Ethnobotanical survey and biological activity of medicinal plants used against *Candida albicans* in Aganang Local Municipality, Limpopo Province

by

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DECLARATION

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

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DEDICATION

I dedicate this work to my family especially to my beloved parents (Alfred and Salome Tlaamela), my sister (Winny Tlaamela), my brothers (Joseph, Frans and Mammone) and my beloved daughter (Phepishi) who sacrificed their energy and time for my project.

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ABSTRACT

The use of medicinal plants is the most preferred traditional medicine in many populations, and their usage is part of an ancient oral-traditional system of knowledge that remains incompletely documented. Medicinal plants can be used following consultations with traditional healers or through self-medication. The current study focused on medicinal plants with potential to treat oral diseases, particularly oral thrush or oral candidiasis. An ethnobotanical survey was conducted to identify medicinal plants used for the treatment of oral thrush/oral candidiasis by the local people and traditional healers in Aganang Local Municipality, Limpopo Province.

Permission to conduct an ethnobotanical survey was obtained from Bakone Ba Matlala A Thaba Traditional Council. The snowball method was used to select and identify traditional healers and local people. Semi-structured questionnaires and guided field walks with the traditional healers were used to obtain data. A questionnaire was designed to gather information on the names of plants used for the treatment of oral candidiasis, the source of the plants, the plant parts used, methods of preparation of medications and other information. The survey revealed that twelve plant species belonging to ten plant families were used by the local people and traditional healers for the treatment of oral candidiasis. The dominating families were Ximeniaceae and Asteraceae. The most frequently used plant species was *Ximenia caffra* Sond. var. *caffra* (65%), followed by *Ximenia caffra* Sond. var. *natalensis* (35%). Noticeably, the roots (43%) and leaves (21.4%) were mostly used by traditional healers to prepare their remedies. The mode of administration for their remedies was mainly orally and decoctions were the most preferred method of preparation. The observed transfer of indigenous knowledge to younger generations was an important practice for preserving our useful medicinal plants.

Nine plant species (*Artemisia afra* Jacq. ex Willd*., Blepharis subvolubilis* subsp. *subvolubilis* C.V. Clarke*., Enicostemma axillare* (Lam.), *Helichrysum caespititium* (DC.) Harv*., Solanum incanum* L.*, Waltheria indica* L.*, Ximenia caffra* Sond. var. *caffra*, *Ximenia caffra* Sond. var. *natalensis* and *Ziziphus mucronata* Willd.*)* were selected based on the information provided by the local people and traditional healers for further phytochemical

investigation and biological assays. The antifungal activity of the crude extracts against *Candida albicans* was determined using microdilution method and bioautography assay. The leaves of *Artemisia afra* and *Solanum incanum* showed excellent antifungal activity against *C. albicans* with minimum inhibitory concentration (MIC) values of 0.02 mg/ml. Plant extracts of *Blepharis subvolubilis* subsp. *subvolubilis* and *Helichrysum caespititium* had moderate to low antifungal activity with MIC values ranging from 0.625 mg/ml to 2.5 mg/ml. Moreover, the good antifungal activity of water extracts against *C. albicans* confirms the efficacy of traditional methods for the treatment of oral candidiasis. In bioautography assay, more compounds were visible in dichloromethane, acetone, hexane, ethanol and ethyl acetate extracts of *Ziziphus mucronata*, *Waltheria indica* roots and *Ximenia caffra* var. *natalensis.* Benzene: ethanol: ammonia (BEA) was the best eluent solvent system, separating more active compounds, particularly in dichloromethane extracts. Based on good antifungal activity, *W. indica* and *X. caffra* var. *natalensis* were the most promising plant species and were selected for further isolation of active compounds.

Solvent-solvent fractionation of acetone extracts of *Waltheria indica* roots and *Ximenia caffra* var. *natalensis* leaves was performed to successively partition the crude extracts with hexane, chloroform, ethyl acetate, butanol and water. This was followed by column chromatography on the most active fractions. Bioassay-guided fractionation of acetone extracts led to isolation of four compounds. Nuclear Magnetic Resonance and Mass Spectrometry were used to identify and characterise the isolated compounds. Compound **1** was identified as epigallocatechin gallate while compound **3** was identified as kaempferol-3-*O*-rhamnoside. Compounds **2** and **4** were not identified due to the presence of mixtures of long chain fatty acids.

ABBREVIATIONS

1D NMR	One-Dimensional Nuclear Magnetic Resonance
2D NMR	Two-Dimensional Nuclear Magnetic Resonance
A	Acetone
Amp B	Amphotericin B
BEA	Benzene: Ethanol: Ammonia
CEF	Chloroform: Ethyl acetate: Formic acid
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
E/ EtOH	Ethanol
EA	Ethyl acetate
EGCG	Epigallocatechin gallate
EMW	Ethyl acetate: Methanol: Water
ESIMS	Electrospray Ionisation Mass Spectrometry
HAART	Highly Active Antiretroviral Therapy
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome
HRESIMS	High Resolution Electroscopy Ionisation Mass Spectrometry
HPLC-HR-ESI-MS	High Performance Liquid Chromatography-Resolution Electroscopy Ionisation -Mass Spectrometry
INT	p-iodonitrotetrazolium violet
M/ MeOH	Methanol
MeOH: DCM	Methanol: dichloromethane
MIC	Minimal inhibitory concentration
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
R_f	Retention factor
Rpm	Revolutions per minute
TLC	Thin layer chromatography
pTLC	Preparative thin layer chromatography

SDA	Sabouraud Dextrose Agar
QTOF	Quadrupole Time-of- Flight
UPLC	Ultra Performance Liquid Chromatography
UV light	Ultra violet light
WHO	World Health Organisation

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CHAPTER 1

BACKGROUND ON MEDICINAL PLANTS

1.1 Introduction

Medicinal plants have been used for centuries, and numerous cultures still rely on these plants for their primary health care (Gurib-Fakim et al., 2010). In South Africa, medicinal plants are used for the treatment of various diseases such as diarrhoea, skin infections, respiratory disorders, venereal diseases and infectious diseases. Approximately 70-95% of the world's population (WHO, 2011) depend on medicinal plants for physical, social, cultural and economic well-being. Approximately 40-45 000 plant species are present in Africa, and 5 000 of these plants are used for medicinal purposes (Eddouks et al., 2017). South Africa consists of over 24 000 species of higher plants and 10% of these species have been found to be used in traditional medicine (Van Wyk et al., 2009).

Traditional medicine is easily accessible and affordable in developing countries (Tshikalange et al., 2016). Recently, traditional medicines are sold in market places due to the increasing demands of medicinal plants and spreading of infectious diseases. The use and commercialisation of medicinal plants for the treatment of various diseases displays the importance of natural resources in traditional medicine (Amiri and Joharchi, 2012). Moreover, trading and consumption of traditional medicine reflects the values embedded in traditions upheld by elders. The expanding trading of medicinal plants in various societies is stimulated by cultural factors (Martins et al., 2001). However, the increasing demand of medicinal plants usually exploits the wild resources for commercial purposes, leading to habitat degradation and possible extinction. Sustainable management of traditional medicine is crucial to ensure future availability of useful medicinal plants.

Plants produce secondary metabolites and some have antifungal and antibacterial activities (Gunatilaka, 2006). In previous studies, South African plants have been reported to have potential for the discovery of structurally diverse metabolites with useful

pharmacological activities (McGaw and Eloff, 2008). The use of natural products in drug discovery may lead to the finding of new bioactive compounds that can produce effective curative agents for microbial infections. The availability of plant-based medicines may combat infectious diseases and alleviate emerging antimicrobial drug resistance. Moreover, the use of medicinal plants in drug development is not only useful in exploiting their constituents themselves for therapeutic purposes, but also as starting materials or lead compounds for drug synthesis (Ahmed, 2016).

Oral candidiasis is one of the fungal infections, mostly caused by *Candida albicans*, which is most prevalent in immuno-compromised patients. It is characterised by an overgrowth of *Candida* species in the epithelium of the oral mucosa. However, treatment of oral candidiasis faces challenges such as drug resistance, high cost and adverse effects that are usually caused by toxicity of antifungal agents (Mehta et al., 2002 and Kathiravan et al., 2012). These complications necessitate research on the bioactive compounds of natural products or traditional medicine to control such infectious diseases.

In this dissertation, antifungal activity of selected medicinal plants used to combat candidiasis in Aganang Local Municipality were investigated. An extensive ethnobotanical survey was conducted to identify various medicinal plant species in the area. This is important for documenting useful plant species which may lead to drug discovery. Pharmacological screening of plants was conducted for validating the safety and efficacy of these medicinal plants. Furthermore, isolation and structure elucidation of pure compounds was also performed.

1.2 Rationale

Candida species comprise the second most frequent cause of fungal infections worldwide (Whibley and Gaffen, 2015)*. Candida albicans* is a pathogenic microorganism that causes oral thrush (Mayer et al., 2013), particularly in immuno-compromised individuals (Samaranayake, 2000 and Jeevitha et al., 2015). The infection of oral candidiasis in HIV/AIDS patients has become a major public health problem (Vazquez, 2000 and Sanguinetti et al., 2015). The increasing incidence of oral diseases due to HIV/AIDS,

coupled with a lack of oral health facilities creates a natural demand for the use of plant products to treat oral diseases (Chinsembu, 2016). Information regarding the treatment of infectious diseases caused by *C. albicans* is not well documented, and not much has been recorded on medicinal plants used for the treatment of candidiasis in rural areas of Limpopo Province. Treatment of candidiasis is complicated due to *Candida* species that are resistant to the currently available antifungal agents (Pfaller et al., 2015 and Sanguinetti et al., 2015), such as amphotericin B, echinocandins and fluconazole. Moreover, some of these antifungal agents are expensive (Mehta et al., 2002), toxic and have low efficacy rates (Mehta et al., 2002 and Kathiravan et al., 2012).

In developed countries, many medicines are produced synthetically in pharmaceutical companies, but the original formulas are derived from plants (Wanjui, 2013). Plants produce secondary metabolites that could lead in the discovery of novel antifungal agents and some of these compounds have antifungal and antibacterial activities which strongly inhibit the growth of other microorganisms (Gunatilaka, 2006). Ethnobotanical surveys play a significant role in identifying and documenting useful plant species. Pharmacological screening of plants is important for the discovery of new, safe, and effective drugs that could combat fungal infections in humans and animals. As such, there is a need for development of new antifungal agents against *Candida* and to combat strains expressing resistance to the available antifungals.

Aim

The aim of the study was to investigate medicinal plants used for the treatment of oral thrush and to determine antifungal activity against *Candida albicans*, and isolate and characterize the compound(s) from selected plant species.

Objectives

The objectives of the study were to:

i. Conduct an ethnobotanical survey to investigate medicinal plant species used for the treatment of oral candidiasis using a semi-structured questionnaire.

ii. Identify the plant parts used by local people and traditional healers to prepare their herbal remedies.
iii. Identify the preparation methods and mode of administration used by traditional healers and local people to combat oral candidiasis.
iv. Select and identify plant species for further phytochemical analysis.
v. Determine the antifungal activity of the selected plant species against *Candida albicans*.
vi. Isolate antifungal compounds from the selected plant species.
vii. Determine the chemical structure of isolated compounds.

1.3 Outline of study

Chapter 1 deals with the general background on medicinal plants, traditional medicine and oral candidiasis. The rationale, aim and objectives of the study are given.

Chapter 2 focuses on the literature review of the medicinal plants and their metabolites, indigenous knowledge systems, oral candidiasis and its management and treatment using both antifungal agents and medicinal plants. The botanical description of the selected plant species is also included.

Chapter 3 is concerned with the ethnobotanical survey on medicinal plants used for the treatment of oral candidiasis. This was based on questionnaires; twelve plant species were identified based on their medicinal uses by local people and traditional healers. Their medicinal uses and applications were highlighted.

Chapter 4 deals with the antifungal activity of nine selected medicinal plants against *Candida albicans*. These plant species were screened for antifungal activity using two different assays: microdilution and bioautography assays.

Chapter 5 focuses on isolation of pure compounds from the leaves of *X. caffra* var. *natalensis*. Bioassay-guided fractionation using solvent-solvent fractionation and column chromatography led to isolation of four compounds.

Chapter 6 deals with the identification of pure compounds isolated from *X. caffra* var. *natalensis*. NMR and MS were used for the identification of pure compounds.

Chapter 7 gives the summary and conclusion of the study. Some recommendations for future work have been suggested. The references used in the study are listed.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter comprises a literature review based on medicinal plants, their uses, traditional medicine and indigenous knowledge. The review is important for understanding the findings of various studies based on medicinal plants. Also, it emphasizes the importance of finding similarities and differences in the uses and applications of various plant species.

2.2 Uses of medicinal plants

Plants that have therapeutic properties and relieve symptomatic problems are defined as medicinal plants. These plant species maintain and promote healthy life by preventing and curing various diseases in humans and animals. Each medicinal plant has its own chemical constituents that may act individually or in synergy to improve wellness. These compounds are used to treat serious acute and chronic conditions, and improve the quality of life in a cost-effective manner. In developed countries, the original formulas of many medicines produced by chemicals in pharmaceutical companies are derived from plants (Wanjui, 2013). Therefore, medicinal plants are major sources of potential drugs. However, these plants should be examined scientifically to better understand the mode of their efficacy and properties. In pharmaceutical trades, most drugs derived from plants are less expensive, safe, and effective; and rarely have side effects (Thite et al., 2013). Despite the availability of different approaches for drug discovery, plants remain the main source of new structures.

2.3 Traditional healthcare system

A traditional healthcare system involves the utilisation of traditional medicine for achieving optimum health and wellness. Traditional medicine has contributed to the improvement of immune systems, especially with critically ill people (Calixto, 2000). It comprises formalised systems with well-documented remedies practiced by lay people as well as

more informal practices handed down orally from generation to generation (Efferth and Greten, 2014). This healthcare system is mostly used by older people because the younger generations have acquired greater reliance on Western medicine through modernised education, religion and cultures. This modernisation results in loss of traditional values as they tend not to appreciate their tradition. Most communities use the traditional healthcare system to combat various diseases since it is easily accessible, cheaper (Rennie, 2001) and due to lack of effective medical services. The preference of traditional medicine over modernised medicine is also driven by strong cultural beliefs and practices, and the extensive local knowledge and expertise among the local communities (Omwenga et al., 2012 and Omwenga et al., 2015). The increase in demand for traditional medicine results from the increasing human population and the inadequate provision of Western medicine in developing countries.

2.4 Indigenous knowledge

Indigenous knowledge can be described as knowledge that is unique to a given culture or society and is passed from generation to generation. It encompasses the skills, experiences and insights of people, applied to maintain or improve their livelihoods (World bank, 1997). Indigenous knowledge on natural resource utilisation of medicinal plants is regarded as an important measure of sustainable plant biodiversity conservation (Kala et al., 2004). Noticeably, loss of traditional knowledge in South Africa is triggered by resulting urbanisation and strong cultural influences from the regions in the world (Van Wyk, 2002).

Traditional knowledge of medicinal plants and their use by indigenous healers and local people is advantageous for community healthcare and future drug development (Pei, 2001). Indigenous knowledge and practices assist in planning conservation strategies for plants and cultural biodiversity. Lack of transfer and documentation of indigenous knowledge results in loss of cultural heritage; and people are forced to change their livelihoods which may lead to severe environmental degradation (Turner et al., 2000).

2.5 Medicinal plants used for oral candidiasis

The utilisation of medicinal plants in the treatment of oral candidiasis is commonly practiced in South Africa. Despite the accessibility of anti-retroviral (ARV) drugs, many people still use traditional medicine for the treatment of opportunistic infections related to HIV/AIDS. Although the ARVs lower the occurrence of opportunistic infections of HIV, there are still drawbacks which lead to the usage of traditional medicine as a means of restoring the immune system (Gail et al., 2015). Traditional healers play an essential role in efforts to address early oral manifestations of HIV/AIDS (Lewis et al., 2004 and Rudolph et al., 2007). It has been reported that most South Africans who visit Western medical health centres first consult traditional healers (Elgorashi et al., 2003).

The majority (90%) of traditional healers interviewed by Lewis et al. (2004) were familiar with oral candidiasis and other mouth problems. Another ethnobotanical study for oral candidiasis was conducted in the Vhembe District (Masevhe et al., 2015). The most common used plant species in the Vhembe region were *Erythrina lysistemon* Hutch, *Solanum panduriforme* E. Mey, *Tagetes minuta* L., *Carpobrotus edulis* (L.) L. Bolus and *Dodonaea angustifolia* L.F. The leaf decoction of *Dodonaea viscosa* was reported to be used for the treatment of oral candidiasis (Naidoo et al., 2012). In Tanzania, *Carica papaya L., Sclerocarya birrea* (A. Rich) Hochst.*, Securidaca longipedunculata* Fresen.*, Ziziphus mucronata* Willd., *Dichrostachys cinerea* (L) Wight & Arn. and others were reported to be used against candidiasis (Runyoro et al., 2006b). *Aloe vera* is used as mouth rinses for mouth thrush and other oral diseases (Subramaniam et al., 2014).

2.6 Conservation status of medicinal plant species

The intensive harvesting of medicinal plants may result in overexploitation, thus threatening the biodiversity. For example, approximately 65% of the medicinal plants used worldwide are tree species, and most of these species are becoming endangered, rare or threatened due to unsustainable harvesting methods (Gates, 2000). Moreover, medicinal plants have uses other than being medicinal sources and over-harvesting for collection purposes poses a threat to the plant species. Examples include *Dichrostachys cinerea* (L) Wight & Arn., *Acacia* species and *Pinus* species. Apart from over-harvesting,

other threats to medicinal plants may results from habitat loss and habitat degradation (Gail et al., 2015). Noticeably, most of plants are collected from the wild. Therefore, the ever-expanding demands of these plants may lead to overexploitation if harvesting is not sustainable.

Medicinal plants also provide a source of income to the livelihood of various people, through the selling of wild-harvested plant material. Therefore, many traded medicinal plants are threatened by unsustainable and destructive harvesting to satisfy human needs (Williams et al., 2013). It was estimated that over 20 000 tons of plant material are sold annually at the informal medicinal markets (Taylor et al., 2001). This may result from the increased demand for cheaper medicines, high unemployment rates and greater incidences of infections from HIV/AIDS (Mabona and Van Vuuren, 2013). Some of the species are *Warbugia salutaris* L., *Cyanella marlothii* J.C. Manning & Goldblatt., *Siphonochilus aethiopicus* (Schweif.) B.L. Burtt., *Euphorbia melanohydrata* Nel. and *Astridia speciosa* L. Bolus (SANBI, 2015).

Cultivation of the most highly used plant species is advised for maintaining the availability of the plants. Van Staden (1999) stated that these plants need to be developed as crops for small-scale farming. Furthermore, plant substitution has been proposed by Zschocke et al. (2000), where the bark or underground part is substituted by the leaves of the same plant.

2.7 Fungal pathogen

2.7.1 *Candida albicans*

Fungal pathogens are the cause of leading infectious diseases across the world. Worldwide, *Candida* species comprise the second most frequent cause of fungal infections (Whibley and Gaffen, 2015)*. Candidial* infections are predominant in warm, moist body areas. *Candida albicans* is the prevalent causative fungal pathogen for oral thrush, particularly in immuno-compromised individuals. It has become a major public health problem as an opportunistic infection of HIV/AIDS (Vazquez, 2000 and Masevhe

et al., 2015). Furthermore, it accounts for about 90% of cases as the main causative agent of oral candidiasis (Edwards, 1995 and Lopez-Ribot et al., 1999).

2.7.2 Oral candidiasis

Infections caused by *Candida* species are known as candidiasis, which can either be oral or vaginal. Oral thrush is a condition where fungus is accumulated in the lining of the mouth. It is characterised by an overgrowth of *Candida* species in the epithelium of the oral mucosa (Melkoumov et al., 2013). More importantly, it reduces the quality of life and increases mortality in infected patients as it leads to life-threatening systemic infections. The occurrence of oral candidiasis is a sign of impaired local or systemic defence mechanism (Magare and Awusthi, 2014) and imbalances in the immune system (Oro et al., 2015). The symptoms of oral candidiasis include creamy white lesions on the tongue, inner cheeks, and sometimes on the roof of the mouth, gums and tonsils, slightly raised lesions with a cottage cheese-like appearance. Asymptomatic characteristics include burning, change of taste, painful sensation, and swallowing difficulty. Infected people often lose weight because of sore throat, which prevents them from eating (Sanne, 2001). Oral candidiasis is severe in immunocompromised patients and people receiving the treatment for HIV (Bonifait et al., 2012). The high incidence of oral candidiasis in HIV/AIDS patients has made candidiasis a leading fungal infection (Vazquez, 2000 and Jankowaska et al., 2001).

2.8 Management and treatment of oral candidiasis

There are several methods for preventing oral thrush, though there is no reliable evidence for its effective treatment (Clarkson et al., 2004). The incidence of oral candidiasis is variable and depends on the nature of the underlying disease and the intensity of the treatment (Clarkson et al., 2009). However, there are a number of difficulties encountered with the management of *Candida* infections such as the limited number of antifungal agents, toxicity, low efficacy rate and high cost of the available antifungal agents (Mehta et al., 2002 and Kathiravan et al., 2012). Another challenge is the interaction of *Candida albicans* with the host especially in the gut or mucosal surfaces, which makes oral candidiasis recurrent.

2.8.1 Antifungal agents

Polyenes were firstly designed as antifungal agents for the treatment of oral candidiasis (Epstein, 1990 and Lewis et al., 1991). To date, only three polyene agents are available: nystatin, natamycin and amphotericin B. Polyenes disrupt the fungal membrane by binding to the lipid bilayer to form a complex with ergosterol to produce a pore (Campoy and Adrio, 2016). This promotes leakage of the contents present in the cytoplasm and oxidative damage leading to fungal cell death. Two of these polyenes (nystatin and natamycin) are only used as topical agents due to their high toxicity to the host. Amphotericin B is active against most fungal pathogens (*Candida*, *Aspergillus*, *Fusarium*, etc.) and is mostly used for more resistant cases of oral candidiasis (Dowd, 2014). It is given intravenously or topically due to its hydrophobicity and adverse side effects such as fever, vomiting and neurological toxicology.

Azoles are fungistatic antifungal agents that also have a broad-spectrum activity against fungal pathogens. They inhibit ergosterol biosynthesis, the main component of the fungal cell membrane. Furthermore, two groups of azoles are available, imidazoles and triazoles. Imidazoles include clotrimazole, miconazole and ketoconazole. The second group (triazoles) were designed to address the high toxicity caused by the imidazoles which include itraconazole and fluconazole. Triazoles have significant safety profiles and a broader spectrum of activity than imidazoles (Campoy and Adrio, 2016).

Echinocandins (caspofungin, anidulafungin and micafungin) are the new antifungal agents that inhibit the β-glucan synthesis. They show a broader spectrum activity against fungal pathogens. They are reported as fungistatic agents against *Aspergillus* species and as fungicidal agents against several *Candida* strains (Campoy and Adrio, 2016). Topical agents including nystatin, amphotericin B, itraconazole, miconazole and clotrimazole are recommended as the first-line treatment for uncomplicated cases of oral candidiasis (Akpan and Morgan, 2002). Fluconazole and itraconazole are appropriate for patients that do not respond to topical treatment and those at high risk of developing systemic infections (Epstein and Polsky,1998).

2.8.2 Antifungal drug resistance

Antifungal resistance is defined as failure of the fungal infection to respond effectively to the antifungal agent. It arises through the emergence of non-susceptible variants within a population of sensitive strains (Kontoyiannis and Lewis, 2002). The development of drug resistance in the treatment of *C. albicans* infections may have risen from the extensive use of a limited number of antifungal agents or the improper handling of antibiotics (Garza et al., 2017). The most important contributing factor to the resistance of *C. albicans* to antifungal drugs is its ability to form biofilms (Cretton et al., 2016). Pathogenic fungi have complex mechanisms of resistance to antifungal drugs (Sanglard, 2002). The cells can develop resistance mechanisms to overcome growth inhibition by antifungal agents or mobilise factors that detect if the agent is fungicidal or fungistatic (Sanglard, 2012). Fungicidal agents kill the fungi while fungistatic agents prevent further growth of the fungi.

Treatment of candidiasis is complicated due to *Candida* species that are resistant to currently available antifungal agents (Pfaller et al., 2015 and Sanguinetti et al., 2015), such as amphotericin B, echinocandins and azoles. Resistance of *Candida* species to azoles is the most prevalent type of antifungal resistance (Sheehan et al., 1999). Resistance of fungi to azoles usually occurs through any of three mechanisms: reduced azole accumulation through the active efflux, alteration/over-expression of the binding site or a loss-of-function downstream mutation in the ergosterol pathway (Kontoyiannis and Lewis, 2002).

