Isolation of antimicrobial and antioxidant compounds from two mistletoes (*Viscum rotundifolium* and *Tapinanthus oleifolius*) and synergistic effects with their hosts

By

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RESEARCH DISSERTATION

Submitted in fulfilment of the requirements of the degree of

MASTER OF SCIENCE

In

MICROBIOLOGY

In the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Molecular and Life Sciences)

At the

UNIVERSITY OF LIMPOPO

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2021

Declaration

I, Mutshidzi Patience Malada, declare that the dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Microbiology has not previously been submitted by me for the degree at this or any other University, and that this is my own work in design and execution. All the materials contained therein have been duly acknowledged.

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Malada, MP (Ms) Date

Dedication

I dedicate this work, firstly, to my mother, Johanna and my late father, Joseph; secondly, to my daughter, Unarine, and my siblings, Rolivhuwa, Faith and Andrea.

Acknowledgements

I thank the Almighty God for giving me the strength and courage to complete my research project.

I also thank Prof. Peter Masoko (Supervisor) and Ms. Mary Mogashoa (Co-Supervisor) for their scientific guidance, encouragement and motivation throughout the project.

My sincere gratitude is extended to Ms. Refilwe Kudumela and Mr Mash Matotoka for their assistance in the laboratory, and Ms. Kholofelo Malemela for her assistance with cytotoxicity.

I would also like to thank Ms. Tselane Ramakgadi for assistance with NMR analysis, and Prof. Ofentse Mazimba at Botswana International University of Science and Technology, for assistance with structure elucidation of the isolated compound.

I acknowledge my mother, family and partner for their prayers, love, encouragements and support.

Thank you to all the people who helped me during my project who have not been mentioned by name here.

Lastly, I thank the University of Limpopo and National Research Foundation for providing financial support to this project.

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List of abbreviations

^{13}C	Carbon- 13
^{1}H	Hydrogen- 1
A	Acetone
BEA	Benzene: Ethanol: Ammonium hydroxide (18:2:0.2)
CEF	Chloroform: Ethyl acetate: Formic acid (10:8:2)
E	Ethyl acetate
DC	*Dichrostachys cinerea*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2- diphenyl-1-picrylhydrazyl radical
EMW	Ethyl acetate: Methanol: Water (40:5.4:5)
GAE	Gallic acid equivalence
g	Gram
H	n-Hexane
INT	p- iodonitrotetrazolium violet
M	Methanol
MA	*Mystroxylon aethiopicum*
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
QE	Quercetin equivalence
R_f	Retardation factor
ROS	Reactive oxygen species

rpm	Revolutions per minute
TB	Tuberculosis
TLC	Thin Layer Chromatography
TO	*Tapinanthus oleifolius*
UV	Ultra violet
VR	*Viscum rotundifolium*
W	Water
WHO	World Health Organization

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Conference

Malada, M.P., Mogashoa, M.M. and Masoko, P. "Evaluation of antimicrobial and antioxidant activity of *Viscum rotundifolium* and *Mystroxylon aethiopicum* leaf extracts". Presented during the 10th annual Faculty of Science and Agriculture Postgraduate Research Day held at Protea Hotel The Ranch Resort, Polokwane, Limpopo, South Africa, September, 2019.

Manuscript

Malada, M.P., Mogashoa, M.M. and Masoko, P. "The evaluation of antimicrobial activity, antioxidant activity and synergistic effects of mistletoe (*Viscum rotundifolium*) and its host (*Mystroxylon aethiopicum*)". Submitted to BMC Complementary and Alternative Medicine and Therapies (BCAM-D-20-00545).

Abstract

The aim of the study was to isolate and characterise the antibacterial and antioxidant compounds from the leaf extracts of the two mistletoes (*Viscum rotundifolium* and *Tapinanthus oleifolius*) and to determine the synergistic effects of the plants with their hosts (*Mystroxylon aethiopicum* and *Dichrostachys cinerea*). The leaves of the selected plants were collected, dried and ground into fine powder. The powdered plant leaves were extracted using n-hexane, ethyl acetate, acetone, methanol and water. The qualitative phytochemical analysis was done using standard chemical tests and thin layer chromatography. The total phenolic, tannin and flavonoid content were estimated using spectrophotometric methods. The qualitative antioxidant activity was determined using 2, 2-Diphenyl-1-pycrylhydryzyl (DPPH) free radical scavenging assay on thin layer chromatography and quantitative antioxidant activity was determined using colorimetric DPPH assay and ferric reducing power assay. The antibacterial activity of extracts was tested against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa* using bioautography and serial broth micro-dilution assay. The cytotoxic effects of the plant extracts were determined using cell viability assay. The active compounds were extracted using serial exhaustive extraction and isolated using the bioassay-guided fractionation and then purified using thin layer chromatography and open column chromatography. The pure compound was identified using the NMR and mass spectroscopy. Methanol was the best extractant with the highest percentage yield. The distinct fluorescing compounds were observed on the CEF and EMW mobile phase. The non-fluorescing compounds detected with vanallin-sulphuric acid spray reagent showed that *V. rotundifolium*, *T. oleifolius* and *D. cinerea* have more polar compounds while *M. aethiopicum* have more non-polar compounds. All the plants revealed the presence of terpenoids, flavonoids, phlobatannin, tannins steroids and cardiac-glycosides and the absence of alkaloids and saponins. The n-hexane extract of *T. oleifolius* was significantly high in flavonoid content (34.801±0.798 mgQE/g of extract) and tannin content (15.367±0.320 mgGAE/g of extract) whereas the ethyl acetate extract of *M. aethiopicum* was high in phenolic content (893.210±3.016 mgGAE/g of extract). The results indicate that the compounds that exhibit antioxidant activity are non-polar to polar, which was confirmed by quantitative tests. *M. aethiopicum* showed activity against all tested bacteria while *V. rotundifolium* only had activity against *E. faecalis*

whereas *T. oleifolius* and *D. cinerea* did not have any activity. The quantitative antibacterial test confirmed the activity of the plant extracts where the MIC values ranged from 0.04-2.5 mg/mL. The combination of *V. rotundifolium* and *M. aethiopicum (*n-hexane, ethyl acetate and acetone extracts) and *T. oleifolius* and *D. cinerea* (n-hexane, acetone and methanol extracts) showed synergistic effects in inhibiting the growth of *S. aureus* whereas the methanol extract of *T. oleifolius* and *D. cinerea* showed antagonistic effects in inhibiting the growth of all tested bacteria. The cell viability assay indicated that acetone extracts of all plants were non-toxic on the human liver (C3A) cells. *M. aethiopicum* was selected for isolation and purification of bioactive compounds. The bioassay-guided fractionation led to the isolation of oleanolic acid acetate. This study demonstrated that the selected plants have antibacterial potential that is ascribed to the phytochemicals present. Further studies including *in vivo* assays are needed in order to support their use in traditional medicine.

Chapter 1: Introduction

1.1. Introduction

The increasing incidence of microbial infections and antibiotic resistance has fostered the generation of new antimicrobial agents. Infectious diseases are among the top causes of death worldwide (WHO, 2018) and a total of 17 million people die annually from bacterial infections (Butler and Buss, 2006). Humans initially depended on plants for their basic needs, including medicine (Gurib-fakim, 2006). The medicinal plant, according to the World Health Organisation (WHO) (2002), is defined as a plant that has organs that contain substances that are useful for therapeutic purposes. Traditional medicinal plants constitute an important element of the indigenous medical system around the world. This involves the ancient and culturally-based health care practices that are passed on verbally from generations (van Wyk *et al*., 2000; Kong *et al*., 2003).

The development of modern health care systems placed an extensive threat to traditional health practices due to their speedy therapeutic effect. As a result, the traditional health practices were underrated, which led to their disappearance. However, due to the increase in population growth, poor supply of synthetic drugs, high costs of treatments, unfavourable side effects, and the development of antibiotic resistance, there was a resurgence of dependence on traditional and complementary or alternative medicine (Karunamoorthi, 2012). About 60% of the population in South Africa consults traditional health practitioners since traditional herbal medicine is affordable, readily available and contains fewer side effects (McGaw and Eloff, 2008). In addition, the medicinal plants are collected by traditional healers and sold in shops and informal markets (Rasethe *et al.,* 2019).

The use of medicinal plants that have been gathered over the years has empowered scientists to isolate different drugs such as aspirin, morphine, and quinine (Butler, 2004). In addition, studies carried out on plants around the world have revealed that medicinal plants contain bioactive secondary metabolites that exhibit various activities such as antimicrobial, antifungal, anticancer and anti-inflammatory properties (Verpoorte, 2000). It is estimated that about 25% of the drugs approved are derived from secondary metabolites from plants (Newman and Cragg, 2016).

The increasing demand for traditional herbal medicine consequently brought challenges with regards to the efficacy, quality, safety, and standardisation of plants. The levels of sanitation, hygiene and living conditions for most of the African people are not proportional to those of developed countries (Taylor *et al*., 2001; Chikezie, 2015). The problem is also aggravated by the lack of proper health care facilities and where these exist, the majority of the population cannot afford to pay for conventional medicines (Matu and van Staden, 2003). In most cases, very little scientific information about the safety, active ingredients and toxicity of such indigenous medicine are available. Therefore, there is a great need for scientific and clinical research to investigate the quality, safety, and efficiency of these herbal therapies (Phillipson, 2001).

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Chapter 2: Literature review

2.1. Medicinal plants

Plants have been used as a source of medicine in traditional healing since ancient times, despite their use as source of food for both animals and humans (Cragg and Newman, 2013; Arulmozhi, 2018). They continuously serve as a source of new therapeutic remedies (Pendota *et al*., 2017). Several scientific research studies have reported that medicinal plants contain a variety of secondary metabolites such as phenols, saponins, alkaloids, and anthocyanins tannins that are highly efficient in treating bacterial infections (McGaw and Eloff, 2008).

It is estimated that about 250 000- 500 000 plant species exist in the world and about 35 000- 70 000 plant species are used as medicine which corresponds to 14- 28% (Verpoorte, 2000; DeLuca *et al*., 2012). Today, over 80% of the population in developing countries depends on herbal traditional medicine because they are readily available and cost-effective since the people are poor and cannot afford the high cost of pharmaceutical drugs although a few are in search of natural products with little or no side effects (Mann *et al*., 2008; Maroyi, 2013).

2.2. The use of medicinal plants in traditional healing

Traditional health practitioners use herbaceous plants, shrubs and climbers to treat infections. The research conducted by Nguyan *et al*. (2014) indicated that 51.5% of herbaceous plant species are the main source of medicine used by the K'ho people due to their abundance. Traditional medicine can be classified into two groups based on the type of therapy required. The first group is the traditional medicine that involves the use of herbal medicine, animal parts and minerals. The second group is the secondary medicine which is the traditional non-medication practice that involves different techniques that exclude the use of medications; this includes, spiritual, mind and body therapy and other related techniques (WHO, 2002).

The different plant parts that are used to treat ailments include: roots, leaves, stems, bulbs, flowers, seeds, fruits, bark, twigs and the whole plant (Tiwari, 2008). These plant materials are prepared in various ways as combinations or single plant parts which include: decoction (boiled teas), infusion (hot teas), concoctions, tinctures

(alcohol and water extracts), macerations (cold-soaking), syrups and paste. In addition, other plant preparations include inhalation of powdered plant parts, steam inhalation of different aromatic plants boiled in water and preparation of plants in hot baths in which the patient is either soaked or bathed with it (Bone and Mills, 2013). The various medicinal plants that have been used in the treatment of infections include *Dissortis perkinsiae* leaves used in Nigeria, Togo and Cameroon to treat skin diseases and malaria (Hutchinson and Dalziel, 1954; Haxaire, 1979). In west Africa, *Futumia africana* is used to treat fever, inflammation, malaria, cancer, amoebic dysentery, urinary incontinence and burns (Ashidi *et al*., 2010). *Alstonia boonei* and *Azadirachta indica* are plant species used in Nigeria to treat malaria (Adebayo and Knettli, 2011).

The developing country of South Africa is estimated to have 59.62 million people according to the statistics South Africa in 2020 (https://www.statssa.gov.za) and about 60% of the population consult traditional health practitioners (McGaw and Eloff, 2008). It is further estimated that there are 200 000 traditional health practitioners in South Africa which include the herbalists, diviners, traditional surgeons who mainly circumcise and serve as traditional birth attendants, compared to the 25 000 Western-trained doctors. The ratio of the healer to the population is 1:200 (Pretorius, 1999; Meissner, 2008; Peltzer, 2009).

The abrupt population growth in urban areas initiated a massive demand for medicinal plants where they are collected and supplied to urban areas to be merchandised in shops and informal markets. This can be observed in Gauteng Province with the largest population of 14.7 million people equivalent to 25.4% of the population in South Africa (https://www.statssa.gov.za). In this province, the health practitioners assemble to sell and market their medicine at the Faraday *muthi* Market. This is a clear indication that traditional health practitioners play a crucial role in the lives of people and due to lack of formal records of the traditional medicine systems, the information is passed on verbally from one generation to the next, which is why it is important to conserve this knowledge (van Wyk *et al*., 2000).

2.3. Medicinal plants in Southern Africa

The use of medicinal plants in southern Africa is strenuously associated with the physiological and pharmacological activities of the active compounds of the plants. South Africa hosts over 30 000 higher plant species which constitute 10% of the plant species around the world of which 3000 plant species are used as therapeutic sources and 350 species are traded medicinal plants (van Wyk *et al*., 2000). The Cape floral kingdom in Cape Town is one of the 6 floral kingdoms acknowledged around the world. It is the most diverse temperate flora worldwide hosting almost 9000 plant species which are a rich source of herbal medicine and food source for animals. It is estimated that 69% of the plant species are endemic (Mulholland, 2005; Odendaal *et al*., 2008; van Wyk *et al*., 2009). The genus *Aloe* is one of the few plant species that contribute to the world's medicine. Approximately 125 *Aloe* species of the documented *Aloe* species are indigenous to South Africa (Raimondo *et al*., 2009), and are mostly used to treat infections, digestive ailments, injuries and as a laxative (Grace *et al*., 2018).

2.4. Medicinal plants as a source of new drug development

Plants represent the greatest source of active substances that can be used in medical therapy ascribed to the large structural variation they exhibit (Cragg and Newman, 2013). These plant medicines initially took the form of crude drugs such as tinctures, powders, and other herbal formulations. In the 19th century, man began to isolate the active compounds of medicinal plants which led to the isolation of early drugs such as aspirin, digitoxin, morphine, quinine and pilocarpine (Butler, 2004). These pharmacologically active compounds are known to exhibit many properties such as anti-inflammatory, aestrogenic, enzyme inhibition, antimicrobial, antiallergic, antioxidant, vascular, cytotoxic, anticancer, and anticoagulant activity (Newman *et al*., 2003).

Today, drug discovery from plants mainly depends on the bioactivity-guided isolation methods. The history of the traditional use of the plants by the indigenous people is essential to the development of natural medicines before the ethnobotanical research. The qualitative and quantitative analytical methods are required to monitor the production of the active compounds of interest and assist in selecting high-yield,

high biomass plants and the optimal season for harvesting (Gericke, 2011). The extracts from the plant materials are prepared using organic solvents and are subjected to phytochemical and biological screening using the relevant assays and further commence the process of isolation, identification and characterisation of the active compounds through the bioassay-guided fractionation (Balunas and Kinghorn, 2005).

Since medicinal plants have been used for thousands of years to treat infections, they are a potential source of new antimicrobial agents and over 74% of pharmacologically active plant-derived compounds have been discovered since the investigation of the use of plants started (Farmsworth and Soejarto, 1991). According to Newman and Cragg (2016), 10 out of 44 approved small molecule drugs are natural product derivatives which accounts for 25% of the 44 approved natural products.

2.5. The bioactive compounds in medicinal plants

Plants produce two groups of compounds, namely; the primary and secondary metabolites. The plant primary metabolites are significant for the growth and development of the plant and they are produced through photosynthesis (Fernie and Pichersky, 2015). The secondary metabolites are the main components of active and potent defence mechanisms against pests and pathogens. In addition, the secondary metabolites are diverse chemical compounds that vary based on the family and species. The limited distribution of the compounds amongst plants enables them to be used as taxonomic markers (Bennet and Wallsgrove, 1994).

