ISOLATION, CHARACTERISATION AND CYTOTOXICITY OF ANTIFUNGAL COMPOUNDS PRESENT IN MEDICINAL PLANTS USED AGAINST *CRYPTOCOCCUS NEOFORMANS* IN VHEMBE DISTRICT, LIMPOPO PROVINCE

By

TAMBUDZANI CAROLINE MACHABA

THESIS

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SUPERVISOR: Prof. SM Mahlo

CO-SUPERVISOR: Prof. JN Eloff

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DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo, for the degree Doctor of Philosophy 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 all material contained herein has been duly acknowledged.

______________________ ______________________

Machaba, TC (Ms) **Date**

DEDICATION

I dedicate this work to my daughter Tshilidzi, my son Dziphathutshedzo, my parents Selaelo and Maria Machaba, my siblings Morwafe, Mokgadi, Kgomotso, Delton and Dennis Machaba.

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LIST OF ABBREVIATION

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
DPPH	1,1-diphenyl-2-picrylhydrazyl
EMW	Ethyl acetate methanol, water (40:5.4:4)
H	Hexane
H_20	Water
INT	p-iodonitrotetrazolium violet
LC_{50}	Lethal Concentration for 50% of the cells
MeOH	Methanol
MTT	(3-(4,5-dimethylthiazol)-2,5 diphenyltetrazolium bromide
NMR	Nuclear Magnetic Resonance
MIC	Minimum inhibitory concentration
TLC	Thin Layer Chromatography
R_f	Retention factor
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDA	Sabouraud Dextrose Agar
TLC	Thin layer chromatography

ABSTRACT

The use of medicinal plants as a source of treatment for various ailments including fungal infections is still practised in South Africa and across the globe. Fungal infections especially of *Cryptococcus, Candida* and *Aspergillus* species are the main cause of morbidity and mortality worldwide, particularly in developing countries. Traditional medicine is used as a source of remedies worldwide and has contributed extensively towards the development of modern medicine. Twelve selected medicinal plants (*Kleinia longiflora* DC*. Berchemia discolor* (Klotzsch) Hemsl., *Persea americana* Mill., *Sansevieria hyacinthoides* (L.) Druce, *Dichrostachys cinerea* (L.) Wright &Arn, *Withania somnifera* Dunal (Ashgandh), *Momordica balsamina* L., *Lonchocarpus capassa, Pappea capensis*, *Rhus lancea* L. fil, *Peltophorum africanum*, *Maytenus heterophylla* (Eckl. & Zeyh.) Robson) were analysed qualitatively for antifungal activities against *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans*.

The plant materials were extracted with solvents of various polarities such as acetone, dichloromethane, methanol, hexane, and water. Methanol extracted the highest amount of crude extracts from all the plant species as compared to other organic solvents. Chemical components of the extracts were analysed using aluminum-backed Thin Layer Chromatography (TLC) plates and developed using three different eluent systems: Ethyl acetate: methanol: water [EMW], Chloroform: ethyl acetate: formic acid [CEF] and Benzene: ethanol: ammonia hydroxide [BEA]. CEF was the best eluent solvent system since it separated more compounds from plant extracts. This indicates that the active compounds were relatively non-polar. More chemical compounds were observed in TLC chromatograms separated with CEF, followed by BEA and EMW. All plant extracts had shown different chemical components when separated from the three solvent systems.

The bioautography and serial dilution assays were used to determine the biological activity of plant extracts against the tested microorganisms, respectively. All the tested plant extracts revealed some varying degrees of fungal inhibition, with minimum inhibitory concentrations (MIC) values ranging between 0.02 mg/ml and 2.5 mg/ml. The aqueous extracts had shown some activity against the tested microorganisms. Noteworthy, antifungal activity was observed in acetone, DCM, hexane, and methanol

root extracts of *D. cinerea* against the three tested microorganisms with MIC values ranging between 0.02 mg/ml and 0.04 mg/ml. Furthermore, acetone extracts of *D. cinerea* and *P. africanum* had excellent activity against three fungal pathogens with MIC values of 0.02 mg/ml and 0.08 mg/ml. Active compounds were observed in dichloromethane extracts of *W. somnifera* with R_f values of 0.40 and 0.64. In TLC chromatograms separated with BEA, active compounds were observed in acetone, hexane, and methanol leaf extract of *P. americana*, this indicates that the fungal compounds were relatively non-polar. No active compounds were observed in plant extracts of *K. longiflora*. Active compounds were visible in all extracts of *P. capensis* in TLC chromatograms developed in CEF and EMW.

The antioxidant present in plants prevents the free radicals from causing various diseases in humans by inhibiting the oxidation of free radicals at the cellular level. The qualitative and quantitative 1,1-diphenyl-2-picrylhydrazyl (DPPH) methods were used to determine the antioxidant activities of plant extracts. The presence of antioxidant compounds was indicated by yellow bands against the purple background on the TLC plates. More antioxidant compounds were observed in acetone and dichloromethane extracts of *S. hyacinthoides* developed in BEA compared to other plant species tested. Methanol, hexane, and water extracts of *L. capassa* revealed good antioxidant activity against DPPH by having a high percentage of inhibition compared to other solvents. Noticeably, extracts of *P. africanum* possess strong antioxidant activity as compared to other plant species.

Solvent-solvent fractionation using column chromatography of the acetone extract led to the isolation of six compounds. The biological activity of the isolated compounds of *L. capassa* was investigated against the tested pathogenic fungi. The isolated compounds revealed some varying degrees of inhibition to the fungal pathogens. The largest quantity was isolated from compound 1 (80 mg), compound 4 (39 mg), compound 3 (27 mg), compounds 2 and 5 (14 mg) and the least was compound 6 (4.8 mg). However only three compounds were successfully identified as Lupeol (compound 1), Friedelin (compound 3) and 6-(γ,γ-Dimethylallyl)-3’,4’-dimethoxy-6”,6”-dimethylpyrano-[2”,3”:7,8]-flavanone (compound 4). Compounds 2, 5 and were not identified due to some impurities.

More importantly, the isolated compounds exhibited good antioxidant activity in qualitative and quantitative scavenging assays, which indicates that isolated compounds of *L. capassa* can scavenge the free radicals causing fungal infections in humans. The results support the traditional use of the selected plants to combat fungal infections and related ailments by the local people and traditional health practitioners in Vhembe District, Limpopo Province.

The (3-(4,5-dimethylthiazol) -2,5-diphenyltetrazoliumbromide) (MTT) assay was used to determine the toxic effects of the plant crude extract and isolated compounds. Lupeol and 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone revealed the same degree of cytotoxicity against the Vero monkey kidney cells. All the compounds were not toxic with an LC_{50} value of > 0.2 mg/ml.

CONFERENCE ORAL PRESENTATIONS

T.C. Machaba, S.M. Mahlo and J.N. Eloff. Antioxidant and in vitro antifungal activities of selected traditional plants used in ethnomedicine. Presented during the 47th Annual South African Association of Botanists (SAAB), South Africa, January 2022.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

South Africa has a long-standing history of traditional healing practices using medicinal plants. Almost 30 000 flowering plant species were documented in South Africa, and 3000 medicinal plant species are used to combat various ailments in humans. More importantly, these plants contain secondary metabolites that may have the ability to combat various diseases in humans (Van wyk et al., 2009; Van Wyk and Gericke, 2000).

Secondary metabolites found in plants have been medicinally used to combat various ailments in humans. Bioactive compounds such as alkaloids, flavonoids, tannins, and phenolic compounds are important raw materials for drug production (Bekele and Hazare, 2017). It has been well-documented that medicinal plants play a vital role in modern drug development (Shakya, 2016). Currently, about 32% of drugs introduced into the international market are derived from plant natural products (Bekele and Hazare, 2017; Kebede et al., 2021).

Fungal infections are a growing burden and threat worldwide, with over a billion people infected globally (Buil et al., 2020). Currently, the number of new antibiotics introduced on the market are derived from natural resources (Shriram et al., 2018; Kebede et al., 2021). Almost 1.6 million people die due to fungal infections (Oladele et al., 2020). In Africa, about 75% of 37 million immuno-compromised individuals are infected by fungal diseases (Oladele et al., 2020). Furthermore, there is an increase in the global rate of fungal infections due to an increasing population receiving immunosuppressive drugs (Buil et al., 2020). *Cryptococcus neoformans* is categorized as an opportunistic fungal pathogen that causes cryptococcosis. It is the common cause of morbidity and mortality in immune-compromised individuals, such as those living with HIV and AIDS worldwide (Bermas and Geddes-McAlister, 2020), while candidiasis and aspergillosis also have a significant contribution (Oladele et al., 2020).

Furthermore, screening for cryptococcosis is recommended in sub-Saharan Africa such as South Africa, Rwanda and Mozambique. These countries have begun with the implementation of cryptococcal screening programs. Since there is a challenge in the treatment of fungal infections due to drug-resistant strains, the development of new pathogens requirement of prolonged treatment management, and a limited variety of clinically effective, non-toxic antifungal treatment options (Wiederhold, 2017). More importantly, a good treatment success rate can be obtained for cryptococcosis among immune-suppressed patients if the symptoms are identified at an early stage. The options for treating fungal infections are extremely limited, only three classes of antifungal drugs are in use compared to ten classes of antibacterial (Montoya et al., 2020).

In this thesis, the study investigated the antifungal activity of medicinal plants used for the treatment of cryptococcosis. In Limpopo Province, the database of medicinal plants used to treat fungal infections is not well documented. Indigenous knowledge surrounding medicinal plants used for the treatment of fungal infections will be preserved and conserved for future generations. In addition, the qualitative and quantitative antioxidant activity of the selected medicinal plants was investigated. Isolation and structure elucidation of chemical structure, which can be used to develop new antifungal agents against *C. neoformans* was investigated. Finally, the cytotoxicity of crude and isolated compounds was studied. Therefore, the findings from the screening of medicinal plants could provide clues to the discovery of novel antifungal agents that can be used to combat fungal infections in humans and animals.

1.2 Rationale

Fungal infections are the leading causes of global morbidity and mortality especially in developing countries (Aumeeruddy-Elalfi et al., 2016). The increase in resistance of fungal pathogens to the currently available antifungal drugs is a major health problem for public health care. Treatment of fungal infections is complicated due to *Candida*, *Aspergillus* and *Cryptococcus* species that are resistant to the available antifungal agents (Sanguinetti et al., 2015). The infection occurs due to the inhalation of blastospores and basidiospores which create a pulmonary infection in humans (Samie et al., 2019). *Aspergillus fumigatus* causes aspergillosis in humans through inhalation of airborne asexual spores called conidia (Kim, 2016). *Candida albicans* cause

candidiasis in humans, and it is found in the oral cavity, the gastrointestinal tract, and the female genital tract (Schmiedel and Zimmerli, 2016). Currently, there are limited drugs such as polyene, itraconazole and fluconazole available to treat fungal infections. However, some of the antifungal drugs are expensive, toxic and have side effects (Zhang et al., 2020). Several antifungal agents such as Amphotericin B are clinically used; however, cure rates are still low, due to the high resistance of fungal pathogens to current drugs (Singh et al., 2016). Furthermore, it is difficult to find new antifungal agents due to resistant strain development among the fungal species against the existing antifungal drugs (Moghadamtousi et al., 2014; Vallabhaneni et al., 2016). Therefore, new antifungal drugs that are cheaper, effective, non-toxic and that are rapidly fungicidal are required to overcome these problems.

Medicinal plants used to treat fungal infections are assumed to be safe due to their long usage in traditional medicine. In particular, the bioactive compounds found in plants could possibly be used as a source of new antifungal agents. These compounds form the basis for drug discovery that may combat various diseases in humans and are commercialised across the world. Drug discovery from medicinal plants led to the isolation of drugs such as vinblastine, vinorelbine and vincristine. efinaconazole and tavaborole are also derived from natural products (Newman and Cragg, 2016). The use of natural products in drug discovery is a lead for finding new novel antifungal agents that may produce effective and curative agents for opportunistic fungal infections (Zhang et al., 2017). The fungal infection rate is increasing and poses burdens on global health care systems (Powers et al., 2018).

In South Africa, *C. neoformans* is an opportunistic fungus that causes cryptococcal meningitis (Miot et al., 2021). Recently, cryptococcal infection is the common cause of meningitis in individuals in developing countries and across the world (Rodrigues and Nosanchuk, 2020). Cryptococcosis is caused by the inhalation of aerosolized particles from the environment and occurs predominantly among immunosuppressed individuals (Mohammed et al 2019). It infects mainly the lungs and brain and later results in life-threatening meningitis (Santos-Gandelman; Machado-Silva, 2019). *Candida albicans* causes candidiasis in human. *Aspergillus fumigatus* causes aspergillosis in the cavities of the lungs in immune-compromised individuals (Liu et al., 2014). Medicinal plants are an effective source of traditional and modern medicines

that are used to treat various medical problems (Mtunzi et al., 2017). Furthermore, these plants contain bioactive compounds such as alkaloids, tannins, and flavonoids, which serve as drugs for various ailments caused by microorganisms (Ramadevi et al., 2016). Dimethylpyrrole and hydroxy-dihydro cornin-aglycones derived from plants are reported to have antifungal activities (Arif et al., 2009). Most azole drugs are fungistatic (Roemer and Krysan, 2014). Some plant extracts have shown notable antifungal activity against opportunistic fungal pathogens and are not toxic to human cells (Powers et al., 2018). Therefore, there is an urgent need to develop novel antifungals that are active with reduced side effects and toxicity to the host (Ravichandran et al., 2018).

Traditional medicine from plants is considered effective, with fewer side effects and more affordable than Western medicine (Shakya, 2016). The ethnobotanical investigation of plants used against fungal infections is not well investigated in Vhembe District. Few studies were conducted and focused only on candidiasis (Masevhe et al., 2015). Therefore, there is a high possibility of finding new bioactive compounds from medicinal plants based on therapeutic effects, then through random screening of natural resources (Ji et al., 2016).

1.3 Aim

The study aims to investigate plant species used for the treatment of cryptococcosis, antioxidant activity, isolate and characterise antifungal compounds, which could be used to develop new antifungal agents against *Cryptococcus neoformans*.

1.4 Objectives

The objectives of the study are to:

i. Select twelve plant species used to treat cryptococcosis from the database of Ethno-medicinal plant species for further phytochemical analysis, antioxidant activity and biological assays.
ii. Determine the antifungal activities of the selected plant extracts against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*.
iii. Isolate antifungal compounds from the most promising plant species and determine their chemical structure.

iv. Determine the qualitative and quantitative antioxidant activity of crude extracts and isolated compounds.
v. Determine the antifungal activity of crude extracts and isolated compounds.

1.5 Hypothesis

Ethnobotanically selected medicinal species have compounds that possess antifungal, and antioxidant activity and are less toxic with the ability to inhibit fungal growth.

1.6 Outline of the thesis

Chapter 1 is concerned with the general introduction to medicinal plants and fungal infections, problem statement, motivation, aim, objectives, and hypotheses of the study.

Chapter 2 dealt with a literature review on fungal infections, and fungal pathogens and a brief description of medicinal plans used in the study.

Chapter 3 focused on the extraction of plant species, and phytochemical analysis of the selected plant species. The materials and methods used to extract plant materials are also discussed. The results of extracted plant materials and phytochemical analysis of plant species are also discussed.

Chapter 4 is concerned with the preliminary screening of the selected plant species for antifungal activities against the tested fungal pathogens. Materials and methods such as serial dilution and bioautography assay are also given. The results for antifungal activity were also given.

Chapter 5 dealt with the qualitative and quantitative antioxidant activity of the plant species. The materials and methods for antioxidant activity are given. The results for antioxidant activity were also given.

Chapter 6 is concerned with the selection of plant species for further screening and isolation of bioactive compounds.

Chapter 7 focused on the antifungal activity of the selected plants for isolation. The serial exhaustive extraction and solvent-solvent fractions methods were given, and the results were discussed.

Chapter 8 dealt with the isolation of antifungal compounds using column chromatography. The methods and results were described in depth.

Chapter 9 is concerned with the structure elucidation of isolated compounds. The methodology was described, and results were given.

Chapter 10 dealt with antifungal activity, antioxidants, and cytotoxicity of the crude extracts and isolated compounds. The methods and results were discussed in depth.

Chapter 11 gives a conclusion that provides a summary of the study, and the conclusions were made based on the results obtained.

Chapter 12 dealt with the references used in this study.

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CHAPTER 2

LITERATURE REVIEW

2.1 Herbal medicine and drug development

Medicinal plants are an important source of bioactive molecules for drug discovery.
In developing countries, conventional drugs are expensive and scarce for the treatment of various ailments and also have side effects. Medicinal plants are a source of conventional and western medicines. Typically, medicinal plants are used due to their affordability and easily available. Furthermore, local people believe and trust the positive effects of using herbal medicine. In addition, plants play an important role as raw materials for the treatment of various human diseases, despite advances in the fields of synthetic drug chemistry and antibiotics (Jain et al., 2019). Furthermore, traditional medicine is used as a source of treatment methods around the world and has made great contributions to the development of modern medicine (Zhang et al., 2017). For this reason, the search for the active compounds presents in medicinal plants began in the nineteenth century leading to the conception of the first drug that we know today (Dutra et al., 2016). Medicinal plants have been used for a long time and many have shown to be effective by laboratory and clinical studies. Therefore, there is a high possibility of finding new bioactive compounds from medicinal plants based on therapeutic effects, then through random screening of natural resources (Ji et al., 2016).

2.2 Fungal infections

Infectious diseases are the main cause of morbidity and mortality worldwide, particularly in developing countries (Mtunzi et al., 2017). Fungal species especially *Cryptococcus, Candida* and *Aspergillus* cause infections with mortality rates exceeding 50%. Despite the currently available antifungal drugs the amount is still increasing (Van Daele et al., 2019). Fungal infections in humans range from common, mild, and superficial infections to life-threatening infections, especially in immune-compromised individuals (Pathakumari et al., 2020). Immunosuppressed people such as HIV patients, premature babies and cancer patients are more susceptible to fungal infections than immunocompetent individuals (Spellberg, 2011)

2.3 Antifungal resistance

Fungal infections are treated using Amphotericin B, and azole compounds such as fluconazole and itraconazole. However, antifungal resistance is increasing with severe side effects associated with these antifungal agents (Pfaller et al., 2013, Powers et al., 2018). The emergence of drug-resistant microorganisms poses threat to the health care system (Mtunzi et al., 2017). Therefore, there is a concern about the mechanisms of resistance to commercial drugs developed by fungal pathogens (Xie et al., 2014; Morais-Braga et al., 2017). There is a limited number of antifungal classes currently available (Morais-Braga et al., 2017). Furthermore, these drugs consist of four classes of antifungal drugs; namely, polyenes, azoles, echinocandins and flucytosine (Van Daele et al., 2019).

2.3.1 Polyenes

Amphotericin B (AmB) is a polyene that acts by binding to ergosterol, the component of fungal cell membrane disintegrates causing leakage of intracellular compounds leading to fungal cell death Figure 2.1 (Bermas and Geddes-McAlister, 2020). AmB is being used for the treatment of fungal infections since it has a broad spectrum of fungicidal activity against *C. neoformans.* Despite its fungicidal activity, AmB has side effects on humans, such as toxicity, and chronic nephrotoxicity due to the interaction with a membrane containing human cholesterol. Despite the side effects, the use of AmB also presents challenges, limited availability, and mode of delivery and AmB administration needs patients to be hospitalised to treat and minimize the toxic side effects, anaemia (Bahr et al., 2014). AmB remains unlicensed in 22 countries and unavailable in 42 countries, resulting in approximately 6.6% of the world’s population not having access to AmB (Kneale et al., 2016). As a result, the availability of antifungal drugs is limited due to their high cost, and insufficient registration in developing countries.

2.3.2 Azoles

Azoles are commonly used for the treatment of invasive fungal infections and are available as oral and intravenous formulations. Azoles include fluconazole, itraconazole, voriconazole and posaconazole. Azoles act by binding or blocking the synthesis of ergosterol through inhibition of lanosterol 14 α-demethylase enzyme

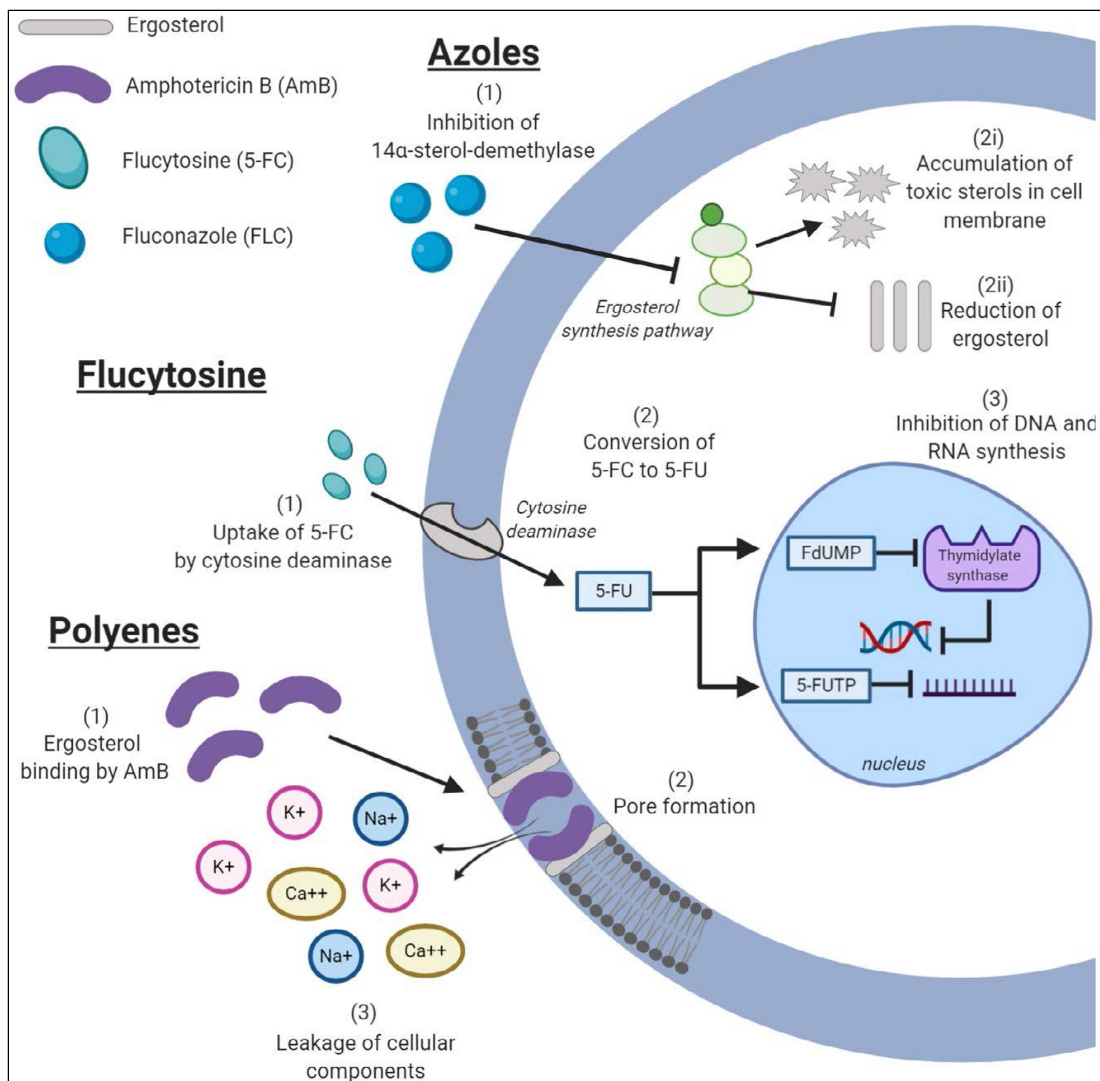
(Figure 2.1) (Van Daele et al., 2019). Despite the use of azoles in the treatment and prevention of *Aspergillosi*s, they also have side effects such as hazardous drug-drug interactions through interaction with the cytochrome p450 enzyme system, erratic absorption of the drug, and saturated pharmacokinetics and prolonged use of azole are associated with toxicity, skin cancer and the emergence of azole resistance in *Aspergillus fumigatus* species (Van Daele et al., 2019).

2.3.3 Echinocandins

The echinocandins class includes caspofungin, anidulafungin, and micafungin and they act by synthesizing the fungal cell wall by inhibiting the fungal 1,3- β- glucan synthase. Echinocandins have very few clinically significant drug-drug interactions and display a very favourable tolerability and toxicity profile. The drugs from the echinocandins class are recommended for the treatment of candidiasis. Unfortunately, these medications are available only as daily intravenous treatment (Van Daele et al., 2019).

2.3.4 Flucytosine

Flucytosine is mostly used to treat Cryptococcal meningitis and is often used in combination with polyenes. Its side effects include bone marrow suppression. Flucytosine interferes with nucleic acid synthesis but is not often used as monotherapy because it is prone to drug resistance (Van Daele et al., 2019).



(Bermas and Geddes-McAlister, 2020)

Figure 2.1 Mechanism of antifungal resistance.

2.4 Plants as antioxidants

Plants with antioxidant activity may be of importance for disease prevention (Alam et al., 2013). Ingesting plants rich in antioxidants can improve healthy life and reduce the risk of degenerative diseases (Do et al., 2014). Plants that possess antioxidants such as flavonoids and vitamin C can improve the body's immune function (Khodadadi, 2015). However, some plant extracts and plant-derived products are related to the inactivation of free radicals and therefore have antioxidant potential (Mtunzi et al., 2017). In addition, antioxidant compounds play a vital role in protecting cells from free radicals and decreasing the oxidative damage of molecular compounds such as lipids, proteins, and nucleic acids.

Antioxidant compounds such as flavonoids, flavones, lignans and isocatechins play an important role in the inhibition of free radicals and oxidative chain reactions within tissues and membranes (Boligon et al., 2012). The active antioxidant compounds possess anti-inflammatory, antitumor, anti-mutagenic, anti-carcinogenic, antifungal, and antibacterial activities (Nazir and Rahman, 2018). Therefore, natural antioxidants from plants are required to cure the disorders caused by free radicals.

Superoxide, hydroxyl, and nitric oxide radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can damage the DNA and lead to the oxidation of lipids and proteins in cells (Xu et al., 2017). The antioxidant derived from medicinal plant materials are mainly polyphenols, carotenoids and vitamins (Baiano and Nobile, 2015). Therefore, the medicinal and nutritional properties present in plants make them a great source of antioxidants. This suggests that a variety of plant bioactive compounds such as flavonoids, sterols, alkaloids and carotenoids possess antioxidant activities (Saranya et al., 2017).

Free radicals are important biochemical development and signify a fundamental part of life and metabolism. Free radicals maybe either oxygen derived which is ROS or nitrogen derived which is RNS. However, they are regarded as a common cause of diseases in humans, such as cell death, cardiovascular disease, tissue damage, ischemic heart diseases, cancer, central nervous system injury, inflammation, obesity, gastritis, arthritis, and aging (Kose et al., 2015). Since various ailments are linked to oxidative stress caused by free radicals. It has been reported that natural antioxidants from plants are capable of defending against the effects of free radicals and showed a broad range of pharmacological activities such as antimutagenic, antimicrobial, antioxidant, and antidiabetic amongst others (Shori, 2015).

Antioxidants can be classified into three types that are primary antioxidants which are involved in the chain-breaking process and become stable components when reacted with lipid radicals (Flieger et al., 2021). Secondary antioxidants are phenolic compounds that stop the chain reactions of free radicals. Furthermore, tertiary antioxidants are involved in the repair of biomolecules damaged by free radicals such as repairing DNA enzymes (Flieger et al., 2021). Antioxidants are further categorised into two groups, enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants

are involved directly or indirectly in fighting against ROS in the body and non-enzymatic antioxidants are acquired from food as a source and include polyphenols, vitamins, carotenoids, and minerals. Furthermore, polyphenols include phenolic acids and flavonoids and are the largest class of antioxidants (Bunaciu et al., 2016).

2.5 Importance of medicinal plants

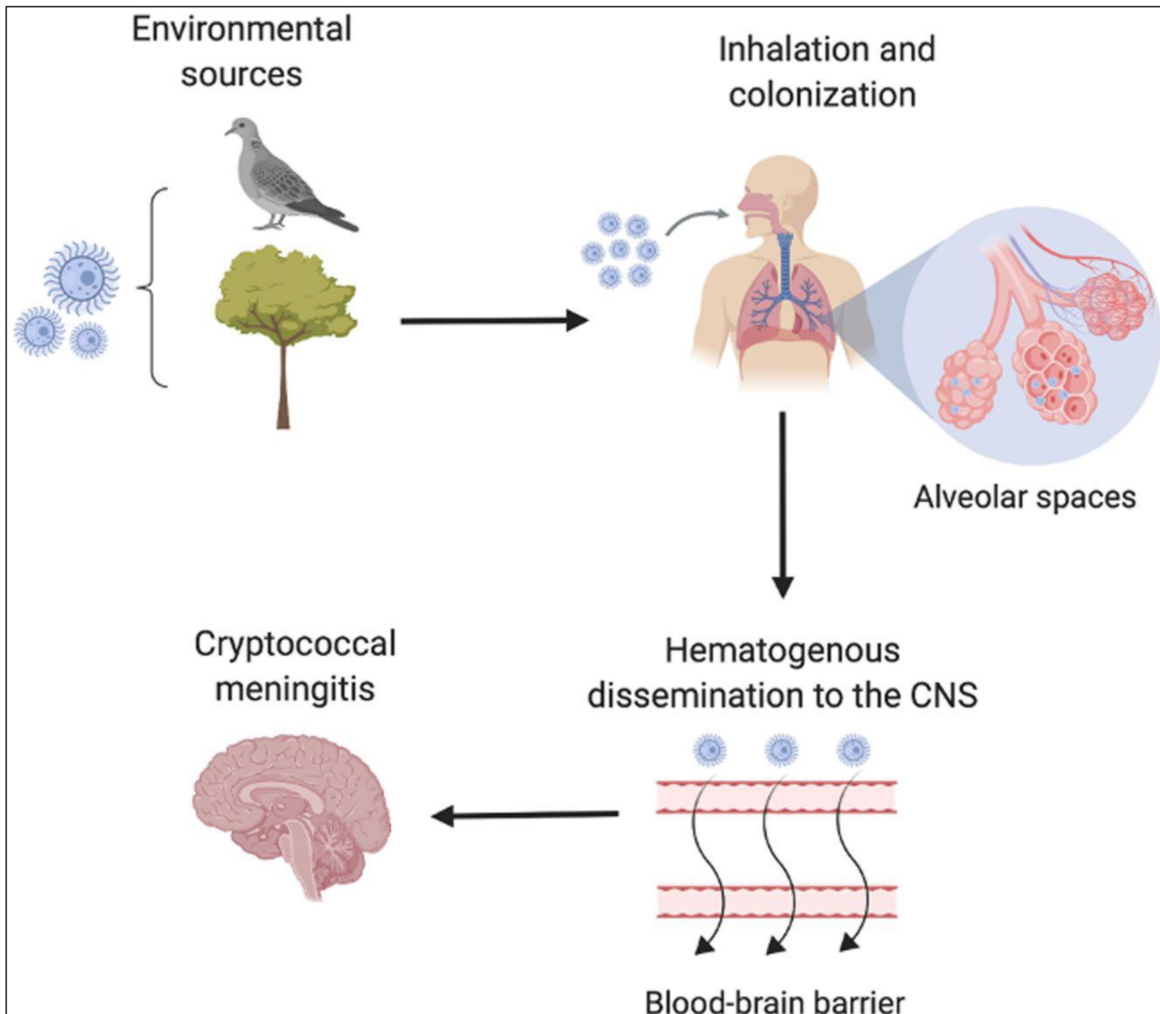
A medicinal plant is any plant that has components that can be used as a therapeutic agent to treat various ailments (Mtunzi et al., 2017; Jain et al., 2019). Furthermore, medicinal plant extracts have been used for the maintenance of health and treatment of diseases such as skin diseases, and diabetes (Mahlo and Shikwambana, 2020). About 80% of people in developing countries rely on medicinal plants for primary health care. In addition, almost 25% of modern medicines in developed countries are derived from plants (Chen et al., 2016). Approximately 14-28 % of higher plant species are used as medicine and about 74 % of plant-derived compounds were discovered after conducting ethnomedicinal uses of plants (Ncube et al., 2008). In South Africa, medicinal plants are used to treat various ailments including infectious and non-infectious diseases (Mtunzi et al., 2017). Healing activities such as antioxidants, antidiarrheal, and antimicrobial have been reported from plant extracts (Sasidharan et al., 2011). Therefore, plant extracts can be a reliable source to find new compounds against pathogens.

2.6 Animal fungal pathogens used in this study

2.6.1 *Cryptococcus neoformans*

Cryptococcus neoformans is an opportunistic fungal pathogen that causes *Cryptoccocca*l meningitis worldwide (Bermas and Geddes-McAlister, 2020). *Cryptococcosis* is an infection caused by pathogens from the genus Cryptococcus. There are only two species (*C. neoformans* and *C. gatti*) from thirty species of Cryptococcus that are commonly pathogenic in humans. *Cryptococcus neoformans* cause cryptococcosis (Powers et al., 2018). It is acquired through the inhalation of aerosolised particles from the environment distributed and exists in high concentrations in pigeons and chickens as illustrated in Figure 2.2 (Mohammed et al., 2019). *Cryptococcosis* is diagnosed by the isolation of *C. neoformans* from a sterile site and the detection of *Cryptococcal* capsular antigen (Mohammed et al., 2019). Approximately 15 % of AIDS-related death are caused by *Cryptococcosi*s worldwide,

however, the majority of death occur in sub-Saharan Africa (Rajasingham et al., 2017). *Cryptococcal* infections account for approximately 63% of meningitis cases in South Africa, due to the high rate of HIV/AIDS (Jarvis et al., 2010).



(Bermas and Geddes-McAlister, 2020)

Figure 2.2 Infection cycle of *Cryptococcus neoformans*.

2.6.2 *Aspergillus fumigatus*

Aspergillus species are opportunistic fungal pathogens that are filamentous and found in the environment. *Aspergillus fumigatus* causes pulmonary aspergillosis in humans. These fungal pathogens are spread by asexual sporulation. Furthermore, it reproduces mainly by asexual means and parasexual cycle resulting from hyphal fusion and inhalation of airborne conidia which are present indoor and outdoor environments. *Aspergillus* species are capable of causing a wide range of infections in humans, such as hypersensitivity reactions, chronic pulmonary infections, and acute

life-threatening infections, especially in immunocompromised individuals (Hoenigl et al., 2018). Of the 40 known *Aspergillus* species, *A. fumigatus* is the most common cause of disease in humans (Jenks and Hoenigl, 2018).

Aspergillosis is primarily found in the lungs, though it may spread to other body organs. Furthermore, the symptoms and signs of *Aspergillosis* depend on the organs affected. The symptoms include cough, chest pain, and skin lesions. However, it is not easy to diagnose *Aspergillosis* through these signs and symptoms, since they may be similar to other ailments (Jenks and Hoenigl, 2018). This result in a delay to diagnose *Aspergillosis*, therefore, it remains associated with a high mortality rate of around 30-40% (Lewis et al., 2013).

2.6.3 *Candida albicans*

Candida albicans is a fungus belonging to the genus candida and can cause *Candidiasis* in humans (Khan et al., 2018). *Candida* infections affect the oropharynx and oesophagus leading to dysfunction in the adaptive immune system. *C. albicans* is a polymorphic fungus and can either grow into ovoid-shaped budding yeast at low pH (<6) and as parallel-walled true hyphae at high pH (>7) (Sawant and Khan, 2017). *Candida albicans* is the leading cause of all healthcare-associated bloodstream infections, with a mortality rate of approximately 40% despite the use of antifungal drugs (Pfaller et al., 2019). *Candida* species are the leading pathogens accountable for nosocomial bloodstream infection with 50% caused by *C. albicans*. As a result, *C. albicans* is an opportunistic fungal infection causing the disease in immune-compromised patients (Ghaddar et al., 2020). Currently, antifungal agents (azole antifungal compounds) are used due to their lower toxicity and perfect efficacy to treat *Candida* infections. However, the long usage of azoles has led to the development of drug resistance in *C. albicans* and other species (Khan et al., 2018). Nonetheless, the fungal agent Amp B is important in the treatment of *Candidiasis* though it is associated with a high rate of toxicity and side effects such as diarrhoea, nausea, vomiting and nephrotoxicity (Hoenigl et al., 2018).

2.7 Plant secondary metabolites

Secondary metabolites are organic compounds from different plant parts that interact with their environment and organisms (Oksma-caldentey and Barz, 2002). Furthermore, these metabolites are metabolic intermediates, which are found as differentiation products in restricted taxa. They are not crucial for the growth and life of production organisms and are biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism (Pagare et al., 2015). The plants produce primary and secondary metabolites comprised of a variety of functions. Secondary metabolites from plants are used by humans as a source of antimicrobial agents since they are suggested to have the ability to inhibit the growth of various microorganisms (Kilonzo et al., 2017). There are three major classes of plant chemicals: terpenoids which is a derivatives of terpenes, phenolic metabolites, and alkaloids (Do et al., 2014). Secondary metabolites belonging to alkaloids, flavonoids and terpenoids are used as drugs for the treatment of various ailments (Pagare et al., 2015).

