IDENTIFICATION AND CHARACTERISATION OF COMPOUNDS WITH ANTIMYCOBACTERIAL ACTIVITY FROM *STOMATOSTEMMA MONTEIROAE*

by

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DISSERTATION

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DECLARATION

I **Nnyadzeni Ramese** declare that the dissertation titled **"Identification and characterisation of compounds with antimycobacterial activity from *stomatostemma monteiroae*"** submitted to the University of Limpopo, for the degree of Master of Science in Microbiology 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.

_____   _____

Ramese Nnyadzeni   Date

DEDICATION

I would like to dedicate this work to the almighty God for granting me this opportunity, my loving family for all the prayers and support they have given me, and my late father may His soul continue to rest in peace.

ACKNOWLEDGEMENTS

"Being confident of this very thing, that He which hath begun a good work in you will perform it until the day of Jesus Christ"

Philippians 1:6

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LIST OF ABBREVIATIONS

^{13}C	Carbon-13
^{1}H	Hydrogen-1
A	Acetone
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
A_c	Absorbance of control
AIDS	Acquired Immune Deficiency Syndrome
$AlCl_3$	Aluminium Chloride
A_s	Absorbance of sample
Asc	Ascorbic acid
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BCG	Bacillus Calmette–Guérin
BEA	Benzene/ethanol/ammonia hydroxide
CD4	Cluster of Differentiation 4
CEF	Chloroform/ethyl acetate/formic
CNS	Central Nervous System
CO_2	Carbon dioxide
COSY	Correlation spectroscopy
D/DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
DOTS	Directly Observed Treatment, Short Course
DPPH	2,2-Diphenyl-1-Picrylhydrazyl

E	Ethanol
EA	Ethyl acetate
EC_{50}	Effective Concentration 50%
EMW	Ethyl acetate/methanol/water
EPTB	Extra Pulmonary Tuberculosis
FRAP	Ferric Reducing Antioxidant Power
FTIR	Fourier-Transform Infrared Spectroscopy
GAE	Gallic acid equivalence
GI	Gastrointestinal tract
H	Hexane
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HIV	Human Immune Virus
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HPTLC	High Performance Thin Layer Chromatography
HSQC	Heteronuclear single quantum coherence
IFN-g	Interferon-gamma
IGRAs	Interferon-gamma release assays
INT	p-Iodonitrotetrazolium violet
LC_{50}	Lethal concentration 50%
LC-MS	Liquid chromatography mass spectrometry
LC-UV	Liquid chromatography ultraviolet
M/MeOH	Methanol

MDRTB	Multidrug resistance tuberculosis
MEM	Minimum essential medium
mg/mL	Milligrams per millilitre
MIC	Minimum Inhibitory Concentration
MR	Magnetic resonance
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
Na_2CO_3	Sodium Carbonate
$NaNO_3$	Sodium Nitrate
NaOH	Sodium Hydroxide
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
OPLC	Overpressured layer chromatography
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDP	Purified protein derivative
QE	Quercetin equivalent
QFT-GIT	QuantiFERON -TB Gold In-Tube test
QFT-GIT	QuantiFERON–TB Gold In-Tube test
Rif	Rifampicin
ROS	Reactive Oxygen species
rpm	Revolutions per minute

SANBI   South African national biodiversity institute

SD   Standard deviation

SEE   Serial exhaustive extraction

SI   Selectivity index

TB   Tuberculosis

TCOSY   Total correlation spectroscopy

TDR-TB   Totally drug resistant tuberculosis

TLC   Thin layer chromatography

TST   Tuberculin skin test

UNIN   University of the north

USD   United states dollars

UV   Ultra violet

v/v   volume per volume

W   Water

w/v   Weight per volume

WHO   World health organisation

XDRTB   Extensively drug-resistant tuberculosis

xg   Multiples of gravitational acceleration

XXDR-TB   Extremely drug resistant tuberculosis

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monteiroae developed in 70% chloroform in ethyl acetate and sprayed with vanillin-sulphuric acid reagent and *M. smegmatis* for antimycobacterial activity respectively.

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CONFERENCES AND PROCEEDINGS

Paper presentations

Ramese, N. and Masoko, P. 2017. Preliminary screening of the phytochemical compounds of *Stomatostemma monteiroae*. University of Limpopo Faculty Research Day, Bolivia Lodge.

Ramese, N. and Masoko, P. 2018. The phytochemical, antimycobacterial and antioxidant activity of *Stomatostemma monteiroae*. Bio Africa convention. Durban, International Convention Centre (ICC).

Ramese, N. and Masoko, P. 2018. The phytochemical, antimycobacterial and antioxidant activity of *Stomatostemma monteiroae*. University of Limpopo Faculty Research Day, Fusion Boutique Hotel. (3rd prize award, Masters category)

Poster presentations

Ramese, N. and Masoko, P. 2018. The phytochemical, antimycobacterial and antioxidant activity of *Stomatostemma monteiroae*. Indigenous Plant Use Forum (IPUF). Surval Boutique Olive Estate, Oudtshoorn.

Ramese, N. and Masoko, P. 2018. The phytochemical, antimycobacterial and antioxidant activity of *Stomatostemma monteiroae.* 1st African Traditional and Natural Product Conference, Protea Hotel Ranch Resort. (1st prize award, poster presentation)

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ABSTRACT

The emergence of drug resistance to the first line drugs complicates the treatment of tuberculosis (TB), especially in parts of sub-Saharan Africa where accessibility to quality health care is limited. The search for alternative medication has been the centre of research for years due to challenges posed by infectious organisms including drug resistance, lengthy treatment periods and lack of quality health care in developing countries. *Stomatostemma monteiroae* is used in traditional medicine to treat TB and related symptoms. The aim of this study was to isolate and characterise compounds with antimycobacterial activity from *Stomatostemma monteiroae*. The plant materials were collected from Ga-Madiga village in Limpopo province of South Africa. Different plant parts namely: leaves, twigs, roots, tuber and tuber-peels were separated, washed, dried and milled to a fine powder. Several solvents (n-hexane, dichloromethane, acetone and methanol) were used to extract the plant material using various extraction methods such as maceration, defatting, and extract enrichment procedure and phytochemical analysis was done using standard chemical tests and thin layer chromatography. The qualitative antioxidant activity was determined by the thin layer chromatography (TLC) based 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and quantitative antioxidant activity was determined using colorimetric DPPH free radical scavenging and ferric reducing power assay. Antimycobacterial activity of the extracts was assessed using bioautography and micro dilution method tested on *Mycobacterium smegmatis* (ATCC 1441), *Mycobacterium tuberculosis* (ATCC 25177) and *M. tuberculosis* H37Rv (ATCC 27294). The cytotoxic effects of the extracts were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on Vero monkey kidney cells. The compounds with antimycobacterial activity were isolated using bioassay-guided fractionation and purified using preparative thin layer chromatography and thereafter identified using NMR spectroscopy to elucidate the structure.

Various phytochemical constituents were detected in different plant parts, with the leaves and twigs possessing more of the phytoconstituents analysed. The TLC profile of *S. monteiroae* indicated that more compounds are non-polar to intermediate in polarity. The antioxidant activity analysis on TLC plates indicated that all the plant parts have low antioxidant activity, this was also confirmed by

quantitative tests. The leaves of *S. monteiroae* had antimycobacterial activity when analysed using bioautography, while other plant parts had no active bands. The minimum inhibitory concentration values were much higher than the positive control rifampicin and the roots (0.31 mg/mL) followed by the leaves (0.83 mg/mL) had lower inhibitory concentrations when tested against *M. smegmatis*. The MIC values of extracts against TB causing strains varied greatly, the leaves and the roots had even higher MIC value. Toxicity analysis indicated that all plant parts were non-toxic towards Vero cells (LC_{50} > 0.02 mg/mL). Bioassay-guided fractionation enabled isolation of one antimycobacterial pure compound from the leaves extracts. The isolated compound was identified using NMR and was found to be a sitosterol derivative 8,9-dehydro-4-methyl-24-vinylobtusifoliol. This compound had a noteworthy activity against *M. smegmatis.* The present study validates the use of *S. monteiroae* in the treatment of TB related symptoms traditionally. Further studies are required to analyse the cytotoxic effects of the isolated compound and also testing the antimycobacterial activity of the isolated compound on TB causing pathogens.

CHAPTER 1

1.1. GENERAL INTRODUCTION

Tuberculosis (TB) is a life-threatening disease that is caused by a pathogenic bacterium, *Mycobacterium tuberculosis* (WHO, 2013). The infection primarily affects the lungs and can be spread when an infected person coughs and releases the tubercle bacilli in the air (Mativandlela *et al.*, 2008). Although tuberculosis is a pulmonary disease, it can also disseminate to other parts of the body such as the bones and joints, central nervous system and lymphatics (Jones *et al.*, 1993). Tuberculosis can progress as a newly acquired infection, reinfection or reactivation of latent infection. However, the progression of the disease can be influenced by the immune system of the individual, chronic diseases, active/passive smoking, diabetes or residing in areas with high TB prevalence (Leung *et al.*, 2004, 2008, 2010).

Tuberculosis is one of the leading causes of deaths accounting for more than 9.6 million new cases and 1.5 million deaths each year worldwide (Raviglione and Sulis, 2016). Worldwide effort to reduce the incidence of TB due to improved therapeutic treatments with a considerable progress, have been achieved due to the intervention of the WHO and STOP-TB partnership (Zager and McNerney, 2008). However, in most parts of sub-Saharan Africa TB cases have been reported to increase as compared to successful treatment rates (Dheda and Migliori, 2012; WHO, 2018). The influencing factors to the increased mortality and morbidity rates in African countries include the HIV epidemic, the emergence of drug resistant TB towards current anti-TB drugs, and unsuccessful treatment regimen (Dheda *et al.*, 2010).

The disease may be cured, provided the specified treatment regimen is followed. The treatment of TB involves drugs such as isoniazid, rifampicin, ethambutol or streptomycin and pyrazinamide taken daily for a period of 6 months (Zumla *et al.*, 2015). Ethambutol or streptomycin is discontinued in the last four months of the treatment if the results show drug susceptibility to the above drugs (Zumla *et al.*, 2015). In the case of Human Immunodeficiency Virus (HIV) related TB and drug resistant TB, fluoroquinolones, rifabutin and other injectable drugs such as amikacin, capreomycin or kanamycin may be employed which may require extended periods of treatment (WHO, 2016).

Eradication of TB with the available drugs has proven to be a challenge, especially in rural areas where treatment accessibility is limited, and emergence of drug resistance continues to be a major threat (Zumla *et al.*, 2015). Therefore finding alternative drugs that are cost effective, readily accessible and highly effective towards the disease is of great importance. This has led to the exploration of plants for alternative therapeutic strategies especially in developing countries were access to quality health care is lacking and herbal remedies being their primary source of health care. Plants have been employed traditionally in treating diseases since time immemorial. This is due to the fact that plants and plant parts have been noted to have disease preventing and healing effects (Komape *et al.*, 2017).

Plants produce secondary metabolites called phytochemicals, these compounds are diverse and have various biological activities (Liu, 2013). Many studies on plants have shown that there are thousands of phytochemicals that are safe and effective alternatives with less adverse effects as compared to modern synthetic drugs (Mujuni, 2014; Zumla *et al.*, 2015; Xego *et al.*, 2016). Such phytochemicals include saponins, phenols, flavonoids, steroids to mention a few and they differ in content and type, according to plant species (Madikizela *et al.*, 2014). This study focused on evaluation of antimycobacterial compounds from a medicinal plant *Stomatostemma monteiroae* which is used traditional to treat TB and related symptoms.

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CHAPTER 2

2. LITERATURE REVIEW

2.1. Traditional medicinal plants

Medicinal plants have been associated with human civilisation since ancient times and forms part of many cultural practices (Xego *et al.*, 2016). The indigenous knowledge of healing from plants is passed down from generations to generations orally within communities (Iwu, 2014). Plants have been used for many purposes and still have many applications in the modern world, such as their use in the making of furniture, shelter, as foods, cosmetics and pharmaceuticals (Schippmann *et al.*, 2002). Globally, up to 60,000 plant species are used for medicinal and nutritional purposes and over 500,000 tons of plant materials are traded, reaching a value of more than 2.5 billion United States Dollars (USD) annually (Xego *et al.*, 2016). A large proportion of plants used in traditional health care systems were selected on the basis of their physical resemblance of an organ in the human body. This phenomenon is referred to as doctrine of signatures by botanists (Iwu, 2014).

Plants used for medicinal purposes are mostly accessed through traditional healers and South Africa alone has up to 200 000 indigenous traditional healers playing a great role in providing accessible health care to the communities (SANBI, 2006). In South Africa local trade of medicinal plants was estimated to be over 20 000 tons each year involving up to 574 plant species (Xego *et al.*, 2016). The trade in medicinal plants provides employment to many individuals, thereby contributing greatly to the economy of South Africa (Liphadzi, 2013). Due to the increase in the harvest of medicinal plants each year (35 000 plant species), most species have become endangered, while most are being used without scientific validation for their safety and efficacy (Xego *et al.*, 2016). Medicinal plant studies seek to find alternative treatments before reaching post-antibiotic era, to validate traditional claims for effective treatments from plants and also to conserve our biodiversity to ensure sustainable development (Mujuni, 2014).

Medicinal plants have proven to be the most diverse bio-resource of therapeutic agents of traditional medicinal systems, pharmaceuticals and chemical intermediates and entities for synthetic drugs (Ncube *et al.*, 2008). The World Health Organization, (2008) reported that up to 80% of people throughout the world depend on traditional medicine to meet their primary health care needs. South African history of the use of medicinal plants in treating a variety of illnesses and ailments dates back thousands of years and has always played a significant role within its health care system (Masoko and Nxumalo, 2013). Diseases for which medicinal plants have been used include cancer, malaria, diabetes and many others, most importantly antimicrobial activities (Masoko and Nxumalo, 2013).

The lack of enough information with regard to dosage taken by adults and those given to children poses a serious challenge as medicinal plants may contain other compounds which are toxic and can cause adverse effects to the patients (Masoko and Nxumalo, 2013). Scientific investigations of medicinal plants not only aim to validate their biological activities, they also include cytotoxic studies to ensure that the medicinal plant(s) are also safe to use (McGaw *et al.*, 2008). Rinne, (2001) reported that local communities consult traditional healers regularly because they are found within a short distance, are familiar with the patient's culture and the affordable costs associated with treatments.

2.2. Medicinal plants used in the treatment of TB-related symptoms

Herbal remedies derived from medicinal plants used to treat TB related symptoms depend on the culture and communities involved and also the availability of plants of interest in that region (Kumar *et al.*, 2010). Some remedies are given as prepared concoctions while others can be given as raw materials with instructions of preparation (Masoko *et al.*, 2010). Medicinal plants such as *Chenopodium ambrosioides* L*., Solanum torvum* Sw*., Cyperus articulatus* L., *Apodytes dimidiata, Lippia javanica, Combretum heroroense, Ardisia japonica, Milletia stulhimannii, Clausena excavate, and Artemisia afra* are used traditionally in the treatment of TB in most African countries (Kumar *et al.*, 2010; Masoko *et al.*, 2010; Nguta *et al.*, 2015 and Komape *et al.*, 2017). Some medicinal plants and their bioactive compounds effective towards Mycobacterium species (Table 2.1) have been studied and tested to validate their safety and efficacy in the treatment of TB related symptoms traditionally. Before the discovery of most antibiotics, species of *Salvia africana-lutea*

were prepared as components of herbal tea mixtures to treat TB and bronchitis (Gerstner, 1941). The traditional healers of Western Cape Province of South Africa prepare decoction of *Salvia africana-lutea* in the treatment of fever, headache, influenza, respiratory and digestive disorders (Amabeoku *et al*., 2001). In Limpopo province of South Africa medicinal plants such as *Citrus lemon, Combretum heroroense* and *Apodytes dimidiata* are currently used in the treatment of TB, and their application is guided by the symptoms that the patient displays, which include coughing up blood, night sweat and rapid weight loss (Komape *et al*., 2017).

Table 2.1: Medicinal plants and compounds used to treat diseases caused by Mycobacterium species (Okunade *et al*., 2004).

Plant species	Compound isolated	Organism model	References
Combretum molle	Phenol: punicalagin	MTB	Asres *et al*, (2001)
C. excavata	Alkaloid: methoxycarbazole	MTB	Sunthitikawinsakul *et al*, (2003)
Cryptolepis sanguinolenta	Alkaloid: cryptolepine hydrochloride	MF	Sunthitikawinsakul *et al*, (2003)
Croton kongensis	Terpenoids: secokauranes	MTB	Thongtan *et al*, (2003)
Morinda citrifolia	Steroid: stigmasterol	MTB	Okunade *et al*, (2004)
Psoralea corylifolia	Phenol: bakuchiol	MTB	Newton *et al*, (2002)
Physalis angulata	Steroid: physalin B	MTB	Januário *et al*, (2002)
Xanthocyparis nootkatensis	Terpenoids: (+)-totarol	MTB	Constatine *et al*, (2001)
Sapium indicum	Terpenoids: phorbol ester	MTB	Chumkaew *et al*, (2003)
Peucedanum ostruthium	Coumarins: ostruthin	MS	Schinkovitz *et al*, (2003)

Key: MTB= *Mycobacterium tuberculosis*, MF= *Mycobacterium fortuitum*, MS= *Mycobacterium smegmatis.*

2.3. Phytochemical constituents

Plants produce various chemical compounds that are generally divided into two types namely primary and secondary metabolites (Kabera *et al.*, 2014). Primary metabolites are those which are involved in the normal growth, reproduction and development of a plant and such are important for survival of the plant. Examples include amino acids, nucleic acids, carbohydrates and many others (Tissier *et al.*, 2014). Secondary metabolites also known as phytochemicals are non-nutritive chemicals that are found in plants (Dillard and German, 2000). They are non-essential nutrients that are not required by the plant for growth and development, but they play a defensive role against any harm in the ecological environment of the plant (Johnson and Williamson, 2003). These chemicals protect the plant from damage or environmental hazards such as drought, attack by pathogenic microorganisms, insects and pests attack, stress, ultraviolet light exposure and pollution (Mathai, 2000). The protective effects of these phytochemicals have been noted to have significant effects when taken in a diet (Liu, 2013).

Phytochemical compounds are extremely diverse, and thousands of them have been documented and classified according to their specific functions and physical and chemical properties (Raskin *et al.*, 2002; Saxena *et al.*, 2013). Most phytochemical compounds have different biological activities, and some have been reported to have antioxidant, antimicrobial, anticancer, anti-inflammatory, anti-malarial and anti-allergic properties (Narasinga, 2003). Phytochemicals can be derived from different parts of the plant such as the roots, bark, flowers, leaves, seeds, stems and fruits (Tiwari *et al.*, 2011). From an ethnobotanical survey (Figure 2.1) of two regions in Limpopo (Lwamondo) and Mpumalanga (Jongilanga) the roots were found to be the most frequently used parts in preparation of herbal remedies (Mahwasane *et al.*, 2013; Tshikalange *et al.*, 2016).
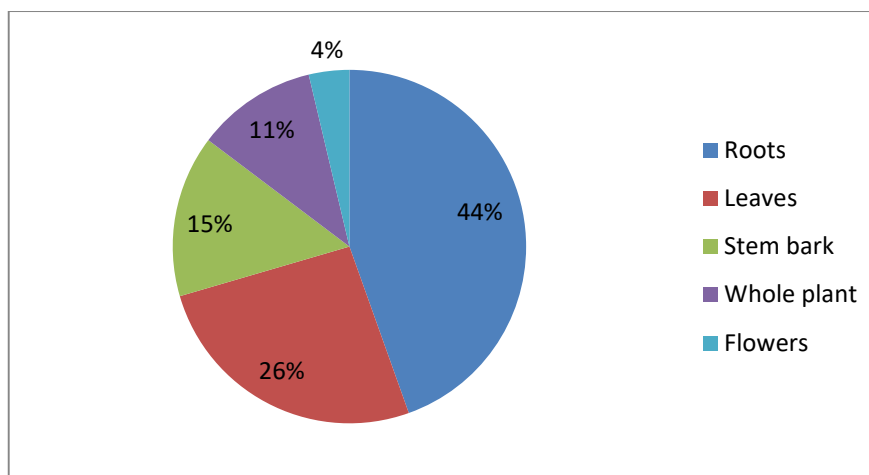
Figure 2.1: Plant parts used in traditional medicine and their percentage of usage (Tshikalange *et al.*, 2016).

Medicinally relevant bioactive compounds from plants are mostly species specific unlike primary metabolites that are present in all plants (Ahmed *et al.*, 2017). Bioactive compounds are useful in a plant that produces them. Other functions include production of colours which attract pollinators in due time. Other medicinal plants have been reported not to have the actual antimicrobial compounds, but they may have stimulant effects on the immune system of an individual which helps fight diseases (Buwa and Afolayan, 2009). Below some medicinally relevant phytochemical compounds are discussed.

2.3.1. Phenolics

Polyphenols are comprised of an aromatic ring having one or more hydroxyl groups (Figure 2.2). They range from simple phenols to higher molecular weights polymerised compounds (Babbar *et al.*, 2015). The main dietary phenolic compounds are flavonoids, tannins and phenolic acids. Flavonoids are low molecular weights phenols containing 15 carbon atoms and constitute the largest group of phenolics from plants (Ashok and Upadhyaya, 2012). Phenolic acids are composed of two subgroups viz the hydroxycinnamic acids which are aromatic containing 3 carbon side chain and the hydroxybenzoic acids that have a 6 carbon structure. Examples include ferulic acid and gallic acid (Babbar *et al.*, 2015). While the tannins are the higher molecular weights phenols, they are divided into condensed also known as proanthocyanidins and hydrolysable tannins, such as esters of gallic acid (Ashok and Upadhyaya, 2012). Phenolic compounds have antioxidant activity due to

their ability to scavenge free radicals. They counteract oxidative stress in the body and consumption of vegetables and fruits has shown beneficial effects because plants have high amounts of antioxidants (Zhao *et al.*, 2014).
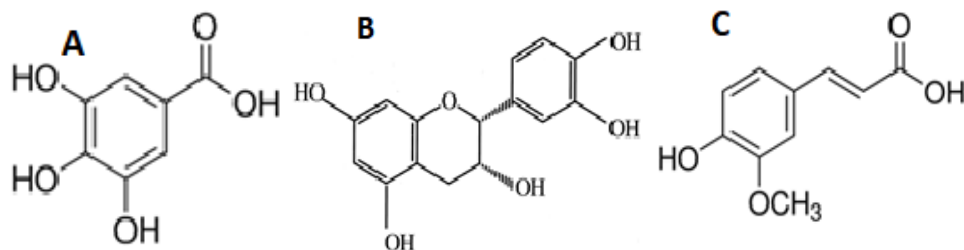
Figure 2.2: Chemical structures of gallic acid (A) (Ashok and Upadhyaya, 2012), epicatechin (B) (Vaidyanathan and Walle, 2002) and ferulic acid (C) Kikuzaki *et al.*, 2002.

2.3.2. Terpenoids

Terpenoids also known as terpenes (when oxidised) are one of the largest group of naturally occurring compounds with more than 40,000 different molecules (Pichersky *et al.*, 1994). They are organic hydrocarbons with a definite number of isoprene units (methylbuta-1,3-diene with 5 carbon atoms). These compounds are important for the growth and survival of the plant in the environment (Aharoni *et al.*, 2005). They have various industrial applications such as fragrances in cosmetics and flavours in foods (Aharoni *et al.*, 2004). Terpenoids are not only useful in agricultural applications but have been reported to have medicinal properties such as antimicrobial, anti-cancer and anti-malarial activities. These activities are attributed to compounds such as glycyrrhizin (Figure 2.3), taxol and artemisinin (Lin *et al.*, 2005; McCaskill and Croteau, 1998; Bertea *et al.*, 2005). Essential oils are an important sub-class of terpenes and are mainly composed of monoterpenes, diterpenes and sesquiterpenes. These compounds have various biological activities including antifungal, antiviral, and antibacterial (Ncube *et al.*, 2008).
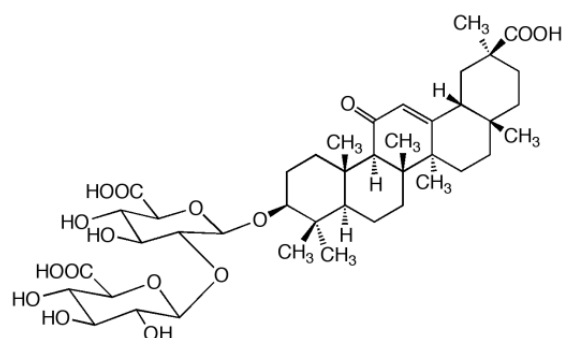
Figure 2.3: The chemical structure of glycyrrhizin a terpenoid (Aharoni *et al.*, 2005).

2.3.3. Alkaloids

Alkaloids are basic and nitrogen containing compounds that are synthesised by many living organisms. They include compounds such as isoquinoline, imidazole, quinoline, atropine and piperdine alkaloids amongst others (Irchhaiya *et al.*, 2015). They are known to be toxic and are mostly used by the plants for defence against attacks from pest, invasion by microbial pathogens and herbivores (Mustafa *et al.*, 2017). Although many alkaloids are toxic, some have antibacterial activities while some are used in the treatment of hypertension i.e. reserpine (Figure 2.4). Chen *et al.* (2014) reported that the plant *Kopsia hainanensis* of the family Apocynaceae contains indole alkaloids with antitumor, antileishmanial and antimitotic activities.
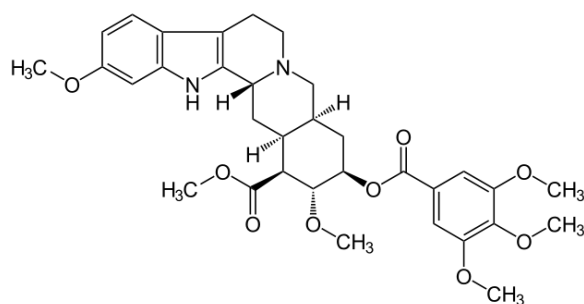
Figure 2.4: The chemical structure of an alkaloid reserpine (Mustafa *et al.*, 2017).

2.3.4. Glycosides

Glycosides are secondary metabolites from plants that are made of glycone, a carbohydrate and aglycone a non-carbohydrate component (Yu *et al.*, 2012). In plants they are mostly derived from post-modification of other secondary metabolites (Bagrov *et al.*, 2009). Aglycone usually contain another secondary metabolite (phenolics, terpernoids or alkaloids) attached to it while the glycone consist of one or more glucose unit (Yagi *et al.*, 2002). Cardiac glycosides have been used for

thousands of years traditionally as arrow poison during hunting and wars. Many cardiac glycosides have been isolated form plants and applied medically. They include digoxin, oleandrin and ouabain which have been isolated from plants such as *Digitalis lanata, Strophanthus gratus* and *Nerium oleander* (Bagrov *et al.*, 2009). Cardiac glycosides have been used to treat cardiac diseases and clinically they have been observed to exert an inotropic effect on the heart muscle, thus improving circulation of blood (Haviv and Karlish, 2013). Aloesin (Figure 2.5) is a glycoside isolated from *Aloe vera* and has been reported to have anti-inflammatory effects and antioxidant activities (Yagi *et al.*, 2002).
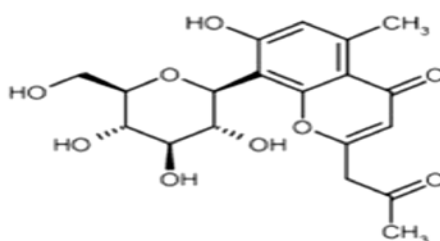
Figure 2.5: Structure of a glycoside Aloesin (Dell-Agli *et al.*, 2007).

