (Ghuman et al., 2016). The study shows that the plants extracts were generally not toxic. Similar results were observed in the studies (Ramadwa et al., 2017) where the plants extracts were not toxic to Vero kidney cells.

Table 5.4 Cytotoxicity of hexane, aqueous and methanol plant extracts

Plant species	Extractants	LC_{50} (mg/ml)	Doxorubin LC_{50} (µM)
Asparagus buchananii	Hexane	0.38	4.34
	Water	> 1.00	4.34
Albuca seineri	Methanol	0.21	4.34
	Water	> 1.00	4.34
Indigofera circinnata	Methanol	0.19	4.34
	Water	0.48	4.34
Maerua juncea	Hexane	0.12	4.34
	Water	> 1.00	4.34
Pentarrhinum insipidum	Ethanol	0.27	4.34
	Water	0.07	4.34

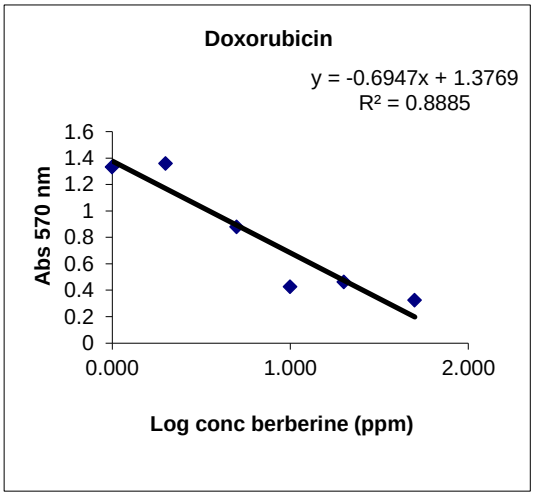
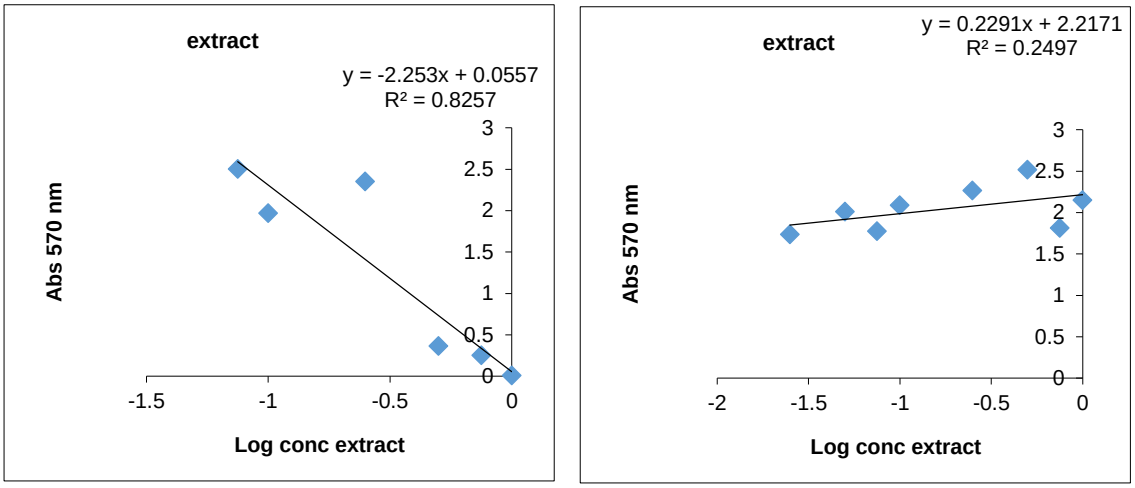
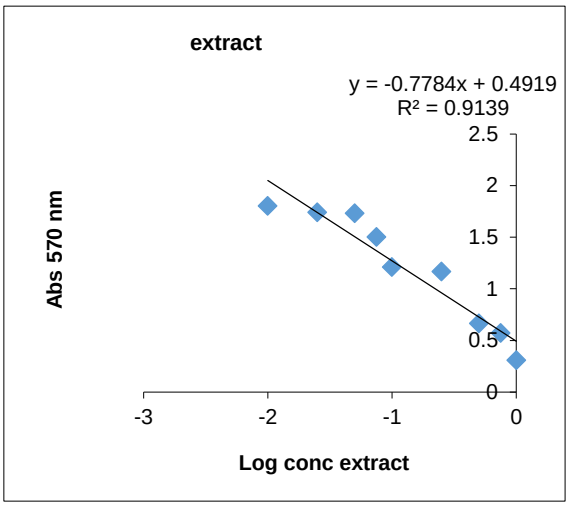