2.7.1 Terpenes

Terpenes are a class of secondary metabolites having more than 30000 known structures (Singh and Sharma, 2015). Terpenes are responsible for the fragrance, taste, and pigment of the plant (Cox-Georgian et al., 2019). They are classified according to the number of isoprene units they possess. Furthermore, terpenes are classified into classes: hemiterpene, monoterpenes, sesquiterpenes and diterpenes. These classes possess biologically active compounds to cure various ailments namely: antimicrobial, anticancer, antimalarial, anti-inflammatory, and insecticidal activities amongst others (Alves et al., 2013). The compounds from monoterpenes are used by pharmaceutical companies since they usually have a strong aroma and odour. Different monoterpenes oils are mixed, and the fragrance is used to make perfumes and other cosmetics. Diterpenes are a source of anticancer drugs such as Taxol (Vasas and Hohmann, 2014).

2.7.2 Alkaloids

Alkaloids are a naturally occurring class of secondary metabolites having diversified compounds possessing nitrogen atoms and are derived from amino acids (Kaur and Arona, 2015). About 3000 alkaloid compounds are known from over 4000 different

plant species. Furthermore, flowering plants are regarded as the main source of alkaloid compounds production (Kurek, 2019). Alkaloids have been used as a source of medicinal agents since they have shown defense mechanisms against pathogenic microbes (Kilonzo et al., 2017). In addition, pharmaceutical properties such as antibacterial, anti-inflammatory, and anti-tumour activities are found in alkaloids amongst others (Kurek, 2019).

2.7.3 Phenolic compounds

Phenolic compounds are secondary metabolites containing benzene rings, with hydroxyl substituents. Phenolic compounds have antioxidant activity which plays a vital role in primary healthcare (Lin et al., 2016). Furthermore, different aspects of the structure of saponins enhance the biological activities found in saponins (Hussain et al., 2019). Phenolic compounds are divided into three categories namely: flavonoids, tannins and saponins (Morales, 2013).

Flavonoids are widely distributed throughout the plant kingdom. Flavonoid compounds possess various biological activities such as antioxidant, anti-inflammatory, antibacterial and antifungal activities (Ceric et al., 2013). Tannins are found in all plant parts and are astringent and have medicinal properties that enable them to inhibit microbial growth causing intestinal disorders and skin diseases (Kilonzo et al., 2017). Saponins are secondary metabolites produced from plant species used traditionally as medicinal to treat various diseases. They are the main constituents found in various plants which are used worldwide as traditional medicines. Saponins are reported to have antibacterial, antifungal, and antiviral activities (Dini et al., 2001).

2.8 Fungal infections and HIV and AIDS

There is an increase in fungal infection globally with increased immunosuppressive infection. However, *Candidiasis* is being reported as the most dominant fungal infection among others (Santosh et al., 2021). Immunocompromised patients especially those with HIV and AIDS have contributed to a high number of fungal infections. Furthermore, approximately 181 000 deaths are concentrated in sub-Saharan Africa due to *Cryptococcal* meningitis (Rajasinghan et al., 2017). The compromised HIV patients with less than 200/mm^3 CD4+ cell counts are at high risk of being infected with fungal infections. Since fungi are opportunistic pathogens

affecting HIV and AIDS individuals. Despite the availability of anti-HIV drugs, fungal infections are still a burden and cause of concern to people living with HIV and AIDS (Rodrigues and Nosanchuk, 2021).

2.9 Fungal infections and COVID-19

The pandemic Covid-19 infection is a contagious disease caused by SARS-CoV-2 and it is regarded as the cause of mortality worldwide (Zhou et al., 2020). The infection has caused great health and public impact in the world since 2019. There is an increase of fungal infections in covid-19 positive patients with other comorbidities such as diabetes, mechanical ventilation, and cytokine storm. The fungi that colonize the respiratory tract and oropharyngeal mucous membrane (*Aspergillus* and *Candida*) species are the most associated with covid-19 infections (Silva et al., 2020). Despite the increase in fungal infections in Covid-19 patients, there is a low rate of fungal infections in hospitalised covid-19 patients (Lansbury et al., 2020). Furthermore, the antifungals were not used widely despite the hospitalisation of Covid-19 patients. Since the treatment of fungal infections is cost-effective for both the public and private sectors annually (Silva et al., 2020).

2.10 Toxicity of medicinal plants

Misidentification, faulty collection, and preparation are the main problems that reduce the effectiveness of medicinal plants (Sen and Chakraborty, 2017). Some of the chemotherapeutic agents currently used are toxic and have side effects. Therefore, there is a need to search for new-infective and chemotherapeutic agents against infectious pathogens, which are highly effective, low toxicity and with minor environmental impact (Mtunzi et al., 2017). Based on antimicrobial studies, some plants have therapeutic potential with fewer side effects than synthetic antibiotics (Ahmad et al., 2012).

Secondary metabolites found in plants contain toxic substances (Mounanga et al., 2015). The toxicity of a particular plant depends on various factors such as the toxicity of the secondary metabolites, the quantity of the plant extract administered, different plant parts used, climate and soil and differences between species (Tulay, 2012). The toxicity of the plant extracts should be investigated using both *in-vitro* and *in-vivo*

models (Mounanga et al., 2015). The use of water extracts to treat various ailments had lower toxicity than the organic solvent extracts.

2.11 Conservation of medicinal plants

Conservation of medicinal plants is important to ensure the availability of medicinal plants in the future. The ethnopharmacological guide is playing an important role in the trading of medicinal plants for the treatment of different diseases. However, the sustainable use and management of these plants are a challenge to all users. The plant parts such as stem, bark and roots of medicinal plants are being harvested in an unsustainable way that may lead to the extinction of plants that are used as a source of medicine. Therefore, it is important to practice sustainable use and good field collection of medicinal plants. Consequently, leaves were investigated as a sustainable resource. Validation and evaluation of the biological activities of the leaf extracts as a possible substitute may provide a viable option for the conservation of medicinal plants (Mtunzi et al., 2017). About 15000 species are threatened with extinction due to overharvesting and habitat destruction (Singh et al., 2019). In South Africa, there is a limited supply of medicinal plants since there is a high demand and a large population (Chen et al., 2016).

2.12 Description of selected medicinal plants used in the study

2.12.1 *Berchemia discolor*

Berchemia discolor is a shrub or tree distributed in Africa characterised by edible fruits and its wood makes charcoal, building material, furniture, and crafts. It is also used as a dye, fodder, ornamental tree, and herbal medicine to treat several human and animal diseases (Cheikhyoussef and Maroyi, 2017). *B. discolor* is commonly known as brown ivory or bird plum, belonging to the family Rhamnaceae. It is a medium to large tree that can grow up to 20 M tall (Figure 2.3). The leaves are opposite to each other, dark green above and paler below. The fruits are fleshy yellow when ripe and edible. The followers are yellowish-green on pedicels. *B. discolor* is an indigenous fruit tree species in Southern Africa. The plant is growing mostly in the northern parts of South Africa, and it is drought tolerant. It is fast-growing with dense shade. The dry fruit pulps are rich in carbohydrates, calcium, sodium iron and potassium. The fruits are green when unripe, become pale yellow when ripe and when dried fruits are dark brown (Van

Wyk, 1972). *B. discolor* is used to treat infertility and menorrhagia (Arnold and Gulumian, 1984).

Figure 2.3 *Berchemia discolor (Munie)* A (whole plant) B (dried fruits).

2.12.2 *Kleinia longiflora*

Kleinia longiflora is a succulent shrub belonging to the family Asteraceae. Its synonym is *Senecio longiflorus* DC Sch.Bip. The shrub can grow up to 1 M tall, it has small fleshy leaves (Figure 2.4). *Kleinia longiflora* is distributed in South Africa, Botswana, and Swaziland. The plant is used medicinally to treat toothaches; chest pains skin diseases and tuberculosis (Semenya and Maroyi, 2019). The root decoction is taken orally to treat STIs (Kumar et al., 2015). Antimicrobial and antioxidant activities were reported for the first time (Asong et al., 2019). Isolated compounds from *Kleinia longiflora* include Lupeol, heptacosane and Dotriacontane (Asong et al., 2019).

Figure 2.4 *Kleinia longiflora (Muvhale).*

2.12.3 *Persia americana*

Persia americana is an evergreen tree of 9-20 M in height from the family Lauraceae and commonly known as avocado, alligator pear and butter pear. It is native to Mexico, Central America and South America (Chen et al., 2009). The fruit of avocado is a berry comprised of a large central seed and pericarp (Figure 2.5). The leaves of avocados vary in shape, they are elliptic, oval and lanceolate. The leaves are reddish and pubescent when young and smooth, leathery, and dark green at maturity.

Persea americana is used traditionally for the treatment of hypertension, anaemia, gastritis, gastroduodenal ulcers, and arthritis. Avocado leaves have been used to treat different ailments including hypertensive, sore throat, diarrhoea, and haemorrhage

(Adeyemi et al., 2002). The seeds are used to treat a variety of infections caused by fungi and bacteria.

P. americana leaves are rich in bioactive compounds such as phenolics, which are natural antioxidants. The leaves contain alkaloids, flavonoids, saponins, tannins and steroids. The plant has shown antifungal activity against *Candida* species and *C. neoformans* from avocado seeds (Leite et al., 2009). However, the leaf extracts have shown fungistatic activity against *C. albicans* and *C. tropicalis* (Ajayi et al., 2017). The ethanol extract of *P. americana* has been reported to lower blood pressure and have antioxidant activity (Owolabi et al., 2010). Furthermore, the flavonoid compounds luteolin, rutin, quercetin, and apigenin were identified (Owolabi et al., 2010).

Figure 2.5 *Persea americana* (*Muafukhada*)A (whole plant) B (fruits).

2.12.4 *Sansevieria hyacinthoides*

Sansevieria hyacinthoides (L.) Druce belongs to the family Asparagaceae and its synonym is *Dracaena hyacinthoides* (L.) Mabb. It is commonly known as mother-in-law. It is native to East, Central and Southern Africa. It is a succulent evergreen and stem less herb which grows up to 60cm in height (Figure 2.6). The leaves are flat and erect arising from the horizontal underground rhizome (Maroyi, 2019). The plant is grown in home gardens in South Africa as an ornamental, medicinal and spiritual plant (Sultana et al., 2011). Different plant parts are used for medicinal purposes to treat various ailments such as sexually transmitted diseases, headaches, earaches, infertility, and respiratory problems amongst others (Maroyi, 2019). The roots and

leaves of *S. hyacinthoides* are used to treat burns and wounds and the infusion of powdered leaves and roots is taken orally to treat diarrhoea (Maroyi and Mosina, 2014). The leaves and roots of *Sansevieria hyacinthoides* are used as herbal medicine for the treatment of ear infections, hemorrhoids, and skin ulcers in South Africa (Van Wyk, 2011). It was reported that *S. hyacinthoides* have anthelmintic, antibacterial, antifungal and antioxidant activities (Sultana et al., 2011).

Figure 2.6 *Sansevieria hyacinthoides (Savha)*.

2.12.5 *Dichrostachys cinerea*

Dichrostachys cinerea is a tree or shrub belonging to the family Mimosaceae, it is commonly known as a sickle bush. The leaves are soft and bipinnately compounds (Figure 2.7). The flowers are spikes that are pink on the top part and yellow below. The fruits are pods that are intertwined and twisted clusters. The plant is used traditionally to treat different ailments such as body pain, backache, toothache, and syphilis. The fruits and bark were reported to treat various ailments. The burnt fruits are applied to a wound for healing. The ground bark is mixed with oil to treat ringworm and the decoction of the bark is used to wash the wounds (Setshego et al., 2020).

The air-dried stem bark of *D. cinerea* is inhaled to treat airway affections. It has been reported to have antimicrobial, antiviral activity (Kambizi and Afolayan, 2001), antibacterial, nephroprotective, anti-diabetic, anti-inflammatory, antidiarrheal and antioxidant activities (Bolleddu et al., 2019).

Figure 2.7 *Dichrostachys cinerea* (*Murenzhe*) A (whole plant) B (pods) C (flowers).

2.12.6 *Withania somnifera*

Withania somnifera is a green shrub belonging to the family Solanaceae, found throughout the drier parts of developing countries including South Africa. The roots are long, short stems, ovate and petiolate leaves (Figure 2.8). It is used as a dietary supplement and the decoction of its root is used as a nutrient and health restorative to pregnant and old people. Furthermore, *W. somnifera* has been used for medicinal purposes to treat various diseases such as lowering inflammation, blood sugar levels, anxiety, and depression (Montalvan et al., 2015) and enhancement of mental and physical conditions (Lopresti and Smith, 2021). The crude water extracts of *W. somnifera* have been evaluated for cytotoxicity effects against the human malignant melanoma cells, A355 and have shown a significant reduction in cell viability (Halder et al., 2015).

Figure 2.8 *Withania somnifera (Musalamarubini)*.

2.12.7 *Momordica balsamina*

Momordica balsamina commonly known as Africa pumpkin is a climber from the family Cucurbitaceae and it is native to the tropical regions of Africa (Kaur et al., 2013). It is widespread in all the provinces of South Africa except the Western Cape Province. It is a perennial tendril-bearing herb (Figure 2.9).

It is a vegetable used as food and for medicinal purposes for the treatment of fever, malaria, and diabetes in Mozambique and South Africa (Ramalhete et al., 2010). The leaves of *M. balsamina* are used traditionally for the treatment of various ailments, such as ulcers of the stomach, inflammations, urinary tract infections, bile disorders, blood cleansers and fever. It is administered orally in the form of tea, which has a bitter taste. The tea is taken orally by young girls to reduce and relieve period pains. It is also taken by mothers of newborn babies to increase milk production (Thakur et al., 2009).

M. balsamina had shown antimalarial activity, anti- hypoglycemic activity, and anti-HIV activity (Kaur et al., 2013). However, it was previously revealed very weak toxicity *in vivo* studies (Ramalhete et al., 2010).

Figure 2.9 *Momordica balsamina* (*Tshibavhe*) A (whole plant) B (fruit and flowers).

2.12.8 *Lonchocarpus capassa*

Lonchocarpus capassa is a tree that grows up to 15 M tall belonging to the family Fabaceae. The tree is drought tolerant, distributed in Eastern Cape, Kwazulu natal, Mpumalanga and Limpopo. The bark is grey, and the leaves are large and rough in texture (Figure 2.10). The plant is used medicinally to treat various diseases. The bark infusion is used to treat diarrhoea, colds, snake bites, intestinal problems, and hookworm, and the burnt root smoke is inhaled to treat colds (Kilonzo et al., 2016). The fine powder of stem bark of *L. capassa* is mixed with porridge for the treatment of wounds (Moshi et al., 2006). The root bark extracts are used to treat stomach aches and hookworm (Kokwaro, 1993). The antifungal activity of the leaf extracts was reported by Kilonzo et al. (2016).

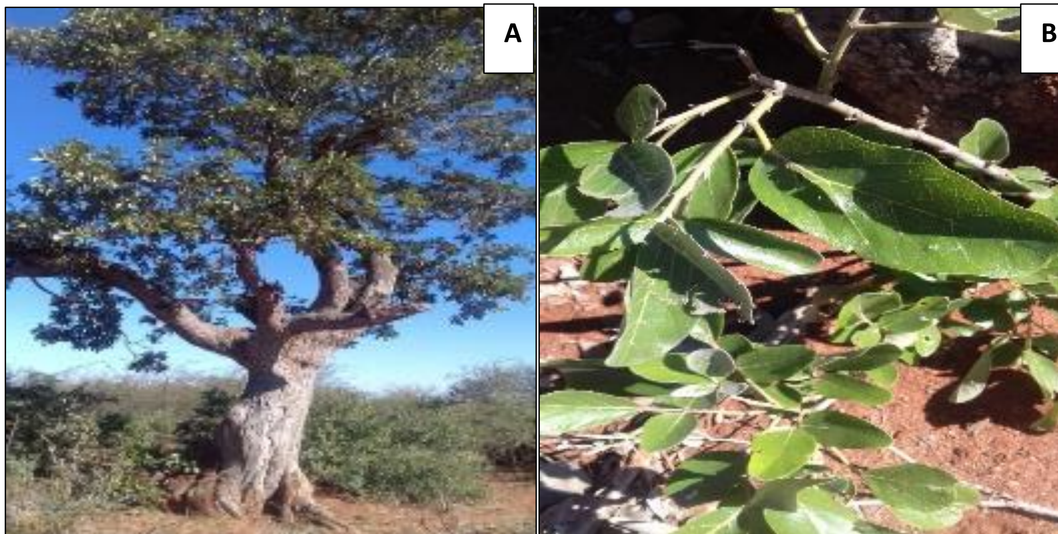


Figure 2.10 *Lonchocarpus capassa (Mufhanda)* A (whole plant) B (leaves).

2.12.9 *Pappea capensis*

Pappea capensis is a tree commonly known as jacket plum belonging to the family Sapindaceae. Its leaves are crowded at the tips of the branches (Figure 2.11). *Pappea capensis* is distributed throughout South Africa from Northern Cape to Kwazulu-Natal (Van Wyk and Gericke, 2000).

The infusion of the root bark is taken orally to treat chronic joint pains (Wambugu et al., 2011). The plant is used traditionally to enhance fertility, and stomach-ache and is also used as a tonic (Mhlongo and Van Wyk, 2019), is used to treat skin infections, and the seed kernel oil is applied topically to treat ringworm (Cock and Van Vuuren, 2020). The leaf extracts are used to treat eye infections and sexually transmitted diseases (Hutchings et al., 1996). The leaves have shown the anti-inflammatory activity of conditions related to venereal diseases (Mulaudzi et al., 2013). *Pappea capensis* showed strong antioxidant activity against DPPH (Ngai et al., 2019). Extracts and fractions of *P. capensis* exhibited good antifungal activity against *C. albicans* (Pendota et al., 2017).

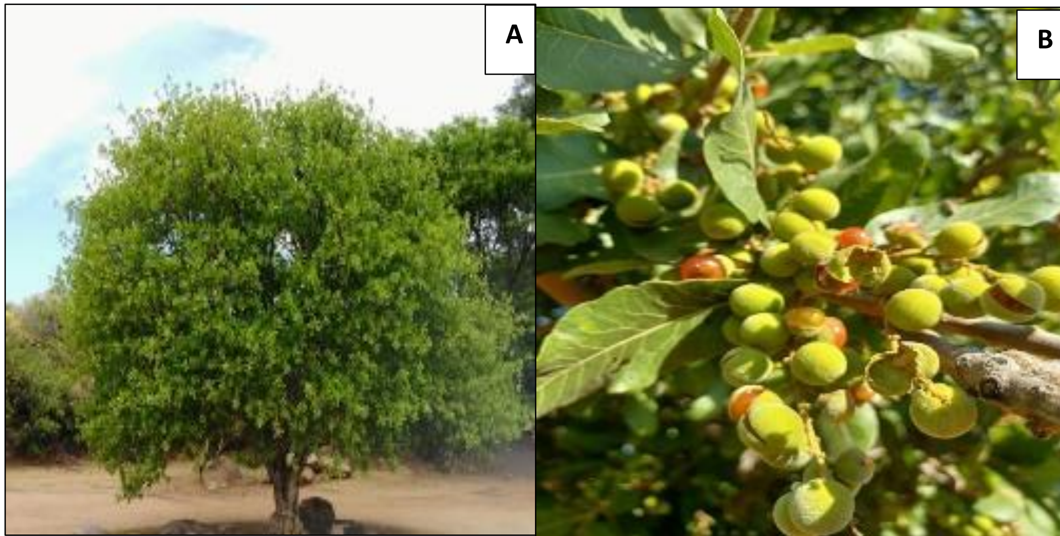


Figure 2.11 *Pappea capensis (Muphinyu*) A (whole plant) B (fruits).

2.12.10 *Searsia lancea*

Searsia lancea previously known as *Rhus lancea* and commonly known as Karree is a small to medium-sized evergreen tree belonging to the family Anacardiaceae. The leaves are alternative spirally arranged lanceolate leaves (Figure 2.12). The bark of *S. lancea* is reddish-brown when young and dark brown in older branches. The followers are yellow, and the fruit is small and round, dull yellow to brown, and slightly flattened (Van Wyk et al., 2008). The tree is native to South Africa. *S. lancea* is used traditionally to treat various ailments. The crushed leaves are mixed with water to clean the skin and for the treatment of measles and rashes (Mahlo and Machaba, 2020). The decoction of leaves is used to remove the pimples (Setshego et al., 2020). The leaves are used to treat diabetes, headaches, fever, and colds (Kose et al., 2015). The plant has been reported to have antifungal, antibacterial, anti-inflammatory and antioxidant activities (Mulaudzi et al., 2012).

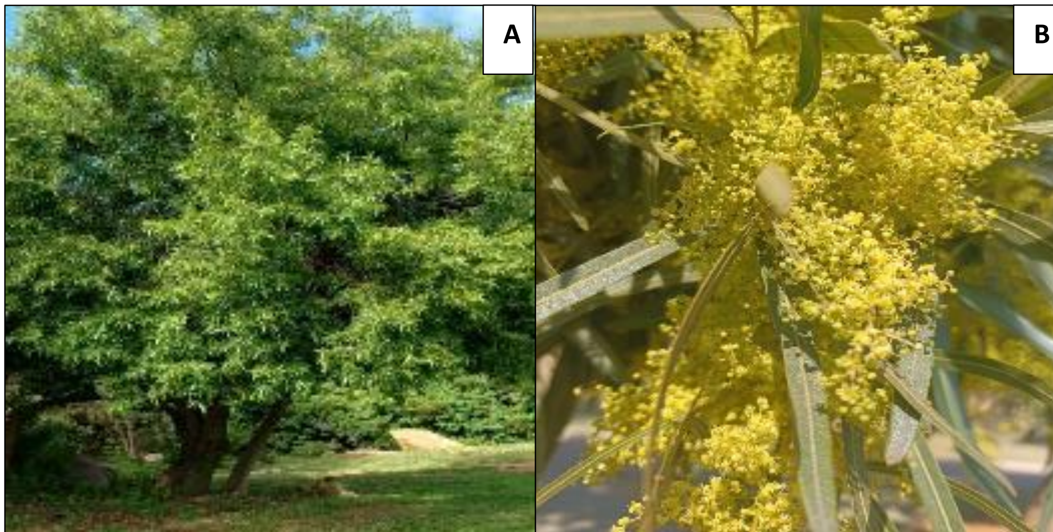


Figure 2.12 *Searsia lancea (Mushakaladza)* A (whole plant) B (flowers).

2.12.11 *Peltophorum africanum*

Peltophorum africanum is a tree that grows up to 5-15 M tall (Figure 2.13). It belongs to the family Fabaceae, and it is commonly known as a weeping wattle. The flowers are yellow, the stem is grey and has pods that are flat and winged (Van Wyk and Van Wyk, 1997). The plant is used traditionally to treat different ailments such as blood cleaning (Maema et al., 2016), tuberculosis, stomach complaints and intestinal parasites (Anochie et al., 2018). Decoction of the bark is used to treat mouth sores (Setshego et al., 2020). The stem bark and root extract of *P. africanum* are used to treat venereal diseases (Van Wyk and Gericke, 2007). The extracts of *P. africaum* are used to treat ailments such as coughs, diarrhoea, painful tooth, sore throat, backache, and fever amongst others. The genus *Peltophorum* is well known for its antibacterial, antimicrobial, and antioxidant activities (Ahmed et al., 2012).

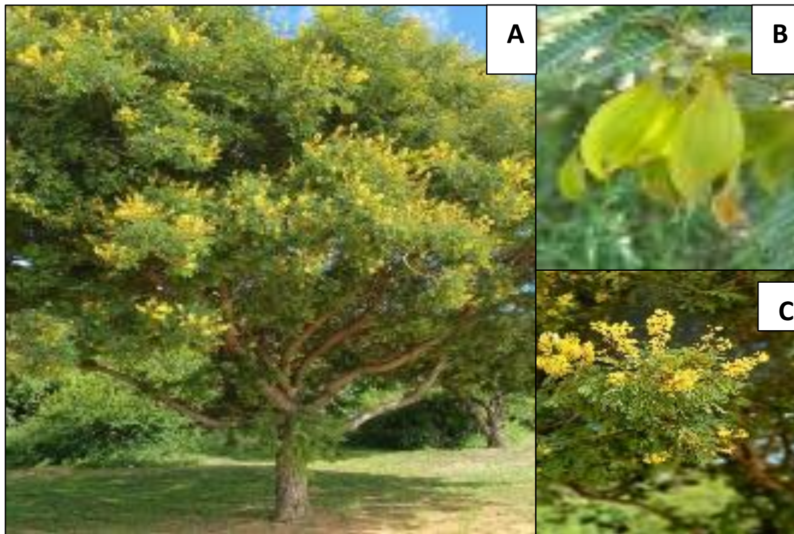


Figure 2.13 *Peltophorum africanum (Musese)* A (whole plant) B (pods) C (flowers).

2.12.12 *Maytenus heterophylla*

Maytenus heterophylla is an evergreen shrub, a tree belonging to the family Celastraceae, which grows up to 9 M high, with green to brown spines up to 24 cm long, glabrous with young branches lined (Figure 2.14). The leaves are alternative and petioled, with dense flowers. It is mainly distributed on the east coast of Africa.

Maytenus heterophylla is used traditionally as an antimicrobial and anti-inflammatory agent. It is used in primary healthcare to treat respiratory ailments and inflammation, snake bites, wounds, and dysentery (Da Silva et al., 2011; Umar et al., 2019). Different secondary metabolites have been identified, flavonoids, alkaloids, terpenoids and triterpenes (Umar et al., 2019). Flavonoid fractions of *M. heterophylla* showed antioxidant activity. Ethanol extracts of *M. heterophylla* exhibited significant anti-inflammatory activity (Da Silva et al., 2011).

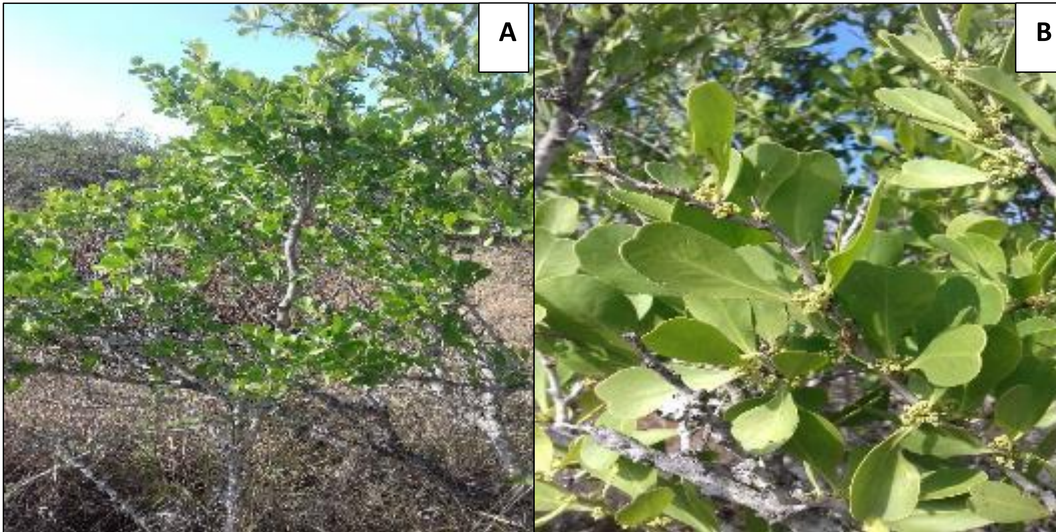


Figure 2.14 *Maytenus heterophylla (Tshiphandwa)* A (whole plant) B (leaves).

CHAPTER 3

PLANT EXTRACTION AND PHYTOCHEMICAL ANALYSIS OF THE SELECTED PLANT SPECIES

3.1 Introduction

Medicinal plants have been used in different countries as sources of medicine. The use of medicinal plants is practised by people residing in both rural and urban areas (Van Vuuren et al., 2014). Almost, 80% of people in developing countries depend on medicinal plants for their primary health care since they are easily accessible, affordable, available, and acceptable (Asgarpanah et al., 2017; Alfa et al., 2018). In South Africa, approximately 3000 medicinal plants are used by traditional health practitioners for healing practices (Van Wyk et al., 2009).

The ability of medicinal plants to cure various ailments is due to various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, and saponins amongst others available in plants (Kadirvelmurugan et al., 2017). Phenolics are responsible for the antioxidant activities of many plant products. Secondary metabolites such as terpenoids, alkaloids and flavonoids are used as drugs and dietary supplements to cure various ailments (Pagare et al., 2015). Therefore, medicinal plants are a potential source for the development of new therapies.

Extraction is the separation of chemical components using selective solvents through standard procedures (Azwanida, 2015). The main aim of the extraction process is to separate the soluble plant metabolites leaving behind the insoluble cellular residue. The bioactive compounds from medicinal plants depend on the proper selection of extraction methods and solvents used during extraction (Sasidharan et al., 2011). During the extraction of medicinal plants, it is important to select solvents of varying polarities to extract various compounds. For example, hexane will extract non-polar compounds and methanol polar compounds. In some instances, hexane is used to remove chlorophyll (Cos et al., 2006). Furthermore, the polar solvents extract phenolic compounds and non-polar extracts of fatty acids and steroids (Dirar et al., 2019). Therefore, the choice of extraction solvents and techniques has an impact on the pharmacological activity of the extracts. Traditional health practitioners use water for

extraction since they do not have access to organic solvents. Water is readily available, cheap, and non-toxic since extraction with it does not generate harmful by-products (Pagare et al., 2015). Water is a polar solvent which makes it dissolve compounds of high polarity.

Phytochemical screening of medicinal plants plays a major role in the quantitative estimation and locating of active components (Suliman, 2018). More importantly, TLC fingerprinting plays a major role in assessing the quality and purity of medicinal plants due to its simplicity, cost-effectiveness and speed (Srivastava and Yadav, 2019). More so it is preferred since the cheapest and most simple method to detect natural product constituents (Nyired and Glowhck, 2001). It was reported that the TLC fingerprinting of medicinal plant extracts can be used for identification and quality control of medicinal plant preparation by retention factor (R_f) values and colour spots (Ahmed, 2012).

3.2 Materials and methods

3.2.1 Plant selection and collection

Twelve plant species *Kleinia longiflora*, *Persea americana*, *Sansevieria hyacinthoides*, *Dichrostachys cinerea*, *Withania somnifera*, *Momordica balsamina*, *Lonchocarpus capassa, Pappea capensis*, *Sersia lancea*, *Peltophorum africanum*, *Maytenus heterophylla* used to treat fungal infections were selected from the currently available database of sixty plants (Ethno-medicinal plants) in the Department of Biodiversity. The plant species were selected based on the treatment of fungal infections from ethnobotanical data and the availability of the plant species.

The plant species were collected from their natural habitat in Muduluni village, Makhado Local Municipality, Limpopo Province. The plants were stored in open mesh orange bags at room temperature of 25°C to ensure efficient drying of the material. Plants were identified at the Larry Leach herbarium at the University of Limpopo. Voucher specimens were prepared and deposited at the Larry Leach Herbarium. Plant materials such as leaves, stems, and bark roots were allowed to dry for 3-5 weeks and ground to a fine powder.

3.2.1 Plant extraction

Three grams (3 g) of each finely ground plant material were extracted in polyester plastic tubes with 30 ml solvents of various polarities such as acetone, dichloromethane, methanol, hexane, and water. The tubes were shaken vigorously for 10 minutes on a shaking machine at a high speed of 3500 rpm. After centrifuging at 3500 rpm for 10 minutes, the extracts were filtered into pre-weighed labelled glass vials. The solvents were removed under a stream of cold air at room temperature. Crude extracts were re-dissolved in acetone prior to phytochemical analysis and biological assays. A freeze dryer was used to remove water from aqueous extracts.

3.2.2 Phytochemical analysis

Aluminium-backed Thin Layer Chromatography (TLC) plates were used to analyse the chemical constituents of each plant extract. Ten microliters of each sample were loaded on TLC plates. The TLC plates were developed in three different eluent solvent systems: Ethyl acetate: methanol: water [EMW], Chloroform: ethyl acetate: formic acid [CEF] and Benzene: ethanol: ammonia hydroxide [BEA] (Kotze and Eloff, 2002). Chemical components were visualized under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600). Vanillin-sulphuric acid spray reagent (Stahl, 1969) was used for the detection of chemical compounds not visible under UV light.

3.3 Results and discussion

3.3.1 Mass extracted

The quantity extracted from 3 g of each plant species is presented in Figure 3.1. Methanol extracted a large quantity of the plant materials compared to other organic solvents with a percentage yield of (11.9 %) followed by dichloromethane (3.4 %), acetone (3.1 %) and the least hexane (2.1 %). The highest quantity was found in methanol extract of *S. lancea* (22 %) followed by the acetone extract of *P. capensis* (8 %), and dichloromethane extract of *D. cinerea* pods (7 %). Hexane extracted the lowest quantity of plant materials (0.3%) as compared to other solvents.

Similar results were observed in other studies where methanol extracted high quantity of plant materials than other solvents (Eloff et al., 2017) while other studies found that water was the best extractant compared to organic solvents with 4.6% yield percentage (Ondua et al., 2019). Various solvent polarities extract different

compounds and have different yields of extraction (Al-Ansari et al., 2019). Furthermore, the percentage yields in the crude extracts of different plant species might be associated with biological compounds responsible for different biological activities (Dikhoba et al., 2019).

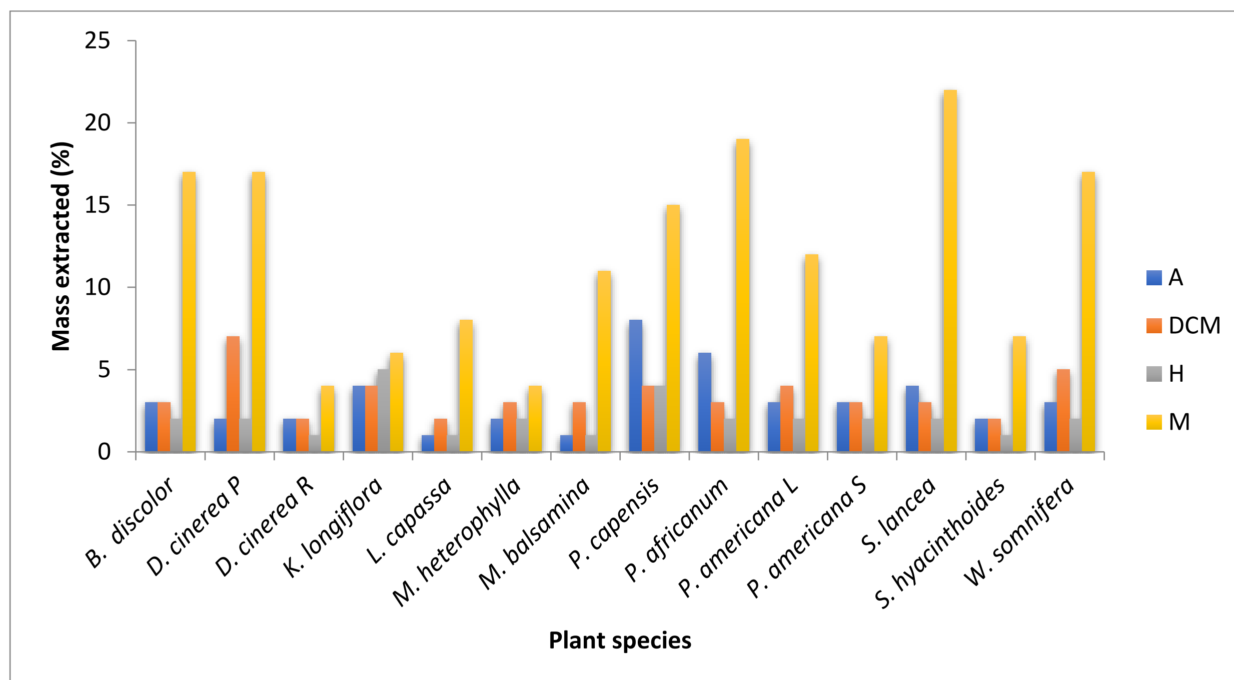


Figure 3.1 Mass extracted (%) from 3 g of *Berchemia discolor, Dichrostachys cinerea pods, Dichrostachys cinerea roots, Kleinia longiflora, Lonchocarpus capassa, Momordica balsamina, Maytenus heterophylla, Persea americana* leaves, *Persea americana* seeds, *Peltophorum africanum, S. lancea, S. hyacinthoides and W. somnifera*. A- acetone, DCM- dichloromethane, H- hexane and M-methanol.

3.3.2 Phytochemical analysis

Thin-layer chromatography was used to determine the number of chemical components in different plant extracts. Chemical components were observed in different extractants separated with Ethyl acetate: methanol: water [EMW], Chloroform: ethyl acetate: formic acid [CEF] and Benzene: ethanol: ammonia hydroxide [BEA]. The TLC chromatograms revealed the presence of different chemical components of plant extracts by various coloured compounds after spraying with vanillin-sulphuric acid (Figure 3.2-3.5). More chemical compounds were visible in methanol extracts in TLC chromatograms separated in CEF compared to other eluent systems. In TLC chromatograms separated with CEF, 34 chemical components were obtained, followed by BEA (33) and the least was EMW (13).