2.4. Biological activities of plants' secondary metabolites in medicine

Numerous studies on the pharmacology of medicinal plants have been done and plants have proven to be a potential source of new medicines (Dahanukar *et al.*, 2000; Bagrov *et al.*, 2009; Saxena *et al.*, 2013). Scientific investigations on phytochemical compounds have revealed that plants are the richest bio-source of therapeutic compounds with biological activities such as hepatoprotective, antimalarial, antifungal, immunostimulant, antibacterial, and anticarcinogenic activity (Saxena *et al.*, 2013). Though plants derived bioactive compounds may belong to the same group, the mechanisms of action (Table 2.2) which brings about the therapeutic effects vary greatly.

Table 2.2: Antibacterial plants derived compounds with their mechanism of action (Silva and Fernandes, 2010).

Plant compound class	Example	Mechanism of action
phenol	Epicatechin	Membrane disruption
Flavonoids	Chrysin	Adhesin binding
Tannins	Ellagitannin	Enzyme inhibition
Alkaloids	Berberine	Intercalation into cell wall and/or DNA
Terpenoids, essential oils	Capsaicin	Membrane disruption
Quinones	Hypericin	enzyme inactivation

Pharmacognosy as practiced has evolved over the years and it is now referred to as the study of physical, chemical, biological and biochemical characteristics of potential drugs and drug substances from natural sources such as plants, bacteria, marine organisms or fungi (Balunas and Kinghorn, 2005). Drug discovery from medicinal plants is an extensive process that follows a number of steps (Figure 2.6) guided by the biological activity of interest. Scientists pursue secondary metabolites from plants as prerequisites for drug development. Despite much work done in synthetic chemistry techniques and combinational chemistry by pharmaceutical companies, medicinal plants remain a great source of new drugs, drug leads and chemical entities (Balunas and Kinghorn, 2005).

```
                    ┌──────────────────────────┐
                    │ Selection of plant species│
                    └────────────┬─────────────┘
                                 ▼
                    ┌──────────────────────────┐
                    │  Collection of the plant  │
                    └────────────┬─────────────┘
                                 ▼
              ┌────────────────────────────────────────┐
              │ Dry the plant material and grind to fine powder │
              └────────────────────┬───────────────────┘
                                   ▼
           ┌──────────────────────────────────────────────┐
           │ Solvents selection and extraction of the plant material │
           └────────────────────────┬─────────────────────┘
                                     ▼
              ┌──────────────────────────────────────┐
              │   Screen plants for phytoconstituents │
              └──────────────────┬───────────────────┘
                                 ▼
                        ┌───────────────┐
                        │   Bioassays   │
                        └───────┬───────┘
                                ▼
      ┌───────────────────────────────────────────────────────────┐
      │ Fractionation and isolation of pure compounds (confirm activity) │
      └──────────┬────────────────────────────────────────────────┘
                 ▼
      ┌────────────────────────────┐        ┌──────────────────┐
      │ Toxicity and safety studies │──────▶│  Clinical tests  │
      └────────────────────────────┘        └─────────┬────────┘
                                                       ╲
                                                        ╲
      ┌────────────────────────────┐        ┌──────────────────────────┐
      │ Mass production in industries │────▶│ Pharmaceutical formulations │
      └────────────────────────────┘        └─────────────┬────────────┘
                                                           ▼
                                            ┌──────────────────────────┐
                                            │     Approval as drugs     │
                                            └──────────────────────────┘
```

Figure 2.6: A flowchart illustrating processes associated with drug discovery procedures from medicinal plants (Pieters and Vlietinck (2005).

2.4.1. Antimicrobial activities

Medicinal plants have a diverse mixture of biologically active compounds that may act in killing or inhibiting the growth of disease causing microorganisms (Alvin *et al.*, 2014). Medicinal plants are investigated with the hope of development of new therapeutic agents and also to ensure minimal toxicity and fewer side effects to the infected individual. Plant derived bioactive compounds have been noted to have less side effects and the evolution of multiple drug resistance necessitates an urgent search for antimicrobial compounds from alternative sources such as plants (Savoia, 2012). Scientific researches not only generate new knowledge and medicinal

alternatives but also help in validating traditional claims on the use of medicinal plants and their healing properties (Cseke *et al.*, 2016). Most of the modern drugs that have been developed were derived from plants and plant parts (Saxena *et al.*, 2013). These studies have a huge impact on our communities due to the reliance of many people on traditional systems for their primary health care needs (Masoko and Nxumalo, 2013). And also considering that in developing countries the status of good quality health care systems is still at an infancy stage and most pharmaceutical drugs are expensive.

2.4.2. Antioxidant activities

Antioxidants are chemical compounds that inhibit oxidation of molecules by scavenging free radicals also called Reactive Oxygen Species (ROS). They prevent oxidative stress by interacting and neutralizing free radicals thereby terminating chain reactions (Lushchak, 2014). Reactive Oxygen Species are highly reactive molecules that are produced in the body as a result of normal cellular metabolism as well as environmental factors (Mittler, 2017). However, these reactive molecules can cause damage when in high concentrations. They can adversely modify cell structures such as lipids membranes, proteins and nucleic acids. Damage to major cell components can result in diseases such as cancer, pulmonary fibrosis, neurological disorders, diabetes and many others (Thanan *et al.*, 2014).

Endogenous antioxidants are formed in the body to counter the effects of ROS. However, their production may not be enough sometimes, and external antioxidants are required (Valko *et al.*, 2007). Exogenous sources of antioxidants also called dietary antioxidants can be derived from fruits, vegetables, and grains and includes vitamin E, vitamin C, vitamin A, glutathione, beta-carotene, and lycopene (Bouayed and Bohn, 2010). Natural sources of antioxidants such as plants have received a great deal of attention due to abundance of these antioxidants (Garhwal, 2010). Medicinal plants from Egypt *Eucalyptus camaldulensis* var. *brevirostris* and *Dorstenia mannii* harvested from Cameroon are used traditionally in the treatment of variety of ailments (Atawodi, 2005). These plants had antioxidants capacity and compounds such as ellagic acid, prenylated flavonoid were isolated from them (Atawodi, 2005).

2.5. Plant interactions

Medicinal plant studies usually end with isolation of the active compounds which then requires confirmation of the activity observed in the initial stages. At times, the biological activity may be lost as a result of fractionation and isolation. This usually indicates the synergistic effects of plant components to produce a desirable activity (Silvia and Fernandes, 2010). The phenomenon of synergism is widely applied since certain medicinal plants are used in combinations and there are reports on conventional antimicrobial drugs being used in combination with plant products to enhance the biological activity. However, combinations (synthetic and natural drug) tested *in vitro* may not necessarily show the same effects on humans due to several factors including the pharmacokinetics involved (Szalek *et al.*, 2006).

2.6. Phytochemistry

Research on plants start with the collection of the medicinal plants of interest. These are usually selected on the basis of their traditional uses in the treatment of illnesses, and ethnobotanical knowledge is very useful as it reveals how people interact with the plants and how these plants are used (Brusotti *et al.*, 2014). After the plants are collected, they are dried at room temperature and standardised laboratory methods of extraction, screening, separation and purification are used with the aim of isolation and identifying compounds with biological activities (Brusotti *et al.*, 2014).

Plants can be used fresh but are usually dried and ground into powder before use, the preference for dried plant material is that secondary metabolites in the plant are mostly produced as a result of the interaction of the plant with the environment and these can be affected by change in the environment (Vongsak *et al.*, 2013), weather changes, and also to reduce the enzymatic action, and to remove excess water in the plant. Grinding lowers particle size and increases surface contact between samples and extraction solvents thereby maximizing phytochemical extraction (Azwanida, 2015). While the whole plant may be used, the roots are mostly used in traditional medicine followed by tubers and rhizomes, generally underground plant parts are extensively used than the above ground parts (Das *et al.*, 2010).

2.7. Extraction techniques

There are various methods that are employed in extraction of phytochemicals. Solvents of varying polarities are used during extraction and the choice of a solvent

is influenced by compounds of interest (Tiwari *et al.*, 2011). Solvents should not be toxic, interfere with the bioassays, destructive to extracts and should evaporate easily. Traditional healers primarily use water to prepare their herbal remedies, however, organic solvents are used in laboratories to extract phytoconstituents since they are specific and help in efficient extraction of phytochemicals that are biologically active (Das *et al.*, 2010). During extraction, solvents of varying polarities are used depending on the compounds being targeted; hexane, chloroform, dichloromethane, acetone, ethanol and methanol are some of the solvents usually used, with preferences given to acetone due to low toxicity towards bioassays (Eloff, 1998). Extraction techniques include maceration, infusion, and decoction.

2.7.1. Maceration

Maceration technique involves pouring of the solvent onto the plant material at room temperature, the mixture is left soaked for a certain period or agitated for maximum extraction. This method can be used for thermolabile components of the plant (Ncube *et al.*, 2008).

2.7.2. Infusion

During infusion the plant material is usually poured with boiling solvent and allowed to stand a certain period of time (Tiwari *et al.*, 2011).

2.7.3. Decoction

This method is useful when the aim is to extract heat stable components. The powdered plant material is boiled with the solvent for a period of 15 minutes then cooled and filtered for further use (Tiwari *et al.*, 2011).

2.8. Separation techniques

Separation and identification of compounds with desirable properties is essential in studying of principles within the plant material (Blessy *et al.*, 2014). Chromatographic techniques have been widely employed to separate many biological molecules and chemical compounds (Sasidharan *et al.*, 2011; Blessy *et al.*, 2014; Armarego, 2017). These are analytical techniques employed in separating sample mixtures under the influence of mobile and stationary phases. There are various chromatographic techniques developed and can be both qualitative and quantitative (Blessy *et al.*, 2014). Selection of a specific technique is influenced by the aim of an experiment at hand, for example simple chromatographic techniques are usually aimed at analysis

for identification and quantification while preparative methods such as prep-TLC requires large samples and focus on isolation of pure compounds (Fanali *et al.*, 2017).

2.8.1. Thin layer chromatography

Thin Layer Chromatography (TLC) is a separation technique where mixture of compounds is separated by distribution between the stationary and mobile phases. This technique is a solid-liquid technique in which a solid is a stationary phase and a liquid phase is the mobile phase that allows separation of compounds with different solubilities. The solids used mostly are silica gel and aluminium oxide on a glass, metal or plastic plate while the mobile phase can be any solvents selected based on the experiment at-hand. The TLC technique involves spotting of the sample at one end of the plate coated with an adsorbent. The plate is then placed in a tank containing the mobile phase in which the solvent will rise up the plate by capillary action. The compounds will show varying affinities either to the mobile or stationery phase, hence compounds with high affinity to the mobile will move up the plate with the mobile phase while those with high affinity for the stationary phase will be immobilised at the bottom of the plate. This technique is inexpensive, simple and fast (Blessy *et al.*, 2014 and Thangaraj, 2016).

2.8.2. High performance thin layer chromatography

High performance thin layer chromatography (HPTLC) is a form of TLC that is enhanced such that different steps are automated, and this allows accurate measurements, proper application of the sample onto the plate and better resolutions (Bernard-Savary and Poole, 2015). The HPTLC offers more advantages over simple TLC, i.e. faster development times, reduced solvent consumption and high sample throughput since many samples can be analysed simultaneously (Bernard-Savary and Poole, 2015).

2.8.3. Column chromatography

Column chromatography is a separation and purification technique similar to TLC but differs in that it is a glass column into which a stationary adsorbent is packed and the mobile phase is poured with the sample on top (Robards *et al.*, 1994). The sample with the solvent is allowed to pass through the column and as the mixture flows down the column, the sample components are adsorbed to the stationary phase

while others pass through freely depending on their affinity for each of the two phases. Components of the mixture can be desorbed and eluted separately by adding more solvents in the column. Column chromatography can allow large scale and small-scale separation and has wide application in many disciplines such as biology and medicine (Giddings, 2002). In phytochemical studies column chromatography is used to isolate the compounds of interest, it can be those with antimicrobial or antioxidant activities amongst others (Harvey, 2008).

2.9. Antimicrobial screening methods

In vitro antimicrobial screening methods are common practice in laboratories for rapid analysis of potential drug candidates (Cragg and Newman, 2013). Selection of a specific method is influenced by the end results desired. Some methods are qualitative which give a simple answer of yes or no as to whether the extracts or compounds have antimicrobial properties, while other methods are quantitative giving the knowledge of how much of the extract/compound is able to inhibit or kill the test organism (Balouiri *et al.*, 2016). Various methods used are diffusion, dilution and bioautographic methods, these methods are used to accurately measure the antibacterial activity and also can be used for routine antibacterial susceptibility testing (Kenny *et al.*, 2015).

2.9.1. Bioautography

Bioautography is a screening method in microbiology that is used to detect the antimicrobial activity of the sample. It is usually coupled to chromatographic techniques such as HPTLC, simple TLC, and overpressured layer chromatography (OPLC) (Sherma, 2010 and Tyihák *et al.*, 2012). Three main bioautographic methods used are contact bioautography, immersion bioautography and direct bioautography. Among the three, the mostly used is direct bioautography (Masoko *et al.*, 2005; Suleiman *et al.*, 2010 and Choma and Grzelak, 2011).

2.9.2. Diffusion methods

There are several standardised diffusion methods used for antimicrobial testing and these methods are mostly used when testing the antimicrobial activity of pure and polar substances (Balouiri *et al.*, 2016). These techniques cannot distinguish between bacteriostatic, bactericidal activity, or be used in quantification of bioactivity, however, they are useful in identification of lead bioactive extracts or compounds

(Ncube *et al.*, 2008). Diffusion methods include disk diffusion, agar well diffusion and the cylinder method. The mostly used technique is the disk diffusion method which is performed by infusing sterile filter paper disks with the test sample, thereafter, the disks are placed on agar plates previously inoculated and incubated with the test organism. The antimicrobial agent diffuses into the agar inhibiting the growth of microorganisms. The plates with the disks are then incubated and zones of inhibition on the plates are observed and measured (Balouiri *et al.*, 2016).

2.9.3. Dilution methods

Antimicrobial dilution methods are aimed at determining the lowest concentration of the antimicrobial agent that inhibits visible growth of the test bacterium, this is termed minimum inhibitory concentration (MIC) that is usually expressed in milligrams per millilitre (mg/mL) or microgram per millilitre (µg/mL) (Klančnik *et al.*, 2010). These techniques are commonly used for antimicrobial agents that can kill or inhibit the growth of the bacteria (Ncube *et al.*, 2008). Antimicrobial susceptibility methods that involve dilution are microbroth dilution and agar dilution methods.

2.10. Phytochemical Structure elucidation

During drug discovery from plant material or any other source, the structure of compounds with biological activities are usually determined to enable further investigations, synthesis and mass production in industries (McChesney *et al.*, 2007). The chemical natures of these plants' constituents are demonstrated using the Nuclear Magnetic resonance and their molecular weight determined using mass spectrometer (Nicholson *et al.*, 2002). These are spectroscopic techniques with which the structures of different compounds are determined based on mass to charge ratio and individual hydrogen and carbon atoms within the structure (Wolfender *et al.*, 1998). Other techniques such as liquid chromatography coupled to mass spectrometer (LC-MS) and liquid chromatography coupled with ultraviolet radiation (LC-UV) can be used, however, these methods lack sensitivities during identification of compounds from natural products but can be used in the initial steps of screening of phytochemicals (Wolfender *et al.*, 1998).

2.11. Medicinal plant used in the study

The medicinal plant *Stomatostemma monteiroae* (Oliv.) N.E.Br (Figure 2.7) belongs to the family Apocynaceae, subfamily periplocaceae. This plant is widely spread in

Angola, Mozambique, Namibia, South Africa (Provinces of KwaZulu–Natal, Mpumalanga and Limpopo) and Zimbabwe. It is commonly known as matasa in Botswana and matamela in Mozambique and South Africa (Venter, 2008). The plant is a vigorous climber or a creeper, and uses its stems to coil around other plants, the roots are long as well as tuberous (Motlhanka *et al.*, 2008). It usually grows in association with plant species of *Sclerocarya* Hochst., *Commiphora Jacq.*, *Brachystegia Benth.*, *Acacia Mill.*, *Terminalia catappa* L., and *Combretum Loefl* and it is found in rocky places, mountains and hills (Venter *et al.*, 1989). It bares strongly scented flowers that are present from November to March and fruits are harvested while green and are used as relish in Botswana. The fruits are present from January till July (Venter and Verhoeven, 1993).

Figure 2.7: Leaves, Twigs and flowers (A) coiled plant (B) of *S. monteiroae* (http://www.zimbabweflora.co.zw/speciesdata/images/14/145310-2.jpg. Accessed on 05 May 2018).

The Apocynaceae family consist of vines, shrubs and tropical trees, and most of these plants species produce milky poisonous juice/milky latex (Venter *et al.*, 1989). The family has five sub-families which include Secamonoideae, Rauvolfioideae, Apocynoideae, Periplocoideae and Asclepiadoideae (Wong *et al.*, 2013). Plants of the Apocynaceae are known to contain steroidal compounds cardenolides and alkaloids that have antitumor and anticancer activity (Wen *et al.*, 2016). However, these compounds have been isolated from genus other than *Stomatostemma*. The genus *Stomatotemma* is only composed of two species *S. monteiroae* (Oliv.) N.E.

Br. and *S. pendulina* (Venter and Field, 1989). *Stomatotemma pendulina* is a shrubby non-succulent species and is only found in Northern Mozambique. The biological activities of both these plants have never been studied (Venter and Verhoeven, 1993).

2.12. Microbial diseases

Pathogenic microorganisms cause many diseases to humans and result in the damage of vital organs, loss of function and even death (Laxminarayan *et al.*, 2016). Viruses, fungi, and bacterial pathogens have for many years become a common cause of morbidity and mortality worldwide (Murray *et al.*, 2015). Microorganisms enter the human body at various sites and cause infection by reaching the target site, attaching and avoiding the immune system response. The site of entry can be respiratory tract, urogenital tract, gastrointestinal tract and through broken skin surfaces (Ribet and Cossart, 2015). The fight against microbial diseases has been the centre of medicine and considerable progress has been made as many drugs have been developed and are useful in treating many illnesses (Masoko and Nxumalo, 2013; Laxminarayan *et al.*, 2016; Komape *et al.*, 2017). Among others, bacterial infections have become the leading cause of death in South Africa and other African countries and inappropriate use of antibiotics have led to the development of antibiotic resistance resulting in low successful treatment rates (Ison and Heldman, 2018; Laxminarayan *et al.*, 2016).

2.13. Tuberculosis

Mycobacterium tuberculosis is an obligate human pathogen that belongs to the Mycobacteriaceae family and is the causative agent of the disease tuberculosis (TB) (Gomez and Mckinney, 2004), this disease may also be caused by *Mycobacterium bovis* and *Mycobacterium africanum* in rare cases. Other species within the Mycobacteriaceae family that have also been noted to be pathogenic to humans include two skin disease causing *Mycobacterium leprae* and *Mycobacterium. ulcerans* (Rastogi *et al.*, 2001). Within the genus Mycobacterium, 85 species are well known, some of these species cause diseases in animals and human while others are ubiquitous in nature (Okunade *et al.*, 2004). *Mycobacterium tuberculosis* bacillus contains the lipid mycolic acid on the cell wall which makes it to be able to survive harsh conditions such as weak disinfectants and dry areas (Ducati *et al.*, 2006). Mycolic acids are long fatty acids chains attached to various functional groups and

the carbon composition differs from species to species. This is the characteristic that makes *M. tuberculosis* to be resistant to most anti-TB medications (Mmushi, 2011).

Unlike other virulent obligate pathogens of Mycobacteria, *Mycobacterium smegmatis* is generally non-pathogenic, acid-fast bacterial species of phylum Actinobacteria and genus Mycobacterium (Jethva *et al.*, 2016). It is a fast-growing bacterium that is usually found in soil and water. It is non-motile and shares many features such as genomic sequences with the pathogenic *M. tuberculosis. Mycobacterium smegmatis* is an environmental microorganism; it can tolerate more diverse conditions than its pathogenic counterparts (Jethva *et al.*, 2016). Although *M. smegmatis* may cause disease in immune-compromised animals, it does not cause tuberculosis (Cayabyab *et al.*, 2006). This makes *M. smegmatis* a potential candidate to study other pathogenic mycobacterial species in laboratory conditions, thus *M. smegmatis* was used as a test organism in this study.

Mycobacterium tuberculosis is slow growing aerobic bacterium that appears as creamy white colonies when cultured (Figure 2.8 A) and can only be distinguished or identified using tests such as acid-fast stain (Figure 2.8 B). During infection it primarily affects the lungs (WHO, 2010). Upon infection, an individual can have latent or active TB. Latent TB occurs when the infected individual shows no symptoms of infection thus the pathogen is limited to its initial site of infection. Latency is achieved when there is early restriction of *M. tuberculosis'* growth in the lungs prior to the onset of the TB disease due to vigorous immune response of the infected persons which arrest the progression of the infection (Rastogi *et al.*, 2001). However, treatment is necessary to completely remove the pathogen, if not treated after some time the infection can be activated and cause an active TB disease (Pfyffer, 2015). Most cases of TB are thought to be reactivation of the latent infection that was never treated. Studies confirm that 5 to 15% of active TB cases are results of latent infection and are even more in immunocompromised individuals (Collins *et al.*, 2014; Getahun *et al.*, 2015; WHO, 2015).
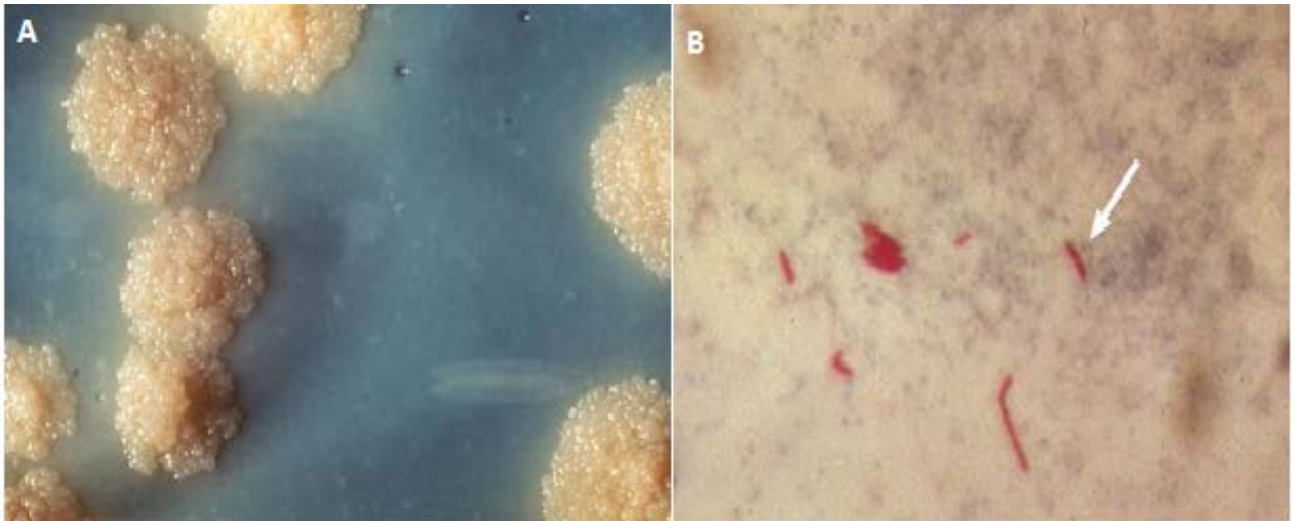
Figure 2.8: *Mycobacterium tuberculosis* colonies (A) and *M. tuberculosis* acid fast stain (B), http://textbookofbacteriology.net/tuberculosis.html. Accessed on 20 April 2017.

Active TB is the opposite of latent infection, and the symptoms of the infection such as unintentional rapid weight loss, coughing for more than two/three weeks, night sweat, fever, coughing out blood, loss of appetite, chest pains and fatigue are apparent (WHO, 2015). In this case the bacterium is able to avoid elimination from the host despite the vigorous immune response towards the infection and the infected person is contagious, usually before the treatment starts and after two weeks of proper treatment the person is no longer contagious (WHO, 2013). Tuberculosis can spread from one person to the next by inhalation of the bacterium. Usually when an infected individual breathes, speaks or sing they can release the bacilli droplets into the air and close people that are exposed can be infected (Collins *et al.*, 2014).

Treatment with anti-TB medications results in a rapid decline in the number of bacilli which then reduces the chances of one spreading the disease to others (Hoppentocht *et al.*, 2014). The manifestation of the disease is largely influenced by the status of the immune system of the individual, pregnant woman, patients with chronic illnesses such as HIV, diabetes, active smokers, just to mention a few, are at a greater risk of developing active TB disease than those without the above mentioned factors (Marais *et al.*, 2013). This is because most diseases weaken the immune system of an individual making the patient vulnerable to many infections.

For example, persons with HIV/AIDS develop TB as an opportunistic infection (Sutariya *et al.*, 2015).

Tuberculosis continues to be a major public health threat and has been noted to be one of the largest cause of mortality worldwide (Cazabon *et al.*, 2017). For many years TB has been associated with humankind, with an estimation of close to one billion deaths having occurred during the last 200 years (Paulson, 2013). In 2015 WHO reported that up to 1.4 million people died due to TB and 10.4 million new cases of active TB were documented, these distressing statistics reveals the true devastating nature of TB. This disease is regarded as "the disease of poverty" due to large number of incidences in rural areas i.e. more than 80% of TB cases occurring in Africa and Asia (McGaw *et al.*, 2008). The incidence of TB is declining very slowly especially in high burden countries where there are higher rates of drug resistance and transmission of the disease, making the End TB strategy of World Health Assembly very unlikely to happen. The End TB Strategy proposed that by the year 2030, they have a target of ending the epidemic of global TB, Which is set to be achieved when 90% of deaths related to TB are reduced and the incidence of active TB is reduced by 80% (WHO, 2016). High burdened countries include India, South Africa, Indonesia, Pakistan, Nigeria and China; these countries contributed 60% of all TB cases reported in 2015 by WHO. With multidrug resistance (MDR) TB and co-infection with HIV contributing 50% of 1. 4 million deaths reported in 2015. As of 2017 10 million people developed TB disease and 1.4 million people died from TB, with 80% of TB cases occurring from sub-Saharan Africa (WHO, 2018)

2.14. Clinical manifestation of TB

The disease TB is primarily a pulmonary disease affecting the lungs of an individual. However, it can affect other parts of the body and is generally referred to as the extrapulmonary (EPTB) disease with a few cases of the disseminated TB (Venturini *et al.*, 2014). Extrapulmonary-TB is the manifestation of TB disease on any site of the body other than the lungs and the disseminated (milliary) form is when the bacteria spread throughout the body (Golden and Vikram, 2005). The most commonly infected sites include the central nervous system (CNS) and meninges, bones and joints, gastrointestinal tract, genitourinary system, pleura and lymph nodes (Sandgren *et al.*, 2013). Although EPTB accounts less on the transmission of the disease, it still contributes to the morbidity and mortality as a result of TB

infection. According to WHO, (2013), of the 5.8 million new cases reported, 0.8 million had EPTB. Individuals who are co-infected with HIV (Human Immunodeficiency Virus) or any other chronic illness appear to have a higher chance of suffering from EPTB than immunocompetent persons. This usually happens when the CD4 count of the infected individuals fall below 100 cells µl/mL, and the diagnosis of extrapulmonary TB is challenging since non-specific signs and symptoms may be observed (Naing *et al*., 2013).

2.14.1. Tuberculosis of the bones and spine

Infection of the spine with *M. tuberculosis* may be characterised by vertebral spondylitis (inflammation of the vertebra), bone and soft tissue swelling and formation of sinus. Bones may be destructed leading to kyphosis and vertebral collapse, also there is spinal tissue granulation and the infected individual experiences progressive back pain. For proper diagnosis, magnetic resonance (MR) imaging is required (Pigrau-Serrallach and Rodríguez-Pardo, 2013).