2.9. Plant bioactive metabolites

Plants have the ability to synthesise organic substances that perform physiological actions in animals and humans. Bioactive compounds can be defined as secondary metabolites that exhibit pharmacological effects in humans and animals. They are produced in a phase subsequent to growth by certain restricted taxonomic groups of microorganisms (Azmir et al., 2013). These substances are known as secondary metabolites since they are not essential for the growth and development of the plant producing them. They are derivatives of primary metabolites produced through

biosynthetic processes, as defence mechanisms against herbivores, pathogenic attack, stress and ultra-violet exposure. The bioactive substances that perform these actions include alkaloids, flavonoids, tannins, steroids, carbohydrates and terpenoids (Edoga et al., 2005). Secondary metabolites accumulate in different plant parts and they also contribute to the plants' colour, aroma and flavour (Thite et al., 2013).

These secondary compounds form the basis for drugs that combat various diseases in humans, and are commercialised across the world. The use of natural products in drug discovery is a lead for finding novel compounds that can produce effective and curative agents for opportunistic fungal infections. Furthermore, the selection of plants is based on the long-term use history through ethnobotany. Examples of plant-derived drugs include vincristine, an anti-cancer agent isolated from *Cantharanthus roseus* (L.) G. Don. and quinine, an anti-malarial agent isolated from the bark of *Cinchona officinalis* L. Currently, about 142 anti-*Candida* active natural compounds have been reported worldwide, and 29 (20.42%) of these compounds were reported in Africa (Zida et al., 2016).

2.10 Botanical description of plant species selected for the study.

Nine plant species used for the treatment of candidiasis/oral thrush in Aganang Local Municipality, Capricorn District were selected based on the information provided by the traditional healers and local people. All pictures of the selected plant species were taken from Boratapelo, Ntlolane and Vlakfontein villages of Aganang Local Municipality (Figures 2.1-2.9).

Artemisia afra Jacq. ex Willd (Asteraceae) is a perennial shrub that grows to up to 2m tall. It is usually characterised by its green leaves that resemble feathers, and an aromatic smell (Figure 2.1). Its conspicuous, pale yellowish flowers are borne along end branches (Van Wyk et al., 2000). In South Africa, it grows in thick, bushy areas and it is distributed throughout the mountainous regions of South Western Cape and extends northwards through the Limpopo Province (Suliman et al., 2010). It globally extends northwards into tropical east Africa as far north as Ethiopia (Van Wyk et al., 2000).

Figure 2.1. *Artemisia afra* Jacq. ex Willd (African wormwood)

Blepharis subvolubilis subsp. *subvolubilis* C.V. Clarke (Acanthaceae) is a semi-deciduous that grows to less than 30 cm tall (Figure 2.2). Its leaves are arranged in whorls of 4, with one leaf greater than the others. The leaves are roughly pubescent and have toothed margins. It has spectacular flowers, with glossy, green to brownish bracts with 4-12 spikes.

Figure 2.2. *Blepharis subvolubilis* subsp. subvolubilis C.V. Clarke
(Eyelash flower)

Enicostemma axillare (Lam.) A. Raynal (Gentianaceae) is an erect, perennial herb that grows up to 5-30 cm tall (Figure 2.3). Its leaves are sessile, sometimes narrowed into a petiole-like base. They are linear to lanceolate or narrowly oblong, entire obtuse and mucronate at the apex. Its inflorescence occurs in many flowered axillary clusters, numerous in the axils of each pair of leaves. The flowers are white with green lines, drying sessile with long bracts (Saranya et al., 2013).

Figure 2.3. *Enicostemma axillare* (Lam.) A. Raynal (White head)

Waltheria indica L., belonging to the family Asteraceae is a short-lived, erect perennial shrub or herb that grows to up to 500 mm high (Figure 2.4). Its stems usually emerge from the ground, with stalked leaves that have irregularly toothed margins. Its yellow flowers occur in clusters. It is distributed in tropical and subtropical regions and in undisturbed soils.

Figure 2.4. *Waltheria indica* L. (monkey bush or velvet leaf)

Solanum incanum L. (Solanaceae) is an herbaceous shrub that grows from 0.5 to 3m tall (Chirchir et al., 2014) (Figure 2.5). The leaves are simple, ovate and elliptic (Indhunamathi and Monandass, 2013). Its stems and underside leaves may be pubescent. It bears a spherical, green and often striped with white fruit that turns yellowish when ripe.

Figure 2.5. *Solanum incanum* L. (Sodom apple)

Helichrysum caespititium (DC.) Harv. (Asteraceae) is a perennial, mat-forming herb with densely tufted branches (Figure 2.6). Leaves are linear, with a broad base and clasping branches. It bears silvery white flowers with yellow centres and which are pale and furry underneath.

Figure 2.6. *Helichrysum caespititium* (DC.) Harv. (Speelwonderboom)

Ximenia caffra Sond. var. *caffra,* belonging to the family Ximeniaceae, is a dioecious shrub or tree that grows up to 6m tall (Figure 2.7). Its leaves are simple, elliptic, alternate, leathery, blue-green with entire margins and rounded apex. The branches and twigs are armed with stout axillary spines. It has a greyish-brown to black rough bark. Its flowers are small, sweet-scented, creamy green to creamy white, borne on a single stem. They occur in clusters on the axils of the spine or on dwarf branchlets. It bears a green drupe with a hard-coated seed that turns orange to reddish when ripe (Palgrave, 2002).

Figure 2.7. *Ximenia caffra* Sond. var. *caffra* (Sour plum)

Ximenia caffra Sond. var. *natalensis* (Ximeniaceae) is a much-branched shrub or small tree with a blue-green appearance (Figure 2.8). Leaves occur often in tufts, oblong blue or grey-green, and are hairless, folded upwards along the midrib. Its green-whitish flowers are clustered in axils of spines. It bears an oval, edible drupe that has a bitter taste. (Van Wyk and Van Wyk, 1997).

Figure 2.8. *Ximenia caffra* Sond. var. *natalensis* (Sour plum)

Ziziphus mucronata Willd. (Rhamnaceae) is a small to medium-sized tree, with a spreading canopy (Figure 2.9). Its bark is rough and greyish-brown. The leaves are bright green and shiny above with three main veins; margins toothed in the upper half. Its small, yellowish-green flowers occur in clusters above each leaf (Van Wyk et al., 2000). It flowers between March and June producing small, red-brown berries with a thin skin. Its berries are available all year but are mostly common between December and June (Van Wyk and Gericke, 2000).

Figure 2.9. *Ziziphus mucronata* Willd. (Buffalo thorn)

CHAPTER 3

ETHNOBOTANICAL SURVEY OF PLANT SPECIES

3.1 Introduction

An ethnobotanical survey was conducted to identify medicinal plants used to treat oral candidiasis by local people and traditional healers in Aganang Local Municipality. This chapter describes the identified plant species, mode of preparation, plant parts used and frequency index.

The use of medicinal plants is mostly preferred in traditional medicine in many African populations. Medicinal plants and their uses in sub-Saharan Africa are part of an ancient oral-traditional system of knowledge that remains incompletely documented (Van Wyk, 2015). The utilisation of medicinal plants can be through consultation with traditional healers or through self-medication. The information on specific plant species used for the treatment of diseases is continuously passed from older generations to the next. Moreover, the utilisation of medicinal plants for various diseases varies from region to region and even from one traditional healer to another (Mahwasane et al., 2013).

Indigenous knowledge has a high value not only for the indigenous cultures, but also for the scientific world (Cussy-Poma et al., 2017). Valuable knowledge about the medicinal plants used lies with the traditional practitioners because they have experience in using a wide range of medicinal plants that address a variety of diseases. It was reported that most Africans rely heavily on traditional medicine and traditional healers are the first and last line of defence against most diseases (Matsheta and Mulaudzi, 2008). Approximately 60% of Africans consult traditional healers in preference or in addition to Western medical doctors (Elgorashi et al., 2003). Therefore, they are targeted for documentation of these useful plants before they are lost due to over-exploitation. Worldwide, there is an increasing interest in traditional healers as a resource for healthcare delivery (Nevin, 2000). Traditional healers have greater credibility than village health workers with respect to social and cultural matters (Kisangau et al., 2011).

Oral diseases including candidiasis are major public health problems (Bonifait et al., 2012). The increasing incidence of oral diseases due to HIV/AIDS increases a demand for the use of medicinal plants. Documenting the indigenous knowledge through ethnobotanical studies is important for sustainable utilisation of medicinal plants in plant discovery (Mbunde et al., 2017). Documentation of this information may also play a key role in conservatory aspects of potential plant species with proven biological activities. Ethnobotanical surveys are important for documenting useful plant species, which may lead to new antimicrobial compounds in the drug discovery process.

3.2 Materials and methods

3.2.1 Description of the study area

3.2.2 The study area

The study was conducted in Aganang Local Municipality, Capricorn District, Limpopo Province, South Africa (Figure 3.1). The area lies between 23°40′S and 29°5′E, covering an area of 1881 km^2. It is a rural municipality situated 45 km west of Polokwane city with a human population size of 131 164, and four traditional councils with 19 wards (Statistics SA, 2011). It receives rainfall during summer (between November and May) with a mean annual precipitation ranging from 454 to 500 mm (Mucina et al., 2005). Annual temperatures range between 26-32°C in summer, and between 7-24°C in winter.

3.2.2 Vegetation

The vegetation types are characterised by Makhado mixed bushveld, small portions of arid sweet veld and Mamabolo mountain bushveld around Mogoshi mountains in Matlala; and Polokwane plateau grassland vegetation (Environmental Management Plan, 2009).

3.3 Data collection

3.3.1 Ethnobotanical survey

Traditional healers and local people were interviewed after permission was granted from the headman of each area. Data was obtained using semi-structured questionnaires (Appendix A) and guided field walks with the traditional healers. Traditional healers and local people were requested to sign a consent form before the interview. Twenty

participants (nine traditional healers and eleven local people) from different villages were chosen using the snowball method. Snowball method is a convenient sampling method whereby the existing participants recruit future participants. A questionnaire was designed to gather information on the names of plants used for the treatment of oral candidiasis, the source of these plants, the part/s of plants used, methods of preparation of medications and other information.

3.3.2 Plant collection and identification

Plant species were collected from their natural habitat during March-May 2016 with the help of traditional healers. Literature and the Larry Leach Herbarium at the University of Limpopo were used to identify the plant species by their scientific names. Voucher specimens of the plant species were prepared and deposited at the herbarium.

3.4 Data analysis

The documented data were analysed using descriptive and inferential statistics with Microsoft Excel 2010. Percentages and frequencies were portrayed in tables and figures to interpret the findings of the study. The frequency index of each plant species was calculated using the formula: FI= FC/N x 100, where FC is the number of traditional healers who mentioned the use of the plant and N is the total number of informants.

3.5 Ethical considerations

The current study was conducted in Aganang Local Municipality, and no samples were collected from animals or human beings. Each traditional healer was requested to sign a consent form approved by the University of Limpopo. The proposal was approved by Turfloop Research Ethics Committee (TREC) for ethical clearance prior to the commencement of the study.

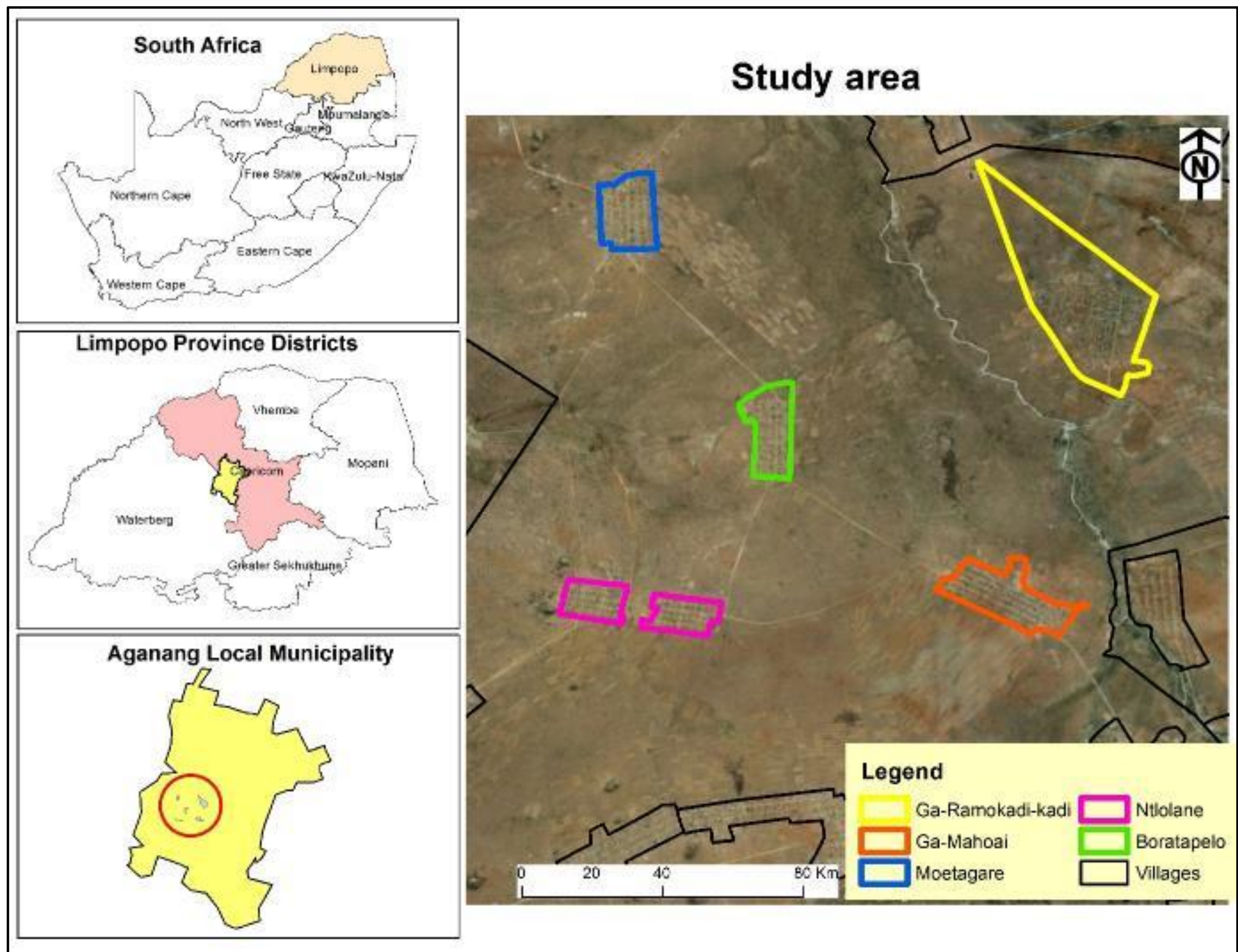

Figure 3.1. Map of Aganang Local Municipality

3.6 Results and discussion

3.6.1 Demographic information

3.6.1.1 Gender

Twenty people were interviewed, 9 males (45%) and 11 females (55%). This suggests that women are more knowledgeable on the usage of medicinal plants for the treatment of oral candidiasis than men. The results were in accordance with previous studies (Gail et al., 2015 and Eddouks et al., 2017). However, some studies indicated the highest contribution of male informants regarding the indigenous knowledge of medicinal plants (Baydoun et al., 2015 and Ahmed, 2016).

3.6.1.2 Age

Many of the participants (60%) fell within the age range of 60-79 years; 25% were 40-59 years and 15% were 20-39 years. Based on our findings, the indigenous knowledge and practice of medicinal plants lie in the older people. This was similar to studies conducted by Kisangau et al. (2011) and Agbor and Naidoo (2011). The percentage of old-age respondents was very low in several studies (De Wet et al., 2013 and Ahmed, 2016).

3.6.1.3 Educational background

The majority (40%) of the participants did not have formal education; 30% have acquired primary education and 30% have received secondary education. The high percentage of non-educated participants might be attributed by the long history of medicinal plants practice, when education was not popular in the area. The results are similar to those obtained by De Wet et al. (2013) and Barkaoui et al. (2017). However, some studies reported a high rate of traditional healers with primary education (Ahmed, 2016 and Eddouks et al., 2017) and a low rate of tertiary education (Barkaoui et al., 2017).

3.6.1.4 Consultation

The traditional healers do not have daily patients compared to Western clinics and hospitals. They usually have less than 10 consultations per month and they also allow further consultations for patients that are not completely healed. Noticeably, some refer their patients to the nearest clinic or hospital. Similar studies reported the traditional healers' referrals to other health care workers where necessary (Lewis et al., 2004 and Agbor and Naidoo, 2011). These traditional healers do not have the same patients coming for consultations throughout the year.

3.6.1.5 Legislation

Most of the traditional healers (77.8%) are registered with the traditional healers' association. However, others mentioned that the association has strict rules and some did not understand the importance of being registered. Related results were obtained by Agbor and Naidoo (2011), where 76% of the traditional healers in the study were registered with the traditional healers' association. Interesting results were obtained by

Gail et al. (2015), which showed that all traditional healers in Mpoza were registered with the local traditional healer's association.

3.6.2 Traditional healing practice

The survey revealed that 45% of the participants use traditional healing practice through ancestral callings and 55% through indigenous knowledge. Baydoun et al. (2015) also reported a low rate of ancestral traditional healers (23%), with 77% without professional practice. The traditional healers in the study area acquired their professional training through the performance of the ritual known as *twasa*. This is important as the community believes that people who go through the ritual before practicing as traditional healers know their work and can be highly recommended for consultation purposes. Most of the informants acquired the traditional knowledge from their elders. One of the traditional healers mentioned that he used to accompany his father for plant collections and assisted him with some remedy preparations such as grinding when he was young. This was an interesting interaction as well as a practical way of sharing indigenous knowledge, cultural and traditional practices with the young generation. According to Cheiskhyoussef et al. (2011), children that accompany their grandparents during medicinal plant collection become motivated and develop an interest in practicing the traditional healthcare system.

3.6.3 Methods of plant collection and storage

All participating traditional healers collected their plants from the wild mainly for specific purposes. However, some of these plant species face seasonal variations. They prefer collecting plant materials in early winter as they believe that they will be matured enough and more active than those collected in summer. Most of the traditional healers (66.67%) prefer growing the commonly used plants in their home gardens to make ease of availability of the plants that grow far from their homes. This similar collection method was reported by Mahwasane et al. (2013). It was worth noting that they also make use of cultivated species. However, they claim that cultivated plants are less effective than wild plant species. They believe that wild plants are much more uncontaminated than those that are exposed to human beings.

For plants that are unable to grow in their homes, they collect them in large quantities and dry, grind and store them in labelled bottles. Similar results were obtained by Mahwasane et al. (2013). This approach is mainly practiced for plant species that grow in mountains and river banks such as *Zanthoxylum humile*, where they have to travel a distance to access them. Some unprocessed materials like roots and bark are dried and stored in their ancestral room (Hut). Masevhe et al. (2015) also reported the storage of plant materials in the hut. However, this approach is not always applicable as some diseases need preparation of fresh materials, especially those that are boiled for inhalation purposes. According to traditional healers, pounded and unprocessed medicines last longer and they have no expiry date. They discourage storing plant materials in a liquid form because it does not last long. A change in appearance and colour may be observed and this suggests that the mixture is perished and should be discarded. More importantly, it was observed that traditional healers in the area buy some plant materials from other traditional healers, street vendors and from the muthi market.

3.6.4 Identified medicinal plants

Based on our results, only twelve plants are used for the treatment of oral thrush by traditional healers of Aganang Local Municipality (Figure 3.2). The documented plant species belong to 10 plant families. The most preferred families were Ximeniaceae and Asteraceae, both with a prevalence of 16.7%. Asteraceae was the dominant family in previous studies (Maema et al., 2016 and Fenetahun et al., 2017). The remaining families were represented by one plant species. The plants' vernacular names, scientific names, family names, plant parts used, methods of preparation and mode of administration are represented in Table 3.1.

Based on our findings, the roots of *Warburgia salutaris* are burnt and applied topically to the mouth. Alternatively, its roots can be ground and drunk with water. *W. salutaris* is an endangered plant species and it is also sold in market places (Coopoosamy and Naidoo, 2012). It was not found in the visited areas of Aganang Local Municipality and the traditional healers always buy the plant from the markets. The leaves of *W. salutaris* have been reported for the treatment of respiratory disorders and venereal disorders (Giday et

al., 2003). Interestingly, a different preparation mode (infusion) of *Carpobrotus edulis* leaves for the treatment of oral candidiasis was reported (Otang et al., 2012). Its leaves are also chewed for the treatment of toothache (Maema et al., 2016). Moreover, the leaves are topically applied for the treatment of skin disorders (Mabona and Van Vuuren, 2013).

The leaves and roots of *Solanum incanum* were reported to be used for the treatment of toothache and skin disorders (Mabona and Van Vuuren, 2013 and Mbunde et al., 2017). The juice of the fruits is used for the treatment of toothache and can also be applied topically to wounds caused by sexually transmitted infections (Kambizi and Afolayan, 2001 and Mbunde et al., 2017). A leaf decoction of *Artemisia afra* is reported for the treatment of oesopharyngeal candidiasis (Otang et al., 2012 and Maroyi, 2014). Its leaf infusion is used for the treatment of respiratory disorders, headache and intestinal worms (Coopoosamy and Naidoo, 2012 and Verschaeve and Van Staden, 2008). The roots of *Waltheria indica* are used for the treatment of cough, diarrhoea, stomach ache, burns, wounds and fever (Ayantunde et al., 2009 and Mabona and Van Vuuren., 2013 and Nciki et al., 2016). Its leaves are consumed as a nutritious vegetable in the Vhembe region (Magwede et al., 2018).

Enicostemma axillare was previously reported for its administration as a tonic for appetite loss and stomach ache (Saranya et al., 2013). The whole plant is also used for the treatment of boils, abdominal ulcers and insect poisoning as well as being a blood purifier (Deore et al., 2008). The whole plant of *Helichrysum caespititium* is crushed and burnt, and its smoke is inhaled to treat headache and chest colds (Mathekga and Meyer, 2001 and Lourens et al., 2008). The root decoction of *Zanthoxylum humile* is reported to be used as a mouth anaesthetic and the powdered roots are topically applied to wounds (Riberio et al., 2010). The roots of *Ziziphus mucronata* are boiled and drunk for uncontrolled menstruation, infertility and indigestion (Maema et al., 2016). Its leaves are chewed for the treatment of nerve pains and a root decoction is administered for improving the treatment (Mabogo, 1990). The leaves were reported for the treatment of boils, wounds, chest problems and diarrhoea (Giday et al., 2003; Nciki et al., 2016 and Maema et al., 2016).

In our study, the fruits of *Ximenia caffra* var. *caffra* are burnt, mixed with petroleum jelly and applied to the mouth. The same application method is applied with *X. caffra* var. *natalensis*. Its stem bark is boiled to prepare soft porridge for the treatment of diarrhoea. The leaves and roots were reported for the treatment of several diseases including infertility, blood in the faeces, cough, fever, venereal diseases, boils and scurvy (Mulaudzi et al., 2011 and Nciki et al., 2016).

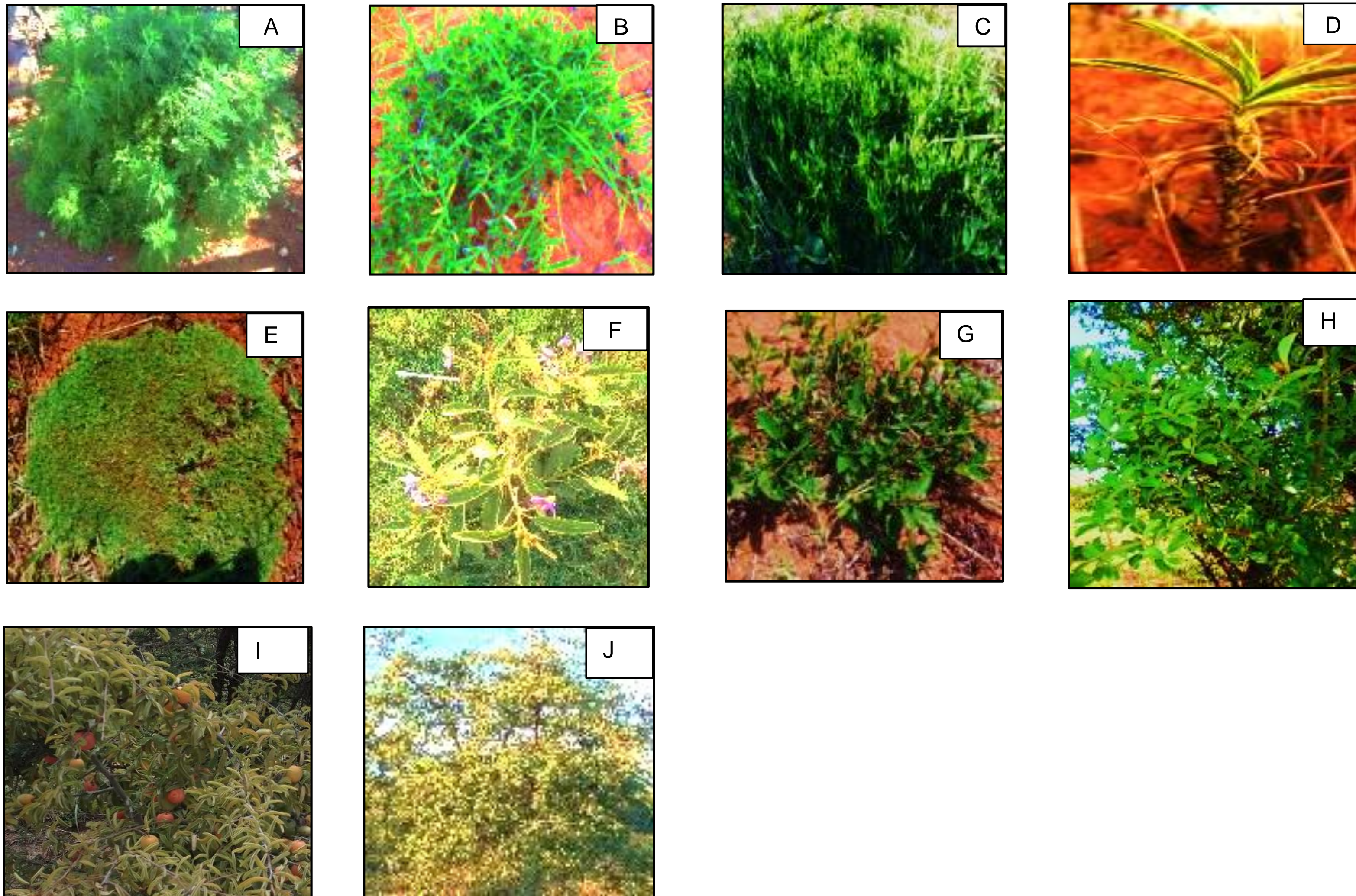

Figure 3.2. Medicinal plants used for the treatment of oral candidiasis in Aganang Local Municipality. A- *Artemisia afra* Jacq. Ex Willd., B- *Blepharis subvolubilis* subsp. *subvolubilis* C.V. Clarke, C- *Carpobrotus edulis* N.E.Br., D- *Enicostemma axillare* (Lam.) A. Raynal, E- *Helichrysum caespititium* (DC.) Harv., F- *Solanum incanum* L., G- *Waltheria indica* L., H- *Ximenia caffra* Sond. var. *caffra*, I- *Ximenia caffra* Sond. var. *natalensis* and J- *Ziziphus mucronata* Willd.