The best separation of compounds with CEF as an eluent system indicates that the extracts contained mainly relatively polar compounds. In TLC chromatograms developed in BEA, similar chemical components with R_f value of 0.16 were observed in acetone extracts of *P. americana* leaves, *P. capensis*, *S. hyacinthoides*, and *W. somnifera*. Methanol extracts of *P. americana* leaves, *S. lancea*, S. *hyacinthoides*, and *W. somnifera* had similar chemical constituents under UV light with an R_f value of 0.64 in chromatograms separated with CEF. Some water extracts of *B. discolor*, *D. cinerea* pods and *P. capensis* had visible chemical components under UV light when separated from all the eluent systems.

Based on our findings, the chromatographic separation is based on different retention of the individual components. The results collaborate with other studies where CEF and EMW solvent systems were not efficient since the compounds moved to the top of the TLC plate making it difficult to count the components (Aderogba et al 2019). Various chemical components were represented by characteristics of different fluorescence, blue and green colour presents the presence of flavonoids (Wagner and Bladt, 1996). Light violet, violet and yellow indicate the presence of steroids, triterpene, terpenoids and flavonoids (Lamola et al., 2017; Kadirvelmurugan et al., 2017). Similarly, CEF was the best solvent system separating more chemical constituents in almost all the plant extracts compared to EMW and BEA (Adebayo et al., 2017; Katerere et al., 2018). In contrast, BEA was the best eluent system, by separating most of the constituents in all the species' crude extracts followed by CEF and the

least was EMW (Meela et al., 2019). Different polarities and ratios in the different solvent systems are capable of showing the best resolution in separating the various components of the plant species investigated (Srivastava and Yadav, 2019)

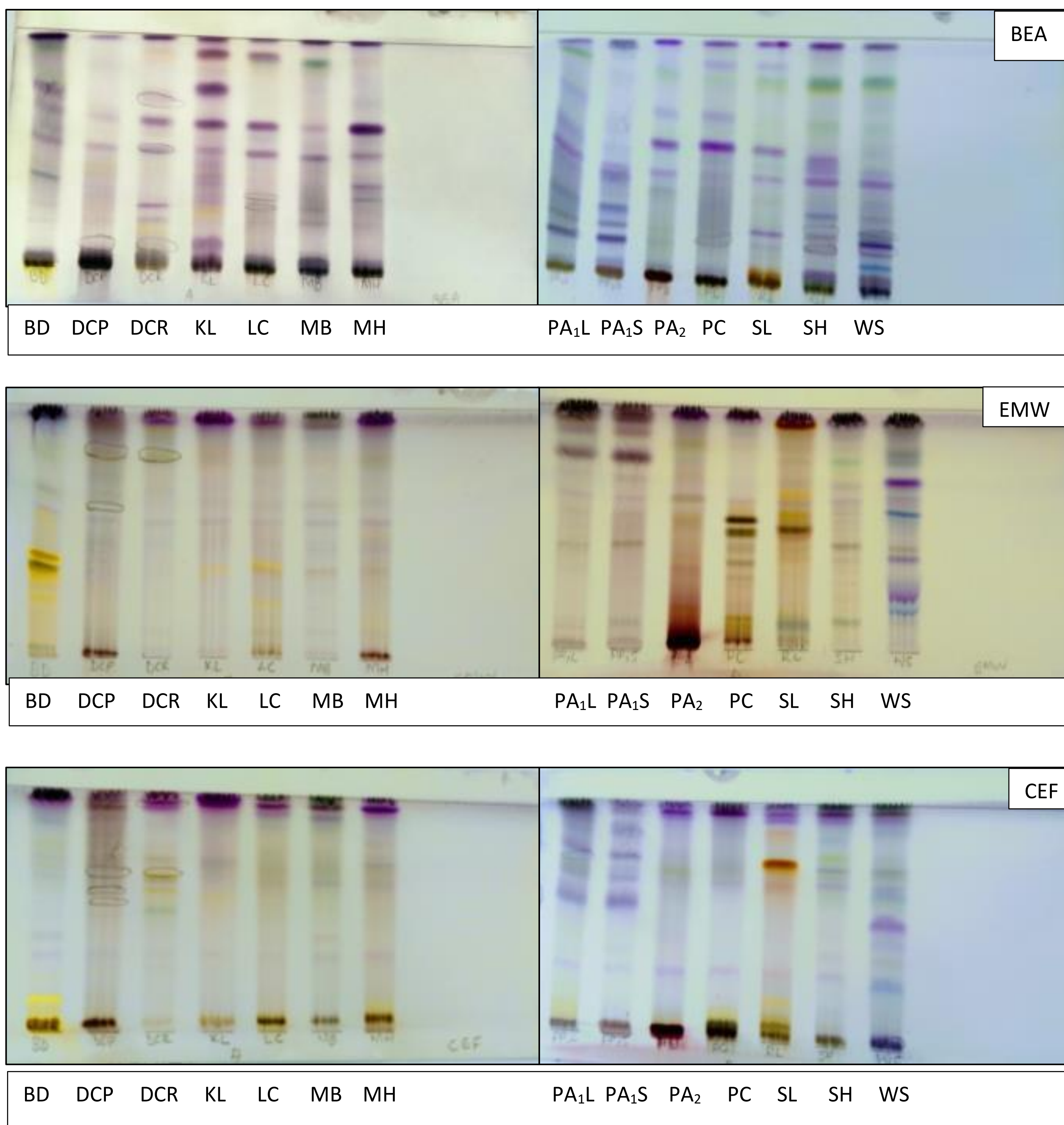


Figure 3.2 TLC fingerprints of Acetone extracts of BD- *B. discolor*, DCP- *D. cinerea* pods, DCR- *D. cinerea* roots, KL- *K. longiflora*, LC- *L. capassa*, MB- *M. balsamina*, MH- *M. heterophylla*, PA1L- *P. americana* leaves, PA1S- *P. americana* seeds, PA2- *P. africanum*, PC- *P. capensis*, SL- *S. lancea*, SH- *S. hyacinthoides*, WS- *W. somnifera* developed in BEA, EMW and CEF solvent systems and sprayed with vanillin sulphuric acid.

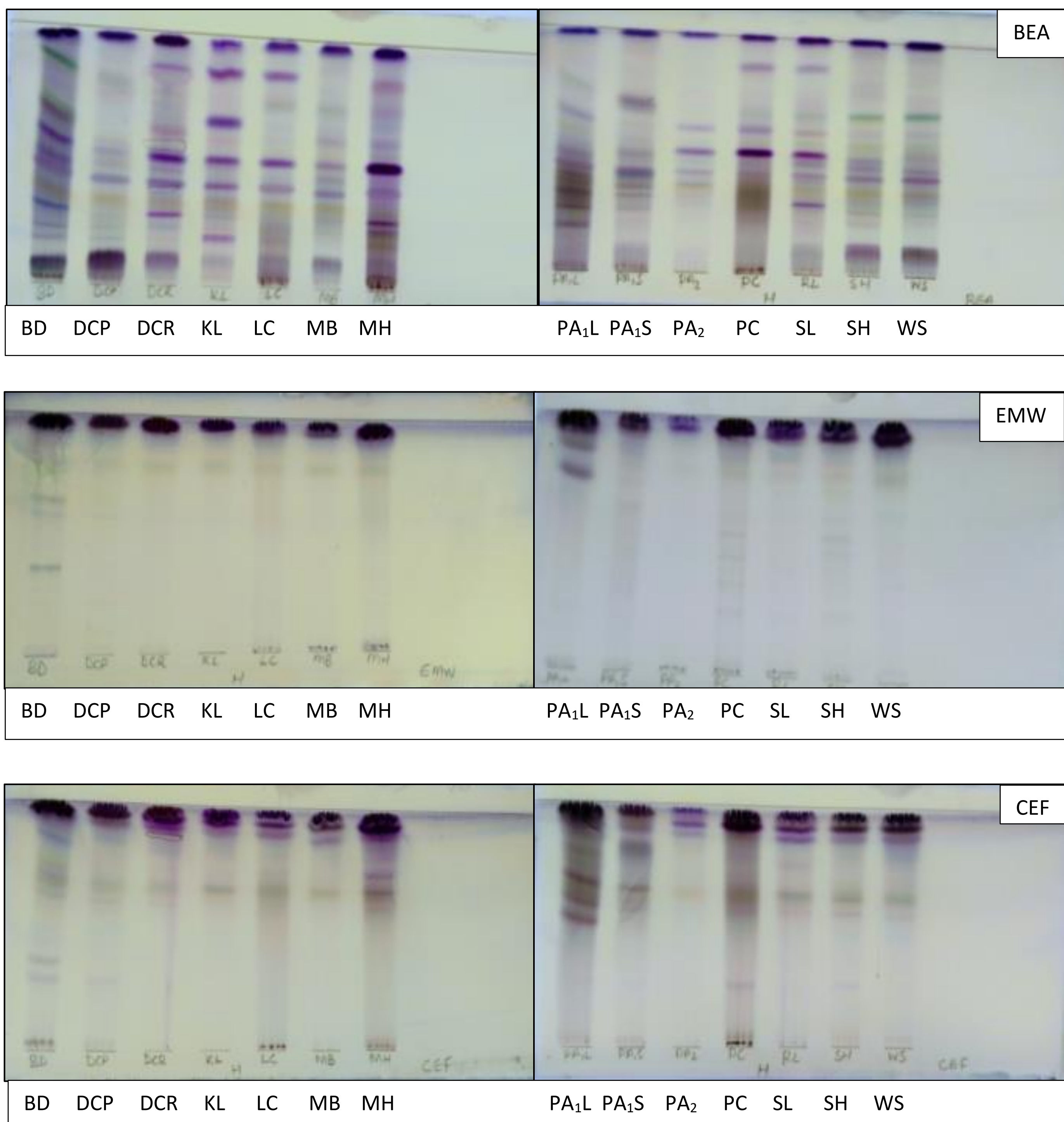


Figure 3.3 TLC fingerprints of hexane extracts of BD- *B. discolor,* DCP- *D. cinerea* pods, DCR- *D. cinerea* roots, KL- *K. longiflora*, LC- *L. capassa*, MB- *M. balsamina*, MH- *M. heterophylla,* PA1L- *P. americana* leaves, PA1S- *P. americana* seeds, PA2- *P. africanum*, PC- *P. capensis*, SL- *S. lancea*, SH- *S. hyacinthoides*, WS- *W. somnifera* developed in BEA, EMW and CEF solvent systems and sprayed with vanillin sulphuric acid.

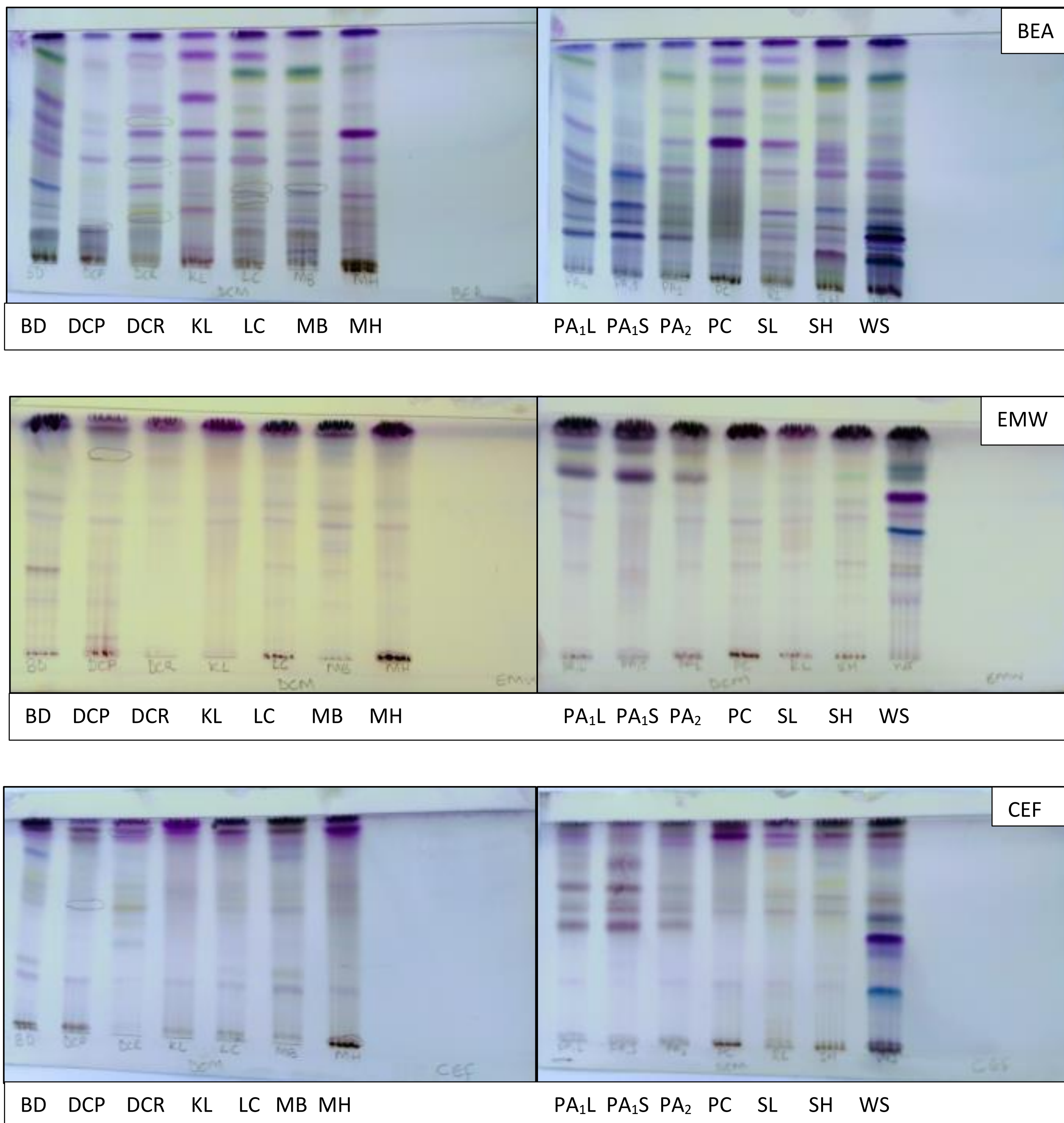


Figure 3.4 TLC fingerprints of Dichloromethane extracts of BD- *B. discolor*, DCP- *D. cinerea* pods, DCR- *D. cinerea* roots, KL- *K. longiflora*, LC- *L. capassa*, MB- *M. balsamina*, MH- *M. heterophylla,* PA1L- *P. americana* leaves, PA1S- *P. americana* seeds, PA2- *P. africanum*,. *P. capensis*, SL- *S. lancea*, SH- *S. hyacinthoides*, WS- *W. somnifera* developed in BEA, EMW and CEF solvent systems and sprayed with vanillin sulphuric acid.

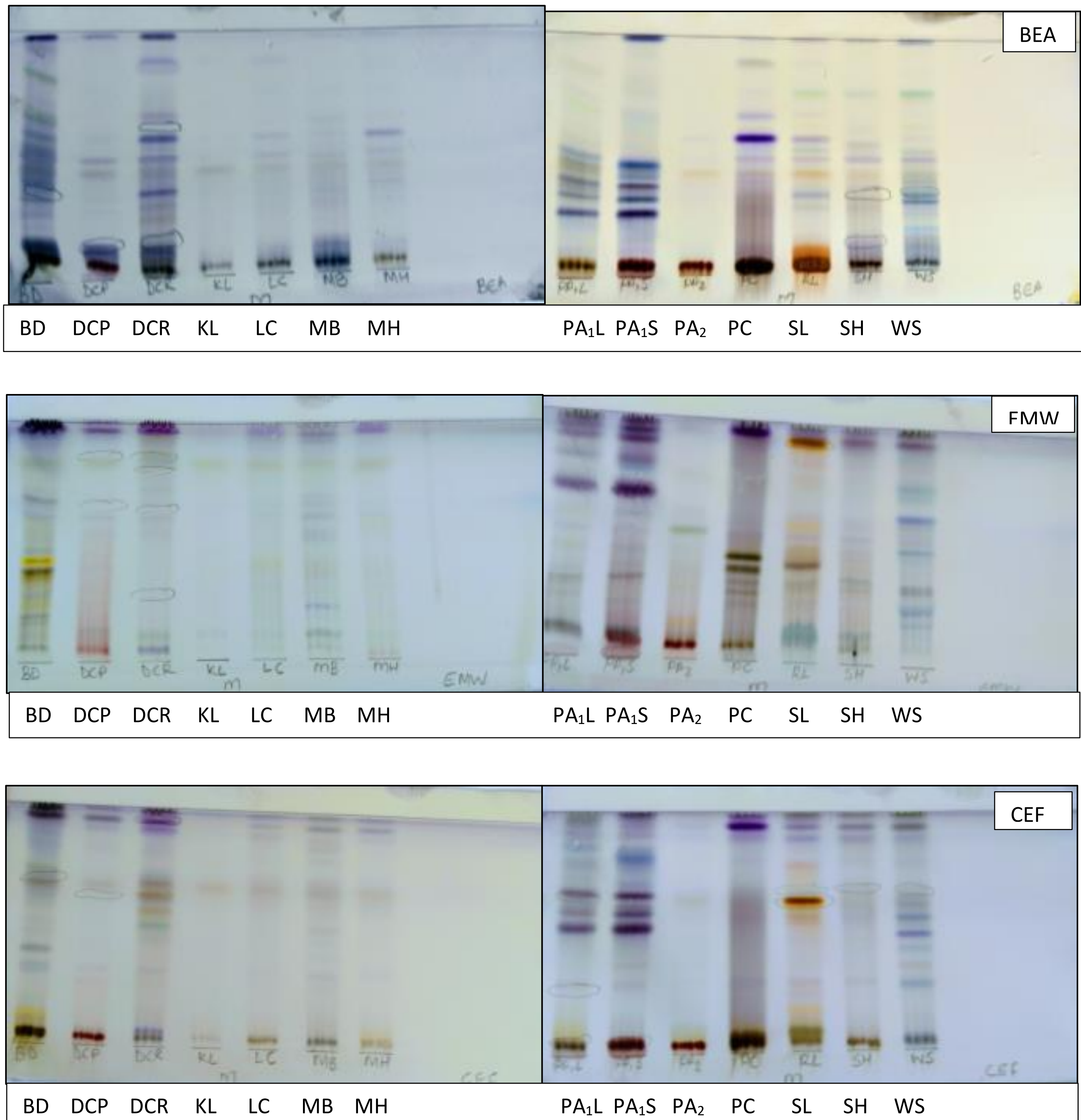


Figure 3.5 TLC fingerprints of Methanol extracts of BD- *B. discolor*, DCP- *D. cinerea* pods, DCR- *D. cinerea* roots, KL- *K. longiflora*, LC- *L. capassa*, MB- *M. balsamina*, MH- *M. heterophylla*, PA1L- *P. americana* leaves, PA1S- *P. americana* seeds, PA2- *P. africanum*, PC- . *capensis*, SL- *S. lancea*, SH- *S. hyacinthoides*, WS- *W. somnifera* developed in BEA, EMW and CEF solvent systems and sprayed with vanillin sulphuric acid.

3.4 Conclusion

Methanol extracted a large quantity of plant materials than other organic solvents. This could be due to differences in the polarity of extraction solvents. Hence, this suggests that the extracted plant species contain mostly polar compounds. Phytochemical analysis of plant species was determined using the TLC plates. All the plant extracts revealed the presence of different chemical components. CEF (34) was the best eluent system by separating more compounds compared to BEA (33) and EMW (13). The results indicate that the extracts contained mainly relatively polar compounds.

In the next chapter, the antifungal activity of the plant crude extracts is investigated against the tested microorganisms.

CHAPTER 4

ANTIFUNGAL ACTIVITY OF THE SELECTED PLANT SPECIES

4.1 Introduction

Medicinal plants have been used for many centuries in developing countries to treat various infectious diseases in humans (Ahmad et al., 2012). These plants contain bioactive compounds that possess antimicrobial properties. The use of plant natural products to search for active compounds serves as a major source to find different therapeutic agents (Morais-Braga et al., 2017). Furthermore, plant-derived compounds are employed in modern medicine, since they play an important role in the synthesis of some complex molecules (Dutra et al., 2016).

Some western medicine has undesirable side effects, and their advantage is its efficacy. Plant extracts are widely used for the treatment of various ailments including fungal infections. Nevertheless, medicinal plants are believed to be safe to use, and comparatively easily available and inexpensive (Kutawa et al., 2018). In addition, many plant extracts have antifungal activity. The biological activity may be due to the secondary metabolites present in the plants (Eruygur et al., 2019). Moreover, plant extracts that possess antimicrobial properties can be important in healing various microbial infections (Ahmad et al., 2012).

Cryptococcus neoformans is the main cause of cryptococcosis in human beings and is capable of producing a couple of virulent compounds playing a major role in the pathogenicity and host invasion (Hassanpour et al., 2020). *Candida albicans* cause nosocomial infection, it colonises most areas of the body such as skin, mucosal membranes, and internal organs (Bojang et al., 2021). *Aspergillus fumigatus* is a common cause of a broad range of human diseases, including aspergillosis, which is associated with the inhalation of *A. fumigatus* spores, and it usually affects the cavities of the lungs. Fungal pathogens cause disease in human beings since they have the ability to grow at or above mammalian body temperature, reaching internal tissues by penetrating the host barriers, lysing

tissues and absorbing their components and can evade host immune defences (Kohler et al., 2017).

The resistance of fungal pathogens to the currently used drugs is one of the great challenges in the treatment of fungal infections (Silva et al., 2020). Furthermore, the current chemotherapeutic options are very limited and far from ideal, mainly owing to their undesirable side effects (Arora et al., 2017). Azole and polyenes are used antifungal agents for the treatment of fungal infections; however, they are susceptible to drug resistance (Hassanpour et al., 2020). Therefore, new antifungal remedies are vital for pathogenic fungi and numerous techniques have to be performed to discover new alternatives.

The current study investigated the antifungal activity of the plant extracts against the three fungal pathogens: *C. neoformans, C. albicans* and *A. fumigatus* using both bioautography and micro-dilution methods.

4.2 Materials and methods

4.2.1 Determining antifungal activity

4.2.1.1 Plant selection and collection

Plant selection, collection and extraction were described in detail in chapter 3.

4.2.1.2 Fungal strains and inoculum quantification

C. albicans (ATCC 10231), and clinical isolates such as *C. neoformans* (ATCC-MYA-4567) and *A. fumigatus* (ATCC 46645) obtained from the Department of Veterinary Tropical Diseases at the University of Pretoria. The haemocytometer cell-counting method described by Aberkane et al. (2002) was used for counting the number of cells for each fungal culture. The sabouraud dextrose (SD) agar slants were used to grow the fungus for seven days at 35°C to prepare the inoculum of each isolate. The sterile loop was used to rub the slant and transfer it to a sterile tube with fresh SD broth (50 ml). After shaking the sterile tubes for 5 min, appropriate dilutions were made to determine the number of cells by microscopic enumeration using a haemocytometer (Neubauer chamber; Merck S.A). The final inoculum concentration was adjusted to approximately 1.0×10^6 cells/ml.

4.2.1.3 Micro-dilution assay

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

4.2.1.4 Bioautography assay

Thin-layer chromatography plates were loaded with 10 µl of each plant extract. 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 overnight to remove the remaining solvent. Fungal cultures were grown on Sabouraud dextrose (SD) agar for 3-5 days. Cultures were transferred into an SD broth from agar with sterile swabs. The plates were incubated at 37 °C in a clean chamber at the humidified chamber for overnight and further sprayed with 2 mg/ml solution of p-iodonitrotetrazolium (INT) violet and incubated for 2-6 hours to allow fungal growth. White areas indicated where reduction of INT to the colored formazan did not take place due to the presence of compounds that inhibited the growth of the fungi.

4.3 Results and discussion

4.3.1 Antifungal activity

4.3.1.1 Micro-dilution assay

The antifungal activity of the plant crude extracts was investigated using the minimum inhibitory concentrations (MIC) values of the plant extract against *C. albicans, C. neoformans* and *A. fumigatu*s. The minimum inhibitory concentration (MIC) is the lowest concentration at which the plant extract inhibits microbial growth Table 4.1-4.3. Antimicrobial activity of the plant extracts has been classified as good (MIC < 0.1 mg/ml) moderate (0.1 ≤ MIC ≤0.32 mg/ml) and weak activity (MIC >0.32 mg/ml) (Famuyide et al., 2019; Eloff, 2021).

Acetone leaf extracts of *B. discolor* had very good activity against *C. albicans* and *A. fumigatus* with a MIC value of 0.08 mg/ml. Dichloromethane, methanol, and aqueous leaf extracts of *B. discolor* had shown good activity against the tested microorganisms. The leaf extracts of *B. discolor* were active against *C. albicans, C. neoformans* and *Candida krusei* (Samie et al., 2010). Despite its activity against fungal pathogens, it was found that the fruit pulp extracts of *B. discolor* showed the highest inhibition activities against the tested microorganisms with MIC value of 6.3 mg/ml (Tshikalange et al., 2017).

Noteworthy antifungal activity was observed in acetone, dichloromethane, hexane, and methanol root extracts of *D. cinerea* against the three tested microorganisms with MIC values ranging between 0.02 mg/ml and 0.04 mg/ml. However, the water extracts had weak activity against *C. albicans* and *A. fumigatus* with MIC values of 2.5 mg/ml and 1.25 mg/ml respectively. The acetone and dichloromethane of *D. cinerea* pods had average MIC values of 0-16 mg/ml against *C. albicans*. The excellent activity was observed in acetone and DCM extracts against *C. neoformans* and *A. fumigatus* with MIC values of 0.02 mg/ml and 0.08 mg/ml.

The methanol and aqueous root extracts of *D. cinerea* had good activity against *C. albicans* (Neondo et al., 2012). It was reported by Mongalo et al. (2015), that an extract with an MIC value of 0.1 mg/ml in an in vitro assay is noteworthy and may be further explored for possible antimicrobial compounds. Methanol extracts of *M. balsamina* showed weak activity against the fungal pathogens tested with MIC values ranging between 0.32 mg/ml and 0.63 mg/ml. Some moderate activity was observed in acetone, DCM, and hexane of *M. balsamina* against all the tested fungal pathogens.

Moderate antifungal activity was observed in DCM extracts of *M. heterophylla* against *C. albicans* and *A. fumigatus* with an MIC value of 0.31 mg/ml. Hexane and methanol extracts of *M. heterophylla* had excellent activity with MIC 0.04 mg/ml against *A. fumigatus*. Very good antifungal activity was observed in acetone, DCM, hexane and methanol extracts of *K. longiflora* against *C. neoformans* and *A. fumigatus* with MIC values ranging between 0.02 mg/ml and 0.08 mg/ml (Table 4.1). The stem-leaf extracts of *K. longiflora* exhibited some degree of antifungal potency against the *Candida* species evaluated and had shown moderate activity against the *Shigella* species with an MIC value of 0.1 mg/ml (Asong et al., 2019).

Acetone, DCM, hexane, methanol, and aqueous leaf extracts of *S. hyacinthoides* showed excellent antifungal activity against *C. neoformans* and *A. fumigatus* with lowest MIC values ranging between 0.02 mg/ml and 0.04 mg/ml. Previously, other studies found that methanol leaf extracts of *S. hyacinthoides* had shown activity against *C. neoformans* with MIC value of 1.67 mg/ml (York et al., 2012). The good activity was observed in acetone and DCM extracts *S. hyacinthoides* with MIC values of 0.08 and 0.04 mg/ml against *C. albicans* respectively. Methanol and hexane extracts of *S. hyacinthoides* were moderately active against *C. albicans* with MIC value of 0.31 mg/ml. In contrast, a high MIC value of 2.5 mg/ml was observed in root methanol extracts of *S. hyacinthoides* against food-borne pathogen (Ceruso et al., 2020). Furthermore, the rhizome ethyl acetate and n-butanol extracts of *S. hyacinthoides* inhibited all the tested pathogenic microorganisms (Islam et al., 2020). The ethanolic leaf extracts of some *Sansevieria* genus had good antibacterial activity against the tested organisms (Maryniuk et al., 2019).

All plant extracts of *W. somnifera* showed very good activity against *A. fumigatus* with MIC value of 0.04 mg/ml. Weak antifungal activity was observed in all extracts of *W. somnifera* against *C. neoformans* with MIC value of 0.63 mg/ml. DCM and methanol leaf extracts of *W. somnifera* had moderate antifungal activity against *C. albicans* with MIC value of 0.16 mg/ml. The biological activities associated with *W. somnifera* are largely attributed to the presence of Withanolides (Khajuria et al., 2004).

Excellent activity was observed in acetone, DCM, hexane, and methanol leaf extracts of *L. capassa* with MIC values between 0.02 mg/ml and 0.08 mg/ml against *C. neoformans* and *A. fumigatus*. Good activity was observed in hexane and methanol leaf extracts of *L. capassa* against *C. albicans* with MIC values of 0.08 mg/ml and 0.16 mg/ml respectively. However, other studies found that the leaf aqueous extracts of *L. capassa* exhibited antifungal activity against *C. albicans* and *C. neoformans* with MIC value of 0.78 mg/ml (Kilonzo et al., 2016).

This might be due to the presence of the alkaloid in *L. capassa* to fight against the pathogenic microbes (Kilonzo et al., 2017).

All the extracts including the aqueous leaf extracts of *P. americana* showed weak activity against *C. neoformans* with MIC values of 0.63 mg/ml and 1.25 mg/ml. Very good antifungal activity was observed in all extracts of *P. americana* seeds against *A. fumigatus* with MIC ranging between 0.02 mg/ml and 0.08 mg/ml. The leaf extracts of *P. americana* were reported to have fungistatic activity against *C. albicans* and *C. tropicalis* (Ajayi et al., 2017). Moderate activity was observed in acetone, hexane, DCM, and methanol extracts of *P. americana* seeds against *C. neoformans* with MIC value of 0.31 mg/ml. The aqueous extracts of *P. americana* seeds showed weak activity against *C. albicans* and *C. neoformans* with MIC values of 0.63 mg/ml and 2.5 mg/ml. Antifungal activity of *P. americana* seeds against Candida species and *C. neoformans* was reported (Leite et al., 2009).

Aqueous extract of *S. lancea* was not active against *C. neoformans* with MIC values of 2.5 mg/ ml. However, all extracts of *S. lancea* showed good activity against *A. fumigatus* and *C. albicans* with lowest MIC values of 0.02 mg/ml and 0.08 mg/ml. DCM leaf extracts of *S. lancea* had moderate activity against the tested microorganisms (Vambe et al., 2018). Acetone and DCM extracts *P. capensis* had excellent activity against *A. fumigatus* and hexane and methanol extracts of *P. capensis* had excellent activity against *C. neoformans* with MIC values of 0.02 mg/ml. The flavonoids glycoside present in *P. capensis* might be responsible for biological activity (Tajuddeen et al., 2021). The weak activity was observed in DCM extracts of *P. capensis* against *C. albicans* and *A. fumigatus* with MIC value of 0.63 mg/ml. In other studies, the leaf extracts of *P. capensis* exhibited a broad- spectrum of antifungal activity against *C. albicans* with MIC values ranging from 0.39 mg/ml to 6.25 mg/ml (Pendota et al., 2017).

All plant extracts of *P. africanum* were active against all tested microorganisms with MIC values ranging between 0.02 and 0.08 mg/ml including the aqueous extracts. Similarly, acetone bark extracts of *P. africanum* had excellent activity

with the lowest MIC value of 0.02 mg/ml (Shikwambana and Mahlo, 2020). Moreover, Acetone leaf and bark extracts of *P. africanum* had moderate MIC values against the tested bacterial strains (Madikizela et al., 2017). It has been reported that water extracts do not have biological activity (Luseba et al., 2007). However, in this study, the water extracts of *P. africanum* had excellent activity against the fungal pathogens tested. Some water extracts had shown good antifungal activity against the tested fungal pathogens (Machaba and Mahlo, 2017). The good activity from water extracts justifies the use of water as an extractant by traditional health practitioners to treat various ailments. The synergistic effects have been found on natural products used to treat various ailments (Jain et al., 2019).

Plant extracts with low MIC values could be a good source of bioactive compounds with antimicrobial strength. Since the lower the MIC, the better the activity. In general, DCM extracts of most plant species showed the best antifungal activity compared to other extracts in the study, which could suggest that the active constituents in most plants tested are more non-polar than polar.

Table 4.1 Minimum inhibitory concentration (MIC) values of the selected plant species against the three tested microorganisms.

Fungi	Time(h)	MIC (mg/ml)															
		Berchemia discolor					*Dichrostachys cinerea* Roots					*Dichrostachys cinerea* Pods					AMP B
		Extractants															
		A	DCM	H	M	H_2O	A	DCM	H	M	H_2O	A	DCM	H	M	H_2O	
Ca	24	0.08	0.16	2.5	0.16	1.25	**0.02**	0.16	0.16	0.04	2.5	0.16	0.16	0.63	0.63	0.63	2.5
	48	0.08	0.16	2.5	0.16	1.25	**0.02**	0.16	0.16	0.04	2.5	0.16	0.16	0.63	0.63	0.63	2.5
	72	0.08	0.16	2.5	0.16	1.25	**0.02**	0.16	0.16	0.04	2.5	0.16	0.16	0.63	0.63	0.63	2.5
Cn	24	0.31	0.31	1.25	1.25	0.16	**0.04**	**0.04**	**0.02**	**0.04**	**0.04**	**0.02**	**0.02**	0.31	0.31	**0.02**	2.5
	48	0.31	0.31	1.25	1.25	0.16	**0.04**	**0.04**	**0.02**	**0.04**	**0.04**	**0.02**	**0.02**	0.31	0.31	**0.02**	2.5
	72	0.31	0.31	1.25	1.25	0.16	**0.04**	**0.04**	**0.02**	**0.04**	**0.04**	**0.02**	**0.02**	0.31	0.31	**0.02**	2.5
Af	24	0.08	0.08	0.31	0.08	0.16	**0.02**	**0.02**	**0.04**	**0.02**	1.25	0.08	0.08	0.31	0.16	0.63	2.5
	48	0.08	0.31	0.63	0.63	0.16	**0.02**	**0.02**	**0.04**	**0.02**	1.25	0.08	0.08	0.31	0.16	0.63	2.5
	72	0.08	0.31	0.63	0.63	0.16	**0.02**	**0.02**	**0.04**	**0.02**	1.25	0.08	0.08	0.31	0.16	0.63	2.5
Average		0.16	0.23	1.42	0.62	0.52	**0.03**	0.07	0.07	0.03	1.26	0.09	0.09	0.42	0.37	0.43	2.5
Fungi	Time(h)	MIC (mg/ml)															
		Mormodica balsamina					*Maytenus heterophylla*					*Kleinia longiflora*					AMP B
		Extractants															
		A	DCM	H	M	H_2O	A	DCM	H	M	H_2O	A	DCM	H	M	H_2O	
Ca	24	0.31	0.31	0.31	0.63	2.5	1.25	0.31	1.25	0.31	2.5	0.16	0.63	0.31	0.31	1.25	2.5
	48	0.31	0.31	0.31	0.63	2.5	1.25	0.31	1.25	0.31	2.5	0.16	0.63	0.31	0.31	1.25	2.5
	72	0.31	0.31	0.31	0.63	2.5	1.25	0.31	1.25	0.31	2.5	0.16	0.63	0.31	0.31	1.25	2.5
Cn	24	**0.02**	**0.02**	0.63	0.63	0.63	1.25	2.5	0.63	0.63	0.16	0.08	0.16	**0.04**	**0.04**	0.31	2.5
	48	**0.02**	**0.02**	0.63	0.63	0.63	1.25	2.5	0.63	0.63	0.16	0.08	0.16	**0.04**	**0.04**	0.31	2.5
	72	**0.02**	**0.02**	0.63	0.63	0.63	1.25	2.5	0.63	0.63	0.16	0.08	0.16	**0.04**	**0.04**	0.31	2.5
Af	24	0.31	0.63	**0.02**	0.31	0.31	0.31	0.31	0.04	**0.04**	1.25	**0.02**	**0.04**	0.63	**0.02**	0.16	2.5
	48	0.31	0.63	0.31	0.31	0.31	0.31	0.31	0.04	**0.04**	1.25	**0.02**	**0.04**	0.63	**0.02**	0.16	2.5
	72	0.31	0.63	0.31	0.31	0.31	0.31	0.31	0.04	**0.04**	1.25	**0.02**	**0.04**	0.63	**0.02**	0.16	2.5
Average		0.21	0.32	0.38	0.52	1.15	0.94	1.04	0.64	0.33	1.30	0.09	0.28	0.33	0.12	0.57	2.5

Abbreviations: C. a- Candida albicans, *C. n- Cryptococcus neoformans*, *A. f- Aspergillus fumigatus,* A- acetone, DCM- dichloromethane, H- hexane, M- methanol and H_2O- water.