2.14.2. Pleural TB

The pleura is a membrane that covers the inside of the chest wall outside the lungs and the surface of the lungs (Gupta *et al*., 2015). The pleural TB is characterised by hypersensitive reaction that generates a local effusion due to direct haematogenous invasion of the pleural region. The effusion develops within few weeks of infection and the disease can represent a primary or reactivation of a previous infection. The symptoms of pleural TB are anorexia, chest pains, shortness of breath and fever. To diagnose pleural TB an examination of the pleural tissue is the most sensitive method because other physical findings can mimic other diseases such as pneumonia (Gupta *et al*., 2015).

2.14.3. Abdominal TB

Abdominal tuberculosis can be acquired in two ways, as a spread from pulmonary disease or the bacilli can be ingested with infected milk (Debi *et al*., 2014). Abdominal TB can be found in several forms, including gastrointestinal (GI) tuberculosis, lymphadenopathy tuberculosis, visceral tuberculosis and peritoneal tuberculosis (Sharma and Bhatia, 2004). General clinical features of abdominal TB are alterations in the bowel movement, loss of weight and appetite, and pain in the abdominal area. Abdominal TB can be managed with normal anti-tubercular

medicines provided that early diagnosis is achieved, however, surgery may be necessary in cases where there's intestinal obstruction, abscess or perforation that cannot be resolved (Piccini *et al.*, 2014).

2.14.4. Meningitis TB

Meningitis TB is a common form of central nervous system (CNS) TB and less likely progress as tuberculous brain abscess, intracranial tuberculoma or tubercular encephalitis (Rock *et al.*, 2008). It is usually due to a haematogenous spread but can also be as a result of the cerebrospinal fluid infection (Marx and Chan, 2011). The disease begins with the development of small tuberculous foci, the Rich foci in the meninges brain, or the spinal cord. The infected individual experiences headache, fever, alterations in the consciousness and meningismus or stiff neck. This is the most devastating form of TB and early diagnosis and treatment may help prevent death (Rock *et al.*, 2008).

2.15. Diagnosis of TB

Similarly to any disease, symptoms are an important tool for the diagnosis of TB. The presence of TB disease can be characterised by a cough that lasts for up to three weeks, hemoptysis, chest pains, fatigue, night sweats and fever (Steingart *et al*, 2013). These symptoms may also depend on the infected area of the body. There are many diagnostic techniques for TB, however, most of them only indicate the presence of an infection which further requires other confirmatory tests to rule out false diagnosis. The first step to determine if an individual has an infection, the TB skin test also called Mantoux tuberculin skin test (TST) or TB blood test can be done (Jaiswal *et al.*, 2013). Other useful factors in determining the TB infection include medical history, and demographic factors such as country of origin, ethnic group, age and occupation that may increase risks of acquiring TB infection. Proper diagnosis is important for reduction of death associated with TB, to prevent the spread of TB and improvement of health of the patients (Steingart *et al.*, 2013).

2.15.1. Diagnostic Microbiology

Microscopic evaluation of a sputum smear is a first step in microbiological diagnosis of TB, the presence of acid fast bacilli when examined under the microscope usually indicates the presence of a TB disease. However, microbial culturing of samples and identification of the organisms should be done to confirm TB disease because other

acid fast bacilli which are not *M. tuberculosis* may be present. This test requires careful examination of cultures before starting the treatment in order to rule out reporting false results since the *M. tuberculosis* grows slowly and may be contaminated with other microorganisms over the period of incubation (Lee, 2015).

2.15.2. TB blood tests

Tuberculosis blood tests also known as interferon-gamma release assays (IGRAs) used are QuantiFERON–TB Gold In-Tube test (QFT-GIT) and the T-SPOT-TB test (T-Spot) (Bae *et al.*, 2016). When IGRAs are conducted they measure an immune response of an individual to *M. tuberculosis*, blood samples are mixed with antigens that have been derived from TB causing bacteria and white blood cells of an infected individual releases interferon-gamma (IFN-g) then the concentrations of IFN-g is measured, this is true for QFT-GIT, T-spot test differs from QFT-GIT in that it enumerates interferon producing cells (Mazurek *et al.*, 2010).

2.15.3. Chest radiograph

This technique is used to check for any abnormalities in the chest. It is an x-ray examination procedure which produces images of the lungs, spine and bones when exposed to a small dose of ionizing radiation (Mandalakas and DiNardo, 2016). Lesions may be visible on the produced image, this may suggest the possibilities of having pulmonary TB, however, this technique may be used as a follow up method after TST or TB blood test was done and not as a definite diagnostic method. This is because patients may have a positive test of TST or TB blood test whilst symptoms of TB are absent (Mandalakas and DiNardo, 2016).

2.15.4. TB skin test

The TST is performed by injecting a tuberculin fluid or a purified protein derivative (PDP) into the skin on the lower part of the arm. The results of TST are examined after 24 to 72 hours, where a trained health care worker looks for a reaction on the area of injection and positive results are indicated by the presence of a swelling, hardness and size of the raised area. However, this results only indicate whether the person is infected or not, other tests must be done to determine if the person has active TB disease or a latent infection (Mandalakas and DiNardo, 2016).

2.15.5. Gene Xpert

Most of diagnostic methods are only able to detect the *M. tuberculosis* bacterial infection. A breakthrough was achieved when an effective and rapid technique (lasting for a period of 2 hours) to detect the presence of *M. tuberculosis* infection and drug resistance was introduced (WHO, 2015). Gene Xpert also called Xpert M. *tuberculosis*/rifampicin (MTB/RIF) allows detection of drug resistance strains of *M. tuberculosis* (resistance to Rifampicin) (WHO, 2015). Gene Xpert is an automated molecular based technique which detects *M. tuberculosis* in a sputum sample. The genome of *M. tuberculosis* is detected, isolated and amplified by polymerase chain reaction (PCR). This technique also detects mutations in the nucleic acid that makes the *M. tuberculosis* to be resistance to Rifampicin (WHO, 2010). Drug resistance to the first line drug Rifampicin can occur alone, however, it might indicate that the patient has multi-drug resistance (MDR) TB especially in areas where MDRTB is prevalent which allows specificity on the proceedings as to which treatment the patient should be given (Steingart *et al.*, 2013). After Xpert MTB/RIF is done, drug susceptibility test and culturing must be done to track the treatment progress and also to check if the strain of *M. tuberculosis* at-hand is resistant to other drugs (WHO, 2010).

2.16. The treatment of TB

The treatment of TB is aimed at curing the disease, prevention of transmission, avert relapse of the disease, preventing activation of latent infection and preventing the development of drug resistance (WHO, 2010). In order to treat TB, multiple drugs are used, this is because each drug plays a specific role in killing and elimination of the tubercle bacilli (Dartois, 2014). There are many drugs used for the treatment of TB and their prescription depend on circumstances such as whether it is a newly infected individual, a drug susceptible or a case of drug resistance, age of an individual, just to mention a few (WHO, 2013). Drugs employed in the treatment of TB can be first line drugs, second line drugs which are taken orally or injectable drugs. First line drugs are the most effective with fewer side effects, however, second line and injectables become necessary in situations where the patient is co-infected with HIV or immunocompromised by other diseases and these drugs have much more adverse effects (Pham *et al.*, 2015).

The treatment regimen of TB is generally referred to as directly observed therapy short-course (DOTS) which is divided into two phases, the intensive phase of 2 months and continuation phase of up to 7 months, these phases involve the use of various medications (Churchyard *et al.*, 2014). Isoniazid, Rifampicin, Ethambutol and Pyrazinamide are administered all together for the first two months and on the second phase only Isoniazid and Rifampin are administered. However, this is applicable for drug susceptible TB, and where there is drug resistance or a relapse, fluoroquinolones, and injectables such as Amikacin or Capreomycin are prescribed for a period of atleast 20 months (Falzon *et al.*, 2017). The latent TB is treated with a single drug, Isoniazid for a period of 9 months and can take up to 36 months in individuals who are co-infected with HIV/AIDS especially to those residing in areas with high prevalence of TB (Churchyard *et al.*, 2014).

2.17. The vaccine of TB

Bacille Calmette-Guérin (BCG) is a live attenuated TB vaccine that was developed in 1921 (Soumya and Banu, 2010). It is prepared from a weakened microorganism that is closely related to *M. tuberculosis*, the *M. bovis* (Soumya and Banu, 2010). The vaccine BCG is given to infants after birth and provides protection from severe forms of TB. However, BCG does not offer protection against EPTB and adult pulmonary TB and it is mostly administered in countries with high prevalence of TB (Soumya and Banu, 2010). Although there are many vaccine candidates still in trial, TB in all age groups especially in developing countries remains a serious threat and the second major cause of death (Dagg *et al.*, 2014).

2.18. Challenges in the treatment of tuberculosis

The success of a TB treatment programme is largely influenced by the patient and the physician involved (Dagg *et al.*, 2014). Prescription of proper medication to the TB disease involved as well as cooperation and adherence of the patient to taking the medication properly has a major impact on the success of eliminating tuberculosis disease (Iribarren *et al.*, 2014). Although TB is a disease that can be completely cured, there are various challenges that determine the outcomes of the treatment. The type of TB case involved, the lack of access to quality medication, and limitations to diagnostic methods in developing countries (Iribarren *et al.*, 2014).

2.18.1. Drug resistance

One of the complicating factors in the treatment of tuberculosis is the emergence of drug resistant strains of *M. tuberculosis* (McGrath *et al.*, 2014). Drug resistance or antimicrobial resistance is the ability of the bacteria to grow and multiply in the presence of an antimicrobial agent (Brauner *et al.*, 2016). Drug resistant TB emerge as a result of wrong prescription of the drug, length or dose of the treatment and incomplete course of the treatment which inadequately expose the bacteria to the drug, thus it undergoes mutation which enables it to tolerate the drug (WHO, 2008).

There are two clinically relevant types of drug resistance TB and they differ in the types of drug to which they are resistance to. Multi-drug-resistance (MDR) TB is when the bacteria become resistant to the most potent first line drugs Isoniazid and Rifampicin (Bastos *et al.*, 2014). While extensively drug resistance (XDR) TB is when the bacteria exert resistance to both Isoniazid and Rifampicin, one (atleast) fluoroquinolone and one of the injectable second-line drugs (Bastos *et al.*, 2014). Another type of drug resistant TB strains has been detected and is usually referred to as extremely drug resistant TB or totally resistant TB (XXDR-TB or TDR-TB), this type is extremely difficult to treat with the current treatment regimens and longer periods of treatment are associated with a greater deal of side effects (Chaudhry *et al.*, 2015).

Drug resistant TB has proven to be a challenge during the course of treatment (WHO, 2016). Due to drug resistant only second line drugs and fluoroquinolones can be used and with XDR surgery may be necessary to remove localized lesions, these drugs are more toxic, expensive and require longer periods of treatment (Caminero *et al.*, 2010). Developing countries are characterised by over-crowded settings, poor health care facilities and malnutrition which even more poses a threat in global TB control and eradication (Chaudhry *et al.*, 2015).

2.18.2. Adverse effects associated with anti-TB medication

Although antiTB medication is available in most countries, it is associated with side effects that usually determine the outcomes of the treatment (Sheng *et al.*, 2014). These side effects reduce the patient acquiescence to the treatment programme, which leads to increased rates of TB recurrence and increased mortality (Sheng *et al.*, 2014). Adverse effects of antiTB drugs mostly experience include neurological

disorders, gastrointestinal disorders, arthralgia, skin reactions and hepatotoxicity (which is the most serious and can be fatal if not addressed early). AntiTB drug-induced hepatotoxicity has been observed to be between 2 – 28% of treated TB cases especially in multidrug resistance cases. This requires discontinuation of the one or more of the drugs used in treatment programme and interruption in the treatment course may result in hospitalization, disability or even death (Lv *et al.*, 2013).

Purpose of the study

 Aim

 The aim of this study was to screen, isolate and characterise antimycobacterial compounds from *Stomatostemma monteiroae*.

Objectives

The specific objectives of the study were to:

i. Perform qualitative phytochemical analysis (extraction and screening) and quantitative phytochemical constituents' analysis.

ii. Determine the presence of antioxidant compounds in the *S. monteiroae* using qualitative antioxidant (2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay) and quantitative antioxidant activity using ferric reducing power assay.

iii. Evaluate the antimycobacterial activity of crude extracts of *S. monteiroae* using minimum inhibitory concentration assay and bioautography.

iv. Isolate bioactive compounds with antimycobacterial activity using column chromatography and preparative TLC.

v. Analyse the effects of the crude extracts and isolated compounds for cytotoxicity on Vero monkey kidney cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

vi. Determine the structure of the isolated compounds using spectroscopic methods such as Nuclear Magnetic Resonance.

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CHAPTER 3

3. EXTRACTION AND PHYTOCHEMICAL SCREENING

3.1. Introduction

Plants have paramount importance in many industries and offer a treasure house of diversity of potential drug candidates (Radulovic *et al.*, 2013). Since antiquity plants have been used to treat diseases. In recent years there has been an increase in awareness of the importance of medicinal plants. People who reside in rural settlements largely depend on traditional medicine (Aleebrahim-Dehkordy *et al.*, 2017). This is because medicinal plants rarely have side effects, are less expensive and are easily accessible compared to modern synthetic drugs (Yadav and Aragwala, 2011).

In traditional health care systems water is used in the preparation of herbal remedies. However, studies have shown that water may not extract the required substances with healing properties (Das *et al.*, 2010). Hence phytochemical studies are very important since they are specific and allow extraction and isolation of the biologically active entities (Yamaji *et al.*, 2005). Scientific investigations have shown that for consistent phytochemical extraction and good antimicrobial activity organic solvents are better than water (Harmala *et al.*, 1992). Das *et al* (2010) reported that water soluble flavonoids have no antimicrobial activity and also phenols that are soluble in water only possess antioxidant activity. Standardised procedures of extraction are used with selective solvents to efficiently extract metabolites of interest. The intended uses of the extracts facilitate the choice of the solvent. When the extractant is aimed at antimicrobial assays solvent with minimal toxicity must be used (Eloff, 1998).

The selected plants for the study are usually screened for possible phytoconstituents within the plant (Atanasov *et al.*, 2015). Standardised tests that used to determine the presence of certain phytochemical constituents have been developed and secondary metabolites such as saponins, alkaloids, terpenoids, glycosides and phenols can be tested by extracting and allowing them to react with certain chemical components or reagents, which by change of colour or foam formation indicates the presence of certain phytoconstituents (Tiwari *et al.*, 2011). The process of extraction

and screening is preliminary to the isolation of bioactive compounds. Testing for phytoconstituents helps in identifying the possible lead compounds within the plant (Wang *et al.*, 2010 and Tiwari *et al.*, 2011).

3.2. Materials and methods

3.2.1. Plant collection

The plant materials were collected from Ga-Madiga village, in the Limpopo Province of South Africa and the study was conducted at the University of Limpopo, Department of Biochemistry, Microbiology and Biotechnology. The plant material was taken to the Larry Leach Herbarium, University of Limpopo for identification and specimen deposit purposes. The plant material was identified by the curator of Larry Leach Herbarium Dr E. Bronwyn (Voucher specimen number UNIN121001). The plant materials were washed and dried at ambient temperature in the dark. The leaves, twigs, roots, tuber and tuber peel of the plant were separated and ground to fine powder using a warring blender and stored in an air tight container in the dark until use.

3.2.2. Extraction procedures

3.2.2.1. Preliminary extraction procedure

Preliminary phytochemical extraction was done by suspending a mass of 1 g of each plant part in 10 mL of solvents of varying polarities namely: hexane, dichloromethane, acetone and methanol. The mixture was agitated for 30 minutes in a shaker incubator (New Brunswick Scientific Co., Inc.) at a speed of 200 rpm. The extracts were filtered using cotton wool into pre-weighed vials and dried at room temperature under a stream of cold air; thereafter, the mass yield of extracts was determined for each plant part and solvent used. The extracts were reconstituted in acetone to a concentration of 10 mg/mL (Kotze *et al.*, 2002).

3.2.2.3. Extraction enrichment procedures

The leaves extracts were chosen for further studies based on preliminary phytochemical and antimycobacterial activities observed. Two enrichment procedures were performed; the first treatment was performed by pre-treating finely ground powdered leaves of *S. monteiroae* with hexane (defatting) followed by extraction of pre-treated plant materials with acetone and ethanol. The second

procedure involved the use of different percentages of acetone and ethanol in water. A mass of 1 g leaves of *S. monteiroae* was extracted with 10 mL of each of the following: acetone, ethanol, water and 20 %, 40%, 60%, and 80% of acetone and ethanol in water. The tubes were shaken for 10 minutes in a shaker incubator and filtered using cotton wool into pre-weighed vials and dried at room temperature under a stream of cold air and the extracted masses were quantified

3.2.2.4. Preliminary screening of phytochemical compounds

The extracts were analysed for their phytochemical profile using thin layer chromatography on aluminium-backed TLC plates (Fluka, silica gel F_{254}). A volume of 10 µL of each extract was loaded on TLC plates and developed in three mobile phases of varying polarities: benzene/ethanol/ammonia hydroxide (BEA) (non-polar/basic) 9:1:0.1, chloroform/ethyl acetate/formic acid (CEF) (intermediate polarity/acidic) (5:4:1), and ethyl acetate/methanol/water (EMW) (polar/neutral) (10:1.35:1) as described by Kotze and Eloff (2002). The separated compounds were viewed under ultraviolet light (UV) at a wavelength of 365 nm for fluorescing compounds and thereafter the TLC plates were sprayed with vanillin-sulphuric acid reagent prepared by dissolving 0.1 g of vanillin in 28 mL methanol and 1 mL of concentrated sulphuric acid. The plates were then heated at 110 ºC for optimal colour development.

3.2.3. Testing for phytoconstituents

The presence of various phytoconstituents such as tannins, saponins, alkaloids, cardiac glycosides, phlobatannins, steroids, reducing sugars, terpenoids, anthraquinones and flavonoids within the plant were determined.

3.2.3.1. Reducing sugars

Fehling's test was used to test for the presence of reducing sugars in the plant extracts. Fehling A was prepared by dissolving 7 g of hydrated copper (II) sulphate into 100 mL of distilled water and Fehling B by dissolving 35 g of potassium sodium tartrate and 10 g of sodium hydroxide in 100 mL of distilled water, equal volumes of Fehling A and B were mixed to form a deep blue solution. The aqueous extract made by dissolving 0.5 g of powdered plant parts in 5 mL of water and filtered was added

to a boiling Fehling's solution. Development of an orange-red precipitate was indicated positive results (Ayoola *et al.*, 2008).

3.2.3.2. Cardiac glycoside

The Keller- Killiani test was employed to test for cardiac glycosides by weighing 0.5 g of the ethanol extract of each plant part of *S. monteiroae* and dilute to 5 mL of water. The diluted plant extracts were mixed with of 2 mL of glacial acetic acid followed by a drop of 0.1% of ferric chloride. One mL of concentrated H_2SO_4 was then added to the mixture and the formation of a brown ring at the interface indicated the presence of cardiac glycosides (Borokini and Omotayo, 2012).

3.2.3.3. Phlobatannins

The presence of phlobatannins was tested by weighing 0.2 g of each plant part of *S. monteiroae* dissolved in 10 mL of distilled water and filtered. Hydrochloric acid (HCl) (2%) was added to the filtrate and boiled, and the formation of a red coloured precipitate indicated the presence of phlobatannins (Borokini and Omotayo, 2012).

3.2.3.4. Steroids

The presence of steroids was tested by adding 2 mL acetic anhydride to ethanol plant extracts. Thereafter 2 mL of absolute H_2SO_4 was added and blue green colour change confirms the presence of steroids in the plant extracts (Borokini and Omotayo, 2012).

3.2.3.5. Terpenoids

Salkowski test was used to test for the terpernoids. A mass of 0.5 g of each of the ethanol extracts was dissolved in 2 mL of chloroform. Then 3 mL of H_2SO_4 was carefully added to form a layer. A reddish-brown colouration at the interface indicated the presence of terpernoids (Borokini and Omotayo, 2012).

3.2.3.6. Anthraquinones

The plant material was boiled with 10 mL of 97% sulphuric acid (H_2SO_4) filtered while hot. The filtrate was shaken with 5 mL of chloroform. The chloroform layer that was formed was transferred to another test tube to which 1 mL of dilute ammonia was added. A pink colour formation indicated positive results (Ayoola *et al.*, 2008).

3.2.3.7. Saponins

The presence of saponins was determined by weighing 0.5 g of powdered plant material and dissolved in 5 mL of distilled water. The mixture was shaken and heated at 100 ºC. Persistent froth formation was observed and indicated the presence of saponins (Odebiyi and Sofowora, 1978).

3.2.3.8. Alkaloids

Dragendorff's test was used to screen for alkaloids. The plant materials were extracted using ethanol (95%) then evaporated to dryness. The plant residues were dissolved in 5 mL of 1% hydrochloric acid (HCl) and then treated with Dragendorff's reagent (Potassium Bismuth Iodide solution). Formation of red precipitates indicated the presence of alkaloids (Harborne, 1973).

3.2.3.9. Flavonoids

A method described by Borokini and Omatayo (2012) was employed to determine the presence of flavonoids in the plant extracts. Aqueous extracts of each plant part of *S. monteiroae* was mixed with 5 mL dilute ammonia solution, followed by addition of 1 mL of concentrated H_2SO_4. The formation of a yellow colour that fades with time indicated the presence of flavonoids.

3.2.3.10. Tannins

The presence of tannins was determined by weighing 0.5 g of each plant material and boiled in 5 mL of distilled water then filtered. A few drops of 0.1% ferric chloride was added to the filtrate and the observed brownish green or a blue-black colouration indicated the presence of tannins (Trease and Evans, 1989).

3.2.4. Phytoconstituents quantification

3.2.4.1. Total phenol content

The concentration of phenolic content in 70% aqueous acetone extracts of the selected plant parts was determined using spectrophotometric method described by Singleton *et al* (1999) with modifications. The determination of the total phenol content employed the Folin-Ciocalteau method, where 0.1 mL of extract and 0.9 mL of distilled water were mixed in a test tube. To this mixture, 0.1 mL of Folin-

Ciocalteau phenol reagent was added and the mixture shaken well. One milliliter of 7% sodium carbonate (Na_2CO_3) solution was added to the mixture after 5 minutes. The volume was made up to 2.5 mL with distilled water. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared as described above. The mixtures were incubated for 90 minutes at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with a UV/visible spectrophotometer (Beckman Coulter-DU730). The total phenol content was expressed as mg of gallic acid equivalents (GAE) per g of the extract and the total phenolic content was determined by the linear regression formula from a gallic acid calibration standard curve (Singleton *et al.*, 1999).

3.2.4.2. Total tannin content

The tannin content was determined using Folin-Ciocalteau method. About 0.1 mL of the 70% aqueous acetone extracts of the selected plant parts was added to a 25-mL volumetric flask with 5 mL of distilled water. To this mixture, 0.2 mL of 2 M Folin-Ciocalteau phenol reagent and 1 mL of 35% Na_2CO_3 solution was added and this was made up to 10 mL with distilled water. The mixture was shaken well and kept at room temperature for 30 minutes. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared in the same manner as described above. Absorbance for test samples and standard solutions were measured against the blank at 725 nm with a UV/visible spectrophotometer (Beckman Coulter-DU730). The tannin content was expressed as mg of GAE/g of extract (Singleton *et al.*, 1999). The tannin content was determined by the linear regression formula from a gallic acid standard calibration curve.

3.2.4.3. Total flavonoid content

Total flavonoid content was determined by the aluminium chloride colorimetric assay. One millilitre of 70% aqueous acetone extracts of the selected plants was mixed with 4 mL of distilled water in a 25 mL volumetric flask. To the flask, 0.30 mL of 5% sodium nitrite was added. About 0.3 mL of 10% aluminium chloride was added to the mixture after 5 minutes and mixed. After 5 minutes, 2 mL of 1 M Sodium hydroxide was added and this was made up to 10 mL with distilled water. A set of reference standard solutions of quercetin (0.0625, 0.125, 0.25, 0.5 and 1 mg/mL) were prepared in the same manner as described above. The absorbance for test and

standard solutions were determined against the reagent blank at 510 nm with a UV/visible spectrophotometer (Beckman Coulter-DU730). The total flavonoid content was expressed as mg of quercetin equivalents (QE) per gram of extract and total flavonoid content was determined by the linear regression formula from a quercetin calibration standard curve (Har and Intan, 2012).

3.3. Results

3.3.1. Preliminary phytochemical extraction

Five plant parts where extracted using 4 different solvents of varying polarities and the mass extracted is indicated in milligrams (mg) (Figure 3.1). Methanol was the best extractant with the highest mass of 56 mg in the leaves extract and hexane extract of peels had the lowest mass extracted (1.3 mg).

3.3.2. Phytochemical screening

Thin layer chromatography was used to analyse the phytochemical composition of different plant parts (Figure 3.2), all the extracts were developed in different solvent systems (BEA, CEF and EMW). Vanillin reactive bands appeared as purple in colour (Figure 3.2 B), while under UV different colours where observed (Figure 3.2 A). More bands were observed in BEA solvent system followed by CEF and EMW had the least number of bands.
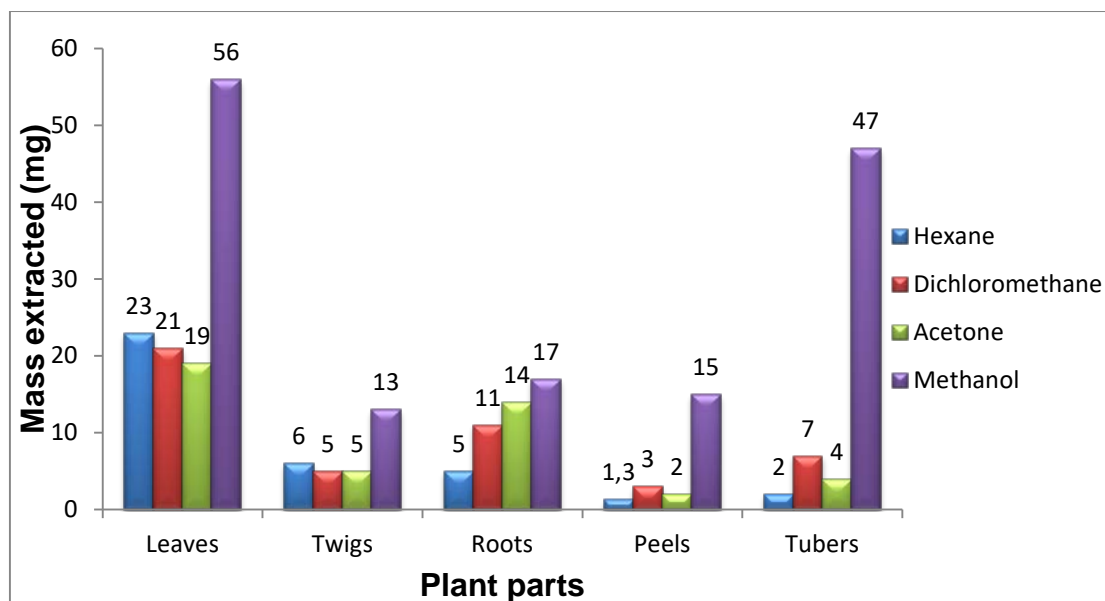
Figure 3.1: The mass extracted from different plant parts of *S. monteiroae* in mg using solvents namely Hexane, Dichloromethane, Acetone and Methanol (non-polar to polar).
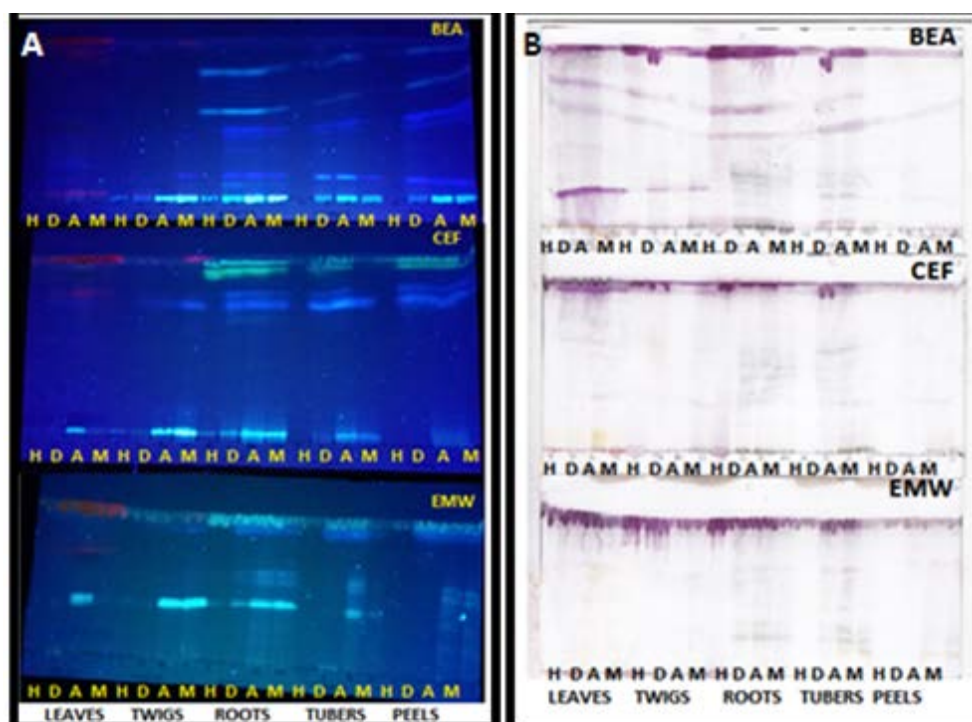
Figure 3.2: TLC profile of the different plant parts of *S. monteiroae* developed in solvent systems of varying polarities. The plates were first viewed under UV (A) and sprayed with vanillin-sulphuric acid (B). Key: H= hexane, D= dichloromethane, A= acetone and M= methanol.