Table 3.1. Medicinal plants used for the treatment of oral candidiasis in Aganang Local Municipality

Scientific name and Family	Voucher specimen #	Vernacular name	Part/s used	Preparation method	Mode of administration	Fidelity index %	Availability status (Red list of South African plants 2017)	Other medicinal uses	References
Artemisia afra Jacq. ex Willd. (Asteraceae)	Tlaamela 05	Lengana	Leaves	Chew / decoction	Oral	5	Least concern	Constipation, intestinal worms, anthelmintic, emetics, coughs, colds, fever, flu, loss of appetite, ear ache, malaria, headache	Verschaeve and Van Staden, 2008; Coopoosamy and Naidoo, 2012.
Blepharis subvolubilis subsp. *subvolubilis* C.V. Clarke (Acanthaceae)	Tlaamela 06	Sehlabatshukudu	Roots	Decoction	Oral	25	Least concern	No report	
Carpobrotus edulis N.E.Br. (Aizoaceae)	Tlaamela 08	Mochips/ Ino	Leaves	Chew	Oral	25	Least concern	Sore throat, mouth infections	Motsei et al., 2003; Otang et al., 2012
Helichrysum caespititium (DC.) Harv. (Asteraceae)	DK 08	Senyabotse	Roots / branches	Decoction	Oral	25	Least concern	Cough, blocked nose, chest colds, headache, wound dressing	Mathekga and Meyer, 2001 and Phungula et al., 2015

Table 3.1 continued. Medicinal plants used for the treatment of oral candidiasis in Aganang local municipality

Scientific name and Family	Voucher specimen #	Vernacular name	Part/s used	Preparation method	Mode of administration	Fidelity index %	Availability status (Red list of South African plants 2017)	Other medicinal uses	References
Enicostemma axillare (Lam.) A. Raynal. (Gentianaceae)	DK 04	Makgonatsohle	Whole plant	Decoction	Oral	10	Least concern	Boils, blood purifier, itches, insect poisoning, appetite loss, stomach ache	Deore et al., 2008 and Saranya et al., 2013
Solanum incanum L. (Solanaceae)	DK 07	Thola	Roots	Decoction mixed branches with *Waltheria indica* L	Oral	5	Least concern	Stomach problems, chest pains, snake bite, tonsillitis, skin disorders	Kambizi and Afolayan, 2001 and Mabona and Van Vuuren, 2013
Waltheria indica L. (Malvaceae)	DK03	Mohutasela	Whole plant	Decoction mixed with roots *Solanum incanum*	Oral	20	Least concern	Skin disorders, urinary tracts infections, infant illnesses, burns, wounds, diarrhoea, vegetable	Nciki et al., 2016 and Magwede et al., 2018

Table 3.1 continued. Medicinal plants used for the treatment of oral candidiasis in Aganang Local Municipality

Scientific name and Family	**Voucher specimen #**	**Vernacular name**	**Part/s used**	**Preparation method**	**Mode of administration**	**Fidelity index %**	**Availability status (Red list of South African plants 2017)**	**Other medicinal uses**	**References**
Warbugia salutaris (G. Bertol.) Chiov. (Canellaceae)	DK 06	Molaka	Roots	Burnt/ground and drunk	Topical or oral	30	Endangered	Abdominal pains, blood disorders, respiratory complaints, rheumatism, stomach ulcers, tooth ache, venereal diseases and malaria	Mabogo, 1990
Ximenia caffra Sond. var. *caffra* (Ximeniaceae)	DK 01	Motshidi	Fruit	Burnt, ground and mixed with lotion	Topical	65	Least concern	Diarrhoea, venereal diseases, blood in faeces, fever, cough, infertility, headache, indigestion and scurvy	Mulaudzi et al., 2011 and Nciki et al., 2016
Ximenia caffra Sond. var. *natalensis* (Ximeniaceae)	DK02	Motshidimphiswane	Fruit	Burnt, ground and mixed with lotion	Topical	35	Least concern		
Zanthoxylum humile (E.A. Bruce) P.G. Waterman (Rutaceae)	Tlaamela 41	Monokwane	Roots	Burnt	Topical	20	Least concern	Mouth anesthetic, burns, pains	Riberio et al., 2010

Table 3.1 continued. Medicinal plants used for the treatment of oral candidiasis in Aganang local municipality

Scientific name and Family	Voucher specimen #	Vernacular name	Part/s used	Preparation method	Mode of administr ation	Fidelity index %	Availability status (Red list of South African plants 2017)	Other medicinal uses	References
Ziziphus mucronata Willd. (Rhamnaceae)	Tlaamela 42	Mokgalo	Roots	Decoction	Oral	15	Least concern	Fertility enhancement, sores, burns, dysentery, boils, glandular swellings, diarrhoea, coughs and chest problems	Mabogo, 1990 and Maema et al., 2016

3.6.5 Frequency index

The frequency index of each plant species was calculated and portrayed in Figure 3.3. The frequency was calculated using this formula: FI = FC÷N x 100 where FC is the number of informants and N is the total number of informants. The most commonly used plant species was *Ximenia caffra* var. *caffra* (65%), followed by *Ximenia caffra* var. *natalensis* (35%) and *Warbugia salutaris* (30%). Solanum *incanum* and *Artemisia afra* were the least preferred plant species (5%). *Artemisia. afra* is mostly used in many parts of South Africa for respiratory disorders, earache, intestinal worms, headaches and loss of appetite and it is sold in market places (Coopoosamy and Naidoo, 2012 and Otang et al., 2012).

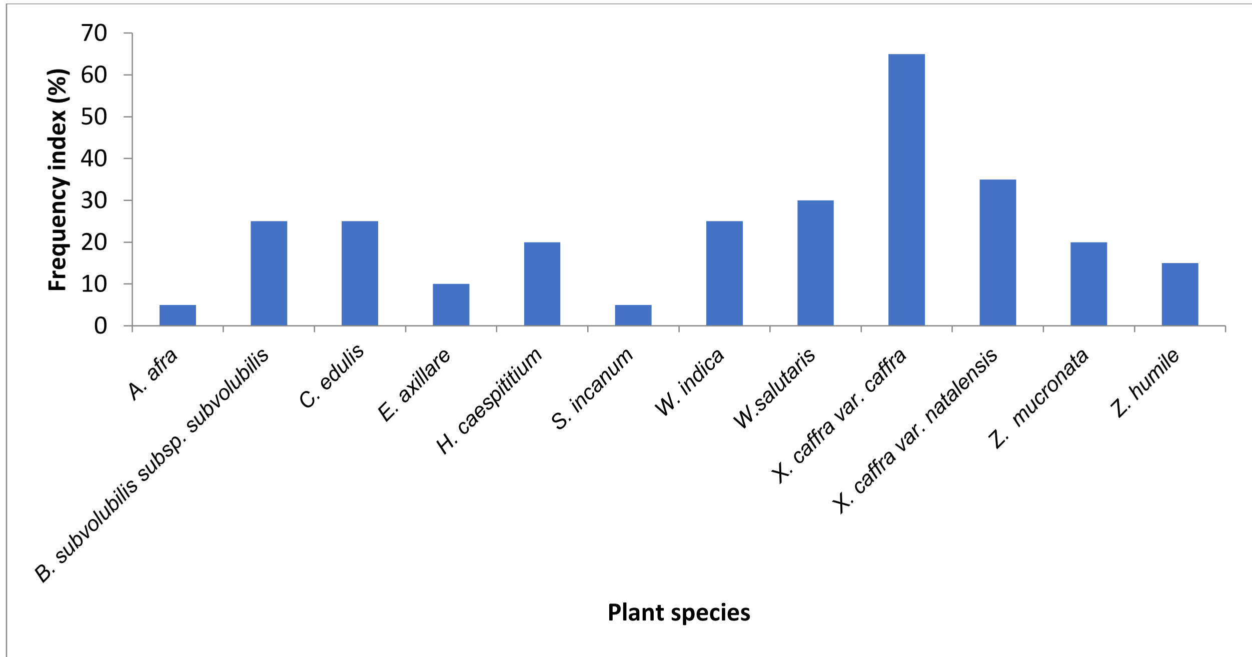

Figure 3.3. Frequency index of plant species used in Aganang Local Municipality

3.6.6 Life forms and plant parts used

In the current study, most of the identified plant species were herbs (45.4%), followed by trees (36.4%) and shrubs (18.2%). Similar results for herbs were obtained by several studies (Ahmed, 2016 and Fenetahum et al., 2017). Trees were the dominating life forms in several studies (Masevhe et al., 2015 and Maema et al., 2016). Shrubs were also listed as the least used plants by Kala et al. (2004) but were reported dominant by Barkaoui et al. (2017). Contrasting results for herbs have also been reported (Otang et al., 2012). Trees were the least dominating in a study conducted by Baydoun et al. (2015).

Traditional healers used various parts of plants to prepare their remedies such as roots (43%), leaves (21.4%), fruits, whole plant (14.3% each) and branches (7%) (Figure 3.4). The roots were also dominant in previous studies (Masevhe et al., 2015 and Maema et al., 2016). Leaves were the most used plant parts in several studies (Ahmed, 2016 and Eddouks et al., 2017). The roots were listed as the least used plant parts in a study conducted by Eddouks et al. (2017). Some studies reported the lowest percentage of usage of the whole plant (Ahmed, 2016 and Barkaoui et al., 2017). The use of the whole plant was generally practiced for small herbaceous plants. Based on our results, the roots were the dominating plant parts used in the area, and this raised concerns on the conservation status of the plant species. Noticeably, traditional healers use different parts of the same plant to prepare herbal remedies for the same disease.

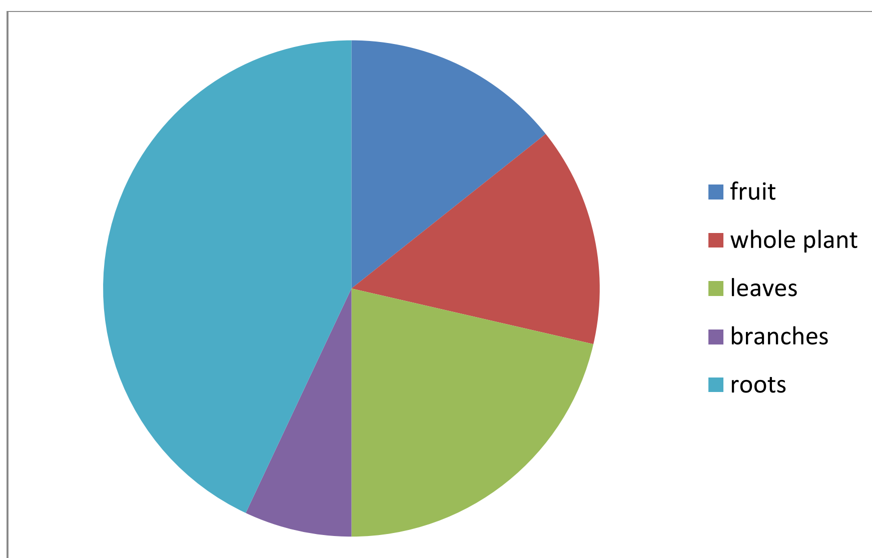

Figure 3.4. Plant parts used for the treatment of oral candidiasis

3.6.7 Preparation methods and mode of administration

The most dominating method of preparation was a decoction (50%), followed by burning (28.6%), chewing (14.3%) and grinding (7.1%) (Figure 3.5). Similar results for decoction have been reported (Barkaoui et al., 2017 and Eddouks et al., 2017). Grinding of plant material was very common in a study conducted by Coopoosamy and Naidoo (2012). Chewing was reported to be the least common method of preparation in a study conducted by Eddouks et al. (2017). In our findings, plant materials are burnt and mixed with petroleum jelly and the mixture is applied on the mouth. Noticeably, some plants work in synergy and this method of preparation is believed to increase the efficacy or activity of the herbal medicine. Synergy is the combination of materials from varying plant species to produce a herbal remedy. For example, *Ziziphus mucronata* is believed to help eliminate all kinds of diseases. Most traditional healers add the leaves of *Z. mucronata* in all their medications to facilitate the recovery of the disease. The roots of *Solanum incanum* are also mixed with branches of *Waltheria indica*. It was reported that the use of more than one plant species to prepare a remedy for diseases is attributed to the additive or synergistic effects that they could have during disease treatment (Bussman and Sharon, 2006 and Mbunde et al., 2017).

All plants mentioned for chewing are eaten raw as fresh materials. However, they did not mention the alternative form for deciduous plants. The survey revealed only two modes of administration for oral candidiasis, oral (69.2%) and topical (30.8%). Oral administration was also dominant in several studies (Coopoosamy and Naidoo, 2012 and Fenetahum et al., 2017).

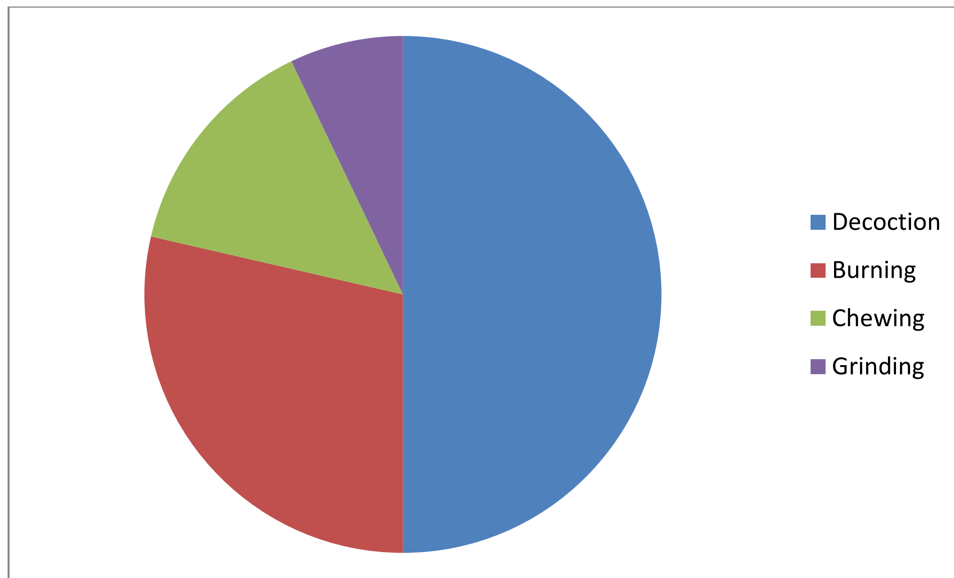

Figure 3.5. Preparation methods for the medicine

3.6.8 Conservation status of identified medicinal plants

The utilisation of roots and the whole plant, accounting for 57.3% collectively, may have a negative impact on the survival and continuity of medicinal plant species. The traditional healers claimed that they harvest their plant material in a sustainable manner. In our findings, the traditional healers harvest the lateral roots to maintain the availability of the plant species. Moreover, future availability of the herbs was maintained by leaving some plants to survive. It was of great interest that the traditional healers know about the conservation measures of harvesting. Unsustainable harvesting is mainly caused by debarking, removal of the whole plant and unregulated harvesting measures. The usage of bark as the herbal remedy was not reported in the current study. Debarking and ring-barking were reported as the main cause of poor, unsustainable harvesting measures (Kisangau et al., 2011). Based on our findings, most of the documented plants were of

least concern under the red list of South African plants and only *W. salutaris* is listed as a threatened or endangered species.

3.7 Conclusions

An ethnobotanical survey is an important parameter for discovering useful medicinal plants in a region. Although traditional medicinal plants are used for primary health care in Aganang Local Municipality, only a few plant species were recorded to be used for the treatment of oral thrush. This suggests that the disease is not yet prevalent in the local villages. *X. caffra* var. *caffra* and *X. caffra* var. *natalensis* were the most frequently used plant species. The use of roots for medicine preparation raises concerns regarding the survival of certain plant species. However, the informants are aware of the sustainable measures of harvesting medicinal plants to ensure future availability of useful plants. Most of the medications are administered orally as decoctions. Noticeably, most of the informants use the information they acquired from their elders to combat various diseases, including oral thrush. The passing on of indigenous knowledge is an important measure for preserving our useful medicinal plants.

In the next chapter, extraction of selected medicinal plants is discussed, and phytochemical analysis and antifungal activity of the crude extracts are reported.

CHAPTER 4

SCREENING NINE SELECTED PLANT SPECIES FOR ANTIFUNGAL ACTIVITY

4.1 Introduction

Nine selected plant species documented in Chapter 3 were screened for antifungal activity using a broth microdilution assay. These plant species are used to treat oral candidiasis and were selected based on information provided by the local people and traditional healers. A bioautography assay was used to determine the number of active compounds present in the plant extracts.

The value of medicinal plants is being rediscovered as some have been proven to be as effective as synthetic medicine with fewer side effects (Saxena et al., 2013). Moreover, the acceptance of medicinal plants is growing increasingly in urban areas. This may be due to the increasing inefficacy of modern drugs and the high cost of prescribed drugs (Jaberian et al., 2013). Furthemore, the extensive use of these antibiotics is associated with adverse effects which may lead to organ damage. Currently, a promising solution to these complications is research on bioactive compounds of natural products or traditional medicine to control the infectious diseases. The discovery of new antifungal drugs will help broaden the spectrum of active agents against *Candida albicans* and combat strains expressing resistance to the available antifungal agents.

Fungal infections are the fourth leading causes of hematogenous infections and the most common involved fungi are *Candida albicans* (Tsai et al., 2013 and Martins et al., 2015). These fungal infections are being increasingly recognised as a major threat to an ever-expanding population of compromised patients (Brown et al., 2012). *Candida albicans* originate from exogenous sources in the environment, and are acquired through breathing, feeding or traumatic implantation (Dorr, 2007). Furthermore, they are still a challenge, particularly in immuno-compromised patients, despite major advances in drug chemotherapy (Mishra and Tiwari, 2011). For instance, the mortality rate of infected patients over two years increased from 10-49% (Pfaller and Diekema, 2007) to 30-50%

(Pappas et al., 2009) even with the use of available drugs such as the highly active antiretroviral therapy (HAART) in HIV/AIDS patients.

Medicinal plants are a major source of a wide variety of chemical constituents which could be developed into drugs. These plants produce primary and secondary metabolites which have significant applications in modern therapy. Phytochemicals are not essential nutrients for humans, but play a major role in preventing or fighting various diseases. However, the active principles of the phytochemicals vary from plant to plant. The biological activities of phytochemicals include antimicrobial activity, anticancer activity, anti-oxidant activity and others.

The use of plant extracts with recognised antimicrobial activities can be of great importance in the healing of microbial infections (Ahmed, 2016). As such, pharmacological screening of plants for antifungal activity may yield novel compounds for new drug development, particularly those that will combat fungal strains expressing resistance to the available antifungals. One major method of plant selection for biological screening is the ethnobotanical/ethnopharmacological approach. The utilization of bioactive plant resources requires extraction, pharmacological screening, isolation and characterization of the compound as well as the toxicological and clinical evaluation (Sasidharan et al., 2011). In this chapter, we focus on extraction of plant material, phytochemical screening and antifungal activity of plant extracts against *Candida albicans*.

4.1.1 Plant extraction

Plant extraction is the first important step in analysing the pharmacological activities of the plant species. In this process, bioactive compounds are separated from inactive compounds of the plant material. The type of solvents and the extraction method depend upon the targeted compounds. Polar solvents are used when extracting hydrophilic compounds. Traditional healers and local people use water as their solvent because of its non-toxicity, availability and effectiveness. However, not all plant constituents are soluble in water. Therefore, organic solvents of varying polarities such as acetone,

hexane, dichloromethane, ethyl acetate and methanol are used to extract varying compounds present in the plant extracts. These compounds may be made available in traditional medicine through chewing or direct application as poultices for example.

4.1.2 Phytochemical analysis

Plant crude extracts contain a variety of different constituents with varying polarities. Therefore, phytochemical screening is necessary to separate the bioactive compounds. TLC fingerprinting is a simple, quick and cost-effective method for detecting compounds present in the plant extracts. It involves developing the plant crude extracts with eluent solvents and thereafter visualizing under UV light or phytochemical spray reagents. Phytochemical spray reagents result in colouration accordingly, showing a great diversity of the chemical constituents. It is also helpful in comparing the R_f values of the unknown compound with the R_f values of known compounds (Sasidharan et al., 2011).

4.1.3 Antimicrobial assays

Agar diffusion method is a technique used to determine antimicrobial activity. It is a well-known method whereby agar plates are inoculated with standardized inoculum of the test microorganism. The antimicrobial agent in this method diffuses into the agar and inhibits growth of the test microorganism (Balouiri et al., 2016). Furthermore, it is easy to read the results obtained by just measuring the diameter of growth inhibition. However, it is not applicable to all microorganisms and it does not distinguish whether the test microorganism is bactericidal/fungicidal or bacteriostatic/fungistatic. The assay is not an appropriate method for determining the minimum inhibitory concentration and may also lead to false positive or false negative results especially when examining extracts of unknown components with differing abilities to move through the water-soluble agar matrix.

Dilution methods are important in estimating the concentration at which the antimicrobial agents trigger complete inhibition of the tested microorganism. The microplate method is a quick, sensitive method that requires a very small amount of the sample (Eloff, 1998). In this method, the known antibiotic is used in one or two series of a 96-well microtitre

plate to reference the minimum inhibitory concentration values of the test organism. This method was used in our study to calculate the minimum inhibitory concentrations of the plant extracts active against *Candida albicans*.

4.1.4 Bioautography assay

Bioautography is a qualitative method that indicates the presence or absence of antimicrobial compounds. This method was used because it is easy to operate, convenient, effective and requires no specialized equipment (Masevhe et al., 2013). Furthermore, its importance in assessing the location of the bioactive compounds is reported (Eloff et al., 2008) and helps decide which of the tested plant species has good potential for further study. The technique involves dipping or spraying the developed TLC plates with the microbial suspension. The latter is then incubated in humid conditions to allow microbial growth. Silica surface serves as the nutritive source, enabling microbial growth. Inhibition zone with reference to the R_f value indicates the locality of the active compound.

4.2 Materials and methods

4.2.1 Plant selection

Nine plant species documented in Chapter 3 used to treat candidiasis were selected for further phytochemical analysis and biological assays. Plants were selected based on the information given by the traditional healers and local people. The availability of the plant species and available literature on the biological activities of these plants were also used for selection.

4.2.2 Plant collection

Plants were collected from Boratapelo, Ntlolane and Vlakfontein villages in Aganang Municipality, with the help of traditional healers and at the University of Limpopo. Collection and identification of plant species is described in chapter 3.

4.2.3 Plant extraction

Plant materials such as the roots, leaves and whole plants were dried at room temperature (25ºC) in the shade for four weeks. The dried material was ground to fine powder using a laboratory grinding mill and stored in airtight bottles. Each finely ground powder (4g) was extracted with 40 ml solvents of varying polarities: acetone, dichloromethane, ethyl acetate, ethanol, hexane, methanol and water. The extracts were shaken with a Labcon Platform shaker at 120 rpm for 10 minutes, and then centrifuged at 2000 rpm for all solvents. The supernatants were filtered into labelled, weighed glass vials. The extracts were placed under a stream of air to evaporate the solvents. The process was repeated three times and the extracts were combined. Aqueous extracts were frozen in a deep freezer. The crude extracts were re-dissolved in acetone prior to biological assay. The experiments were repeated three times to confirm the results.