Table 4.2 Minimum inhibitory concentration (MIC) values of the selected plant species against three tested microorganisms.

Fungi	Time(h)	MIC (mg/ml)															
		Lonchocarpus capassa					*Persea americana* leaves					*Persea americana* seeds					AMP B
		Extractants															
		A	DCM	H	M	H_2O	A	DCM	H	M	H_2O	A	DCM	H	M	H_2O	
Ca	24	0.31	0.31	0.08	0.16	2.5	**0.02**	**0.02**	**0.04**	**0.04**	2.5	**0.04**	**0.04**	0.63	0.16	2.5	2.5
	48	0.31	0.31	0.08	0.16	2.5	**0.02**	**0.02**	**0.04**	**0.04**	2.5	**0.04**	**0.04**	0.63	0.16	2.5	2.5
	72	0.31	0.31	0.08	0.16	2.5	**0.02**	**0.02**	**0.04**	**0.04**	2.5	**0.04**	**0.04**	0.63	0.16	2.5	2.5
Cn	24	**0.04**	**0.04**	**0.02**	**0.02**	**0.02**	1.25	1.25	0.63	0.63	1.25	0.31	0.31	0.31	0.31	0.63	2.5
	48	**0.04**	**0.04**	**0.02**	**0.02**	**0.02**	1.25	1.25	0.63	0.63	1.25	0.31	0.31	0.31	0.31	0.63	2.5
	72	**0.04**	**0.04**	**0.02**	**0.02**	**0.02**	1.25	1.25	0.63	0.63	1.25	0.31	0.31	0.31	0.31	0.63	2.5
Af	24	**0.02**	**0.02**	0.08	**0.02**	0.63	**0.02**	**0.02**	0.31	**0.02**	0.31	**0.04**	0.08	**0.02**	**0.02**	0.08	2.5
	48	**0.02**	**0.02**	0.08	**0.02**	0.63	**0.02**	**0.02**	0.31	**0.02**	0.31	**0.04**	0.08	**0.02**	**0.02**	0.08	2.5
	72	**0.02**	**0.02**	0.08	**0.02**	0.63	**0.02**	**0.02**	0.31	**0.02**	0.31	**0.04**	0.08	**0.02**	**0.02**	0.08	2.5
Average		0.12	0.12	0.06	0.07	1.05	0.43	0.43	0.33	0.23	1.35	0.13	0.14	0.32	0.16	1.07	2.5
Fungi	Time(h)	MIC (mg/ml)															
		Searsia lancea					*Pappea capensis*					*Peltophorum africanum*					AMP B
		Extractants															
		A	DCM	H	M	H_2O	A	DCM	H	M	H_2O	A	DCM	H	M	H_2O	
Ca	24	**0.02**	**0.02**	0.16	0.08	0.31	0.16	0.63	0.31	1.25	0.63	0.08	0.08	0.16	0.31	0.31	2.5
	48	**0.02**	**0.02**	0.16	0.08	0.31	0.16	0.63	0.31	1.25	0.63	0.08	0.08	0.16	0.31	0.31	2.5
	72	**0.02**	**0.02**	0.16	0.08	0.31	0.16	0.63	0.31	1.25	0.63	0.08	0.08	0.16	0.31	0.31	2.5
Cn	24	0.63	1.25	0.63	0.63	2.5	0.63	0.63	**0.02**	**0.02**	0.08	**0.02**	**0.02**	**0.02**	0.08	**0.02**	2.5
	48	0.63	1.25	0.63	0.63	2.5	0.63	0.63	**0.02**	**0.02**	0.08	**0.02**	**0.02**	**0.02**	0.08	**0.02**	2.5
	72	0.63	1.25	0.63	0.63	2.5	0.63	0.63	**0.02**	**0.02**	0.08	**0.02**	**0.02**	**0.02**	0.08	**0.02**	2.5
Af	24	**0.02**	**0.02**	**0.04**	**0.02**	**0.02**	**0.02**	**0.02**	0.31	1.25	0.31	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	2.5
	48	**0.02**	**0.02**	**0.04**	**0.04**	0.08	**0.02**	**0.02**	0.31	1.25	0.31	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	2.5
	72	**0.02**	**0.02**	**0.04**	**0.04**	0.08	**0.02**	**0.02**	0.31	1.25	0.31	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	2.5
Average		0.22	0.43	0.28	0.25	0.96	0.27	0.43	0.21	0.84	0.34	0.04	0.04	0.07	0.14	0.12	2.5

Table 4.3 Minimum inhibitory concentration (MIC) values of the selected plant species against three tested microorganisms.

Fungi	Time(h)	MIC (mg/ml)										AMP B
		Sanseviera Hyacinthoides					*Withania somnifera*					
		Extractants										
		A	DCM	H	M	H_2O	A	DCM	H	M	H_2O	
Ca	24	0.08	0.04	0.31	0.31	2.5	**0.02**	0.16	2.5	0.16	2.5	2.5
	48	0.08	0.04	0.31	0.31	2.5	**0.02**	0.16	2.5	0.16	2.5	2.5
	72	0.08	0.04	0.31	0.31	2.5	**0.02**	0.16	2.5	0.16	2.5	2.5
Cn	24	**0.02**	**0.02**	0.63	0.31	**0.02**	0.63	0.63	0.63	0.63	0.63	2.5
	48	**0.02**	**0.02**	0.63	0.31	**0.02**	0.63	0.63	0.63	0.63	0.63	2.5
	72	**0.02**	**0.02**	0.63	0.31	**0.02**	0.63	0.63	0.63	0.63	0.63	2.5
Af	24	**0.02**	**0.02**	**0.02**	**0.02**	0.04	0.04	0.04	0.04	**0.02**	0.04	2.5
	48	**0.02**	**0.02**	**0.02**	**0.02**	0.04	0.04	0.04	0.04	**0.02**	0.04	2.5
	72	**0.02**	**0.02**	**0.02**	**0.02**	0.04	0.04	0.04	0.04	**0.02**	0.04	2.5
Average		0.04	0.03	0.32	0.21	0.85	0.23	0.28	1.06	0.27	1.06	2.5

Abbreviations: C. a- Candida albicans, *C. n- Cryptococcus neoformans*, *A. f- Aspergillus fumigatus,* A- acetone, DCM- dichloromethane, H- hexane, M- methanol and H_2O- water.

4.3.1.2 Average MIC value of extractants

The average MICs of the plant extracts were investigated against the three tested microorganisms. The average MIC values are presented in Figure 4.1. Acetone, dichloromethane, hexane, and methanol root extracts of *D. cinerea* had very good activity ranging between 0.03 mg/ml and 0.07 mg/ml. All the extracts of *M. balsamina* and *M. heterophylla* had shown weak average activity against the tested microorganisms with MIC values greater than 0.32 mg/ml. Acetone and DCM extracts of *L. capassa* had an average activity of 0.12 mg/ml. Hexane and methanol extracts had an average MIC value of 0.06 mg/ml and 0.07 mg/ml respectively. As a result, the lowest average MIC value indicates the highest antifungal potential. The average MIC ≤1 mg/ ml against the tested microorganism was considered to have broad-spectrum properties (Anokwuru et al., 2021).

Acetone, DCM, hexane, and methanol extracts of *P. americana* seeds revealed average activity against the test microorganisms with MIC values ranging between 0.13 mg/ml and 0.32 mg/ml. Despite the good activity of *P. americana* seeds extracts, the leaf extracts of *P. americana* revealed moderate activity with an average MIC between 0.33 mg/ml and 0.43 mg/ml. Acetone, DCM, and hexane extracts of *P. africanum* had a very good activity with average MIC values between 0.04 and 0.08 mg/ml. Furthermore, methanol and aqueous extracts had moderate average MIC values of 0.14 mg/ml and 0.12 mg/ml respectively. However, some methanol extracts of *Helichrysum* species had an average MIC value ranging between 0.65-3.4 mg/ml against the bacterial strains tested (Serabele et al., 2021).

The average activity was revealed in hexane and methanol extracts of *S. hyacinthoides* with average MIC values of 0.32 mg/ml and 0.21 mg/ml. All the aqueous plant extracts were active with an average MIC between 0.34 mg/ml and 1.36 mg/ml against the tested microorganism. However, the aqueous extracts of *P. africanum.* Were active against the tested fungal pathogens. The average MIC value of 1 mg/ml indicates the possible presence of overlapping antimicrobial active compounds in the extracts (Serabele et al., 2021). The choice of solvent used during extraction had a strong effect on the antimicrobial activity of the extracts since there is a variation in average MIC values.

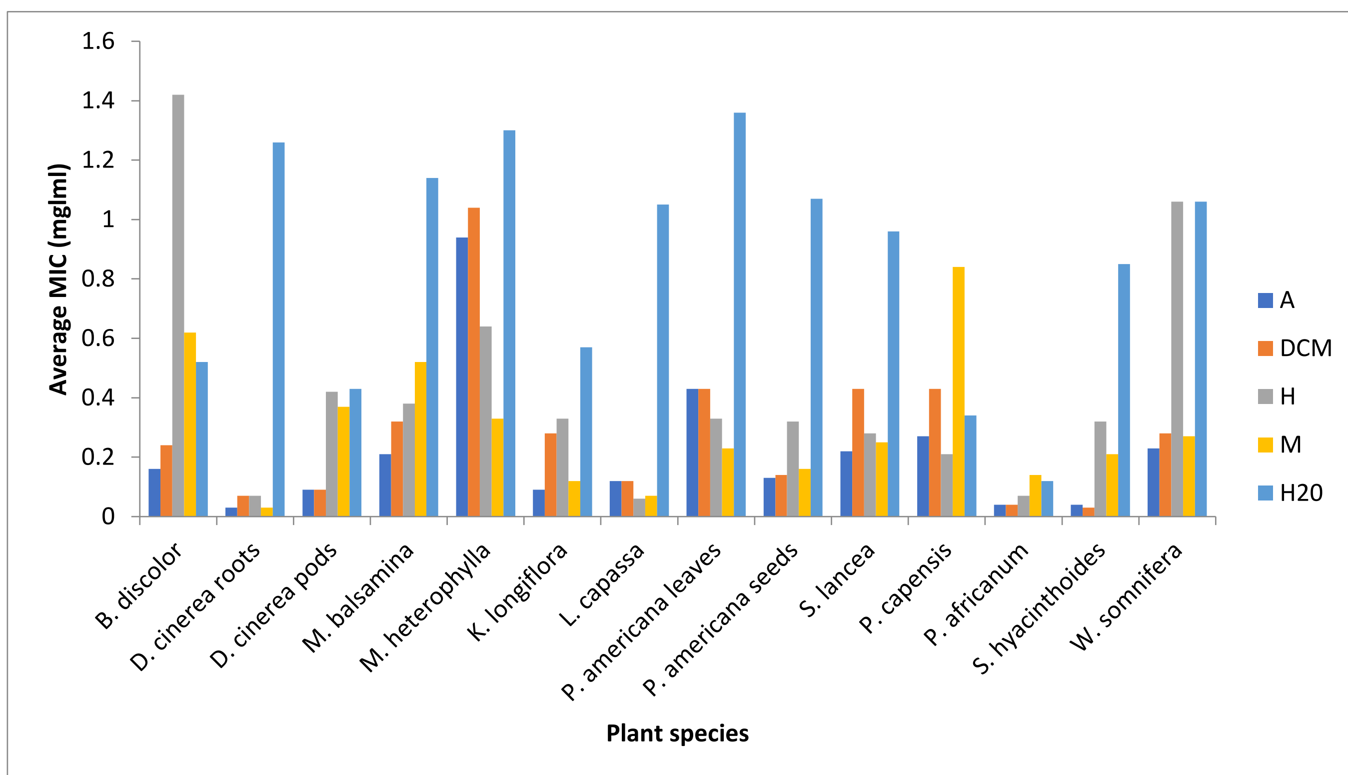


Figure 4.1 The average MIC values in mg/ml of acetone, Dichloromethane, hexane, methanol, and water extracts of twelve plants against *C. albicans, C. neoformans* and *A. fumigatus.*

4.3.1.3 Total activity

The total activity indicates the volume to which the bio-active compounds extracted from 1 g of plant material can be diluted and still inhibits the growth of a microorganism (Eloff, 2000). In this study, the total activity of the plants was calculated as follows: The total antifungal activity was obtained by dividing the quantity extracted from 1 g of each plant extract by the MIC value. The results are presented in Table 4.4-4.5. The highest total activity was observed in acetone extracts of *Momordica balsamina* (10334 ml/g) against *C. neoformans*. Similarly, acetone had the highest total activity against the tested microorganisms (Katerere et al., 2018; Erhabor et al., 2022). The lowest total activity was found in hexane extracts of *W. somnifera* and *M. heterophylla* against *C. albicans* with total activity of 7 ml/g and 8 ml/g respectively. The higher the total activity, the higher the potency (Arora et al., 2017).

The lowest average total activity was observed in acetone extracts of *Maytenus heterophylla* and hexane extracts of *D. cinerea* pods with total activity of 16 ml/g and 18 ml/g respectively. In contrast, acetone had the highest average total activity compared to other extracts and hexane was the least (Mtunzi et al., 2017). It is important to note that the total activity is dependent on the solubility of the plant materials in a specific solvent and the activity of such extract on the selected microorganism (Dikhoba et al., 2019).

The highest average total activity means that less plant material is required to inhibit microbial growth (Saddiqe et al., 2020). The average total activity was observed in methanol extracts followed by n-hexane of *Hypericum* species extracts against pathogenic bacteria (Saddiqe et al., 2020). Therefore, extracts possessing higher total activity value in ml/g are considered the best for the isolation of potentially bioactive compounds (Mtunzi et al., 2017).

Table 4.4 Total activity (ml/g) values of the selected plant species against three tested microorganisms.

Fungi	Time	Total activity ml/g															
		Berchemia discolor				*Dichrostachys cinerea* Roots				*Dichrostachys cinerea* Pods				*Mormodica balsamina*			
		Extractants															
		A	DCM	H	M	A	DCM	H	M	A	DCM	H	M	A	DCM	H	M
Ca	24	375	146	65	146	834	125	83	3250	104	104	11	69	667	54	86	159
	48	375	146	65	146	834	125	83	3250	104	104	11	69	667	54	86	159
	72	375	146	65	146	834	125	83	3250	104	104	11	69	667	54	86	159
Cn	24	97	75	19	131	417	500	667	3250	834	834	22	140	10334	834	42	159
	48	97	75	19	131	417	500	667	3250	834	834	22	140	10334	834	42	159
	72	97	75	19	131	417	500	667	3250	834	834	22	140	10334	834	42	159
Af	24	373	292	75	2042	834	1000	333	6500	208	208	22	271	667	27	1334	323
	48	373	75	37	259	834	1000	333	6500	208	208	22	271	667	27	86	323
	72	373	75	37	259	834	1000	333	6500	208	208	22	271	667	27	86	323
Average		282	123	45	377	695	542	361	4333	382	382	18	160	3889	305	210	214
	Time	*Maytenus heterophylla*				*Kleinia longiflora*				*Lonchocapus capassa*				*Persea americana* leaf			
		Extractants															
		A	DCM	H	M	A	DCM	H	M	A	DCM	H	M	A	DCM	H	M
Ca	24	8	75	8	204	167	48	75	129	43	43	125	458	1500	1500	833	2750
	48	8	75	8	204	167	48	75	129	43	43	125	458	1500	1500	833	2750
	72	8	75	8	204	167	48	75	129	43	43	125	458	1500	1500	833	2750
Cn	24	8	9	16	101	333	188	583	1000	333	333	500	3667	24	24	53	175
	48	8	9	16	101	333	188	583	1000	333	333	500	3667	24	24	53	175
	72	8	9	16	101	333	188	583	1000	333	333	500	3667	24	24	53	175
Af	24	32	75	250	1583	1334	750	37	2000	667	667	125	3667	1500	1500	1667	5500
	48	32	75	250	1583	1334	750	37	2000	667	667	125	3667	1500	1500	1667	5500
	72	32	75	250	1583	1334	750	37	2000	667	667	125	3667	1500	1500	1667	5500
Average		16	53	91	629	611	329	232	1043	348	348	250	2597	1008	1008	851	2808

Abbreviations: C. a- Candida albicans, *C. n- Cryptococcus neoformans*, *A. f- Aspergillus fumigatus,* A- acetone, DCM- dichloromethane, H- hexane, M- methanol and H_2O- water.

Table 4.5 Total activity (ml/g) values of the selected plant species against three tested microorganisms.

Fungi	Time (h)	Total activity ml/g											
		Persea americana seeds				*Searsia lancea*				*Pappea capensis*			
		Extractants											
		A	DCM	H	M	A	DCM	H	M	A	DCM	H	M
Ca	24	917	500	16	438	3000	1334	208	2292	379	48	97	120
	48	917	500	16	438	3000	1334	208	2292	379	48	97	120
	72	917	500	16	438	3000	1334	208	2292	379	48	97	120
Cn	24	118	65	32	226	95	21	53	291	96	48	1500	7500
	48	118	65	32	226	95	21	53	291	96	48	1500	7500
	72	118	65	32	226	95	21	53	291	96	48	1500	7500
Af	24	917	250	500	3500	3000	1334	833	9167	3034	1500	97	120
	48	917	250	500	3500	3000	1334	833	4583	3034	1500	97	120
	72	917	250	500	3500	3000	1334	833	4583	3034	1500	97	120
Average		651	272	183	1388	2032	896	365	2898	1170	532	565	2580
	Time (h)	Extractants											
		Peltophorum africanum				*Withania somnifera*				*Sanceviera hyathinthoides*			
		A	DCM	H	M	A	DCM	H	M	A	DCM	H	M
Ca	24	875	250	83	484	1667	229	7	792	250	500	43	366
	48	875	250	83	484	1667	229	7	792	250	500	43	366
	72	875	250	83	484	1667	229	7	792	250	500	43	366
Cn	24	3500	1000	667	1875	53	58	27	201	1000	1000	21	366
	48	3500	1000	667	1875	53	58	27	201	1000	1000	21	366
	72	3500	1000	667	1875	53	58	27	201	1000	1000	21	366
Af	24	3500	1000	667	7500	833	917	417	6334	1000	1000	667	5667
	48	3500	1000	667	7500	833	917	417	6334	1000	1000	667	5667
	72	3500	1000	667	7500	833	917	417	6334	1000	1000	667	5667
Average		2625	750	472	3286	851	401	150	2442	750	833	243	2133

Abbreviations: C. a- Candida albicans, *C. n- Cryptococcus neoformans*, *A. f- Aspergillus fumigatus,* A- acetone, DCM- dichloromethane, H- hexane, M- methanol and H_2O- water.

4.4 TLC bioautograms of fungal species

The bioautography assay was used to determine the number of antifungal compounds on plant extracts using the TLC plates. The TLC chromatograms revealed different compounds present in the plant extracts in Figures 4.2 and 4.3. The retention factor values are presented in Tables 4.4 and 4.5. More compounds were observed in BEA (41) compared to other eluent systems CEF (30) and EMW (37). In contrast, more compounds were visible in TLC chromatograms developed in EMW against the dermatophytes (Shikwambana and Mahlo, 2020). In other studies, CEF and EMW separated more compounds compared to the least polar BEA mobile phase (Katerere et al., 2018).

4.4.1 TLC chromatograms of *C. neoformans*

The active compounds were observed in DCM extracts of *P. americana seeds, S. lancea, P. capensis, P. africanum, S. hyacinthoides* and *W. somnifera* separated with BEA against *C. neoformans*, with similar R_f values 0.25 and 0.33. More compounds were observed in methanol extracts of *P. americana* seeds separated with BEA against *C. neoformans* with R_f values 0.23, 0.29, 0.34 and 0.4. Antifungal compounds were observed in DCM extracts of *D. cinerea* pods, *D. cinerea* roots, *K. longiflora*, *M. heterophylla, P. americana* seeds, *P. americana* leaves, *P. africanum, S. lancea* and *W. somnifera* developed in CEF against *C. neoformans* with R_f value of 0.58.

Compounds were visible in chromatograms of dichloromethane leaf extracts of *W. somnifera*, *S. hyacinthoides, P. americana* and *P. americana* pods developed in EMW with R_f value 0.80 against *C. neoformans*. Three compounds were observed in hexane extracts of *M. balsamina* and *M. heterophylla* separated in BEA against *C. neoformans* with R_f values 0.37, 0.47 and 0.56. A compound with similar R_f value of 0.91 was observed in hexane extracts of *P. capensis, P. africanum, S. lancea, P. americana* seeds and *S. hyacinthoides* separated in EMW against *C. neoformans*. Other researchers found that there were no active compounds observed in extracts of *W. somnifera* against *C. albicans* (Masevhe, 2013).

4.4.2 TLC chromatograms of *A. fumigatus*

The active compound with a similar R_f value of 0.9 was observed in acetone, dichloromethane, methanol, and hexane leaf extracts of *S. lancea, P. capensis, K. longiflora, P. americana*, *D. cinerea pods* and *D. cinerea* roots against *A. fumigatus* separated in EMW eluent system. Antifungal compounds were visible in methanol and DCM leaf extracts of *S. hyacinthoides* with R_f values 0.88 and 0.91 separated in EMW and CEF against *A. fumigatus*, respectively.

Three compounds with R_f values 0.63, 0.53 and 0.56 were visible in DCM leaf extracts of *W. somnifera* separated in CEF against *A. fumigatus*. Similarly, the leaf ethyl acetate extract of *W. somnifera* showed three active bands against *Fusarium* pathogens (Seepe et al., 2021). Acetone and hexane extracts of *P. americana* seeds and *P. americana* leaves had similar compounds with R_f values 0.69 and 0.9 separated in CEF against *A. fumigatus*. Compounds with R_f values 0.91 and 0.92 were visible in the hexane leaf extract of *L. capassa* and the DCM leaf extract of *S. hyacinthoides* separated in CEF *A. fumigatus*. No antifungal compounds were observed in some plant extracts with good antifungal activity when using a micro-dilution assay, this indicates that there is a possible synergism.

Active compounds in the plant extracts were mostly present in the intermediate polarity extracts. In most of the extracts, more than one active compound was observed. No clear zone was observed in all plant extracts against *C. albicans* indicating that no separated compound had activity in the bioautography assay. Furthermore, the lack of activity of extracts of some species in bioautography assay could be due to the evaporation of active compounds during the removal of the TLC eluent system.

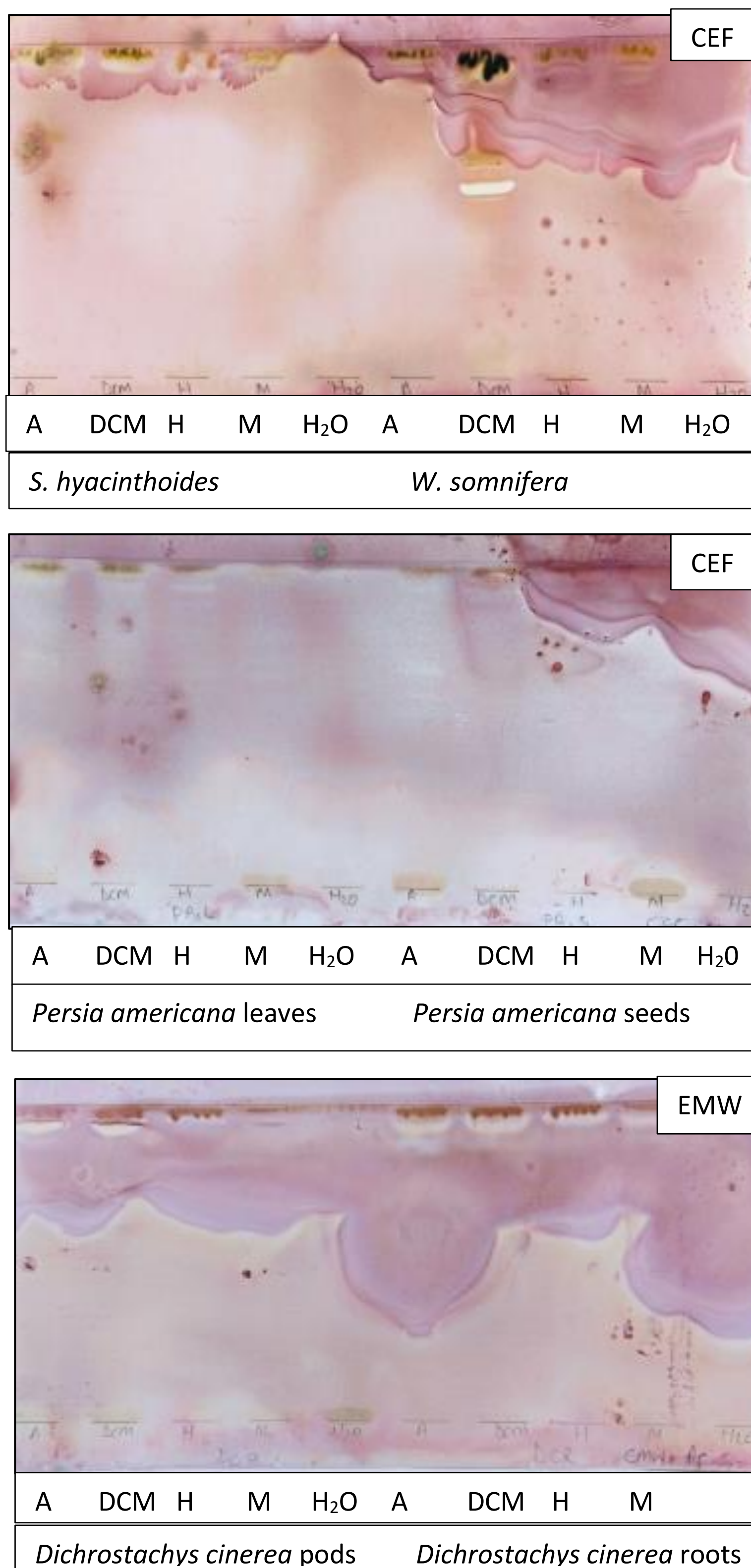


Figure 4.2 Bioautograms of extracts of *Sanseviera hyacinthoides*, *W. somnifera, Persea americana* leaves, *Persea americana* seeds, *Dichrostachys cinerea* pods and *Dichrostachys cinerea* roots. TLC plates developed in CEF and EMW sprayed with *A. fumigatus*. White areas indicate inhibition of fungal growth. Lanes from left to right: acetone=A, dichloromethane=DCM, hexane=H, methanol=M and aqueous extracts=H_2O.

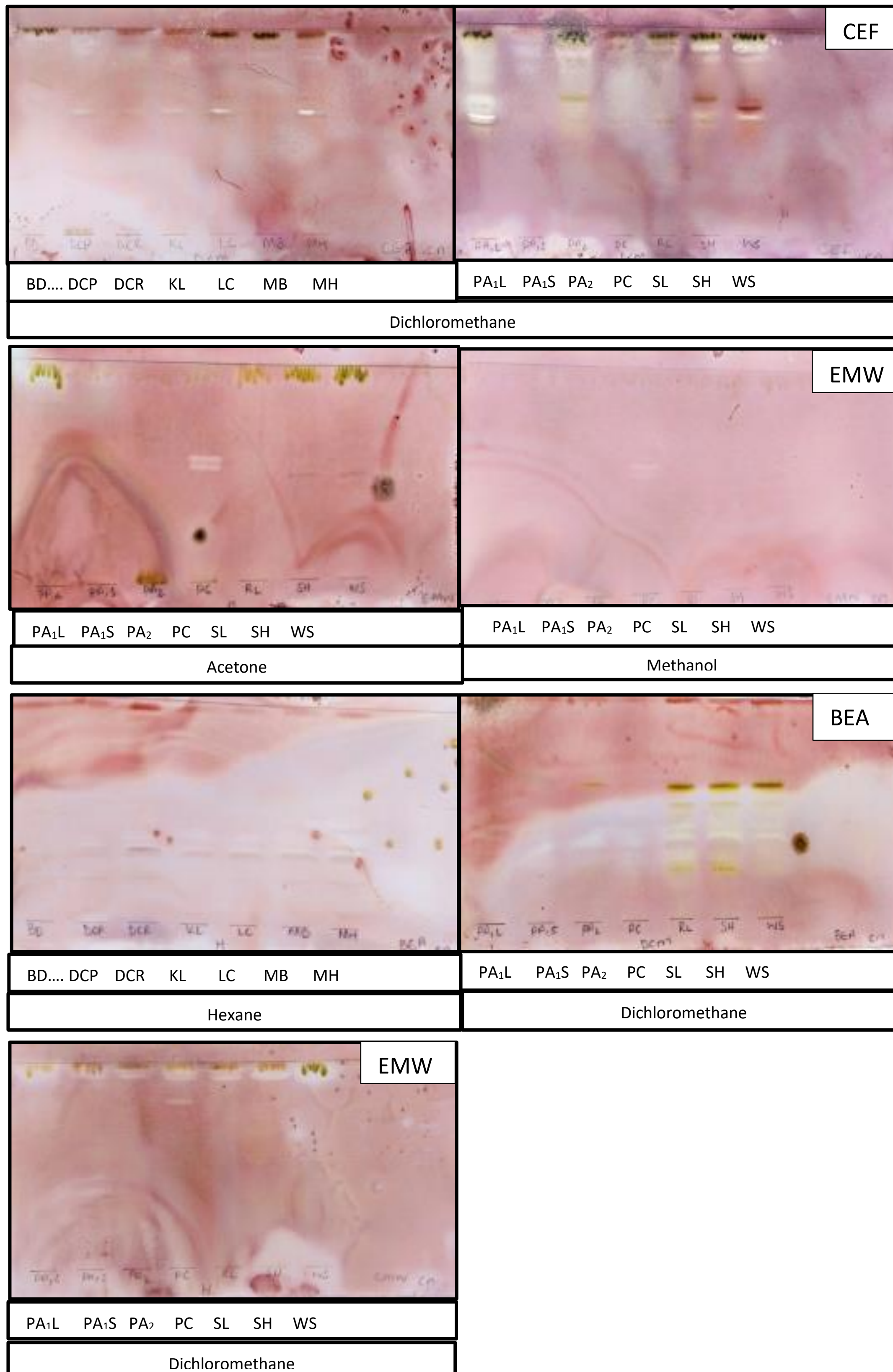


Figure 4.3 Bioautograms of Acetone, methanol, hexane and dichloromethane extracts of BD- *B. discolor*, DCP- *D. cinerea* pods, DCR- *D. cinerea* roots, KL- *K. longiflora*, LC- *L. capassa,* MB- *M. balsamina*, MH- *M. heterophylla*, PA1L- *P. americana* leaves, PA1S- P. *americana* seeds, PA_2- *P. africanum*, PC- *P. capensis*, SL- *S. lancea*, SH- *S. hyacinthoides,* WS- *W. somnifera* developed in CEF, EMW and BEA solvent systems and sprayed with *Cryptococcus neoformans.* White areas indicate inhibition of fungal growth.

Table 4.6 R_f values of twelve plant species against *C. neoformans*. BD= *B. discolor*, DCR= *D. cinerea* roots, DCP= *D. cinerea* pods, MB= *M. balsamina*, MH= *M. heterophylla,* KL= *K. longiflora,* LC= *L. capassa*, PAL= *P. americana* leaf, PAS= *P. americana* seeds, SL= S. *lancea*, PC= *P. capensis*, PA2= *P. africanum*, SH= *S. hyacinthoides*, WS= *W. somnifera*.

			Species name													
Extractant	Eluent system	R_f values	BD	DCR	DCP	MB	MH	KL	LC	PAL	PAS	SL	PC	PA2	SH	WS
Acetone	BEA	0.29										√				
		0.53										√				
	EMW	0.51											√			
		0.56											√			
DCM	BEA	0.25								√		√	√	√	√	√
		0.31														√
		0.33									√	√	√	√	√	√
		0.35		√	√	√	√		√							
		0.42										√				
		0.44												√		
		0.54											√			
	CEF	0.58		√	√		√	√	√	√	√	√		√	√	√
		0.9			√											√
	EMW	0.8								√	√				√	√
Hexane	BEA	0.37		√	√	√	√		√							
		0.47				√	√									
		0.56				√	√									
	EMW	0.79											√			
		0.91									√	√	√	√	√	
Methanol	BEA	0.13										√				
		0.21														√
		0.23									√					
		0.29									v					
		0.34									√				√	
		0.38														
		0.4									√					
		0.5											√			

Table 4.7 R_f values of twelve plant species against *A. fumigatus*. BD= *B. discolor*, DCR= *D. cinerea* roots, DCP= *D. cinerea* pods, MB= *M. balsamina*, MH= *M. heterophylla*, KL= *K. longiflora*, LC= *L. capassa*, PAL= *P. americana* leaf, PAS= *P. americana* seeds, SL= S. *lancea*, PC= *P. capensis,* PA2= *P. africanum*, SH= *S. hyacinthoides*, WS= *W. somnifera*.

Extractant	Eluent system	R_f values	Species name													
			BD	DCR	DCP	MB	MH	KL	LC	PAL	PAS	SL	PC	PA2	SH	WS
DCM	CEF	0.53														√
		0.56														√
		0.55					√									
		0.59					√							√		
		0.86														
		0.88										√				
		0.9									√					
		0.91													√	
		0.63														√
Acetone	CEF	0.58									√					
		0.69								√	√					
Hexane	CEF	0.88										√	√			
		0.9								√						
		0.92							√							
Methanol	CEF	0.88										√				
Acetone	EMW	0.88			√							√				
		0.81									√	√				
		0.9			√	√		√					√			
		0.7						√								
DCM	EMW	0.82										√	√			√
		0.9		√	√			√					√			
Hexane	EMW	0.9		√				√		√						
Methanol	EMW	0.81									√					
		0.82														√
		0.88													√	

4.5 Conclusion

All tested plant extracts revealed some varying degrees of fungal inhibition, with MIC values ranging between 0.02 and 2.5 mg/ml. Some water extracts had shown some activity against the tested microorganisms. The TLC chromatograms revealed different compounds' presence in the plant extracts. More compounds were observed in BEA compared to CEF and EMW eluent systems. The antifungal extracts of the twelve selected medicinal plants support the traditional use of the plants in the treatment of fungal infections. The antifungal activity of the plant extracts indicated in most plant species indicates that the plant can be used to treat fungal infections. Based on the results, this suggests that these plants could be used as a new potential source of antifungal agents. Further screening and isolation of the most promising plant species will be undertaken.

In the next chapter, the qualitative and quantitative antioxidant activity of the plant extracts will be investigated.

CHAPTER 5

ANTIOXIDANT ACTIVITY OF TWELVE SELECTED PLANT SPECIES

5.1 Introduction

Antioxidants are a group of compounds of enormous interest for the biochemists and pharmaceutical industries and are unknown for their capacity to diminish harm, resulting from some reactive species of oxygen and nitrogen (Ayoub et al., 2017). Antioxidant plays an important role to protect the human body from damage by excessive reactive oxygen species (Aswiri et al., 2018). The presence of antioxidants in plants prevents free radicals from causing diseases like cancer, and cardiovascular disease by inhibiting oxidation at the cellular level (Moonmun et al., 2017). Natural antioxidants are required to cure disorders caused by excessive free radicals (Ahmed et al., 2015). They cure the disorders by suppressing and preventing diseases associated with oxidative stress. Active antioxidant compounds are flavonoids, flavones, lignans and isocatechins, which play an important role in the inhibition of free radicals and oxidative chain reactions within tissues and membranes (Boligon et al., 2012).

Many antioxidant compounds also possess anti-inflammatory, anti-tumour, antimutagenic, anticarcinogenic, antibacterial, antifungal, and antiviral activities (Nazir and Rahman, 2018). The body accumulates the free radicals leading to oxidative stress causing diseases in humans such as cancer, and cardio-vascular disease amongst others. Natural antioxidants from plants are used as potential scavengers of free radicals in the body and protect the body from diseases and aging (Mangole and Afolayan, 2020).

The DPPH method is used for measuring the ability of different compounds to act as free radical scavengers and to evaluate the antioxidant activity of food and beverages. The method is used since it is a rapid, simple, accurate, inexpensive, and convenient method independent of sample polarity for screening many samples for radical scavenging activity. Scavenging activity for free radicals of DPPH has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources due to its shortness of analysis time compared to other methods (Eruygur et

al., 2019). In this chapter, the antioxidant activity of plant extracts will be determined using qualitative and quantitative DPPH free radical scavenging assays.

5.1 Materials and methods

5.1.1 Plant selection and extraction

Plant selection, collection and extraction were described in detail in chapter 3.

5.1.2 Determining antioxidant activity

5.1.2.1 Qualitative DPPH free radical scavenging assay

The qualitative 1,1-diphenyl-2-picrylhydrazyl (DPPH) method was used to determine the antioxidant activities of plant extracts (Fatimi et al., 1993). Ten microliters of each plant extract were loaded on TLC plates and developed using different eluent solvent systems: CEF, BEA and EMW. The prepared TLC chromatograms were visualized under UV light and the compounds were identified. The TLC chromatograms were sprayed with a solution of 0.2% DPPH in methanol and dried in a fume cupboard. The presence of antioxidant compounds was indicated by yellow bands against the purple background.