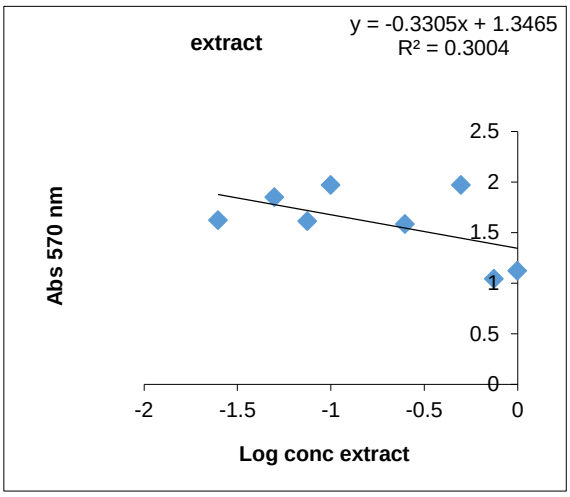
Figure 5.5 Cytotoxicity of Doxorubicin with LC_{50} = 4.3405 uM



(a) (b)

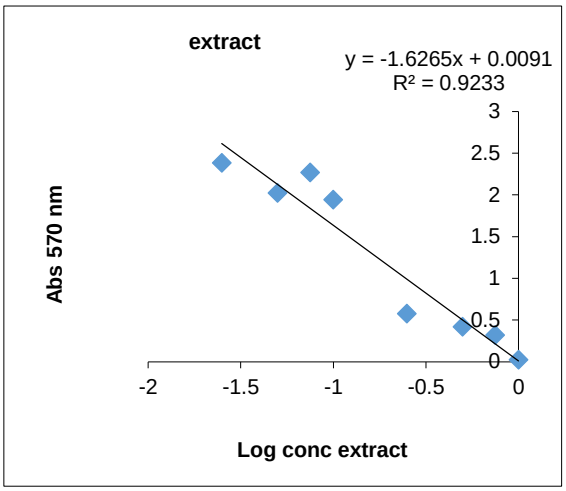
Figure 5.6 Cytotoxicity of (a) hexane and (b) aqueous exacts of *A. buchananii* with LC_{50}= 0.35 mg/ml and LC_{50}>1 mg/ml respectively**.**

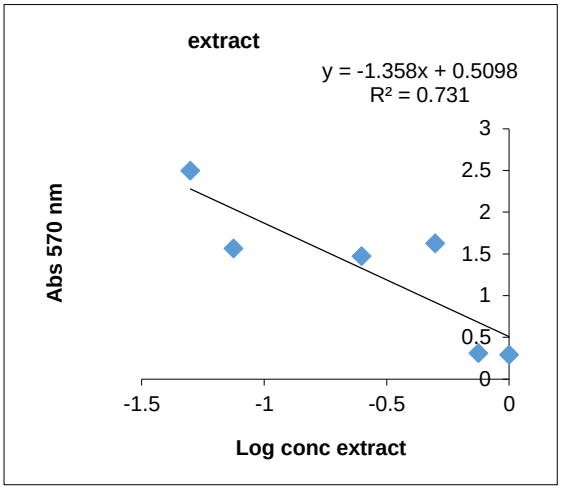




(a) (b)

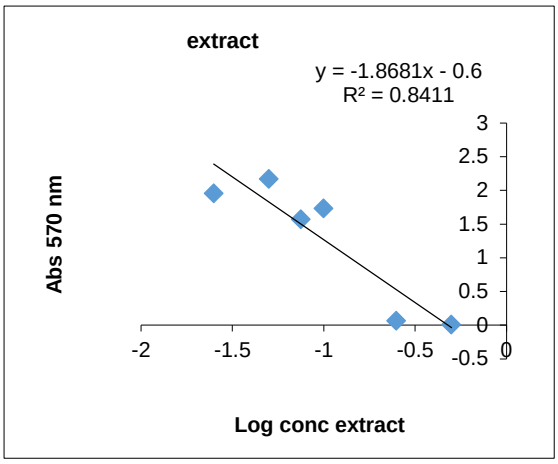
Figure 5.7 Cytotoxicity of (a) methanol and aqueous extracts of *A. seineri* with LC_{50}= 0.21mg/ml and LC_{50}= >1 mg/ml