3.3.3. Extract enrichment procedures

3.3.3.1. Hexane wash (defatted)

The plant material was defatted with hexane followed by subsequent extraction with ethanol and acetone of the same plant material and the resultant masses are shown in Figure 3.3. Ethanol was the best extractant with a mass of 63 mg and both hexane for ethanol and hexane for acetone extracted the least.
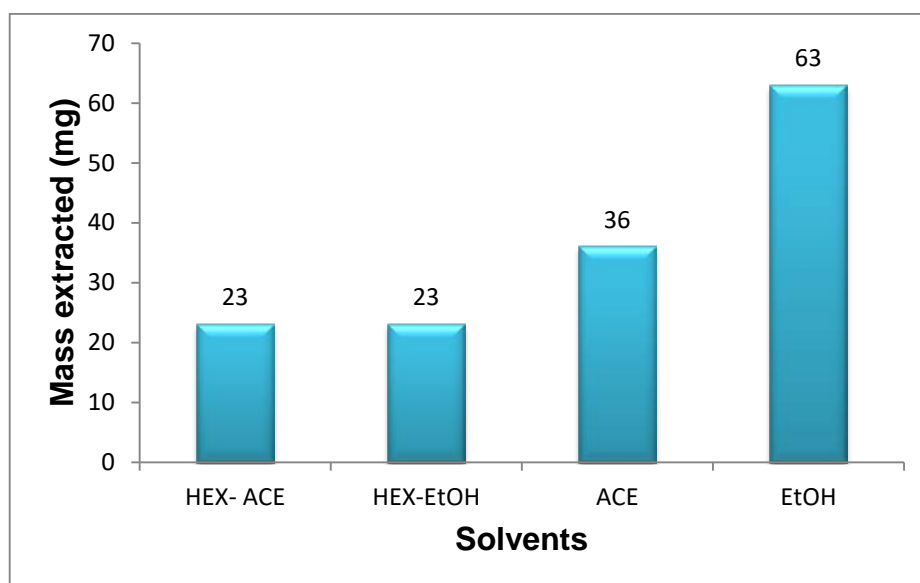
Figure 3.3: Mass extracted from the leaves of *S. monteiroae* with solvents of different polarities: hexane for acetone (HEX-ACE), hexane for ethanol (HEX-EtOH), acetone (ACE) and ethanol (EtOH).

3.3.3.2. Extract enrichment procedures

The defatted extracts were loaded on TLC plates and developed in BEA, CEF and EMW mobile phases in order to analyse phytochemical components of the extracts. The separated compounds were then viewed under UV light to check for fluorescing compounds and later sprayed with vanillin-sulphuric acid reagent for optimal colour development. The separated compounds had few UV reactive compounds (Figure 3.4 A). More compounds were observed after spraying with the vanillin-sulphuric acid reagent. Among the three solvent systems used, more bands were observed in BEA followed by CEF and EMW had the least (Figure 3.4 B).
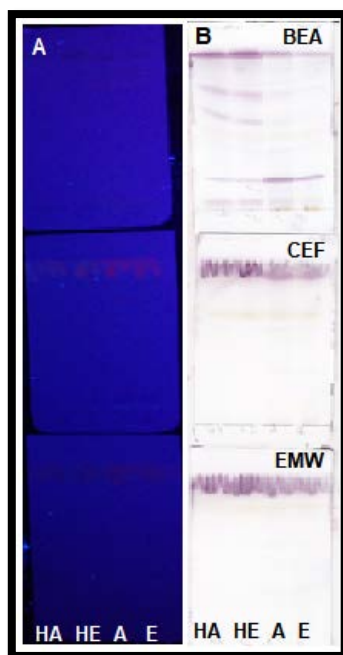
Figure 3.4: The TLC fingerprint of extracts of the leaves of *S. monteiroae* after hexane wash, developed in three eluent systems (BEA, CEF and EMW) and viewed under UV at 365 nm (A) and sprayed with vanillin-sulphuric acid reagent (B).

The leaves of *S. monteiroae* were extracted with solvents of varying polarities and percentages (Figure 3.5). Extracting with 60% aqueous acetone yield highest mass (197 mg) while absolute acetone yield the least mass (27 mg). All ethanol extracts had least masses as compared to those of acetone with all the percentages (20%, 40%, 60% and 80%). Theses extracts were also analysed for their phytochemical compounds on TLC plates. Vanillin-sulphuric acid reagent was used for optimal colour development to reveal different phytoconstituents in the plant extracts, and acetone, ethanol and 80% of aqueous acetone and ethanol were showing purple bands in all eluent system, while other extracts had no bands. In general BEA solvent system had more bands followed by CEF and EMW had the least (Figure 3.6)
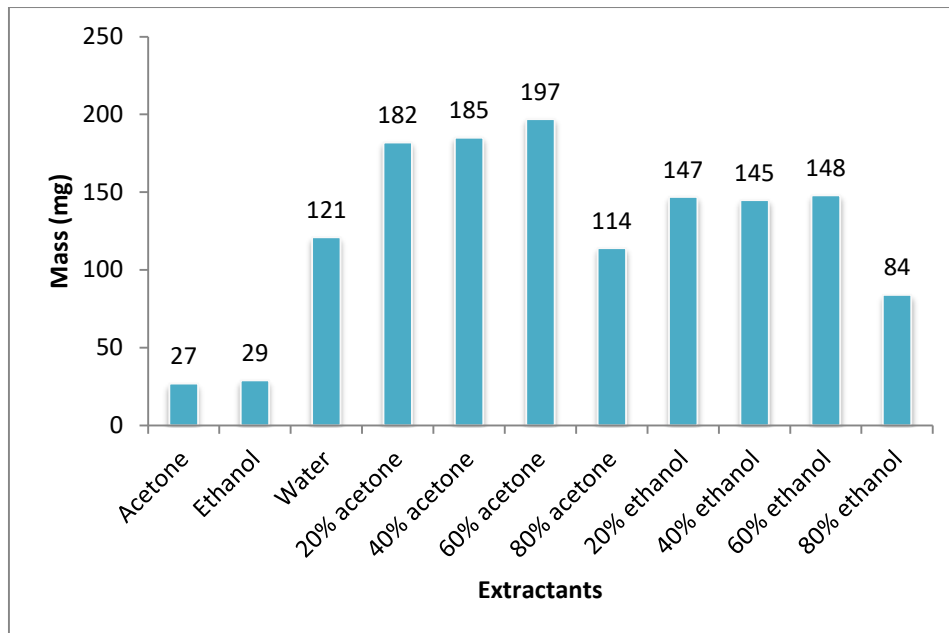
Figure 3.5: Mass extracted from *S. monteiroae* leaves in mg by solvents of varying polarities: Acetone, Ethanol, Water and different percentages (20%, 40%, 60% and 80%) of acetone and ethanol in water.
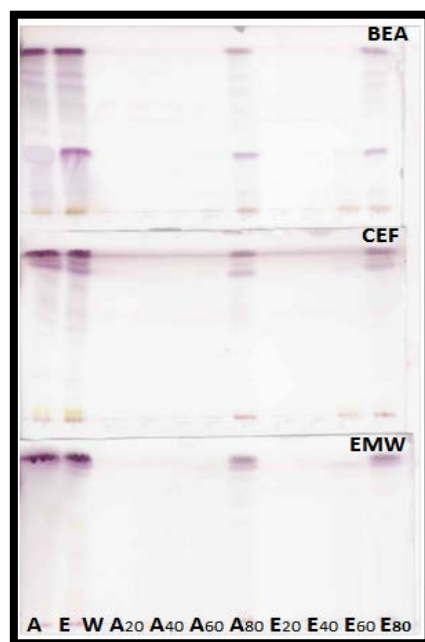
Figure 3.6: Chromatograms of *S. monteiroae* leaves extracts after treatment with aqueous ethanol and aqueous acetone at different percentages. Key: Acetone (**A**), Ethanol (**E**), Water (**W**), 20% Acetone (**A$_{20}$**), 40% Acetone (**A$_{40}$**), 60% Acetone (**A$_{60}$**), 80% Acetone (**A$_{80}$**), 20% Ethanol (**E$_{20}$**), 40% Ethanol (**E$_{40}$**), 60% Ethanol (**E$_{60}$**), and 80% Ethanol (**E$_{80}$**).

3.3.4. Phytochemical screening

Standardised methods were used to test for the presence of secondary metabolites and the results are represented in Table 3.1. The twigs had the most phytoconstituents followed by the leaves while the roots had the least phytoconstituents tested. Terpenoids, cardiac glycoside, reducing sugars and tannins were present in all plant parts, while alkaloids and steroids were not detected.

Table 3.1: The major phytoconstituents detected in different plant parts of *S. monteiroae*

Phytoconstituents	Plant parts				
	Leaves	Twigs	Roots	Tuber	Peel
Reducing sugars	+	+	+	+	+
Alkaloids	-	-	-	-	-
Tannins	+	+	+	+	+
Flavonoids	+	+	+	+	+
Anthraquines	-	+	-	-	-
Phlobatannins	+	+	-	-	-
Cardiac glycosides	+	+	+	+	+
steroids	-	-	-	-	-
Saponins	+	+	-	+	+
Terpenoids	+	+	+	+	+

Key: **+** = Present, **-** = Absent.

3.3.5. Quantification of phytoconstituents

The total phenols, tannins, and flavonoids of *S. monteiroae* of selected plant parts were measured and quantified from standard curves (Figure 3.7 to 3.9). Each value being an average of 3 analyses ± standard deviation. The total phenols were the most abundant in the analysed plant parts, followed by the tannins and the least were the flavonoids (Table 3.2). Among all extracts, the leaf extracts showed to have more phenols and tannins and the twig extract showed to have the most flavonoid content.
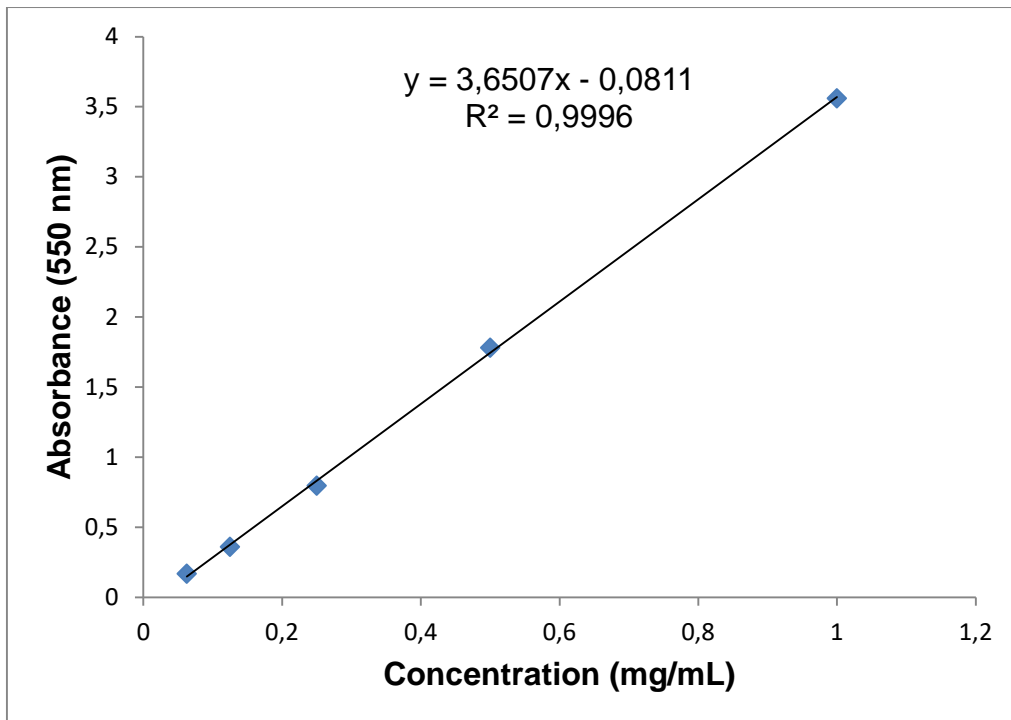
Figure 3.7: The gallic acid standard curve for total phenol
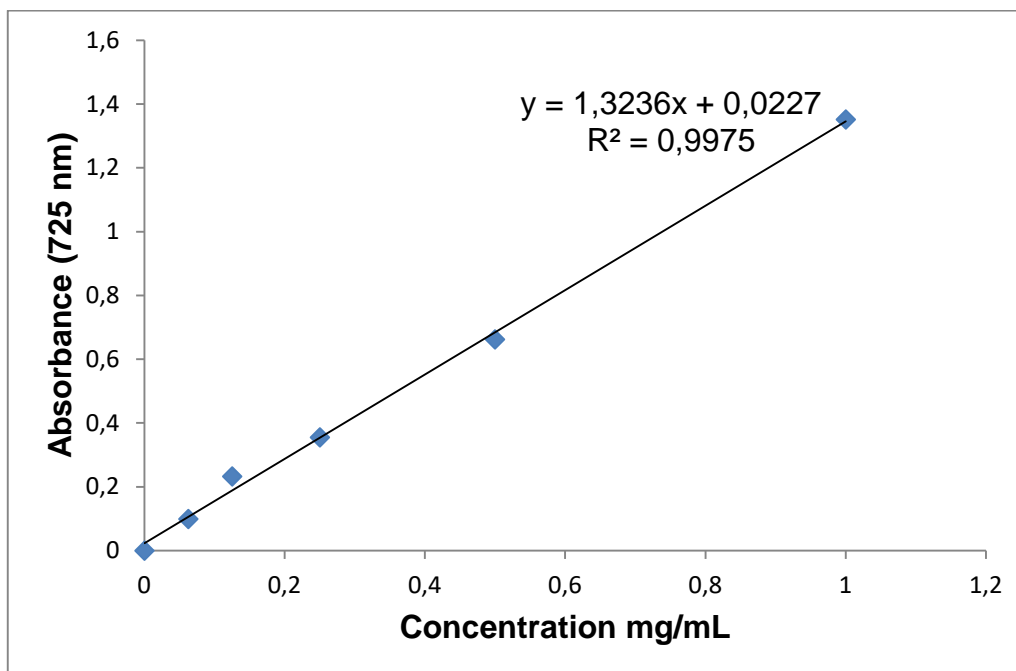
Figure 3.8: The gallic acid standard curve for total tannin analysis
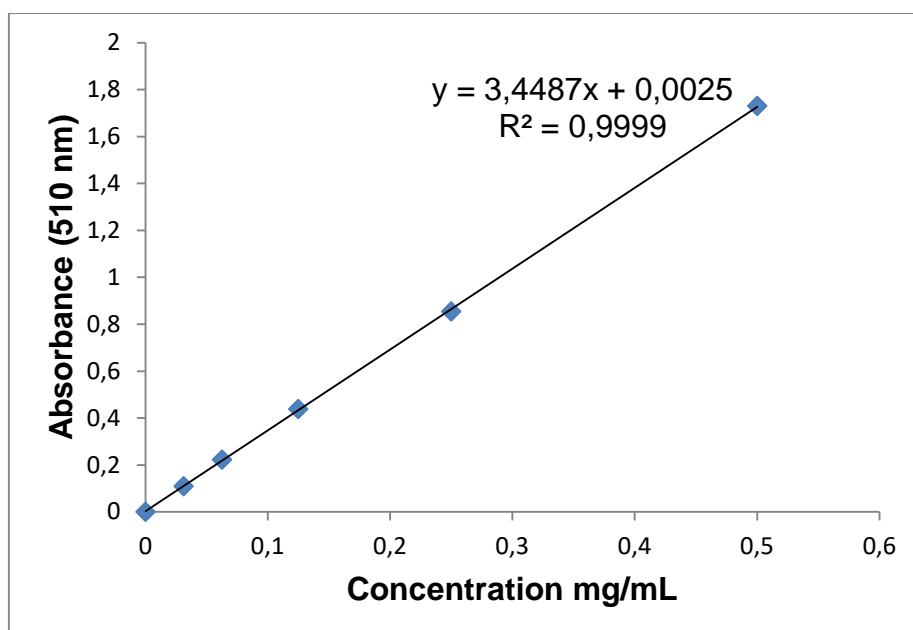
Figure 3.9: Quercetin calibration curve for total flavonoids analysis.

Table 3.2: The total phenol, tannins and flavonoids contents of different plant parts.

Sample	Total phenols (mg of GAE/g of sample)	Tannins (mg of GAE/g of sample	Flavonoids (mg of QE/g of sample)
Leaves	151,00 ± 3,56	193,33 ± 7,93	9,90 ± 0,14
Twigs	83,67 ± 0,58	48,33 ± 2,31	14,67 ± 1,53
Roots	36,67 ± 1,53	21,00 ± 1,73	8,57 ± 0,55
Peels	56,67 ± 5,77	26,67 ± 0,58	10,67 ± 1,15

Key: GAE = Gallic acid equivalence, QE = Quercetin equivalence.

3.4. Discussion

Medicinal plants provide a large pool of biologically active entities that are of great therapeutic value (Atanasov *et al.*, 2015). The knowledge of the use of traditional medicinal plants is usually derived from traditional healers and also elders within the communities (Kumar *et al.*, 2015). The traditional methods of preparation of herbal concoctions mostly involve the use of water as a solvent which is advantageous to them because it is readily available and has no side effects. However studies on medicinal plants bioactive constituents have shown that water is not an efficient extractant since some phytoconstituents extracted by this solvent may not have desired biological activities (Parekh and Chanda, 2007; Das *et al.*, 2010). Extraction

and separation of the bioactive components within the plant material is done to remove the non-active components, hence organic solvents are used to allow maximum extraction of entities with various biological activities (Eloff, 1998).

Preliminary phytochemical extraction involved the use of four solvents of varying polarities namely hexane, dichloromethane, acetone and methanol from five different plant parts. Methanol was found to be the best extractant with the highest yield in all plant parts, with a highest mass of 56 mg from the leaves, followed by dichloromethane and hexane the least. This is in-line to previous reports, where methanol extracted the most phytochemical compounds (Masoko *et al.*, 2005; Parekh and Chanda, 2007). Methanol is polar in nature and it extracts more polar compounds, however it can also extract non-polar due to its amphiphilic character (Parekh and Chanda, 2007). Hexane extracted the least from all the plant parts with the lowest mass of 1.3 mg. Hexane is selective and extracts non-polar compounds. The leaves of *S. monteiroae* were found to have more phytochemicals extracted and the peels of the tubers had the least. The plant extracts were reconstituted in acetone for further analysis such as antioxidant and antimycobacterial assays. Acetone was selected due to its ability to extract both polar and non-polar compounds and also because it is non-toxic in bioassays or test organisms (Eloff, 1998).

Thin layer chromatography was used to separate the compounds extracted by different solvents. Different compounds were detected both under UV and after spraying with vanillin-sulphuric acid reagent. With all the solvent systems used, more compounds were observed in BEA eluent system which is a non-polar solvent system. This implies that more non-polar compounds were separated on the plates. Under the UV light at the wavelength of 365 different coloured bands were observed showing diversity of compounds at different regions on the TLC plates. Under UV visualisation, more bands were present on tuber and peels extract, while more of vanillin reactive compounds were present within the leaves extracts. Secondary metabolites that fluoresce are known to possess antimicrobial and antioxidant properties. This method had low mass yield when compared to other methods, however, more compounds were observed on the TLC profile of these extracts followed by the hexane wash of the leaves extracts. These compounds were also better resolved in the BEA solvent system which suggests that more of non-polar

compounds are abundant in the leaves of *S. monteiroae*. Thin layer chromatography helps in revealing compounds that can be targeted for further analysis such as antimicrobial assays and isolation of the respective compounds (Móricz *et al.*, 2017).

Various extraction methods and solvents percentages may help in maximizing the extraction of the desired phytochemical constituents when comparing with absolute solvents (Tiwari *et al.*, 2011). Hexane is widely used in industries to extract vegetable oils from plants (Potty, 2009). The leaves of *S. monteiroae* were treated with hexane to remove chlorophyll and oils with subsequent extraction with acetone and ethanol separately. After extraction of the phytochemicals, the highest yield was from ethanol extract (63 mg), followed by acetone (36 mg) and lastly defatted extracts with 23 mg for each. The phytochemical analysis of these extracts revealed UV reactive bands in extracts eluted in CEF (Figure 3.4 A). The CEF solvent system is of intermediate polarity and efficiently separates compounds that are intermediately polar, and the bands observed were for those extracts of acetone and ethanol. To the contrary, TLC plates sprayed with vanillin reagent showed that BEA solvent system consistently separated more compounds that are non-polar as compared to all the other solvents used. Another pre-treatment procedure was used where the leaves of *S. monteiroae* were extracted with different percentages of solvents (Figure 3.4B). The yield of extracted phytochemicals is presented in figure 3.5 and high masses were obtained for 20%, 40% and 60% (masses of 182 mg, 185 mg and 197 mg) of aqueous acetone followed by 20%, 40% and 60% (147 mg, 145 mg and 148 mg) of aqueous ethanol. The lowest yield being that of acetone and ethanol. The water extract also had a high mass of 121 mg. Amongst all the extraction methods used, the extract enrichment procedure of using different solvent percentages was the best extracting method with a highest mass of 197 mg for 60% acetone, while the preliminary extraction had low yield masses. The TLC profile of *S. monteiroae* leaves extract (Figure 3. 6) after spraying with vanillin-sulphuric acid reagent revealed bands showing different compounds on the TLC plates. Acetone and ethanol extracts had more compounds and that of 80% for both acetone and ethanol as compared to other extracts, while the water extract did not show any bands in all solvent systems. Although water is mostly used in traditional health care systems, organic solvents appear to give more consistent antimicrobial active compounds (Parekh *et al.,* 2006; Yamaji *et al.*, 2005). Amongst all the solvent

systems, BEA had more compounds, followed by the CEF and EMW. This indicated that more non-polar compounds dominate within the leaves and less compounds that are polar in nature as observed in the EMW solvent system (Kotze and Eloff, 2002).

Qualitative analysis of the phytoconstituents within different plant parts involved the use of standard chemical tests. Major phytoconstituents were analysed within different plant parts. Secondary metabolites such as terpenoids, tannins, cardiac glycosides, flavonoids and reducing sugars were found to be present in all plant parts while steroids and alkaloids were found to be absent. From all the plant parts, the twigs and the leaves of the plant had more phytoconstituents as compared to other plant parts with the leaves showing almost all the major phytoconstituents tested. The secondary metabolites may be distributed differently within the plant parts, since they play various roles and are produced in the plant in response to the interaction with the environment (Singh, 2012; Azmir *et al.*, 2013). Also the presence of phytochemical compounds may be influenced by the time of harvest of the plant and season of collection (Azmir *et al.*, 2013). The tested phytoconstituents are generally known to have various biological roles. Therefore knowing the plant's phytochemical composition is vital in identifying compounds with desired effects (Das *et al.*, 2010; Mehmood *et al.*, 2015).

The quantity of selected phytochemical compounds within selected parts based on the phytochemical composition was determined. All the phytoconstituents quantified varied greatly amongst all the plant parts. The total phenols were the most abundant in all plant parts and mostly in the leaves extract (151 mg GAE/g ± 3 56) followed by the tannins and flavonoids were the least abundant especially in the roots (8,57 mg QE/g ± 0,55). The leaves extract generally had the most phytoconstituents, except that it had less flavonoids which were found to be high in the twig while roots had the least quantity of all the phytochemicals quantified. Secondary metabolites from plants such as polyphenols possess numerous medicinal properties including antimicrobial, anticarcinogenic, and antioxidant activities (Singh *et al.*, 2003).

3.5. Conclusion

Phytochemical diversity was observed from the analysis of *S. monteiroae* both qualitatively and quantitatively. The presence of phytochemical compounds such as phenols, tannins, and flavonoids indicate that *S. monteiroae* may have potential therapeutic properties which are important for drug development from plants. The next chapter therefore seeks to evaluate the antioxidant activity of extracts of different plant parts as they are imperative in the treatment of tuberculosis.

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CHAPTER 4

4. ANTIOXIDANT ACTIVITY ASSAYS

4.1. Introduction

The production of free radicals following metabolism and other external factors can result in the development of diseases such as cancer, cardiovascular and neurodegenerative ailments (Gupta *et al.*, 2014). It has been demonstrated that consumption of fruits and vegetables offers protective effects against diseases that may arise due to oxidative stress (Zhang *et al.*, 2015). The protective role being attributed to phytochemical compounds within them, which have numerous biological activities (Schmidt *et a*l., 2015). Such biologically active compounds have their activities attributed to properties such as the antioxidant properties; these properties help combat free radicals that may have been the debilitating element to the progressive ailment (Yew *et al.*, 2018). Although synthetic antioxidants have been developed, most people residing in rural areas of African countries may not be able to afford them. Thus the use of natural antioxidants from vegetation caters for their needs (Schmidt *et al.*, 2015). Dietary antioxidants from fruits, vegetables and herbal remedies have been utilised for centuries and researches has been directed towards antioxidant studies of natural products (Chan *et al.*, 2010; Yew *et al.*, 2018).

Infection with *Mycobacterium tuberculosis* induces reactive oxygen species production by phagocytes in the body in attempt to eliminate the TB infection (Yew *et al.*, 2018). However the TB bacilli have several antioxidant defence mechanisms including production of protective enzymes, a thick cell wall, and small molecule thiols, which enables the pathogen to thrive in the infected persons (Oosthuizen *et al.*, 2018). The generation of ROS may promote tissue injury and inflammatory responses in affected individuals, which further contributes to immunosuppression, particularly in those with compromised antioxidant capacity, such as co-infection of TB with HIV and other diseases (Yew *et al.*, 2018). Hence therapeutic agents effective against TB especially those that offers antioxidant activity may boost the treatment of TB and the immune system of the individual in fighting diseases. Research on plants helps in bringing more understanding about the quality and quantity of antioxidant compounds present within the plant (Tabart *et al.*, 2009).