The secondary metabolites demonstrate many biological activities and are recently the main focus of many scientific research studies. These biological activities include antimicrobial, antifungal, anticancer and anti-inflammatory (Vaghasiya *et al*., 2011). Most occurring secondary metabolites are aromatic substances. The major types are phenols, terpenes and nitrogen/sulfur containing compounds with phenols being abundant (Madiha *et al*., 2018). It has been reported that about 12000 secondary metabolites have been isolated (Basumatary, 2016).

2.5.1. Phenolics

Phenolics are the simplest bioactive phytochemicals that consist of one or more phenol groups (Figure 2.1). The phenyl group is a common characteristic of phenols that range from simple structures with one aromatic ring to highly complex polymeric structures (Ruhab and Amira, 2019). The site and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that, increased hydroxylation results in increased toxicity (Geissman, 1963). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidised compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman, 1987). Gallic acid is a well-known phenolic acid that demonstrates diverse pharmacological activities *in vitro*, including antibacterial, antiviral, antifungal, anti-inflammatory, antitumor, antianaphylactic, antimutagenic, choleretic and bronchodilatory (Ruhab and Amira, 2019).

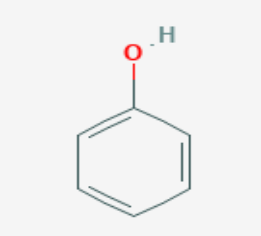


Figure 2.1: The structure of phenol (https://pubchem.ncbi.nlm.nih.gov)

2.5.2. Alkaloids

Alkaloids are a class of naturally occurring organic compounds that mostly contain basic nitrogen atoms in a heterocyclic ring. In addition to the carbon, hydrogen, and nitrogen, alkaloids may also contain oxygen, sulphur and more rarely, other elements such as chlorine, bromine, and phosphorus. Alkaloids are produced in a variety of organisms such as fungi, plants, and animals. Several classes of alkaloids include phenylalkylamines, pyrrolidines, tropane alkaloids, pyrrolizidines and purine alkaloids (Savoia, 2012). These compounds have a wide range of pharmacological activities including analgesia, local anesthesia, cardiac stimulation, respiratory stimulation and relaxation, vasoconstriction, muscle relaxation, and toxicity, as well

as antineoplastic, hypertensive and hypotensive properties (Ruhab and Amira, 2019).

The first medically useful alkaloid was morphine which was isolated from the opium poppy *Papaver somniferum* in 1805 (Fessenden and Fessenden, 1982). Berberine (Figure 2.2) is an important representative of the alkaloid group that is abundant in roots, rhizoids, stems, and bark of plants (Komal *et al*., 2011). It is potentially effective against trypanosomes and plasmodia (Omulokoli *et al*., 1997). The continuous study on the pharmacological activities of berberine over the years has revealed a broad spectrum of activities such as antiarrhythmic, antihyperglycemiic, anticancer, antidepressant, anxiolytic, neuroprotective, antioxidant, anti-inflammatory, analgestic, and hypolipidemic (Battu *et al*., 2010; Kulkarni and Dhir, 2010). The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA (Phillipson and O'Neill, 1987).

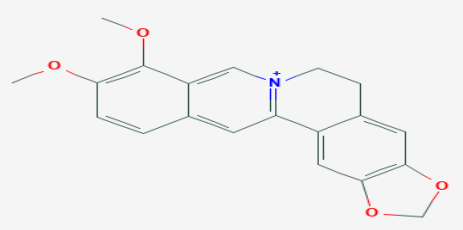


Figure 2.2: The structure of berberine (https://pubchem.ncbi.nlm.nih.gov)

2.5.3. Tannins

Tannins are a group of polymeric phenolic substances that are found in almost every part of the plant such as, bark, wood, leaves, fruits, and roots (Scalbert, 1991). They are divided into two groups; hydrolysable and condensed tannins. Hydrolysable tannins are based on Gallic acid, usually as multiple esters with D-glucose. The condensed tannins (often called proanthocyanidins) (Figure 2.3) are more numerous and derived from flavonoid monomers. Tannins may be formed by condensations of flavan derivatives which have been transported to woody tissues of plants or may be formed by polymerisation of quinone units (Geissman, 1963). This group of compounds is responsible for human physiological activities such as stimulation of

phagocytic cells, host-mediated tumour, and antimicrobial activity. The mode of antimicrobial action of tannins may be related to their ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins (Haslam, 1996).

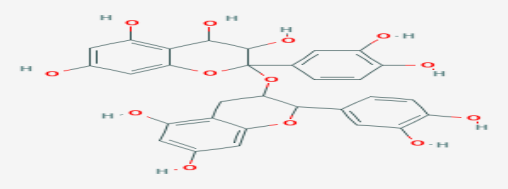
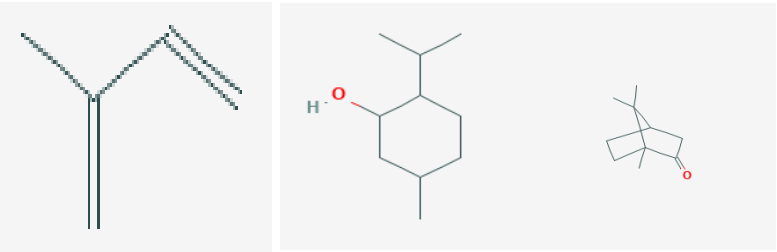


Figure 2.3: Procyanidin (condensed tannin)

(https://pubchem.ncbi.nlm.nih.gov)

2.5.4. Terpenoids and essential oils

The fragrance of plants is carried in the essential oil fractions that are secondary metabolites highly enriched in compounds based on an isoprene structure. They are referred to as terpenes and are formed by fusions of two or more isoprene (C_5H_8) (Figure 2.4A). The terpenes occur as monoterpenes, diterpenes, triterpenes, tetraterpenes, hemiterpene, and sesquiterpenes. When the compounds contain additional elements, usually oxygen, they are called terpenoids (Langenheim, 1994). Terpenoids are synthesised from acetate units, and they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclised. Terpenes or terpenoids are active against bacteria, fungi, viruses and protozoa. Their mechanism of action involves membrane disruption by the lipophilic compounds (Ghoshal *et al*., 1996). The common examples of terpenoids are menthol (Figure 2.4B) and camphor (monoterpenes) (Figure 2.4C).



(**A**) (**B**) (**C**)

Figure 2.4: The structures of terpenoids; isoprene (**A**), menthol (**B**) and camphor (**C**) (https://pubchem.ncbi.nlm.nih.gov)

2.5.5. Flavonoids

Flavonoids are hydroxylated phenolic substances that occur as a C6 -C3 unit linked to an aromatic ring. They are divided into different classes based on the level of oxidation of the central carbon ring (Figure 2.5). The common classes include anthocyanins, flavones, flavonones and flavonols (Iwashina, 2000). The flavonoids have been reported to have antibacterial, anti-inflammatory, antiallergic, antithrombotic and vasoprotective, anti-tumour, anti-viral properties and ability to protect the gastric mucosa (Harborne, 1996; Serafini *et al*., 2010). Some flavonoids are formed as antimicrobial agents in plants' response to pathogens (Deliorman, 2010). The activity of flavonoids is due to their ability to complex with extracellular and soluble proteins and bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1996).

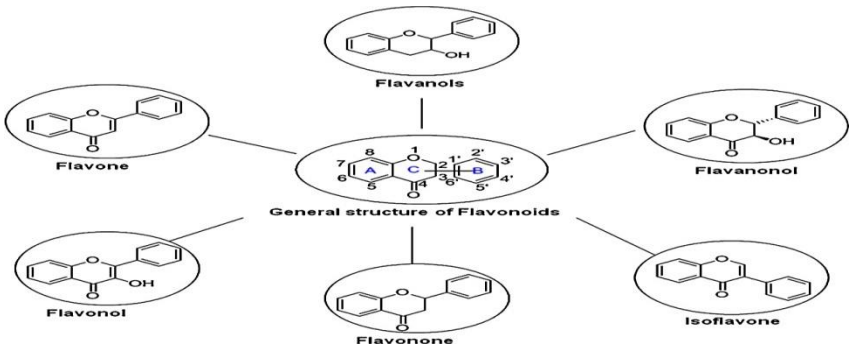
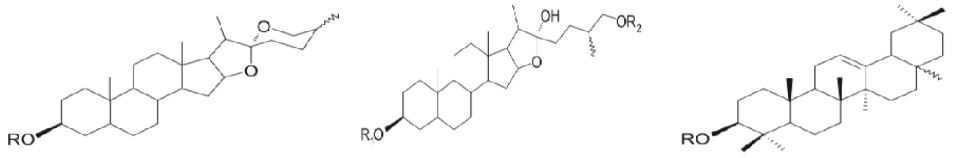


Figure 2.5: The general structure of flavonoids and the different classes (Ravishankar *et al*., 2013).

2.5.6. Saponins

Saponins are the second widely distributed secondary metabolites in plants including the ones used in traditional medicine (Choon *et al*., 2014). Their name was derived from their strong foaming potential in an aqueous solution (Milgat and Roberts, 1995). The saponins are classified into two groups, namely; triterpenoids which are abundant in dicotyledonous angiosperms and steroidal glycosides which are most common in monocotyledonous angiosperms. The two classes vary based on the number of sugar units that are attached to their structure (aglycone skeleton) (Figure 2.6) (Bruneton, 1995; Hostettman and Marston, 1995). The biological and pharmacological activity of saponins include haemolytic, molluscicidal, anti-

inflammatory, antifungal, antimicrobial, antiparasitic, cytotoxicity, anticancer and antiviral (Sparg *et al*., 2004). Mandal *et al*. (2005) isolated the acylated bisglycoside saponins (acaciaside A and B) from the funicles of *Acacia auriculiformis* which were proven to exhibit anthelmintic, antibacterial and antifungal activity.



(**A**) (**B**) (**C**)

Figure 2.6: Skeletons of different aglycone saponins (**A**) steroidal spirostane, (**B**) steroidal furostane and (**C**) triterpenoid saponin. R group is the sugar moiety (Sparg *et al*., 2004).

2.6. Interaction among the plant defense compounds

Plants contain a large variety and quantity of secondary metabolites. Some of these secondary metabolites play a role in defence against microorganisms and pathogens (Nelson and Kursar, 1999). There is a greater probability of the interaction between these compounds due to their diversity. This includes, synergistic, antagonistic and additive or non-interactive. Additive or non-interactive is the interaction of the compounds that results in an activity that is equal to the sum of their individual activity while antagonistic is the interaction that results in the activity that is less than the additive effect (Efferth and Koch, 2011; Pemovska *et al*., 2018). Synergistic interaction is the interaction of the compounds which results in enhanced activity that is greater than the sum of their individual activities.

The antimicrobial potential of the plant compounds gives them the ability to function synergistically with synthetic antibiotics. Therefore, the activity of the antibiotic is enhanced making it active against the resistant microorganisms (Aiyegoro and Okoh, 2009). The study conducted by Ahmad and Aqil (2007) showed synergistic effects between two antibacterial drugs (tetracycline and ciprofloxacin) and the extracts of *Acorus calamus*, *Holarrhena antidysenterica,* and *Delonix regia*.

2.7. Antioxidants

The free radicals and other reactive species are produced as a result of aerobic metabolism in the body. Overproduction of free radicals causes oxidative damage to biomolecules (lipids, proteins, DNA), which successively leads to different chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans (Halliwell, 1995). Antioxidants are substances that prevent damage to cellular components arising from chemical reactions that involve free radicals (Fernandes *et al*., 2004). In addition, they react with free radicals and other oxygen species within the body to protect it from damaging oxidation reactions thus hindering the process of oxidation (Sharma *et al*., 2004). Antioxidant supply is limited because it can react with a single free radical and during the reaction, the antioxidant becomes oxidised. Therefore, there is a constant need to replace the antioxidant source. The endogenous and exogenous antioxidant defenses are present to protect cellular components since free radicals can react randomly leading to the damage of cellular components.

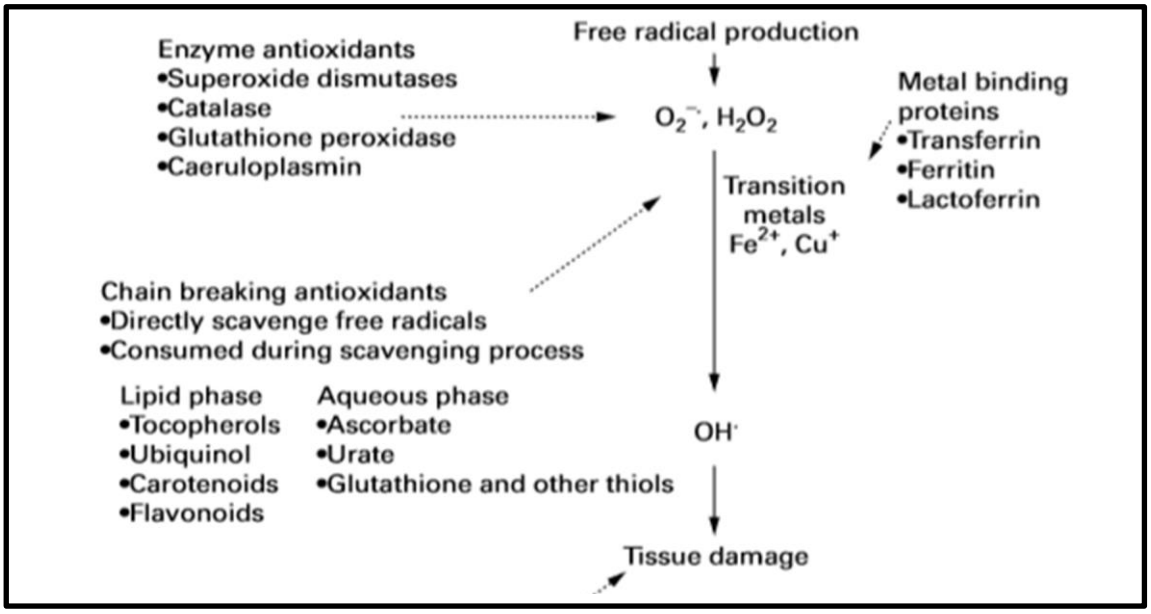


Figure 2.7: Antioxidant defences against free radical attack (Young and Woodside, 2001).

Antioxidants are divided into 3 main groups: antioxidant enzymes, chain-breaking antioxidants and transition metal-binding proteins (Young and Woodside, 2001). Antioxidant enzymes catalyse the breakdown of free radical species before they attack cellular components, they act by reducing the energy of free radical species by giving up some of their electrons for its use thereby stabilising it (Takahashi and Cohwen, 1986). Transition metal-binding proteins prevent the interaction of transition metals such as copper and iron with hydrogen peroxide and superoxide producing highly reactive hydroxyl radicals. Chain-breaking antioxidants act as electron donors and react with free radicals before they can damage the target molecules. In the process, the antioxidant is oxidised and replaced (Halliwell, 1995).

The plant-derived antioxidants such as vitamin E, vitamin C, and polyphenols including phenolic acids, phenolic diterpenes, flavonoids, catechins, procyanidins, and anthocyanins are considered important dietary sources. They can act as free radical scavengers, neutralising dangerous reactive oxygen species and metal ion chelators (Hashim *et al*., 2005). Research studies have proven that these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, anti-mutagenic, anticarcinogenic, antibacterial and antiviral activities to a greater extent (Halliwel, 1995). The elevated attention in the replacement of synthetic food antioxidants with natural ones has strengthened the research on plant sources and the screening of raw materials to identify new antioxidants (Luximon-Ramma *et al*., 2002).

2.8. Antimicrobial activity

Plants are used in traditional medicine to treat various ailments in many countries around the world because they manifest antimicrobial properties (Ribeiro *et al*., 2010). The antimicrobial activity of plant leaves, flowers, stems, roots, and fruits has been investigated over the years. Antimicrobial/antibacterial substances are the substances that either kill bacteria (bactericidal) or inhibit the growth of bacteria (bacteriostatic). Several scientific research studies have reported on the variety of secondary metabolites in plants that have both the antioxidant and antimicrobial properties making them highly efficient in treating bacterial infections (McGaw and Eloff, 2008). Pendota *et al*. (2017) reported on the antimicrobial activity of *Pappea*

capensis against *Basillus subtilis*, *Staphylococcus aureus*, *Escherichia coli,* and *Klebsiella pneumonia*.