4.2.4 Fungal strains and inoculum quantification

Candida albicans (ATCC 10231) was obtained from the culture collection of the Department of Veterinary Tropical Diseases at the University of Pretoria. For quantification of fungi, the haemocytometer cell-counting method described by Aberkane et al. (2002) with some modifications was used for counting the number of cells for each fungal culture prior to conducting the assays. The inoculum of each isolate was prepared by growing the fungus on Sabouraud Dextrose (SD) agar slants for 24 hours at 37°C. The colony was picked with a sterile loop from the slant and transferred to a sterile bottle with fresh SD broth (80 ml). The final inoculum concentration was adjusted to approximately 1.0×10^6 cells/ml.

4.2.5 Phytochemical analysis

Thin Layer Chromatography (TLC) was used to analyse the chemical constituents of plant extracts. Each plant extracts (10µl) were loaded on TLC plates and developed using three different eluent systems: chloroform: ethyl acetate: formic acid: 20:16:4 [CEF], ethyl acetate: methanol: water: 40:5.4:4 [EMW] and benzene: ethanol: ammonia hydroxide: 90:10:1 [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 was used for chemical compounds not visible under UV light (Stahl, 1969).

4.2.6 Antifungal activity

4.2.6.1 Microdilution method

The microdilution method of Eloff (1998) was used to determine the minimum inhibitory concentration (MIC) values of the selected plant extracts. The plant extracts (100µl) were serially diluted (50%) with distilled water in 96-well microtitre plates and fungal culture (100µl) was added to each well. Amphotericin B (160 µg/ml) was used as a positive control and 100% acetone was the negative control. After overnight incubation, 40µl of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) was added to the microplate wells as an indicator of fungal growth. The covered plates were incubated at 37°C for 24 to 48 hrs.

4.2.6.2 Bioautography

TLC plates were loaded with 10µl of each plant extract. The plates were developed in different eluent solvent systems such as EMW, CEF and BEA. The chromatograms were dried under a stream of air to evaporate solvents. The developed plates were sprayed with overnight cultures of *C. albicans* until they were completely wet. The plates were incubated at 37°C in a clean chamber in a humidified incubator for overnight and further sprayed with a solution of 2 mg/ml p-iodonitrotetrazolium (INT) violet and incubated for 2-6 hours for fungal growth. White areas were indicated where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the fungi.

4.3 Results and discussion

4.3.1 Plant extraction

Methanol extracted a large quantity of plant material (34.66%), followed by acetone (16.23%) except for *H. caespititium,* where acetone yielded the lowest quantity (11%). The highest yield was obtained from methanol extracts of *X. caffra* var. *caffra* (40%) followed by *E. axillare* (36.5%) (Figure 4.1). Acetone yielded the greatest quantity of crude

extracts in previous studies (Eloff, 1998 and Mahlo et al., 2010). The lowest yield was obtained from hexane extract of *B. subvolubilis* subsp. *subvolubilis*.

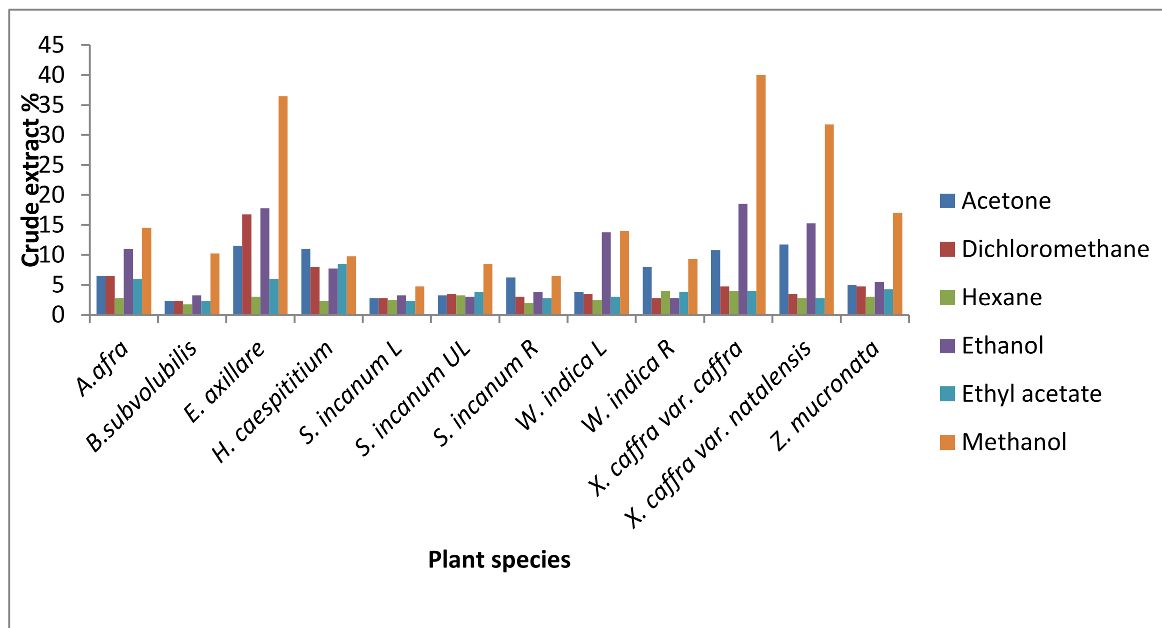

Figure 4.1. Mass of crude extract (%) obtained from 4g of powdered plant material

4.3.2 Phytochemical analysis

The chemical constituents of the plant extracts were investigated using TLC. The chromatograms were visualised using UV light before spraying with the vanillin-sulphuric acid spray reagent (Table 4.1). The observed bands on the TLC chromatograms indicated the diversity of chemical constituents in the crude plant extracts. Based on our findings, hundred-and-seven (107) bands were visible under UV light. More bands were visible in EMW (51.4%) followed by CEF (32.7%) and BEA (15.7%). Amongst all plant extracts, *W. indica* had more bands followed by *A. afra* and *H. caespititium*. There were no visible bands in plant extracts of *B. subvolubilis* subsp. *subvolubilis*. Fewer bands were visible in DCM, ethanol, ethyl acetate and methanol extracts of *X. caffra* var. *caffra*. Chemical compounds were also visible in DCM, ethanol, ethyl acetate and methanol extracts of *H. caespititium* developed in CEF with the same R_f value of 0.08. In TLC chromatograms separated with EMW, bands were observed in acetone, DCM and ethyl acetate extracts of *X. caffra* var. *natalensis* and *Z. mucronata*.

Acetone and ethyl acetate extracts of *S. incanum* developed in CEF and EMW had similar compound with the same R_f value of 0.68. Chemical components were observed in acetone, DCM and ethyl acetate extracts of *W. indica* developed in BEA with R_f value of 0.94. Amongst all solvents, acetone had the most bands visible under UV, followed by ethyl acetate. No visible bands were observed in hexane extracts of all selected plant species. After spraying with vanillin-sulphuric acid spray reagent and heating at 110°C, more compounds were visible in CEF followed by EMW (Figures 4.2-4.3). However, Masevhe et al. (2013) reported more compounds in BEA followed by EMW. More importantly, the use of different eluent solvent systems makes it possible to separate compounds of varying polarities present in the plant extracts. Noticeably, separation of compounds was visible in *B. subvolubilis* subsp. *subvolubilis* hexane extracts in BEA, CEF and EMW.

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

Eluent solvent	Plant species name	Plant part	Solvent	No. of compounds	R_f value
CEF	*H. caespititium*	Whole plant	A	4	0.11
					0.48
					0.67
					0.81
			DCM	1	0.80
			E	3	0.13
					0.48
					0.80
			EA	1	0.80
			M	1	0.80
			H_2O	1	0.68
	S. incanum	Leaves	A	1	0.80
			DCM	1	0.77
			EA	1	0.76
	S. incanum UL	Leaves	A	2	0.68
					0.75
			E	1	0.67
			EA	1	0.68
			H_2O	1	0.68
	W. indica	Leaves	A	2	0.09
					0.67
			D	1	0.67
			EA	1	0.67
			M	1	0.09
	W. indica	Roots	A	3	0.03
					0.53
					0.70
			DCM	2	0.50
					0.67
			E	1	0.50
			EA	1	0.50
	X. caffra var. *natalensis*	Leaves	H_2O	1	0.35
	Z. mucronata	Leaves	A	1	0.63
			E	1	0.65
			EA	1	0.65

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

Eluent solvent	Plant species name	Plant part	Solvent	No. of compounds	R_f value
BEA	*A. afra*	Leaves	A	1	0.16
			DCM	1	0.14
			E	2	0.10
					0.14
			EA	1	0.12
			M	3	0.08
					0.12
					0.16
	W. indica	Leaves	A	1	0.94
			DCM	1	0.94
			EA	1	0.94
	X. caffra var. *natalensis*	Leaves	A	2	0.81
					0.93
			DCM	1	0.93
			EA	1	0.89
	Z. mucronata	Leaves	A	1	0.89
			EA	1	0.91
EMW	*A. afra*	Leaves	A	2	0.35
					0.85
			DCM	1	0.85
			E	1	0.85
			EA	1	0.85
			M	2	0.33
					0.85
			H_2O	1	0.85
	E. axillare	Whole plant	A	2	0.23
					0.78
			DCM	1	0.77
			E	1	0.24
			EA	1	0.77
			M	1	0.23
			H_2O	1	0.44
	H. caespititium	Whole plant	A	1	0.10
			E	1	0.10
			M	1	0.10
			H_2O	1	0.88

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

Eluent solvent	Plant species name	Plant part	Solvent	No. of compounds	R_f value
EMW	*S. incanum*	Leaves	A	2	0.69
					0.79
			DCM	1	0.68
			E	2	0.68
					0.79
			EA	2	0.68
					0.79
	S. incanum	Leaves	A	2	0.69
					0.79
	W. indica	Leaves	A	4	0.32
					0.51
					0.79
					0.85
			DCM	2	0.79
					0.85
			EA	2	0.79
					0.85
			H_2O	1	0.85
	W. indica	Roots	A	1	0.28
			DCM	1	0.83
			E	1	0.29
			EA	2	0.29
					0.83
			M	1	0.28
			H_2O	1	0.29
	X. caffra var. *caffra*	Leaves	A	1	0.77
			DCM	1	0.77
			EA	1	0.77
			H_2O	2	0.29
					0.47
	X. caffra var. *natalensis*	Leaves	A	1	0.64
			DCM	1	0.80
			EA	2	0.62
					0.80
	Z. mucronata	Leaves	A	1	0.80
			EA	1	0.80

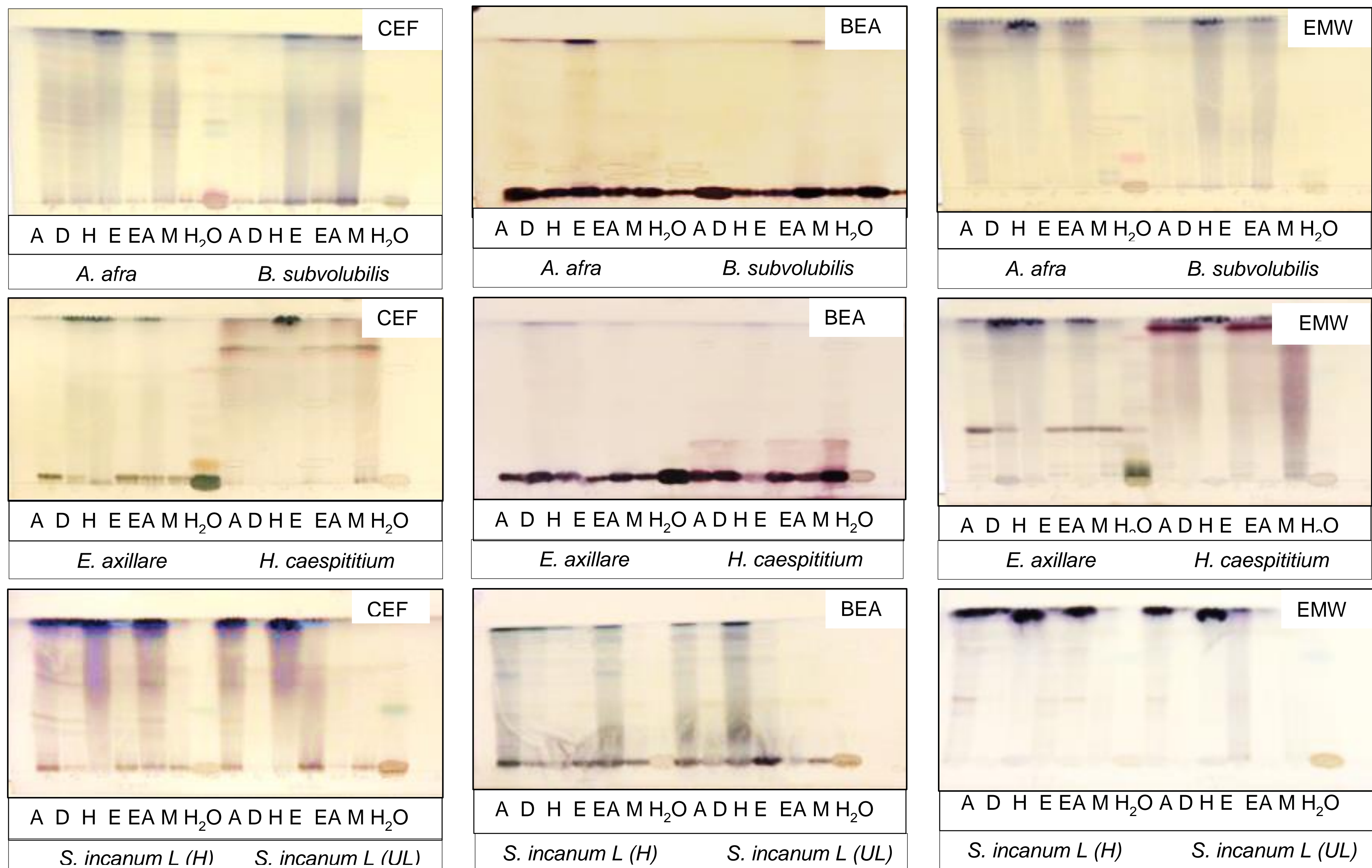

Figure 4.2. TLC chromatograms of *A. afra, B. subvolubilis* subsp. *subvolubilis*, *E. axillare, H. caespititium* and *S. incanum* developed in CEF, BEA and EMW after spraying with vanillin-sulphuric acid spray reagent. Lanes from left to right: A=acetone, D=dichloromethane, H=hexane, E=ethanol, EA=ethyl acetate, M=methanol and H_2O=aqueous extract.

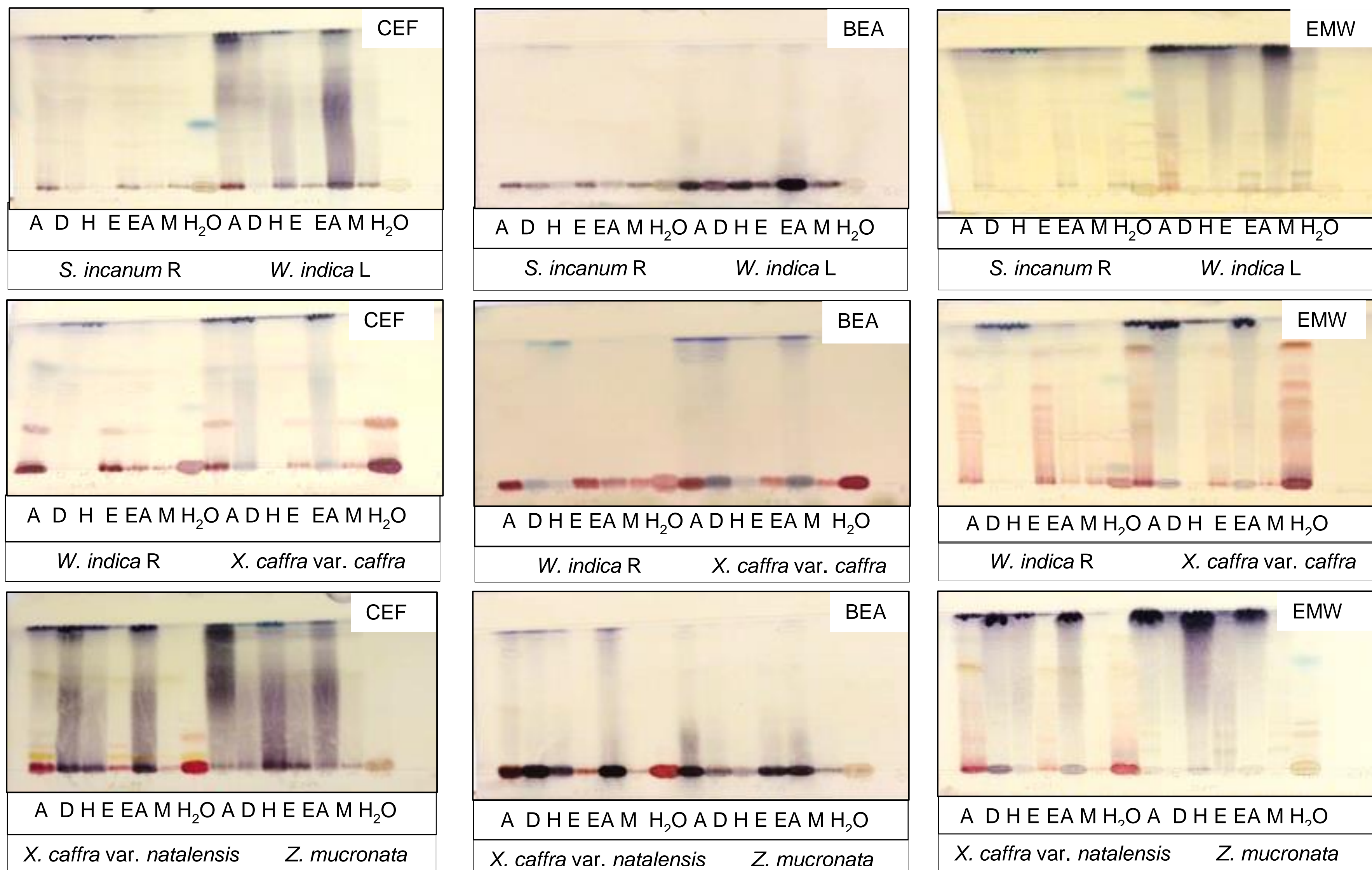

Figure 4.3. TLC chromatograms of *S. incanum* roots, *W. indica* leaves and roots, *X. caffra* var. *caffra*, *X. caffra* var. *natalensis*, and *Z. mucronata* developed in CEF, BEA and EMW after spraying with vanillin-sulphuric acid spray reagent. Lanes from left to right: A=acetone, D=dichloromethane, H=hexane, E=ethanol, EA=ethyl acetate, M=methanol and H_2O=aqueous extract.

4.3.4 Antifungal activity

4.3.4.1 Microdilution method

Antifungal activity of twelve plant extracts was determined against *C. albicans.* All plant extracts were active against the tested fungal pathogen (Table 4.2). *C. albicans* was resistant to the positive control, amphotericin B. Excellent anti-*Candida* activity was observed in leaves of *A. afra* and *S. incanum* with MIC value of 0.02 mg/ml. However, More et al. (2012) obtained moderate activity with ethanolic extracts of *A. afra* against *C. albicans.* Previously, it was reported that methanolic extracts of *S. incanum* leaves were inactive against *C. albicans* (Hamza et al., 2006). Aqueous extract of *A. afra* was also active against *Cryptococcus neoformans* (Suliman et al., 2010). However, aqueous extracts of *A. afra* (Hübsch et al., 2014) previously showed moderate activity against *C. albicans.*

Acetone, hexane and ethyl acetate leaf extracts of *W. indica* had excellent antifungal activity with MIC value of 0.02 mg/ml. The dichloromethane extract had good antifungal activity against *C. albicans* with MIC value of 0.08 mg/ml. Similar results for the DCM leaf extracts of *W. indica* were obtained by Cretton et al. (2016). Nciki et al. (2016) found similar results for poorly active aqueous extracts. Acetone and DCM extracts of *Z. mucronata* exhibited good antifungal activity against *C. albicans* with MIC value of 0.02 mg/ml while ethanol, ethyl acetate, methanol, hexane and aqueous extracts were active with MIC value of 0.08 mg/ml. Ilonga et al. (2012) reported that DCM extracts of *Z. mucronata* were poorly active while ethanol, hexane and methanol extracts had moderate antifungal activity. However, acetone and hexane extracts of *Z. mucronata* were inactive against *C. albicans* in a study conducted by Samie et al. (2010). Furthermore, previous studies reported poor antifungal activity of the aqueous extracts of its leaves (Mabona et al., 2013 and Nciki et al., 2016).

Interestingly, DCM, ethanol, hexane, ethyl acetate, methanol and aqueous extracts *of X. caffra* var. *caffra* had excellent antifungal activity with MIC value of 0.02 mg/ml. Acetone extracts also had good antifungal activity with MIC value of 0.04 mg/ml. Similar results for aqueous extracts were reported (Nciki et al., 2016). A previous study by Mulaudzi et al.

(2011) also reported that ethanol extracts were active against *C. albicans*. However, weak anti-*Candida* activity of the aqueous extract was reported by Naidoo et al. (2013). Samie et al. (2010) reported weak antifungal activity of hexane extracts of the roots against *C. albicans*.

Acetone, DCM, hexane and ethyl acetate extracts of *E. axillare* had good anti-*Candida* activity while aqueous and methanol extracts had moderate activity. Deore et al. (2008) reported good antifungal activity of ethyl acetate, methanol and aqueous extracts of *E. axillare* against *C. albicans*. Excellent antifungal activity of *H. caespititium* was observed in DCM, hexane, methanol and aqueous extracts. Acetone and ethyl acetate extracts had moderate antifungal activity and ethanol extracts had weak activity against *C. albicans*. However, acetone extracts had good activity against other fungal pathogens such as *Aspergillus niger, Cladosporium* sp. and *Phytopthora capsici* (Mathekga and Meyer, 2001).

No literature was found on the biological activity of *B. subvolubilis* and *X. caffra* var. *natalensis*. Based on our findings, acetone, DCM and hexane extracts of *B. subvolubilis* had good antifungal activity against *C. albicans with* MIC value of 0.02 mg/ml. Moderate antifungal activity was observed in ethyl acetate and ethanol extracts while weak anti-*Candida* activity was detected in methanol and aqueous extracts. Noteworthy antifungal activity of *X. caffra* var. *natalensis* was observed in acetone, DCM, hexane, ethanol, ethyl acetate and methanol extracts. There is a lack of information on the biological activities of *X. caffra* var. *natalensis*. This might be attributed to the fact that many researchers do not separate *X. caffra* into their specific varieties. The currently available varieties of *X. caffra* were classified in a study conducted by Maroyi et al., 2016. This provides a need for further determining the other biological activities and thereafter isolating and identifying active compounds, especially in extracts that had good antifungal activity.

Due to relatively slow growth of fungi, the MIC value of plant extracts against *C. albicans* was determined after 24hr and 48hr incubation. Moreover, incubation time is one of the key factors that influences the vulnerability of test organisms. An increase in incubation

time resulted in decreased antifungal activity of some plant extracts. Ethanol and ethyl acetate extracts of *A. afra* lost activities from 0.02 mg/ml to 0.156 mg/ml. Similar activity loss was observed in the methanol extract of *X. caffra* var. *natalensis*. *Z. mucronata* also lost activity in hexane, ethanol, ethyl acetate and methanol extracts from 0.02 mg/ml to 0.08 mg/ml. This might be due to prolonged incubation of sufficient growth of *C. albicans*. Prolonged incubation also has little effect on the activity of amphotericin B, but consistently raises the MIC value (Tornatore et al., 1997). In contrast, acetone, DCM and ethanol extracts of *W. indica* roots increased activity after 48hr incubation. These extracts had excellent antifungal activity with MIC values of 0.02 mg/ml. No change of activity was observed in *B. subvolubilis* subsp. *subvolubilis*, *E. axillare*, *H. caespititium*, *S. incanum*, *W. indica* leaves and *X. caffra* var. *caffra*.

Hexane and DCM extracts exhibited strong antifungal activity against *C. albicans*. Noticeably, aqueous extracts had the lowest MIC values in most plant extracts, except for *B. subvolubilis*, *X. caffra* var. *natalensis* and *W. indica* (1.25 mg/ml and 0.156 mg/ml respectively). This was of great interest since traditional healers and local people use water extracts such as infusions and decoctions. However, literature reported that aqueous extracts have no or poor activity against microorganisms (Eloff et al., 2008 and Masevhe et al., 2013). Moreover, different parts of the plant exhibit varying antifungal activities. Based on our findings, the leaves of *S. incanum* had lower MIC values than the roots.