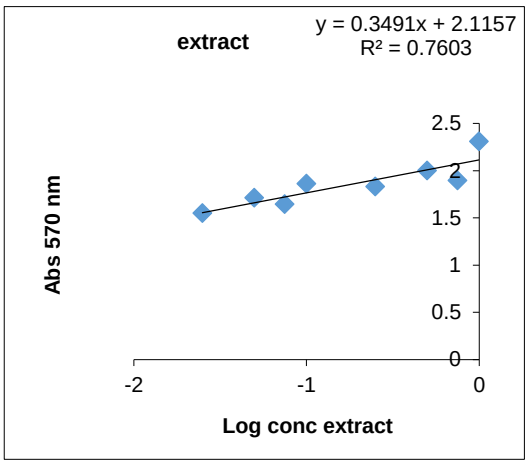




(a) (b)

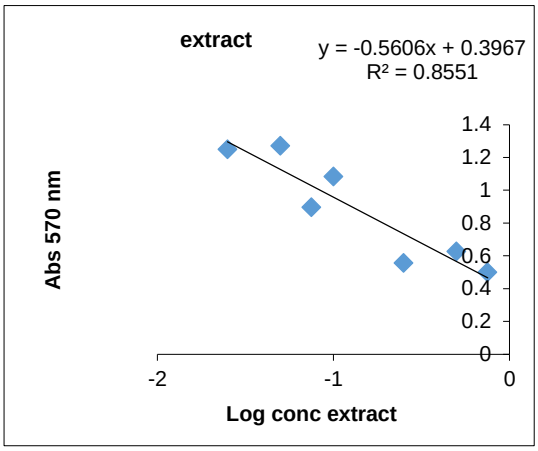
Figure 5.8 Cytotoxicity of (a) methanol and (b) aqueous extracts of *I. circinnata* with LC_{50}= 0. 20 mg/ml and LC_{50}= 0.49 mg/ml respectively.



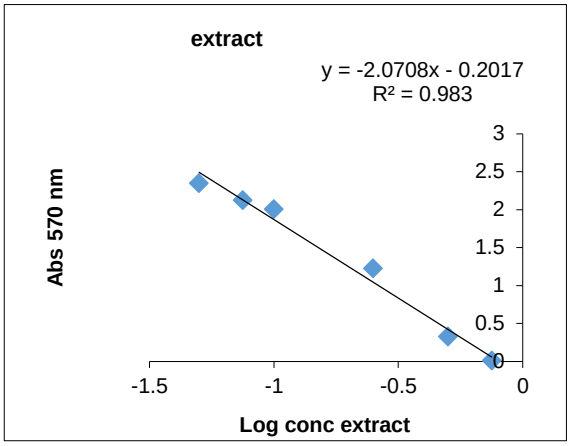


(a) (b)

Figure 5.9 Cytotoxicity of (a) hexane and (b) aqueous extracts of *M. juncea* with LC_{50}=0.13 mg/ml and LC_{50}=>1 mg/m/ respectively.



(a)



(a) (b)

Figure 5.10 Cytotoxicity of (a) ethanol and (b) aqueous extracts of *P. insipidum* with LC_{50}= 0.28 mg/ml and LC_{50}= 0.65 mg/m respectively.

6. Conclusion

In Vhembe District, no information regarding the biological activity of *A. buchananii, A. seineri, I. circinnata, P. insipidum* has been recorded. These plant species were investigated for the first time against fungal infections. The results from the study support the use of these plants by the traditional healers and the local people for the treatment of different ailments related to fungal infections using the water extracts. Ethnobotanical surveys and indigenous knowledge on medicinal plants should be taken into consideration because they may provide a lead to new antifungal agents.

Bioautography was used to determine the number of active compounds in the plant extracts. In TLC chromatograms developed in BEA more active compounds were observed (16) in *S. molle* leave extracts than in CEF (6) and EMW (2) against *A. fumigatus*.

Cytotoxicity of the plant extracts that had excellent antifungal activity was determined against the Vero kidney cells. All plant extracts tested were relatively not toxic against the cells with LC_{50} ranging between 0.13 mg/ml and $>$ 1 mg/ml. Water extracts of *A. buchananii, A. seineri* and *M. juncea* had $LC_{50} > 1$ mg/ml.