The different bioassays that are used to test for the *in vitro* antibacterial activity of plants and antimicrobial agents are diffusion, micro/macro dilution and bioautography methods. Over the years, the diffusion method played an important role in the search for new antimicrobial compounds due to its simplicity, ability to analyse a wide range of antimicrobial agents and low costs (Balouiri *et al*., 2016). However, this method is not appropriate to determine the minimum inhibitory concentration and does not distinguish between the bactericidal and bacteriostatic effects. Therefore, to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the antimicrobial agents, a dilution method is employed. It is possible to estimate the concentration of the tested antimicrobial agent in agar (agar dilution) or broth (broth dilution). The MIC is described as the lowest concentration of the antimicrobial agent that inhibits microbial growth (Jorgensen and Ferraro, 2009). The bioautography is a method that combines the Thin Layer Chromatography (TLC) with bioassay *in situ* and enables the localisation of the active compounds (Marston, 2011).

2.9. Disease-causing bacteria

Bacteria are microscopic organisms that survive in a wide range of habitat including soil, water, and air. Unlike the animal cells and other eukaryotes, bacterial cells lack true nucleus and membrane-bound organelles, hence they are regarded as prokaryotes. Due to their size and simplicity, they can survive in complex associations with other organisms. The largest population of bacteria reside in the gut flora in humans and the majority of the bacteria found in the human body are regarded as harmless (Sears, 2005). However, distinct bacterial species secrete pathogenic toxins that are responsible for a majority of human infections and death (Wollina, 2017). The formation of highly resistant inactive spores called endospores which enables bacteria to adapt and survive in extremely harsh conditions (Nicholson *et al*., 2000).

The most disease causing-bacterial pathogens chosen for the study are two Gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus*) and two Gram-negative

bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). These microorganisms represent the pathogenic species that are commonly associated with nosocomial infections and are selected based on the recommendation of the Clinical Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, 1990).

2.9.1. *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive, facultative anaerobe that appears as oval *cocci* belonging to the Enterococcaceae family. Despite its survival in the gastrointestinal tract of human beings and other mammals, *E. faecalis* can cause life-threatening infections such as endocarditis and septicaemia, urinary tract infections and meningitis (Kayauglu and Orstavik, 2004). In addition to its possession of virulence factors including enzymes, cytolysin, aggregation substances, and pheromones, it can suppress the action of lymphocytes in the blood resulting in endodontic failure (Lee *et al*., 2004). However, its survival and ability to cause diseases are contributed by its ability to transfer or share this virulence trait among its species (Jett *et al*., 1994).

2.9.2. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, coccal shaped, facultative anaerobe that belongs to the Staphylococcaceae family. Although *S. aureus* is not always pathogenic as a commensal of the human microbiota, it is likely to be an opportunistic pathogen causing skin infections, respiratory tract infections and food poisoning (Lowy, 1998). The pathogenic strains of *S. aureus* have created a burden in the treatment of infections due to its ability to produce and express distinct surface proteins that act as virulence factors that bind and inactivate antibiotics (Manukumar and Umesha, 2017). *S. aureus* also produces various enzymes such as the bound and free coagulases which form clots on plasma and the bacterial cell to prevent phagocytosis. The methicillin-resistant *Staphylococcus aureus* (MRSA) is identified as the leading cause of nosocomial and as well as community-acquired infections (DeLeo *et al*., 2010).

2.9.3. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, facultative anaerobe bacterium that belongs to the Pseudominaceae family. *P. aeruginosa* survives in a wide range of environments such as in soil, water, skin flora and most of the made environments (Silby *et al*., 2011). It is recognised as a pathogenic bacterium that causes infections in both plants and animals, and humans. Its ability to adapt in animal cells enables it to attack and infect the damaged tissues and tissues with reduced immunity resulting in inflammation and sepsis (Sadikot *et al*., 2005). It is an opportunistic pathogen that is associated with nosocomial infections and ventilator-associated infections. It is estimated that over 5-10% of infectious exacerbations in patients with chronic obstructive pulmonary diseases are caused by *P. aeruginosa* (Murphy, 2009). *P. aeruginosa* display resistance to a wide variety of antibiotics including aminoglycosides, quinolones, and B- lactams (Hencock and Speert, 2000).

2.9.4. *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped, motile, facultative anaerobe, non-sporulating bacteria that belongs to the Enterococcaceae family. It is widely distributed in the lower intestines of humans and animals and also on the natural environments (Russo and Johnson, 2000). Despite its commensalism with the host body, some distinct variants can cause gastroenteritis when sufficient quantities are consumed by the host through contaminated food (Ruso and Johnson, 2000; Poolman, 2017). The Shiga toxin produced by 0157:H7 *E. coli* is a well-known family of cytotoxins that represent the main virulence of *E. coli* responsible for a life-threatening Haemolytic Uremic Syndrome (HUS) (Svennerholm, 2011; Gomes *at al*., 2016).

2.10. Antibiotic resistance of microorganisms

Antibiotics are substances or compounds that are produced by microorganisms, especially fungi that can inhibit the growth of bacteria (Wakson and Woodruff, 1940). The antibiotic penicillin was the first antibiotic developed in 1928 paving a way for the production of other antibiotics such as chloramphenicol and streptomycin which were used to treat infections and saving millions of lives. Although countless antibiotics have been produced by pharmaceutical companies for decades, we are now faced

with a crisis where many antibiotics are no longer effective in fighting the simplest infections. This resulted in an elevated number of hospitalisations and more treatment failures, including high mortality and the persistence of drug-resistant bacterial pathogens (Nascimento *et al*., 2000). These include microbial pathogens such as methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Staphylococcus pneumonia* and vancomycin-resistant *enterococci* and bacteria that produce extended-spectrum β-lactamases (Tally, 1999; Wright, 2014; Martens and Demain, 2017).

Antibiotic resistance is influenced by the overuse and misuse of antibiotics including overprescribing antibiotics for minor and self-limiting bacterial infections such as acute bronchitis and common cold (Straad *et al*., 2017). The resistance is further complicated by the emergence of immune compromising infections such as HIV/AIDS, tuberculosis (TB), typhoid, pneumonia, and candidiasis (Hazara *et al*., 2014). To make matters adverse, the antibiotic resistance continues to increase while the pipeline for the development of new antibiotics is drying up. This is mainly influenced by several reasons, among them being; pharmaceutical companies have abandoned the production of new antibiotics due to poor financial returns and investments as antibiotics are used for a short period and are more likely to lose their activity secondary to resistance. Furthermore, the development of new antibiotics is more expensive due to increasing regulatory requirements and lengthy process of clinical trials (Bax and Green, 2015; Martens and Demain, 2017).

Today, antibiotics are not only used in the treatment of infections but are extensively used in livestock farms, agricultural sectors, aquaculture and sewage treatment. This misuse and overuse of antibiotics poses a threat to the human as well as the natural microbial system (Qiola *et al*., 2017). The presence of antibiotics in microbial systems influences genetic change to sensitive bacteria, allowing them to survive and produce antibiotic-resistant strains that carry antibiotic-resistant genes (Martinez, 2009).

Antibiotic resistance has remarkable consequences on both the health of the people and the economic implication. Around the world, a total of 17 million people worldwide die annually from bacterial infections (Butler and Buss, 2006). It is estimated that about 2300 out of 2 million people who are infected with bacterial

pathogens that are resistant to antibiotics, die due to these infections (https://www.cdc.gov/drugresistance/). According to the report of the European Antimicrobial Resistance Surveillance (EARS) in 2015, around 25 thousand people in Europe died from hospital-acquired infections caused by antibiotic resistant bacteria which cost the healthcare EUR (€) 1.5 billion including the loss of productivity. Therefore, there is an urgent need to develop advanced and effective antibiotics to reduce the danger of fatal outcomes. The screening of medicinal plants for antimicrobial activity has revealed that higher plants manifest a potential source of new anti-infective compounds (Press, 1996).

2.11. Plants selected for the study

The two host and parasitic plants, namely; *Mystroxylon aethiopicum* and *Viscum rotundifolium*, *Dichrostachys cinerea* and *Tapinanthus oleifolius* were selected for the study based on their use in traditional medicine to treat various ailments.

2.11.1. Mistletoes

Mistletoes are parasitic plants that belong to the Santalales order and Viscaceae family also known as Santalaceae family. They are known for their white or translucent berries that can be quite sticky (Nickrent and Musselman, 2004). There are two plants with mistletoe as their common name is the American mistletoe (*Phoradendron serotinum* or *Phoradendron flavescens*) and the European mistletoe (*Viscum album*). Although *Phoradendron* and *Viscum* species both contain the common name mistletoe, the *Viscum* species are highly toxic when compared to *Phoradendron* species. They cause notable gastrointestinal irritation following ingestion (Schaller, 1998). The two main families of mistletoes in Southern Africa are *Viscaceae* and *Loronthaceae* and they use *Acacia* and *Combretum* as their main host plants (Poulin *et al*., 2011). All the parts of mistletoes are potentially harmful, but their toxicity varies with the type of host tree on which they grow. The major components of mistletoes are gastric-irritating alkaloids, cardiac toxins (viscotoxins, phoratoxins) and lectins. The cardiac effects hypothetically exist based on the presence of cardiotoxins in mistletoe, but there is insufficient information on human responses to indicate that these cardiotoxins may cause clinically significant cardiac abnormalities following causal ingestions (Hall *et al*., 1986).

Mistletoes are adapted to attach to branches of host trees and access the vascular tissue of the host tree using their specialised structure called haustorium (Glatzel and Geils, 2009). They sometimes require chemicals called Haustorium- Inducing Factors (HIF) or a contact signal to initiate the development of the haustorium (Rödl and Ward, 2002). The haustorium requires adaptive flexibility so that the mistletoe can access nutrients and water from the host species as they switch amongst different host species (Gonzáles *et al*., 2007). The parasitic plants selected for the study are *Viscum rotundifolium* and *Tapinanthus oleifolius*.

2.11.1.1. *Viscum rotundifolium*

Viscum rotundifolium as a parasitic plant that belongs to the Santalaceae family. *V. rotundifolium* is a red-berry mistletoe (English) of southern Africa that is variable, wide-ranging and monoecious. It is widely distributed in southern Africa and grows on different plants. It is adapted to attach to branches of host trees and access the vascular tissue of the host tree using its specialised structure called haustorium (Glatzel and Geils, 2009). The haustorium requires adaptive flexibility so that it can access nutrients and water from the host species as it switches amongst different host species (Gonzáles *et al*., 2007). *V. rotundifolium* is used traditionally to treat various ailments such as epilepsy, infertility, hypertension, arthritis, and cancer.

(http:/www.google.co.za/images)

Figure 2.8: Fully grown *V. rotundifolium* (**A**), leaves, flowers (**B**) and fruits (**C**)

2.11.1.2. *Tapinanthus oleifolius*

Tapinanthus oleifolius is a hemi parasitic plant that belongs to the Loranthaceae family. It is widely distributed in the drier parts of southern Africa throughout Namibia, Free State, Botswana, Gauteng, Mpumalanga, Limpopo, and North West. It grows

on diverse host species such as species of *Acasia*, Aloe, *Mytenus*, *Combretum*, *Diospyros*, *Ziziphus*, *Rhus* and *Melianthus*. It is used traditionally to treat epilepsy, diarrhoea, dysentery, wounds, cancer and brain disorders (Polhil and Wiens, 2006).

(http:/www.google.co.za/images)

Figure 2.9: *Tapinanthus oleifolius* leaves (**A**) flowers (**B**) and fruits (**C**)

2.11.2. Pollination and seed dispersal of mistletoes

The pollination of mistletoe is carried out by insects, birds and rarely by the wind. The birds act as the primary dispersers of mistletoe (Okubamichael *et al*., 2011). The fruits of mistletoes are large, high in sugar concentration and brightly coloured to attract the birds. The fruits act as the major source of food for bird dispersers during winter when other food sources are few in the ecosystem (Watson, 2001). The sticky substance called viscin that coats mistletoe seeds also influences the behaviour of birds by creating difficulties for birds to expel seeds that are attached to their body parts (Aukema, 2004). The pectic material found in the viscin has the water holding capacity and ability to resist repeated drying which accounts for the ability of mistletoe seeds to attach strongly to host twigs and form haustorium (Roxburgh and Nicolson, 2005).

2.11.3. Host specificity in parasitic plants

Parasitic plants are very distinctive and comprise of 3500 to 4000 species that display variation in host-specificity. In a similar way to plant and animal parasites, the mistletoes live in a close association with their hosts to derive nutrients (Watson, 2001). A limited number of parasites are known to infect only a single type of host species and this helps to sustain the parasite population and genetic variation. The parasite that infects a larger number of host species are not completely unrestricted in their host range but are more likely to show a preference for some host species

above others (Norton and Lange, 1999). It is estimated that in Africa, 70% of mistletoes are generalist species of parasites that infect host from several families, 12% are specific on hosts from one family but can grow on a few genera of other families and 18% are specific on hosts from one or few other host species of a single genus (Poulin *et al*., 2011).

Several factors such as the seed dispersal vectors, host availability, host abundance, host compatibility and suitable niche for the parasite determine the host specificity in mistletoes (Okubamichael *et al*., 2016). Over and above all, the seed dispersal by animal or bird vectors determines mistletoe–host interactions over time and space, which successively affects the geographic arrangement of mistletoes and their hosts. Birds can transfer mistletoe seeds over large distances and possibly disperse seeds to hosts that are distantly related as well (Aukema and Martìnez del Rio, 2002). The host plants for the selected mistletoes are *Mystroxylon aethiopicum* (host for *V. rotundifolium*) and *Dichrostachys cinerea* (host for *Tapinanthus oleifolius*).

2.11.3.1. *Mystroxylon aethiopicum*

Mystroxylon aethiopicum (Thumb.) Loes subsp. *schlechteri* (loes.) R.H. Archer commonly known as bushveld kooboob berry (English), and a member of the Celastracea family. *M. aethiopicum* is a variable evergreen shrub/small to medium sized tree that is found in bushveld and forests (Burrows and Willis, 2005). *M. aethiopicum* is mostly abundant in Ethiopia, Sudan, South Africa, Namibia, Angola, Cameroon, Madagascar, Seychelles and Comoro (Cutis and Mannheime, 2005). Traditionally, the plant is consumed in Africa to treat haemorrhagic diarrhoea, stomach and respiratory tract infections, coughs, and anaemia (lwu, 2014). In Kenya, the root bark extract of this plant is reported to be used in making tea which is consumed as stomach medicine by children (Kokwaro, 1993). The toxicity of the root bark aqueous extract was tested in albino mice and was found to be safe when administered orally (Kilonzo *et al*., 2016a). However, the root bark extracts were found to be toxic against brine shrimp larvae (Kilonzo *et al*., 2016b).

(http:/www.google.co.za/images)

Figure 2.10: *M. aethiopicum* fully grown tree (**A**), leaves, flowers (**B**) and fruits (**C**)

2.11.3.2. *Dichrostachys cinerea*

Dichrostachys cinerea commonly known as the sickle bush is a plant that belongs to the Fabaceae family. It is indigenous to North West India, Central India, Rajasthan and North Australia (Vijayalakshimi *et al*., 2013). The barks of *Dichrostachys cinerea* are used for the treatment of headache, toothache, dysentery, and elephantiasis; the roots infusions are used to treat leprosy (Hansen's disease), syphilis, coughs, bites/ stings, the leaves are used for the treatment of epilepsy and as a diuretic and laxative, the powdered form of the leaves is used to massage the limbs with bone fractures (Eisa *et al*., 2000). Vijayalakshimi *et al*. (2013) reported on the phytochemical analysis of the ethanolic extracts.

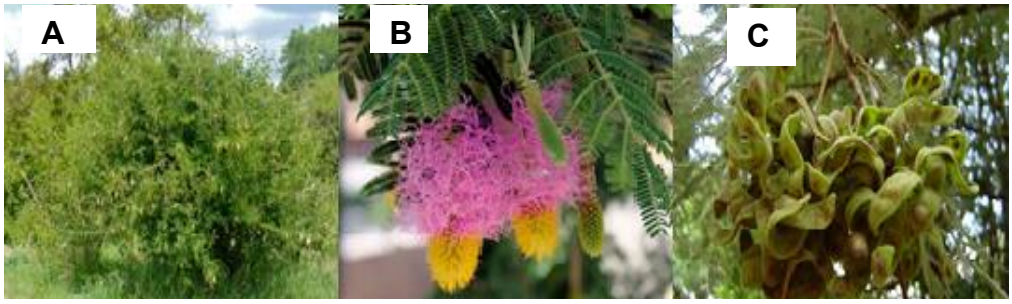


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Figure 2.11: Fully grown *Dichrostachys cinerea* (**A**), leaves, flowers (**B**) and fruits (**C**)

2.2.1. Aim

The aim of the study was to isolate the antimicrobial and antioxidant compounds from two mistletoes (*Viscum rotundifolium* and *Tapinanthus oleifolius*) and synergistic effects with their hosts (*Mystroxylon aethiopicum* and *Dichrostachys cinerea*).