The roots of *W. indica* also had higher MIC values than the leaves, especially in acetone, ethanol and aqueous extracts. The similarities were only found in DCM extracts with MIC value of 0.08 mg/ml. Moreover, it was observed that plant species from varying geographical regions exhibited varying antifungal activity. Two species of *S. incanum* were screened against *C. albicans* (collected from Matlala villages and the other from University of Limpopo). Variations were observed in ethanol and water extracts, where *S. incanum* from Matlala village was highly active.

The total activity of the crude plant extracts was calculated by dividing the mass extracted from one gram (mg) by the MIC value in mg/ml (Table 4.2). This is an important parameter for comparing the activity of different plant extracts. It indicates the degree to which the active compounds in 1g can be diluted and still inhibit the growth of microorganism and assist in selecting promising plants (Eloff et al., 2008). Amongst all extracts, methanol extracts had the highest total activity. The highest activity was observed in all plant extracts except for *B. subvolubilis* subsp. *subvolubilis* and *W. indica* leaves. Methanol extracts of *X. caffra* var. *caffra* had the highest total activity (20 000 ml/g) followed by methanol extracts of *E. axillare* (18 250 ml/g). The average lowest total activity of ethanol extracts was observed in *H. caespititium* against *C. albicans* with 62 ml/g. However, Masevhe at al. (2013) obtained the highest total activity with hexane extract. The lowest total activity was observed in the ethanol extract of *W. indica* roots (11 ml/g).

Table 4.2 The minimum inhibitory concentrations (mg/ml) and total activity (ml/g) of different plant extracts against *C. albicans*.

Artemisia afra (L)								*Blepharis subvolubilis* subsp. *subvolubilis* (L)							Amp B
MIC values (mg/ml															
TIME (Hrs)	A	D	H	EtOH	EA	M	H_2O	A	D	H	EtOH	EA	M	H_2O	
24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.156	0.156	1.25	1.25	<0.02
48	0.02	0.02	0.02	0.156	0.156	0.02	0.02	0.02	0.02	0.02	0.156	0.156	1.25	1.25	<0.02
Average	0.02	0.02	0.02	0.088	0.088	0.02	0.02	0.02	0.02	0.02	0.156	0.156	1.25	1.25	<0.02
Total activity (ml/g)															
24	3250	3250	1375	5500	3000	7250		1125	1125	875	208.33	144.239	82		
48	3250	3250	1375	705.128	384.615	7250		1125	1125	875	208.33	144.239	82		
Average	3250	3250	1375	1250	681.818	7250		1125	1125	875	208.33	144.239	82		
Enicostemma axillare (W)								*Helichrysum caespititium* (W)							Amp B
MIC values (mg/ml)															
TIME (Hrs)	A	D	H	EtOH	EA	M	H_2O	A	D	H	EtOH	EA	M	H_2O	
24	0.02	0.02	0.02	0.02	0.02	0.02	0.078	0.625	0.02	0.02	1.25	0.625	0.02	0.02	<0.02
48	0.02	0.02	0.02	0.02	0.02	0.02	0.078	0.625	0.02	0.02	1.25	0.625	0.02	0.02	<0.02
Average	0.02	0.02	0.02	0.02	0.02	0.02	0.078	0.625	0.02	0.02	1.25	0.625	0.02	0.02	<0.02
Total activity (ml/g)															
24	5750	8375	1500	8885	3000	18250		176	4000	1125	62	136	4875		
48	5750	8375	1500	8885	3000	18250		176	4000	1125	62	136	4875		
Average	5750	8375	1500	8885	3000	18250		176	4000	1125	62	136	4875		

Amphotericin B (Amp B) was used as a positive control. Abbreviations: L-leaf, R-root; W-whole plant; A-acetone; D-dichloromethane; H-hexane; EtOH-ethanol; EA-ethyl acetate; M-methanol; H_20-water.

Table 4.2 continued. The minimum inhibitory concentrations (mg/ml) and total activity (ml/g) of different plant extracts against *C. albicans*.

	Solanum incanum (L)							*Solanum incanum* R							Amp B
	MIC values (mg/ml)														
Time (h)	A	D	H	EtOH	EA	M	H_2O	A	D	H	EtOH	EA	M	H_2O	
24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.078	0.02	0.02	<0.02
48	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.078	0.02	0.02	<0.02
Average	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.078	0.02	0.02	<0.02
	Total activity (ml/g)														
24	1375	1375	1250	1625	1125	2375		3125	1500	1000	1875	352.564	3250		
48	1375	1375	1250	1625	1125	2375		3125	1500	1000	1875	352.564	3250		
Average	1375	1375	1250	1625	1125	2375		3125	1500	1000	1875	352.564	3250		

	Solanum incanum (L) UL							*Waltheria indica* (L)							Amp B
	MIC values (mg/ml)														
Time (h	A	D	H	EtOH	EA	M	H_2O	A	D	H	EtOH	EA	M	H_2O	
24	0.02	0.02	0.02	0.039	0.02	0.02	0.039	0.078	0.078	0.02	0.156	0.02	0.313	0.02	<0.02
48	0.02	0.02	0.02	0.039	0.02	0.02	0.039	0.078	0.078	0.02	0.156	0.02	0.313	0.02	<0.02
Average	0.02	0.02	0.02	0.039	0.02	0.02	0.039	0.078	0.078	0.02	0.156	0.02	0.313	0.02	<0.02
	Total activity (ml/g)														
24	1625	1750	1625	769.231	1875	4250		480.769	448.718	1250	881.41	1500	447.284		
48	1625	1750	1625	769.231	1875	4250		480.769	448.718	1250	881.41	1500	447.284		
Average	1625	1750	1625	769.231	1875	4250		480.769	448.718	1250	881.41	1500	447.284		

Amphotericin B (Amp B) was used as a positive control. Abbreviations: L-leaf, R-root; W-whole plant; A-acetone; D-dichloromethane; H-hexane; EtOH-ethanol; EA-ethyl acetate; M-methanol; H_20-water.

Table 4.2 continued. The minimum inhibitory concentrations (mg/ml) and total activity (ml/g) of different plant extracts against *C. albicans*.

	Waltheria indica (R)							*Ximenia caffra* var. *caffra* (L)							Amp B
MIC values (mg/ml)															
Time(h)	A	D	H	EtOH	EA	M	H_2O	A	D	H	E	EA	M	H_2O	
24	1.25	0.156	0.02	2.5	0.02	0.02	1.25	0.039	0.02	0.02	0.02	0.02	0.02	0.02	<0.02
48	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.039	0.02	0.02	0.02	0.02	0.02	0.02	<0.02
Average	0.635	0.088	0.02	1.26	0.02	0.02	0.635	0.039	0.02	0.02	0.02	0.02	0.02	0.02	<0.02
Total activity (ml/g)															
24	64	176.28	2000	11	1875	4625		2756.4	2375	2000	9250	2000	20000		
48	4000	1375	2000	1375	1875	4625		2756.4	2375	2000	9250	2000	20000		
Average	125.984	312.5	2000	21.8254	1875	4625		2756.4	2375	2000	9250	2000	20000		
	Ximenia caffra var. *natalensis* (L)							*Ziziphus mucronata* (L)							Amp B
MIC values (mg/ml)															
Time(h)	A	D	H	EtOH	EA	M	H_2O	A	D	H	EtOH	EA	M	H_2O	
24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	<0.02
48	0.02	0.02	0.02	0.02	0.02	0.02	0.156	0.02	0.02	0.078	0.078	0.078	0.078	0.039	<0.02
Average	0.02	0.02	0.02	0.02	0.02	0.02	0.088	0.02	0.02	0.049	0.049	0.049	0.049	0.0295	<0.02
Total activity (ml/g)															
24	5875	1750	1375	7625	1375	15875		2500	2375	1500	2750	2125	8500		
48	5875	1750	1375	7625	1375	15875		2500	2375	384.615	705.128	544.872	2179.5		
Average	5875	1750	1375	7625	1375	15875		2500	2375	612.25	1122.45	867.347	3469.4		

Amphotericin B (Amp B) was used as a positive control Abbreviations: L-leaf, R-root; W-whole plant; A-acetone; D-dichloromethane; H-hexane; EtOH-ethanol; EA-ethyl acetate; M-methanol; H_20-water.

The average MIC values of the plant extracts tested against *C. albicans* is represented in Figure 4.4. Good antifungal activity is shown by the lowest average MIC value against *C. albicans*. The lowest average MIC values were observed in *A. afra*, *S. incanum* and *X. caffra* var. *caffra* with MIC value ranging between 0.02 and 0.08 mg/ml. *E. axillare* had the lowest average MIC in acetone, DCM, hexane and ethyl acetate extracts. *B subvolubilis* had the highest average MIC in acetone, DCM and hexane extracts. Ethanol of *H. caespititium* had shown the highest average MIC value of 1.25 mg/ml while the aqueous extract of *A. caffra* and *S. incanum* had the lowest MIC value of 0.01 mg/ml. Acetone, ethanol and water extracts of *W. indica* were in active with highest average MIC value of 1.26 mg/ml against the tested fungal pathogen.

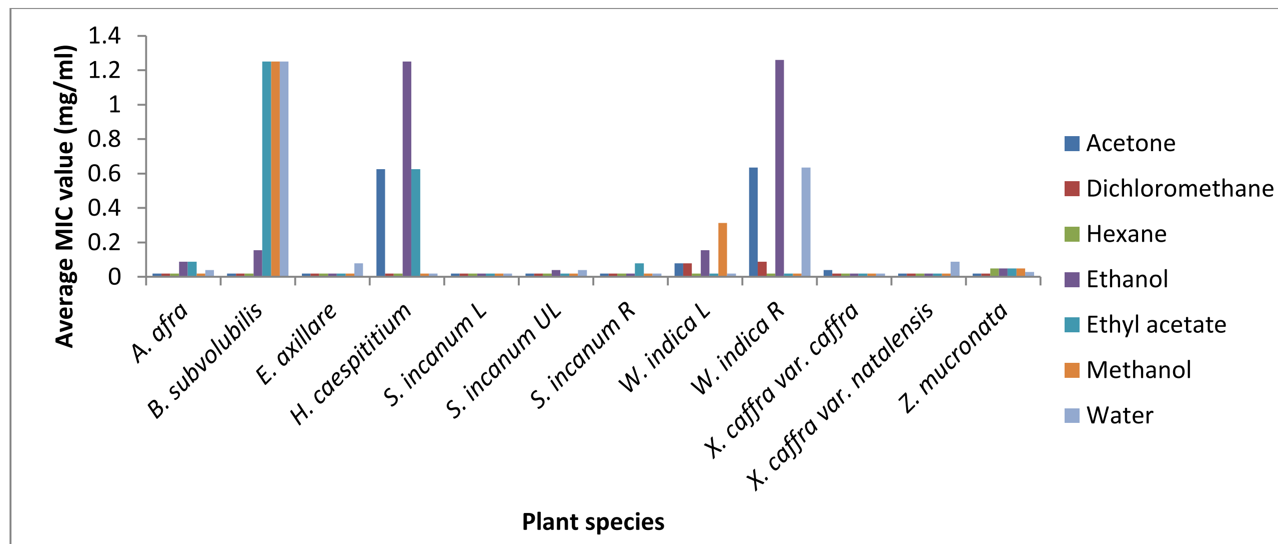

Figure 4.4. The average MIC values (mg/ml) of plant extracts tested against *C. albicans*

4.3.4.2 Bioautography

The bioautography assay was used to determine the number of active compounds against *C. albicans* (Table 4.3). Sixty-three active compounds were represented by BEA (non-polar), CEF (intermediate) and EMW (polar). Amongst all active compounds, 52.4% were separated by BEA, 34.9% by CEF and 12.7% by EMW. Antifungal compounds were observed in leaf extracts of *A. afra*, *B. subvolubilis* subsp. *subvolubilis*, *S. incanum* and *W. indica* (Figure 4.5). In TLC chromatograms developed in BEA, active compounds were visible in extracts of *S. incanum*, *W. indica* and *X. caffra* var. *caffra* developed in EMW (Figure 4.7). This suggests that most of the active compounds present in the tested plant extracts are relatively non-polar. Previous study indicated more active compounds separated with EMW and few with BEA (Masevhe et al., 2013).

Noticeably, most of the active compounds were visible in DCM extracts. Methanol extracts had the lowest number of active compounds with one compound in *S. incanum* developed in CEF and one developed in EMW (Figure 4.6). *Z. mucronata* had more antifungal compounds (19.05%), where 7 of these were visible in BEA, 4 in CEF and 1 in EMW. Acetone, DCM, ethanol and hexane extracts of *Z. mucronata* developed in CEF had compounds with similar R_f value of 0.68. Runyoro et al. (2006a) reported less than four inhibition zones of chloroform and methanol root extracts of *Z. mucronata* against *C. albicans*.

Noteworthy results were also obtained with *W. indica* root extracts which had 10 active compounds (15.9%). Five of these compounds were visible in CEF with R_f values ranging between 0.45 and 0.68. Acetone, DCM, hexane and ethyl acetate extracts of *S. incanum* developed in BEA had compounds with the same R_f value of 0.26. Antifungal compounds were also observed in DCM, hexane and ethyl acetate extracts of *X. caffra* var. *natalensis* with R_f values ranging between 0.2 and 0.27. In TLC chromatograms separated with CEF, two compounds were visible in DCM extracts of *S. incanum* with R_f values of 0.61 and 0.63. No active compounds were observed in all plant extracts of *H. caespititium*. However, it was noted that this plant species had good activity in the microdilution method. The absence of active compounds in some plant species was observed in

previous studies (Mahlo et al., 2010 and Masevhe et al., 2013). This confirms that the compounds in this plant extract worked in synergy to inhibit fungal growth. The separation of these compounds on TLC plates therefore disrupted the activity of these compounds. Moreover, water extracts had visible activity only in *A. afra* in BEA (R_f value, 0.04), though there was good antifungal activity in other plant species. This may be due to insolubility of some active compounds in water. Previous studies reported no active compounds for aqueous extracts (Eloff, 2008 and Mahlo et al., 2013). Variations in bioautography of tested plant species may result from different plant parts, type of solvent used for extraction and geographical location from which the plant material was collected. *W. indica* and *X. caffra* var. *natalensis* were selected as the most promising plant species.

Table 4.3. The R_f values of active compounds separated with BEA, CEF and EMW eluent systems, extracted with acetone (A), dichloromethane (D), hexane (H), ethanol (E), ethyl acetate (EA), methanol (M) and water (H_2O) sprayed with *Candida albicans.*

Eluent solvent	Plant species	Plant part	Solvent	No. of compounds	R_f value
CEF	*A. afra*	Leaves	D	1	0.88
	B. subvolubilis	Leaves	D	1	0.64
	E. axillare	Whole plant	D	2	0.63
					0.68
			E	1	0.68
			EA	1	0.66
	S. incanum	Roots	D	2	0.61
					0.63
	W. indica	Leaves	D	2	0.61
					0.63
	W. indica	Roots	D	2	0.64
					0.65
			H	2	0.68
					0.45
			EA	1	0.45
	X. caffra var. *natalensis*	Leaves	D	2	0.68
					0.64
	Z. mucronata	Leaves	A	1	0.68
			D	1	0.68
			E	1	0.68
			H	1	0.68
BEA	*A. afra*	Leaves	D	1	0.14
			H	1	0.14
			H_2O	1	0.04
	B. subvolubilis	Leaves	A	1	0.08
			D	1	0.08
	S. incanum	Leaves	D	2	0.28
					0.13
	S. incanum UL	Leaves	A	1	0.01
			D	1	0.01
			H	2	0.13
					0.82
			EA	1	0.01

Table 4.3 continued. The R_f values of active compounds separated with BEA, CEF and EMW eluent systems, extracted with acetone (A), dichloromethane (D), hexane (H), ethanol (E), ethyl acetate (EA), methanol (M) and water (H_2O) sprayed with *Candida albicans*.

Eluent solvent	Plant species	Plant part	Solvent	No. of compounds	R_f value
BEA	*S. incanum*	Roots	A	1	0.26
			D	1	0.26
			H	1	0.26
			EA	1	0.26
	W. indica	Leaves	EA	2	0.19
			D		0.28
	W. indica	Roots	D	1	0.83
	X. caffra var. *caffra*	Leaves	D	1	0.81
	X. caffra var. *natalensis*	Leaves	D	2	0.27
					0.20
			H		0.27
				2	0.20
			EA	2	0.20
					0.27
	Z. mucronata	Leaves	A	1	0.32
			D	1	0.22
			H	1	0.15
			E	2	0.34
				2	0.22
			EA		0.34
					0.22
EMW	*S. incanum*	Leaves	D	1	0.86
			M	1	0.34
	W. indica	Leaves	D	1	0.83
	W. indica	Roots	D	3	0.85
					0.70
					0.61
			E	1	0.69
	Z. mucronata	Leaves	D	1	0.82

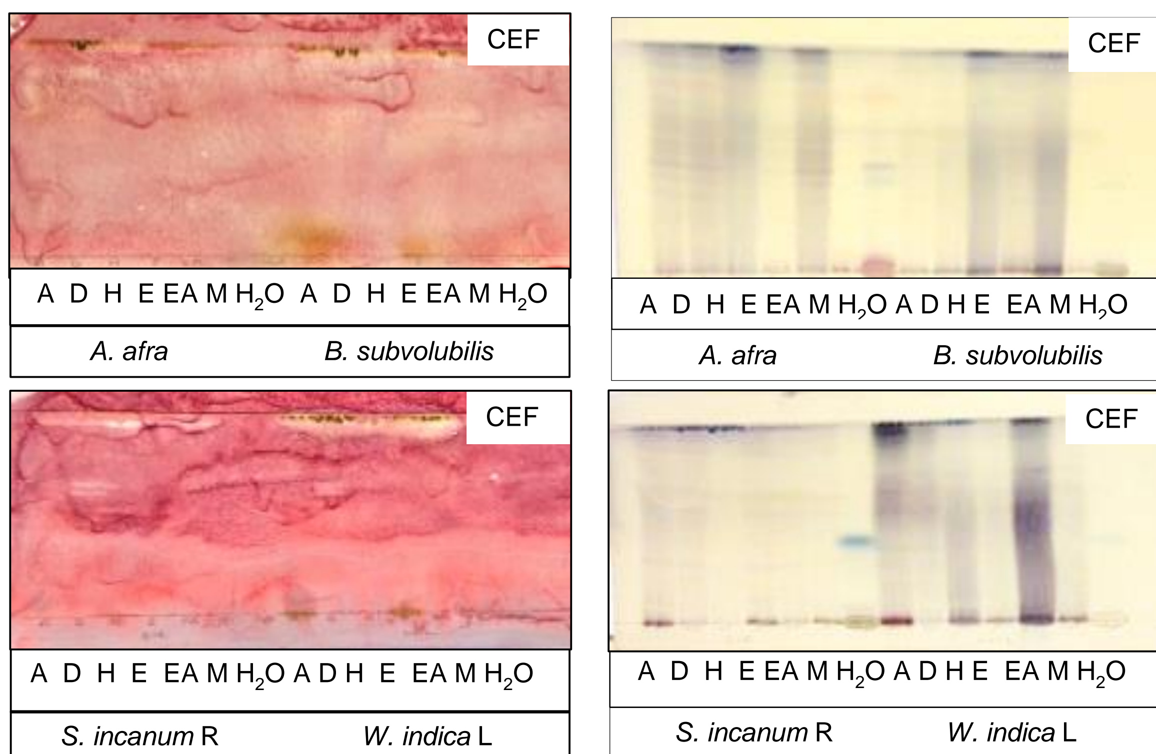

Figure 4.5. Bioautograms (left) and phytochemical analysis (right) from extracts of *A. afra*, *B. subvolubilis*, *S. incanum* and *W. indica* developed in CEF were sprayed with *Candida albicans*. White areas indicate inhibition of fungal growth. Lanes from left to right: A=acetone, D=dichloromethane, H=hexane, E=ethanol, EA=ethyl acetate, M=methanol and =H_2O aqueous extract.

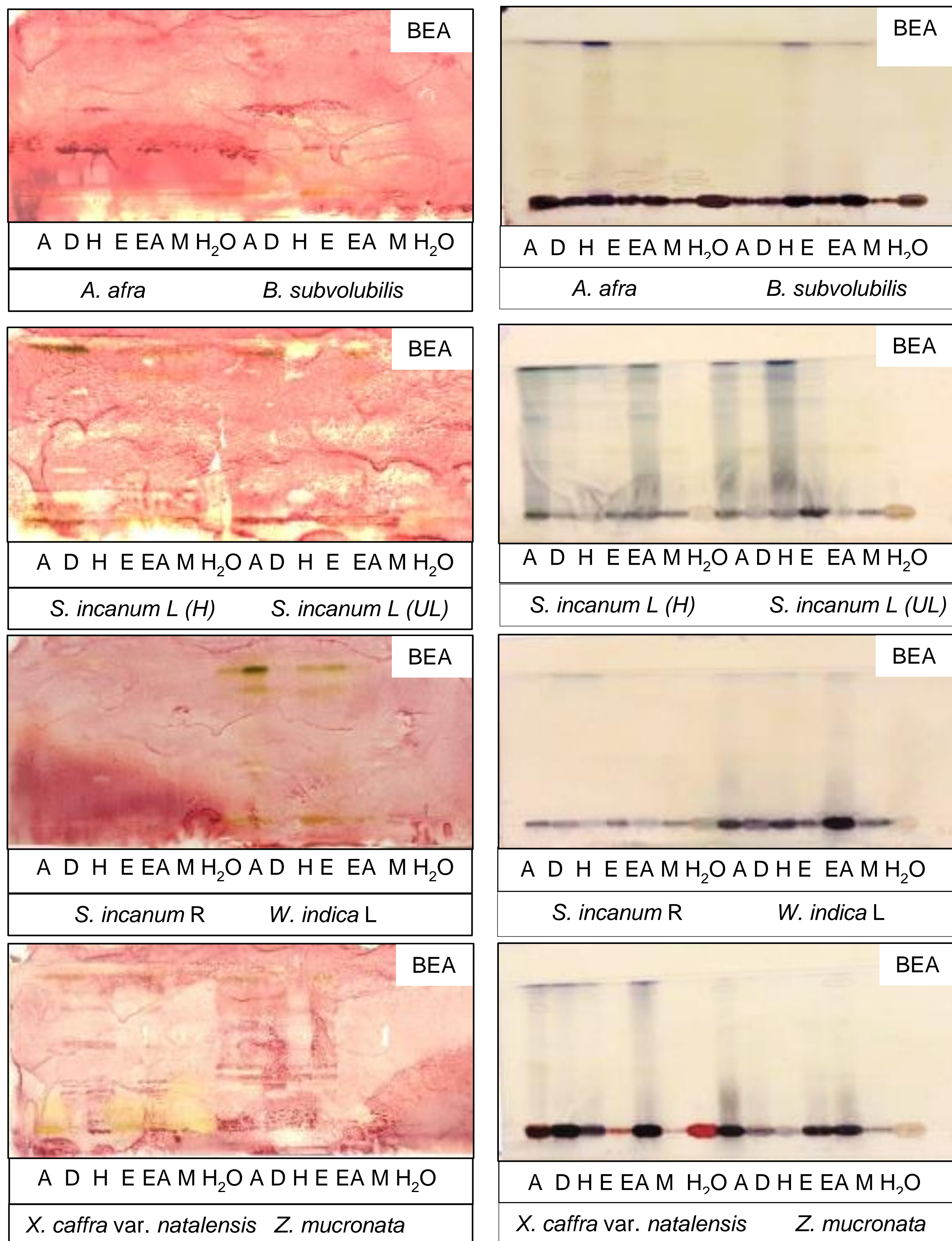

Figure 4.6. Bioautograms (left) and phytochemical analysis (right) from extracts of *A. afra*, *B. subvolubilis* subsp. *subvolubilis*, *S. incanum*, *W. indica*, *Z. mucronata* and *X. caffra* var. *natalensis* developed in BEA were sprayed with *Candida albicans*. White areas indicate inhibition of fungal growth. Lanes from left to right: A=acetone, D=dichloromethane, H=hexane, E=ethanol, EA=ethyl acetate, M=methanol and H_2O=aqueous extract.