5.1.2.2 Quantitative DPPH free radical scavenging assay

The antioxidant activity of the plant extracts was evaluated using the free radical-scavenging method (DPPH) as described by Brand-Williams et al. (1995). Various concentrations of plant extracts prepared in methanol were added to 1 ml of methanolic solution of 0.2 mmol/L DPPH and kept in the dark at room temperature for 30 minutes. Methanol was used as a blank and was prepared in the same manner as the plant extracts. L-ascorbic acid (vitamin C) was used as a standard in the quantification of antioxidant activity and was prepared in the same concentration range as the plant extracts. The absorbance of the resulting mixture was read using a spectrophotometer at 517 nm. The percentage of antioxidant activity of both the plant species and the ascorbic acid was calculated using the following formula:

$$\%\ \text{inhibition} = \frac{Ab - Ac}{Ab}\ X100$$

Where: Ab= absorbance of the blank and Ac= absorbance of the samples.

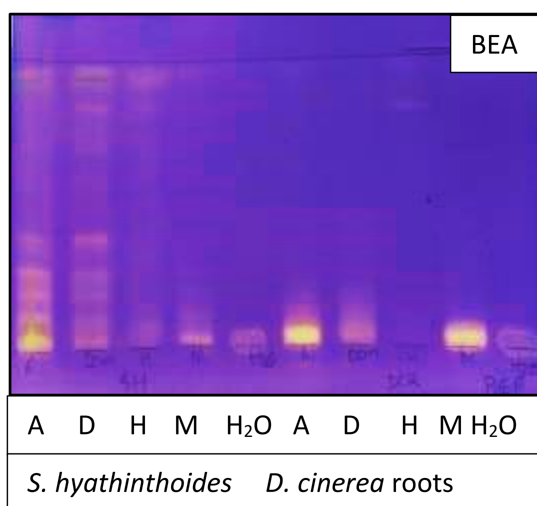
5.2 Results and discussion

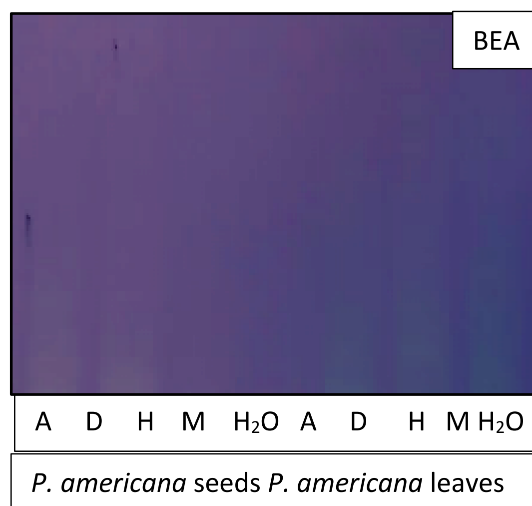
5.2.1 Qualitative DPPH free radical scavenging assay

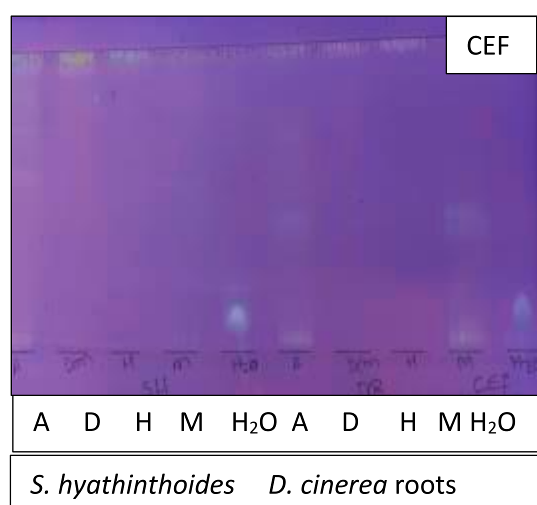
The antioxidant activity of the plant extracts was conducted using the TLC DPPH method. All plant species showed antioxidant activities represented by the inhibition of DPPH radical scavenging Figure 5.1-5.3. The presence of antioxidant compounds was detected by yellow spots against the purple background. More antioxidant compounds were observed in BEA (56) than in EMW (26) and CEF (26) eluent systems. The best antioxidant activity was observed in the non-polar of tested extracts (Adebayo et al., 2019). However, all the solvent systems showed strong antioxidant compounds in the five plant species evaluated (Mamabolo et al., 2017). The retention factor values are presented in Tables 5.1-5.3.

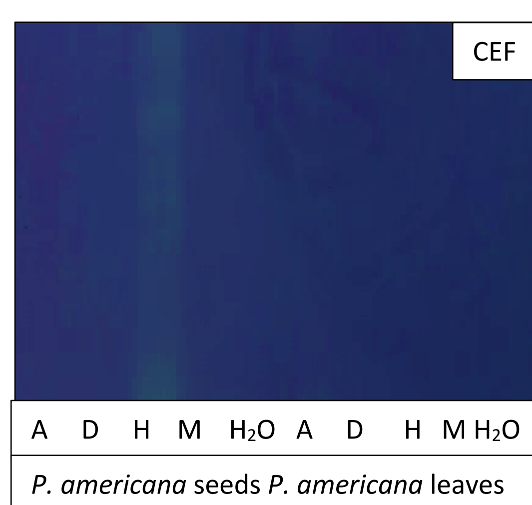
Of the extracts evaluated, the acetone extracts of *S. hyacinthoides* displayed more antioxidant compounds. Acetone extracts have more antioxidant compounds than other solvent extracts because acetone extracts both polar and non-polar compounds (Eloff, 1998a). A compound with a similar R_f value of 0.15 was observed in acetone, dichloromethane, and hexane extracts of *P. americana* seeds and *P. americana* leaf separated in the BEA solvent system. It was found that acetone and methanol extracts of *P. americana* possess antioxidant activity (Bertling et al., 2007). Furthermore, the phytochemical constituents such as vitamin C, alkaloids, flavonoids, steroids, and triterpenoids presence in *P. americana* fruit can reduce the potential risk of various diseases (Rafique and Akhtar, 2018).

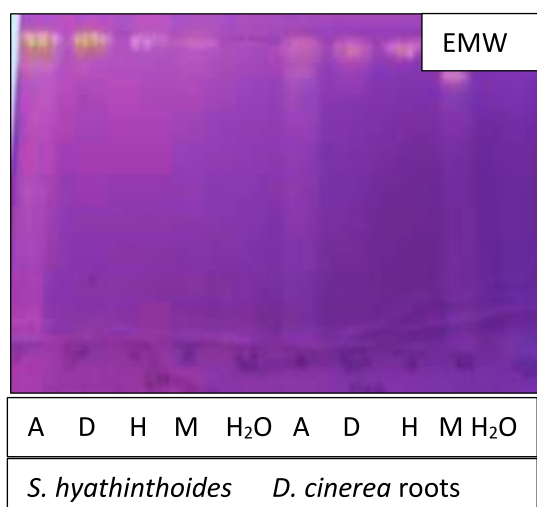
In TLC chromatograms separated with CEF, two compounds were observed in the aqueous extracts of *K. longiflora*. All the plant species evaluated revealed noteworthy antioxidant activity including *K. longiflora* (Asong et al., 2019). The antioxidant activity of the plant extracts might be attributed to the presence of different secondary metabolites such as terpenoids, alkaloids, saponins and tannins present in the plant extracts.











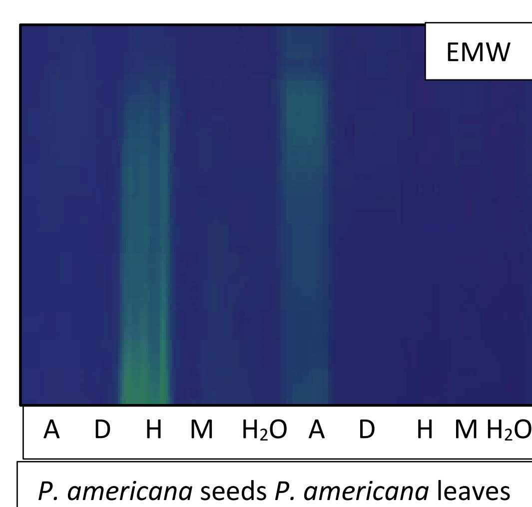
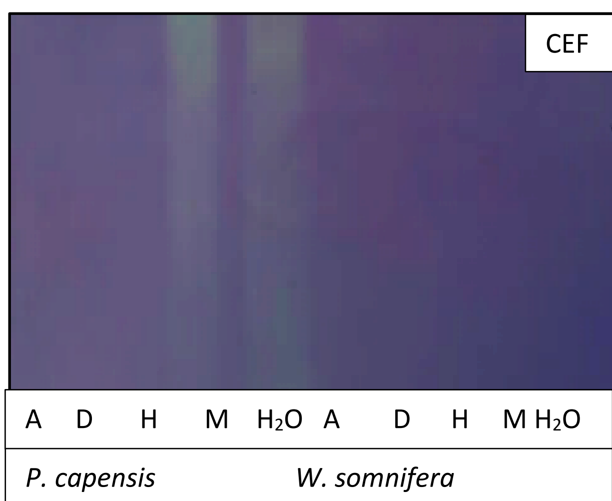
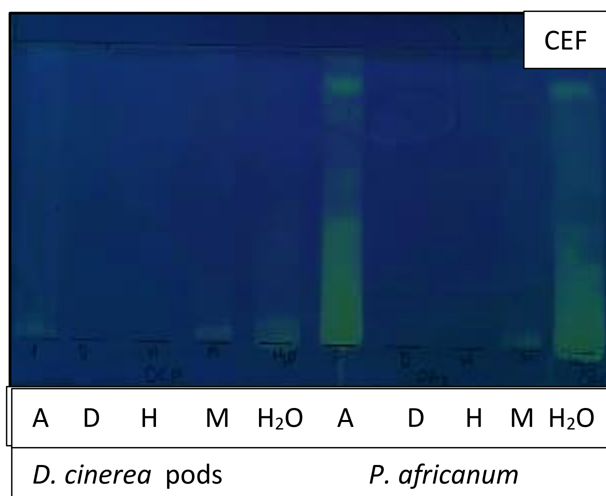
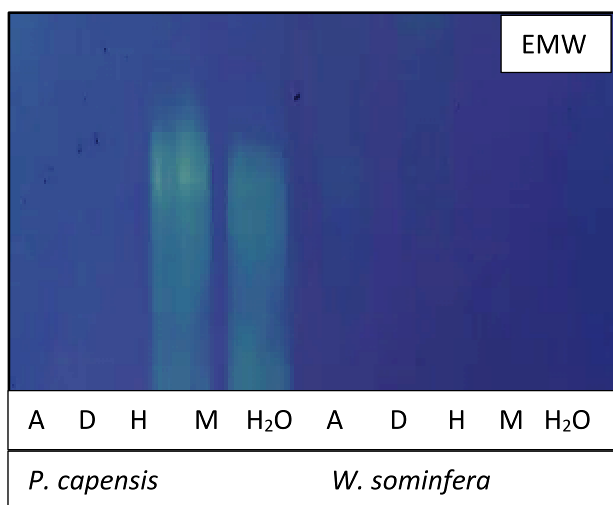


Figure 5.1 TLC chromatograms of *S. hyacinthoides*, *D. cinerea* roots, *P. americana* seeds and *P. americana* leaves, extracted with acetone, dichloromethane, hexane, methanol, and water. Developed in BEA, CEF and EMW, Sprayed with 0.2% DPPH in methanol.







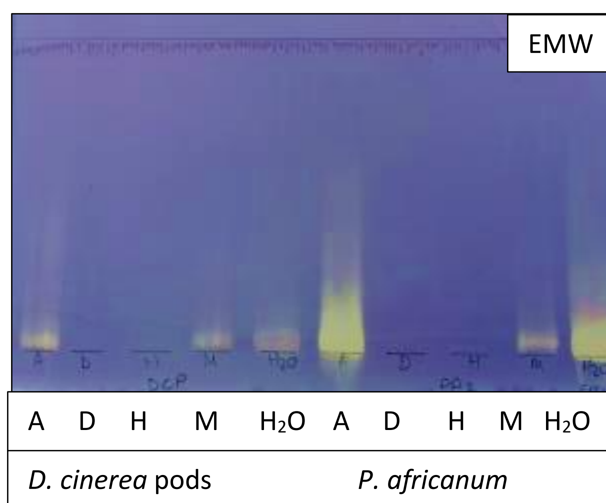
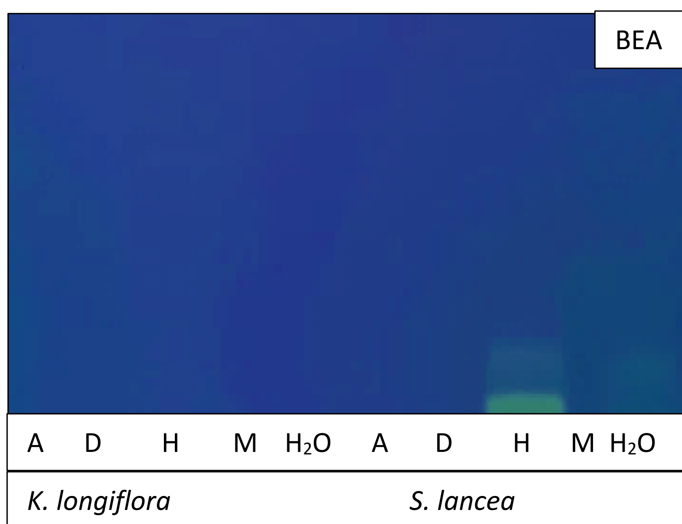
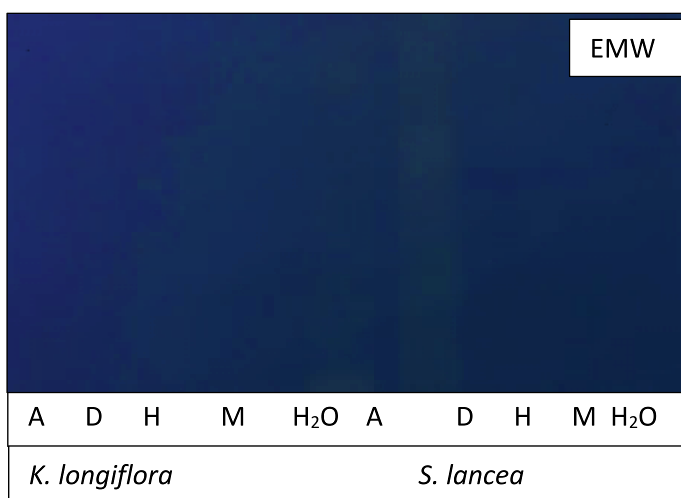


Figure 5.1 TLC chromatograms of *P. capensis, W. sominfera*, *D. cinerea* pods and *P. africanum* extracted with acetone, dichloromethane, hexane, methanol, and water. Developed in BEA, CEF and EMW, Sprayed with 0.2% DPPH in methanol.





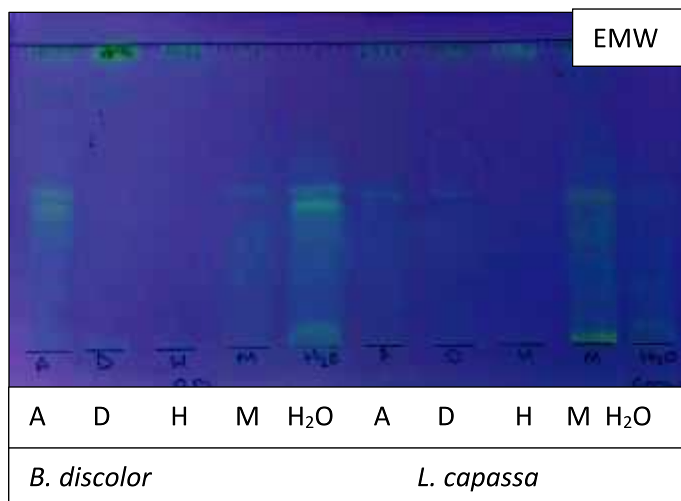


Figure 5.2 TLC chromatograms of *K. longiflora, S. lancea, B. discolor* and *L. capassa* extracted with acetone, dichloromethane, hexane, methanol, and water. Developed in BEA, and EMW, Sprayed with 0.2% DPPH in methanol.

Table 5.1 R_f values of plant species extracted by (acetone, dichloromethane, hexane, methanol, and water) and developed in, BEA and sprayed with DPPH solution.

Solvent systems		BEA				
Plant species	R_f values	Extractants				
		A	D	H	M	H_2O
S. hyacinthoides	0.08	√		√	√	
	0.14	√	√	√	√	
	0.18	√	√			
	0.22					
	0.26	√	√			
	0.27	√	√			
	0.34	√	√			
	0.36	√	√			
	0.7	√	√			
	0.89	√	√	√		
P. americana (S)	0.15	√	√	√	√	
	0.29	√	√	√		
	0.44	√	√	√		
P. americana (L)	0.15	√	√	√		
W. somnifera	0.16	√	√	√	√	
S. lancea	0.12	√	√	√		
B. discolor	0.2	√	√			
	0.3		√			
	0.92		√			
L. cappasa	0.2	√	√	√		
	0.92		√			
M. balsamina	0.19	√	√	√	√	

Table 5.2 R_f values of plant species extracted by (Acetone, Dichloromethane, hexane, methanol, and water) and developed in, CEF and sprayed with DPPH solution.

Solvent system		CEF				
Plant species	R_f values	Extractants				
		A	D	H	M	H_2O
K. longiflora	0.48					√
	0.6					√
S. lancea	0.3					√
P. africanum	0.8	√				√
B. discolor	0.79	√				√
L. cappasa	0.63					√
P. americana	0.92				√	
S. hyacinthoides	0.31	√	√			
	0.49	√	√			
	0.64	√				
	0.12				√	
	0.87				√	
D. cinerea roots	0.12					√
	0.85				√	
	0.87	√				

Table 5.3 R_f values of plant species extracted by (Acetone, Dichloromethane, hexane, methanol, and water) and developed in, EMW and sprayed with DPPH solution.

Solvent system		EMW				
Plant species	R_f values	Extractants				
		A	D	H	M	H2O
B. discolor	0.45	√				√
	0.51	√			√	√
L. cappasa	0.45	√	√		√	
	0.51	√				
W. somnifera	0.26					√
P. capensis	0.58				√	√
	0.64	√				
S. hyacinthoides	0.13					√
	0.63	√				
D. cinerea roots	0.13					√
	0.37	√			√	
	0.46	√			√	
P. africanum	0.13	√				
	0.25					√
	0.63	√				√
S. lancea	0.53	√				
	0.93	√			√	

5.2.2 Quantitative DPPH free radical scavenging assay

The free radical scavenging activity assay (DPPH) was used to quantify the antioxidant activity of the plant extract. L-ascorbic acid was used as a positive control. Three plant species were selected for quantification since they had shown good antioxidant activity on the qualitative assay. The results are presented in Figure 5.4-5.6 as the percentage of inhibition. The values used are the mean of triplicates ± standard deviation.

Methanol, hexane, and water extracts of *L. capassa* revealed good antioxidant activity against DPPH by having a high percentage of inhibition compared to other solvents. Dichloromethane extracts of *L. capassa* had the lowest antioxidant activities with a low percentage of inhibition. The leaf methanol, DCM, and water extracts of *S. hyacinthoides* inhibited good antioxidant activity. Similarly, the leaf extracts of *S. hyacinthoides* revealed good activity by exhibiting over 80% DPPH activity. Acetone

and methanol extracts from the same plant exhibited a high percentage of DPPH scavenging activities (Aliero et al., 2008).

Noticeably, all the extracts of *P. africanum* had possess antioxidant activity against the DPPH, with some extractants having good activity than the ascorbic acid. The synergistic effects on the plant extracts may enhance their antioxidant activity. The presence of antioxidant activity in *P. africanum* might be due to the presence of polyphenols. The root and bark polar extracts have shown high antioxidant activity (Bizimenyera et al., 2005). In addition, acetone, and methanol extracts of *P. africanum* revealed good antioxidants against DPPH compared to other plant species and ascorbic acid (Masuku et al., 2020). Extracts of *P. africanum* had the best antioxidant activity using DPPH and ABTS assay (Adebayo et al., 2015).

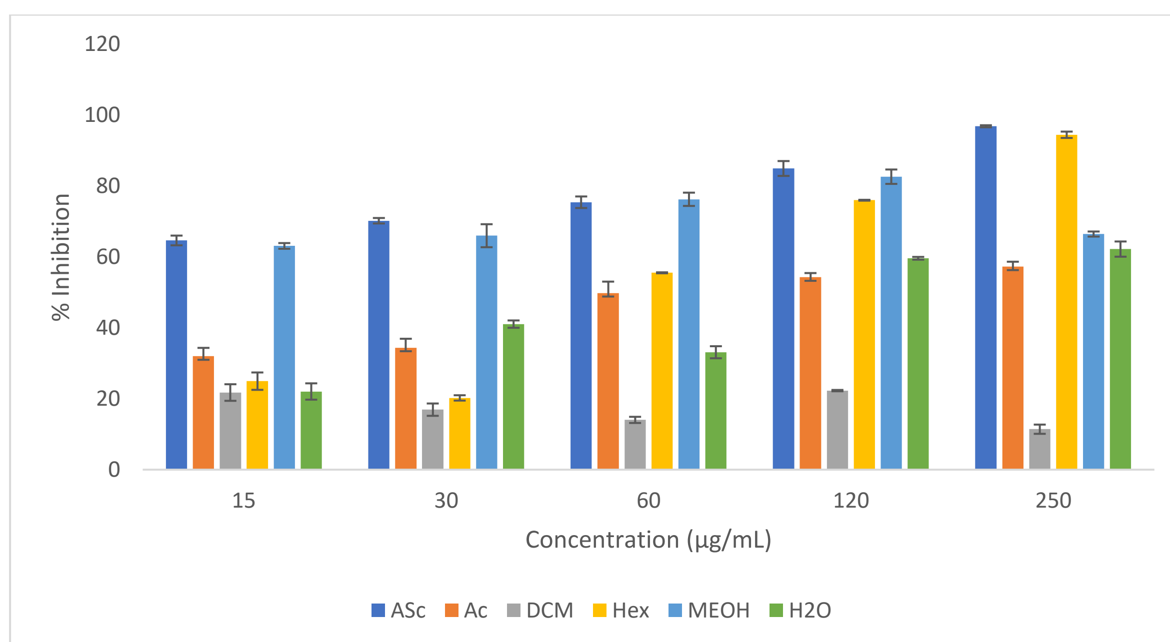


Figure 5.3 Percentage inhibition antioxidant activity of *L. capassa* crude extracts, Ascorbic acid (Vit C) was used as a positive control.

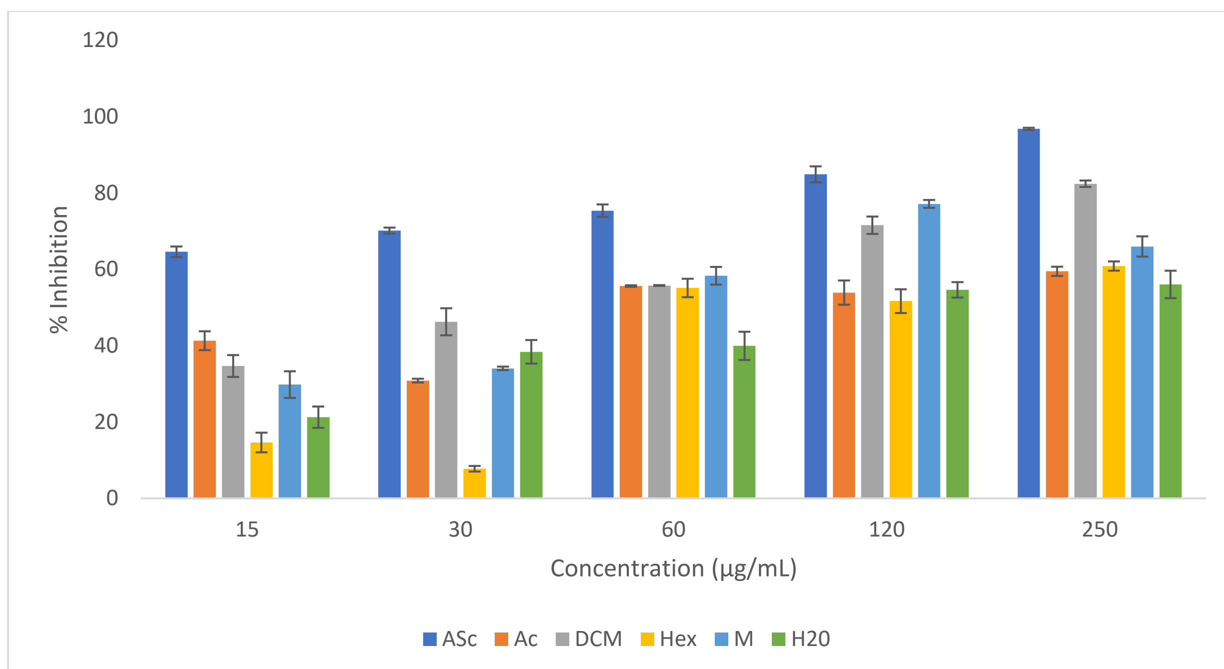


Figure 5.4 Percentage inhibition antioxidant activity of *S. hyacinthoides* crude extracts, Ascorbic acid (Vit C) was used as a positive control.

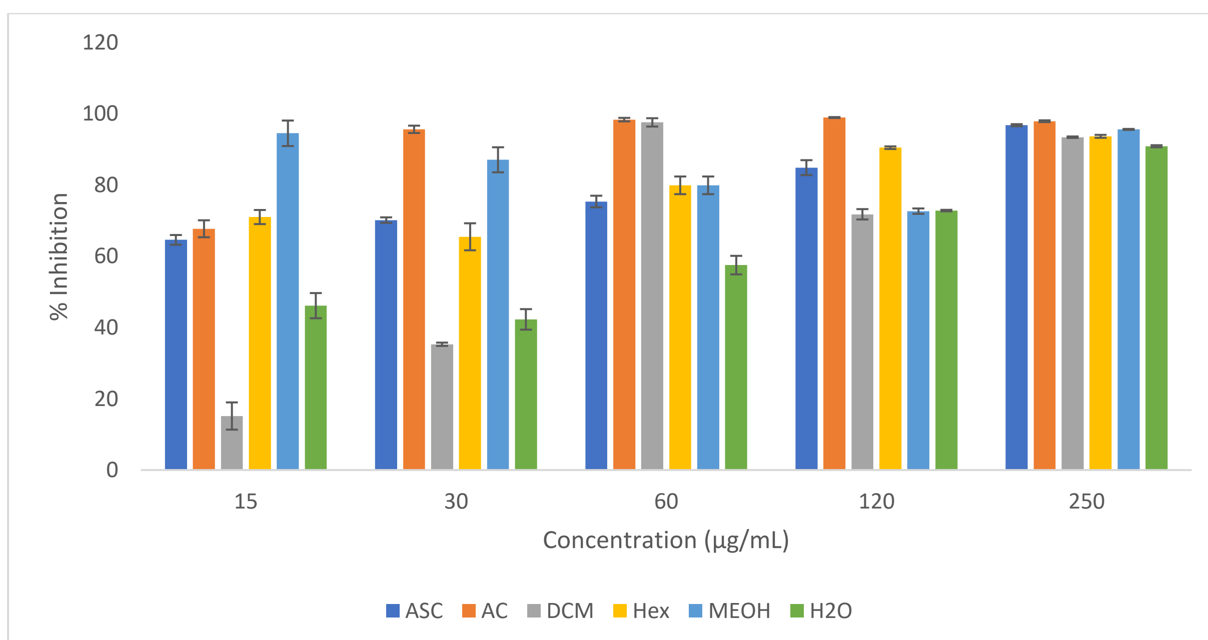


Figure 5.5 Percentage inhibition antioxidant activity of *P. africanum* crude extracts, Ascorbic acid (Vit C) was used as a positive control.

5.3 Conclusion

Most plant species investigated displayed noteworthy antioxidant activity, which provides scientific evidence for their utilisation by traditional health practitioners to treat diseases possibly including fungal infections. As a result, plant species with antioxidant compounds could be used with a wider potential use than only for antifungal drugs. Since many secondary metabolites have antimicrobial and antioxidant activity. The antioxidant presence in plant species investigated in this study means that there is a possible synergism. Strong antioxidant compounds were observed in some plant extracts developed in BEA, suggesting that antioxidant compounds can be isolated based on the polarity of the solvents. Plant extracts of P. *africanum* showed strong antioxidant activity by inhibiting DPPH, compared with the standard ascorbic acid. The results from this study show that the extract can be used as an easily accessible source of natural antioxidants.

In the next chapter, the selection of plant species for screening and isolation of active compounds will be discussed.

CHAPTER 6

PLANT SELECTION FOR FURTHER SCREENING AND ISOLATION OF BIOACTIVE COMPOUNDS

6.1 Introduction

Porras et al. (2021) claim that 95 % of humans in the growing global population depend on herbal medicine as a source of primary healthcare to treat various infections in humans. The verified scientific effectiveness of plant extracts in long-standing conventional medicinal practices throughout the world is crucial (Gurib-Fakim, 2006), to their ease of access and the ability for synergistic interactions (Caesar and Cech, 2019). In addition, plants contain certain compounds that could be used to derive potential drugs to combat fungal infections (Christenhusz and Byng, 2016). The pharmaceutical enterprise is in the know of this capacity, and future added screening programmes for vegetation from tropical areas.

The ethnobotanical technique is probably one of the important methods that may be implemented in selecting plants for pharmacological studies. Since the random selection of medicinal plants may yield relatively few new drug possibilities. The ethnobotanical method assumes that the indigenous knowledge of the use of medicinal plants can provide strong leads to the biological activities of the medicinal plants. Plants were selected based on the ethnopharmacological use by traditional health practitioners and the local people.

The problem of antibiotic resistance necessitates creative and revolutionary approaches, from chemical identity and evaluation of biological activities of medicinal plants. Plant secondary metabolites constitute a promising supply of antimicrobial lead compounds that would assist in drug discovery due to the current antibiotic resistance. The overuse and misuse of antibiotics in human and animal health and the shortage of improvement of recent antibiotics cause problems in primary health care (Porras et al., 2021). Plant natural products can act as antimicrobials through the diverse mode of action. (Harvey et al., 2015).

6.2 Selection of plant species for screening

Twelve plant species used to treat fungal infections were selected from the list of plant species identified on a database (unpublished data) of the University of Limpopo. The plant species selected for screening were selected based on the information provided by traditional health practitioners on the treatment of ailments related to the symptoms of the three fungal pathogens used in this study. Symptoms related to ailments caused by *A. fumigatus*, *C. albicans* and *C. neoformans* were considered for selection. The availability and accessibility of the plant species selected were also checked.

6.3 Selection of plant species for isolation

Two plant species (*L. capassa* and *S. hyacinthoides*) had promising results from chapter 4 with good MIC, and more compounds in the bioautography assay. These plant species were selected further for the isolation of antifungal compounds. The MIC value which is commonly used as an indicator of antimicrobial potency was used for the selection of plants for isolation. The MIC is the lowest concentration of an antimicrobial agent that inhibits the growth of the microorganism.

Searching for new compounds in plants implies a screening of extracts for the presence of novel bioactive compounds that are normally remoted so that one can continue to structure elucidation and carry out similar organic and toxicological testing. The route which leads from the intact plant to its natural materials is long (Hostettmann and Terreaux, 2000). Target compounds, both active in a single bioassay and detected as potential new chemical entities, will then be isolated and their structure elucidated. This is achieved through fractionation using different chromatographic techniques.

CHAPTER 7

FURTHER INVESTIGATION OF TWO PLANT SPECIES SELECTED FOR ISOLATION OF ACTIVE COMPOUNDS

7.1 Introduction

Almost 80 % of the world ‘s population depends on herbal drug treatments. Plants contain an extensive variety of chemical compounds that can be used to treat chronic as well as infectious diseases (Ingle et al., 2017). Bioactive components present in medicinal plants may play an important role as a source of therapeutic agents for various diseases (Van Wyk and Wink, 2015). The increasing interest in medicinal plants to find novel therapeutic agents is motivated by the increasing development of fungal resistance to the currently available drugs. Some drugs are harmful and have side effects.

Extraction is an important step in determining the structural integrity of the bioactive compounds present in the plant (Zhu et al., 2017). Soxhlet extraction has been used to obtain various bioactive compounds from plant materials (Li et al., 2019). However, the method takes time and has high energy consumption and may destroy sensitive metabolites due to the high temperature used (Ramos et al., 2019). During extraction, the choice of the solvent is crucial, since it selectively extracts different compounds (Kotze and Eloff, 2002).

7.2 Selected plant species for solvent-solvent fractionation

S. hyacinthoides, and *L. capassa* (chapter 4) were selected for further phytochemical analysis, isolation of antifungal compounds, and determining antifungal and antioxidant activity.

S. hyacinthoides is a succulent perennial herb plant (Figure 7.1) belonging to the family Asparagaceae. It is commonly known as “*Savha”* by Vhavenda-speaking people. The synonym of *S. hyacinthoides* is *D. hyacinthoides* (L.) Mabb.

However, *S. hyacinthoides* is the accepted name. The plant is native to East, central and southern Africa. However, other countries have introduced it as an ornamental and fiber crop (Newton, 2018). The leaves of *S. hyacinthoides* are rigid, fibrous, flat, and arising from a horizontal underground rhizome and they have pale green bands leathery and dull green and pale green bands (Maroyi, 2019).

The plant is used traditionally as medicine to treat various ailments by traditional health practitioners and the local people. The leaves and roots of *S. hyacinthoides* have the potential as medicine to treat various ailments such as ear infections, respiratory problems, hemorrhoids, fever, magical purposes, skin infections toothache and sexually transmitted diseases. Several compounds have been identified in the plant species *S. hyacinthoides*. The steroids 25S-ruscogenin and 1β,3β-dihydroxy-5,16-prenadien-20-one was identified by Gamboa-Angulo et al. (1996) from the leaves of *S. hyacinthoides*.

Various biological activities of *S. hyacinthoides* have been reported from the leaf, root, and rhizome extracts. Antifungal activity was reported by Sultana et al. (2011) against *Candida albicans*. Akhalwaya et al. (2018) evaluated the antifungal activity of leaf extracts against candida species. Antibacterial activities of the aqueous, ethanol and hexane leaf extracts of *S. hyacinthoides* were investigated against *Escherichia coli* (McGaw et al., 2000). The antioxidant activities of acetone and methanol leaf and root extracts of *S. hyacinthoides* were evaluated using 1,1-diphenyl-2-picrylhydrazyl free radical (DDPH) scavenging assay. Several compounds have been isolated from the leaves of *S. hyacinthoides*. Sultana et al. (2011) identified β-sitosterol, daucosterol and isokaemferide which are steroids and flavonoids.

Lonchocarpus capassa is a tree (Figure 7.2) belonging to the family Fabaceae, it is commonly known as *Mufhanda* in Tshivenda. The plant is used to treat sexually transmitted diseases. The leaves are used to treat respiratory problems, chest pains fever, and sexually transmitted diseases. The plant has been reported to

have antifungal, and antibacterial activities (Kilonzo et al., 2017). However, no compounds have been isolated from the plant species.

The plants of the genus *Lonchocarpus* have been reported to have the ability to produce compounds such as alkaloids, triterpenoids derived from benzoic acids, and flavonoids. Flavonoids are phenolic compounds consisting of two benzene rings linked through a heterocyclic pyrimidine ring. Furthermore, flavonoids have been reported to have anti-inflammatory, antiallergic, antitumor, antioxidant and antimicrobial activities (Vasconcelos et al., 2014). The antifungal and cytotoxicity activities of *L. capassa* were reported by Kilonzo et al. (2016).

In this chapter, solvent-solvent fractionation will be carried out using solvents of various polarities. The biological activity of the fractions will be determined using serial dilution and bioautography assays against the selected fungal pathogens.

Figure 7.1 *S. hyacinthoides* A- fresh plant B- dried leaves.

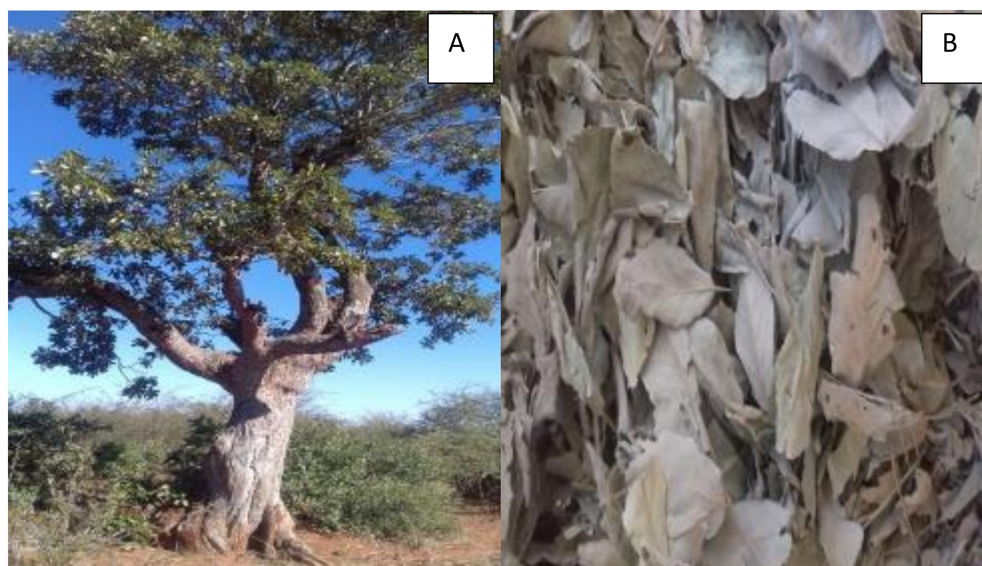


Figure 7.2 *L. capassa* A- fresh whole plant B- dried leaves.