Finally, it was found that *A. buchananii, A. seineri*, *I. circinnata, P. insipidum* and *M. juncea* had promising antifungal activity and not toxic. Thus, future studies are required to isolate and identify the bioactive compounds present in these plant species.

CHAPTER 6

Summary and conclusion

A thorough ethnobotanical survey has been conducted using a structured questionnaire which included information from the local people and traditional healers. A total of 63 plant species were identified in Makhado Local Municipality. The antifungal activity and toxicity of eight selected plant species has been investigated using serial dilution and bioautography assay. Cytotoxicity assay was used to determine the toxic effects of the crude extracts against the Vero kidney cells.

The findings of the survey indicate that local people and traditional healers in Vhembe District still rely on medicinal plants as a source of primary health care. It was established that most of the medicinal plants used to combat various diseases have not been documented in Makhado Local Municipality. The most common methods of preparation of medicinal plants were decoction (65%) and infusion (35%). Decoction is mostly preferred by traditional healers when extracting tougher and more fibrous bark and root, since they possess more water soluble chemicals. It was also found that traditional healers prepare their medicine in some form of aqueous extract since they do not have access to lipophilic solvents and is their cultural tradition to use water to extracts plant materials. *Warburgia salutaris* was the frequently used plant to treat various ailments (64.7%) and the least was *Crassula ovata* (5.9%). Trees were the most predominant plant form used and the least were herbs and climbers. Trees are available throughout the year and they are relatively drought resistant.

The traditional healers of Makhado Local Municipality prefers the bark and roots to prepare their traditional medicines. However, this could result in extinction of some of plants due to overexploitation and deforestation. Traditional healers and local people should be taught about the sustainability and conservation of medicinal plants that are indigenous to South Africa. The traditional healers and the local people who are knowledgeable about medicinal plants should pass the indigenous knowledge to the next generation.

Eight plant species used as medicine by the traditional healers and local people were selected for further phytochemical analysis and microbiological assay. The selected plant species were extracted with seven solvents of varying polarities' that is hexane, DCM, acetone, ethanol, ethyl acetate, methanol and water. Methanol extracted large quantity of plant material (13%) as compared to other organic solvents. The highest amount was extracted from leaves of *E. elephantina*. Ethyl acetate and hexane were the least solvent extracting low quantity of plant material (0.75%) from leaves of *Senna italica*.

Antifungal activities of eight plant species were determined against *C. albicans*, *C. neoformans* and *A. fumigatus* using serial dilution method. Dichloromethane leaf extracts of the all plant species, *M. juncea*, *S. italica*, *A. seineri,* A*. buchananii, P. insipidum, S. molle* and root extract of *I. circinnata* had shown excellent antifungal activities against *C. albicans, C. neoformans* and *A. fumigatus* with MIC values ranging between 0.02 mg/ml and 0.08mg/ml. Of the tested plant species, hexane leaf extracts of *M. juncea*, ethyl acetate leaf extracts of *S. italica*, *A. buchananii* and *E. elephantin*a had excellent activities with MIC values as low as 0.02 mg/ml and 0.08 mg/ml against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Acetone extracts had moderate antifungal activities against fungal pathogens. *Aspergillus fumigatus* was relatively resistant against plant extracts highest MIC value of 1.25 mg/ml. The plant extracts screened against these microorganisms might have the secondary metabolites that enabled the inhibition of the fungal growth.

The total activity of the plant extracts was used to determine the extract volume an extract from 1 gram of the plant material that could be diluted and still inhibit the growth of the test organisms. The highest total activity was observed in methanol leaf extracts *E. elephantina* against *C. neoformans*. The lowest total activity was observed in hexane root extracts of *I. circinnata* against *C. neoformans*.

Bioautography was used to determine the number of active compounds present in the plant extracts. More active compounds were observed in TLC chromatograms developed in BEA (87), followed by CEF (38) and the least was EMW (23). The active compounds were mostly non-polar. More active compounds were observed in

DCM of most plant extracts. All extracts of *A. seineri, E. elephantina, P. insipidum, S. molle*, and *S. italica* separated in CEF eluent system had no active compounds against *C. neoformans*. The formic acid might have inhibited the fungal growth and also possible synergistic effects between secondary metabolites. No active compounds were observed in TLC chromatograms eluted with BEA against *C. neoformans* in *E. elephantina*, *I. circinnata* and *S. molle* plant extracts.