2.2.2. Objectives

The objectives of this study are:

i. To analyse the plant extracts for phytochemicals using Thin Layer Chromatography (TLC) analysis.
ii. To compare and contrast the chemical profile of the host and parasite.
iii. To determine the antioxidant activity of plant extracts using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and ferric reducing power.
iv. To analyse the antibacterial activity of plant extracts against disease-causing bacteria using bioautographic assay and broth microdilution assay.
v. To perform synergistic/antagonistic antibacterial study between the parasite and host plants.
vi. To isolate the bioactive compounds detected in the plants.
vii. To purify and analyse the structure using Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS).
viii. To determine the cytotoxicity effects of the plant extracts and isolated compounds using cell viability assay.

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Chapter 3: Extraction and phytochemical analysis

3.1. Introduction

Plants continue to play an important role in the healthcare system. The research on medicinal plants has gained strength since plants contain compounds that exhibit various biological activities. The four plant species, namely; *Viscum rotundifolium*, *Mystroxylon aethiopicum*, *Tapinanthus oleifolius,* and *Dichrostachys cinerea* were selected for the study based on the information from literature as they are used traditionally to treat various ailments. The leaves for each plant were used to ensure conservation and sustainability.

The primary steps employed when determining the biologically active compounds from the plants are extraction, pharmacological screening, isolation and characterisation of biological compounds, toxicological evaluation and clinical evaluation (Sisadharan *et al.*, 2011). The extraction procedure is the most important step in processing the bioactive constituents from the plant material. The dried plant material is often favoured by scientists for various reasons, with one of them being; there are limited problems associated with large scale extraction of dried plant material as compared to fresh material. The water content in the fresh plant material is likely to affect the solubility and separation during liquid-liquid extraction. The secondary metabolic compounds in plants are stable and preserved in dried form, making them potential sources of antimicrobial agents (Eloff, 1998; Azwanida, 2015). The various organic solvents used during extraction vary from polar to non-polar and the choice of solvents depends on what is intended with the extract.

The secondary metabolites present in the different plant extracts are predominantly complex matrices at low levels. Thin Layer Chromatography (TLC) is one of the chromatographic purification methods that are essential for the identification and characterisation which separates the mixture of compounds in the plant extracts. This is a conventional method for the analysis of extracts due to its simplicity, rapidity and cost-effectiveness (Bele and Khale, 2011). The secondary metabolites present in the plants are detected by different chemical tests. These include the frothing test for saponins, salkowski test for terpenoids, keller-killian and Liebermann's test for cardiac glycosides, Wagner's and Mayer's alkaloid reagent test for alkaloids. Alkaline

reagent and shinoda test for flavonoids (Iraqui *et al*., 2013). The chapter is aimed at determining the presence of different phytochemicals in the plant extracts using Thin Layer Chromatography and standard chemical tests and further quantify them using Folin-Ciocalteau and aluminium chloride calorimetric assay.

3.2. Materials and methods

3.2.1. Plant collection

The plant leaves of *Mystroxylon aethiopicum* (UNIN 121992), *Viscum rotundifolium* (UNIN 122441), *Tapinanthus oleifolius* (UNIN 121991) and *Dichrostachys cinerea* (UNIN 121990) were collected in summer at the University of Limpopo and deposited at Larry Leach Herbarium (UNIN) for voucher specimen. The collected leaves were examined for colour change and infection. The leaves were then dried at room temperature for about a week until they were easy to break using hands. The dried plant leaves were ground to fine powder using a blender and stored in airtight bottles in the dark until extraction.

3.2.2. Preliminary extraction

Finely ground leaf material (1 g) was extracted with 10 mL of different solvents, namely; n-hexane, acetone, ethyl acetate, methanol, and water. Each solvent was allowed to extract the ground plant material for 30 minutes on a shaking incubator (Labotec model 20.2) at 200 rpm. The extracts were filtered through Whatman no. 1 filter paper (Lasec) using a Buchner funnel. The resulting filtrates were evaporated under vacuum using Buchi rotavaporator R-114 (Labotec). The concentrated extracts were poured into pre-weighed glass vials. The extraction procedure was repeated twice to exclusively extract the plant material. The filtered solvent was allowed to dry under a stream of cold air (at room temperature). The masses of the extracts' yields were determined. The extracts were then reconstituted to a final concentration of 10 mg/mL in acetone.

3.2.3. Qualitative phytochemical analysis

Chemical constituents of each of the extracts obtained were analysed using aluminium-backed Thin Layer Chromatography (TLC) plates (ALUGRAM® SILg/ UV

254- MACHEREY- NAGEL, Merck) that were developed in either one of the 3 eluent systems developed in the Phytomedicine (Kotze and Eloff, 2002): Ethyl acetate: methanol: water: 40:5.4:5 [EMW] (polar), Chloroform: ethyl acetate: formic acid:10:8:2 [CEF] (intermediate polarity: acidic), Benzene: ethanol: ammonium hydroxide:18:2:0.2 [BEA] (nonpolar/basic). Exactly 10 µL of the extracts were loaded in a line 1 cm wide on the TLC plate and developed without delay to minimise the possibility of photo-oxidative change. The TLC plate was dried under a stream of air to evaporate the solvents. The separated components were visualised under a visible and ultraviolet light (245 and 365 nm, Camec Universal UV lamp TL-600). For the detection of chemical compounds not visible on UV light, the chromatograms were sprayed with vanillin-sulphuric acid reagent [0.1 g vanillin (Sigma ®): 28 mL methanol: 1 mL concentrated sulphuric acid] and heated at 110°C for 1-2 minutes for colour development.

3.2.4. Preliminary screening for phytoconstituents

3.2.4.1. Saponins

The presence of saponins in the plant material was tested using a persistent frothing test described by Odebiyi and Sofowora (1978). Exactly, 0.5 g of the powdered leaf sample was suspended in 5 mL tap water. The mixture was vigorously shaken and heated. The sample was observed for the formation of forth to draw an inference.

3.2.4.2. Terpenes/ terpenoids

The presence of terpenoids in the plant material was determined using the Salkowski test Odebiyi and Sofowora (1978). The powdered leaf sample (0.5 g) was mixed with 2 mL of chloroform and 3 mL concentrated sulphuric acid (H_2SO_4) was carefully added to form a later. The mixture was observed for a reddish-brown colour change to draw an inference.

3.2.4.3. Phlobatannin

The presence of phlobatannin in the plant material was determined using the method described by Borokini and Omotayo (2012). The powdered leaf material (0.5 g) was dissolved in 5 mL of distilled water and filtered. The filtrate was boiled with 2 mL of

1% hydrochloric acid (HCL). The sample was observed for the formation of a red coloured precipitate to draw an inference.

3.2.4.4. Tannins

The method described by Borokini and Omotayo (2012) was used to test for the presence of tannins in the plant material. The powdered leaf sample (0.5 g) was dissolved in 5 mL of distilled water, then boiled gently and cooled. The solution (1 mL) was added in a test tube and 3 drops of 1% ferric chloride solution was added. The sample was observed for colour change (blue-black, brown-green, green or blue-green) to draw an inference.

3.2.4.5. Cardiac glycosides

The Keller-Killiani test described by Odebiyi and Sofowora (1978) was used to determine the presence of cardiac glycosides in the plant material. The powdered leaf material was treated with 2 mL of glacial acetic acid, containing one drop of 0.1% ferric chloride solution. The mixture was underplayed with 1 mL of concentrated sulphuric acid (H_2SO_4). The sample was observed for colour change to draw an inference.

3.2.4.6. Flavonoids

The method described by Borokini and Omotayo (2012) was used to determine the presence of flavonoids in the plant material. Diluted ammonia (5 mL) was added to a portion of the aqueous filtrate of the plant extract, followed by the addition of concentrated sulphuric acid (H_2SO_4). The sample was observed for colour changes to draw an inference.

3.2.4.7. Steroids

The method described by Borokini and Omotayo (2012) was used to determine the presence of steroids in the plant material. Acetic anhydride (2 mL) was added to a 0.5 g powdered leaf sample, followed by the addition of 2 mL of sulphuric acid (H_2SO_4). The sample was observed for colour change to draw an inference.

3.2.4.8. Alkaloids

Drangendoff's reagent method described by Odebiyi and Sofowora (1978) was used to test the presence of alkaloids in the plant material. Finely ground leaf material (0.5 g) was extracted with 95% ethanol in a shaking incubator (Labotec model 20.2) at 200 rpm for 10 minutes and filtered through Whatman no. 1 filter paper (Lasec) using a Buchner funnel. The resulting filtrates were evaporated under vacuum using Büchi rotavaporator R-114(Labotec). The residue was re-dissolved in 5 mL of 1% Hydrochloric acid (HCL), followed by the addition of 5 drops of Drangendoff's reagent. A colour change was observed to draw an inference.

3.2.5. Quantification of major phytochemicals

3.2.5.1. Total phenolic content

The quantity of phenolics present in each plant extract was determined by using the Folin-Ciocaltleu reagent method (Velioglu *et al*., 1998) with minor modifications (Humadi and Istudor, 2008). The plant material (10 mg/mL) was reduced to a concentration of 5 mg/mL in a test tube. Exactly 100 µL of 5 mg/mL was transferred to a new test tube and diluted with 900 µL of distilled water, followed by the addition of 100 µL of Folin-Ciocaltleu reagent. To stop the reaction, 1 mL 7% sodium carbonate was added and the mixture was incubated in the dark at room temperature for 90 minutes. A blank prepared similarly, except that the plant extracts were replaced with acetone. The absorbance of the mixtures was determined using an ultraviolet/visible (UV/VIS) spectrophotometer (Thermo Scientific) at 550 nm. Gallic acid (Sigma-Aldrich) was used as a standard and reference standard solutions (1.25, 0. 63, 0.31, 0.16, 0.08 mg/mL) were prepared and absorbance for the solutions was measured against the blank. The results were expressed as milligram gallic acid equivalence/gram of extract (mg of GAE/g extract) calculated using the equation obtained from Gallic acid standard curve ($y = 0.6814x + 0.0057$, $R^2 = 0.9983$). The experiment was conducted in triplicates and independently repeated three times for each plant extract.

3.2.5.2. Total tannin content

The Folin–Ciocalteu method described by Tambe and Bhambar (2014) was used to determine the tannin content in the plant extract. Briefly, 50 µL of 10 mg/mL plant extract was added to a clean test tube containing 3.8 mL of distilled water. The Folin–Ciocalteu reagent (0.25 mL) was added to the mixture and vortexed, followed by the addition of 0.5 mL of a 35% solution of sodium carbonate (Na_2CO_3) into the mixture. The mixture in the tube was transferred to a 10 mL volumetric flask and the volume of the mixture was made up to 10 mL by the addition of distilled water. The mixture was vortexed and incubated at room temperature for 30 minutes in the dark. A blank was prepared in the same manner as the test solutions without adding any extract. The absorbance of the mixture was measured using UV/VIS spectrophotometer (Thermo Scientific) at 725 nm. Gallic acid (Sigma-Aldrich) was used as a standard and reference standard solutions (1–0.625 mg/mL) were prepared and the absorbance for the solutions was measured against the blank. Tannin content was expressed as milligram gallic acid equivalence/gram of extract (mg GAE/g extract) calculated using the equation obtained from the Gallic acid standard curve ($y = 3.8869x – 0.023$, $R^2 = 0.9993$). The experiment was conducted in triplicates and independently repeated three times for each plant extract.

3.2.5.3. Total flavonoid content

Total flavonoid content was determined by the aluminium chloride colorimetric assay described by Tambe and Bhamber (2014). Briefly,100 µL of 10 mg/mL plant extract was added to 4.9 mL of distilled water in a clean test tube, followed by an addition of 300 µL of 5% sodium nitrite ($NaNO_2$) dissolved in distilled water. The mixture was left at room temperature for 5 minutes. After the 5 minutes elapsed, 300 mL of 10% aluminium chloride ($AlCl_3$) (dissolved in distilled water) was added to the reaction mixture. The reaction was allowed to stand for 5 minutes at room temperature, after which 2 mL of 1 M sodium hydroxide (NaOH) was added to the solution. The mixture in the test tube was then made up to 10 mL with distilled water. Quercetin (Sigma-Aldrich) was used as a standard. Different concentrations (500–31.5 µg/mL) of the quercetin were prepared in the same method as the extracts. The absorbance of the experimental samples and the standard were determined using a UV/VIS spectrophotometer (Thermo Scientific) at a wavelength of 510 nm. The blank was

prepared in the same manner as the experimental and standard samples; however, 100 µL of distilled water was added instead of the plant extracts. The total flavonoid content of the samples was expressed as milligram quercetin equivalence/gram of extract (mg QE/g extract) calculated using the equation obtained from the quercetin standard curve ($y = 3.4379x + 0.0017$, $R^2 = 0,9996$). The experiment was conducted in triplicates and independently repeated three times for each plant extract.

3.2.5.4. Statistical analysis

The experiments were performed in triplicates and the results were presented as mean with ± standard deviation. Calculations were carried out using Microsoft Office Excel 2010. All data were calculated using a linear regression formula (y=mx+c) obtained from each standard curve.

3.3. Results

3.3.1. Preliminary extraction

The mass extracted from each solvent, namely; n-hexane, ethyl acetate, acetone, methanol and water from 1 g leaf material of *Viscum rotundifolium*, *Mystroxylon aethiopicum*, *Tapinanthus oleifolius* and *Dichrostachys cinerea* are shown in figure 3.1. The methanol solvent was the best extractant in all the plant materials. It extracted 370 mg (*T. oleifolius*), 310 mg (*V. rotundifolium*), 310 mg (*D. cinerea*) and 270 mg (*M. aethiopicum*).

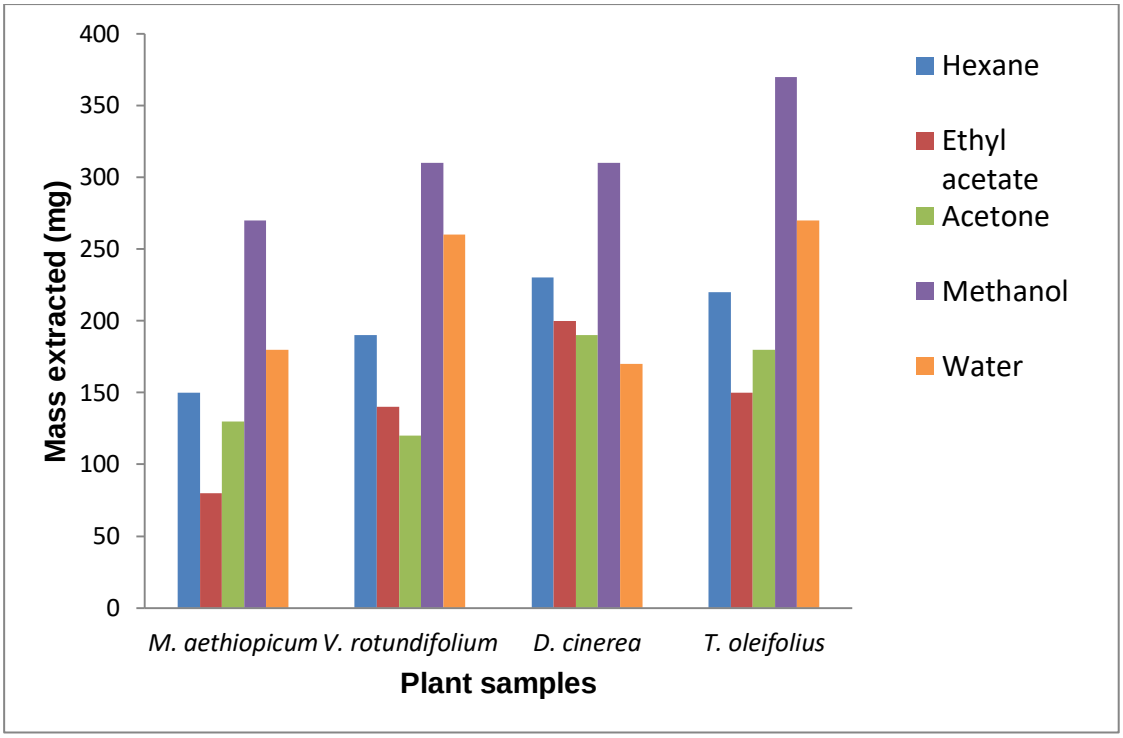

Figure 3.1: The mass of *V. rotundifolium*, *M. aethiopicum*, *T. oleifolius* and *D. cinerea* extracted from 1 g dried leaves using n-hexane, ethyl acetate, acetone, methanol and water.