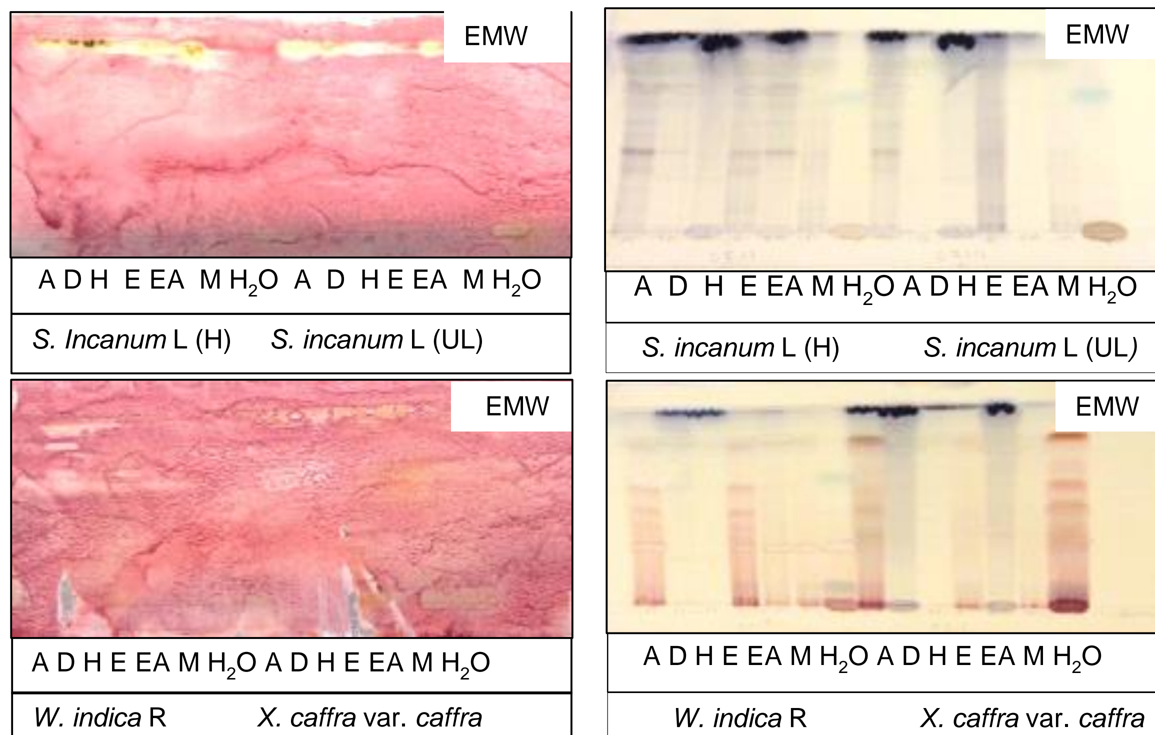

Figure 4.7. Bioautograms (left) and phytochemical analysis (right) of *S. incanum*, *W. indica* and *X. caffra* var. *caffra* extracts developed in EMW and sprayed with *Candida albicans*. White areas indicate inhibition of fungal growth. Lanes from left to right: A=acetone, D=dichloromethane, H=hexane, E=ethanol, EA=ethyl acetate, M=methanol and =H_2O aqueous extract.

4.4 Conclusions

Methanol and acetone extracted larger quantities of plant material compared to other organic solvents. In phytochemical analysis, TLC chromatograms developed in EMW had more chemical compounds than CEF and BEA. In the microdilution assay, extracts of *A. afra*, *E. axillare*, *S. incanum, X. cafffra* var. *caffra, X. caffra* var. *natalensis and Z. mucronata* had excellent activity with MIC values ranging between 0.02 and 0.08 mg/ml. The resistance of amphotericin B to *C. albicans* necessitates the need for new antifungal agents. The effectiveness of oral administrations of medicinal plants (decoctions and infusions) was confirmed by the excellent antifungal activity of the aqueous extracts.

In the bioautography assay, more active compounds were observed in TLC chromatograms developed in BEA than other eluent solvent systems. The absence of active compounds, particularly those that had good antifungal activity in the microdilution method indicates possible synergism. Following the good antifungal activity in both microdilution and bioautography assays, the roots of *W. indica* and the leaves of *X. caffra* var. *natalensis* were the most promising plant species.

In the next chapter, isolation of antifungal compounds from roots of *W. indica* and leaves of *X. caffra* var. *natalensis*, and their activity against *C. albicans* will be discussed.

CHAPTER 5

ISOLATION OF ANTIFUNGAL COMPOUNDS

5.1 Introduction

In Chapter 4, roots of *W. indica* and *X. caffra* var. *natalensis* leaves were selected as the best plant parts for further phytochemical investigation and isolation of antifungal compounds (Figure 5.1). This chapter focuses on isolation of antifungal compounds from leaves and roots of these two plant species.

W. indica is a short-lived perennial herb as described in Chapter 2, section 2.10. The medicinal usage of these plant species is described in Chapter 3. The roots are used widely for the treatment of diarrhoea, wounds, fever and stomach ache (Ayantunde et al., 2009). Previous work indicates that antifungal quinolone alkaloids and triterpene derivatives have been isolated from its root extracts (Cretton et al., 2014 and Cretton et al., 2015). Antibacterial activity of the roots against selected bacterial strains such as *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were reported (Olajuyigbe et al., 2011 and Mongalo, 2015). Also, the antimicrobial activity of the root extract against bacterial and fungal strains including *Candida albicans* was previously reported (Nciki et al., 2016). The botanical description of *X. caffra* var. *natalensis* was explained in Chapter 2. The medicinal usage of this plant species is described in chapter 3. To the best of our knowledge, no literature has been reported on the biological activity of this plant species.

Plants are among the most important sources of potentially valuable new drugs (Koné et al., 2004). The use of medicinal plants in drug development is not only useful in exploiting their constituents directly but using them as starting materials for drug synthesis (Ahmed, 2016). They provide potential ingredients for drugs from either direct extraction or transformation of the chemicals found within them. Furthermore, plants are recognized to possess pharmacological effects or other information gained from observations or experiments such as cultural contexts of medicinal plant usage (Pedrollo et al., 2016).

Plant extracts occur as a combination of various compounds with varying polarities that display more than one physiological effect. Therefore, isolation of compounds is important in knowing and assessing the potential of individual bioactive compounds. Isolated active compounds from plants with a long history of use are likely to be safer than synthetic compounds (Ginovyan et al., 2017).

Bioassay-guided fraction is the process of fractionating the extract further and further until a pure compound is isolated. It is applied to reduce a complexity of fractions, avoiding simultaneous availability of compounds in the bioactive site. The extracts are separated chromatographically using column chromatography, thin layer chromatography, gel chromatography, liquid chromatography, and others. The selection of a type of chromatography depends on the material to be purified and several forms can be used sequentially to achieve purification of a compound (Williams and Wilson, 1975). Thin layer chromatography separates molecules based on polarity and column chromatography separates molecules based on physical properties such as size and shape. In gel chromatography, molecules are separated based on their molecular sizes and an inert gas is used as a mobile phase (Weller, 2012). Each fraction collected is evaluated for bioactivity and fractionated further if it has interesting activity.

The ethnobotanical survey described in Chapter 3 was conducted to identify medicinal plants used for the treatment of candidiasis. *Ximenia caffra* var. *natalensis* was selected based on excellent antifungal activity in Chapter 4 for further isolation of pure compounds. The focus of this chapter is to isolate pure compounds from the leaves of *X. caffra* var. *natalensis* and the roots of *Waltheria indica* following bioassay-guided fractionation.

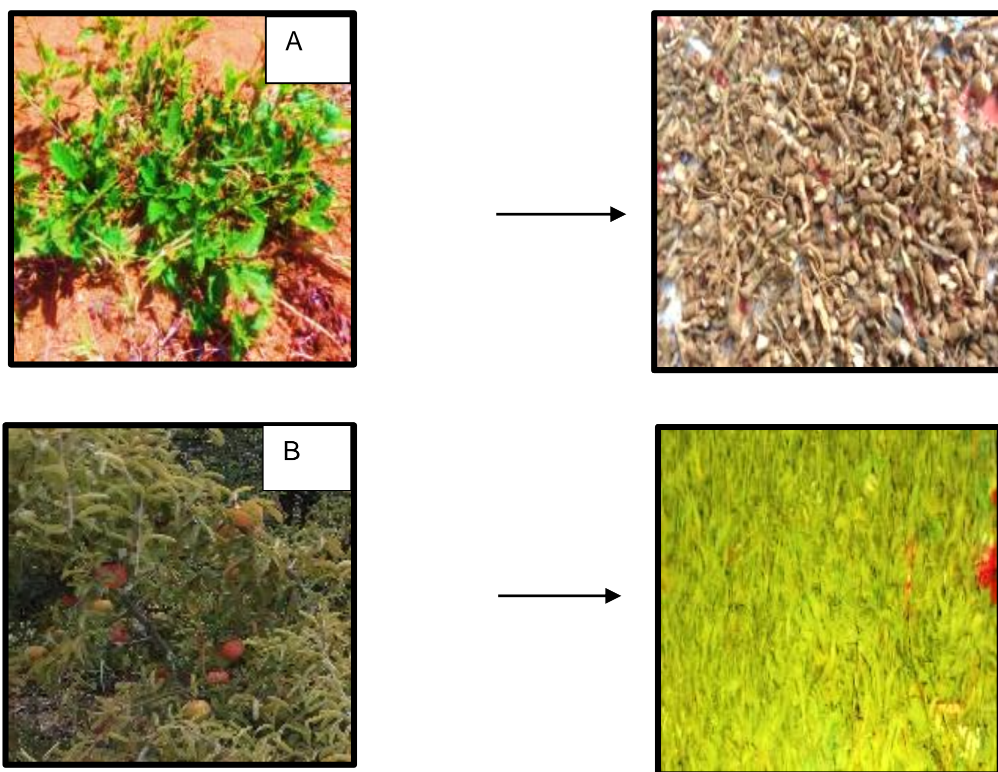

Figure 5.1. A picture of (A) whole plant and roots of *Waltheria indica* and (B) whole plant and drying leaf material of *Ximenia caffra* var. *natalensis* were taken from Aganang Local Municipality.

5.2 Materials and methods

5.2.1 Plant extraction

The roots of *W. indica* and the leaves of *X. caffra* var. *natalensis* were collected from Boratapelo village, Aganang Local Municipality. Plant materials were allowed to dry for three to four weeks and ground to a fine powder. Powdered plant materials of *X. caffra* var. *natalensis* and *W. indica* (828.38g and 764.90g respectively) were extracted with acetone (1:10). Plant extracts were shaken vigorously for 30 minutes, filtered and then allowed to evaporate. The resulting filtrates were dried under reduced pressure at 40ºC using a rotavapor (Büchi rotary evaporator) and the extracts were transferred into pre-weighed glass jars. The extracts were placed under a stream of cold air to allow complete evaporation of the solvents. The plant materials were washed three times with acetone and the crude extracts were combined.

5.2.2 Solvent-solvent fractionation

Dried crude extracts of *X. caffra* var. *natalensis* and *W. indica* (95.14g and 78.66g) were partitioned five times with hexane, chloroform, ethyl acetate, butanol and water respectively. The crude extracts of *X. caffra* var. *natalensis* and *W. indica* were dissolved in 800 ml hexane and mixed with an equal amount of distilled water in a 2 L separating funnel. After the occurrence of two layers in the separatory funnel, the bottom layer was collected to yield the hexane fraction. The process was repeated three times by extracting the water fraction with chloroform. After separation, the bottom layer was collected to yield the chloroform fraction and the residue was further mixed with an equal amount of ethyl acetate and allowed to separate into two layers. The ethyl acetate fraction was collected and the residue was further mixed with an equal amount of butanol and allowed to separate. After separation, the top layer was collected yielding a butanol fraction and the bottom layer yielded the water fraction. The fractions were collected in pre-weighed jars and evaporated to dryness at 45ºC under reduced pressure using a Büchi Rotavapor. The water fraction was evaporated using a Specht Scientific freeze dryer.

5.2.3 Phytochemical analysis

The phytochemical constituents of the fractions obtained from solvent-solvent fractionation were investigated using phytochemical analysis as explained in Chapter 4, under section 4.2.5.

5.2.4 Antifungal activity

5.2.4.1 Microdilution method

The minimum inhibitory concentration of the fractions obtained from solvent-solvent fractionation was determined against *C. albicans* using the microdilution method as described in Chapter 4, under section 4.2.6.1.

5.2.4.2 Bioautography

All fractions obtained from solvent-solvent fractionation were analysed using thin layer chromatography and antifungal activity was determined against *C. albicans* as described in Chapter 4, under section 4.2.6.2.

5.2.5 Isolation of antifungal compounds

The procedure for solvent-solvent fractionation of the selected plant species is described in a schematic diagram in Figure 5.2. The procedure for the compound isolation from *Ximenia caffra* var. *natalensis* is also described in a schematic diagram in Figure 5.2.

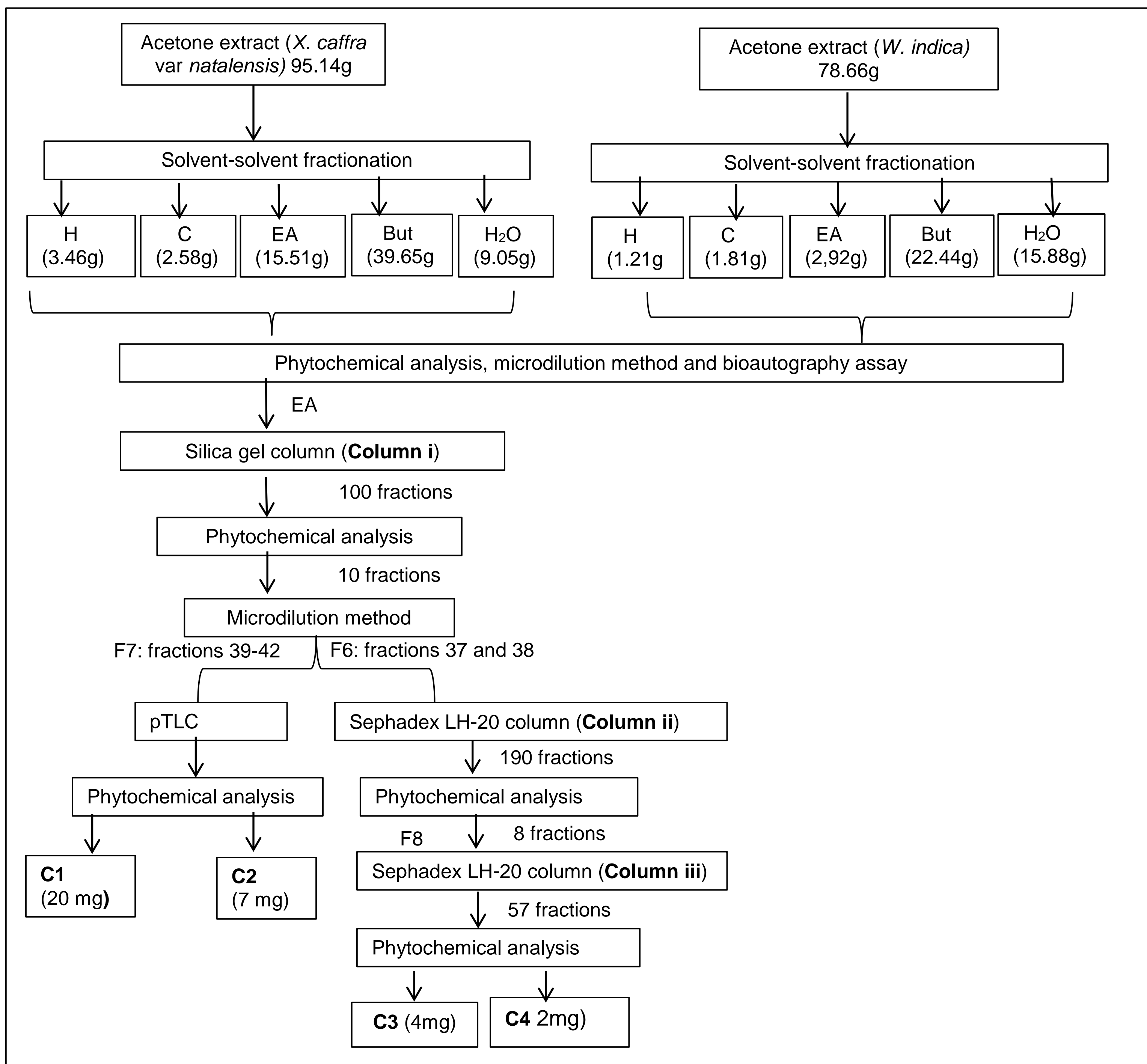

Figure 5.2. A schematic diagram showing solvent-solvent fractionation of the acetone extracts; compound isolation from ethyl acetate fraction of *X. caffra* var. *natalensis* using column chromatography and preparative thin layer chromatography (pTLC).

5.2.5.1 Column chromatography (column i)

The ethyl acetate fraction (15.15g) obtained from solvent-solvent fractionation was further separated using column chromatography. The fraction was dissolved in a minimal amount of ethyl acetate and mixed with 5g of silica gel and evaporated to dryness, forming a powder. The mixture of 200g silica gel and 500 ml of DCM was prepared and packed in a glass column (7.5 x 60cm). The ethyl acetate mixture was then loaded on top in the column. The column was initially eluted with 100:0 of DCM: MeOH; and polarity of the mixture was sequentially increased to 0:100. Hundred fractions were collected in conical flasks (100 ml), evaporated under reduced pressure and subjected to phytochemical analysis. The fractions with similar spots were pooled together, resulting in ten fractions and evaporated under reduced pressure. Fractions 37-38 and 39-42 obtained from first column (column i) were loaded on TLC plates and developed using EMW [40:5.4:4].

Fractions 39-42 were combined and dried under a stream of cold air at room temperature. The phytochemical analysis of this fraction contained two compounds and they were further separated by preparative thin layer chromatography (pTLC). The fraction was dissolved in methanol, loaded on pTLC plate and developed in EMW. The chromatogram was visualized under UV light. Visible compounds were scraped off the plate with a blade into jars. The latter was dissolved with 10% MeOH: DCM and 100% methanol. They were then filtered into pre-weighed vials, concentrated and subjected to phytochemical analysis, yielding compound **1** and compound **2**. Compound **2** was repeatedly subjected to pTLC for purification. The fractions were tested for antifungal activity using the microdilution method described in section 4.2.6.1.

5.2.5.2 Sephadex LH-20 column chromatography (column ii)

Fractions 37-38 obtained from column i were pooled together and further purified using Sephadex LH-20 column chromatography. The fraction was dissolved in methanol and loaded into a Sephadex-containing glass column. Methanol (100%) was used to elute the column until the stationary phase was clear. Collected fractions (190) were evaporated under reduced pressure and subjected to phytochemical analysis. Fractions containing the same spot were combined and resulted in sixteen fractions. Fractions F14-16 were

further separated by Sephadex LH-20 column following the same procedure (column iii). Fractions of 20 ml were collected. TLC chromatograms of these fractions highlighted some impurities and it was purified further. Fractions 1-2 were pooled together and purified further using pTLC analysis to yield compound **3** and **4**.

5.3 Results and discussion

5.3.1 Solvent-solvent fractionation

The acetone extract of each plant species yielded five fractions. The quantity of the fractions extracted with hexane, chloroform, ethyl acetate, butanol and water is presented in Figure 5.3. The highest quantity of plant material was extracted with butanol of *X. caffra* var. *natalensis* (39.64g), followed by the butanol fraction of *W. indica* (22.44g). The lowest quantity was obtained from the hexane fraction of *W. indica* (1.21g). Similar results for the hexane fraction were obtained by Mokoka et al. (2013).

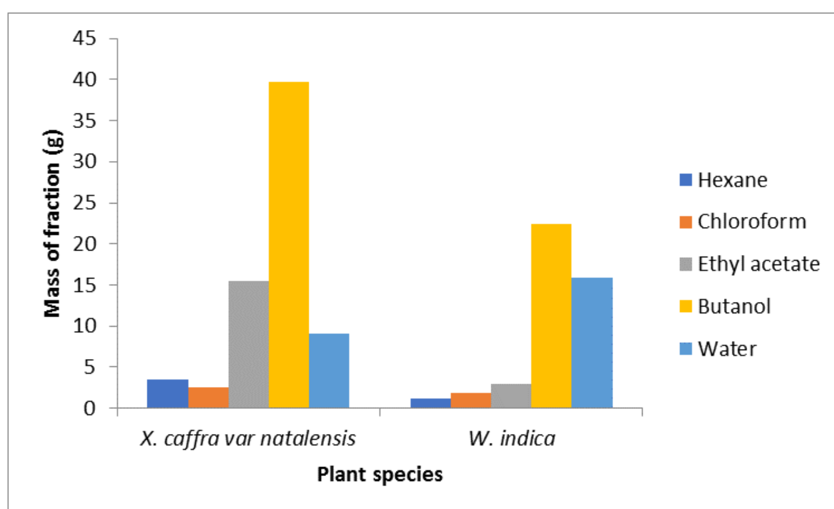

Figure 5.3. Mass of fractions obtained from solvent-solvent fraction of acetone extracts of *Ximenia caffra* var. *natalensis* leaves and *Waltheria indica* roots.

5.3.2 Antifungal activity of fractions

5.3.2.1 Microdilution method

All fractions were screened for activity against *C. albicans* using the microdilution method. The varying relative activities of fractions against *C. albicans* are presented in Table 5.1. Noteworthy antifungal activity was exhibited by *X. caffra* var. *natalensis*, where ethyl

acetate and butanol fractions were active with MIC values of 0.08 mg/ml. Hexane and chloroform fractions of *W. indica* fractions had moderate activity with MIC value was 0.313 mg/ml. This confirms the synergistic effects of compounds active against *C. albicans*. However, Mokoka et al. (2013) reported excellent antifungal activity of the hexane fraction of *Maytenus undata* (Thunb.) Blakelock against *Cryptococcus neoformans.* Ramadwa et al. (2017) reported excellent antifungal activity of hexane and chloroform fractions of *Funtumia africana* (Benth.) Stapf against *C. albicans.* Moreover, water fractions of both plant species had moderate activity with 0.625 mg/ml.

Table 5.1. MIC values (mg/ml) of fractions of *X. caffra* var. *natalensis* and *W. indica* against *C. albicans*

Plant species	Fraction	MIC (mg/ml)
W. indica	Hexane	0.313
	Chloroform	0.313
	Ethyl acetate	1.25
	Butanol	1.25
	Water	0.625
X caffra var. *natalensis*	Hexane	0.313
	Chloroform	0.625
	Ethyl acetate	0.078
	Butanol	0.078
	Water	0.625

5.3.2.2 Bioautography assay

In the current study, the highest number of active compounds (94.12%) were visible in the TLC chromatograms of *W. indica* and only one active compound was observed in *X. caffra* var. *natalensis*. Amongst all active compounds, 47.06% was separated by BEA, followed by EMW (35.3 %) and CEF (17.64 %). This suggests that most of the active compounds present in the tested plant extracts are relatively non-polar. Chloroform fractions had more antifungal compounds (7) compared to other fractions. Table 5.2 displays the R_f values of visible compounds in the fractions after spraying with *C. albicans*. Interestingly, hexane, chloroform and ethyl acetate fractions of *W. indica* had similar compound with R_f value of 0.24 in the chromatograms separated with EMW (Figure 5.4). All fractions of *X. caffra* var. *natalensis* were not active against *C. albicans*, except one compound observed with hexane fraction with R_f value of 0.025 (Figure 5.5). Noticeably, there were no visible compounds in butanol and water fractions. The absence of compounds in fractions suggests possible synergistic effects of compounds against *C. albicans*. The separation of these compounds through solvent-solvent fractionation and further TLC analysis resulted in lack of activity.

Table 5.2. The R_f values of active compounds against *C. albicans* present in fractions of *W. indica* and *X. caffra* var. *natalensis*.

Eluent solvent	Plant species	Fraction	Number of compounds	R_f value
BEA	*W. indica*	Hexane	2	0.16
				0.28
		Chloroform	3	0.16
				0.28
				0.43
		Ethyl acetate	2	0.16
				0.28
	X. caffra var. *natalensis*	Hexane	1	0.025
EMW	*W. indica*	Hexane	1	0.63
		Chloroform	3	0.59
				0.68
				0.83
		Ethyl acetate	2	0.59
				0.68
CEF	*W. indica*	Hexane	1	0.24
		Chloroform	1	0.24
		Ethyl acetate	1	0.24

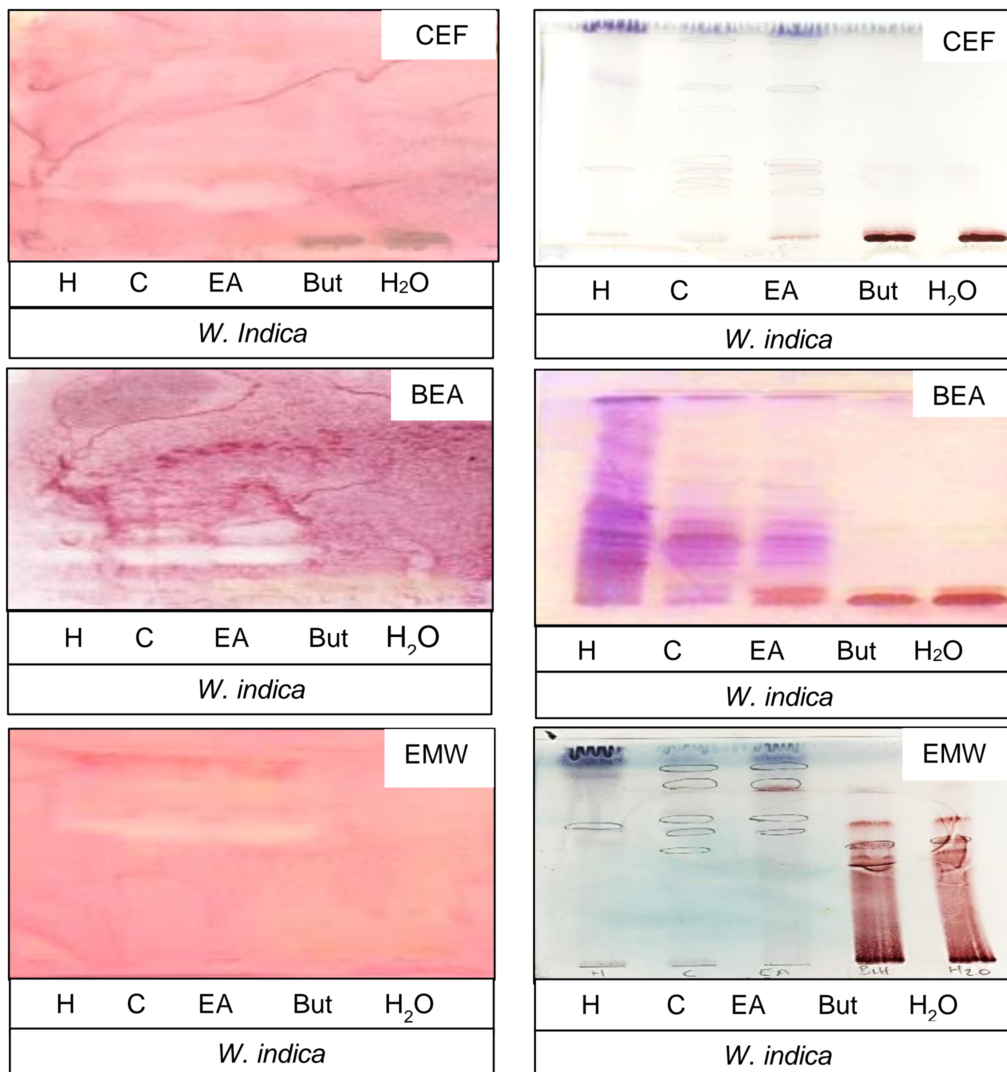

Figure 5.4. Bioautograms and phytochemical analysis of *W. indica* fractions developed in CEF, BEA and EMW. Lanes from left to right: H=hexane, C=chloroform, EA=ethyl acetate, But=butanol and H_2O=water fraction.