7.3 Materials and methods

7.3.1 Serial exhaustive extraction

A mass of 500g of *S. hyacinthoides* plant material was serially extracted with 1500 ml of solvents of varying polarities, starting with hexane, dichloromethane, acetone, ethyl acetate and methanol. The plant material was washed three times. The extracts were filtered using Whatman No. 1 filter paper into pre-weighed beakers. The solvents were concentrated using a rotary evaporator at 40°C. The concentrated solvents were transferred into pre-weighed beakers and placed under a stream of cold air in a fume hood for complete dryness.

7.3.2 Phytochemical analysis

The chemical components of the plant extracts were analysed using TLC plates. The method is described in chapter 3 section 3.2.3.

7.3.3 Antifungal activity

7.3.3.1 Micro-dilution assay

The microplate method described in chapter 4 section 4.2.1.3 was used to determine the minimum inhibitory concentration (MIC) of the plant extracts.

7.3.3.2 Bioautography assay

The number of active compounds present in the plant extracts was determined using the bioautography method described in chapter 4 section 4.2.1.4.

7.3.4 Solvent-solvent fractionation

Solvent-solvent fractionation method described by Adaramola and Onigbinde, (2017) was used to separate fractions. The acetone crude extracts of *L. capassa* were partitioned using various solvents such as chloroform, n-butanol, acetone, and water. The acetone crude extracts were redissolved in acetone and were transferred into a separatory funnel. A 500 ml of chloroform was added into the separatory funnel and shaken then allowed to settle. The two layers were formed, and the bottom of the separatory funnel was opened to collect the aqueous fraction. The chloroform fraction was collected. Then an equal volume of butanol

was added to the water fraction into the separatory funnel and the butanol fractions were collected. In each step, the fractions were tested for antifungal activity using a serial dilution assay. The bioautography assay was used to determine the number of active compounds present in the fractions after each stage of solvent–solvent fractionation.

7.3.5 TLC fingerprinting

The chemical constituents of the fractions were determined using TLC plates described in section 3.2.3.

7.3.6 Antifungal activity of the fractions

7.3.6.1 Serial dilution

The microplate method was used to determine the MIC of fractions the method was described in section 4.2.1.3.

7.3.6.2 Bioautography assay

To determine the antifungal activity of the extract and fractions a TLC bioautography was used. The method is described in detail in chapter 4 under section 4.2.1.4.

7.4 Results and discussion

7.4.1 Mass extracted and phytochemical analysis

Mass extracted from 500 g of the two selected plant species (*S. hyacinthoides* and *L. capassa*) is presented in Figure 7.3. The plant materials were serially extracted with solvents of various polarities, starting with hexane, dichloromethane, acetone, ethyl acetate and methanol. The highest quantity of plant materials was extracted from methanol (5.6%), then followed by acetone (2.3%), ethyl acetate (1.2%), hexane (0.5%) and dichloromethane (0.3%). Methanol is used as an extractant because it has the ability to extract metabolites of varying polarities from plants. Acetone was used as an extractant since, it was reported that acetone can extract both polar and non-polar compounds from plants and it is not toxic to microorganisms in bioassays (Eloff, 1998a).

The number of chemical components was determined using the TLC plates and visualised under UV light. BEA extracted more compounds than other eluent systems (CEF and EMW) this indicates that the active compounds are relatively non-polar. No compounds were visible in EMW solvent system

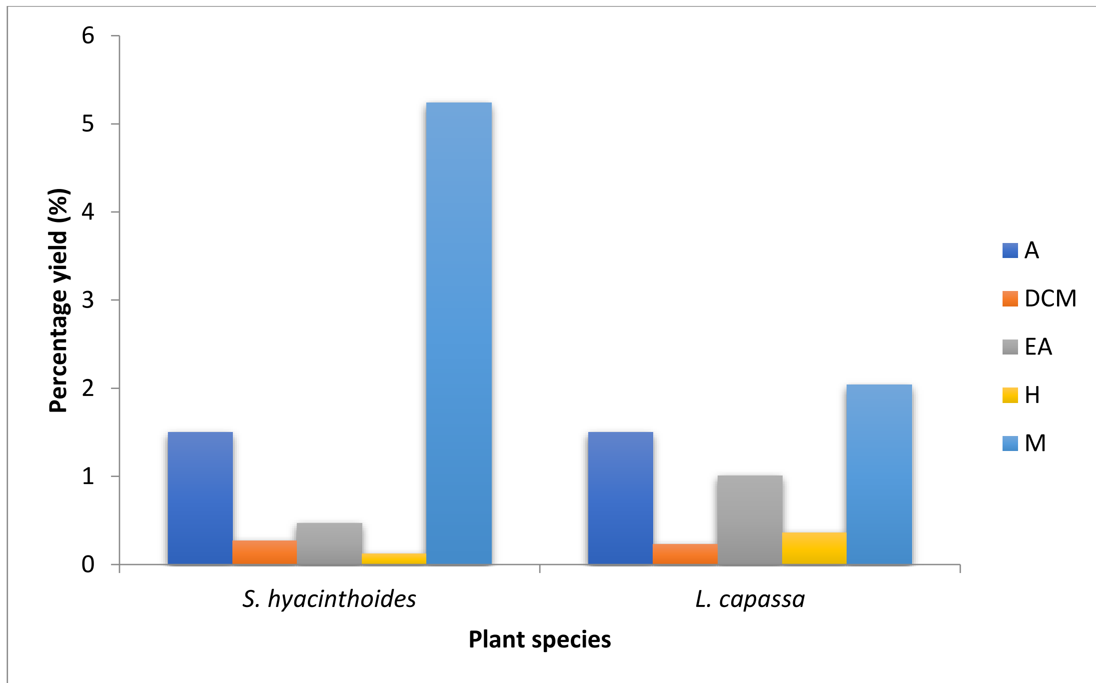


Figure 7.3 Percentage of quantity of plant material extracted with acetone, dichloromethane, ethyl acetate, hexane, and methanol in percentage (%) from 500 g of *Lonchocarpus capassa* and *Sansevieria hyacinthoides*

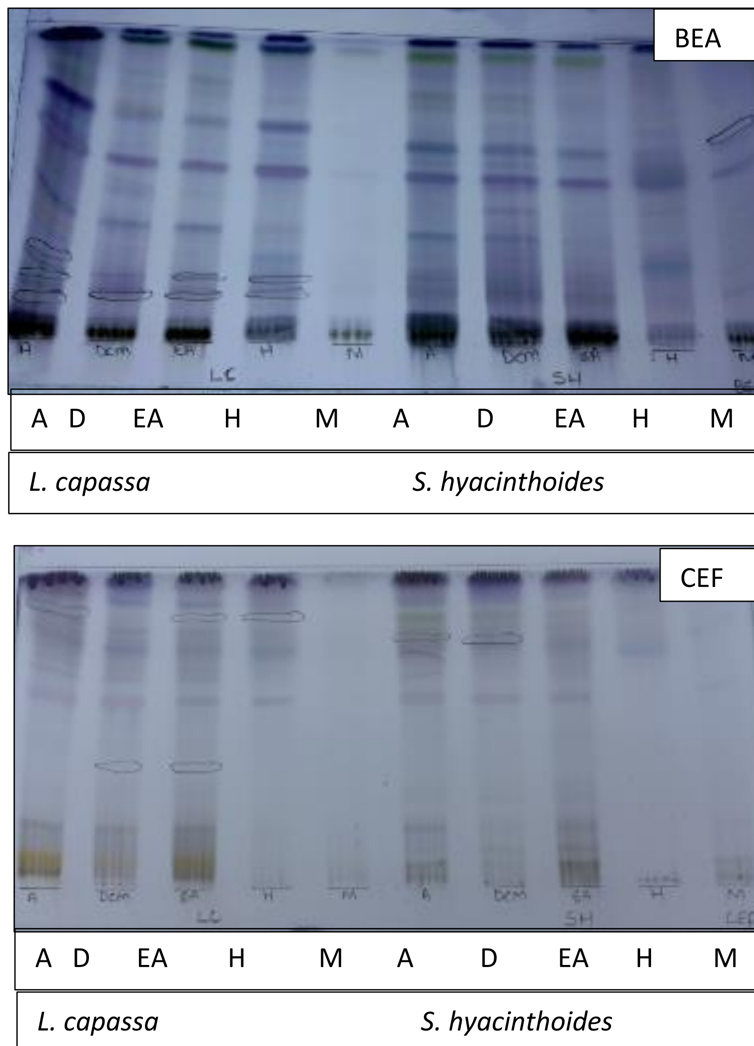


Figure 7.4 TLC chromatograms of *L. capassa* and *S. hyacinthoides* extracted with acetone, dichloromethane, ethyl acetate, hexane, and methanol developed in BEA, CEF, and EMW, Sprayed with Vanillin sulphuric acid reagent spray.

7.4.2 Antifungal activity of the crude extracts

7.4.2.1 Micro-dilution method

The micro-dilution assay was used to determine the minimum inhibitory concentrations of the plant extracts against the tested pathogens. The results are presented in Table 7.1. Acetone and methanol leaf extracts of *L. capassa* had excellent activity against *C. neoformans* and *C. albicans* with an MIC value of 0.02 mg/ml. Dichloromethane and hexane extracts had good activity against the tested microorganisms with MIC values ranging between 0.02 mg/ml and 0.31 mg/ml.

Noteworthy results were observed in acetone leaf extracts of *S. hyacinthoides* against *C. albicans* and *C. neoformans* with an MIC value of 0.02 mg/ml. Dichloromethane, hexane and methanol extracts of *S. hyacinthoides* inhibited the growth of the tested pathogens with MIC values between 0.02 mg/ml and 0.31 mg/ml. Acetone and ethyl acetate extracts of both *L. capassa* and *S. hyacinthoides* showed good activity against *A. fumigatus* with MIC values between 0.16 mg/ml and 0.31 mg/ml. Hexane extracts of both plant species had poor activity against *A. fumigatus* with an MIC value of 2.5 mg/ml. The aqueous extracts showed good MIC values against *C. neoformans*, *C. albicans* and *A. fumigatus* which support the use of water as a good extractant by traditional health practitioners.

Table 7.1 Minimum inhibitory concentration (MIC) values of the selected plants species against the *C. neoformans* and *C. albicans*.

Fungi	Time (h)	MIC (mg/ml)										AMP B
		Lonchocarpus capassa					*Sanseviera hyacinthoides*					
		Extractants										
		A	DCM	EA	H	M	A	DCM	EA	H	M	
	24	**0.02**	**0.02**	1.25	0.08	**0.02**	**0.02**	**0.02**	0.63	0.31	0.31	0.02
	48	**0.02**	**0.08**	1.25	0.08	**0.02**	**0.02**	0.39	0.63	0.31	0.31	0.02
C. neoformans	72	**0.02**	**0.08**	1.25	0.08	**0.02**	**0.02**	0,39	0.63	0.31	0.31	0.02
	24	**0.02**	**0.02**	0.31	0.31	**0.02**	**0.02**	**0.02**	0.08	**0.02**	**0.02**	0.02
	48	**0.02**	**0.02**	0.31	0.31	**0.02**	**0.02**	**0.02**	0.08	**0.02**	**0.02**	0.02
C. albicans	72	**0.02**	**0.02**	0.31	0.31	**0.02**	**0.02**	**0.02**	0.08	**0.02**	**0.02**	0.02
	24	0.31	0.63	0.31	2.5	1.25	0.16	0.63	0.31	1.25	0.63	0.02
	48	0.31	0.63	0.31	2.5	2.5	0.16	1.25	0.31	2.5	0.63	0.02
A. fumigatus	72	0.31	0.63	0.31	2.5	2.5	0.16	1.25	0.31	2.5	2.5	0.02
Average		0.12	0.24	0.62	0.96	0.74	0.07	0.44	0.34	0.80	0.53	0.02

7.4.2.2 Bioautography assay

Bioautography was used to determine the number of active compounds in the plant extracts. Three compounds with similar R_f values (0.13; 0.5 and 0.63) were observed in acetone, dichloromethane, ethyl-acetate, hexane, and methanol extracts of *S. hyacinthoides* and *L. capassa* against *C. albicans* separated in BEA eluent systems (Figure 7.5 and 7.6).

Similar compounds with an R_f value of 0.25 was visible in acetone, dichloromethane, ethyl-acetate, and hexane extracts of *L. capassa* against *C. neoformans* in TLC chromatograms developed in the CEF solvent system. A compound with the same R_f value was observed in acetone, dichloromethane, ethyl-acetate, hexane, and methanol extracts of *S. hyacinthoides* against *C. neoformans* separated in the CEF solvent system (Figure 7.3).

An active compound with an R_f value of 0.5 was observed in acetone, dichloromethane, ethyl-acetate, and hexane extracts of *L. capassa* separated in the BEA eluent system against *C. neoformans*. A compound of 0.13 R_f value was visible in acetone, dichloromethane, ethyl-acetate, hexane, and methanol extracts of *S. hyacinthoides* eluted in the BEA mobile phase against *C. neoformans* Figure 7.6.

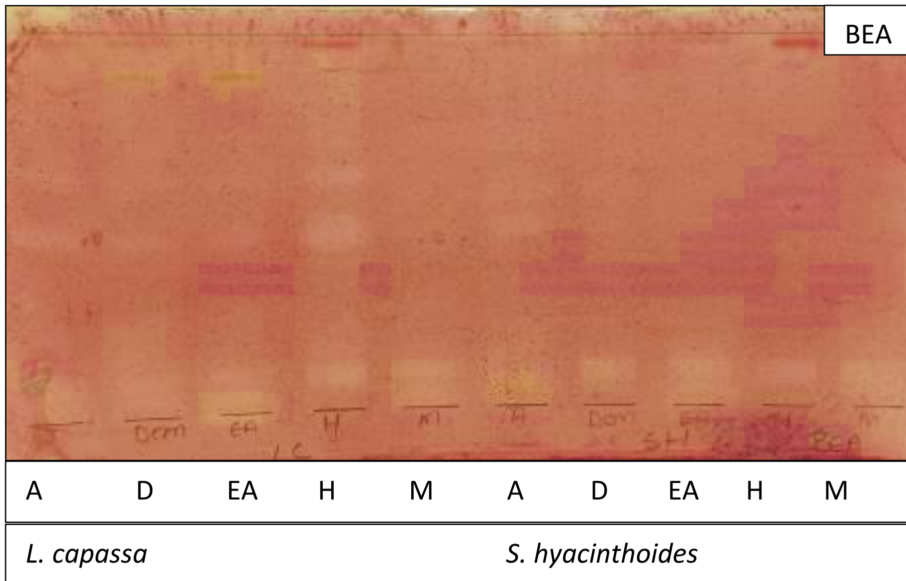


Figure 7.5 TLC chromatograms of *L. capassa* and *S. hyacinthoides,* extracted with acetone, dichloromethane, ethyl acetate, hexane, and methanol. Developed in BEA, Sprayed with *C. albicans*. White areas indicate the

Table 7.2 R_f values of plant species extracted by (acetone, dichloromethane, ethyl acetate, hexane, and methanol) and developed in, BEA and sprayed with *C. albicans.*

BEA eluent system						
		Extractants				
Plant species	R_f values	A	DCM	EA	H	M
L. capassa	0.13	√	√	√	√	√
	0.5	√	√	√	√	√
	0.63	√	√	√	√	√
S. hyacinthoides	0.13	√	√	√	√	√
	0.50	√		√	√	
	0.63	√		√	√	

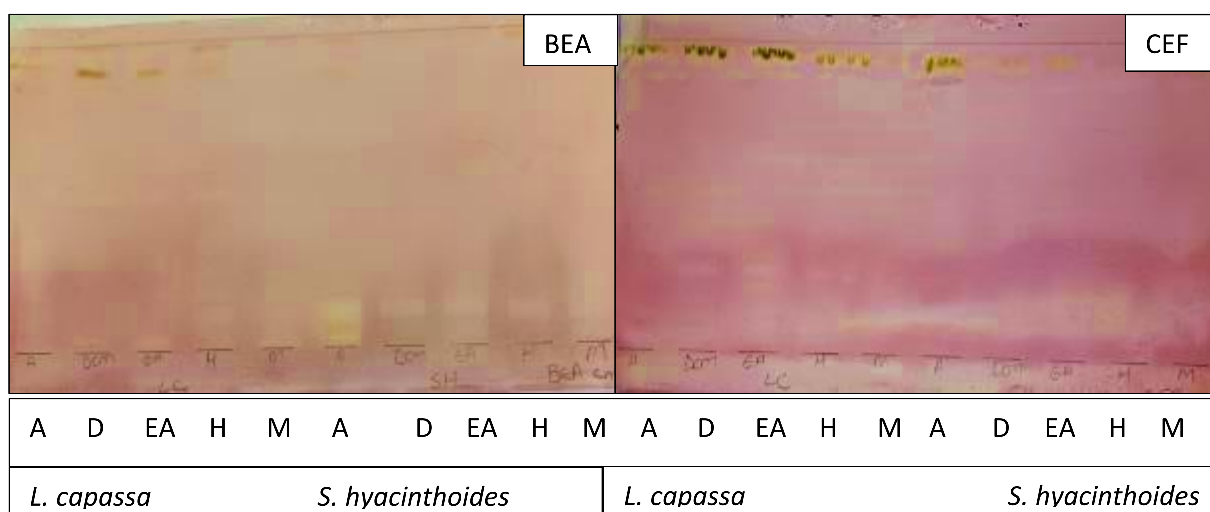


Figure 7.6 TLC chromatograms of *L. capassa* and *S. hyacinthoides*, extracted with acetone, dichloromethane, ethyl acetate, hexane, and methanol. Developed in BEA and CEF, Sprayed with *C. neoformans.*

Table 7.3 R_f values of plant species extracted by (acetone, dichloromethane, ethyl acetate, hexane, and methanol) developed in, BEA and CEF and sprayed with *C. neoformans*.

		Extractants				
Plant species	R_f values		DCM	EA	H	M
			BEA			
L. capassa	0.13				√	
	0.5	√	√	√	√	
S. hyacinthoides	0.13	√	√	√	√	√
			CEF eluent system			
L. capassa	0.25	√	√	√	√	
S. hyacinthoides	0.13	√				
	0.88	√	√	√	√	√

7.4.3 Solvent-solvent fractionation

L. capassa acetone fractions extracted the highest quantity of plant material from 7.41g of acetone crude extracts. Acetone fractions were extracted (80%) as compared to other solvents. The mass extracted in percentages is illustrated in Figure 7.7.

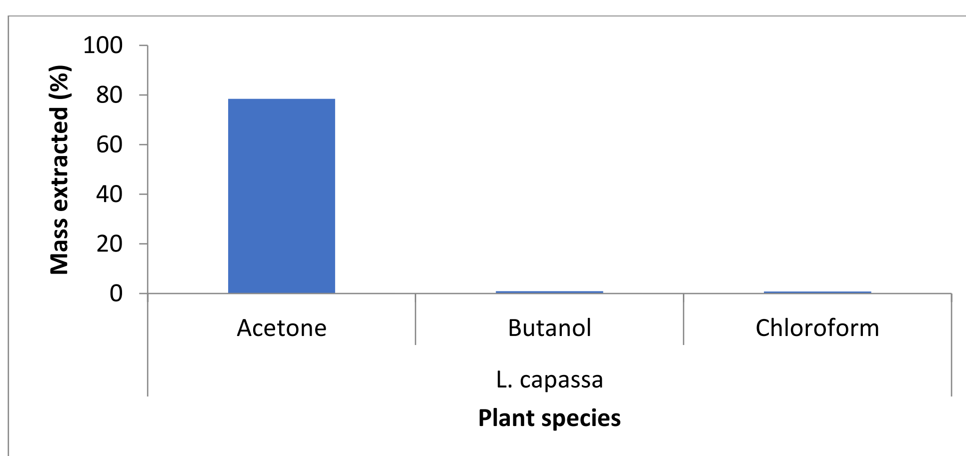


Figure 7.7 Percentage of plant material extracted (%) from 500 g of *Lonchocarpus capassa.*

7.4.4 Phytochemical analysis of fraction

The chemical components of the fractions were determined using the TLC plates and then sprayed with vanillin sulphuric acid reagent spray. More compounds were observed in the BEA solvent system followed by EMW and the least was CEF. This shows that the active compounds were relatively non-polar (Figure 7.8).

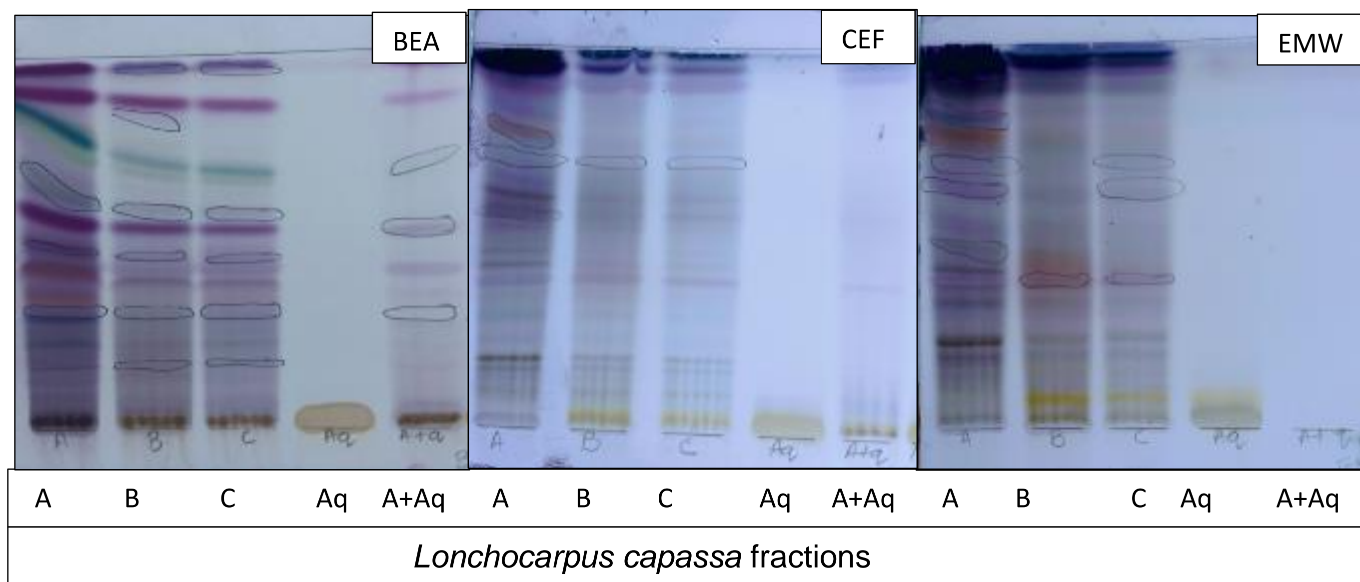


Figure 7.8 TLC chromatograms of *L. capassa* fractions, lines from left to right A-acetone, B-butanol, C-chloroform, Aq- aqueous, and A+Aq- acetone and aqueous fractions. Developed in BEA, CEF, and EMW, Sprayed with Vanillin sulphuric acid reagent spray.

7.4.5 Antifungal activity of the fractions

7.4.5.1 Micro-dilution method

All the extractants from solvent-solvent fractionation showed varying degrees of inhibition by fungal pathogens as presented in Table 7.4. Excellent activity was observed in acetone and butanol fractions of *L. capassa* against *C. neoformans* with an MIC value of 0.02 mg/ml. Poor activity was observed in chloroform fractions against the three tested microorganisms with MIC values ranging between 1.25 mg/ml and 2.5 mg/ml. In contrast, non-polar fractions were more active than the polar fractions from leaf extracts of *Ptaeroxylon obiquum* (Ramadwa et al., 2022).

The water fraction of *L. capassa* had poor activity against all tested microorganisms. Similarly, water fractions showed the poor activity of all the microorganisms investigated (Kotze and Eloff, 2002; Mtunzi et al., 2017). The results are a contradiction with the use of water as extractant by traditional health practitioners.

Acetone fractions of *L. capassa* were the most promising fraction presenting the best yield against the tested microorganisms with the MIC values ranging between 0.02 mg/ml and 0.63 mg/ml. Similarly, the acetone fractions from *B. tomentosa* extracts inhibited good antifungal activity against *C. albicans* with MIC values between 0.019 mg/ml and 2.5 mg/ml (Teodoro et al., 2018). Ramadwa et al. (2019) also found that the polar fractions were not active against the tested microorganisms, and the non-polar fractions (n-hexane and chloroform) had good MIC values. The acetone fraction from *L. capassa* was selected further for the isolation of bioactive compounds using column chromatography.

Table 7.4 Minimum inhibitory concentration (MIC) values of the *Lonchocarpus capassa* fractions against the *C. neoformans, C. albicans* and *A. fumigatus.*

Fungi	Time (h)	MIC (mg/ml)					
		Lonchocarpus capassa					
		Extractants					
		A	B	C	Aq	A+Aq	AMP B
C. neoformans	24	**0.02**	**0.02**	2.5	2.5	**0.02**	2.5
	48	**0.02**	**0.02**	2.5	2.5	**0.02**	2.5
	72	**0.02**	**0.02**	2.5	2.5	**0.02**	2.5
C. albicans	24	**0.16**	0.63	2.5	2.5	2.5	2.5
	48	**0.16**	0.63	2.5	2.5	2.5	2.5
	72	**0.16**	0.63	2.5	2.5	2.5	2.5
A. fumigatus	24	0.63	2.5	1.25	2.5	2.5	2.5
	48	0.63	2.5	1.25	2.5	2.5	2.5
	72	0.63	2.5	1.25	2.5	2.5	2.5
Average		0.27	1.05	2.08	2.5	1.67	2.5

7.4.5.2 Bioautography assay

The bioautography was used to determine the number of active compounds in acetone butanol, chloroform, and water fractions against *C. neoformans, C. albicans,* and *A. fumigatus* as displayed in Figure 7.9-7.11. BEA was the best extractant by separating more compounds in all the pathogens, then followed by the CEF solvent system. The R_f values of the active compounds are presented in Table 7.7. More compounds with different R_f values were observed in acetone fractions (10) followed by chloroform (5) then the least was butanol (4). Three compounds were observed in acetone fraction against *C. neoformans* with R_f values 0.19, 0.71 and 0.77. Previously, it was found that other hexane fractions have the highest number of antibacterial compounds in all the tested microorganisms (Mtunzi et al., 2017).

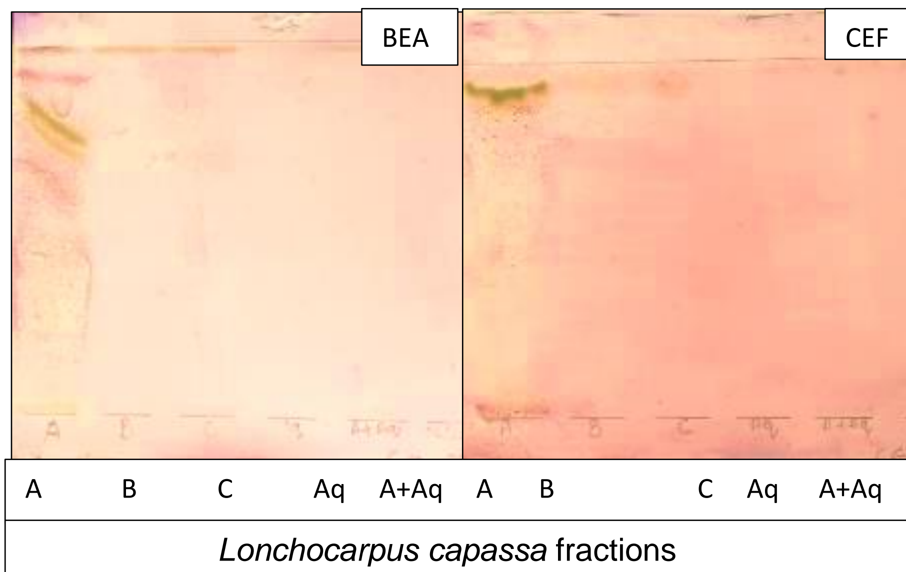


Figure 7.9 TLC chromatograms of *L. capassa* fractions, lines from left to right A-acetone, B-butanol, C-chloroform, Aq- aqueous, and A+Aq- acetone and aqueous fractions. Developed in BEA and CEF, Sprayed with *C. neoformans*. White areas indicate inhibition of fungal growth.

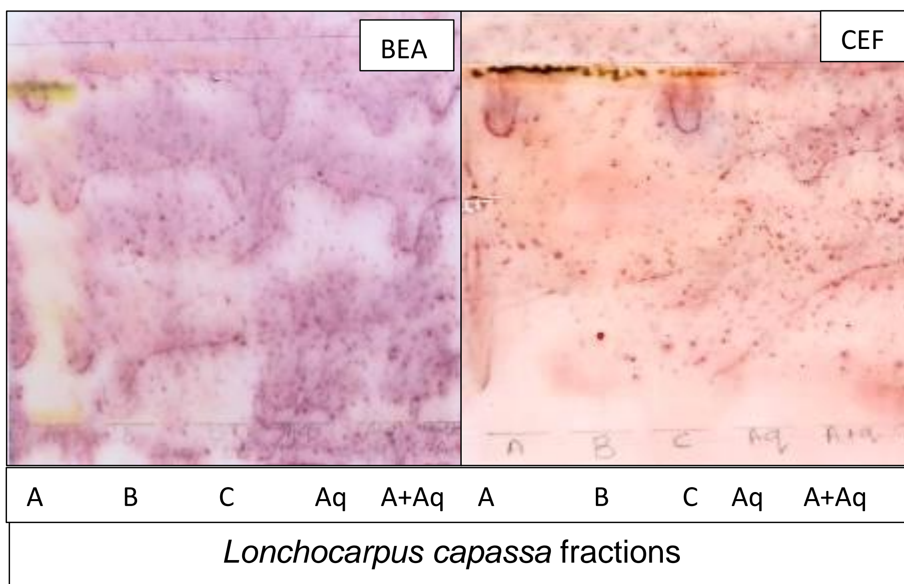


Figure 7.10 TLC chromatograms of *L. capassa* fractions, lines from left to right A-acetone, B-butanol, C-chloroform, Aq- aqueous, and A+Aq- acetone and aqueous fractions. Developed in BEA and CEF, Sprayed with *C. albicans.* White areas indicate inhibition of fungal growth.

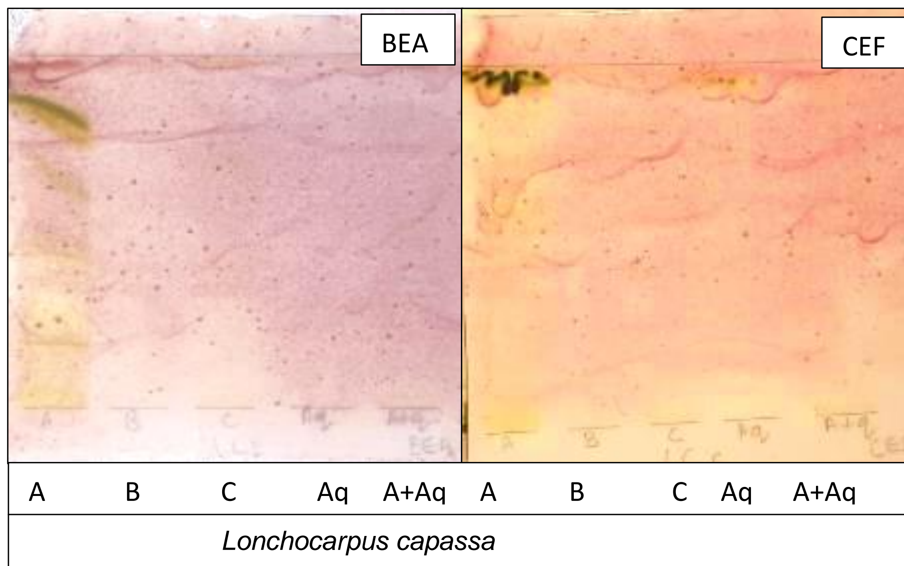


Figure 7.11 TLC chromatograms of *L. capassa* fractions, lines from left to right A-acetone, B-butanol, C-chloroform, Aq- aqueous, and A+Aq- acetone and aqueous fractions. Developed in BEA and CEF, Sprayed with *A. fumigatus.* White areas indicate inhibition of fungal growth.

Table 7.5 R_f values of A-acetone, B-butanol, C-chloroform, and Aq-aqueous, fractions of *L. capassa*. Developed in BEA and CEF, against *C. neoformans, C. albicans* and *A. fumigatus*.

		Extractants							
Solvent systems		BEA				CEF			
Fungal species	R_f values	A	B	C	H20	A	B	C	H_20
C. n	0.15					√			
	0.19					√			
	0.32						√		
	0.44		√	√					
	0.6	√							
	0.63		√	√					
	0.77					√			
	0.81	√							
	0.89	√	√	√			√		
C. a	0.13			√					
	0.19	√				√			
	0.44	√				√			
	0.67	√							
	0.81	√							
	0.89	√	√						
A. f	0.2			√					
	0.28	√							
	0.41	√							
	0.56					√			
Total		10	4	5	0	6	2	0	0

7.5 Conclusion

The plant extracts had varying degrees of inhibition against the tested microorganisms. *L. capassa* is a promising plant after solvent-solvent fractionation. Methanol extracted the highest quantity of plant material from serial exhaustive extraction in all plant species. Both *S. hyacinthoides* and *L. capassa* had varying degrees of inhibition of fungal pathogens. With acetone leaf extracts of *L. capassa* showed good antifungal activity against *C. albicans* and *C. neoformans* with MIC of 0.02 mg/ml. In the bioautography assay, BEA was the best solvent system by separating more antifungal compounds against the tested microorganism than other eluent systems.

Solvent-solvent fractionation was used to separate the fractions from acetone crude extracts of *L. capassa*. Butanol and acetone fractions of *L. capassa* had excellent activity against the fungal pathogens tested using both micro-dilution and bioautography assays. More active compounds were visible in acetone fractions of *L. capassa*. Therefore, acetone fractions of *L. capassa* had shown promising results in both assays, then it was selected for isolation using column chromatography.

In the next chapter acetone fraction of *L. capassa* resulting from solvent-solvent fractionation will be isolated using a column chromatography

CHAPTER 8

ISOLATION OF ANTIFUNGAL COMPOUNDS FROM ACETONE LEAF EXTRACTS OF *L. CAPASSA*

8.1 Introduction

L. capassa (chapter 7) was selected as the most promising plant species for further phytochemical analysis and isolation of bioactive compounds.

Plant natural products play an important role in the drug discovery and development process (Newman and Cragg, 2016). Bioassay-guided fractionation has been used to identify bioactive natural products (Brusotti et al., 2013). Furthermore, pure compounds are isolated based on their biological activity. In addition, the use of bioassay-guided isolation strategies in the discovery of new therapeutic agents is common among many researchers. The technique is preferred due to less time-consuming, particularly in the chemical profiling of extracts and the activity of fractionated compounds (Scrivasta et al., 2021).

Column chromatography may be used for the isolation and purification of bioactive compounds (Altemimi et al., 2017). Many bioactive molecules were isolated and purified by using different paper, thin-layer and column chromatography methods. A combination of solvent-solvent fractionation and column chromatography is widely used, and the efficacy of separation is determined by thin-layer chromatography. Bioautography identifies the active compounds for isolation. The pure compounds are then used for structure elucidation (Ingle et al., 2017). Increasing the polarity of the eluent leads to better separation than an isocratic system.

In this chapter, column chromatography was used to isolate compounds from acetone fractions of *L. capassa*

8.2 Materials and methods

The summary of the methodology is presented in Figure 8.1. Ground-powdered leaves (500 g) of *L. capassa* was serially extracted with a solvent of increasing polarities. The solvents were concentrated using a rotary evaporator and transferred into pre-weighed beakers allowing complete dryness under a stream of cold air. The crude extracts were analysed using TLC fingerprinting and tested for antifungal activity using both microdilution and bioautography assays.

Acetone crude extracts of *L. capassa* were partitioned using solvents such as chloroform, n-butanol, acetone, and water. The fractions were collected and concentrated and tested for antifungal activity. The acetone fraction was subjected to column I chromatography using solvents of increasing polarities. Forty fractions in 50 ml tubes were collected, analysed for antifungal activity using bioautography assay and phytochemical analysis. The active compounds visible on TLC plates with similar R_f values were combined. This resulted in pooling out compounds 1 and 2. Furthermore, fractions 20-22 were subjected to open column ii chromatography. Seventy-seven fractions were collected and analysed using phytochemical analysis. Compounds 3, 4, 5 and 6 were pooled.