Plant extracts are mixtures of numerous compounds with various biological activities and compounds with antioxidant activities include phenolic compounds, flavonoids, and tannins (Kaur and Mondal, 2014). These compounds can be evaluated using methods such as 2,2-diphenyl-1-picrylhydrazyl (DDPH) assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay and ferric reducing antioxidant power (FRAP), phosphomolybdenum assay and oxygen radical absorbance capacity (ORAC) method (Tabart *et al.*, 2009). Analysis of antioxidant activity using DPPH assay is based on the principle that DPPH˙ can accept hydrogen atom from the antioxidant molecule and get reduced to $DPPH_2$ and its original purple colour changes to yellow, which can be measured using a spectrophotometer to determine the antioxidant activity (Mishra *et al.*, 2012). The ferric reducing power assay is based on the ability of the extracts/compounds to reduce the ferric iron to the ferrous form at low pH. The reduction of iron of monitored by using the spectrophotometer and the absorbance are compared to that of the standard (Alam *et al.*, 2013).

4.2. Materials and methods

4.2.1. Qualitative antioxidant activity assay

The antioxidant activity of the plant extracts was evaluated using a method described by Deby and Margotteaux, (1970). All extracts from section 3.2.2.1 and 3.2.2.3 were tested for their antioxidant activity. TLC plates were spotted and developed in BEA, CEF and EMW solvent systems, and then dried at room temperature. The developed TLC plates were then sprayed with 0.2% (w/v) of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma®) in methanol as an indicator. The presence of antioxidant activity was observed as yellow spots on the plate against a purple background.

4.2.2. Quantitative antioxidant activity assay

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay was used to quantify the antioxidant activity of the 70% aqueous acetone extracts of different plant parts. In test tubes, the plant extracts were serially diluted with distilled water to make a volume of 1 mL at different concentrations (1 mg/mL to 0.0625 mg/mL) and then mixed with 1 mL of 0.2% DPPH solution in methanol. The method was further modified by diluting the samples with 10 mL of methanol. Methanol was used as blank and DPPH solution a standard control (all samples were done in triplicates). The mixtures were then incubated for 20 minutes in the dark and the

absorbance was measured at 725 nm using a spectrophotometer (Beckman Coulter-DU730) and ascorbic acid was used as a reference control. The EC_{50} value of ascorbic acid was compared with that of the extracts (Brand-Williams *et al.*, 1995). The radical scavenging activity was calculated from the linear regression formula.

4.2.3. Ferric reducing power assay

A method described by Oyaizu (1986) was used to determine antioxidant capacity using the reducing power assay. Seventy percent aqueous acetone plant extracts were serially diluted with 50% aqueous methanol at various concentrations (1 mg/mL to 0.0625 mg/mL). Methanol was used as blank and ascorbic acid was used as positive control. In a test tube a volume of 2.5 mL plant extract, 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1% w/v in distilled water) were added and mixed well. The mixture was incubated at 50 °C for a period of 20 minutes. Thereafter, 2.5 mL of trichloroacetic acid (10% w/v in distilled water) was added to the mixture and centrifuged at 650 rpm for 10 minutes. Five millilitres of the supernatant was transferred to another test tube with 5 mL distilled water and 1 mL ferric chloride (0.1% w/v in distilled water) solution and mixed (all samples were done in triplicates). The absorbance was then measured after 60 minutes of incubation at 700 nm using a spectrophotometer (Beckman Coulter-DU730) and recorded for all samples.

4.3. Results

4.3.1. Qualitative antioxidant activities

The presence of antioxidant compounds was observed as yellow spots on TLC plates against a purple background. Generally the plant had very low antioxidant activity in all eluent systems used, with the tuber peel extract showing moderate antioxidant activity followed by the twigs and leaves extracts, while the roots had no antioxidant activity (Figure 4.1). The CEF eluent system had more antioxidant compounds, followed by the BEA solvent system while EMW had the least. The leaves of the plant were defatted with hexane and the leaves had little to no antioxidant activities in all solvent systems (BEA, CEF and EMW) used (Figure 4.2).

The chromatograms of the leaves after pre-treatment with different percentages of solvents are presented in figure 4.3. The antioxidants activities were observed in

absolute acetone and ethanol and also in 80% aqueous acetone, 60% aqueous ethanol and 80% aqueous ethanol in all eluent systems used.
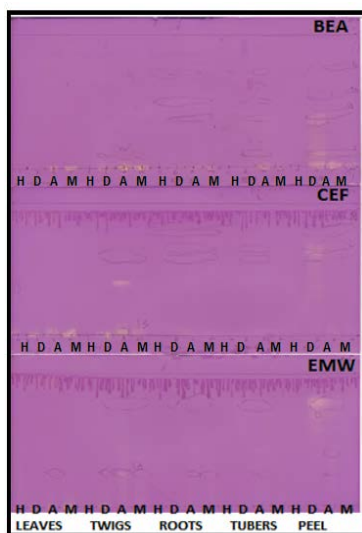
Figure 4.1: Chromatograms of plant parts of *S. monteiroae* extracted with Hexane (H), Dichloromethane (D), Acetone (A) and Methanol (M) from left to right. The plates were then sprayed with DPPH for antioxidant activity.

Figure 4.2: Chromatograms of *S. monteiroae* leaves after pre-treatment with hexane (HA=hexane for acetone and HE=hexane for ethanol) and then extracted with acetone (A) and ethanol (E).
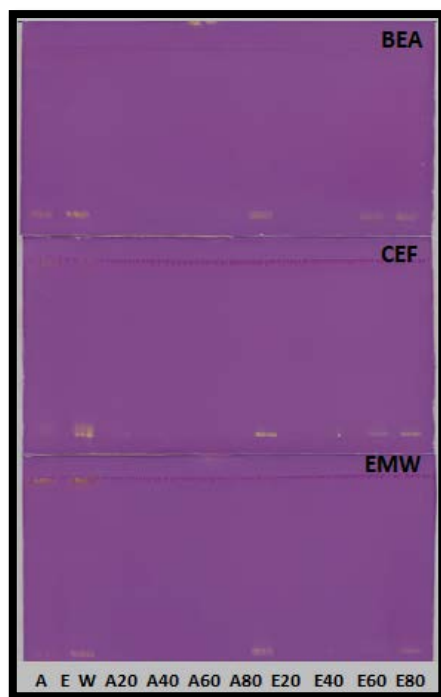
Figure 4.3: Chromatograms of *S. monteiroae* leaves crude extracts developed in solvent systems of varying polarities (BEA, CEF and EMW) and sprayed with 0.2% DPPH in methanol as an indicator. Key: Acetone (A), Ethanol (E), Water (W), 20% Acetone (A20), 40% Acetone (A40), 60% Acetone (A60), 80% Acetone (A80), 20% Ethanol (E20), 40% Ethanol (E40), 60% Ethanol (E60), and 80% Ethanol (E80).

4.3.2. Quantitative antioxidant activities

The radical scavenging activity of the plant extracts was quantified from a standard curve (Figure 4.4) and presented as half maximal effective concentration (EC_{50}) which is the concentration of the extract causing 50% loss of the DPPH activity (colour). The leaves had the highest radical scavenging potential and the roots had the least radical scavenging potential (Table 4.1.) the positive control had the highest activity when compared to all the plant parts analysed.
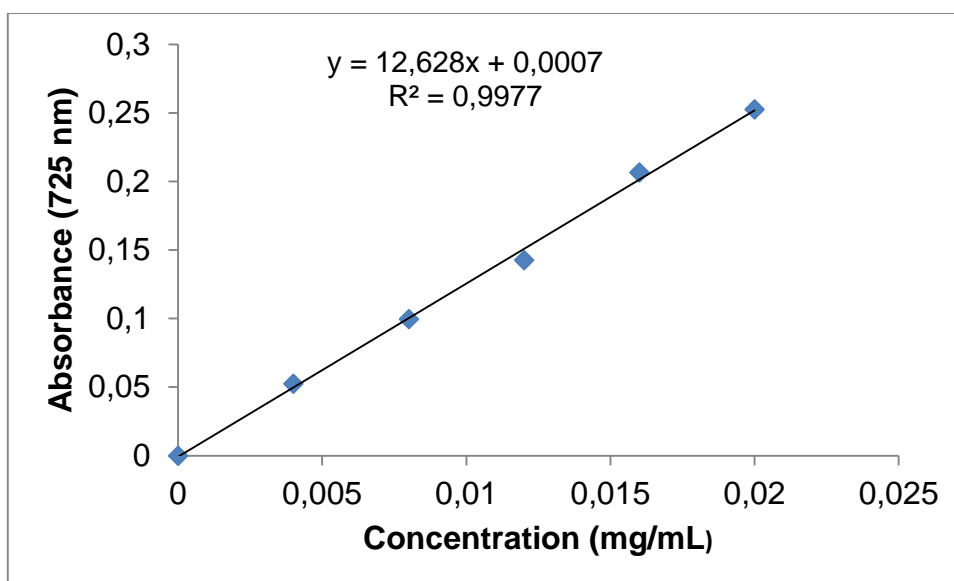
Figure 4.4: The DPPH standard curve generated from measuring the absorbance at 725 nm using different concentrations of DPPH solution.

Table 4.1: The EC_{50} values for the DPPH scavenging potential representing antioxidant capacity of different plant parts.

Sample name	DPPH scavenging potential EC_{50}
Leaves	$0,35 \pm 0,0001$
Twigs	$2,53 \pm 0,0092$
Roots	$3,70 \pm 0,0212$
Peels	$1,76 \pm 0,0014$
Ascorbic acid	$0,10 \pm 0,0014$

4.3.3. The ferric reducing capacity of plant extracts

The reducing power of the plant materials were determined using ferric reducing antioxidant potential (FRAP) assay. Ascorbic acid was used as standard and generally all the plant parts tested had very low ferric reducing power/ antioxidant capacity when compared to the standard reference (Figure 4.5). The leaves of *S. monteiroae* had a high ferric reducing capacity as compared to other plant parts, while the roots had a very low ferric reducing power.
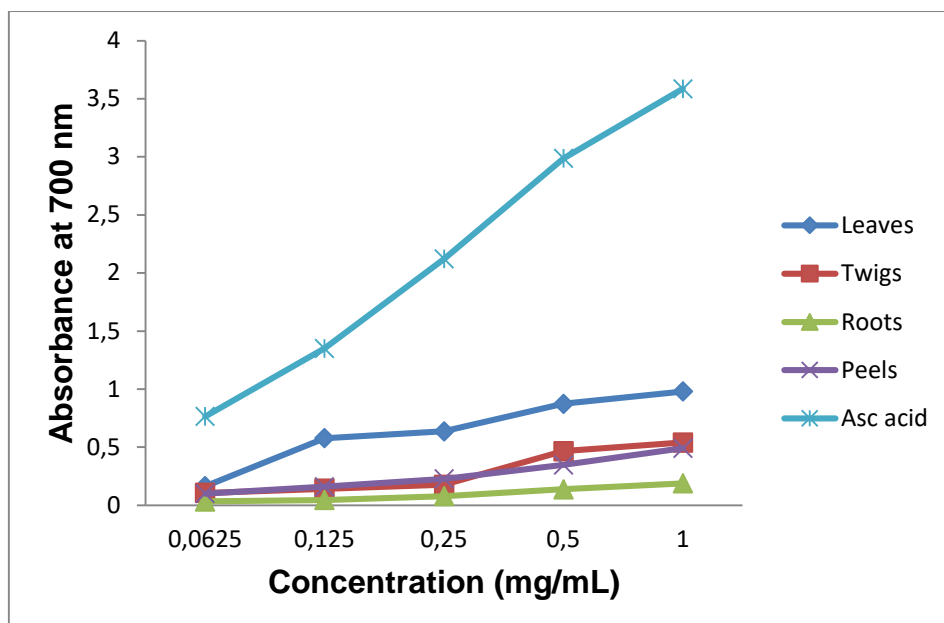
Figure 4.5: Reducing potential of different plant parts of *S. monteiroae* measured at 700 nm. Key: Asc acid= ascorbic acid.

4.4. Discussion

Many disorders including cancer, arthritis, gastritis, reperfusion injury of many tissues and atherosclerosis may occur as a result of the overload of free radicals in the human body overpowering the natural antioxidants within the system (Pourmorad *et al.*, 2006). The antioxidant activity of *S. monteiroae* was investigated using the qualitative DPPH-TLC assay. The reagent DPPH is a dark-purple coloured crystalline powder which is composed of free radicals that upon encounter with antioxidant get reduced to a yellow coloured substance (Gonçalves *et al.*, 2013). The plant extracts had very little antioxidant activity in all plant parts (Figure 4.1). After defatting (hexane-wash) the leaves extracts of *S. monteiroae* with hexane and subsequent extraction with acetone and ethanol indicated that the antioxidant activity was very minimal on all TLC plates developed in 3 eluent systems. The extract enrichment procedure was also performed for the leaves extract of *S. monteiroae* to enhance the extraction of compounds with antioxidant activities. The compounds with antioxidant potential had little to no migration on the TLC plates indicating that they have more affinity for the stationery phase than the mobile phase used. Hence the developing solvent is of low polarity than the compounds of the extract. More of the yellow bands (Figure 4.3) were observed in the BEA and CEF eluent systems than the EMW, which indicated that more compounds that had antioxidant activity

are non-polar and intermediately polar. The extract enrichment procedure of different solvent percentages also had more antioxidant activity than other methods used. Compounds that are known for their antioxidant activities include phenols, flavonoids and tannins (Heim *et al.*, 2002), and with the qualitative tests, these compounds tested positive within the leaves extract. Shamar and Bhat, (2009) reported that polar compounds have better antioxidant activities when compared to the non-polar compounds.

The radical scavenging potential of different plant parts was analysed and quantified using the quantitative-DPPH assay and ascorbic acid as a positive control. The leaves had the lowest EC_{50} value following the ascorbic acid (Table 4.1), indicating high scavenging potential while the roots had the least. The lower the concentration the higher the radical scavenging potential of the extract (Xie and Schaich, 2014). The reducing power of a plant material indicated its antioxidant abilities (Sinha and Varma, 2017). Similarly, the ferric ion reducing power for all the plant extracts was lower than the control with the leaf and root extracts showing high and low potential, respectively. The four plant parts (leaves, twigs, roots and peels) of *S. monteiroae* were investigated for their ferric iron reducing power with ascorbic acid as a standard. All the plant parts had low ferric reducing power when compared with the standard reference, ascorbic acid. However, amongst all the plant parts the leaves appeared to have a better ferric reducing power while the roots had the least ferric reducing power. Phytochemical analysis indicated that there are phenols, flavonoids and tannins in all the plant parts which explained the antioxidant activities observed, and also the phytochemical quantification on different plant parts also disclosed that the leaves extracts had a high quantity of phenols and tannins than the other plant parts which correlate with what was observed in this section. *Picralima nitida* Stapf of the Apocynaceae family has also been assessed for the antioxidant activity and low antioxidant activity was observed (EC_{50} of 2.69 mg/mL) (Ngaïssona *et al.*, 2016). Phytochemical compounds can have synergistic or antagonistic effects when used together, which may justify the variation of the low antioxidant activity on TLC plates where compounds are separated when comparing to the quantitative analysis. Previous antioxidant analysis of *S. monteiroae* has not been done before, hence there is not much to compare with.

4.5. Conclusion

Antioxidant analysis of *S. monteiroae* indicated that it does have some antioxidant activity. However, it may not be an excellent source of antioxidant compounds. This study lays a foundation for further studies of *S. monteiroae* as a source of antioxidants and brings about new knowledge about *S. monteiroae* as a medicinal plant and its bioactive principles in relation to TB treatment. Hence, the following chapter evaluates the antimycobacterial activity of *S. monteiroae*.

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CHAPTER 5

5. BIOLOGICAL ACTIVITY ASSAYS

5.1. Introduction

Microbial diseases have threatened human lives since ancient times and TB has been one of the most deaths causing infection observed throughout the world (Komape *et al.*, 2014; Andam *et al.*, 2016). The increase in microbial infections and antibiotic resistance strains continues to complicate the treatment of diseases. With the available treatment regimens, certain TB cases have proved complicated to treat and eliminate, thereby increasing the chances of spreading such infectious disease (WHO, 2018). In African countries, the lack of easy access to modern treatment programmes especially in rural areas makes it even more difficult to end the TB epidemic (Masoko and Nxumalo, 2013).

Side effects associated with synthetic drugs, drug resistance and expensive medications resulted in sourcing for alternative therapeutic agents from plants that are used in traditional health care systems (Brusotti *et al.*, 2014). Antimicrobials from the plant kingdom represent a diverse source of medicine that requires further investigation to counter the effects of pathogens on humans as well as animals. Plants provide secondary metabolites with many mechanisms of action for protecting the plant against herbivores and microorganisms (Elisha *et al.*, 2017). Thus, humans take advantage of the protective effects of these secondary metabolites during research and drug development (Mithöfer and Maffei, 2017). Many reports on plant extracts and plant derived compounds regarding their antimicrobial activity indicate the potential of medicinal plants as a source of new medicines (Elisha *et al.*, 2017; Malik *et al.*, 2016; Akthar *et al.*, 2014; Komape *et al.*, 2017).

Medicinal plants have shown much bio-activity and fewer side effects when compared to most modern synthetic drugs, however, their safety is not guaranteed. Some traditional medicinal plants contain toxic compounds which can threaten the lives of those using them; therefore, cytotoxicity tests must be done (Arunkumar and Muthuselvam, 2009). Many methods are used to study plant extracts and are tested against pathogenic organisms for their potential to kill or inhibit the pathogens as well as their toxic effects. Bioautography, 3-[4,5-dimethylthiazole-2-yl]-2,5-

diphenyltetrazolium bromide (MTT) assay, adenosine triphosphate (ATP) assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and microbroth dilution method are some of the methods used in laboratories to evaluate the bioactivity of plant extracts (Balouiri *et al.*, 2016).

Bioautography involves the use of TLC plate that has been developed and allowed to dry for a period of at least 4 to 5 days and sprayed with the microbial suspension grown in broth, and the plates are incubated in a humid atmosphere. The inhibition of microbial growth is observed when the plates are sprayed with an indicator of growth after incubation (Suleiman *et al.*, 2010). Usually tetrazolium salts are used and when sprayed (incubated for 2-3 hours at appropriate temperatures) the viable microorganisms' dehydrogenase enzyme will convert these salts into a purple/pink coloured formazan, while the inhibited growth on the plate will be observed as white bands (Suleiman *et al.*, 2010). Micro broth-dilution methods employ liquid medium for microbial growth, where varying concentrations of the antimicrobial agent is added in an increasing order, to which a defined number of bacterial cells are inoculated with the use of microtiter plates. After incubation the growth of the organism is indicated by turbidity of the solution (Wiegand *et al.*, 2008). In agar dilution methods, a known number of bacterial cells are spotted onto the nutrient agar plates that have been previously inoculated with the extracts of different concentrations. The plates are then incubated at appropriate temperatures for a defined period and after incubation, the growth of the organism as colonies will indicate the sensitivity or resistance of the organism to the test sample (Wiegand *et al.*, 2008).

The MTT assay is a colorimetric assay for analysing cell metabolic activity, hence cell viability assay. It is based on the ability of nicotinamide adenine dinucleotide phosphate dependent oxidoreductase enzymes to reduce the tetrazolium dye, MTT to its insoluble formazan that has a purple colour (Arunkumar and Muthuselvam, 2009). In MTT assay a solubilisation solution dimethyl sulfoxide (DMSO) or acidified ethanol solution is usually added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) using a spectrophotometer. The MTT method is one of the most widely used methods to analyse cell proliferation and cell viability (Kuete, 2017). Another cytotoxicity assay

that is widely used is Brine shrimp assay. It is a simple and high throughput lethality bioassay based on the killing ability of the test compounds on a simple zoological organism-brine shrimp *Artemia salina* (Karchesy *et al.*, 2016). Brine shrimp also known as sea monkeys are crustaceans that live in sea water, their eggs hatch rapidly and their larvae/nauplius are very sensitive to lower concentrations of bioactive agents (Karchesy *et al.*, 2016). One main disadvantage of this assay is that one cannot extrapolate the results of toxicity to mammals, hence MTT assay was used in this study (McGaw and Eloff, 2005). These are basic laboratory tests that pave a way in understanding the biological activity and cytotoxicity associated with the plant extracts or compounds (Balouiri *et al.*, 2016).

5.2. Materials and methods

5.2.1. The test organisms

Mycobacterium smegmatis (ATCC 1441), *Mycobacterium tuberculosis* (ATCC 25177) and *Mycobacterium tuberculosis* H37RV (ATCC 27294) were obtained from the University of Pretoria, Faculty of Veterinary Sciences, Department of Para-clinical Sciences.

5.2.2. Qualitative antimycobacterial assay against *M. smegmatis*

Qualitative antimycobacterial analysis was done using bioautography method as described by Begue and Kline, (1972) and this was tested against *M. smegmatis.* A volume of 20 µL of the extracts was loaded on TLC plates and developed in 3 solvent systems (BEA, CEF and EMW) as described in section 3.2.2.4. The extract enrichment procedures described in section 3.2.2.3 were also tested for their antimycobacterial activity. The plates were dried at room temperature for a period of 4 to 5 days to allow the solvents to evaporate. The developed plates were sprayed with an overnight culture until they were fully saturated with the organism and incubated at 37 °C at 100% humidity for 24 hours. After incubation the plates were sprayed with 2 mg/mL of ρ-iodonitrotetrazolium violet INT (Sigma®) until wet and incubated further for 3 hours. The plates were observed for mycobacterial growth inhibition. A clear zone on the TLC plate against a pink background indicated antimicrobial activities.

5.2.3. Quantitative antimycobacterial activity assay against *M. smegmatis*

The serial microbroth dilution method developed by Eloff, (1998) was used to determine the minimum inhibitory concentration (MIC) of the extracts. The test organism *M. smegmatis* was inoculated into 100 mL of middle brook 7H9 broth, incubated for 24 hours at 37 °C and used as the stock culture. A volume of 10 mL of the organism from the stock culture was further inoculated into 100 mL of middle brook 7H9 broth and incubated at 37 °C for 24 hours. The dried plant extracts were reconstituted with acetone to a concentration of 10 mg/mL and 100 μL of the plant extract was serially diluted (50%) with water in a 96 well microtiter plate. Bacterial culture (100 μL) was added to each well and the plates were incubated for 24 hours at 37 °C. Rifampicin and acetone were included as positive and negative controls respectively. To detect microbial growth a volume of 40 μL of 0.2 mg/mL INT was added to each well as an indicator for growth. All tests were performed in triplicates. The plates were incubated for further 1 hour at 37 °C and observed for colour development. A pink colour formation indicated the reduction of INT by live microorganisms whereas clear wells indicated that there is microbial inhibition. The MIC was recorded as the lowest concentration of the extract that inhibited the growth of the test organism. The total activity in mL/g was calculated by dividing the MIC value with the quantity extracted from 1 g of the plant material, which indicates the volume to which the extract can be diluted and still be able to inhibit the growth of the organisms (Eloff, 2004).

5.2.4. The antimycobacterial activity of the extracts against *M. tuberculosis*

The plant extracts were evaluated for their antimycobacterial activity testing on pathogenic microorganisms, *M. tuberculosis* H37RV ATCC 27294 and *M. tuberculosis* ATCC 25177 strains from the American Type Culture Collection (ATCC). The microorganisms were maintained on Lowenstein-Jensen (LJ) slants supplemented with glycerol for a period of one month, thereafter the colonies were transferred into 3 ml of oleic acid, albumin, dextrose and catalase (OADC)-supplemented Middlebrook broth, homogenized by vortexing, and the larger particles were allowed to settle. The test inoculums were prepared in OADC- supplemented Middlebrook 7H9 broth, adjusted to the McFarland standard 1 equivalent to 3.0×10^8 and diluted to a final density of 5×10^5 CFU/ml in the medium. The microdilution assay (Eloff, 1998; Jadaun *et al.*, 2007) was used to determine the MIC values of the

different plants extracts against the MTB strains. In a 96 well microplate plate, the plant extracts were prepared in 10% dimethylsulfoxide (DMSO) and serially diluted twofold (100 µL) going down the wells of the plate with OADC-supplemented Middlebrook 7H9 broth. Ten percent of DMSO, the inoculum and OADC-supplemented Middlebrook 7H9 broth were used as negative controls, whereas first line antiTB drugs such as streptomycin, isoniazid and rifampicin were used as positive controls. Thereafter, mycobacteria culture were added in all wells excluding wells containing the OADC-supplemented Middlebrook 7H9 broth only. The plates were sealed with parafilm and incubated for 7-10 days at 37°C. After incubation, 40 µl of 0.2 mg/ml of freshly prepared p-iodonitro-tetrazolium chloride (INT) solution was added into the wells of the plate to determine the MIC values. The colour change after adding INT was observed and the MIC values were read as the lowest concentration of the extract inhibiting mycobacterial growth. The experiments were tested in triplicate. The study was done under the biosafety cabinet level 2 and all precautionary measures were followed to avoid infections in the laboratory.

5.2.5. Cell viability assay

Colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide/MTT assay was used to determine viability of African green monkey kidney (Vero) cells treated with the plant extract (the leaves, twigs and roots, selected based on their low MIC values). A culture of sub-confluent cells were harvested and centrifuged at 200 xg for 5 minutes and resuspended in Minimal Essential Medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). Cell density used in the assay was 5 x 10^4 cells/mL. Into a sterile 96-well microtiter plate a total of 200 µL of the cell suspension was plated per well and a volume of 200 µL of MEM was added to the wells at the edges to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 hours at 37 °C in a humidified incubator with 5% CO_2, until the cells were in the exponential phase of growth. The growth media was aspirated from the cells, and the cells were washed with 150 µL of 1 X phosphate buffered saline (PBS, Whitehead Scientific) and replaced with the test extract (200 µL) at differing concentrations in quadruplicate. The MEM was used to prepare serial dilutions of the extracts ensuring very little disturbance of the cells during aspiration of the medium and addition of the extracts. Untreated cells and positive control (doxorubicin

chloride, Pfizer Laboratories) were included and the plates were incubated for 48 hours at 37 °C in a 5% CO_2 incubator. After incubation, 30 µL of MTT reagent (Sigma, stock solution of 5 mg/mL in PBS) was added to each well of the plates and then incubated for 4 more hours at 37 °C. The medium was carefully removed from each well after incubation to avoid disturbing the MTT crystals in the wells. To dissolve the MTT formazan crystals, 50 µL of Dimethyl Sulfoxide (DMSO) was added to each well and the plates were shaken gently until the MTT was dissolved. The reduction of MTT was measured by reading the absorbance in a microplate reader (BioTek Synergy) at a wavelength of 570 nm. Negative control wells that contained the medium and MTT but no cells were used as blank. All the determinations were carried out in triplicates. The LC_{50} values were calculated as the concentration of test compound resulting in a 50% reduction of the absorbance when compared to untreated cells (Mosmann, 1983). In order to calculate the selectivity index (SI), the LC_{50} values greater than 1 mg/mL were taken as being 1000 mg/mL. The SI was calculated as follows:

$$\text{Selectivity Index (SI)} = LC_{50} \, (\text{mg/mL}) / \text{MIC (mg/mL)}$$

5.3. Results

5.3.1. Antimycobacterial activity

The bioautographic assay of all the plant parts from preliminary extraction procedures revealed the presence of antimycobacterial activity on the leaves extracts of dichloromethane, acetone and methanol in the non-polar solvent system BEA and very little activity on the CEF solvent system in the acetone extract (Figure 5.1). The minimum inhibitory concentrations and the total activity of the plant extracts were determined by testing on *M. smegmatis*. The roots had an average low MIC value of 0.31 mg/mL with a high total activity of 57 mL/g, followed by the leaves extracts with MIC of 0.83 mg/mL and the tubers had an average high MIC value of 0.98 mg/mL and a low total activity of 36 mL/g (Table 5.1 and Table 5.2). All the samples had high MIC values as compared to the positive control rifampicin. The plant extracts were evaluated for their antimycobacterial activity on the pathogenic *M. tuberculosis* strains, and compared with first line antiTB drugs rifampicin, isoniazid and streptomycin (Table 5.3). Low MIC values were observed for the tuber extract (0.15 mg/mL) while the leaves (0.25 mg/mL). Roots (0.58 mg/mL) and the

twigs (0.25 mg/mL) had high MIC values. All the plant extracts had high MIC values as compared to the antiTB drugs.