3.3.2. Qualitative phytochemical analysis

The Thin Layer Chromatography (TLC) was used to separate the chemical constituents to create phytochemical fingerprints of different extracts of *V. rotundifolium*, *M. aethiopicum, T. oleifolius* and *D. cinerea*. The chromatograms were developed in three mobile phase systems based on their polarity, BEA (non- polar), CEF (intermediate polarity) and EMW (polar). The different extractants used were: n-hexane, acetone, ethyl acetate, methanol and water. The separated fluorescing compounds were visualised using ultraviolet light at the wavelength of 365 nm and 254 nm as illustrated by figure 3.2. The different colours observed on the chromatograms indicate the different chemical compounds present on the plant material. The distinct fluorescing compounds were observed on the CEF and EMW mobile phase.

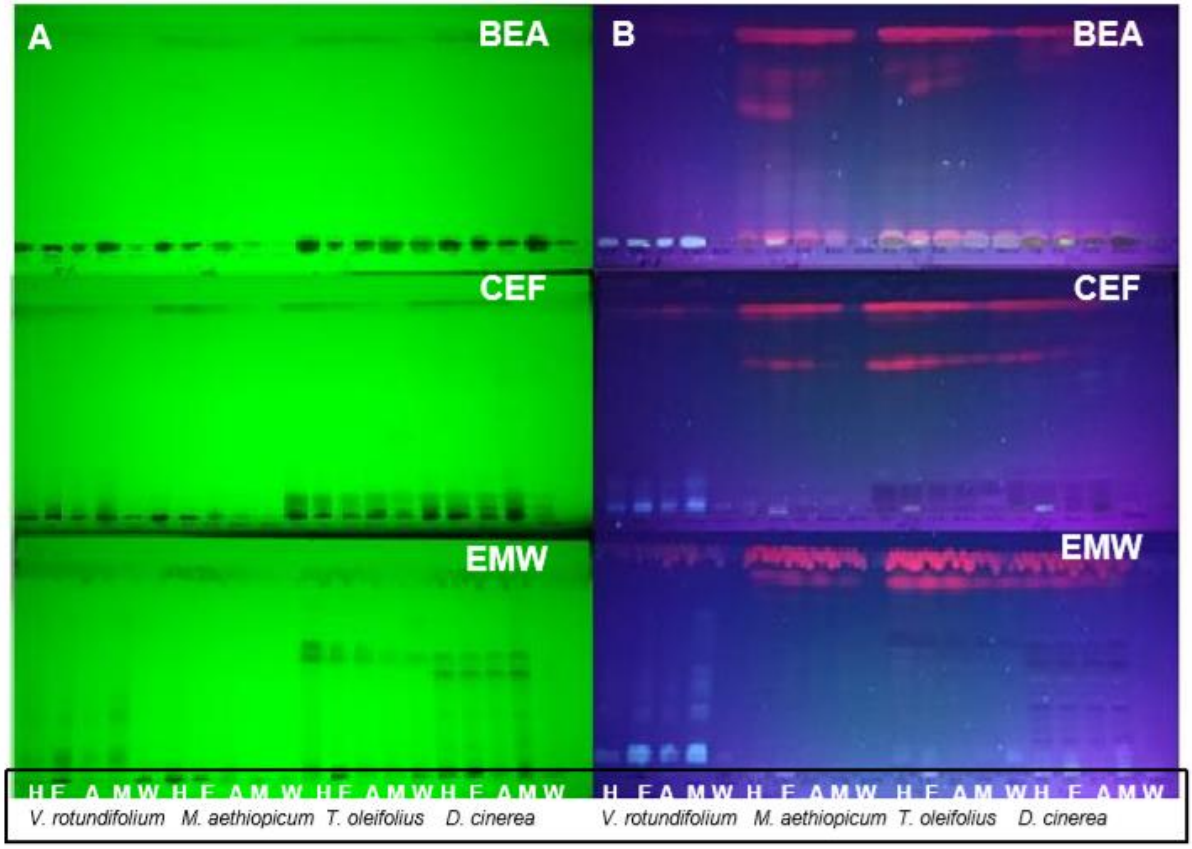

Figure 3.2: The chromatograms of *V. rotundifolium*, *M. aethiopicum*, *T. oleifolius* and *D. cinerea* dried leaves extracted with different solvents and developed in BEA, CEF and EMW mobile systems then visualised under UV light at 365 nm (**A**) and 254 nm (**B**).

The separated compounds that do not fluoresce were detected with vanillin-sulphuric acid spray reagent. The compounds were separated based on their polarities as illustrated by figure 3.3. The compounds separated on the polar (EMW) mobile phase had high number of bands than any other mobile phase. The EMW separated most compounds in the *V. rotundifolium*, *T. oleifolius* and *D. cinerea* leaf extracts while the compounds in *M. aethiopicum* leaf extracts were most separated on the BEA mobile phase. All the mobile phases show similar compounds between the hosts and the parasites plants.

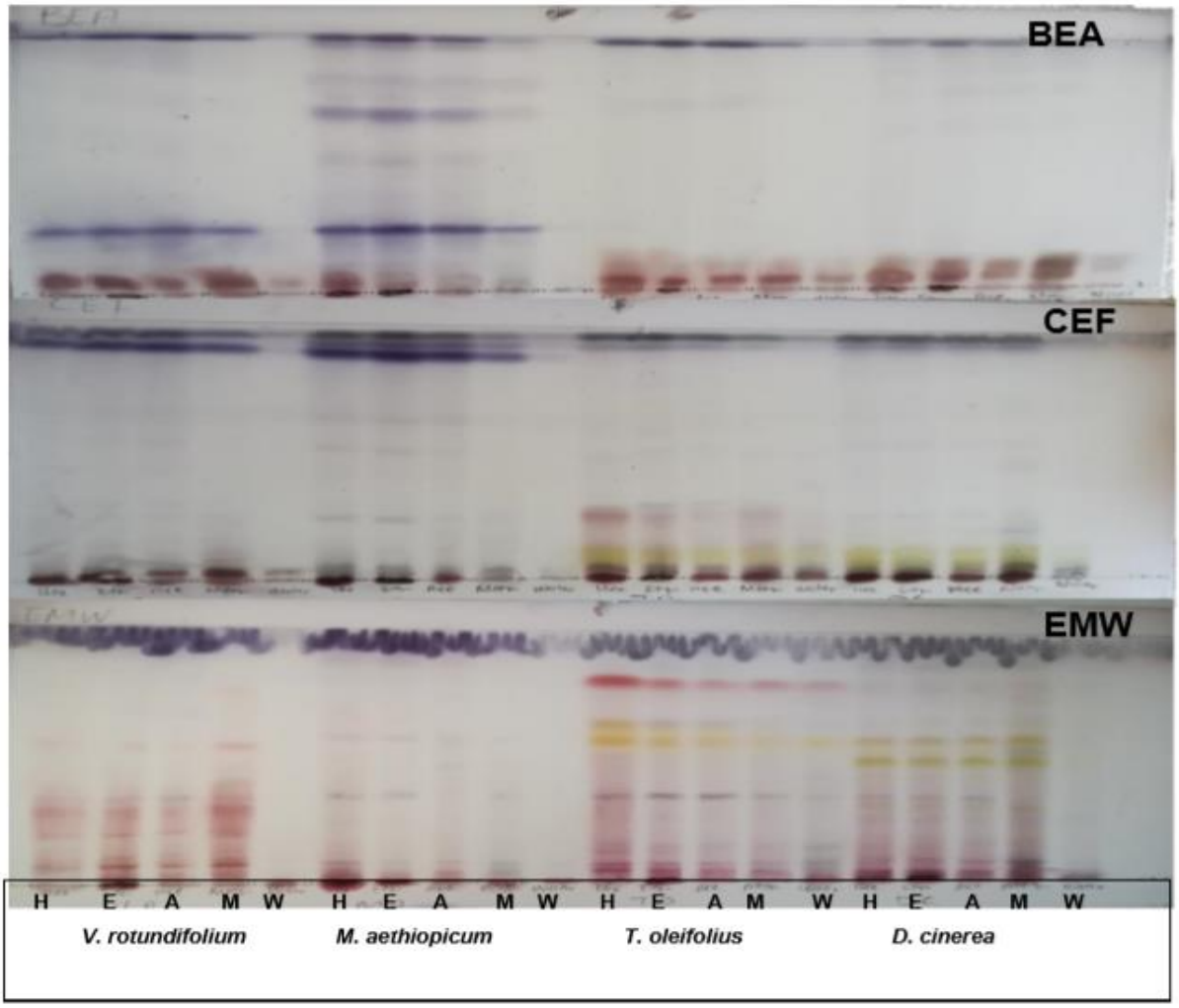

Figure 3.3: The chromatograms of *V. rotundifolium*, *M. aethiopicum*, *T. oleifolius* and *D. cinerea* dried leaves extracted with different solvents and developed in BEA, CEF and EMW mobile systems then sprayed with vanillin-sulphuric acid reagent.

Key: H= n-Hexane, E= Ethyl acetate, A= Acetone, M= Methanol and W= Water

3.3.3. Preliminary screening for phytoconstituents

The different secondary metabolites present in the plant extracts are represented in Table 3.1. All the plants revealed the presence of terpenoids, flavonoids, phlobatannin, tannins steroids and cardiac-glycosides; the absence of alkaloids and saponins. Therefore, it was observed that similar phytochemicals are present in both the parasite and host plants.

Table 3. 1: Phytoconstituents of *V. rotundifolium*, *M. aethiopicum*, *T. oleifolius* and *D. cinerea* leaf extracts.

Phytoconstituents	*V. rotundifolium*	*M. aethiopicum*	*T. oleifolius*	*D. cinerea*
Terpenoids	**+**	**+**	**+**	**+**
Alkaloids	**-**	**-**	**-**	**-**
Saponins	**-**	**-**	**-**	**-**
Flavonoids	**+**	**+**	**+**	**+**
Phlobatannin	**+**	**+**	**+**	**+**
Cardiac-glycosides	**+**	**+**	**+**	**+**
Tannins	**+**	**+**	**+**	**+**
Steroids	**+**	**+**	**+**	**+**

Key**:** (**+**) present, (**-**) absent

3.3.4. Quantification of major phytochemicals

The total concentration of the phenolic, tannin and flavonoid content present on the plant extracts were estimated using the equation (y=mx+c) obtained from the standard curves of gallic acid (Figure 3.4 and 3.5) and quercetin (Figure 3.6) at different concentrations. The linear curves indicate a positive linear relationship between the concentration of the phytochemicals and the absorbance.

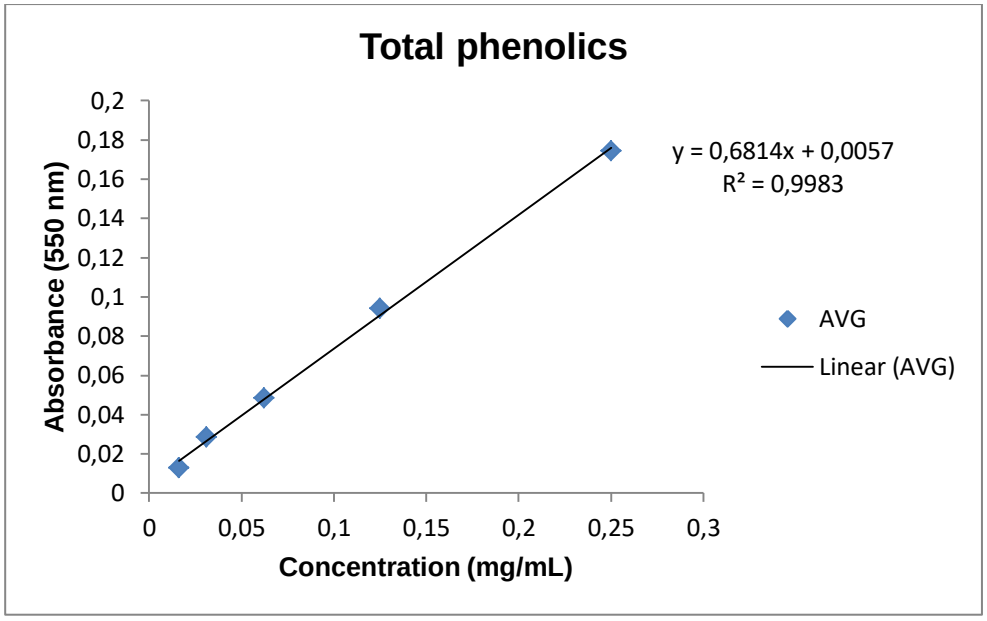

Figure 3.4: The gallic acid standard curve for total phenolic content determination.

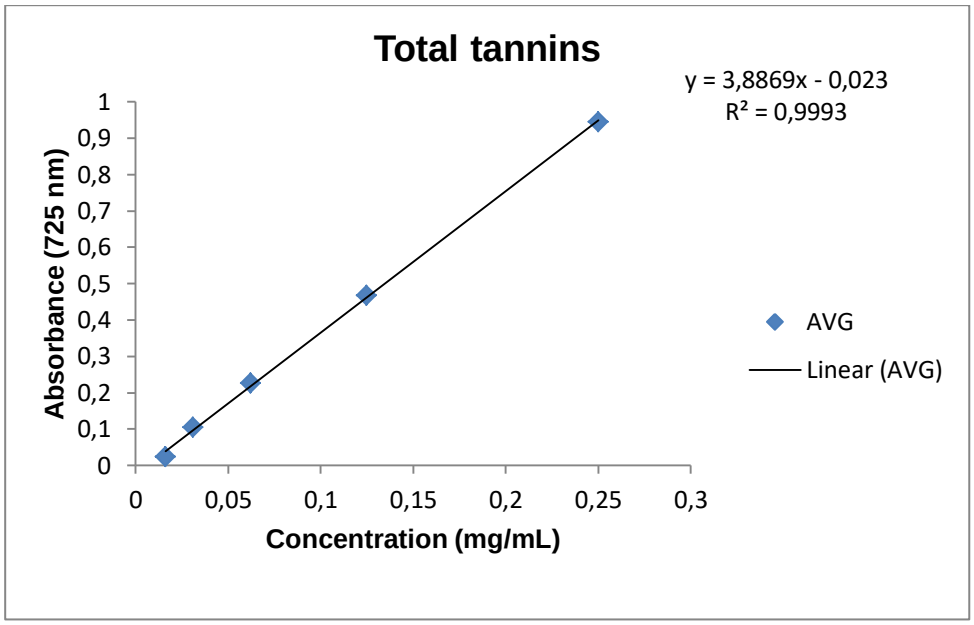

Figure 3.5: The gallic acid standard curve for total tannin content determination.

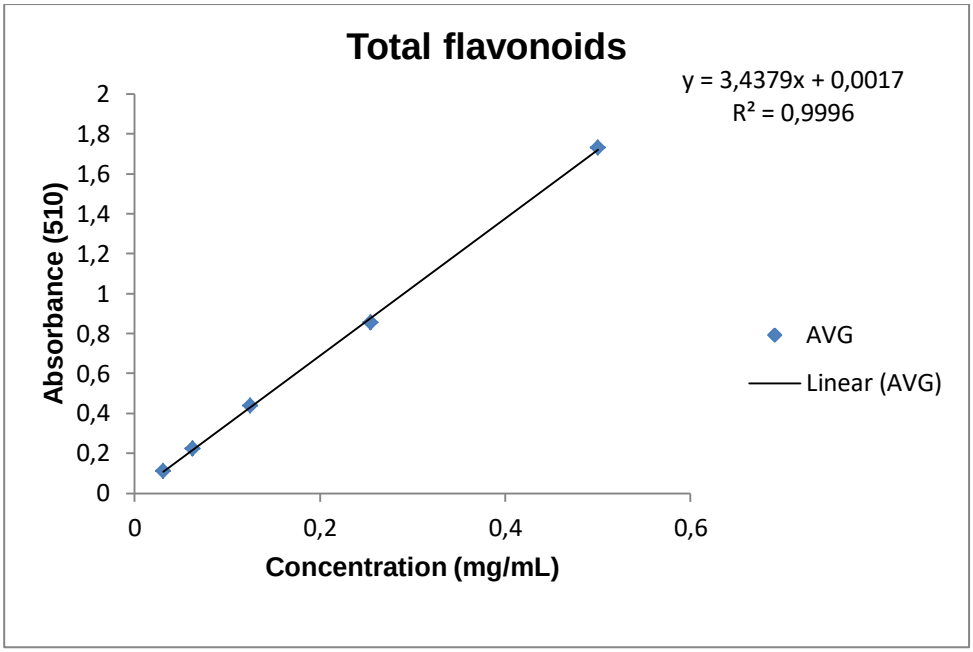

Figure 3.6: The quercetin standard curve for total flavonoid content determination.