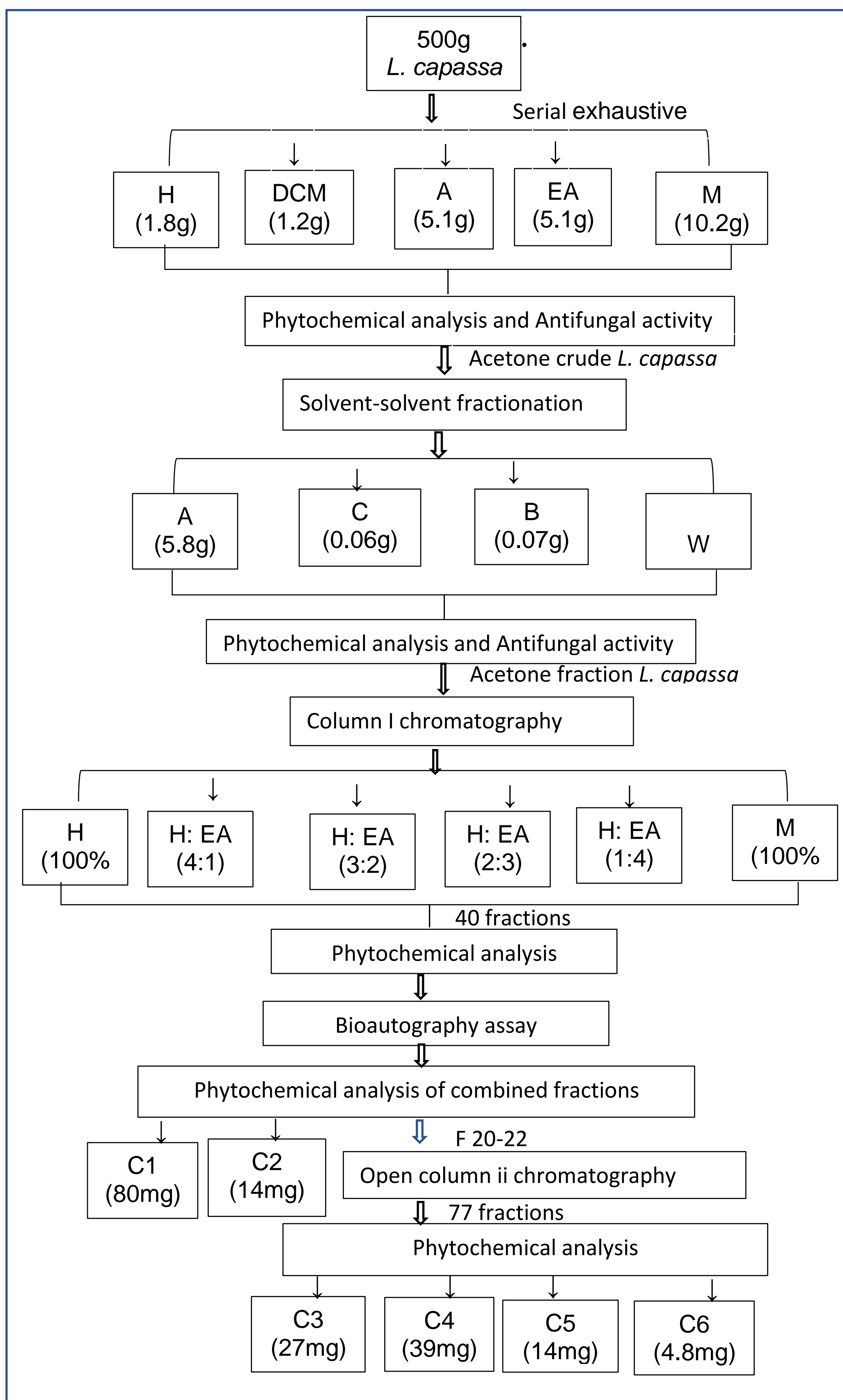


Figure 8.1 Schematic diagram of serial exhaustive extraction of *L. capassa* leaves, solvent-solvent fractionation of acetone extract, and isolation of compounds from column chromatography.

8.3 Isolation of antifungal compounds

8.3.1 Column I chromatography

The *L. capassa* acetone active fractions resulting from solvent–solvent fractionation was subjected to column chromatography using silica gel as a stationary phase and various eluent solvent systems. A column of 50 cm height and 3 cm diameter was used. The fraction was redissolved in different solvent mixtures such as hexane: ethyl acetate (4:1), (3:2), (2:3) and (1:4) and 100% methanol was used to elute the column. The column fractions were collected in test tubes and analysed on TLC plates. The prepared TLC plates were sprayed with vanillin-sulphuric acid spray reagent. The fractions were further analysed using a bioautography assay against the pathogenic fungi. Fractions with similar pure spots on TLC chromatograms were grouped together and evaporated to dryness under reduced pressure 45 °C. The combined fractions were further analysed using TLC fingerprinting.

8.3.2 Column ii chromatography

The combined fraction 20-22 resulting from column I chromatography was subjected to column ii chromatography using silica gel as a stationary phase and various eluent systems. A column of 30 cm in height and 2 cm in diameter was used to pack a column. The fraction was re-dissolved in different solvent mixtures such as hexane: ethyl acetate (4:1), (3:2), (2:3), (1:4) (3:1) (96:4), (95:3) (94:2), (92:1), and (80:20). The fractions were collected into test tubes and analysed for TLC fingerprinting. The prepared TLC plates were sprayed with vanillin-sulphuric acid spray reagent.

8.4 Results and discussion

8.4.1 Column I fractions

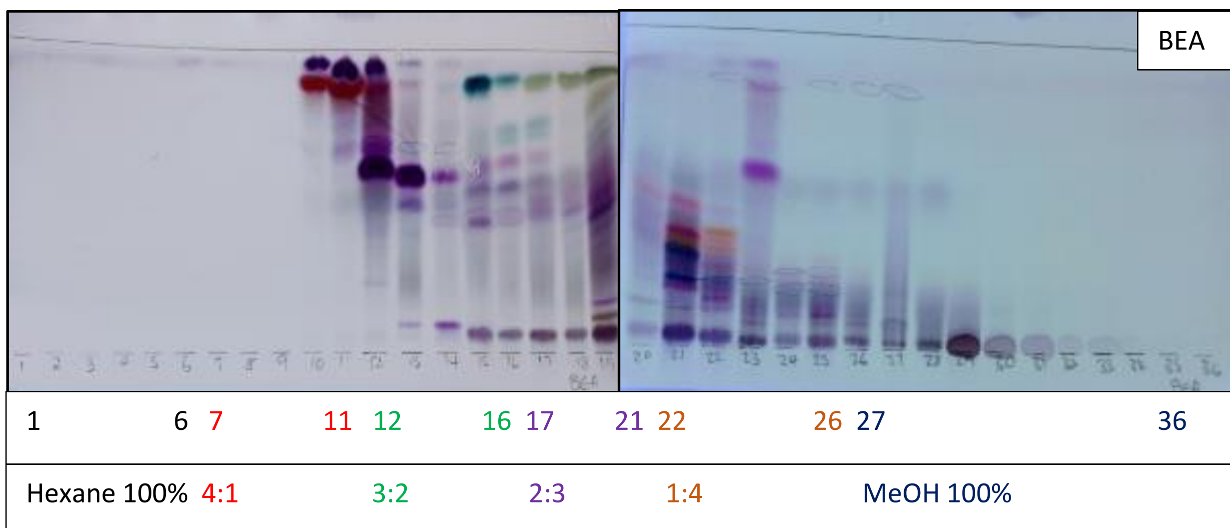
Bioautography was used to determine the number of active compounds from column I fractions. BEA TLC had shown some promising results by indicating more compounds in tested microorganisms Figures 8.3 and 9.4. In TLC chromatograms separated with BEA, a compound with an R_f value of 0.38 was observed in fractions 14-19 against *A. fumigatus* and *C. neoformans* separated. A compound with an R_f value of 0.88 was observed in fractions 14-19 separated in EMW and CEF eluent systems against both *A. fumigatus* and *C. neoformans*. Furthermore, fractions with

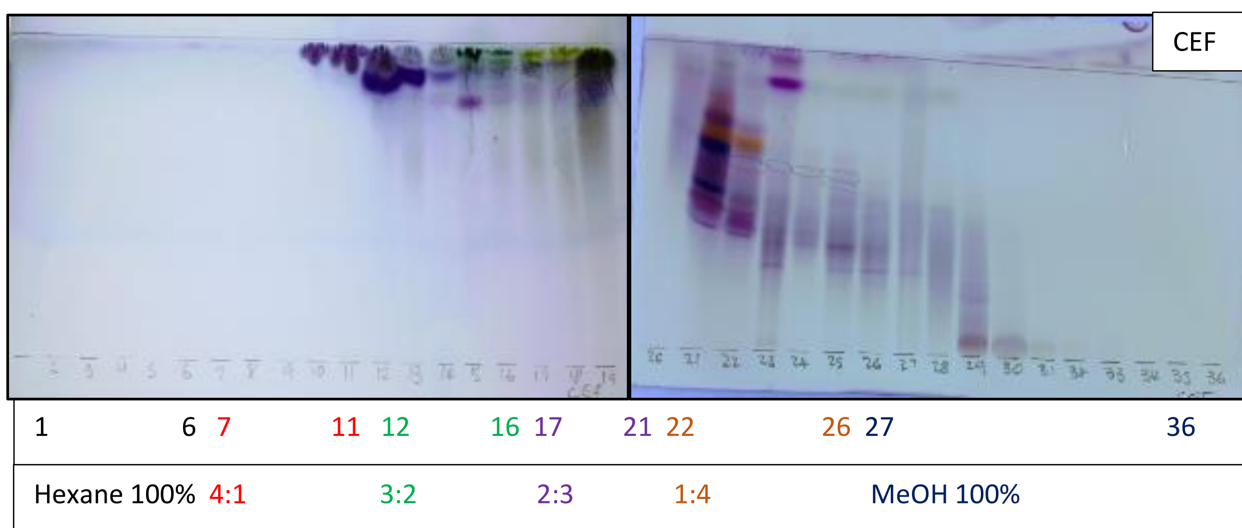
similar compounds from bioautography were combined and analysed for phytochemical analysis.

Fractions with similar antifungal compounds from bioautography assay were combined. Fractions 12-13 resulted in compound 1 (C1), which was concentrated in a rotavapor under vacuum at 45⁰C and transferred into a pre-weighed vial and put under a stream of cold air to complete dryness. Fractions 10-11, 20-22 and 14-19 were combined separately.

8.4.2 Open Column ii fractions

Fraction 20-22 (230 mg) figure 8.5 was subjected to column ii chromatography using different solvent mixtures such as hexane: ethyl acetate (4:1), (3:2), (2:3), (1:4) (3:1) (96:4), (95:3) (94:2), (92:1), and (80:20). Open column ii chromatography resulted in a collection of 77 fractions and isolation of four compounds. Fraction 8-11 Hexane: Ethyl acetate 3:2 resulted in the isolation of compound 3. Fraction 12-13 Hexane: Ethyl acetate 2:3 resulted in the isolation of compound 4. Fraction 20-22 Hexane: Ethyl acetate 1:4 resulted in the isolation of compound 5. Fraction 42-45 Hexane: Ethyl acetate 96:4 resulted in the isolation of compound 6, however, compound 6 was not concentrated on the TLC plate. All the isolated compounds were concentrated with vacuum pressure at 45°C and transferred into a pre-weighed vial and allowed to dry under a stream of cold air.





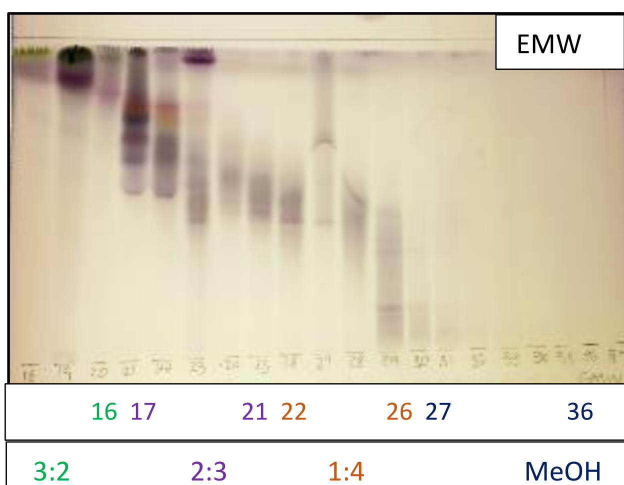
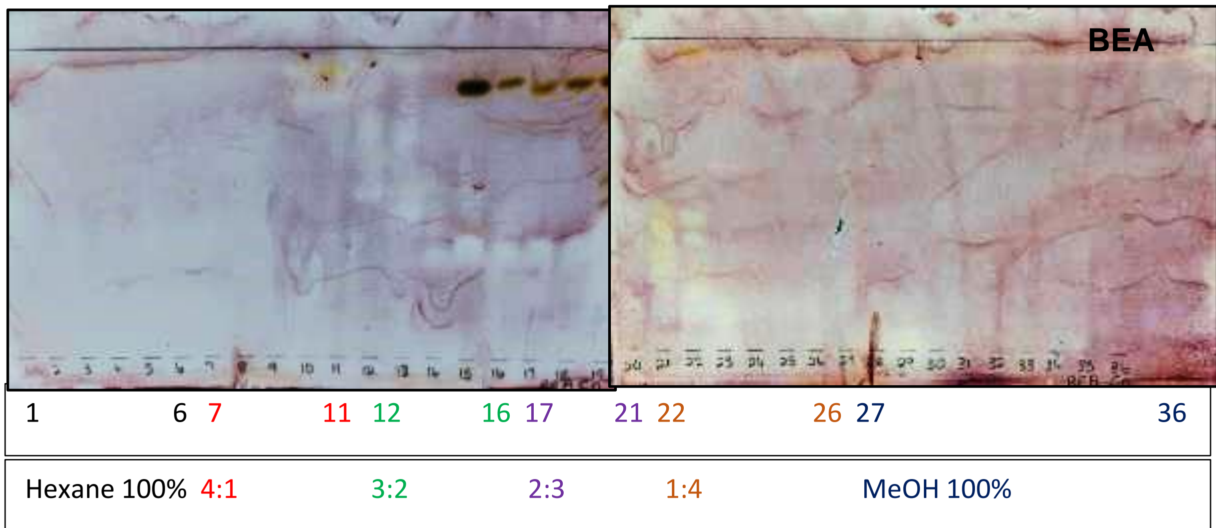
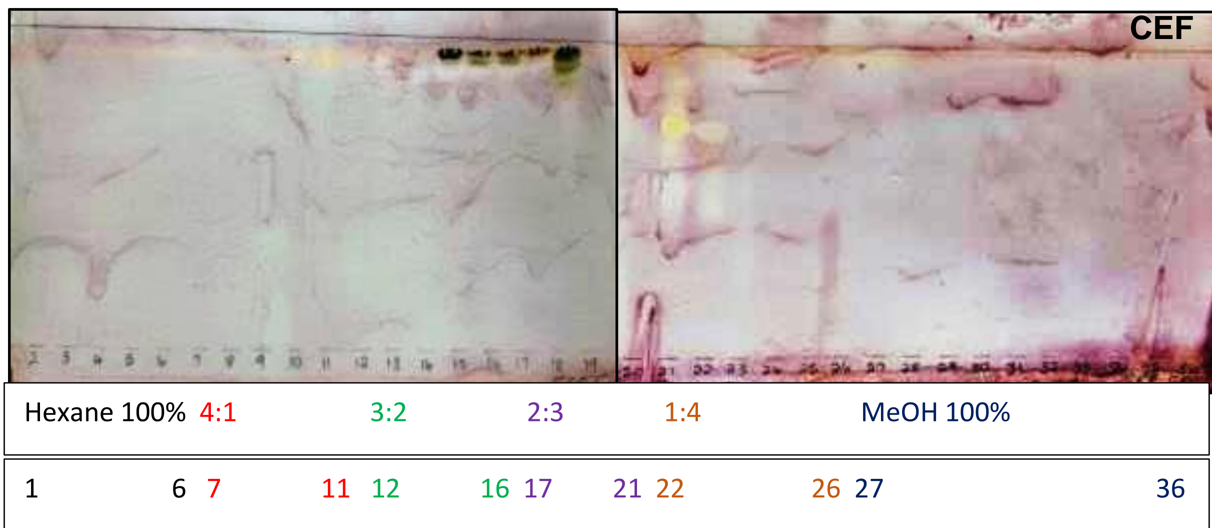


Figure 8.2 TLC chromatograms of *L. capassa* fractions developed in BEA, CEF and EMW solvent systems then sprayed with vanillin sulphuric acid.





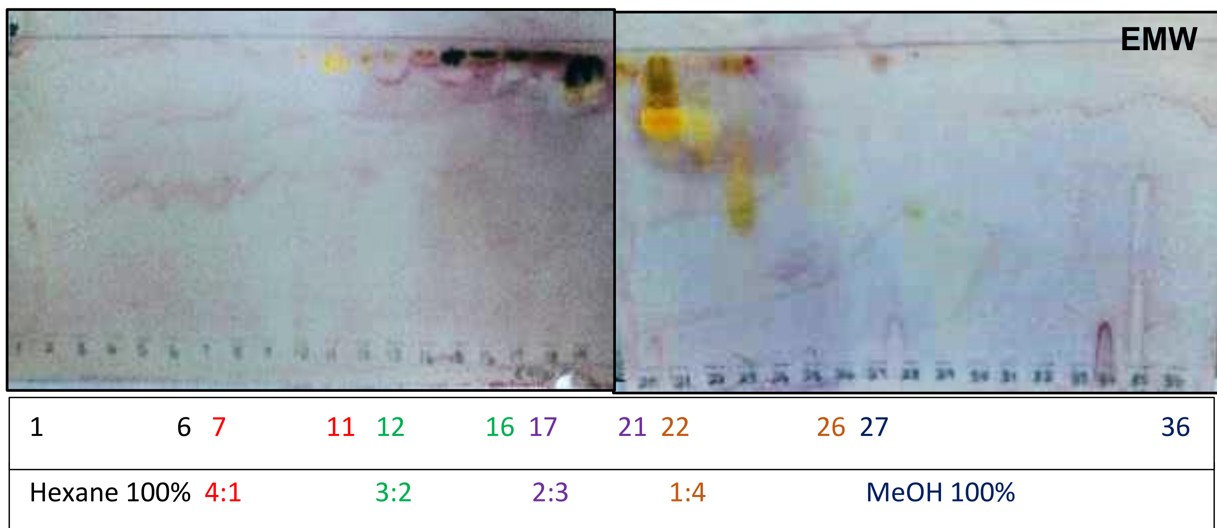
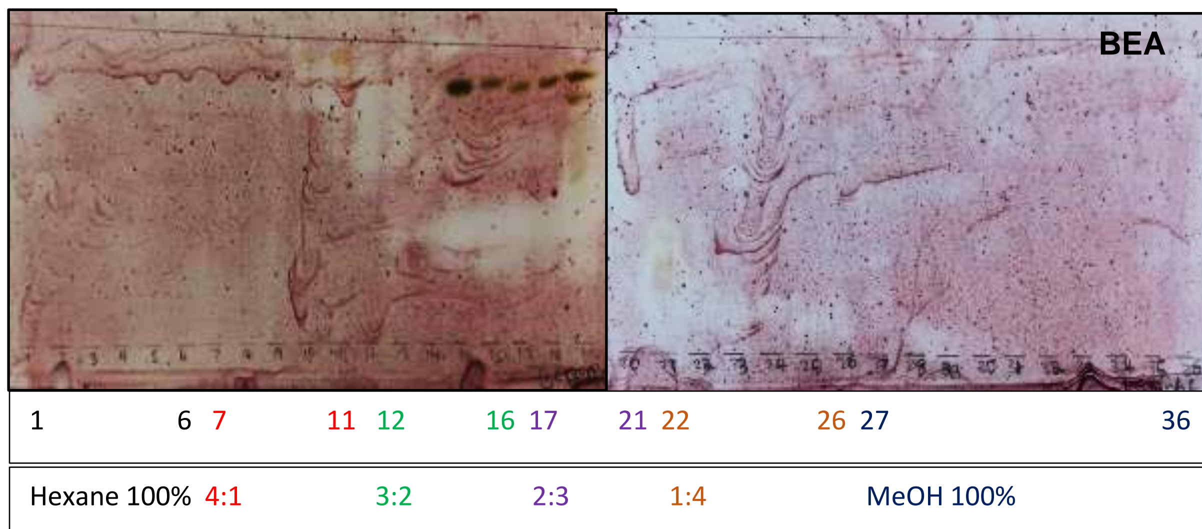
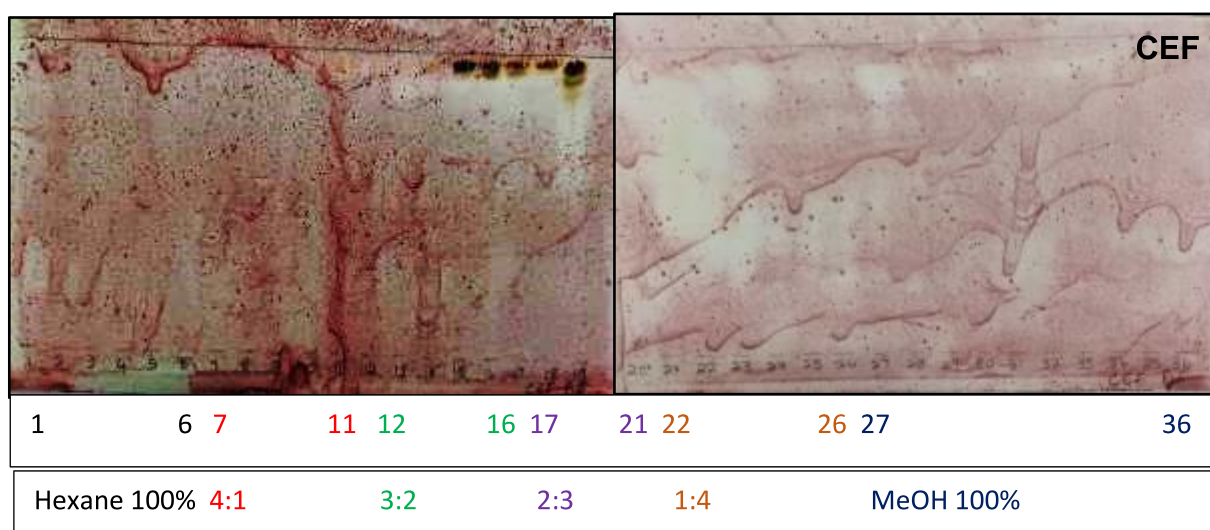


Figure 8.3 TLC chromatograms of *L. capassa* fractions developed in BEA, CEF and EMW solvent systems then sprayed with *C. neoformans*.





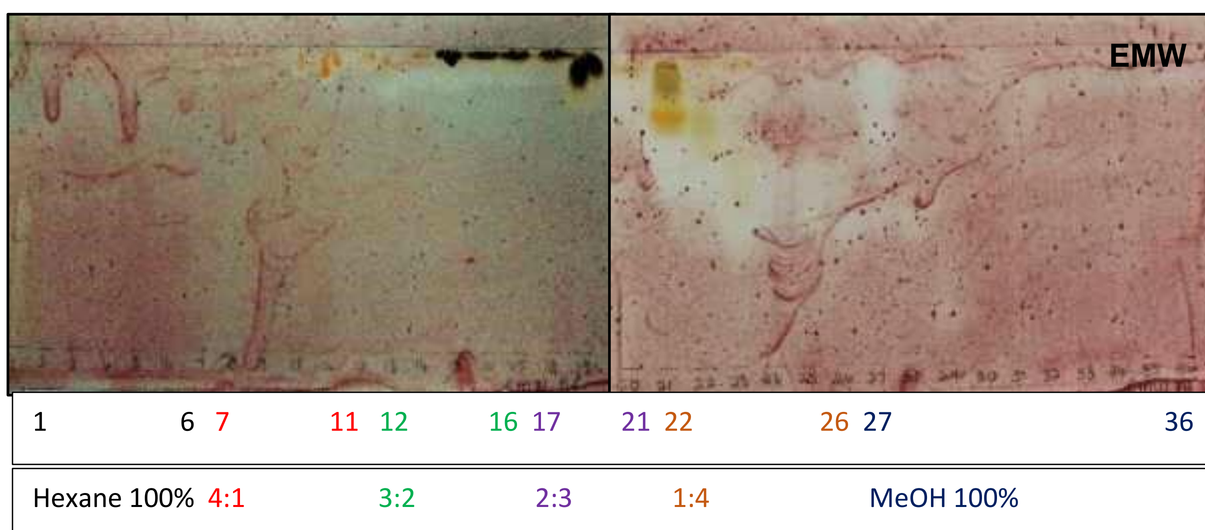


Figure 8.4 TLC chromatograms of *L. capassa* fractions developed in BEA, CEF and EMW solvent systems then sprayed with *A. fumigatus*

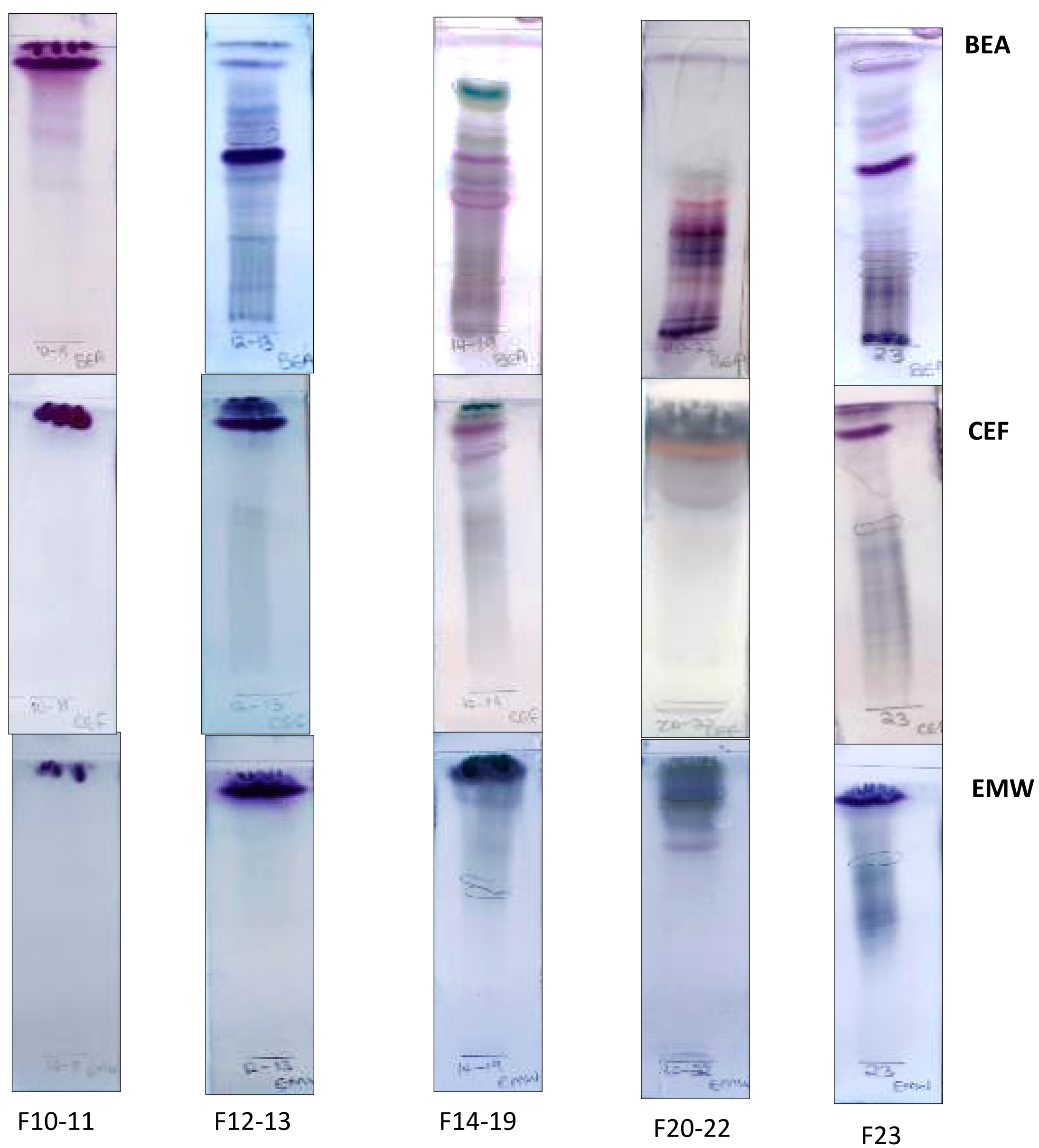


Figure 8.5 TLC chromatograms of *L. capassa* fractions developed in BEA, CEF and EMW solvent systems then sprayed with vanillin sulphuric acid.

8.5 Conclusion

Six different antifungal compounds were isolated. Column one fractionations resulted in the isolation of compounds 1 and 2. Column ii fractions resulted in the isolation of compounds 3, 4, 5 and 6. Different quantities were isolated of compound 1 (80 mg), compound 4 (39 mg), compound 3 (27 mg), compound 2 and 5 (14 mg) and the least was compound 6 (4.8 mg).

In the next chapter, the structure elucidation of the isolated compounds will be discussed.

CHAPTER 9

STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS

9.1 Introduction

It is important to determine the structure of isolated compounds and also investigate if the biological activity of the compound is already known. Nuclear magnetic resonance (NMR) and mass spectroscopy (MS) play an important role in distinguishing between known compounds and new molecules directly from crude plant extracts (Hostettmann et al., 2001). NMR makes it possible to determine the structure, and physical, chemical, and organic properties of compounds. The one-dimensional approach may be sufficient for simple known molecules. More importantly, the structure elucidation of complex and novel molecules requires two-dimensional NMR techniques. (Ingle et al., 2017).

Mass spectrometry is a powerful analytical approach for the identification of unknown compounds, quantification of recognised compounds and elucidation of the structure and chemical properties of molecules (Gupta et al., 2021). Through MS spectrometry the molecular weight of the sample can be determined. This technique is employed for the structural elucidation of the organic compound, for peptide or oligonucleotide sequencing and for monitoring the existence of until now characterises compounds with high specificity through defining both the molecular weight and diagnostic fragment of the molecule (Ingle et al., 2017). Furthermore, using mass spectrometry, relative molecular mass can be determined with high accuracy and a specific molecular formula can be determined with information on locations where the molecule has been fragmented (Najmi et al., 2022). The strategies of UV-visible, IR, NMR and MS have been employed to represent the structure of the bioactive molecule. Furthermore, molecules can be hydrolysed, and their derivatives characterised. Mass spectrometry offers considerable data for the structural elucidation of the compounds whilst tandem mass spectrometry is applied. Therefore, the mixture of HPLC and MS enables fast and correct identification of chemicals in medicinal plants, whilst a natural standard is unavailable (Altemimi et al., 2017). In this chapter, the structure of the isolated compounds will be determined using NMR.

9.2 Materials and methods

9.3 Structure elucidation

The structure of the compounds was determined using nuclear magnetic spectroscopy (NMR). Nuclear Magnetic Resonance (NMR) was used for the identification of isolated antifungal compounds. All samples were sent to the Department of Chemistry at the University of Limpopo for NMR analysis and structure elucidation.

9.4 Results and discussion

9.4.1 **Compound 1**

The 1H NMR spectrum showed the presence of seven methyl protons at δ_H 0.77, 0.81, 0.86, 0.92, 0.95, 1.05 and 1.66 (integrated for 3H-each). A sextet of one proton at δ_H 2.38 was assigned as 19H. The H-3 proton showed a doublet of doublets at δ_H 3.19 while a pair of broad singlets at δ_H 4.55 and δ_H 4.67 (1H, each) was indicative of olefinic protons at (H-29 a & b).

The structural assignment of **Compound 1** was further substantiated by the 13C NMR experiments which showed seven methyl groups at [δc: 27.39 (C-23), 18.02 (C-28), 16.15 (C-25), 15.97 (C-26), 15.40 (C-24), 14.20 (C-27) and 19.31 (C-30)]; the signals due to an exomethylene group at [δc: 109.36 (C-29) and 151.04 (C-20)]; ten methylene, five methine and five quaternary carbons were assigned with the aid of DEPT experiment. The de-shielded signal at δc 79.04 was due to C-3 with a hydroxyl group attached to it.

The confirmation of the structure of M-C1 was accomplished through the 2D NMR experiments (COSY, HSQC, and HMBC).

The HSQC spectrum of **Compound 1** showed a strong correlation between two olefinic protons (H_{29} a,b) at δ_H 4.55 and 4.70 with a carbon peak at δc 109.36 (C-29). The proton resonance at δ_H 3.19 (C-3) corresponded with a carbon peak at δC 79.04 (C-3) ppm in the HSQC. Through HSQC a sextet of one proton at δ 2.38 assigned as 19H showed a good correlation with carbon δc 48.00 (C-19). The COSY spectrum of **Compound 1** exhibited some correlation between δ_H 2.37, H-19 and methylene proton signal (δ_H 1.37, H-21) also another methine proton signal (δ_H 1.89, H-18). Correlation

between the oxygenated methine proton signal (δ_H 3.2, H-3) and methylene signal (δ_H 1.60, H-2) was also observed through COSY.

In the HMBC spectrum, the methine proton signal at δ_H 3.2 (H-3) showed cross peaks with a methyl carbon signal (δ_C 27.39, C-23) and a methyl carbon signal (δ_C 18.32, C-6). The sextet methyl signal at δ_H 2.37 (H-19) showed cross peaks with a methyl carbon signal [δ_C 19.31 (C-30)] and a quaternary carbon signal [δ_C 151.04 (C-20)]. The pair of broad olefinic proton at δ_H 4.55 and 4.70 showed cross peaks with a methylene carbon signal [δ_C 48.00 (C-19) and δ_C 19.31 (C-30)]

Lupeol belongs to bioactive triterpenoids with great therapeutic agents (Saxena et al., 2022). The isolated chemical structure of Lupeol (Figure 9.1) is in good agreement with the structure isolated by Jain and Bari, (2010) from *Wightia tinctoria*. Lupeol was previously isolated from *Holarhena floribunda* (Fotie et al., 2006) and *Axinella infundibuliformis* (Lutta et al., 2008). The compound Lupeol was currently isolated from *Sideroxylon mascatense* (Ali et., 2022), *Croton echioides* (Vendruscolo et al., 2022). Lupeol has shown good antioxidant activity and antibacterial activity was also reported (Ali et al., 2022). The cytotoxicity of Lupeol isolated from *C. echiodes* was reported (Vendruscolo et al., 2022). The anti-inflammatory, anti-tumor, anti-prostate, and antiprotozoal activities of Lupeol were reported (Saxena et al., 2017; Margareth and Miranda, 2009; Wal et al., 2011).

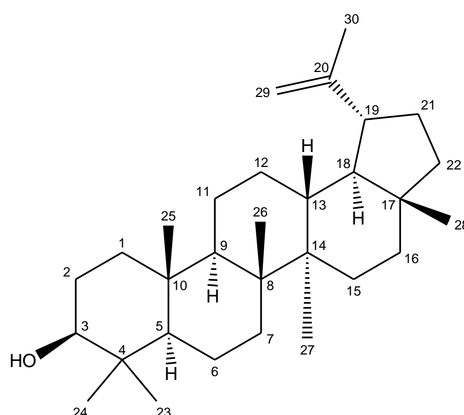


Figure 9.1 Lupeol isolated from *L. capassa*.

Table 9.1 ^{1}H and ^{13}C NMR data of the isolated compound

No	1H	13C	1H (Literature)	13 C (Literature)
1		38.87		38.7
2		27.39		27.2
3	3.19 dd	79.04	3.20 dd	77.7
4		38.87		38.7
5		55.14		54.0
6		18.32		18.6
7		43.01		43.3
8		40.81		41.7
9		50.40		49.2
10		37.15		37.5
11		20.92		21.3
12		38.02		37.9
13		38.68		38.0
14		40.81		41.6
15		28.00		28.6
16		35.57		35.9
17		42.82		42.9
18	1.89	50.40		49.2
19	2.37	48.00		48.0
20		151.04		149.7
21	1.37	29.41		30.0
22		40.01		39.6
23	0.77	27.39	0.76 s	27.3
24	0.81	14.5	0.79 s	14.9
25	0.86	16.15	0.83 s	16.0
26	0.95	15.97	0.94 s	15.8
27	1.05	14.2	1.02 s	14.5
28	0.77	18.02	0.76 s	17.4
29	4.55 d	109.36	4.57 d	108.7
29	4.67 d		4.67 d	
30		19.31		19.9

9.4.2 **Compound 3**

The ^{1}H, $_{13}C$ NMR had a signal for H_a-1 at δ_H 1.61 and H_b-2 at δ 2.27 integrating for 1 proton each. A signal for H-4 was observed integrating for one proton at δ_H 2.33, while H-6 was at δ 1.24 with corresponding C-2 and C-6 at δ_C 33.65 and 31.95, respectively. Eight signals typical of methyl protons were also observed at δ 0.80, 0.84, 0.92, 0.95, 1.00,1.02 and 1.03, 1.23 with corresponding $_{13}C$ NMR signals at δ_C 29.73, 29.47 for Me-29 and Me-30, and 22.65 for Me-26. Signals typical of methylene carbons were also observed at δ 56.00, 55.50 and 59.52 for C-4, C-8 and C-10, while quaternary carbon signals were observed at δ 29.27 and 29.39 for C-20 and C-17. Also, observed signals typical of methylene carbons at δ 14.16, 22.65, 28.00, 29.09, 31.95, 33.65, 35.64, 35.92, 36.33, 39.51. The cross correlation between a methyl signal at δ 1.24 and a quartet of methine at δ 2.27 in the ^{1}H-^{1}H COSY spectrum strongly supported the structure of the compound. The C=O carbon was confirmed by signal at 177.63 typical of a ketonic carbon. Thus, based on spectroscopic evidence, compound **3** was identified as friedelin.