Figure 5.1: The TLC profile of different plant parts of *S. monteiroae* developed in 3 solvent systems BEA, CEF and EMW and then sprayed with *M. smegmatis* and INT as an indicator of growth.

Table 5.1: The minimum inhibitory concentration (MIC) of the extracts of different plant parts in mg/mL.

Solvent	Leaves	Twigs	Roots	Tubers	Peels	Averages
Hexane	>2.5	>2.5	>2.5	>2.5	>2.5	2.5
Dichloromethane	1,04	1,67	0,52	1,25	0,63	1,02
Acetone	0,73	0,84	0,21	2.5	2,08	0,97
Methanol	0,73	0,27	0,21	0,71	0,13	0,41
Averages	0,83	0,93	0,31	0,98	0,95	
Rifampicin	0.08					

Key: Red coloured values= noteworthy activity

Table 5.2: The total activity (mL/g) of different plant parts of *S. monteiroae*

Solvent	Leaves	Twigs	Roots	Tubers	Peels	Averages
Hexane	n/a	n/a	n/a	n/a	n/a	n/a
Dichloromethane	20	3	21	6	5	11
Acetone	26	6	67	n/a	1	20
Methanol	77	48	81	66	120	78
Averages	41	19	57	36	42	

The hexane wash was performed for the leaves extract of *S. monteiroae*. Antimycobacterial activity was also observed for this extract on plates developed in the non-polar solvent system BEA within the acetone extract as shown in Figure 5.2. Another enrichment procedure was performed on the leaves extract of *S. monteiroae* using different solvent percentages and analysed for their antimycobacterial activity, and very little microbial inhibition was observed in the extracts of acetone and ethanol within the BEA solvent system (Figure 5.3).
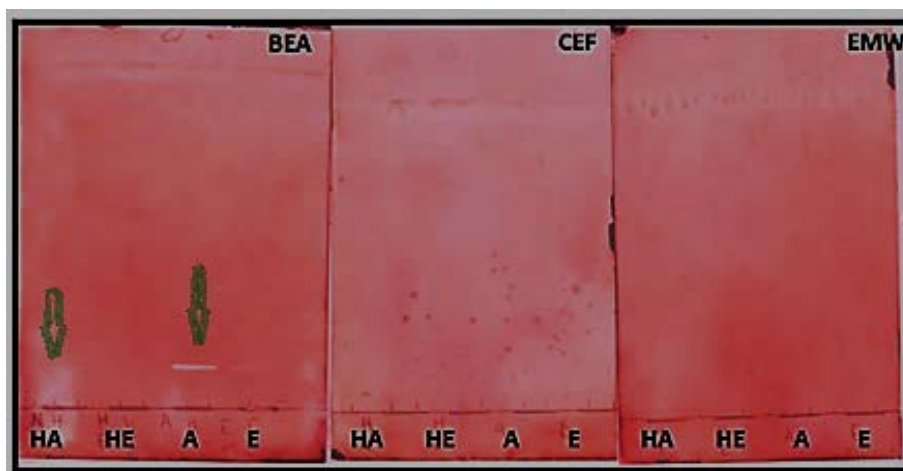
Figure 5.2: Bioautograms of *S. monteiroae* leaves defatted extracts and separated on 3 solvent systems namely BEA, CEF and EMW then sprayed with *M. smegmatis* and INT as an indicator of growth. Key: hexane for acetone (HA), hexane for ethanol, acetone (A) and ethanol (E).
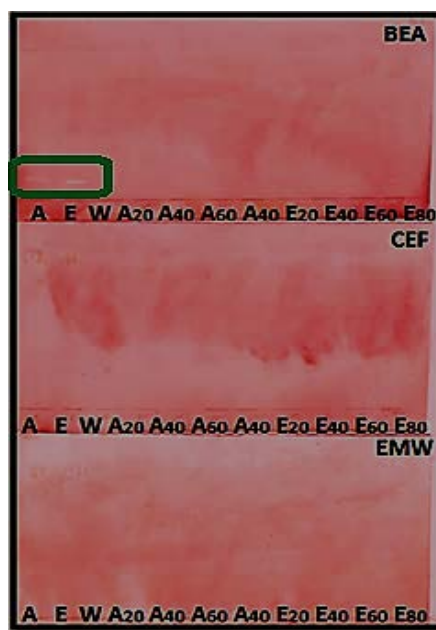
Figure 5.3: Bioautograms of *S. monteiroae* leaves extracts separated in three solvent systems of increasing polarities namely BEA, CEF and EMW, and sprayed with *M. smegmatis* and INT for bacterial growth inhibition. Key: acetone (**A**), ethanol (**E**), water (**W**), 20% acetone (**A$_{20}$**), 40% acetone (**A$_{40}$**), 60% acetone (**A$_{60}$**), 80% acetone (**A$_{80}$**), 20% ethanol (**E$_{20}$**), 40% ethanol (**E$_{40}$**), 60% ethanol (**E$_{60}$**) and 80% ethanol (**E$_{80}$**).

Table 5.3: The MIC values of the extracts tested on *Mycobacteria tuberculosis* strains in mg/mL.

Sample	*M. tuberculosis* ATCC 25177	*M. tuberculosis* H37Rv ATCC 27294
Leaves	0.25	0.25
Roots	0.575	0.575
Tuber	0.15	0.15
Twigs	0.25	0.25
Rifampicin	0.0002	0.0002
Isoniazid	0.0001	0.0001
Streptomycin	0.0005	0.0005

5.3.2. The cytotoxicity of the plant extracts

The leaves, twigs and roots extracts of *S. monteiroae* were assessed for their cytotoxicity using the MTT assay, these plant parts were selected based on the low MIC values observed. The extracts with LC_{50} more than 0.02 mg/mL were considered relatively non-cytotoxic. All the plant parts analysed for cytotoxicity were found to be non-cytotoxic, with the leaves extracts being the most non-cytotoxic with the LC_{50} of more than 1 mg/mL (Table 5.4). The selectivity index which is indicative of the selective toxicity of the extract between the bacterial pathogen and the mammalian cells was also calculated and the leaves extracts were found to have a high SI of 1.4, while the other plant parts had low SI values.

Table 5.4: The cytotoxicity presented as LC_{50} and selectivity index of acetone extracts of different plant parts of *S. monteiroae.*

Sample Extracts	Cytotoxicity in LC_{50} ± SD (mg/mL)	Selectivity Index
Leaves	1	1.4
Twigs	0,175019 ± 0.010117	0.2
Roots	0,119732 ± 0.010062	0.6

Key: LC_{50} = Lethal concentration capable of killing half population of the bacteria; SD= Standard deviation.

5.4. Discussion

Toxicological and antimicrobial studies are essential in discovering and development of safe and new drugs from medicinal plants (Taylor *et al.*, 2001). Indigenous people of Africa largely depend on local plants to restore their health (Cordier and Steenkamp, 2015). Mainly because of their traditional values, therefore it is worthwhile to investigate plants used traditionally as medicines to ascertain as to whether their medicinal value can be validate by showing antimicrobial activity (Taylor *et al.*, 2001). The antimycobacterial activity of *S. monteiroae* was analysed qualitatively and quantitatively using bioautography and micro-dilution method respectively. In this study *M. smegmatis* which is not pathogenic to humans was used as a model. This microorganism is similar to the pathogenic microorganism *M. tuberculosis* in genomic sequences and is preferable to use because it is fast

growing than the pathogenic *M. tuberculosis* (Namouchi *et al.*, 2017). First all the plant parts were screened for antimycobacterial activities using bioautography assay. In bioautography the inhibition of the test organism is observed as white spots on the pink background of the TLC-plate (Begue and Kline, 1972). The leaves extracts of *S. monteiroae* had qualitative microbial inhibition in a non-polar solvent system developed plates (BEA), which indicated that the compounds responsible for the observed antimycobacterial activities are non-polar. The TLC plates developed in CEF eluent system had little antimycobacterial activity on the leaves extract, the formic acid used in the solvent system might still be in the TLC-plates thus causing the organism not to grow since the formic acid is toxic to bacteria (Masoko and Eloff, 2005). After hexane wash of the leaves of *S. monteiroae,* the acetone and ethanol leaves extract had a good antimycobacterial activity as similarly observed in the initial bioautography assay, within the extracts separated in the BEA non-polar solvent system. Enrichment extracts of ethanol and acetone were analysed for qualitative antimycobacterial activity using bioautography. These extracts had some antimycobacterial activity towards the test organism *M. smegmatis* in the extract of absolute acetone and ethanol, while no activity was observed in the aqueous-mixture extracts. The enrichment procedures did not enhance the antimycobacterial activity of the extracts*,* while defatted extracts had better antimycobacterial activity than un-defatted extracts, which correlates with what Parekh *et al.* (2006) reported on a study of *Bauhinia variegata* L. bark*.*

The microdilution assay involves quantitative analysis of the antimycobacterial activity observed. All the plant parts were analysed for minimum inhibitory concentrations against *M. smegmatis*. It is noteworthy that in all analyses the extracts were reconstituted in acetone for further analyses because other solvents are toxic to cells and acetone is much safer to use (Eloff *et al.*, 2007). The extracts with minimum inhibitory concentrations of less than 1 mg/mL were considered to have a good antimycobacterial activity. For comparison purposes of the activity among different plant parts, it is important to take into account the quantity extracted from the plant material (Elisha *et al.*, 2017). And this is done by calculating the total activity of the extracts, which is indicative of the volume of solvent that can be added to the mass extracted from 1 g of the plant and still inhibit mycobacterial growth. From all the analyses the roots had an average lowest MIC value of 0.31 mg/mL and

a good total activity of 57 mL/g. The leaves extract had the second lowest MIC value (0.83 mg/mL) and amongst all the plant parts the tubers had the highest inhibitory concentration. However, all the plants parts had higher MIC values when compared to a known antimycobacterial drug rifampicin which was found to be 0.08 mg/mL. Phytochemical constituents such as phenols, tannins, flavonoids were found to be present in different plant including the leaves. These phytochemicals are known to have antimicrobial activities, which may be responsible for the antimycobacterial activity observed (Das *et al.*, 2010). The bioautography findings did not resonate with that of the MIC findings; leaf extracts exhibited activity that the extract (root) with the best MIC activity did not poses, the reason for this we are not sure of, however, it can be speculated that the activity within the leaf extract is masked by other compounds in the extracts, therefore, separation through TLC reveals an individual compounds ability to exhibit antimicrobial activity. To the contrary, extracts like that from the roots exhibited good MIC values but poor bioautogram result, this could indicate that the activity is synergistic. It is however logical that the leaf extract is chosen for isolation of bioactive compounds, otherwise activity during isolation of compounds from roots has no bio-guided basis and activity can be lost as the isolation process proceeds, despite its potential activity. (Taylor *et al.*, 2001).

Based on the results observed against *M. smegmatis*, the extracts were further tested for their anti-TB activity against the pathogenic strains *M. tuberculosis* (ATCC 25177) and *M. tuberculosis* (H37Rv) using the microdilution method. Three first line drugs were used as positive controls and the drugs had very low inhibitory concentrations ranging between 0.0001 and 0.0005 mg/mL while the test extracts had high activity of up to 2.5 mg/mL, with the tubers having the lowest activity followed by the roots and the leaves extracts having a higher activity. However these results were contradicting with what was observed initially when *M. smegmatis* was used as a test organism where the roots possessed a higher activity when compared with other plant parts. These variations may be attributed to the differences in the morphological compositions of these organisms; *M. tuberculosis* has a tough cell wall that is difficult to penetrate by most drugs which also contribute to its slow growth rate, drug resistance and nutrition up take (Van der Geize *et al.*, 2007). Also, the higher activities of the plant extracts observed may not necessarily indicate that the plant extracts are not active but rather that they are useful in treating the

symptoms of TB traditionally, thus, exhibiting healing-like effects (Green *et al.*, 2010). Ngemenya *et al.* (2006) reported that weak activity of plant extracts like some other drugs observed *in vitro* may be enhanced *in vivo* due to metabolic transformation of their components into highly active entities. These observations correlate with what Green *et al* (2010) reported on a study of medicinal plants used in Venda in the treatment on TB and tested on the pathogenic strains, but they did not show any good activity *in vitro*.

Although plant extracts are often non-toxic and have less side effects when compared to modern synthetic drugs, some are however cytotoxic and can be dangerous to human health if not thoroughly investigated (Elisha *et al.*, 2017). *In vitro* studies such as MTT assay is used to determine the cytotoxicity of plant extracts and their phytoconstituents (Cordier and Steenkamp, 2015). This is because plant derived compounds and extracts may have no selectivity towards microorganisms and biological cells (mammalian cells), thus it is important to determine their levels of cytotoxicity and selectivity indices. The MTT assay was used to investigate the cytotoxic effects of the plant parts of *S. monteiroae* (leaves, twigs and roots). The LC_{50} values were calculated as the concentration of the test sample that resulted in 50% reduction of absorbance compared to untreated cells, while the selectivity index (SI) which is obtained by dividing the LC_{50} (mg/mL) by the MIC (mg/mL) value indicates whether the plant extract is more toxic to the pathogen or to the host cells (mammalian cells). If the SI is greater than 1 it is considered to be less toxic to the host cells than to the bacteria thus indicating that the plant extracts will cause less damage to the mammalian cells and more damage to the target pathogen when applied as a therapeutic agent (Kuete, 2010; Elisha *et al.*, 2017). All the plant parts were observed to be generally non-cytotoxic with the LC_{50} of greater than 1 mg/mL for the leaves, 0.18 mg/mL for the twigs and 0.12 mg/mL for the roots extracts, the leaves extracts appeared to be more non-cytotoxic (LC_{50} = 1 mg/mL). However, the selectivity index of other plant parts (except the leaves extracts) was found to be very low i.e. less than 1 which indicates that they are less toxic to the target microorganism than they are to the host cell.

A good SI indicates that there's a large safety margin between the mammalian Vero cells toxic concentrations of the extract and the minimum inhibitory concentration of the extract to the mycobacteria (Elisha *et al.*, 2017). Thus, a higher selectivity index

indicates that the plant extracts activity is not due to a metabolic toxin within the extracts, therefore it is safe to use as a medicinal plant (Dzoyem *et al.*, 2016). The bioautography results indicated that leaves extracts had antimycobacterial activity while the other plants had no antimycobacterial activities, and also the leaves extracts appeared to be less cytotoxic to Vero cells than they are to the bacterial cells. The phytochemical constituents within the plant may work together to produce a synergistic effect. Thus the observed activity (SI) may be different as the individual phytoconstituents get isolated to individual component. *In vitro* activity does not directly indicate true *in vivo* response to the extracts upon administration to mammalian cells/body due to physiological responses and pharmacokinetics (Dzoyem *et al.*, 2016). Therefore further investigations are necessary especially on TB causing strains.

5.5. Conclusion

The leaves extracts of *S. monteiroae* had a good antimycobacterial activity with bioautography assay and a moderate activity with the microdilution method. This indicated the efficacy of the leaves of plant as used by traditional health care practitioners in the treatment of TB related symptoms. Low toxicity was also observed which indicated the safety of this plant as used in traditional medicine. The present study demonstrated for the first time the antimycobacterial activity of *S. monteiroae* plant parts with the use of both *M. smegmatis* and *M. tuberculosis*.

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CHAPTER 6

6. ISOLATION AND PURIFICATION OF BIOACTIVE COMPOUNDS

6.1. Introduction

Medicinal plants in the form of standardised plant extracts or pure compounds provide a diversity of potential drug leads (Cos *et al.*, 2006). Medicinal plants can be developed into drugs or used in conjunction with synthetic drugs to enhance patient's recovery (Masoko *et al.*, 2016), for example drugs such as codeine, morphine, quinine and cocaine have been isolated form medicinal plants. Drug discovery has evolved throughout the years from medicinal plants, synthetic drugs to other natural products and medicinal plants have remained a reliable source as many drugs continue to be developed from them (Eloff, 1998). Indole alkaloids that have antitumor activity have been isolated from *Kopsia hainanensis* of Apocynaceae, which is the family of *S. monteiroae* (Chen *et al.*, 2014).

Screening of crude extracts allows localization of the bioactive compounds that may be targeted for isolation. Plant extracts exist as a combination of different bioactive compounds that requires isolation and identification. Preliminary steps of phytochemical extraction, screening, separation and evaluation of bioactivity are necessary as they provide some information on the process of isolation (Newton *et al.*, 2002). These tests are easy and simple to perform and they are followed by Serial Exhaustive Extraction (SEE) which involves extraction of the same plant material using solvents of varying polarities from non-polar to polar in order to ensure that different compounds are completely extracted (Das *et al.*, 2010; Tiwari *et al.*, 2011). The resulting extracts are analysed for their biological activity and once the biological activity has been determined, the extracts must be purified for the purpose of isolation and characterisation of bioactive compounds. Isolation of compounds from medicinal plants provide single active entities that may further be developed into new drugs, however, due to synergism between compounds within the extracts, the activity may be lost. Antagonistic effects also play a role in the bioactivity of new compounds which also influence the isolation of compounds and bring new knowledge about the medicinal plants in use (Newton *et al.*, 2002).

Several techniques of isolation and purification such as TLC, HPTLC preparative TLC and column chromatography are used. Amongst others column chromatography is one of the most useful techniques for separation of various mixtures. Its principle is based on differential adsorption of substance by the adsorbent. The adsorbents usually employed in column chromatography are silica, alumina, calcium carbonate, and calcium phosphate, while the solvent used is dependent on the sample and the adsorbent. The rate at which the components of a mixture are separated depends on the activity of adsorbent and polarity of the solvent (Gini and Jothi, 2018). And if the activity of the adsorbent is very high and polarity of the solvent is very low, the separation is very slow but gives a good separation of the compounds. On the other hand, if the activity of adsorbent is low and polarity of the solvent is high the separation is rapid but gives only a poor separation (the components separated will not be 100% pure) (Gini and Jothi, 2018).

Bioassay guided fractionation has been used for many years to direct the isolation process of bioactive components of plants (Newton *et al.*, 2002; Brusotti *et al.*, 2014). It involves repetitive fractionation of extracts with evaluation of the specific biological activity until the isolation of pure compounds (Brusotti *et al.*, 2014). Bioassay guided fraction has many advantages: however, a large amount of the plant material is required for the fractionation process and there is a risk of losing the bioactivity and the process is labour intensive and time consuming (Brusotti *et al.*, 2014). Bioassay guided fraction was used in this study to isolate and purify the bioactive compounds in column chromatography and preparative-TLC.

6.2. Materials and methods

6.2.1. Preparation of crude extracts

The serial exhaustive extraction was used to extract 300 g of *S. monteiroae* leaves with two litres of four different solvents namely: hexane, dichloromethane, acetone and methanol. The bottles were shaken at 200 rpm and the extraction process was repeated three times for each solvent to completely/exhaustively extract the plant material. The extracts were filtered into pre-weighed beakers and the solvents were evaporated at room temperature under the fan and weighed. The phytochemical analysis was performed as described in section 3.2.2.4.

6.2.2. Qualitative antioxidant activity assay

The antioxidant activities of the leaves extracts were evaluated using DPPH-TLC as described in section 4.2.1.

6.2.3. Antimycobacterial activity assays

Bioautography and microdilution methods were used to assess the antimycobacterial activities and the procedure was done as described in sections 5.2.2 and 5.2.3.

6.2.4. Isolation of antimycobacterial compounds

6.2.4.1. Open column chromatography

Bioassay guided fractionation was used to guide the isolation, separation and purification of bioactive compounds using column chromatography. An open column (38 x 3 cm) was packed with silica gel 60 (particles size 0.063-0.200 mm) (Fluka) using 100% hexane. The finely ground hexane extract was thinly spread on top of the packed silica gel and then covered with cotton wool and eluted with solvents of different percentages as outlined in Table 6.1. Collected fractions were concentrated using rotary evaporator at 50°C water bath, dried and weighed. The fractions were tested for antimycobacterial activity using bioautography and microdilution method (Section 5.2.2 and 5.2.3).

Table 6.1: Different solvents and their percentages used for elution of open column chromatography.

Solvents	Percentages %
Hexane	100
Hexane: Ethyl acetate	90:10
	80:20
	70:30
	60:40
	50:50
	40:60
	30:70
	20:80
Ethyl acetate	100
Ethyl acetate: Methanol	90:10
	80:20
	70:30
	60:40
	50:50
	40:60
	30:70
	20:80
Methanol	100

6.2.4.2. Small column chromatography

The fractions obtained above (Table 6.1) with antibacterial activities were combined to make one sub-fraction. This fraction was then subjected to a small open column chromatography (34 x 3 cm) packed with silica gel 60 (particles size 0.063-0.200 mm) (Fluka) using 100% chloroform. The finely ground hexane extract was thinly spread on top of the packed silica gel and then covered with cotton wool and eluted with 70% chloroform in ethyl acetate. The sub-fractions were collected in test tubes and concentrated under the stream of cold air and tested on TLC plates for bioactive compounds.

6.2.4.3. Preparative TLC

The sub-fractions named A1 and A2 was further separated on TLC silica gel glass plates (Merck Silica gel 60 F$_{254}$) using 70% chloroform in ethyl acetate for antimycobacterial compounds. To detect UV reactive bands, the plates were visualized under UV light at 360 nm to locate the bands on the TLC plates and also a small portion on the side of the glass plates was sprayed with vanillin-sulphuric acid reagent while the rest of the plates were covered with an aluminium foil. Visualised bands with vanillin sulphuric acid reagent were used as referenced line for scraping off active bands from developed TLC plates. The active compounds were immersed in the chloroform, filtered using cotton wool and separated from silica gel. Purity of isolated compounds was confirmed by developing TLC plates in 70% chloroform in ethyl acetate and spraying TLC with vanillin-sulphuric acid and heated at 110 ºC until colour developed. The compounds were also analysed for antimycobacterial activity using bioautography assay, with *M. smegmatis* as the test organism.

6.3. Results

6.3.1. Serial exhaustive extraction

The leaves extracts of *S. monteiroae* were extracted using serial exhaustive extraction with solvents namely hexane, dichloromethane (DCM), acetone and methanol and methanol had the highest yield followed by hexane (Table 6.2).

Table 6.2: The mass of extracts derived from 300 g of the leaves of *S. monteiroae* using solvents of varying polarities.

Extraction solvent		Mass yield (g)	Total mass yield (g)
Hexane	I	11,6	16
	II	3,5	
	III	1	
DCM	I	1,5	2
	II	0,5	
	III	0,1	
Acetone	I	1,3	2
	II	0,5	
	III	0,2	
Methanol	I	23	35.2
	II	8,7	
	III	3,5	
Total			55.2

6.3.2. Phytochemical analysis

The leaves extracts were analysed for their phytochemical components on TLC plates which after developing were viewed under UV light (Figure 6.1 A) and also visualised by using vanillin-sulphuric acid reagent (Figure 6.1.B). In all used solvents fluorescing compounds were observed in acetone and dichloromethane extracts with less fluorescence on hexane extract in CEF solvent system. After spraying the TLC plates with vanillin-sulphuric acid agents, more compounds were observed in BEA and CEF eluent system.
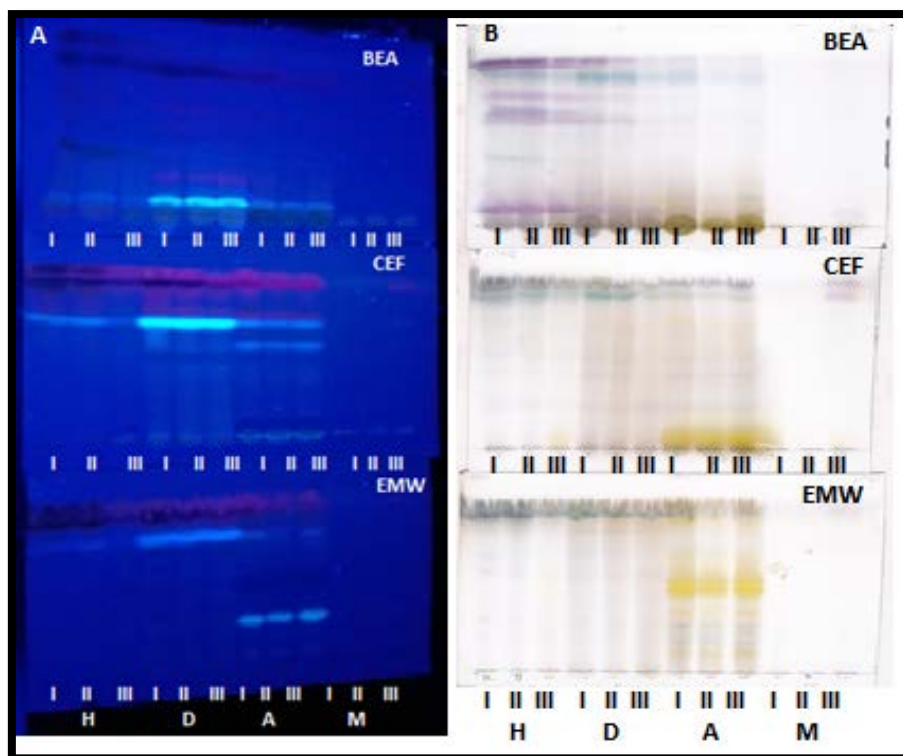
Figure 6.1: The chromatograms of the leaves extracts of *S. monteiroae* extracted with solvents of varying polarities and developed in different solvent systems (BEA, CEF and EMW), and visualised under UV light (A) at 365 nm and sprayed with vanillin-sulphuric acid reagent (B). Key: H = Hexane, D = Dichloromethane, A = Acetone, M = Methanol.

6.3.3. Qualitative antioxidant activity

The leaves extracts obtained by SEE method were also analysed for antioxidant activity which was observed to be more visible in acetone extracts within all the solvent systems used (Figure 6.2), followed by dichloromethane extracts, while hexane and methanol had no antioxidant activities.
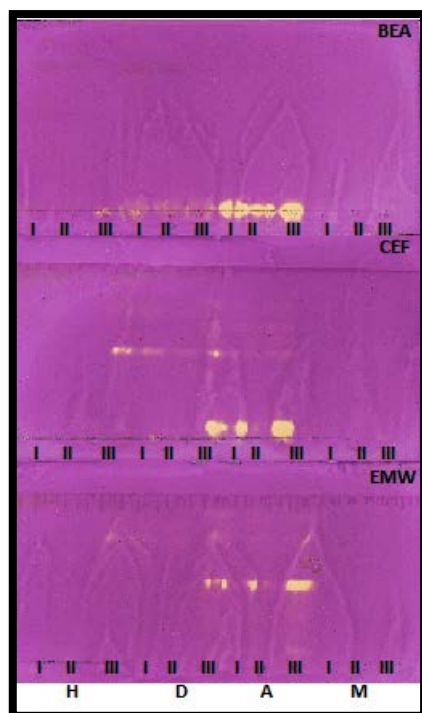
Figure 6.2: The TLC plates of *S. monteiroae* leaves extracts developed in solvent systems of different polarities and sprayed with 0.2% of DPPH solution as an indicator of antioxidant activity. Key: H = Hexane, D = Dichloromethane, A = Acetone, M = Methanol.