The estimated concentration of the total phenolic content ranged from 15.605 to 893.210 mgGAE/g of extract, the total tannin content ranged from 2.135 to 34.801 mgGAE/g of extract and the total flavonoid content ranged from 0.048 to 15.367 mgQE/g of extract (Table 2). The n-hexane extract of *T. oleifolius* was significantly high in flavonoid content (34.801±0.798 mgQE/g of extract) and tannin content (15.367±0.320 mgGAE/g of extract) whereas the ethyl acetate extract of *M. aethiopicum* was high in phenolic content (893.210±3.016 mgGAE/g of extract).

Table 3.2: The total phenol, tannin and flavonoid content of *M. aethiopicum*, *V. rotundifolium*, *T. oleifolius* and *D. cinerea* leaves.

Solvents	Phenolic content (mg GAE/g extract)	Flavonoid content (mg QE/g extract)	Tannin content (mg GAE/ g extract)
	V. rotundifolium		
n-Hexane	702.43 ± 3.66	10.32 ± 0.87	16.76 ± 0.36
Ethyl acetate	187.31 ± 2.49	6.09 ± 0.78	15.51 ± 0.44
Acetone	338.96 ± 3.02	6.63 ± 0.82	7.77 ± 0.48
Methanol	748.90 ± 3.17	2.87 ± 0.40	10.87 ± 0.25
Water	28.32 ± 3.17	2.39 ± 0.24	7.27 ± 0.52
	M. aethiopicum		
n-Hexane	553.71 ± 4.32	3.98 ± 0.69	13.18 ± 0.07
Ethyl acetate	893.21 ± 3.02	6.07 ± 0.12	18.44 ± 0.07
Acetone	191.22 ± 2.08	2.28 ± 0.56	3.62 ± 0.14
Methanol	322.82 ± 1.83	0.05 ± 0.05	4.05 ± 0.66
Water	86.05 ± 3.02	0.85 ± 0.11	2.14 ± 0.24
	T. oleifolius		
n-Hexane	173.61 ± 5.49	15.37 ± 0.32	34.80 ± 0.80
Ethyl acetate	42.51 ± 4.21	12.03 ± 0.55	28.78 ± 0.42
Acetone	31.26 ± 3.17	5.40 ± 0.19	12.88 ± 0.84
Methanol	147.20 ± 3.59	2.72 ± 0.11	12.34 ± 0.83
Water	15.61 ± 3.66	3.29 ± 0.06	12.96 ± 0.73
	D. cinerea		
n-Hexane	54.74 ± 5.22	5.91 ± 0.53	22.98 ± 0.18
Ethyl acetate	71.86 ± 4.99	6.50 ± 0.14	25.20 ± 0.35
Acetone	28.32 ± 2.40	4.82 ± 0.71	13.87 ± 0.57
Methanol	158.94 ± 4.79	3.00 ± 0.44	18.99 ± 0.60
Water	59.63 ± 3.66	1.21 ± 0.20	6.03 ± 0.73

Results are represented as mean of triplicates ± standard deviation

Key: GAE = gallic acid equivalence, QE = quercetin equivalence

3.4. Discussion

Plants have been used as a source of medicine to treat various ailments. These health benefits are mostly ascribed to the presence of many active phytochemicals in the plants (Dhawan and Gupta, 2017). Hence, the current study aimed to evaluate the possible bioactive chemical compounds from the leaves of two hosts plants and their parasites; *Mystroxylon aethiopicum*, *Viscum rotundifolium*, *Dichrostachys cinerea,* and *Tapinanthus oleifolius* used to treat bacterial infections. The four selected plants were screened for phytochemicals with the desire of isolating and purifying active compounds that can be used to develop novel antibacterial drugs.

The dried and ground leaves of *M. aethiopicum*, *V. rotundifolium*, *D. cinerea,* and *T. oleifolius* were extracted with solvents of varying polarity from non-polar to polar, namely; n-hexane, ethyl acetate, acetone, methanol, and water. The mass extracted from each plant material using the different solvents are represented in figure 3.1. The results showed that methanol extracted most of the plant material in all plants as compared to the other solvents; *T. oleifolius* (370 mg), *D. cinerea* (310 mg), *V. rotundifolium* (310 mg) and *M. aethiopicum* (270 mg). Methanol is a good extractant resulting in a high amount of mass extracted since it has the ability to extract both the polar and non-polar compounds present in the plant materials (Dhawan and Gupta, 2017).

The Thin-Layer Chromatography (TLC) was used to separate the chemical constituents to create phytochemical fingerprints of different extracts of *V. rotundifolium*, *M. aethiopicum, T. oleifolius,* and *D. cinerea* (Figure 3.3). The separated fluorescing compounds were visualised using ultraviolet light at the wavelength of 365 nm and 245 nm. Distinct fluorescent compounds were observed on the CEF and EMW mobile phase (Figure 3.3). This indicates that compounds of diverse structural conformations react differently and fluoresce at different wavelengths (Matotoka and Masoko, 2018). The non-fluorescent separated compounds were detected with vanillin-sulfuric acid spray reagent. The compounds separated on the polar (EMW) mobile phase had a high number of bands with distinct colours than any other mobile phase for all the leaf extracts. Furthermore, similar compounds between the hosts and the parasites were observed.

Previous research studies on plants indicated that various secondary metabolites are potential antimicrobial agents although in many cases they serve as defence mechanisms against microorganisms in plants (Vaghasiya *et al*., 2011). Therefore, it was important to evaluate and screen the different secondary metabolites. Preliminary phytochemical screening (Table 3.1) revealed the presence of terpenoids, flavonoids, phlobatannins, tannins, steroids and cardiac-glycosides and the absence of alkaloids and saponins in all the plants. Therefore, it was observed that similar phytochemicals are present in both the parasite and host plants, this is because the pharmacologically active compounds may pass from the host tree to the parasite plants (Khwaja *et al*., 1984). The phytochemical results of the leaves of *D. cinerea* in this study confirm the results obtained by Eisa *et al*. (2000), Neondo *et al*. (2012), Vijayalakshmi *et al*. (2013) and Shandukani *et al*. (2018) except for the saponins and alkaloids which were absent. The variation on the phytochemicals may be due to the seasonal and geographical location, nature of the soil, growth conditions and the biochemical factors within the individual species and plant parts which include genetic variation and plant age (Prance, 1994; Bopana and Sexena, 2007). Furthermore, these variations may also occur due to the treatment of the plants after collection which includes storage and preparation (Houghton and Raman, 1998; Stafford *et al*., 2005). Therefore, the same medicinal plants collected from various locations may not always produce the same phytochemicals.

The quantitative measure of the tannin content, phenolic content and flavonoid content was performed to estimate the concentration of the major phytochemicals in the plants' crude extracts. The total phenolic content ranged from 15.605 to 893.210 mgGAE/g of extract, while the total tannin content ranged from 2.135 to 34.801 mgGAE/g of extract and the total flavonoid content ranged from 0.048 to 15.367 mgQE/g of extract. Between the parasites, the ethyl acetate extract of *T. oleifolius* was high in flavonoid (34.801±0.798 mgQE/g of extract) and tannin content (15.367±0.320 mgGAE/g of extract) whereas the methanol extract of *V. rotundifolium* was high in phenolic content. Between the hosts, *M. aethiopicum* ethyl acetate extract had the highest concentration of phenolic content (893.210±3.016 mgGAE/g of extract) while the *D. cinerea* ethyl acetate extract had the highest concentration of tannin (25.204±0.345 mgGAE/g of extract) and flavonoid content (6.505 ±0.143 mgQE/g of extract). Relative to *M. aethiopicum*, methanol extract of *V. rotundifolium*

had a high concentration of phenolic content while the n-hexane extract had a high concentration of flavonoid and tannin content. In *M. aethiopicum*, the ethyl acetate extract was high in all quantified phytochemicals (Table 3.2). Relative to *D. cinerea,* the n-hexane extract of *T. oleifolius* was significantly higher with all the quantified phytochemicals. Whereas the methanol extract of *D. cinerea* was high in phenolic content, the ethyl acetate extract was high in both the flavonoid and tannin content. The total phenolic content in all the plants is higher than the flavonoid and tannin content. This is because the phenolic phytochemicals include flavonoids and tannins (Stankovic, 2011). The study conducted by Shandukani *et al*. (2018) showed that the total phenolic content in *D. cinerea* increase with an increase in the polarity of the solvent. This variation in the amount of phenolic content may be influenced by the different location in which the plant leaves were collected.

3.5. Conclusion

The extraction of the plant materials with different solvents resulted in the separation of various phytochemicals that were similar in both the parasite and host plants on the TLC. This was confirmed by the screening of major phytoconstituents, where the terpenoids, flavonoids, phlobatannins, cardiac glycoside, tannins, and steroids were present in all plants. Furthermore, the screened major phytoconstituents are known to possess both the antibacterial and antioxidant activities. Therefore, the plants will be analysed further for their potential as antioxidant and antimicrobial agents.

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Chapter 4: Antioxidant analysis

4.1. Introduction

The key factor in the pathophysiology of most diseases is oxidative stress. Oxidative stress results from the imbalance between Reactive Oxygen Species (ROS) production and the elimination of cellular disturbance by the biological system (Nita and Grzybowski, 2016). Although the ROS has beneficial effects on several physiological processes in the body, excessive productions of ROS possess a serious problem to the body's homeostasis and cause oxidative tissue damage (Bhattacharyya *et al*., 2014). This causes chronic diseases such as cancer, aging, diabetes, cardiovascular and neurodegenerative diseases (Poulson *et al*., 1998).

Despite the continuous generation of free radicals, the body is well suited to fight against the harmful effects of the ROS with the help of antioxidants. Due to the limitation of the antioxidants produced in the body, there is a continuous need for antioxidant sources in the body (Bhattacharyya *et al*., 2014). The synthetic antioxidants have been used to eliminate the free radicals in the body but due to their side effects, research on the natural antioxidants that are safe and effective has gained momentum (El Jemli *et al*., 2016). Several studies have reported that medicinal plants contain compounds such as phenolic, flavonoids, and tannins which are known for their health benefits as antioxidants (Cherrat *et al*., 2014).

Amongst the different methods for measuring the antioxidant activity, the recommended *in vitro* assays to analyse the antioxidant activity of the plants include DPPH radical scavenging assay, phosphomolybdenum and reducing power (Chahmi *et al*., 2015). In this study, two different antioxidant assays: 2, 2, diphenyl-1-picrylhydrazyl (DPPH) assay and ferric reducing power were used to determine the antioxidant activity of *V. rotundifolium*, *M. aethiopicum*, *T. oleifolius* and *D. cinerea*. Given the reports in chapter 2 of how these plants are extensively used in combating some oxidative stress-mediated diseases, the aim of this chapter was to evaluate the antioxidant activity of the leaves qualitatively using the DPPH radical scavenging assay on Thin Layer Chromatography (TLC) and quantitatively, using the DPPH radical scavenging assay and the ferric reducing power assay.

4.2. Materials and methods

4.2.1. Qualitative DPPH free radical scavenging activity assay on TLC plates

The DPPH assay, as previously used by Braca *et al*. (2002), was employed with a colour change of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) (Sigma-Aldrich). DPPH is purple and is reduced to diphenyl-1-picryl hydrazine which is yellow. The extracts were reconstituted in acetone to 10 mg/mL and 10 µL of each extract was loaded in a 1 cm wide line on the TLC plate and developed in EMW, CEF, and BEA eluent systems. The plate was dried in a stream of air to evaporate the solvents and later sprayed with 0.2% DPPH in methanol and the colour change was observed.

4.2.2. Quantitative antioxidant activity assay

4.2.2.1. DPPH free radical scavenging assay

The free radical scavenging activity of the plant extracts was quantified using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) method reported by Gyamfi *et al*. (1999) with slight modifications (Chigayo *et al*., 2016). Briefly, different concentrations of the plant extract (250–15.63 µg/mL) were prepared to a volume of 1 mL of the solution. L-Ascorbic acid was used as standard by preparing the same concentration range as the plant extracts. To these 1 mL solutions, 1 mL of 0.2 mmol/L DPPH solution dissolved in methanol was added and vortexed thoroughly. All the prepared mixtures were incubated in the dark for 30 minutes. The blank was prepared in the same manner as the experimental solutions, however, 1 mL of acetone was added instead of the plant extracts. The control solution was prepared by adding 2 mL of 0.2 mmol/L DPPH to 1 mL of distilled water. After the elapsed time, the solutions were analysed with a UV/VIS spectrophotometer (Thermo Scientific). The absorbance of the solutions was read at 517 nm and the percentage inhibition was calculated using the formula below where, **Ac** is the absorbance of the control solution, **As** is the absorbance of the plant extract. The experiment was run in duplicate and repeated three times.

$$\textbf{\% inhibition} = \frac{Ac - As}{Ac} \times 100$$

4.2.2.2. Ferric reducing power assay

The ferric reducing power of the plant extracts was determined using the methods of Oyaizu (1986). Five different concentrations of the plant extracts (625–39 µg/mL) were prepared by serially diluting a stock solution of 1250 µg/mL. The different concentrations (2.5 mL) were mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v in distilled water), in a test tube. The mixtures were vortexed after the addition of solutions. The mixtures were incubated at 50°C for 20 minutes. Two millimeters of trichloroacetic acid (10% w/v in distilled water) was added to the test tubes after incubation. The mixtures were centrifuged at 3000 rpm for 10 min and 5 mL of the resulting supernatant was transferred to a clean test tube. To this solution, 5 mL of distilled water and 1 mL ferric chloride (0, 1% w/v in distilled water) were added consecutively with thorough vortexing after each addition. A UV/VIS spectrophotometer (Thermo Scientific) was used to read the absorbance of solutions at 700 nm wavelength. The blank for this procedure was prepared in the same manner, however, the plant extracts were replaced by an equal amount of acetone. L-Ascorbic acid (625–39 µg/mL) was used as a positive control and was prepared similar to the plant extracts. The experiments were performed in triplicates and repeated three times.

4.3. Results

4.3.1. Qualitative DPPH free radical scavenging activity assay on TLC plates

The 2, 2, diphenyl-1-picrylhydrazyl (DPPH) assay on the TLC plates was used to test for the radical scavenging activity of the different compounds present in the plant extracts. The antioxidant activity of the plant extracts was observed by the presence of yellow bands on the chromatogram resulting from the reduction of the purple DPPH as illustrated in figure 4.1. The results indicated that the compounds that exhibit antioxidant activity are non-polar to polar as observed on the BEA, CEF and EMW mobile phase. The *T. oleifolius* and *D. cinerea* plant extracts had prominent antioxidant compounds.