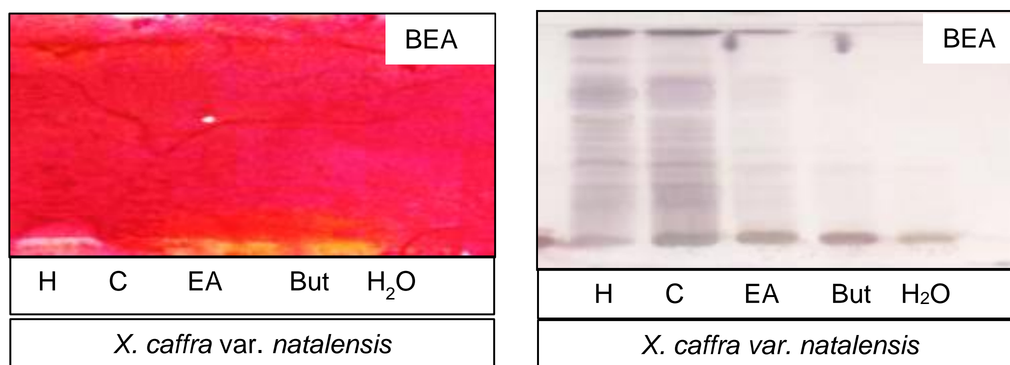

Figure 5.5. Bioautograms and phytochemical analysis of *X. caffra* var. *natalensis* fractions developed in BEA. Lanes from left to right: H=hexane, C=chloroform, EA=ethyl acetate, But=butanol and H_2O=water fraction.

5.3.3 Isolation of compounds

Column i led to collection of one hundred fractions (100). Phytochemical analysis of the fractions is shown in Figure 5.6. Fractions 39-42 contained two bands after spraying with vanillin-sulphuric acid spray reagent (Figure 5.7a). Further purification of this fraction using pTLC resulted in compound 1 (20mg) as a red powder and compound 2 as a yellow powder. The visualisation of the pTLC chromatogram of F39-42 under UV light is presented in Figure 5.7b. However, compound 2 had some impurities and it was further purified with pTLC to yield a single band (7mg).

Fractions 37-38 obtained from column i were further fractionated by column chromatography using Sephadex as a stationary phase (column ii). Following phytochemical analysis, combination of fractions with the same compounds resulted in sixteen fractions as shown in Figure 5.8a. Figure 5.8b presents further combination of the sixteen fractions, resulting in eight fractions. Sephadex LH-20 column chromatography of F14-16 (column iii) led to collection of 53 fractions. Fractions 20-29 contained single bands. Fraction 1 was obtained from the combination of F20-F25, fraction 2 from F26-F29 and fraction 3 from F30-F38 (Figure 5.9). Fractions 1 and 2 were further combined to form compound **3**. The phytochemical analysis of compound **3** resulted in two bands and it was therefore purified by pTLC analysis to yield compound **3** (4 mg) and **4** (2 mg).

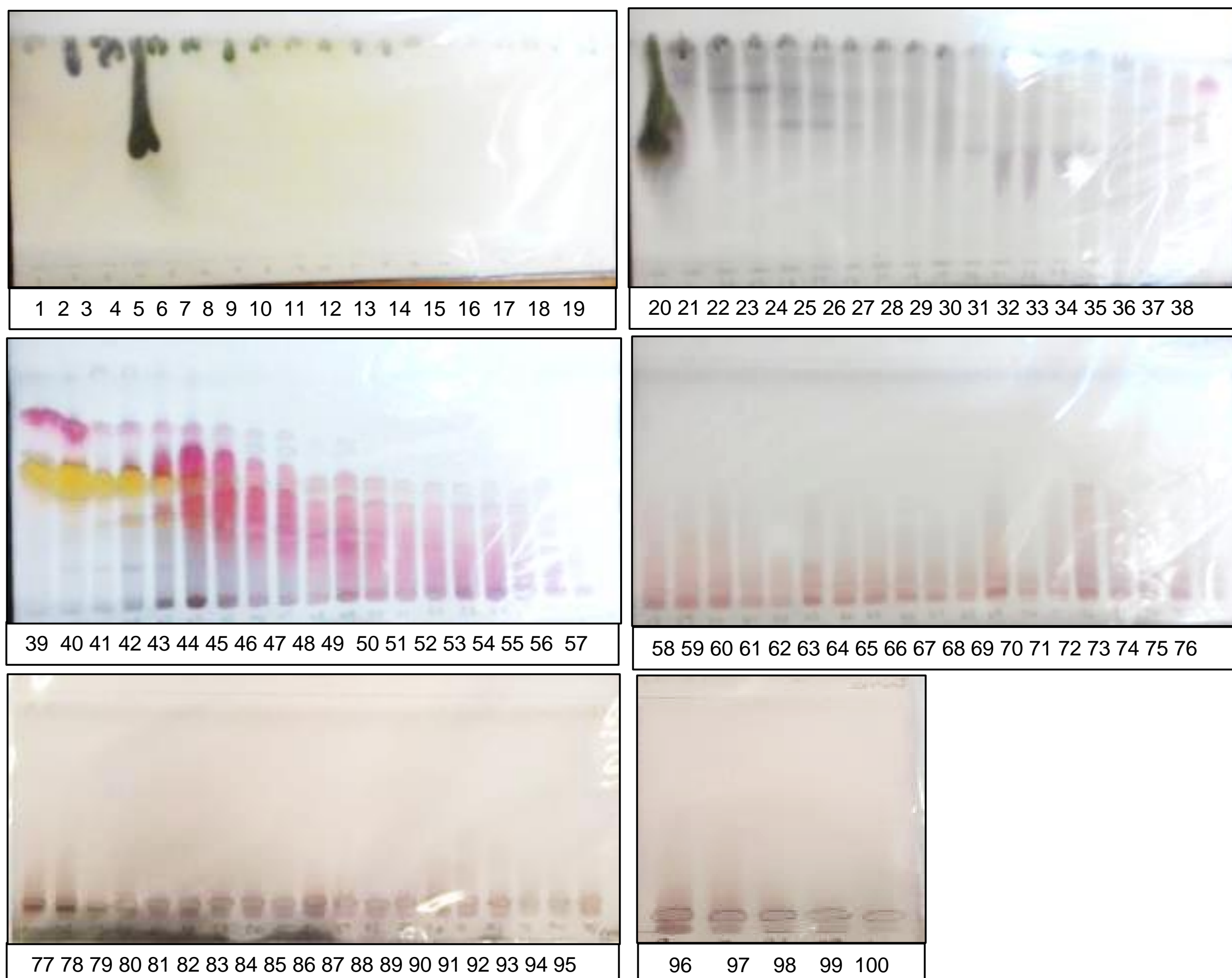

Figure 5.6. Fractions collected from column i developed in EMW after spraying with vanillin-sulphuric acid spray reagent.

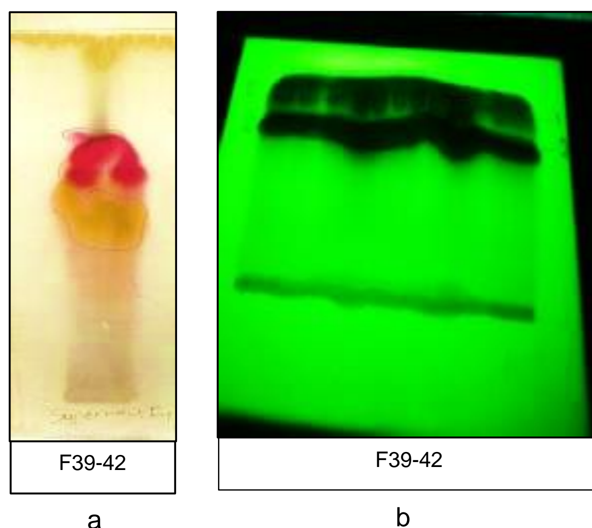

Figure 5.7. Fractions resulting from the combination of F39-42 developed in EMW [40:5:4:4] after spraying with vanillin-sulphuric acid spray. (a). Preparative TLC chromatogram of F39-42 developed in EMW under UV light (b).

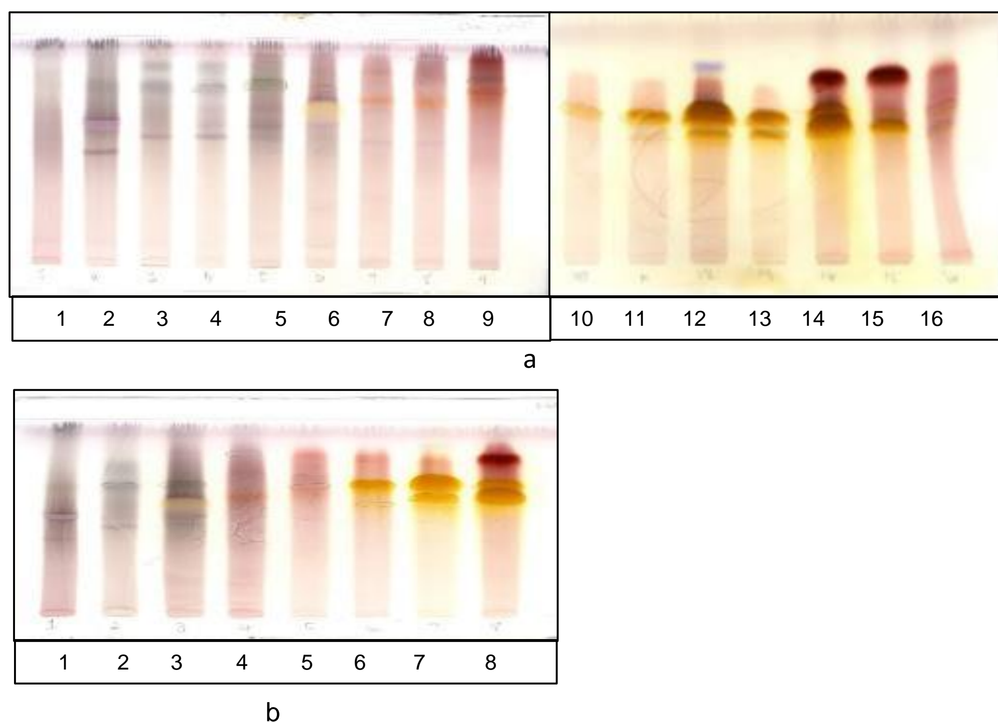

Figure 5.8. TLC chromatograms of fractions obtained from column ii. Fractions obtained from the combination of 190 fractions in column ii after spraying with vanillin-sulphuric acid spray reagent (a). Eight fractions obtained from further combination of the sixteen fractions after spraying with vanillin-sulphuric acid spray reagent (b).

Figure 5.9. Fractions obtained from column iii. Fraction 1 was obtained from F20-F25, fraction 2 from F26-F29 and fraction 3 from F30-F38.

5.3.4 Microdilution method

The ten fractions obtained from the first column (column i) were tested for antifungal activity against *C. albicans*. The varying minimum inhibitory concentrations are presented in Table 5.3. Noticeably, the tested fractions had moderate to low antifungal activity with the lowest MIC value of 0.313 mg/ml in fractions 4 and 7. Fractions 2 and 5 had the highest MIC value of 2.5 mg/ml.

Table 5.3. Minimum inhibitory concentrations of fractions obtained from column i tested against *C. albicans*

Fraction #	MIC (mg/ml)
1	1.14
2	2.5
3	1.67
4	0.313
5	2.5
6	1.25
7	0.313
8	0.625
9	0.625
10	1.25

5.3.5 Phytochemical analysis of isolated compounds

The phytochemical analysis of the four isolated compounds (10µl) resulted in one band in each chromatogram visualized under UV light and after spraying with vanillin-sulphuric acid spray reagent. Compound 1 appeared as a red spot on the chromatogram with R_f value of 0.83 (Figure 5.10a). Compound 2 appeared as a yellow band with R_f value of 0.84 (Figure 5.10b). Noticeably, fraction 1 and 2 obtained from column iii contained single yellow spots with same R_f value of 0.69. Purification of the compound by pTLC led to compound 3 and compound 4 had no band (Figure 5.10c). This may be caused by lower concentrations of the compound.

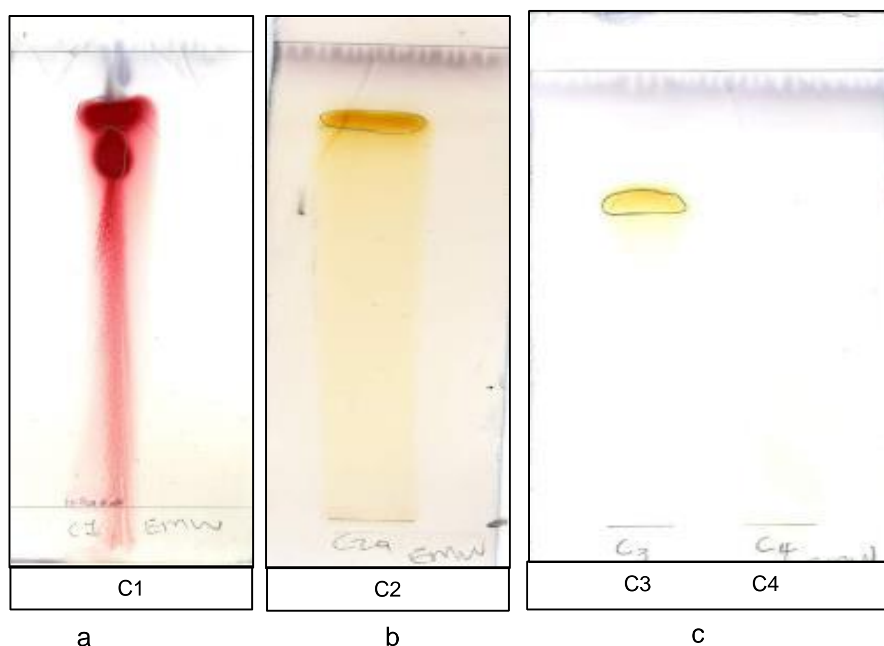

Figure 5.10. TLC chromatograms of compound 1 (a), compound 2 (b) and compound 3 (c) and 4 (c) isolated from the ethyl acetate fraction of *Ximenia caffra* var. *natalensis* developed in EMW after spraying with vanillin-sulphuric acid spray reagent.

5.4 Conclusions

Butanol fractions yielded the largest quantity of plant material upon extraction of the two plant species compared to other solvents. Amongst all fractions, butanol and ethyl acetate fractions of *X. caffra* var. *natalensis* exhibited good antifungal activity against *C. albicans* with MIC value of 0.08 mg/ml. However, only one active compound was visible in TLC chromatograms separated with BEA. *W. indica* had more active compounds visible when separated in TLC using CEF and EMW solvent systems. Further fractionation of the ethyl acetate fraction of *X. caffra* var. *natalensis* led to isolation of four compounds.

In the next chapter, the four isolated compounds will be identified and characterized using nuclear magnetic resonance and mass spectrometry.

CHAPTER 6

STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS

6.1 Introduction

The chemistry of natural products is important in the search for bioactive compounds. Structure elucidation is the full *de novo* procedure of structure identification, resulting in a complete molecular connection with correct stereo chemical assignments (Kind and Fiehn, 2010). Compounds isolated from plants are identified using techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR). These techniques will give complete structure elucidation of plant natural products.

Nuclear magnetic resonance is a technique used to determine the physical and chemical properties of atoms within a molecule. It is a highly quantitative and reproducible technique categorised into two groups, 1-dimensional NMR (1D-NMR) and 2-dimensional NMR (2D-NMR). 1D-NMR is used for identification of known compounds from their physiochemical data. 1D ^{1}H and ^{13}C NMR spectra are the commonly used databases for storing physiochemical data together with the subset of their NMR data. 2D-NMR is characterised by its longer acquisition time and superior resolution.

Mass spectrometry is a technique used for analytical investigation of the metabolites. It determines the elemental composition of the compound, giving information about the parent compound.

In this chapter, NMR and MS were used for the identification of four compounds isolated from the leaves of *Ximenia caffra* var. *natalensis*. The structures were elucidated from the spectroscopic data in collaboration with Dr M.A Abdalla (University of Pretoria).

6.2 Materials and methods

6.2.1 Structure identification

6.2.2. Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) (1D and 2D) spectroscopy and mass spectrometry were used to identify the isolated compounds. ^{1}H NMR and 2D NMR experiments data were acquired on a 400 MHz NMR spectrometer (Bruker Avance III 400 MHz). HPLC-HR-ESI-MS was performed on Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) system hyphenated to a quadrupole-time-of-flight (QTOF) instrument. All samples were sent to the Department of Chemistry, University of Pretoria for NMR analysis. Each sample was dissolved in 0.7 ml DMSO and transferred into NMR tubes (5 mm).

6.2.3 Mass Spectrometry preparation

Approximately 2 mg of each isolated compound was dried, placed into a glass vial and sent to the University of the Pretoria, Department of Chemistry for MS analysis.

6.3 Results and discussion

6.3.1 Compound **1**

Compound 1 was isolated as a red powder (20mg). The mass spectrum showed the *pseudo*molecular peak at m/z 443.0941 $[M+H]^+$ and m/z 441.0821 $[M-H]^-$, consistent with the molecular formula $C_{22}H_{18}O_{10}$ (Table 6.1). A search in dictionary of natural products and chemical abstracts services (Scifinder) using the spectroscopic information led to the structure of **1** as epigallocatechin gallate (EGCG) as shown in Figure 6.1, which was confirmed by comparing with the literature data (Zhang et al., 2016).

Epigallocatechin gallate (ECGC) is a polyphenolic compound which is abundant in green tea extracts (*Camellia sinensis)*. It constitutes 35-52% of catechins in green tea with strong physiological activities including antimicrobial activity, antioxidant activity and induction of breast cancer apoptosis (Kim et al., 2014 and Zhang et al., 2016). Kim et al. (2014) also reported the role of ECGC in lipid metabolism of the whole body as well as the cellular level. Weber et al. (2015) reported its antiviral activity against *Chikungunya*

virus. The methanol root extracts of *X. americana* var. *caffra* are rich in catechins, including ECGC (Sobeh et al., 2017). ECGC isolated from the chloroform-ethyl acetate fraction of *Terminalia bellerica* fruits showed good antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* (Gangadhar et al., 2011).

Table 6.1. ^{1}H-NMR and ^{13}C-NMR data of epigallocatechin gallate (EGCG) (1) in DMSO-d_6

Position	δ_C	C type	δ_H (mult.; J in [Hz])
2	77.0	CH	5.04 (s)
3	68.2	CH	5.35 (br s)
4	26.0	CH_2	2.95 (d, ^{3}J = 16.5, 4.1 Hz) 2.69 (d, ^{3}J = 16.5 Hz)
10	97.8	C_q	
5	156.9	C_q	
6	95.2	CH	5.84 (d ^{3}J = 2.3Hz)
7	156.6	C_q	
8	96.0	CH	5.90 (d ^{3}J = 2.3 Hz)
9	155.9	C_q	
1'	131.0	C_q	
2'	118.0	CH	6.68 (s)
3'	145.3	C_q	
4'	144.5	C_q	
5'	118.2	CH	6.77 (dd ^{3}J = 8.2, 2.1 Hz)
6'	115.1	CH	6.87 (d ^{3}J = 2.1 Hz)
1''	122.8	C_q	
2'', 6''	108.5	CH	6.83 (s)
3'', 5''	145.0	C_q	
4''	140.0	C_q	
COO	166.5	C_q	

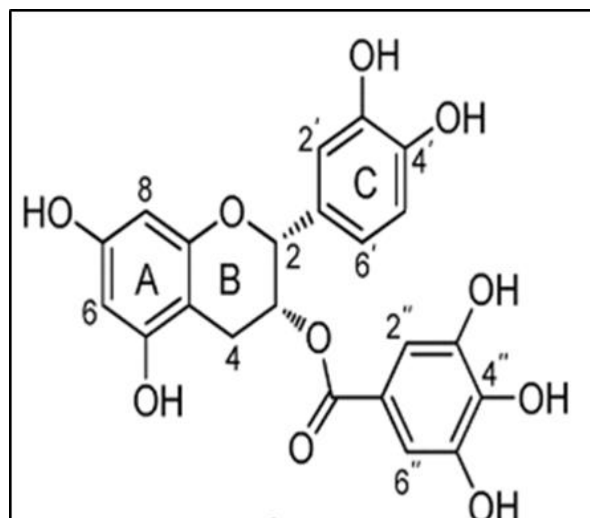

Figure 6.1. Structure of epigallocatechin gallate (ECGC) (**1**)

6.3.2 Compound **3**

Compound **3** was isolated as a yellow powder (4mg). The mass spectrum of kaempferol 3-O-rhamnoside (**3**) afforded the *pseudo*molecular peak at m/z 455.0950 $[M+Na]^+$ and m/z 431.0996 $[M-H]^-$, which gave the molecular weight 432 and the molecular formula $C_{21}H_{20}O_{10}$ by HRESIMS (Table 6.2). A search in dictionary of natural products and chemical abstracts services (Scifinder) using the spectroscopic information led to identification of compound **3 (**Figure 6.2). This was in agreement with^{1}H NMR and ^{13}C NMR spectroscopic data (Zhang et al., 2014).

Kaempferol-3-*O*-rhamnoside was isolated from *Schima wallichii*, *Zanthoxylum bungeanum* and *Tetraclinis articulata* (Diantini et al., 2011, Zhang et al., 2014 and Rached et al., 2018). Barliana et al. (2013) reported its anti-plasmodial activity against *Plasmodium falciparum*. Previously, it was reported that it has antioxidant activity and anti-cancer activity (Diantini et al., 2011 and Rached et al., 2018). Moreover, it was reported to inhibit the polar auxin transport in *Arabidopsis* shoots (Yin et al., 2014).

Table 6.2. ^{1}H NMR and ^{13}C NMR data of kaempferol -3-*O*-rhamnoside (3) in DMSO-*d_6*

Position	Compound	
	H	C
2	-	157.2
3	-	135.0
4	-	178.2
5		160.9
6	6.11 (s)	99.7
7	-	166.0
8	6.30 (s)	94.6
9	-	155.8
10	-	103.0
1'	-	121.5
2', 6'	7.72 (d, *J* = 8.2 Hz)	130.7
3', 5'	6.90 (d, *J* = 8.2 Hz)	115.9
4'	-	160.4
1''	5.30 (br s)	102.2
2''	3.98 (m)	70.5
3''	3.48 (ddd, *J* = 2.8, 8.7, 9.6 Hz)	70.8
4''	3.10 (dd, *J* = 3.5, 9.6 Hz)	71.5
5''	3.13 (dd, *J* = 6.3, 9.6 Hz)	71.0
6''	0.79 (d, *J* = 6.1 Hz)	17.9

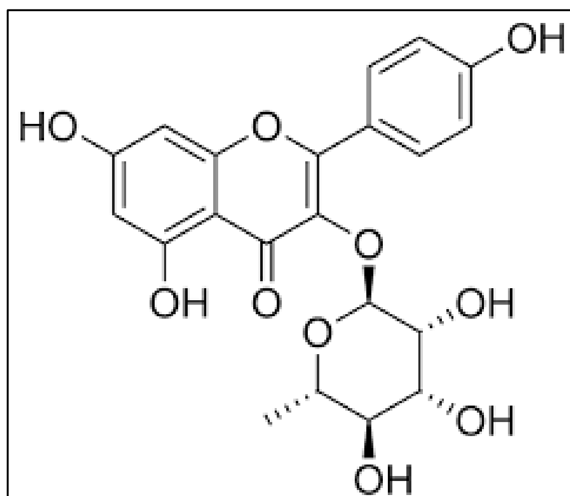

Figure 6.2. Structure of kaempferol 3-*O*-rhamnoside (**3**)

6.3.3 Compounds **2** and **4**

Compound **2** and **4** were isolated as yellow powder. The NMR analysis showed some impurities for both compounds. Based on mass spectra results and the low quantity of material available, the compounds were not analysed further by NMR.