^{1}H ($CDCl_3$, 300 MHz, ppm): δ_H 1.61 (H-1, 2H,), 2.27 (H_b-2), 2.31 (H_a-2), 2.25 (H-4, 1H, m), 1.24 (H-6,), 0.84 (H-23, 3H), 0.80 (H-24, 3H), 0.84 (H-25, 3H), 1.00 (H-26, 3H), 1.02 (H-27, 3H), 1.03 (H-28, 3H), 1.20 (H-29, 3H).

13C NMR ($CDCl_3$, 75 MHz, ppm): δ_C 22.65 (C-1), 33.65 (C-2), 177.63 (C-3), 56.00 (C-4), 39.50 (C-5), 36.33 (C-6), 14.16 (C-7), 55.50 (C-8), 39.5 (C-9), 59.52 (C-10), 33.65 (C-11), 30.8 (C-12), 35.64 (C-13), 39.52 (C-14), 31.95 (C-15), 39.51 (C-16), 29.39 (C-17), 43.1 (C-18), 35.9 (C-19), 29.27 (C-20), 33.65 (C- 21), 39.51 (C-22), 7.1 (C-23), 14.16(C-24), 22.73 (C-25), 22.65 C-26), 22.72 (C-27), 24.73 (C-28), 29.73 (C-29), 29.47 (C-30).

The chemical structure of Friedelin isolated from *L. capassa* is presented in Figure 9.2. Friedelin was previously isolated from *Syzygium jambos* (Majidul et al., 2015), and *Maytenus robusta* (Grasiely et al., 2012). Friedelin was reported to have the potential in the treatment of ulcerative colitis (Shi et al., 2021).

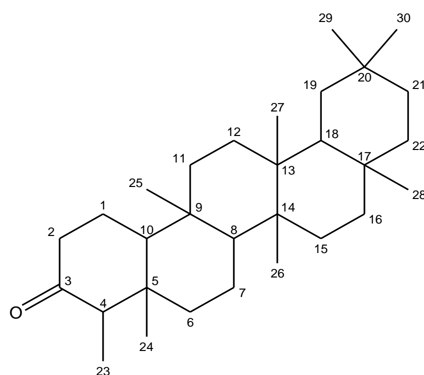


Figure 9.2 Friedelin isolated from *L. capassa*.

9.4.3 **Compound 4**

1H NMR: 5.47 (H-2), 3.61 (1H, H-3ax), 2.27 (H-3eq), 7.39 (1H, H-5), 6. (1H,H-6), 6.53 (2H, m, H-2',6'), 6.41 (1H, H-5'), 6.08 (H-3"), 6.89 (1H, H-4"), 1.82, 1.81 (2 X 3H, 2 x s, 2"-Me2), 3.80, 3.83 (2 X 3H, 2 x s, 3'-OMe, 4'-OMe).

13C NMR: 79.49 (C-2), 39.37 (C-3), 157.25 (C-4), 132.25 (C-5), 125.91 (C-6), 156.84 (C-7), 103.66 (C-8), 152.21 (C-9), 112.51 (C-10), 135.59(C-1'), 106.90 (C-2'), 149.46, 150.73 (C-3', C-4'), 112.51 (C-5'), 120.51 (C-6'), 77.36 (C-2"), 125.91 (C-3"), 109.65 (C-4"),31.94 (C-5''), 125.91 (C-6'') 28.83, 27.99 (2"-OMe), 55.61 (3'-OMe, 4'-OMe).

The compound 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone in Figure 9.3 isolated from *L. capassa* is a new compound, it has never been isolated before and it is reported for the first in this study.

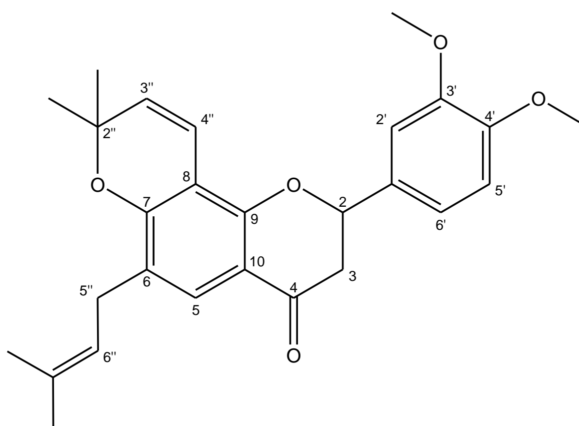


Figure 9.3 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone isolated from *L. capassa*

9.5 Conclusion

Nuclear Magnetic Resonance (NMR) was used for the identification of six isolated antifungal compounds from *L. capassa* leaves. However only three compounds were successfully identified as Lupeol (compound 1), Friedelin (compound 3) and 6-(γ,γ-Dimethylallyl)-3’,4’-dimethoxy-6”,6”-dimethylpyrano-[2”,3”:7,8]-flavanone (compound 4). Furthermore,6-(γ,γ-Dimethylallyl)-3’,4’-dimethoxy-6”,6”-dimethylpyrano-[2”,3”:7,8]-flavanone was reported for the first time in this study. HPLC-MS is required to confirm the identified chemical structure.

In the next chapter, the antifungal and antioxidant activity of the isolated compounds will be determined.

CHAPTER 10

ANTIFUNGAL, ANTIOXIDANT AND CYTOTOXICITY ACTIVITIES OF ISOLATED COMPOUNDS

10.1 Introduction

Medicinal plants have long been used to cure and prevent various diseases including fungal infections (Sohaib et al., 2022). Drugs that are used to treat fungal infections have problems such as the development of fungal resistance and toxicity to humans (Arif et al., 2009). Medicinal plants can be a good approach to fight against the limitations facing conventional drugs. Therefore, screening and isolating compounds that can be used to treat fungal infections are crucial. Traditional medicines are generally safe, however, that does not mean that they are not toxic. Since medicinal plants vary with chemical constituents' presence in a particular plant. Traditional health practitioners often used medicinally in combination with other herbs to reduce their toxicity. Furthermore, they administer plants in low doses. Toxic medicinal plants have been used traditionally to treat various ailments.

Despite the useful effects of medicinal plants, the toxicity of the crude and isolated compounds to treat fungal infections should be investigated, since the traditional use of toxic plants is associated with dysfunctions of the liver and kidneys in humans (Anywar et al., 2021). Furthermore, if toxicity tests of the plants are not conducted, continued usage of the medicinal plants may lead to acute and chronic diseases. The cytotoxicity of *L. capassa* was investigated by (Kilonzo et al., 2016).

The antioxidant activities of the plant extracts rely on the polarity of the solvent, the extraction process, and the way in which the antioxidant is evaluated (Wanasundana and Shahichi, 1998). Furthermore, any medicinal plant that manages stress-associated illnesses have the ability to avoid oxidative stress (Muniyandi et al., 2017). Phenols are the most considerable group in plant extracts since they can protect the organisms against oxidative stress conditions. Oxidative stress conditions occur when the antioxidant disequilibrium in living organisms, in which free radical molecules become stable by electron combinations with other molecules and cause various diseases in humans (Sohaib et al., 2022). Estimation of the antioxidant activity using

one antioxidant assay is not recommended (Rafat et al., 2010). Therefore, in the current study, the antioxidant activity of the isolated compounds was determined using qualitative and quantitative DPPH assays.

10.2 Materials and methods

10.2.1 TLC fingerprinting

The chemical components of the isolated compounds were analysed using TLC plates. The method is described in chapter 3 section 3.2.3.

10.2.2 Antifungal activity

10.2.2.1 Micro-dilution assay

The microplate method described in chapter 4 section 4.2.1.3 was used to determine the minimum inhibitory concentration (MIC) of the isolated compounds.

10.2.2.2 Bioautography assay

The number of active isolated compounds was determined using the bioautography method described in chapter 4 section 4.2.1.4.

10.2.3 Antioxidant activity of the isolated compounds

10.2.3.1 Qualitative DPPH free radical scavenging assay

The qualitative 1,1-diphenyl-2-picrylhydrazyl (DPPH) method described in chapter 5 section 5.2.2.1 was used to determine the antioxidant activities of the isolated compounds

10.2.3.2 Quantitative DPPH free radical scavenging assay

The antioxidant activity of the isolated compounds was evaluated using the free radical-scavenging method (DPPH) described in chapter 5 section 5.2.2.2

10.2.4 Cytotoxicity activity

The cytotoxicity of plant crude extracts and isolated compounds were determined using the (3-(4,5-dimethylthiazol) -2,5-diphenyltetrazoliumbromide) (MTT) assay described by Mosmann, (1983) and (McGaw et al., 2007) against African green monkey Vero kidney cells. The cells were 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 plate.

The microtitre plates were incubated at 37 °C in a 5% CO_2 incubator for 48 h with a test compound or extract. After incubation of 4h in 30 µl of 5 mg/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. Doxorubicin chloride was used as a positive control. The LC_{50} values were calculated as the concentration of the test compound resulting in a 50% reduction of absorbance compared to untreated cells.

10.3 Results and discussion

10.3.1 TLC fingerprinting

The TLC fingerprinting of the isolated compounds was determined using the tree solvent system. The TLC chromatograms are presented in Figure 10.1. Compounds 1 (Lupeol) and 2 were pure in all the eluent systems. However, compounds 3 (Friedelin), compound 4 (6-(γ,γ-Dimethylallyl)-3’,4’-dimethoxy-6”,6”-dimethylpyrano-[2”,3”:7,8]-flavanone), and 5 had some inseparable compounds and also some impurities.

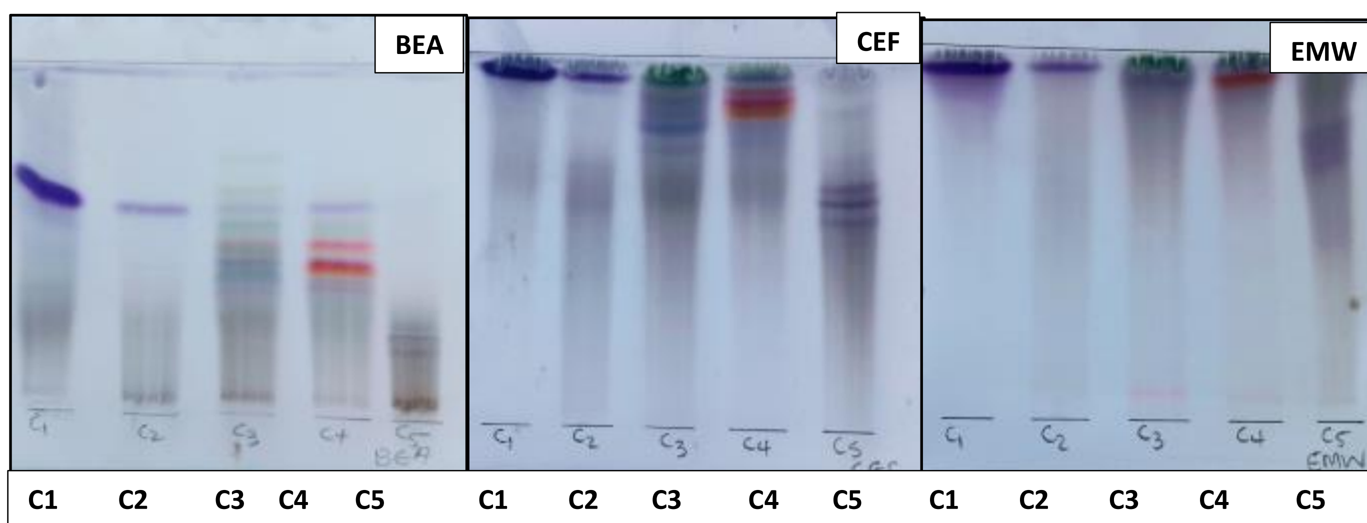


Figure 10.1 TLC chromatograms of isolated compounds from *L. capassa* developed in BEA, CEF and EMW solvent systems then sprayed with vanillin sulphuric acid reagent spray

10.3.2 Antifungal activity of isolated compounds

10.3.2.1 Micro-dilution assay

The findings presented as minimum inhibition concentrations (MIC) indicated that the isolated compounds have varying antifungal activity as summarised in table 10.1. Lupeol showed good activity against *C. albicans, C. neoformans*, and *A. fumigatus* with MIC values of 0.039 to 0.078 mg/ml. Compounds Friedelin and 6-(γ,γ-Dimethylallyl)-3’,4’-dimethoxy-6”,6”-dimethylpyrano-[2”,3”:7,8]-flavanone and compound 5 inhibited good antifungal activity against *C. neoformans* with MIC value of 0.078 mg/ml. However, compound 5 had low activity against *C. albicans* and *A. fumigatus* with high MIC values of 2.5 mg/ml ad 1.25 mg/ml respectively. Compound 2 revealed moderate activity against the tested fungal strains with an MIC range of 0.16 to 0.63 mg/ml.

Table 10.1 Minimum inhibitory concentration (MIC) values of isolated compounds against the *C. neoformans, C. albicans* and *A. fumigatus*.

Fungal species	Time (hrs)	Compounds					
		C1	C2	C3	C4	C5	AMP B
C. neoformans	24	**0.039**	0.16	**0.078**	**0.078**	**0.078**	0.02
	48	**0.039**	0.16	**0.078**	**0.078**	**0.078**	0.02
	72	**0.039**	0.16	**0.078**	**0.078**	**0.078**	0.02
C. albicans	24	**0.039**	0.313	0.16	**0.02**	2.5	0.02
	48	**0.039**	0.313	0.16	**0.02**	2.5	0.02
	72	**0.039**	0.313	0.16	**0.02**	2.5	0.02
A. fumigatus	24	**0.078**	0.63	0.313	**0.078**	1.25	0.02
	48	**0.078**	0.63	0.313	**0.078**	1.25	0.02
	72	**0.078**	0.63	0.313	**0.078**	1.25	0.02
Average		**0.05**	0.37	0.183	0.058	1.28	0.02

C1=Lupeol, C3= Friedelin, C4=6-(γ,γ-Dimethylallyl)-3’,4’-dimethoxy-6”,6”-dimethylpyrano-[2”,3”:7,8]-flavanone

10.3.2.2 Bioautography assay

Bioautography was used to determine the activity of the isolated compounds. The bioautograms of the isolated compounds are presented in figure 10.2 -10.4 and the R_f values of the active compounds against *A. fumigatus, C. neoformans* and *C. albicans* are presented in Table 10.2. Lupeol was active against all the fungal pathogens. A compound with an R_f value of 0.63 was present in TLC chromatograms developed in BEA against *A. fumigatus, C. neoformans* and *C. albicans.* A compound with a similar R_f value of 0.88 was visible in Lupeol, Friedelin and 6-(γ,γ-Dimethylallyl)-3’,4’-dimethoxy-6”,6”-dimethylpyrano-[2”,3”:7,8]-flavanone against *C. neoformans.* Compound 6 was active against *C. neoformans* with the compound of 0.93 and 0.92 R_f values developed in CEF and EMW solvent systems respectively. No compound was observed in compound 6 against *C. albicans* and *A. fumigatus.*

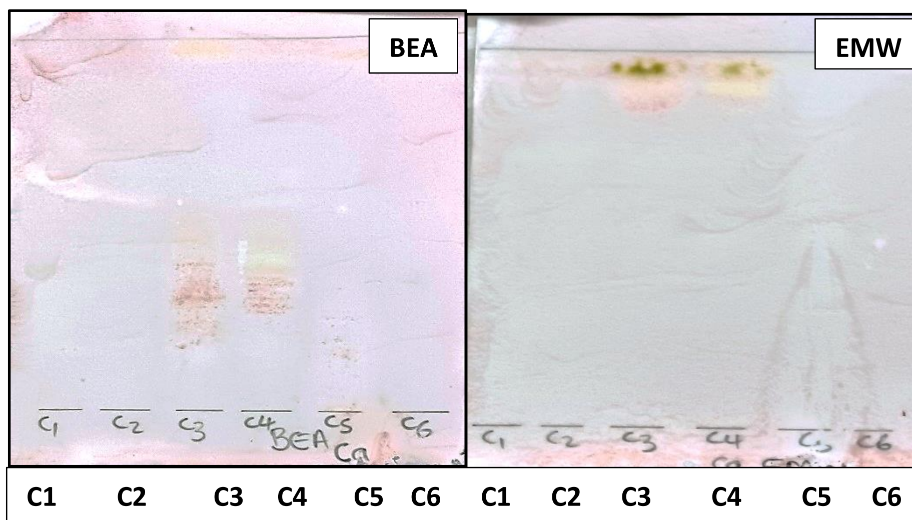


Figure 10.2 TLC chromatograms of isolated compounds from *L. capassa* developed in BEA, CEF and EMW solvent systems then sprayed with *C. albicans*.

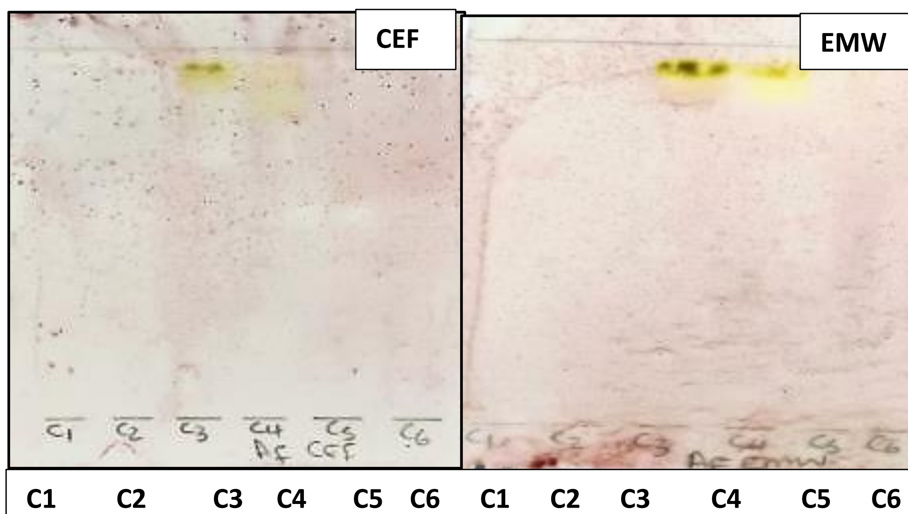


Figure 10.3 TLC chromatograms of isolated compounds from *L. capassa* developed in CEF and EMW solvent systems then sprayed with *A. fumigatus*.

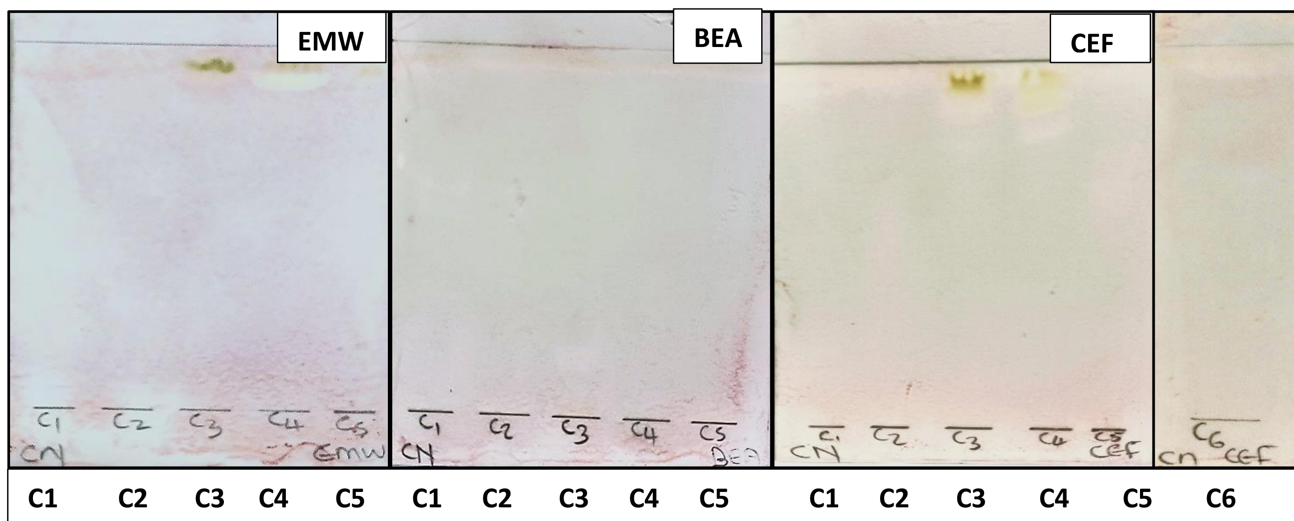


Figure 10.4 TLC chromatograms of isolated compounds from *L. capassa* developed in BEA, CEF and EMW solvent systems then sprayed with *C. neoformans.*

Table 10.2 R_f values of isolated compounds

Fungal species	solvent system	Rf values	C1	C2	C3	C4	C5	C6
A. F	BEA	0.63	√					
	CEF	0.6					√	
		0.9				√		
		0.93	√		√			
	EMW	0.9				√		
C.A	BEA	0.25			√			
		0.38				√		
		0.56		√				
		0.63	√					
	EMW	0.93			√	√		
		0.94	√					
C. N	BEA	0.23			√			
		0.63	√					
	CEF	0.88			√	√		
		0.93						√
	EMW	0.88	√		√	√		
		0.92						√

C1=Lupeol, C3= Friedelin, C4=6-(γ,γ-Dimethylallyl)-3’,4’-dimethoxy-6”,6”-dimethylpyrano-[2”,3”:7,8]-flavanone.

10.3.3 Antioxidant activity

10.3.3.1 Qualitative antioxidant activity

The tested compounds in this study showed antioxidant activity in the qualitative assay as presented in Figure 10.5. Strong antioxidant activity was visible in compounds Friedelin and 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone when sprayed with 0.2 % of DPPH in methanol, the compounds were visible in all the three-eluent systems BEA, CEF and CEF. No activity was observed in compound Lupeol, C2 and C5 compounds. Therefore, compounds Friedelin and 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone were further analysed using the quantitative assay. It was not necessary to quantify compounds lupeol, compound 2, and 5 since no activity was observed on the TLC plate.

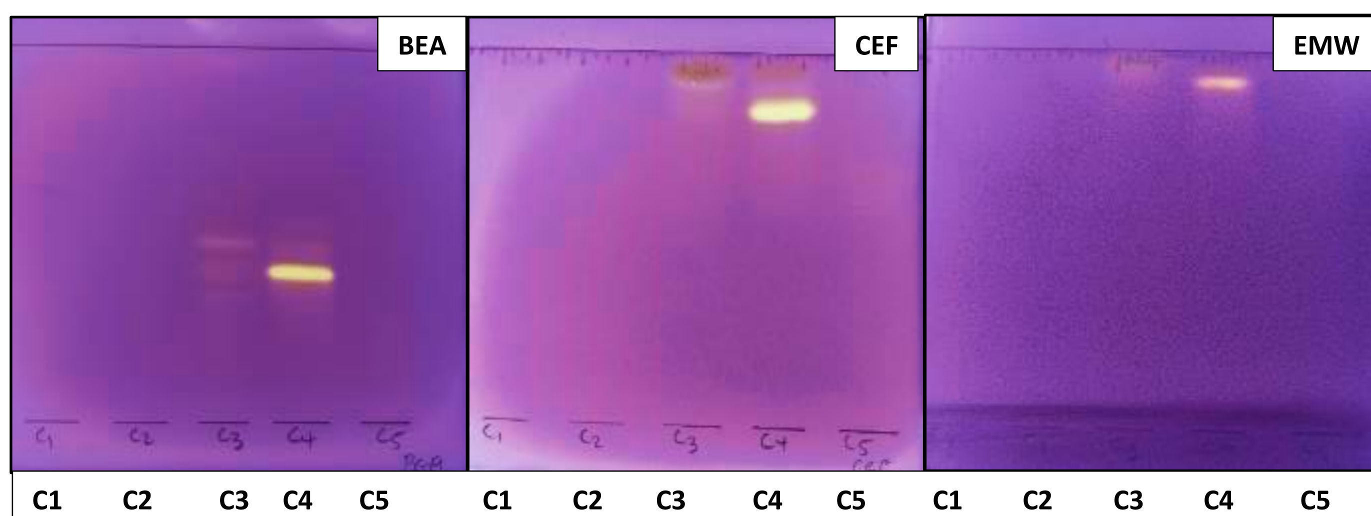


Figure 9.5 TLC chromatograms isolated compounds from *L. capassa* Developed in BEA, CEF and EMW, Sprayed with 0.2% DPPH in methanol.

10.3.3.2 Quantitative antioxidant activity

There was a significant positive correlation between quantitative and qualitative assay of compounds Friedelin and 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone. The isolated compounds were active against the DPPH by showing a high percentage of inhibition as presented in Figure 10.6. Compound 4 had percentage inhibition of 67 % and 77% in 120 µg/ml and 250 µg/ml concentrations respectively. Compound Friedelin also had a good inhibition percentage of 53% in 120 µg/ml and 54 % in 250 µg/ml. However, it was low compared

to that of compound 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone. This shows that 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone is highly active than the compound Friedelin in the quantitative assay.

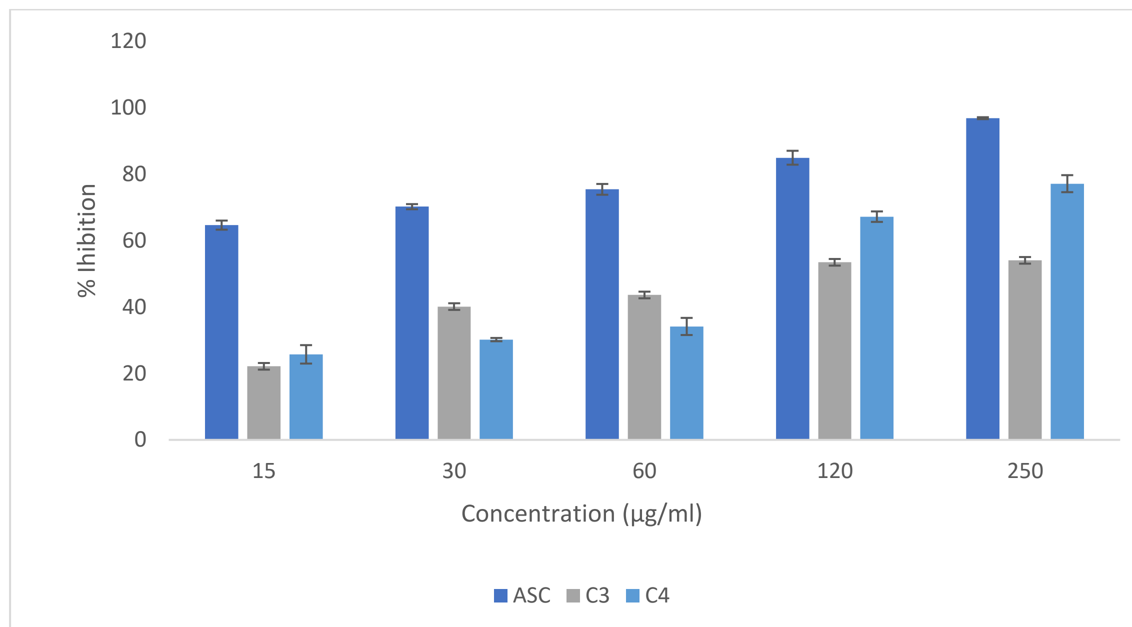


Figure 10.6 Percentage antioxidant activity of isolated compounds and ascorbic acid.

10.3.4 Cytotoxicity activity

The toxic effects of the acetone crude extract and isolated compounds were investigated using the MTT assay against the Vero monkey kidney cells. The results are presented in Table 10.3. The acetone leaf extract was less toxic with LC_{50} of 0.23 mg/ml. Previously, it was reported that ethyl acetate bark and root extracts of *L. capassa* were toxic against brine shrimp LC_{50} of 17.86 µg/ml (Kilonzo et al., 2016). Compound 1 (Lupeol) and compound 4 (6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone) were not toxic with LC_{50} > 0.2 mg/ml against the Vero monkey cells. Elisha et al. (2017) reported that plant extracts with LC_{50} > 0.2 mg/ml are relatively non-toxic.

Table 10.3 Half maximal cytotoxicity (LC_{50}) of compounds and extract against Vero monkey kidney cells using MTT assay.

Sample	Mean	SD
Acetone crude extract	0.23	0.02
Lupeol	>0.2	NA
6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone	>0.2	NA

NA= Not applicable.

10.4 Conclusion

The antifungal activity of the isolated compounds of *L. capassa* against the *C. albicans, C. neoformans*, and *A. fumigatus* which have been implicated as the cause of death to immune compromised individuals, such as those living with HIV and AIDS is a novel finding. It is interesting that the antifungal activity of the isolated compounds was lower than that of the crude extracts indicating a possible synergistic effect between different compounds. The isolated compounds were not pure based on TLC, yet the chemists at the University of Limpopo were able to determine the structures unambiguously, possibly by crystallisation of further purification. Furthermore, some isolated compounds revealed good antioxidant activity in both scavenging assays, which indicates that isolated compounds of *L. capassa* can scavenge the free radicals causing fungal infections in humans. The study indicated that isolated compounds from *L. capassa* can be used as a source of therapeutic agents. The isolated compounds and crude extract of *L. capassa* were not toxic against the Vero monkey kidney cells, which ascertains the use of the plant extracts as medicine to treat fungal infections by traditional health practitioners.

CHAPTER 11

SUMMARY, CONCLUSION AND RECOMMENDATION

11.1 Summary and conclusion

The aim of the study was to investigate plant species used traditionally for the treatment of diseases that could be associated with cryptococcosis and to isolate and characterise antifungal compounds, which could potentially be used to develop new antifungal agents against *Cryptococcus neoformans*.

The following objectives were achieved in the recent study:

i. **Select twelve plant species used to treat cryptococcosis from the database of Ethno-medicinal plant species for further phytochemical analysis, antioxidant activity and biological assays.**

   The selected twelve plant species were extracted using extractants of varying polarities such as acetone, dichloromethane, methanol, hexane, and water. Different extraction solvents resulted in various extraction percentage yields. In terms of quantity extracted, methanol was the best. This shows that the extracted plant species contain high concentrations of polar compounds. The chemical components of plant extracts were determined by TLC. All the plant extracts revealed the presence of different chemical constituents. CEF (34) was the best eluent system by separating more compounds compared to BEA (33) and EMW (13). The results indicate that the extracts contained polar and non-polar compounds.

ii. **Determine the antifungal activities of the selected plant extracts against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*.**

   The antifungal activity of the plant extracts was investigated using microdilution and bioautography assays. All the tested plant extracts revealed some varying degrees of fungal inhibition, with MIC values ranging between 0.02 mg/ml and 2.5 mg/ml. In a few cases, water extracts had some activity against the tested microorganisms. This may validate the use of water as an extractant by traditional health practitioners to treat fungal

infections, but the major antifungal activity was obtained by using non or intermediate polarity extractants. The TLC chromatograms revealed different compounds' presence in the plant extracts. More compounds were visible in BEA compared to CEF and EMW eluent systems. The antifungal activity of the twelve selected medicinal plants supports the traditional use of the plants in the treatment of fungal infections. The antifungal activity of the plant extracts indicated in most plant species indicates that the plant extracts can be used to treat cryptococcosis. Based on the results, this suggests that these plants could be used as a new potential source of antifungal agents.

iii. Screen plant extracts for qualitative and quantitative antioxidant activity

The qualitative and quantitative free radical scavenging activity assays (DPPH) were used to determine the antioxidant activity of the plant extract. Most plant species investigated displayed noteworthy antioxidant activity, which provides scientific evidence for their utilisation by traditional health practitioners to treat fungal infections. As a result, plant species with antioxidant compounds could be used as potentials for antifungal drugs. Since many secondary metabolites have antimicrobial and antioxidant activity, the antioxidant presents in plant species investigated in this study mean that there is a possible synergism. Strong antioxidant compounds were observed in some plant extracts developed in BEA, suggesting that antioxidant compounds can be isolated based on the polarity of the solvents. All the extracts of *P. africanum* had noticeable results against the DPPH, with some extractants having good activity than the ascorbic acid. The results from this study show that the extract can be used as an easily accessible source of natural antioxidants.

iv. Isolate antifungal compounds from the most promising plant species and determine their chemical structure.

S. hyacinthoides and *L. capassa* were selected as promising plants from the preliminary screening against the tested fungal pathogens. The two selected species were serially extracted with hexane, dichloromethane,

acetone, ethyl acetate and methanol. Methanol was the best extractant by extracting more quantity of the plant material than other solvents from serial exhaustive extraction in all plant species compared to other solvents. The two plant species investigated revealed some varying degrees of inhibition of fungal pathogens. The acetone leaf extracts of *L. capassa* inhibited the growth of *C. albicans* and *C. neoformans* with an MIC value of 0.02 mg/ml. The bioautography assay was used to determine the number of active compounds from the plant extracts. BEA was the best solvent system by separating more antifungal compounds against the tested fungal pathogens than other eluent systems. This indicates that the active compounds were relatively non-polar.

Solvent-solvent fractionation was used to separate the fractions from acetone crude extracts of *L. capassa*. Butanol and acetone fractions of *L. capassa* had excellent activity against the fungal pathogens tested using both microdilution and bioautography assays. More active compounds were visible in acetone fractions of *L. capassa*. Therefore, acetone fractions of *L. capassa* exhibited promising results in both assays, then it was selected for isolation using column chromatography.

Bioassay-guided fractionation using chromatography of some extracts led to the isolation of 6 antifungal compounds. Column one fractions resulted in the isolation of compound 1 (Lupeol) and compound 2. Column ii fractions resulted in the isolation of compound 3 (Friedelin), compound 4 (6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone). The structures of compounds 5 and 6 could not be elucidated due to the low quantities available and the presence of impurities. The largest quantity was isolated from compound Lupeol (80 mg), 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8]-flavanone (39 mg), compound Friedelin (27 mg), compound 2 and 5 (14 mg) each and the least was compound 6 (4.8 mg).

Compound 6-(γ,γ-Dimethylallyl)-3',4'-dimethoxy-6",6"-dimethylpyrano-[2",3":7,8] flavanone was reported for the first time in the current study.

Therefore, isolated compounds from the medicinal plant may possess leads to novel antifungal drugs

v. **Determine the antioxidant and antifungal activity of the isolated compounds**

The antifungal activity of the isolated compounds of *L. capassa* against the *C. albicans, C. neoformans*, and *A. fumigatus* which have been implicated as the cause of death to immune-compromised individuals, such as those living with HIV and AIDS is a novel finding. Since most isolated compounds exhibited good activity against *Cryptococcus neoformans* compared to *C. albicans* and *A. fumigatus*. Furthermore, some isolated compounds revealed good antioxidant activity in both scavenging assays, which indicates that isolated compounds of *L. capassa* have the ability to scavenge the free radicals causing cryptococcosis and other fungal infections in humans. The study indicated that isolated compounds from *L. capassa* can be used as a source of therapeutic agents

vi. **Determine the cytotoxicity of the crude extracts and isolated compounds against Vero monkey kidney cells.**

The isolated compounds and crude extract were not toxic against the Vero monkey kidney cells. They had the same degree of cytotoxicity with LC_{50} of > 0.2 mg/ml. The isolated compounds can further be evaluated using *in vivo* studies since they are not toxic.

11.2 Recommendations

- *In-vivo* studies of the isolated compounds

  The *in vivo* research provides treasured information concerning the effects of the isolated compound from plant species in a living organism. Furthermore, *in vivo* studies especially animal studies uncover the mechanisms that underlie various disease processes and evaluate the safety of the isolated compounds. In addition, various biological effects of the isolated compounds may be monitored within the living organism.

It is worthwhile to investigate the potential use of crude extracts and isolated compounds using animal studies. If the extracts and pure compounds are active and not toxic, therefore, a new antifungal agent may be developed from the most promising plant species which could be used to combat fungal infections in humans.

- Investigate the crude and isolated compounds against other microorganisms.

  Testing the isolated compounds and crude to other microorganisms will validate the activity of the compounds to kill several microorganisms not only the selected ones.

  The plant extracts and isolated compounds are active against the tested fungal pathogens. The local community must be taught the conservation measures of harvesting medicinal plants, especially the roots and bark to ensure the future availability of the studied plants.

CHAPTER 12

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