The cytotoxicity activities of *A. buchananii, A. seineri*, *I. circinnata, P. insipidum* and *M. juncea* extracts were investigated against Vero kidney cells. All plant extracts tested were relatively not toxic against the cells with LC_{50} ranging between 0.13 mg/ml and $>$ 1 mg/ml. Water extracts of *A. buchananii, A. seineri* and *M. juncea* had $LC_{50} >$ 1 mg/ml. The water extracts were not toxic and this support their use in traditional medicine.

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No information regarding the antifungal activities of *A. buchananii, A. seineri, I. circinnata, P. insipidum* has been recorded. These plant species were investigated for the first time against fungal infections in Makhado Local Municipality. The results from the study support the use of these plants by the traditional healers and the local people for the treatment of different ailments caused by fungal infections. Ethnobotanical surveys and indigenous knowledge on medicinal plants should be taken into consideration because they may provide a lead to new antifungal agents. Finally, it was found that *A. buchananii, A. seineri*, *I. circinnata, P. insipidum* and *M. juncea* had promising antifungal activity using both microdilution and bioautography assays with low MIC values and active compounds and toxicity activity. Thus, further investigation is required to isolate and identify the bioactive compounds present in these plant species, and can provide a lead to new antifungal agents.

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APPENDIX A: Ethnobotanical survey of medicinal plants use by traditional healers, in Makhado Local Municipality, Limpopo Province, South Africa.

QUESTIONNAIRE NUMBER

1. Geographical information									
Villages		Villages		Villages		Villages		Villages	
1		1		1		1		1	
2		2		2		2		2	
3		3		3		3			

2. Personal information								
2.1	Gender							
	Female				Male			
2.2	Age							
	1	15-30	2	31-45	3	46-60	4	61-85
2.3	Level of education							
	1	No formal education	2	Primary schooling	3	Secondary schooling		
2.4	How many years' experience in traditional healing?							
	1	<10	2	11-20	3	21-30	4	>30
3. Consultation								
3.1	How many patients do you see per month?							
	1	<10	2	11-20	3	21-30	4	>30
3.2	Do you only have same patients who consult or different patients?							
	1	Yes			2	No		

4. Collection	
4.1	Do you collect plants?

	1	Yes		2	No			
4.2	If no how do you get plant materials?							
	1	Get from other traditional healers.	2	Buy from the chemist/market.	3	Send someone to collect for me.	4	Other (Specify).
4.3	How are plants collected?							
	1	For a specific purpose.	2	General collection.	3	For a specific plant species.	4	Other (Specify).
4.4	Which plant parts do you prefer for treatment of fungal infections?							
	1	Leaves	2	Roots	3	Bark	4	Other (Specify)
4.5	How do you identify the symptoms of fungal infections?							
4.6	Which plant species do you use?							
4.7	What is the local name/vernacular name of the plant? (Specify language)							
4.8	How do you prepare your medicine?							
	1	Decoction	2	Infusion		Maceration	4	Other (Specify)
4.9	How is it administered?							
4.10	For how long is it administered?							
4.11	How do you know if the plant species is toxic or not?							
4.12	What other diseases can this plant used for?							
4.13	Any other relevant information?							
5.	**Legislation**							
5.1	Do you know of any protected plants?							
	1	Yes		2	No			
5.2	Are you registered with the Traditional Association for Healers?							
	1	Yes		2	No			
5.3	If no, why not?							

	1	Very strict requirements	2	Don't see any reason to be within the association	3	I do not know about the association	4	Others (Specify)
6.	**Conservation**							
6.1	Have you noticed a decline of certain plant species that you use in the area where you collect species?							
	1	Yes			2	No		
6.2	If yes, please name those species.							
	1		5		9			
	2		6		10			
	3		7		11			
	4		8		12			
6.3	Does the decline have an impact on your practice?							
	1	Yes			2	No		
6.4	If yes, explain the impact.							
6.5	Do you use cultivated plants?							
	1	Yes			2	No		
6.6	If no, explain why?							
6.7	Are there any plants that are not available in your area?							
	1	Yes			2	No		
6.8	If yes, how do you get them?							
	1	Get them from other healers	2	Buy them from the market	3	Buy them from street vendors	4	Others (Specify)