6.3.4. The antimycobacterial activities of the SEE extracts

The SEE extracts were also assessed for antimycobacterial activity using bioautography and microdilution method. From bioautography analysis the hexane extracts (**I**, **II** and **III**) had antimycobacterial activity within the BEA eluent system and some activity was also observed on the CEF eluent system within the hexane III extract (Figure 6.3).

The MIC values of the SEE extracts were determined and the DCM and ACE extracts both had the lowest MIC of 0.93 mg/mL, and the Hexane extracts having the highest MIC values of 2.5 mg/mL, while the methanol extracts did not show any antimycobacterial activity (Table 6.3).
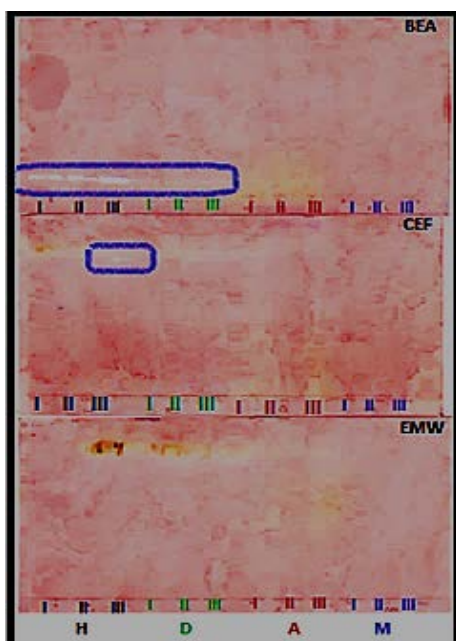
Figure 6.3: Bioautograms of *S. monteiroae* leaves extracts obtained by SEE method, separated in three eluent systems (BEA, CEF, and EMW), and sprayed with *M. smegmatis* and INT for microbial growth inhibition**.**

Table 6.3: The minimum inhibitory activity of *S. monteiroae* leaves extracts obtained by SEE method.

Solvents	MIC (mg/mL)			Average
H I	2,5	2,5	2,5	2,5
H II	2,5	2,5	2,5	2,5
H III	2,5	2,5	2,5	2,5
D I	1,25	1,25	1,25	1,25
D II	1,25	0,63	1,25	1,04
D III	0,63	0,63	0,63	0,63
A I	1,25	1,25	1,25	1,25
A II	1,25	1,25	1,25	1,25
A III	0,63	0,63	0,63	0,63
M I	n/a	n/a	n/a	n/a
M II	n/a	n/a	n/a	n/a
M III	n/a	n/a	n/a	n/a

n/a= no activity, H = hexane, D = dichloromethane, A = acetone, M = methanol

6.3.5. Isolation of antimycobacterial compounds from hexane extract

6.3.5.1. The mass yield from the first open column chromatography

Open column chromatography was used to fractionate the hexane extract; the column was eluted with solvents of varying polarities as outlined in Table 6.1. The mass of the fractions was recorded and 70% hexane in ethyl acetate had a highest mass of 4.33 g, while the 100% hexane had a lowest mass of 0.02 g. The total mass obtained was 15.79 g for all fractions (Table 6.4).

Table 6.4: The mass (g) of *S. monteiroae* leaves hexane extract fractions collected from the first column chromatography with different solvents.

Elution solvent	Percentages (%)	Mass (g)
Hexane	100	0.02
Hexane: Ethyl acetate	90:10	0.06
	80:20	1.21
	70:30	3.33
	60:40	1.69
	50:50	1.38
	40:60	0.97
	30:70	0.80
	20:80	0.64
Ethyl acetate	100	0.37
Ethyl acetate: Methanol	90:10	0.39
	80:20	0.97
	70:30	0.64
	60:40	0.46
	50:50	0.30
	40:60	0.15
	30:70	0.13
	20:80	0.09
Methanol	100	0.21
Total		**13.79**

6.3.5.2. Phytochemical analysis of collected fractions

The collected fractions from column chromatography were analysed for their phytochemical finger print. The UV analysis of the TLC plates showed fluorescing compounds within the 20% hexane in ethyl acetate fraction and 100% ethyl acetate fraction in all eluent systems used, with varying Rf values of 0.2 in BEA, 0.9 in CEF and 0.96 in EMW (Figure 6.4 A). Vanillin reactive compounds were found to be more visible in the BEA eluent system (with a dominating band from 60% hexane in ethyl acetate to 100% methanol of the same Rf value of 0.2) followed by EMW and CEF had the least (Figure 6.4 B).
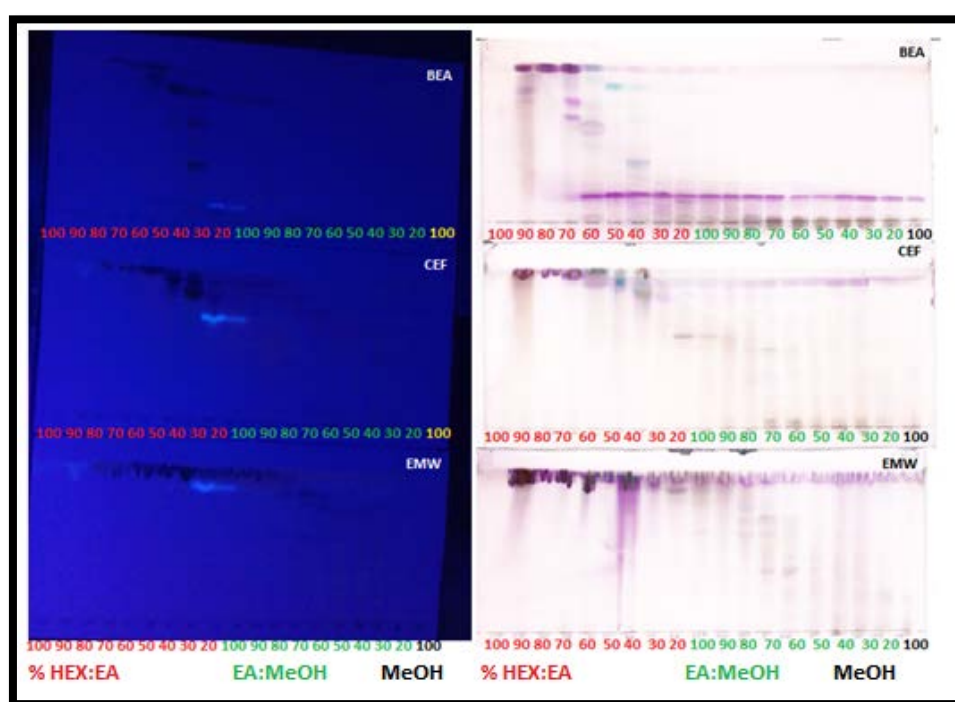

Figure 6.4: The TLC profile of *S. monteiroae* hexane leaves extract fractions separated in BEA, CEF and EMW. The plates were viewed under UV at 365 nm (A) and sprayed with vanillin-sulphuric acid reagent (B).

6.3.5.3. The antimycobacterial activity of the hexane fractions

The qualitative and quantitative antimycobacterial assays were conducted for the fractions collected from first open column chromatography. From the qualitative bioautography test, the antimycobacterial activity was observed mostly on the BEA eluent systems in the fractions from 60% hexane in ethyl acetate up to 100% methanol, with the same Rf value of 0.2 and within the CEF eluent system on the

fractions from 60% hexane in ethyl acetate up to 100% methanol with the same Rf value of 0.9, and EMW had very little antimycobacterial activity similar to the fractions of CEF (Figure 6.5).
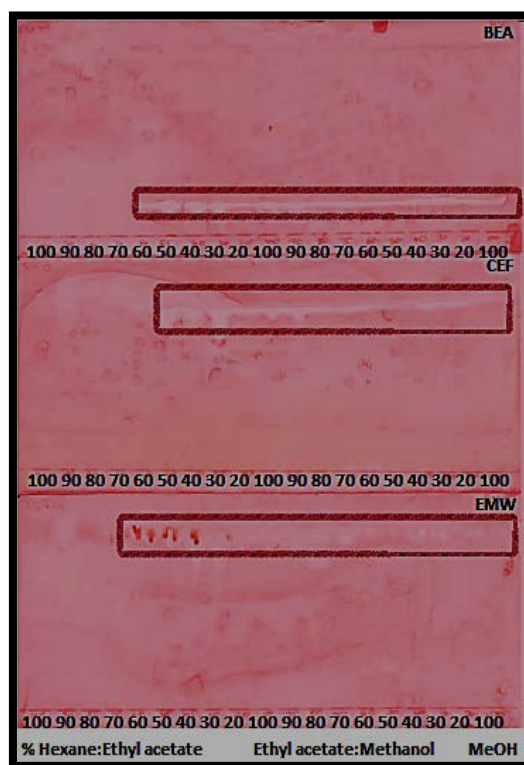

Figure 6.5: The Bioautograms of the leaves extracts of *S. monteiroae* hexane extract fractions, separated with the eluent systems: BEA, CEF and EMW, sprayed with *M. smegmatis* and INT for mycobacterial inhibition.

The minimum inhibitory concentrations of the fractions of the hexane extracts were determined using the micro-dilution method. The 50% hexane in ethyl acetate had the lowest concentration (0.21 mg/mL) inhibiting the mycobacterial growth, followed by 60% hexane in ethyl acetate (0.42 mg/mL), while the 100%, 90% and 80% of hexane in ethyl acetate had no antimycobacterial activity (Table 6.5). Generally, all the fractions had an average high MIC value of 1.02 mg/mL.

Table 6.5: The minimum inhibitory concentrations of the fractions of the leaves extracts of *S. monteiroae*.

Solvent	Percentage (%)	MIC (mg/mL)
Hexane	100	n/a
Hexane: Ethyl acetate	90:10	n/a
	80:20	n/a
	70:30	1,25
	60:40	0,42
	50:50	0,21
	40:60	0,26
	30:70	0,47
	20:80	0,84
Ethyl acetate	100	0,94
Ethyl acetate: Methanol	90:10	1,04
	80:20	1,04
	70:30	1,04
	60:40	0,84
	50:50	1,04
	40:60	1,67
	30:70	2,5
	20:80	2,5
Methanol	100	1,25

Key: recoloured values= noteworthy activity

6.3.5.4. Combination of fractions with bioactivity

The fractions that had antimycobacterial activity were combined to create 3 individual fractions in the following order: number 1 = 60 and 50% hexane in ethyl acetate; 2 = 40, 30 and 20% hexane in ethyl acetate; 3 = 100% ethyl acetate and 90, 80, 70, 60, 50, 40, 30, 20% ethyl acetate in methanol and 100% methanol. The combined fractions were dried and weighed (Table 6.6). Combination 3 had the most mass (3.7 g) while combination 1 had the least mass (2.4 g).

Table 6.6: The mass of combined fractions based on their antimycobacterial activity.

Fractions	Mass (g)	Total mass (g)
Combination 1	2.7	
Combination 2	2.4	8.8
Combination 3	3.7	

The combined fractions were analysed on TLC plates and treated with vanillin-sulphuric acid reagent to track the bioactive compounds. From the initial analysis of the extracts, the bioactive compounds within the plant extracts were noted to be purple in colour and not visible under UV light (Figure 6.6). The purple colour was observed in plates developed in BEA (Rf = 0.1) and CEF (Rf = 0.9) eluent systems.
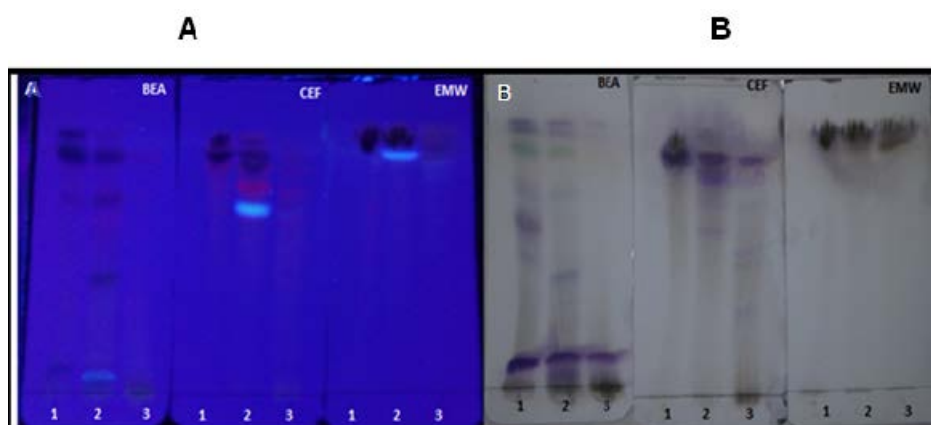

Figure 6.6: The TLC fingerprint of the combined fractions of the leaves extracts of *S. monteiroae* developed in three solvent systems and viewed under UV at 365 nm (A), then sprayed with vanillin-sulphuric reagent in methanol (B) for visualisation of compounds.

The combined fractions were analysed using different solvent combinations on TLC plates to find the eluent system that gave a desirable Rf value of 0.5 and based on the TLC finger print of 80% chloroform in ethyl acetate and 60% chloroform in ethyl acetate (Figure 6.7), 70% chloroform in ethyl acetate was selected for further fractionation.
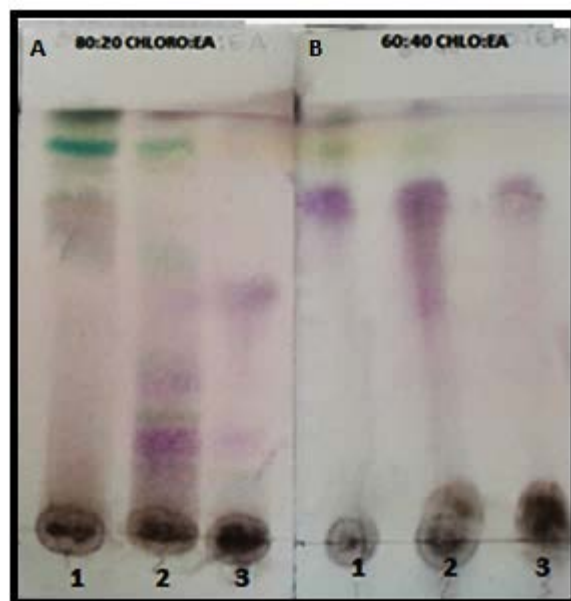
Figure 6.7: The chromatograms of *S. monteiroae* leaves hexane combined fractions developed in 80% chloroform in ethyl acetate (A) and 60% chloroform in ethyl acetate (B) and sprayed with vanillin-sulphuric acid reagent.

6.3.5.5. Second open column chromatography

The fractions obtained in section 6.3.4.1 that were combined (combination 1, 2 & 3), were mixed to make one target fraction that was eluted with 70% chloroform in ethyl acetate, the sub-fractions were collected in test tubes and selected tubes were analysed on TLC plates to trace the target purple compounds. The fractions in test tubes 178 to 196 and 229 to 235 were found to have traces of the purple compounds (Figure 6.8).
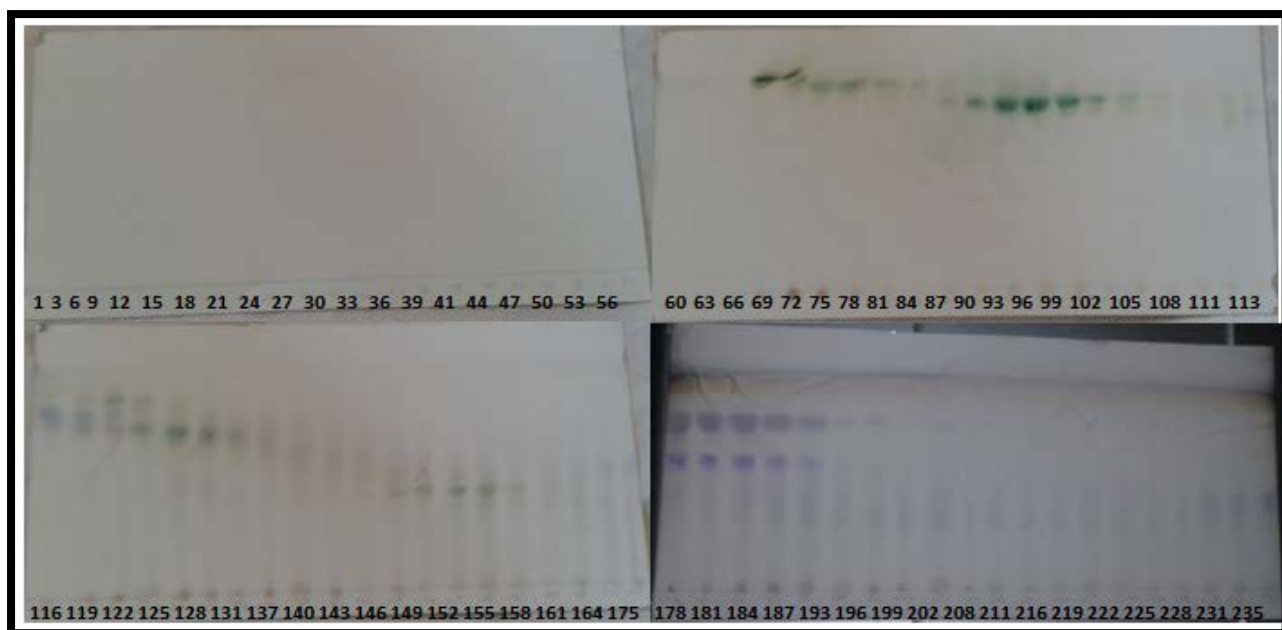
Figure 6.8: The chromatograms of *S. monteiroae* leaves fractions collected in test tubes, concentrated and analysed on TLC plates (developed in 70% chloroform in ethyl acetate) and sprayed with vanillin-sulphuric acid reagent.

6.3.5.6. Analysis of the fractions

The sub-fractions (from test tubes 178-196 and 229-235) were dried as two separate sub-fractions namely A and B and weighed (Table 6.7), and fraction A generated from tubes 178-196 had a highest mass (0.257 g), while fraction B was low with a mass of 0.026 g. These fractions were analysed on TLC plates for vanillin reactive compounds and also their bioactivity using TLC-bioautography, and fraction A was found to have significant antimycobacterial activity while fraction B had no antimycobacterial activity (Figure 6.9).

Table 6.7: The mass of fractions pooled together.

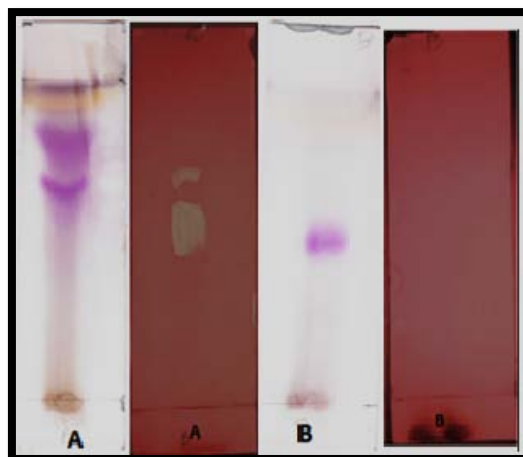| Sub-fraction	Mass (g)	Total mass (g)
A	0.257	
		0.282
B	0.026	

Figure 6.9: The chromatograms of the fractions pooled together make two fractions, analysed by spraying with vanillin-sulphuric acid reagent for purple compounds visualisation and also sprayed with *M. smegmatis* for antimycobacterial activity.

6.3.5.7. Third open column chromatography

Fraction A was subjected to the third column and analysed on TLC plates to track the purple compounds using vanillin-sulphuric acid reagent. On the very first set of collection (test tubes 1-73), the target compounds were observed and after subsequent analyses of sub-fractions, no purple coloured compounds were observed (Figure 6.10). The sub-fractions pooled together were divided into A1 (test tubes 1-13), A2 (test tubes 17-41) and A3 (test tubes 45-73) based on their TLC profile, and A3 was found to have a high mass of 0.1 g and all the sub-fractions had a total mass of 0.245 g (Table 6.8). The TLC profile and bioautography analysis of A1, A2 and A3 indicated that A1 and A3 had a similar profile and antimycobacterial activity, while A2 had two separated bands (Figure 6.11).
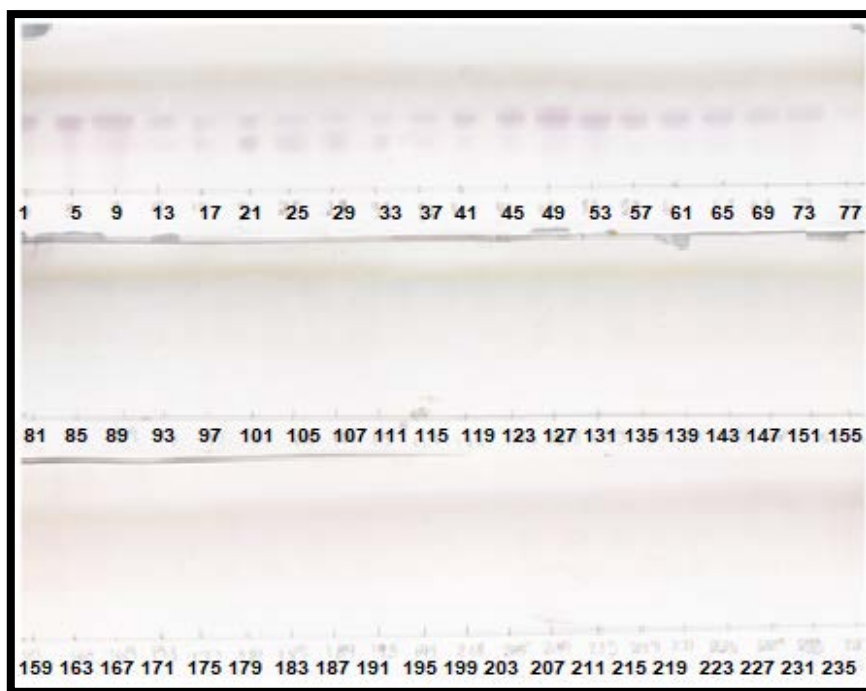
Figure 6.10: The TLC finger print of the sub-fraction A of the *S. monteiroae* leaves extract eluted with 70% chloroform in ethyl acetate in a column chromatography and sprayed with vanillin-sulphuric acid reagent.

Table 6.8: The mass of sub-fractions pooled together from fraction A in a column chromatography.

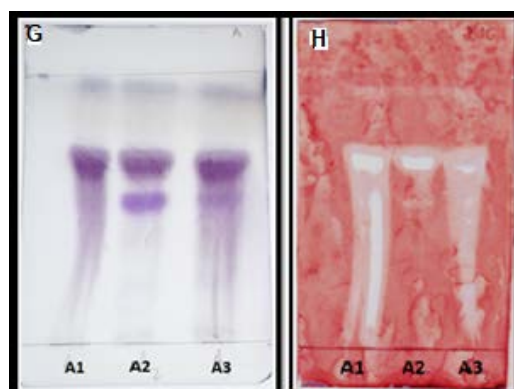| Fraction	Mass (g)	Total mass (g)
A1	0.0461	
A2	0.0984	0.245
A3	0.1	

Figure 6.11: The TLC profile (G) and Bioautograms (H) of sub-fraction of fraction A of the leaves of *S. monteiroae* developed in 70% chloroform in ethyl acetate and sprayed with vanillin-sulphuric acid reagent and *M. smegmatis* for antimycobacterial activity.

6.3.5.8. Prep-TLC

Sub-fractions A1 and A2 were further purified on preparative TLC and a combination of several compounds were observed for the scrapped compounds (A2) which were also purple in colour Figure 6.12K. These compounds were observed to have antimycobacterial activity (Figure 6.12 L). Sub-fraction A1 was found to be a pure smear as indicated on Figure 6.11.
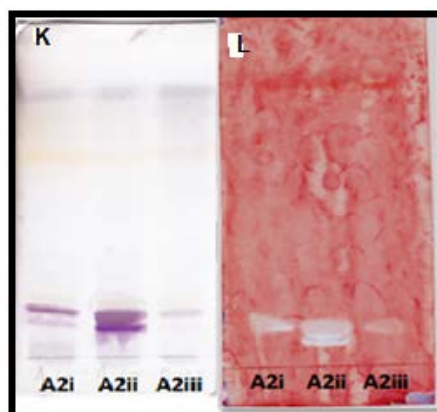

Figure 6.12: The TLC profile (K) and Bioautograms (L) after further fractionation of sub-fraction of fraction A2 of the leaves extracts of *S. monteiroae* developed in 70% chloroform in ethyl acetate and sprayed with vanillin-sulphuric acid reagent and *M. smegmatis* for antimycobacterial activity.

The flow diagram below (Figure 6.13) represents the isolation process of antimycobacterial compounds from the hexane leaves extracts of *S. monteiroae.* Bioassay guided fractionation of hexane extracts through a series of 3 open column chromatography was used to isolate compound with antimycobacterial activity and the isolated compound was identified using NMR.
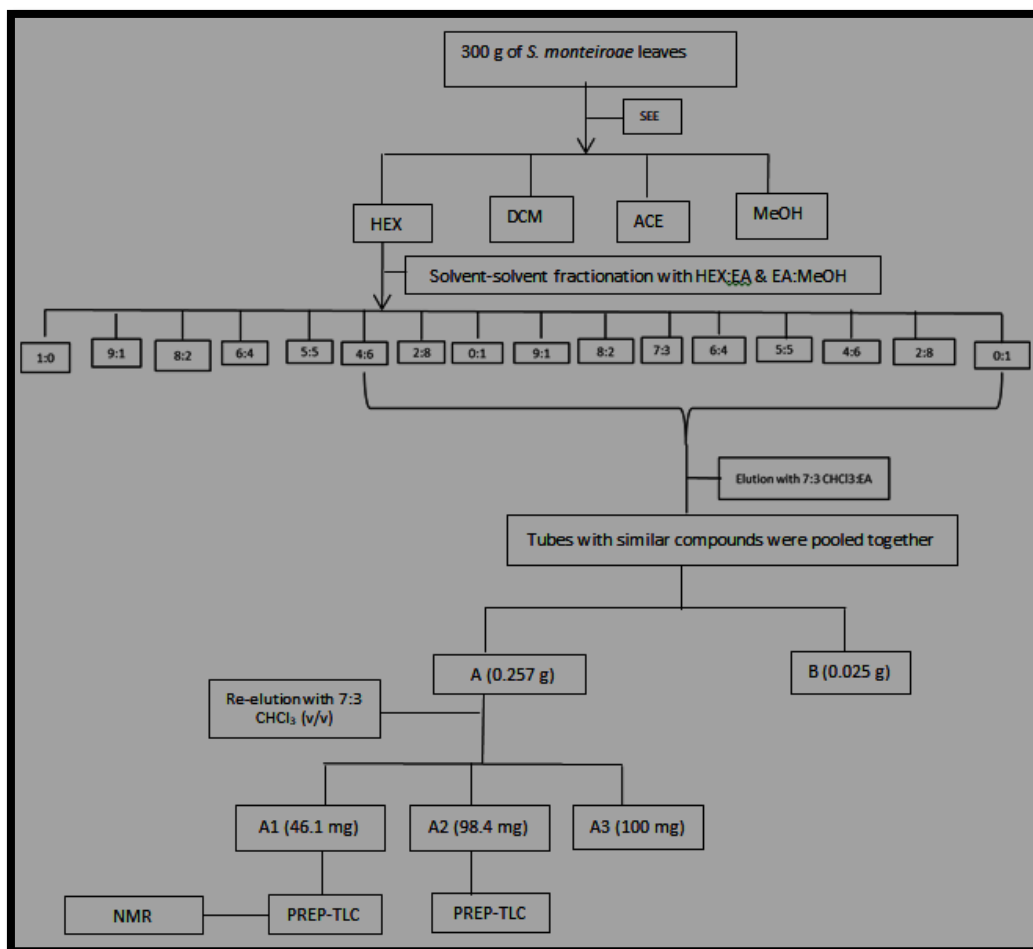

Figure 6.13: The flow diagram of the isolation process of antimycobacterial compound from *S. monteiroae* leaves.