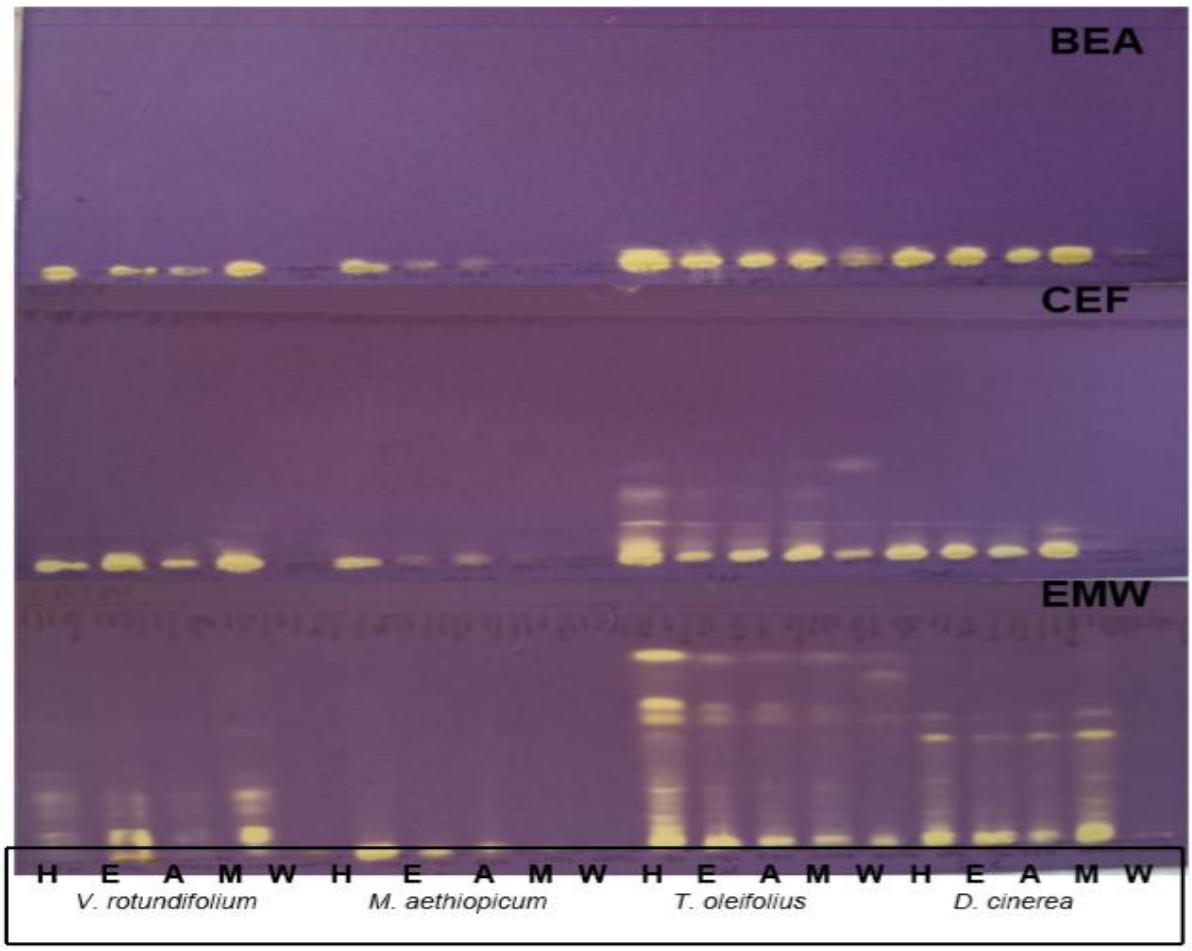

Figure 4.1: The chromatograms of *V. rotundifolium*, *M. aethiopicum*, *T. oleifolius* and *D. cinerea* dried leaves extracted with different solvents and developed in BEA, CEF and EMW mobile systems and sprayed with 0.2% DPPH in methanol. The yellow zones indicate the antioxidant activity.

Key: H= n-Hexane, E= Ethyl acetate, A= Acetone, M= Methanol and W= Water

4.3.2. Quantitative antioxidant activity assay

The quantitative antioxidant activity of the plant extract was performed using free radical scavenging assay and ferric reducing power assay.

4.3.2.1. DPPH free radical scavenging activity assay

The antioxidant activity of the plant extracts was quantified using 2, 2, diphenyl-1-picrylhydrazyl (DPPH) reduction and compared with ascorbic acid as illustrated in figure 4.2A and 4.2B. The antioxidant activity of the plant extracts was expressed as the percentage inhibition. The antioxidant activity increases with increase in

concentration of the plant extract. The ethyl acetate extract of *M. aethiopicum* (Figure 4.2A) had the highest activity while the methanol extract had the lowest activity. The methanol extract of *V. rotundifolium* (Figure 4.2B) had the highest antioxidant activity while the acetone and water extract had the lowest activity.

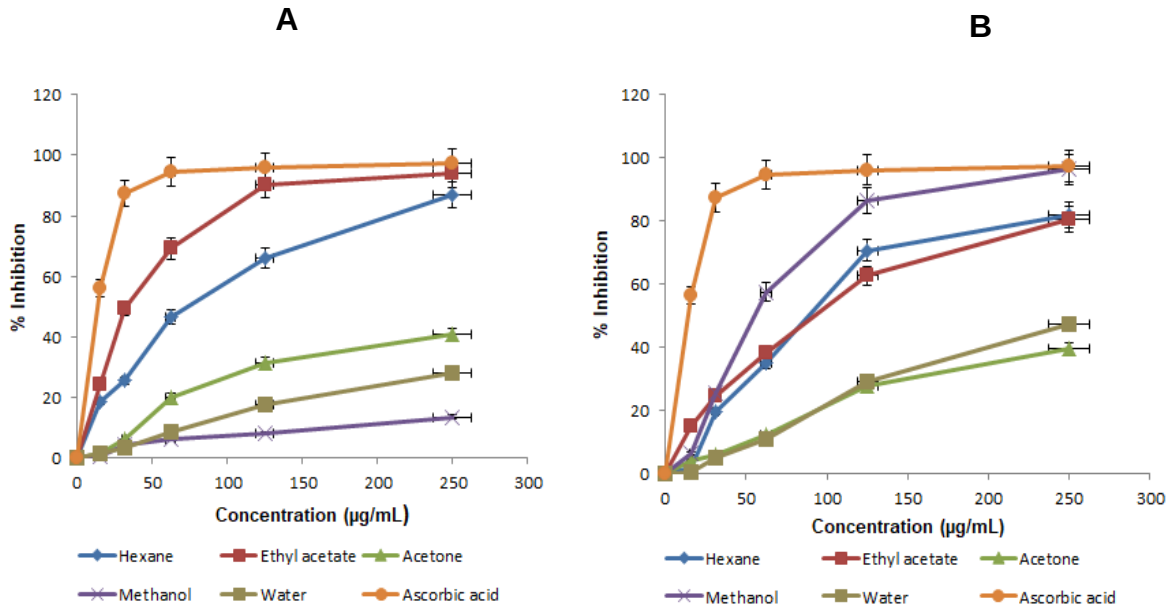

Figure 4.2: The percentage free radical (DPPH) inhibition of *M. aethiopicum* (**A**) and *V. rotundifolium* (**B**).

The values used are mean of triplicates ± standard deviation

The antioxidant activity of methanol, ethyl acetate and n-hexane extracts of *D. cinerea* (Figure 4.3A) were high while the antioxidant activity of the water extract was the lowest. The methanol extract of *T. oleifolius* (Figure 4.3B) had the highest antioxidant activity while the water and acetone extract had the lowest activity.

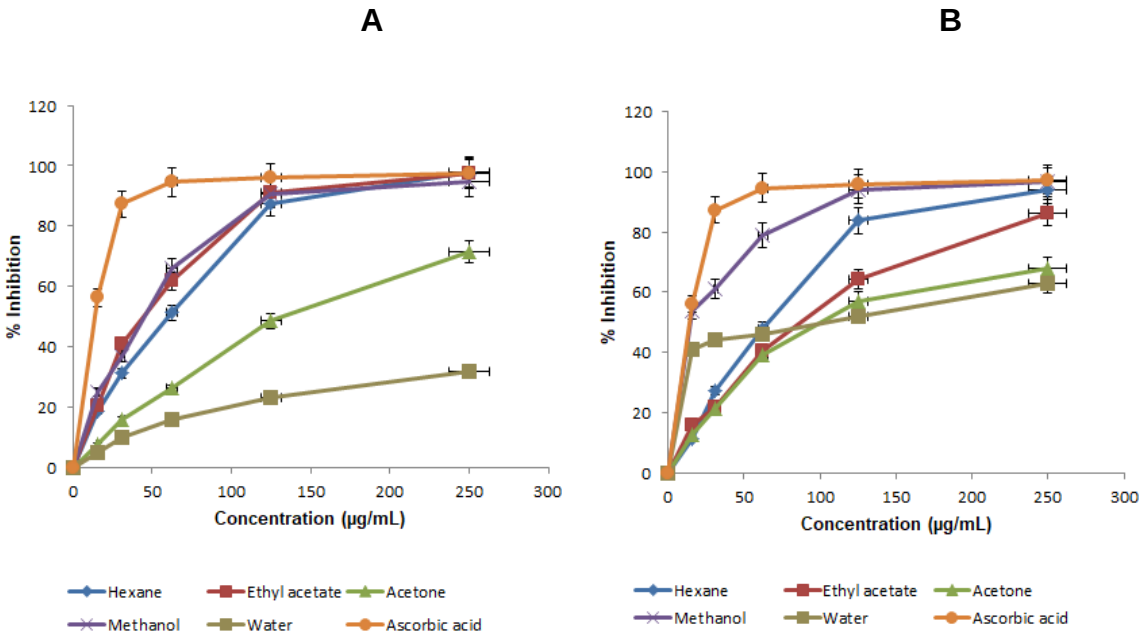

Figure 4.3: The percentage free radical (DPPH) inhibition *D. cinerea* (**A**) and *T. oleifolius* (**B**).

The values used are mean of triplicates ± standard deviation

4.3.2.2. Ferric reducing power

The antioxidant activity of the plant extracts was also evaluated using the ferric reducing power method. The antioxidant activity of the plant extracts was compared to the standard (ascorbic acid) in a concentration dependent manner. The increase of the absorbance with the increase in concentration indicated the degree of the reducing power of the extract. The reducing power of the leaves of *M. aethiopicum* was found remarkable where the ethyl acetate and n-hexane extracts had high reducing ability as compared to ascorbic acid and other extracts (Figure 4.4A). The methanol extract of *V. rotundifolium* had the highest reducing power as compared to ascorbic acid and other extracts (Figure 4.4B).

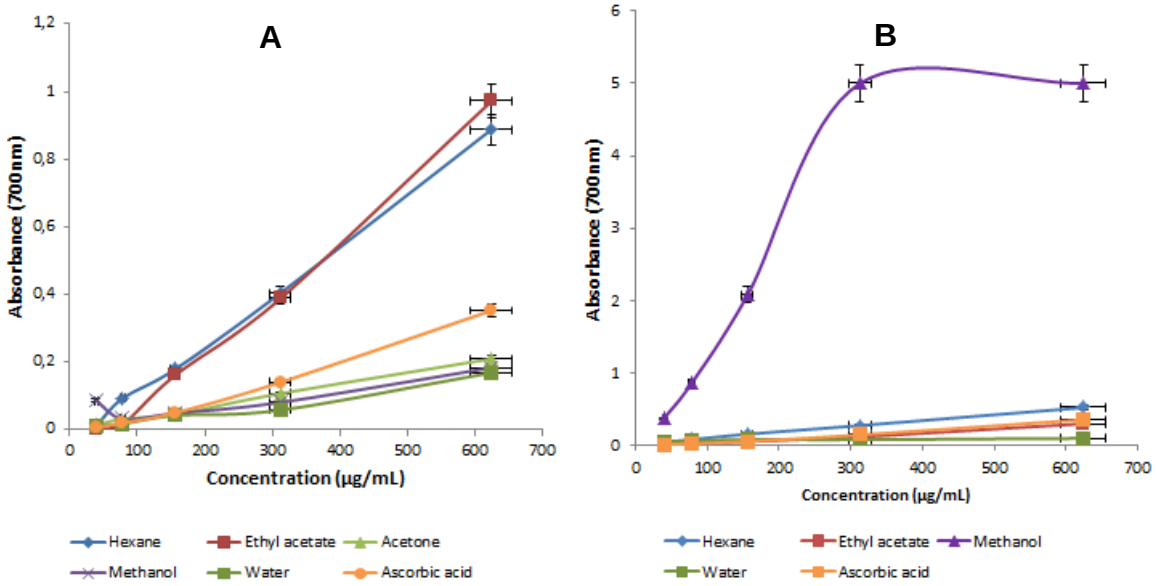

Figure 4.4: The ferric reducing power of *M. aethiopicum* (**A**) and *V. aethiopicum* (**B**).

The values used are mean of triplicates ± standard deviation.

The reducing power of the ethyl acetate and n-hexane leaf extracts of *D. cinerea* was higher than ascorbic acid and other extracts (Figure 4.5A). The methanol, ethyl acetate and n-hexane leaf extracts of the *T. oleifolius* had the highest activity than ascorbic acid and water (Figure 4.5B).

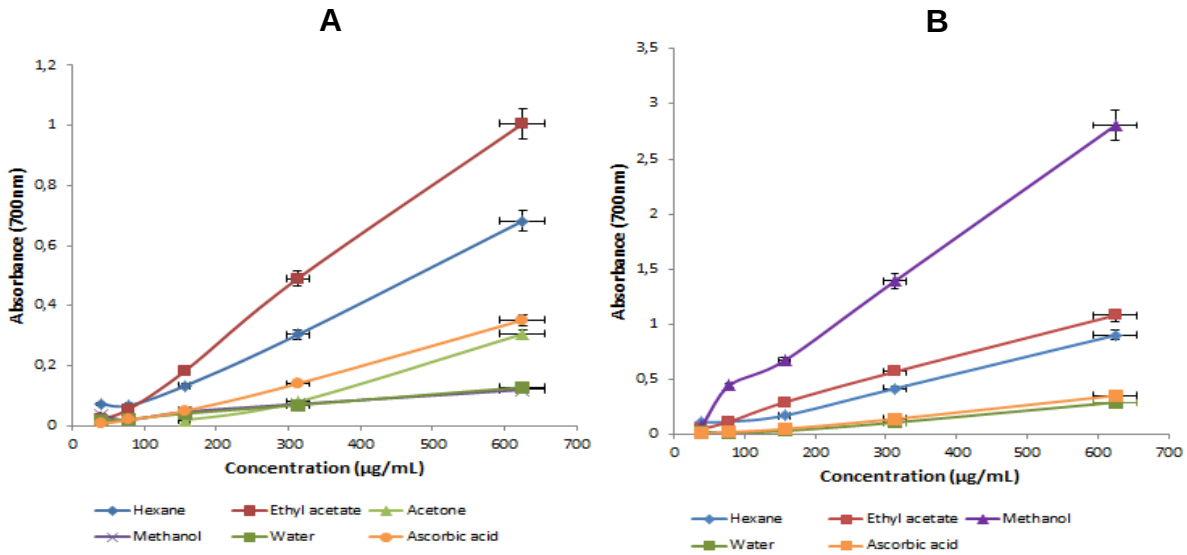

Figure 4.5: The ferric reducing power of *D. cinerea* (**A**) and *T. oleifolius* (**B**).

The values used are mean of triplicates ± standard deviation.

4.4. Discussion

There has been a rapid increase on the interest of finding naturally occurring antioxidants for use as therapeutic agents or food source to replace the synthetic antioxidant based on their risk of causing cancer (Mohammad *et al*., 2019). In this study, two different antioxidant assays (DPPH and ferric reducing power) were used since different antioxidant compounds act through various and distinct mechanisms against the oxidising agents. Furthermore, it is difficult to correctly evaluate the antioxidant efficacy of plant extracts using one method (Xiao *et al*., 2015). The 2, 2, diphenyl-1-picrylhydrazyl (DPPH) assay on the TLC plates was used to test for the radical scavenging activity of the different compounds present on the plant extracts. The DPPH is a purple and stable free radical that is reduced in the presence of an antioxidant molecule based on electron transfer (Huang *et al*., 2005).

Antioxidant activity of the plant extracts was observed by the presence of yellow bands on the chromatograms resulting from the reduction of the purple DPPH as illustrated in figure 4.1. The compounds that exhibit antioxidant activity are non-polar to polar as observed on the BEA, CEF and EMW mobile phase. The *T. oleifolius* and *D. cinerea* plant extracts had prominent antioxidant compounds on the CEF and EMW mobile phase which are intermediate and polar mobile phases. Similar results on *D. cinerea* were observed by Shandukani *et al*. (2018). The antioxidant compounds of *M. aethiopicum* and *V. rotundifolium* were observed on the EMW but did not separate well in the BEA and CEF mobile phase. This is mainly caused by the polarity of the compounds that are present. The compounds may be polar than the mobile phase, for this reason, they tend to stick more tightly to the plate than moving along with the solvent (Bele and Khale, 2010).

The quantitative antioxidant activity of the plant extract was performed spectrophotometrically using 2, 2, diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and ferric reducing power method. The DPPH free radical scavenging assay is the rapid, simple and frequently used method to measure the antioxidant potential of the plant extracts (Prakash, 2011). The antioxidant activity of the plant extracts was expressed as the percentage inhibition (Figure 4.2 and 4.3B). The antioxidant activity increases with increase in concentration of the plant extract. The ethyl acetate extract of *M. aethiopicum* (Figure 4.2A) had the highest activity

while the methanol extract had the lowest activity. The methanol extract of *V. rotundifolium* (Figure 4.2B) had the highest antioxidant activity while the acetone and water extract had the lowest activity. The antioxidant activity of methanol, ethyl acetate and n-hexane extracts of *D. cinerea* were high while the antioxidant activity of the water extract was the lowest (Figure 4.3A). The methanol extract of *T. oleifolius* had the highest antioxidant activity while the water and acetone extract had the lowest activity (Figure 4.3B).