6.4 Conclusions

Structure elucidation of the four isolated compounds from the leaves of *X. caffra* var. *natalensis* was determined using Nuclear Magnetic Resonance spectroscopy and Mass Spectrometry. Amongst the four compounds, only two compounds were successfully identified as epigallocatechin gallate (**1**) and kaempferol 3-*O*-rhamnoside (**3**). Compounds 2 and 4 appeared to consist of long chain fatty acids as shown by MS.

In the next chapter, the summary and conclusions of the study are given. Recommendations are also given.

CHAPTER 7

Summary and conclusion

An ethnobotanical survey was conducted in depth using a semi-structured questionnaire to identify plant species used by the local people and traditional healers to treat oral candidiasis in selected villages of Aganang Local Municipality. Antifungal activity of nine plant species was determined using microdilution against *C. albicans*. *Ximenia caffra* var. *natalensis* was the promising plant species for further phytochemical analysis. Solvent-solvent fractionation of the leaf extract of *X. caffra* var. *natalensis* was carried out and resulted in five fractions: hexane, chloroform, ethyl acetate, butanol and water fractions. Isolation of antifungal compounds using column chromatography led to the isolation of four compounds. Nuclear Magnetic Resonance and Mass Spectrometry techniques were used to identify the isolated compounds.

The survey identified twelve medicinal plants used for the treatment of oral candidiasis belonging to ten plant families. The families to which most species belonged were the Asteraceae and Ximeniaceae. *Ximenia caffra* var. *caffra* and *Ximenia caffra* var. *natalensis* were mostly the preferred plant species. *Warbugia salutaris* was the only endangered plant species in the current study. The least used plant species were *Artemisia afra* and *Solanum incanum*. Roots and leaves were the most used plant parts for the preparation of traditional medicine. Decoction was the preferred preparation method. The dominant life form of the used medicinal plants were herbs followed by trees.

Antifungal activity of nine plant species (*Artemisia afra, Blepharis subvolubilis* subsp. *subvolubilis, Enicostemma axillare, Helichrysum caespititium, Solanum incanum, Waltheria indica, Ximenia caffra* var. *caffra*, *Ximenia caffra* var. *natalensis* and *Ziziphus mucronata)* was determined against *C. albicans*. Excellent antifungal activity was observed in leaf extracts of *Artemisia afra* and *Solanum incanum* against *C. albicans*. Ethanol, methanol and water extracts of *Blepharis subvolubilis* subsp. *subvolubilis* had weak antifungal activity. The antifungal activity exhibited by the water extracts confirms

the effectiveness of the preparation methods used by the traditional healers and indigenous knowledge holders. The total activity of the plant extracts was calculated, and the best activity was observed in methanol extracts of *X. caffra* var. *caffra* followed by *E. axillare.* Noticeably, highest total activity was observed mostly in methanol extracts. Hexane extracts had the lowest total activity in several plant species including *E. axillare*, *S. incanum* and *Z. mucronata*.

Amongst the three eluent solvent systems used for the bioautography assay, BEA separated more active compounds. More active compounds were visible in *Ziziphus mucronata* extracts with R_f values ranging between 0.15 and 0.86 separated with BEA, CEF and EMW. Extracts of *W. indica* and *X. caffra* var. *natalensis* also had more active compounds with R_f values ranging from 0.19 to 0.70. The absence of active compounds, particularly in those extracts that had high antifungal activity in the microdilution method, indicate possible synergism.

W. indica roots and *X. caffra* var. *natalensis* leaves were the most promising plants for further phytochemical analysis and isolation of pure compounds. Acetone extracts were partitioned five times with solvents of varying polarities (hexane, chloroform, ethyl acetate, butanol and water respectively). Based on good antifungal activity, the ethyl acetate fraction of *X. caffra* var. *natalensis* was selected for further fractionation. Bioassay-guided fractionation using column chromatography led to isolation of four compounds. NMR and MS were used for the identification of the isolated compounds. Compound 1 was identified as epigallocatechin gallate (**1**) and compound 3 as kaempferol 3-*O*-rhamnoside (**3**). Compounds **2** and **4** were not identified conclusively as they appeared to be mixtures of long chain fatty acids which are difficult to separate. The results have shown the potential bioactivity of medicinal plants in the treatment of oral candidiasis.

Recommendations

Based on the obtained results, the following bioassays are recommended for future work:

(i) *In vitro* antifungal activity of the isolated compounds

The antifungal activity of the isolated compounds should be determined using the microdilution method and bioautography assay.

(ii) Cytotoxicity of the crude extracts and isolated compounds

In vitro cytotoxic activity of the isolated compounds and active plant extracts should be determined using various cell lines to ensure that they are not toxic to mammalian cells.

(iii) Antifungal activity against other microorganisms

Plant extracts and isolated compounds could be tested against other microorganisms to determine the extent of their antifungal activity.

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APPENDIX A: Ethnobotanical survey of medicinal plants used by traditional healers in Aganang Local Municipality, Capricorn district, Limpopo province, South Africa.

QUESTIONNAIRE NUMBER

1

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

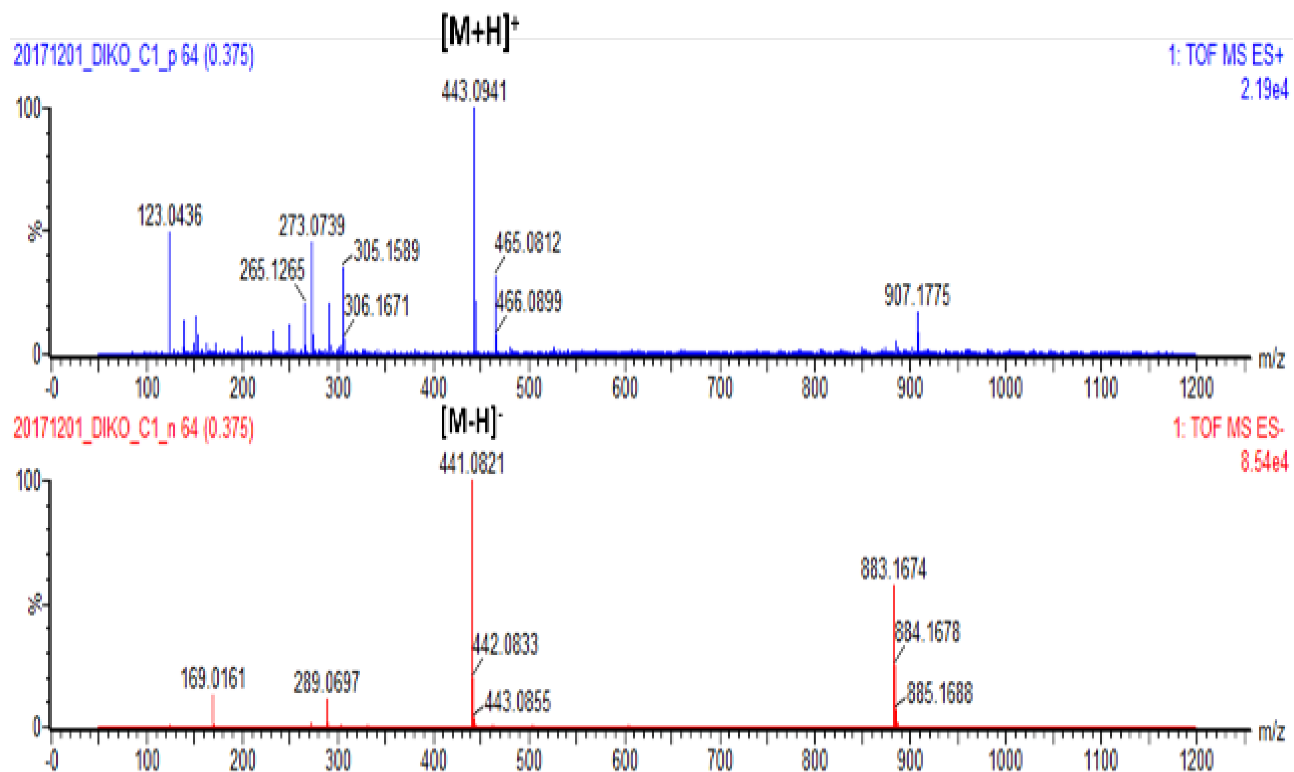
2. Personal information								
2.1	Level of education							
	1	No formal education	2	Primary schooling	3	Secondary schooling		
2.2	How many years' experience in traditional healing?							
	1	<10	2	11-20	3	21-30	4	>30

3. Consultation								
3.1	How many patients do you see per month?							
	1	<10	2	11-20	3	21-30	4	>30
3.2	Do you have the same or different patients coming for consultation?							
	1	Yes, the same patients	2	No, different patients				

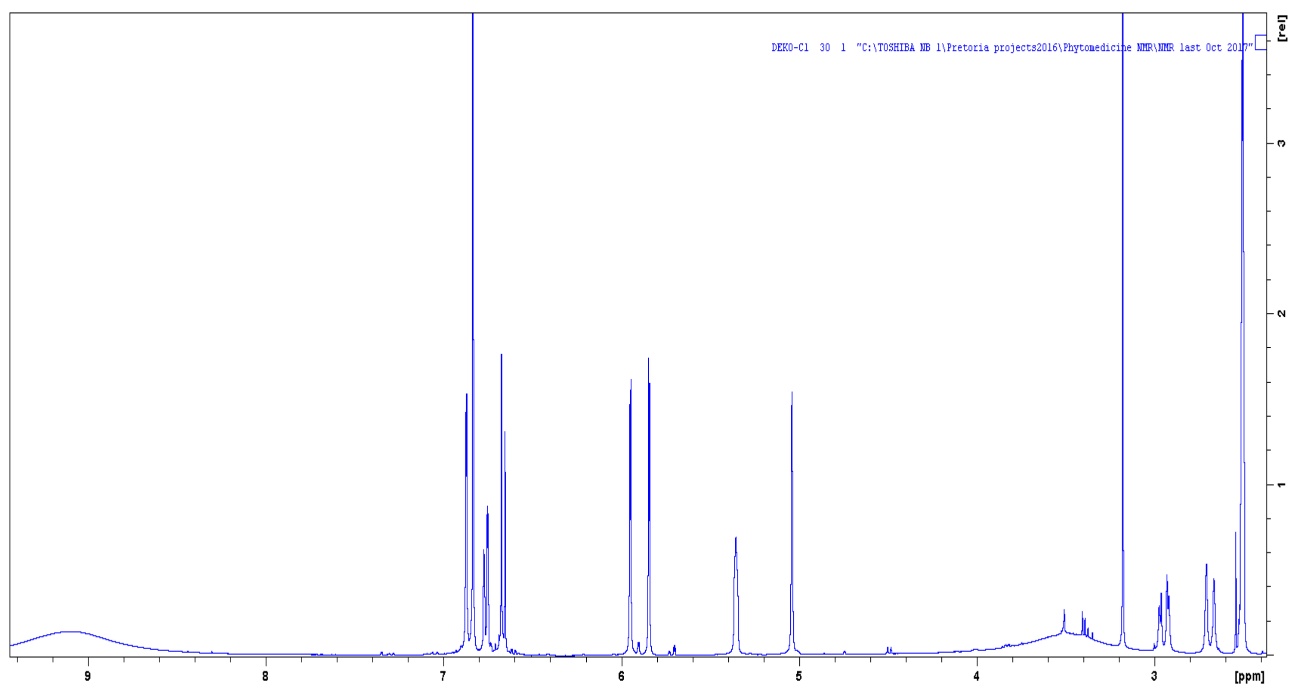
4.	Collection							
4.1	Do you collect plants?							
	1	Yes		2	No			
4.2	If no how do you get plant materials?							
	1	Get from other traditional healers.	2	Buy from the chemist/market.	3	Send someone to collect for me.	4	Other (Specify).
4.3	How are plants collected?							
	1	For a specific purpose.	2	General collection.	3	For a specific plant species.	4	Other (Specify).
4.4	Have you treated patients with the following symptoms of candidiasis?							
	1	Creamy white lesions on the tongue, inner cheeks, roof of the mouth, gums and tonsils.	2	Slightly raised lesions with cheese-like appearance in the mouth	3	Mouth ulcers	4	Other (Specify).
4.5	Which plant parts do you prefer for treatment of candidiasis?							
	1	Leaves	2	Roots	3	Bark	4	Other (Specify)
4.6	What is the local name/vernacular name of the plant? (Specify language)							
	1		4		7			
	2		5		8			
	3		6		9			

4.7	How do you prepare your medicine?							
	1	Decoction	2	Infusion		Maceration	4	Other (Specify)
4.8	How is it administered?							
4.9	For how long is it administered?							
4.10	How do you know if the plant /plant part is toxic?							
	Any other information?							

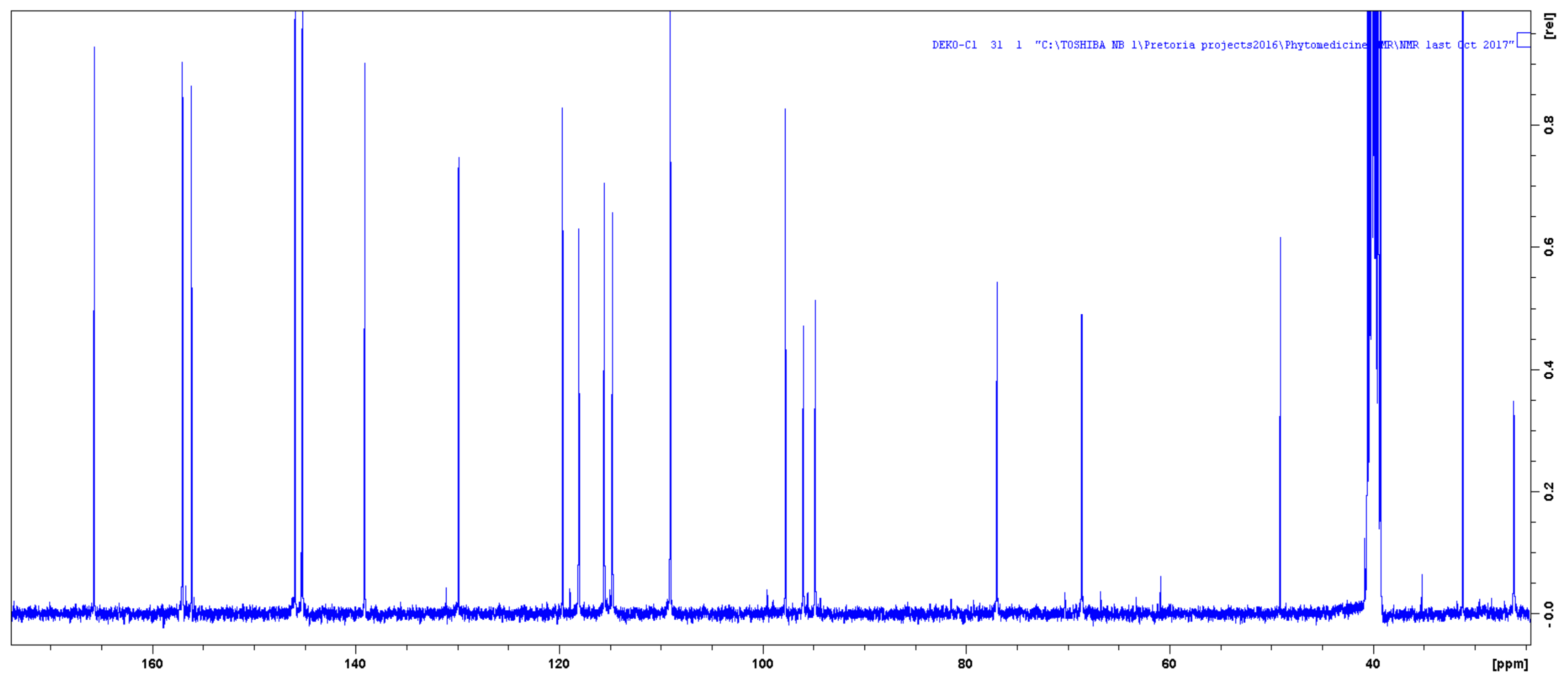
APPENDIX B: ESI/MS spectrum of epigallocatechin gallate (EGCG) (1)



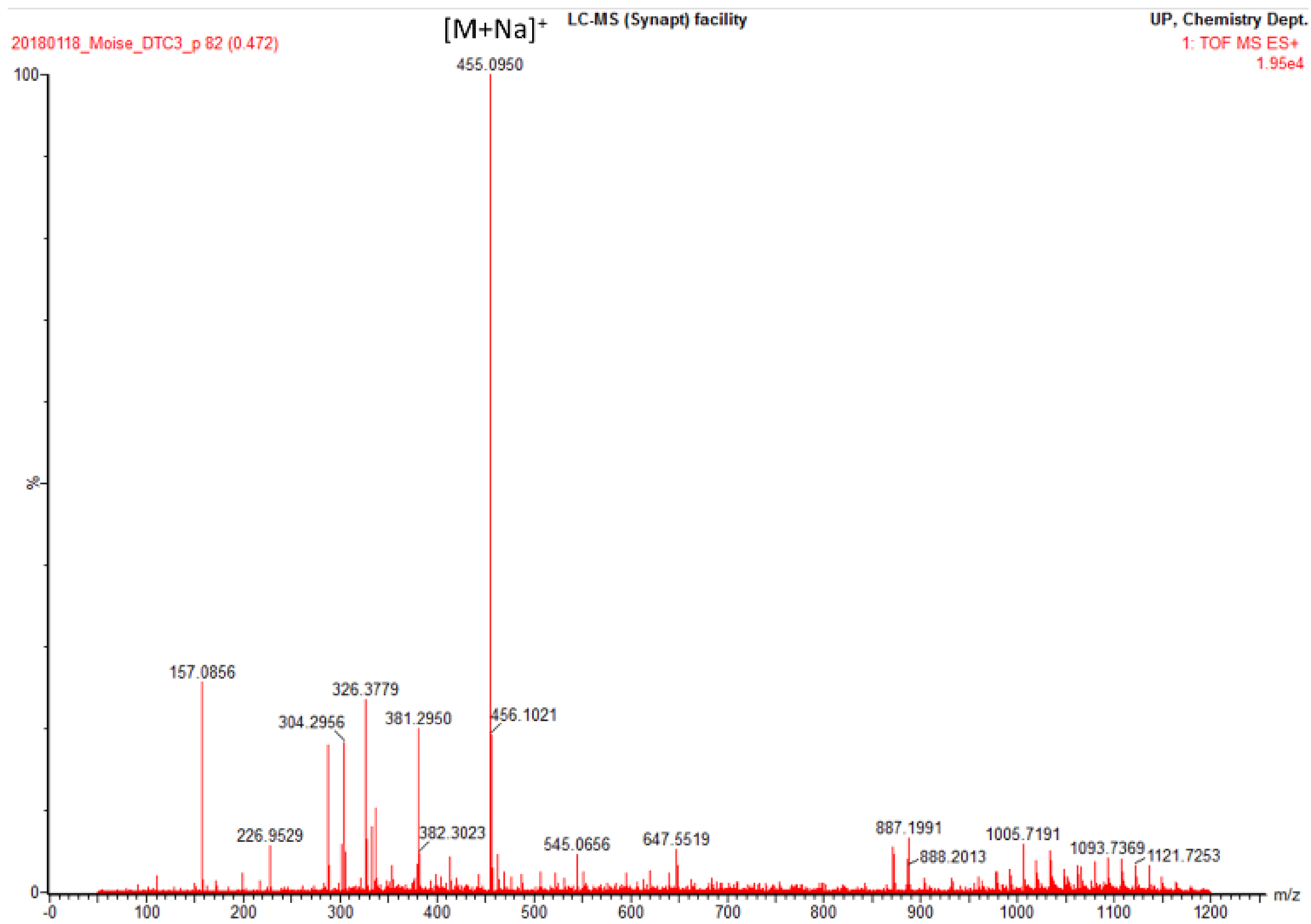
APPENDIX C: 1H NMR spectrum (DMSO-d6, 500 MHz) of epigallocatechin gallate (EGCG) 1



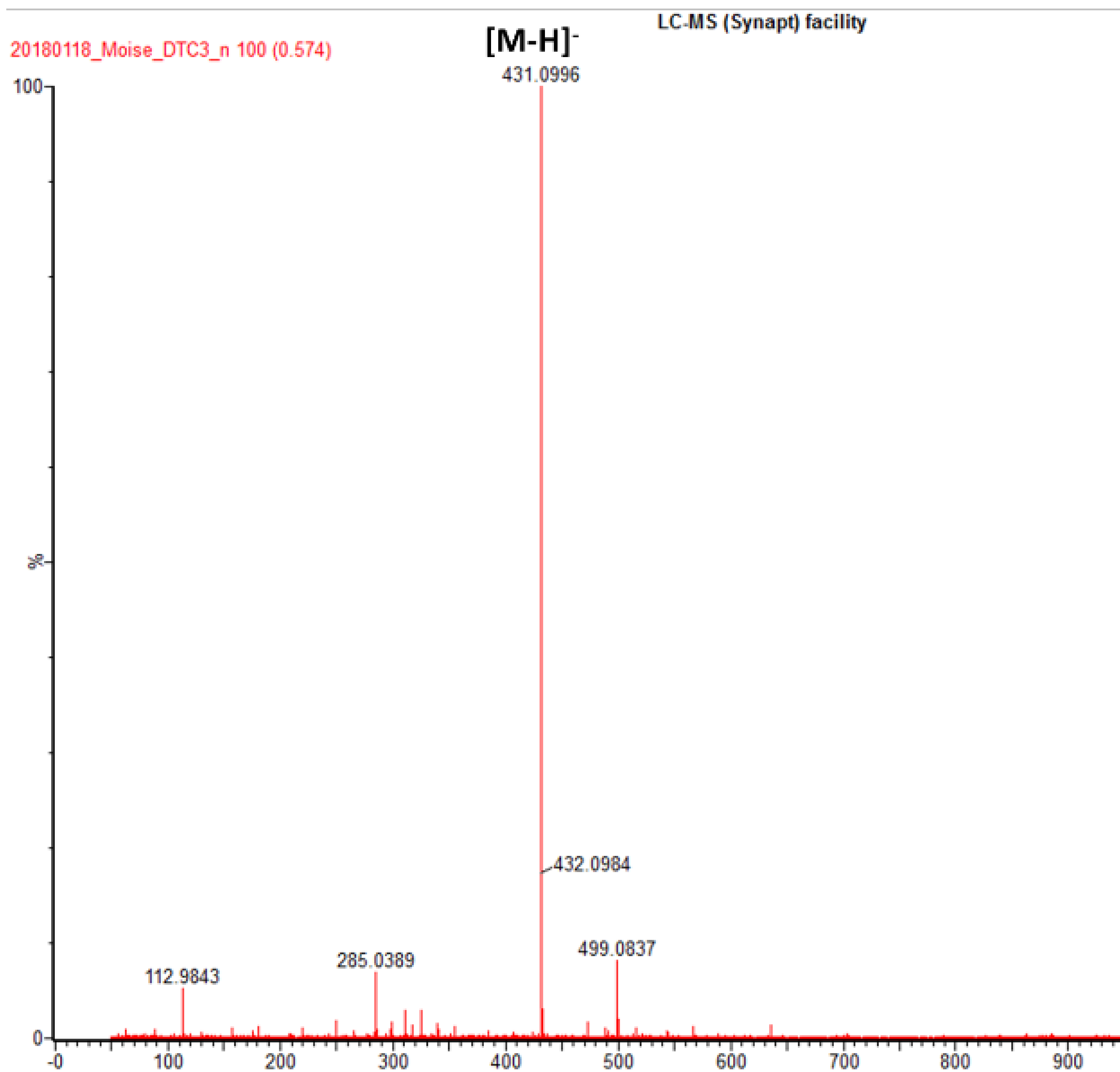
APPENDIX D: 13C NMR spectrum (DMSO-d6, 125 MHz) of epigallocatechin gallate (EGCG) (1)



APPENDIX E: ESIMS for kaempferol 3-*O*-rhamnoside (3) on positive mode



APPENDIX F: ESIMS for kaempferol 3-*O*-rhamnoside (3) on negative mode



APPENDIX G: 1H NMR spectrum (DMSO-d6, 400 MHz) of kaempferol 3-*O*-rhamnoside (3)