6.4. Discussion

Medicinal plants consist of a large pool of potential therapeutic components against many ailments, which in research mostly require the isolation processes to acquire the active compounds (Atanasov *et al.*, 2015). The isolation of phytochemical compounds allows in-depth investigations of the bioactivity by identifying the responsible compounds and possible mechanisms of action. In traditional health care systems medicinal plants are usually used in combination to heighten the

biological activity of the extracts however, compounds within a plant may have either antagonistic effects or synergistic effects when applied together (Dzoyem *et al.*, 2016). Therefore, isolated compounds may or may not show the biological activity that was exhibited by the extracts. In this study the isolation process was guided by the antimycobacterial activity of the extract and it began with serial exhaustive extraction to allow maximum extraction of the compounds within the plant material. A high mass was obtained from methanol extract (35.5 g) followed by hexane extract (16 g) while acetone and dichloromethane had lower masses (2 g). These extracts were analysed for their TLC profile visualised by UV light and vanillin-sulphuric acid reagent. More compounds were observed with the CEF developed TLC plates under UV light, while visualisation with vanillin-sulphuric acid reagent, more compounds were observed in the BEA eluent system.

Antimycobacterial activity of the SEE extracts was observed on the hexane extracts only, however their MIC values were found to be higher than those of acetone and dichloromethane extract, and methanol extract had no antimycobacterial activity. In a previous study (Leon-Diaz *et al.,* 2010). A hexane extracted compound Licarin A from a medicinal plant *Aristolochia taliscana* was found to have even lower inhibitory concentrations against 12 clinical isolates of *M. tuberculosis,* which support our findings, since hexane extracts had antimycobacterial activity and also initial findings were indicative of extracts from non-polar eluent system having microbial growth inhibition. Licarin A is a lignan compound which falls under polyphenols group that in fighting diseases act by destabilizing the cytoplasmic membrane or by inhibiting synthesis of extracellular membranes of bacteria (Saleem *et al.*, 2005). Based on bioautography results the hexane extract was selected for further fractionated in column chromatography, and after fractionation of the hexane extract (16 g) a total mass of 13.79 g was recovered and individual fractions had lower masses with 70% hexane in ethyl acetate having a higher mass than other extracts.

The TLC analysis of the fractions with visualisation by UV light and vanillin-sulphuric acid reagent indicated some UV reactive compounds on the fractions of 20 and 30% hexane in ethyl acetate for the 3 solvent systems used, while vanillin sprayed plates indicated more compounds in the BEA solvent system and EMW had the least. Antimycobacterial analysis of the fractions with bioautography indicated a similar

activity for CEF and EMW solvent systems with Rf values of 0.9 while the fractions (from 60% hexane in ethyl acetate up to 100% methanol) that had antimycobacterial activity in CEF and EMW also had the activity in BEA with an Rf value of 0.2. These variations are due to the affinity of the compounds for the mobile systems used based on the polarity, i.e. compounds with polarity similar to the solvent system in use, they will move with the solvent, while those which have polarity that varying polarity to the solvent system will tend to have little movement. The lowest inhibitory concentration for these fractions was observed to be 0.21 mg/mL which was higher than any antimycobacterial drugs used. The observed bands indicating compounds with bioactivity were noted to be purple in colour from the TLC profile of the fractions. The active fractions were combined to give 3 individual fractions and fraction 3 had more mass (3.7 g) which was expected since more fractions were combined. The combined fractions were analysed on TLC plates to tract the purple compounds and had similar profile and were therefore mixed to create one major fraction.

Column chromatography was used to further fractionate the fraction to remove other non-active components. After elution the test tubes with traces of purple compounds were pooled together to give sub-fractions A and B, which after weighing gave a total mass of 0.282 g. Sub-fraction A and B were analysed on TLC for their profile and antimycobacterial activity and sub-fraction A had two bands with bioactivity while B had one band which had no bioactivity. Therefore, sub-fraction A was further separated into A1, A2 and A3, and analysed on TLC plates for both their bioactivity and phytochemical fingerprint. Similar bands/smear of compounds was observed on A1 and A3 sub-fractions with the Rf value of 0.7, while A2 appeared have two bands that were finely separated (Rf values of 0.5 and 0.7). The antimycobacterial activity was also observed to be similar for A1 and A3, while A2 appeared to have one active band. Sub-fraction A1 was observed as a whitish and oily powder after fractionation, and it was purified on prep-TLC and taken for NMR analyses, while A2 was subjected to prep-TLC also to remove other non-active bands. After prep-TLC more bands of compounds were observed indicating that the fraction was not pure and had lower mass, which could not be taken for NMR analysis. Compounds that are closely related maybe difficult to separate and slower rates on column chromatography can help further separation. The challenge with isolation of the compounds with antimycobacterial activity was that the compounds were from a

hexane extract, and hexane is well known to remove fats from plant extracts, which contributed to the isolated compounds appearing as smears and not distinctive bands (Potty, 2009).

Bioassay guided fraction has been used previously in the isolation of antimycobacterial compounds from other plants such as *Curtisia dentata* and *Aloe vera* (Chandran *et al.*, 2017; Fadipe *et al.*, 2017) *Curtisia dentata* is a medicinal plant used in the treatment of TB, sexually transmitted diseases and stomach ailments. Three compounds were isolated from *Curtisia dentata* namely: β sitosterol, betulinic acid, ursolic acid and lupeol and were found to be active against *M. tuberculosis* (Fadipe *et al.*, 2017). *Aloe vera* has been investigated through bioassay guided fractionation and the isolated compound was observed to have a noteworthy activity against *M. smegmatis* (Chandran *et al.*, 2017). Anti-TB compounds from another medicinal plant *Lantana hispida* was also isolated using bioassay guided fractionation from a hexane extract and it was effective against *M. tuberculosis* H37Rv (Jiménez-Arellanes, *et al.*, 2007). These reports indicate the usefulness and effectiveness of bioassay-guided fractionation in column chromatography for isolation of bioactive compounds. In this study bioassay-guided fractionation was employed to isolate antimycobacterial compounds and 1 compound was successfully isolated and identified.

6.5. Conclusion

The isolation of antimycobacterial compounds was successful with the use of column chromatography, and for the first time here we report the isolation of bioactive compounds from *S. monteiroae* leaves extract. The isolated compound has a potential in the development of anti-TB drugs, due to the antimycobacterial activity that was observed through the isolation process, hence the following chapter focused on identification of the isolated compound that had antimycobacterial activity.

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CHAPTER 7

7. Structure elucidation using NMR spectroscopy

7.1. Introduction

The biological activity observed when medicinal plants are evaluated is mainly due to secondary metabolites such as terpenoids, steroids, alkaloids, phenols and flavonoids (Das *et al.*, 2010). Structural elucidation is a crucial process in evaluating the biological activity of the natural compounds as it is well-known that biological activity depends to a large extent on the 3-Dimensional (3-D) arrangement of functional groups on the molecules (Ngobeni, 2012). Compounds isolated from medicinal plants must be identified and characterised using techniques such as Fourier-transform infrared spectroscopy (FTIR), mass spectrometry (MS) and nuclear magnetic resonance (NMR) (Pauli *et al.*, 2005).

Nuclear Magnetic Resonance-spectroscopy is a useful tool in structure elucidation of unknown natural or synthetic compounds; it also provides information about the quantitative and qualitative properties of the compounds. The detailed information about structure, chemical environments and reaction state of the molecules can be determined (Halabalaki *et al.*, 2014). One dimensional NMR include proton ^{1}H, Nitrogen and carbon-13, while 2 dimensional NMR correlation spectroscopy are correlation spectroscopy (COSY), Total correlation spectroscopy (TCOSY), distortionless enhancement by polarization transfer (DEPT), nuclear overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), and Heteronuclear multiple-bond correlation spectroscopy (HMBC) (Halabalaki *et al.*, 2014). These techniques allow identification of neighbouring substituents of the observed functional groups. Three dimensional and 4 dimensional NMR-spectroscopy are complex methods which suppresses or amplify specific types of resonances and are usually used in protein NMR (Pauli *et al.*, 2005).

Mass spectrometry is also a powerful technique that can be used to determine the molecular weight of compounds. The process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances of each ion type (Bouslimani *et al.*, 2014). Mass spectrometry is much more sensitive than NMR but

requires inclusion of other separation techniques due to low molecular weight diversity of metabolites and is limited to detecting metabolites that readily ionize (Sparkman, 2000). After identification and characterisation of the bioactive compounds, their biological activities and cytotoxic effects must be determined before proceeding to *in vivo* studies. Once the compounds have been chemically defined then laboratory synthesis, analogues and derivatives as well as mechanisms of action may be determined. In this study NMR was used to determine the structure and characterise the isolated compounds.

7.2. Material and methods

7.2.1. Identification of the isolated compound using NMR

The isolated compound from *S. monteiroae* leaves extract was sent to the Chemistry Department, University of Limpopo for NMR analysis. The sample (compound A1) was dissolved in deuterated chloroform (Chloroform-d) and characterised using ^{1}H NMR and ^{13}C NMR spectra. Samples were run using 400 MHz NMR Spectrometer (Bruker) at 400 MHz, with chloroform-d as a reference signal solvent. Prof Mazimba O, of Botswana International University of Science and Technology assisted with the analysis of the NMR spectrums and structure elucidation of the compound.

7.3. Results

Bioassay-guided fractionation of the hexane leaves extract led to the isolation of one compound, and the NMR spectral analysis of this compound is shown ie. ^{1}H NMR (Figure 7.1), ^{13}C NMR (Figure 7.2), gCOSY (Figure 7.3), DEPT-NMR (Figure 7.4). Compound A1 was identified as a sitosterol derivative 8,9-dehydro-4-methyl-24-vinylobtusifoliol (Figure 7.5).
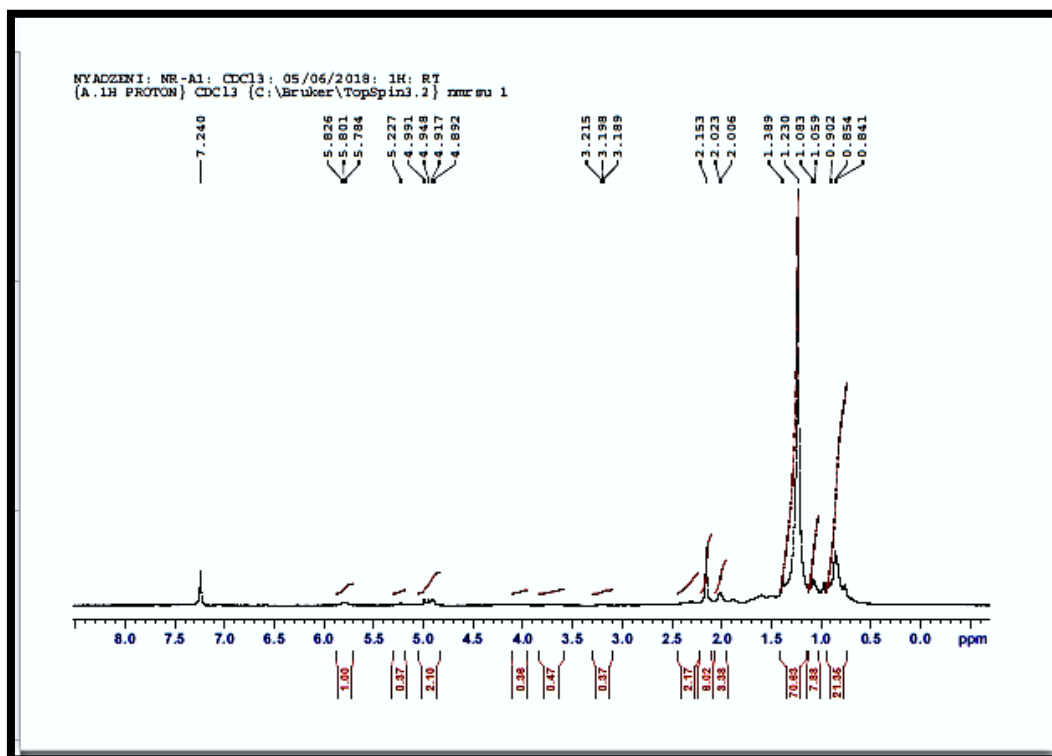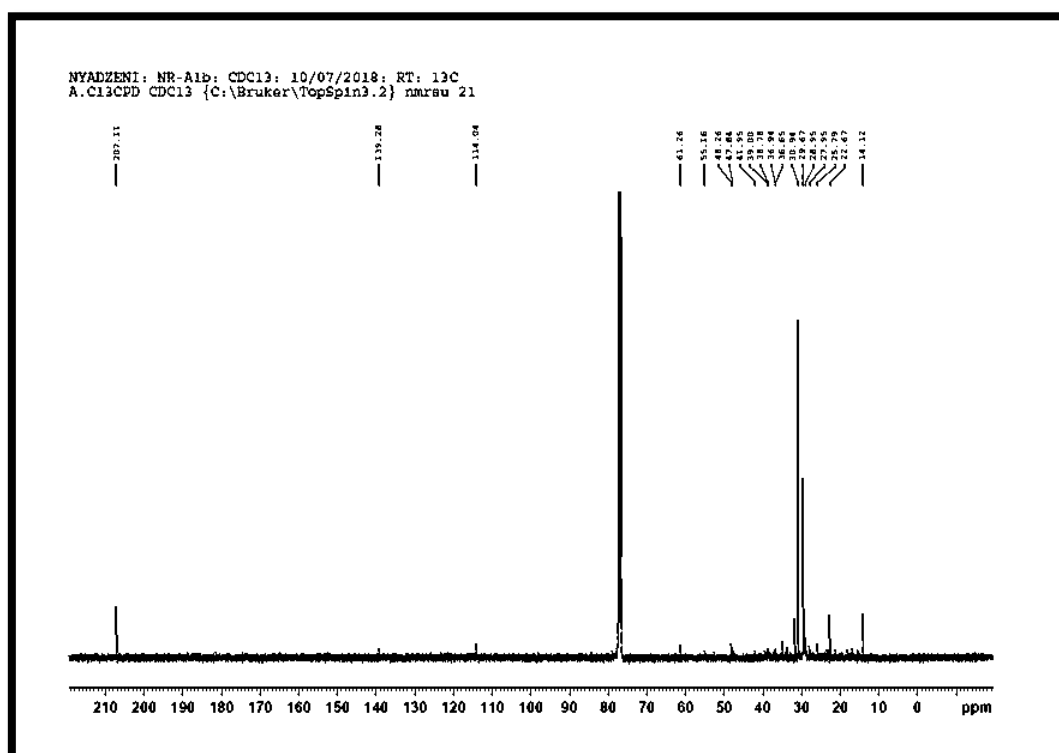
Figure 7.1: ^{1}H NMR spectrum of Compound A1

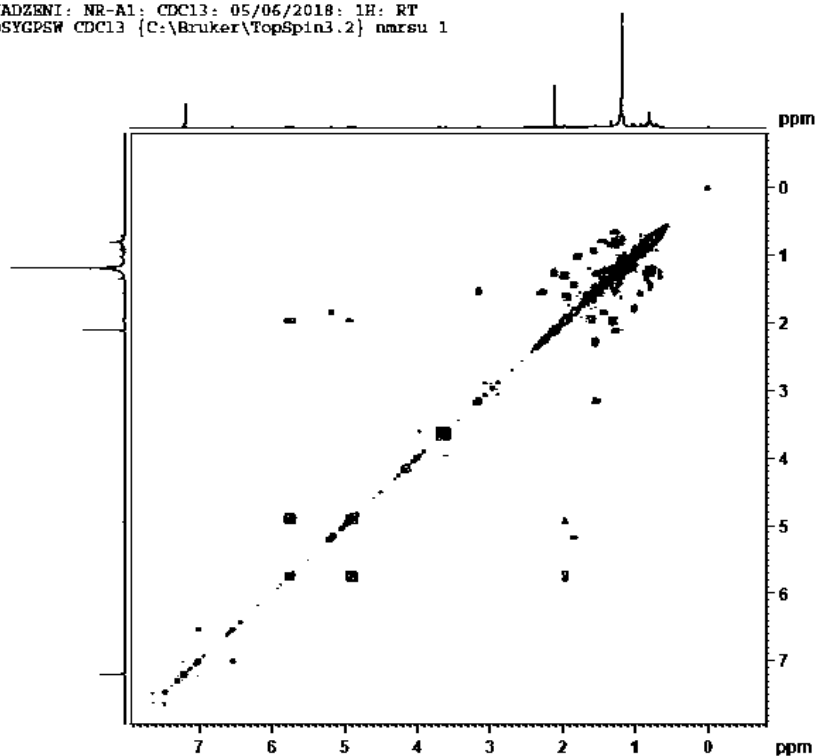**Figure 7.2:** ^{13}C NMR spectrum of the isolated compound A1.

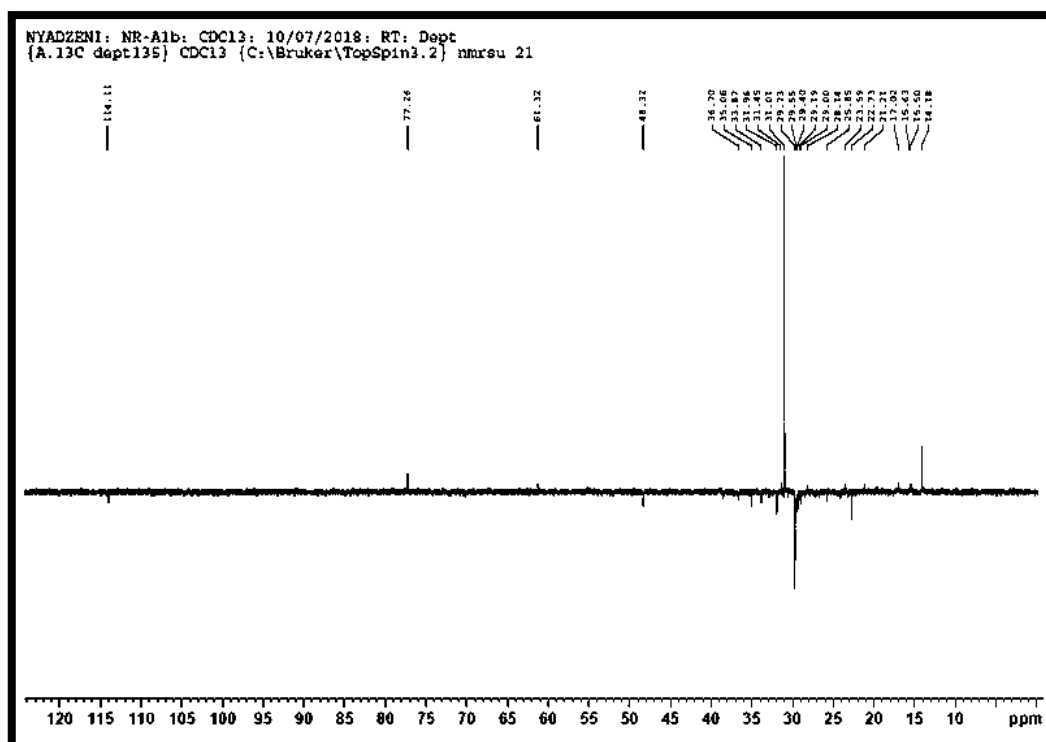**Figure 7.3:** gCOSY spectrum of isolated compound in CDC13.

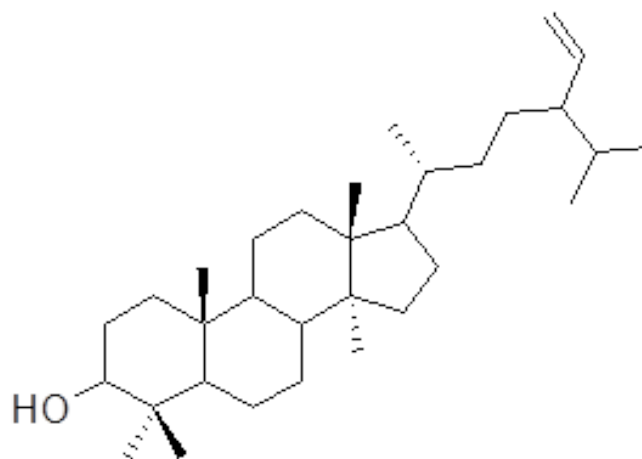**Figure 7.4:** 13C DEPT-NMR spectrum of compound A1.

Figure 7.5: The structure of 8,9-dehydro-4-methyl-24-vinylobtusifoliol isolated from the leaves of *S. monteiroae.*

7.4. Discussion

The NMR spectroscopic technique is useful in identifying new compounds especially those of plant origin, for example *Aloe vera* is used in the ayuverdic medicinal system to treat cough, colds, piles, debility, dyspnoea, asthma and jaundice (Chandran *et al.*, 2017). Compound isolated from *A. vera* was effective against *M. smegmatis* and found to be aloverose isolated and identified using NMR (Chandran *et al.*, 2017). Bioassay-guided fractionation in column chromatography was used to isolate the compound with antimycobacterial activity from the leaves extract. Which through NMR analysis was identified as a sitosterol derivative 8,9-dehydro-4-methyl-24-vinylobtusifoliol ($C_{32}H_{56}O$) that has an unusual vinyl group at the regions of carbon 24, 31 and 32.

Sitosterols are a type of phytosterols that have been derived from plants, their structure is similar to that of cholesterol and are usually used in the treatment of hypercholesterolemia and synthesis of steroid hormones (Lei *et al.*, 2017). Several studies have reported that sitosterol compounds are difficult to isolate to pureness, because they often have the same Rf value and difficult to them separate even when using different solvents. These include stigmasterol, beta sitosterol and campesterol (Farlane, 1972). It was interesting to note that in the screening of phytochemical constituents' steroids compounds were not detected, this may be due to lower concentrations of the compounds in the crude extracts. The isolated compound had

good activity towards *M. smegmatis* on bioautography, however due to lower yields of the isolated compound, further bioactivity evaluations could not be carried out.

7.5. Conclusion

The bioactivity of the isolated compound was noteworthy when tested against *M. smegmatis* with bioautography assay. In this study, we report for the first time the isolation of a beta-sitosterol derivative from *S. monteiroae* leaves extract. The isolation of the beta-sitosterol compound lays a foundation for further investigation of its activity against tuberculosis and development of antiTB medication; hence it is recommended that further investigations of the cytotoxic effects and *in vitro* antiTB of the compound be carried out.

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CHAPTER 8

8.1. General discussion

The potential therapeutic value of *S. monteiroae* was evaluated through phytochemical, antioxidant and antimycobacterial activity analysis. The phytochemical constituents of different plant parts were found to vary from plant part to plant part while common phytoconstituents such as flavonoids, tannins, saponins, and terpenoids were detected. The concentrations of phytochemical compounds may be affected by season of collection, climate changes and other environmental conditions, which could be the reason why the other phytochemical compounds were not detected. Thin layer chromatography indicated that more compounds are non-polar, since numerous bands were observed in plates developed in the BEA eluent system. The TLC profile of phytochemicals helps in identifying possible leads of compounds that may be targeted for isolation.

Polyphenols in plant extracts are known to have therapeutic effects such as antioxidant, antimicrobial and anti-inflammatory activity. The total phenol, tannin and flavonoids were quantified and the leaves extracts were found to have abundant of all these phytoconstituents while the flavonoids were low in content within all the extracts analysed. Oxidative stress due to mycobacterial infections may result in suppression of the immune system of the infected individual causing the active diseases TB, hence the antioxidant activity of *S. monteiroae* was evaluated. Different plant parts analysis indicated that *S. monteiroae* had a low antioxidant potential both qualitative and quantitative. The leaves extracts of *S. monteiroae* had better antioxidant activities when compared with other plant parts, which was further elaborated by the DPPH free radical scavenging activity and by ferric reducing power that were found to be higher than other plant parts analysed. The antioxidant potential of the leaves is due to the phytoconstituents detected and quantified i.e. phenols, flavonoids and tannins.

The antimycobacterial activity of selected plants was determined using bioautography and micro dilution method, and from initial analysis the leaves had antimycobacterial activity through analysis by qualitative test. The quantitative test of antimycobacterial activity using *M. smegmatis* indicated that the roots had lower MIC values followed by the leaves. The MIC determination method has been noted to

have short comings due to lower volumes and dilution of the extracts while the bioautography method involves the use of higher extract concentrations, other factors include the synergistic effects of compounds when applied together and volatile compounds that may be absent in bioautography since the plates are dried for up to 5 days under a fan. Higher inhibitory concentrations were observed when extracts were tested on TB causing pathogens comparing with positive controls, which may be due to the composition of the cell wall of *M. tuberculosis* causing resistance of the penetration of the extracts. To rule out cytotoxicity of the extracts towards the test organism and the mammalian cells, MTT assay with Vero monkey kidney cells was used to assess the cytotoxic effects of the extracts. Plant parts analysed for cytotoxicity were found to be non-cytotoxic towards Vero cells which indicated the safety of the plant extracts towards mammalian cells. The leaves extracts were the most non-cytotoxic with a high LC_{50} of 1 mg/mL. The relative safety margin was determined because these plant parts are used in the treatment of TB traditionally hence their safety is important. The leaves extracts were found to be non-toxic, which indicated that the relative safety margin (SI) was large, this allows for large amounts of the leaves to be included in the treatment without causing toxic effects. Based on the qualitative results of antimycobacterial activity on bioautography and low cytotoxic effects, the leaves were selected for isolation of bioactive compounds.

The SEE method was used to extract the leaves of *S. monteiroae* and a high mass of phytochemical compounds was observed for methanol and hexane. Phytochemical analysis of these extracts indicated that more of the non-polar compounds were present, consistent to the initial analysis where the leaves extract had more compounds observed in the BEA eluent system. It was also observed that the leaves extracts had antimycobacterial activity on the hexane extracts only in bioautography. However, these results were contradicting with the MIC values, were acetone and dichloromethane had better minimum inhibitory concentrations, which may be due to hexane extract being concentrated with fats hence unable to dissolve well in the solvent. The hexane extract was further analysed on column chromatography to isolate compounds with antimycobacterial activity and a mass recovery of 13.79 g was obtained from a 16 g extract. Phytochemical analysis on TLC indicated that BEA eluent system separated more compounds, which was

expected since the extract used was that of a non-polar solvent. The antimycobacterial activity was observed for all the solvent systems used, and the inhibitory concentration not correlating with the bioautography results. Further fractionation, antimycobacterial analysis and purification on pre-TLC led to the isolation of a whitish powder that in deuterated chloroform appeared oily and partitioned into two phases. The isolated compound was identified using NMR and it was found to be a beta-sitosterol derivative with an unusual vinyl group. Other compounds from medicinal plants with antimycobacterial potential have been isolated previously. In this study we reported for the first time the phytochemical composition of different plant parts of *S. monteiroae,* its antioxidant activity and antimycobacterial activity. Preliminary analysis of this plant paved a path for the isolation of an antimycobacterial active compound from the leaves extract of *S. monteiroae.* The inhibitory properties of *S. monteiroae* leaves extract indicate its potential in the development of therapeutic medication against TB and related symptoms.

8.2. Conclusion

This study indicated that *S. monteiroae* possess considerable antimycobacterial properties. It also validates the use of this plant in traditional medicine in the treatment of tuberculosis and related symptoms. This study demonstrated different phytochemical compounds composition of different plant parts of *S. monteiroae,* which are also related to the biological activities observed. The aims of this project were achieved and it is recommended that further study be carried out to assess the antimycobacterial activity of the isolated compound on TB causing pathogens and its cytotoxic effects.