The antioxidant activity of the plant extracts was also evaluated using the ferric reducing power method. The ferric reducing power measures the reduction of ferric ion (fe^{3+}) to ferrous ion (fe^{2+}) in the presence of antioxidants where the yellow colour of the test solution changes to green or blue depending on the reducing power of the samples (Benzie and Strain, 1996). When the pH is low, the ferric/ferricyanide complex is reduced to ferrous form and this results in the formation of an intense Perl's Prussian that is measured at 700 nm (Gülçin *et al*., 2012). The antioxidant activity of the plant extracts was compared to the standard (ascorbic acid) in a concentration dependent manner. The leaves of *M. aethiopicum* were found remarkable where the ethyl acetate and n-hexane extracts had a high reducing ability as compared to ascorbic acid and other extracts (Figure 4.4A) while methanol extract of *V. rotundifolium* had the highest reducing power as compared to ascorbic acid and other extracts (Figure 4.4B). The reducing power of the ethyl acetate and n-hexane leaf extracts of *D. cinerea* was higher than ascorbic acid and other extracts (Figure 4.5A) while the methanol, ethyl acetate and n-hexane leaf extracts of *T. oleifolius* had the highest activity than ascorbic acid and water extract (Figure 4.5B). The quantification of the major phytochemicals in chapter 3 revealed that the total phenolic content in all the plants is higher than the flavonoid and tannin content. Therefore, since the phenolic compounds, including the flavonoids, are known to have antioxidant activity, it is likely that the antioxidant activity of these plants is attributed to these compounds (Tepe *et al*., 2006). In addition, it was observed that the plant extracts with high phenolic content have high antioxidant activity. Despite the fact that different antioxidant assays (DPPH and Ferric reducing power) were used in this study, they have different reaction mechanisms and do not necessarily measure the same activity (Prior *et al*., 2005). For this reason, the extracts did not have the same activity in all assays.

4.5. Conclusion

The chapter demonstrated the antioxidant potential of the plants which may be viewed as a result of the high amount of phenolic content present. The wide range of antioxidant compounds were extracted during the during extraction process which resulted in different antioxidants quantified in all the plants. Therefore, further pharmacological studies and isolation of these antioxidant active compounds is important for them to act as sources of natural antioxidant supplements.

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Chapter 5: Antibacterial assays

5.1. Introduction

Some of the antibiotics developed thousands of years ago are now ineffective in treating infections due to a number of limitations. These include high cost and poor supply in developing countries, antibiotic resistance by microorganisms and unfavourable side effects (Walsh *et al*., 2004; Suleiman *et al*., 2010). This has increased the search for and development of new antimicrobial agents. Several scientific research studies have reported that medicinal plants contain a variety of secondary metabolites that exhibit various biological activities. This led to the isolation of drugs such as aspirin, digitoxin, morphine, quinine and pilocarpine (Butler, 2004). It is also estimated that 25% of the drugs approved are derived from secondary metabolites from plants (Newman and Cragg, 2016). Therefore, in order to screen the plants for compounds that exhibit antimicrobial and antifungal activity, various bioassays were developed, namely; dilution methods, diffusion methods and bioautography methods (Balouiri *et al*., 2016).

In this study, in order to test the plant extracts for antimicrobial agents the bioautography assay and serial broth microdilution, assays are selected. Bioautography is one of the microbiological screening methods that are commonly used to detect the antimicrobial activity of organic extracts mostly in plants. This is the first procedure used to determine the presence or absence of bioactive compounds within plant extracts (Aerts *et al*., 1995). Despite the fact that the screening methods are highly sensitive as compared to other methods, they are simple, inexpensive, save time and require simple equipment. The bioautography detection method can be coupled with other chromatographic techniques such as Thin Layer Chromatography (TLC), High Performance Thin-Layer Chromatography (HPTLC), Over Pressured-Layer Chromatography (OPLC) and Planer Chromatography (PEC) (Choma and Grzelak, 2011).

There are three bioautographic techniques, namely; agar/contact diffusion, direct bioautography and agar overlay/immersion assays. In direct bioautography, the microorganisms grow directly on Thin-Layer Chromatography (TLC) plates in a humid chamber in contact with bioautography, where the antimicrobial compounds

are transferred from the TLC plate to an inoculated agar plate, and in agar overlay or immersion bioautography, where an inoculated agar medium is applied onto the TLC plate (Balouiri, 2016). In this study, the direct bioautography method was used. The TLC plates that are developed are sprayed with a microbial suspension and then incubated under humid conditions (Dwanjee *et al*., 2015). After incubation, the microbial growth is visualised by spraying the bioautograms with a tetrazolium salt which is converted to an intensely coloured formazan by dehydrogenase enzyme in living microorganisms (Grare, 2008). Cream white zones against the purple background on the surface of the TLC demonstrate the antimicrobial activity of the sample (Silva, 2005; Runyoro, 2006; Choma and Grzelak, 2011; Cieśla, 2015).

Serial broth microdilution assay is one of the dilution methods used to quantify the antimicrobial activity of the antimicrobial agents (plant extracts) against the test microorganisms. It is used to determine the lowest concentration of the antimicrobial agents that inhibit the tested microorganism completely. This concentration is commonly known as Minimum Inhibitory Concentration (MIC) and is expressed in mg/mL or µg/mL (Pfaller *et al*., 2004; Balouiri *et al*., 2016). To determine the MIC value, the two-fold dilution of the antimicrobial agent is carried out in a 96-well microtitre plate. Then, the test microorganism that is grown in a broth is added and the mixture is incubated under the suitable conditions for the microorganism. To determine the antimicrobial activity, the tetrazolium salt is added in wells where it is converted to a formazan colour and indicates growth of the microorganisms while clear wells indicate the activity of the antimicrobial agent (Eloff, 1998). The total activity of the plant extracts depends on the quantity extracted from the plant material. This total activity indicates the volume in which an extract from 1 g of the plant material can be diluted and still inhibit the growth of the test microorganism. Therefore, to determine the total activity, the quantity extracted (in mg) from 1 g of plant material is divided by the MIC (in mg/mL) (Eloff, 2000; Adamu *et al*., 2014).

The antimicrobial compounds in plants may be less effective when they are acting on their own. However, when combined with compounds from other plants, they become more effective. This interaction is known as synergy. In a situation where one compound inhibits the activity of another compound, the interaction is called antagonism (Hemaiswarya *et al*., 2008; Rani *et al*., 2009). Therefore, the interaction between the compounds is achieved by determining the MIC of the combinations

exhibiting antibacterial activity. The aim of this chapter was to evaluate the antimicrobial activity of the selected plants against the bacterial strains using bioautography and broth micro-dilution assay. This chapter also aimed at determining the antagonistic and synergistic effects of the parasite and host plants.

5.2. Materials and methods

5.2.1. Bacterial cultures

The pathogenic bacterial species selected were: Gram-positive (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212) and Gram-negative (*P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922). These microorganisms represent the pathogenic species that are commonly associated with nosocomial infections and are selected based on the recommendation of the Clinical Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, 1990). These bacterial species were maintained on a nutrient agar at 4°C. The cultures will later be inoculated in nutrient broth and incubated at 37°C for 12 hours prior to the screening procedures.

5.2.2. Qualitative antibacterial activity assay

5.2.2.1. Bioautographic assay

The qualitative analysis of the number of antimicrobial compounds found in the extracts was determined by the bioautographic method (Begue and Kline, 1972). The TLC plates were loaded with 20 µL of each extract (10 mg/mL) and developed in EMW/ CEF/ BEA as described in chapter 3. The plates were then dried at room temperature under a stream of air for 5 days to remove the remaining solvent. The developed TLC plates were sprayed with concentrated suspension of bacterial cultures of *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* which were grown overnight in nutrient broth at 37°C until completely moist. This process was carried out in a laminar flow cabinet (Labotec). Thereafter, the plates were incubated overnight at 37°C in 100% humidity. The plates were then sprayed with a 2 mg/mL solution of p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich) and further incubated for 2-6 hours. Bacterial growth led to the emergence of a purple-pink colour resulting

from the reduction of INT into the corresponding formazan salt. White bands indicated the inhibition of the bacteria by the active compounds present.

5.2.3. Quantitative antibacterial activity assay

5.2.3.1. Serial broth microdilution assay

A serial microdilution assay (Eloff, 1998) was used determine the Minimum Inhibitory Concentration (MIC) of the extracts, using INT reduction as an indicator. This was determined against Gram-positive *Staphylococcus aureus*, *Enterococcus faecalis*, and Gram-negative *Pseudomonas aeruginosa* and *Escherichia coli*. Aliquots of the extracts were dissolved in acetone to a final concentration of 10 mg/mL. Two-fold serial dilutions of extracts (2.5-0.02 mg/mL) were prepared in 96-well microtitre plate and 100 µL of the microbial culture was added to each well. Chloramphenicol (Sigma-Aldrich) was used as positive control and acetone was used as a negative control. As the indicator of growth, 40 µL of 0.2 mg/mL p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich) was added to the microtitre wells. The covered microtitre plates were incubated and examined after 30 minutes at 37°C at 100% relative humidity. Where bacterial growth was inhibited, the solution in the well showed a marked reduction in intensity of the colour after incubation with INT and where bacterial growth occurred, it was seen by the presence of a pink/purple colour on the wells. The MIC was recorded as the lowest concentration of the extract that inhibited bacterial growth. All samples were assayed in triplicates. The total activity was calculated by dividing the quantity extracted (in mg) from 1 g of plant material by the MIC (in mg/mL).

5.2.3.2. Antibacterial interaction effects of the selected plants

The stock solutions (10 mg/mL) of n-hexane, ethyl acetate, acetone and methanol extracts of each plant were prepared by reconstituting the extracts in acetone. Three different host and parasite combinations were performed. For 1:1 test combinations, 50 µL of each of the two extracts were mixed to make up a volume of 100 µL in the first wells of a 96-well microtitre plate. Each extract contributed 33.3 µL and 66.6 µL for the 1:2 and 66.6 µL and 33.3 µL for the 2:1 combinations, respectively, to make up 100 µL in the first wells of a 96-well microtitre plate. MIC values were determined for each of these combinations to establish any interaction effect following the

antibacterial assays described in section 5.2.3.1. Following investigations of the independent MIC of the selected plants, the synergistic or antagonistic interactions between the plants were investigated. This was achieved by determining the MIC of the combinations exhibiting antibacterial activity to establish any interaction effect. The Fractional Inhibitory Concentration (FIC) was calculated for the 1:1 combinations of the plants. This was determined with the equation below, where (i) and (ii) represented the different 1:1 plant combinations (Mabona *et al*., 2013). The FIC index was expressed as the sum of FIC (i) and FIC (ii) and this was used to classify the interaction as either synergistic (≤0.50), additive (0.50-1.00), indifferent (>1.00-4.00) or antagonistic (>4.00) (van Vuuren and Viljoen, 2008).

$$FIC(i) = \frac{\text{MIC of (a) in combination with (b)}}{\text{MIC of (a) independently}}$$

$$FIC(ii) = \frac{\text{MIC of (b) in combination with (a)}}{\text{MIC of (b) independently}}$$

5.3. Results

5.3.1. Bioautography assay

The qualitative antibacterial screening of the plant extracts against both the Gram-negative and Gram-positive bacteria as test microorganisms are represented in figure 5.1 and 5.2 below. The TLC plates were developed in three mobile phases based on their polarity, namely; BEA, CEF and EMW. Clear bands on the chromatograms indicate antibacterial activities of the plant extracts, which inhibited the growth of bacteria. The activity of the plant extracts against Gram-negative (*P. aeruginosa* and *E. coli*) are presented in figure 5.1. In the BEA mobile phase, the n-hexane, ethyl acetate, acetone and methanol extracts of *M. aethiopicum* showed activity against both *P. aeruginosa* and *E. coli* at the R_f values of 0.23, 0.3 and 0.18, respectively. Whereas the *V. rotundifolium* leaf extracts did not show any activity. No activity in both the *T. oleifolius* and *D. cinerea* extracts was observed against Gram-negative bacteria.

From the antibacterial activity of the plant extracts against Gram-positive bacteria (Figure 5.2), the *M. aethiopicum* n-hexane, ethyl acetate, acetone and methanol

extracts also showed activity against the tested bacteria (*S. aureus* and *E. faecalis*) at the R_f values of 0.23, 0.86 and 0.39, respectively. While the n-hexane, ethyl acetate, acetone and methanol of *V. rotundifolium* had activity against *E. faecalis* at the R_f value of 0.39. *T. oleifolius* and *D. cinerea* leaf extracts did not have antibacterial activity on the tested bacteria.

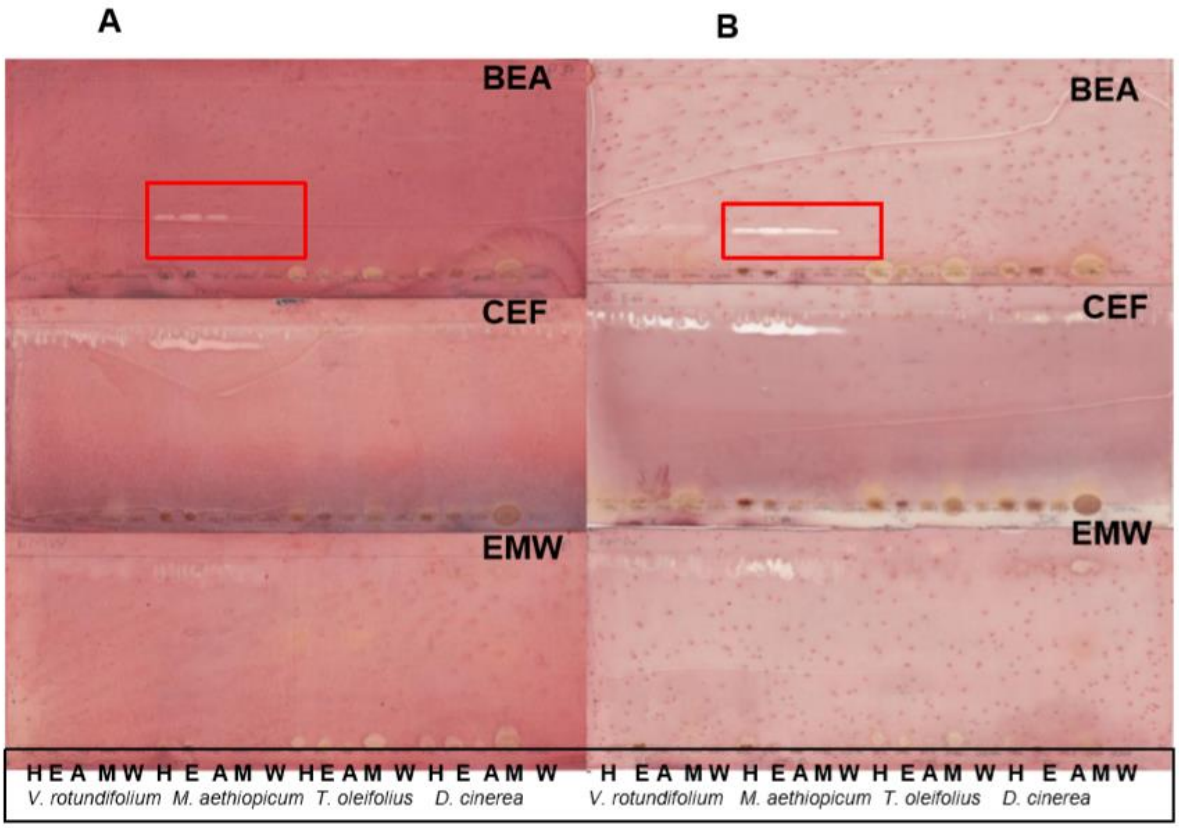

Figure 5.1: The chromatograms of *V. rotundifolium, M. aethiopicum, T. oleifolius* and *D. cinerea* plant extracts separated in BEA, CEF and EMW and sprayed with Gram-negative *P. aeruginosa* (**A**) and *E. coli* (**B**). White areas indicate zones of inhibition.

Key: H= n-Hexane, E= Ethyl acetate, A= Acetone, M= Methanol and W= Water.

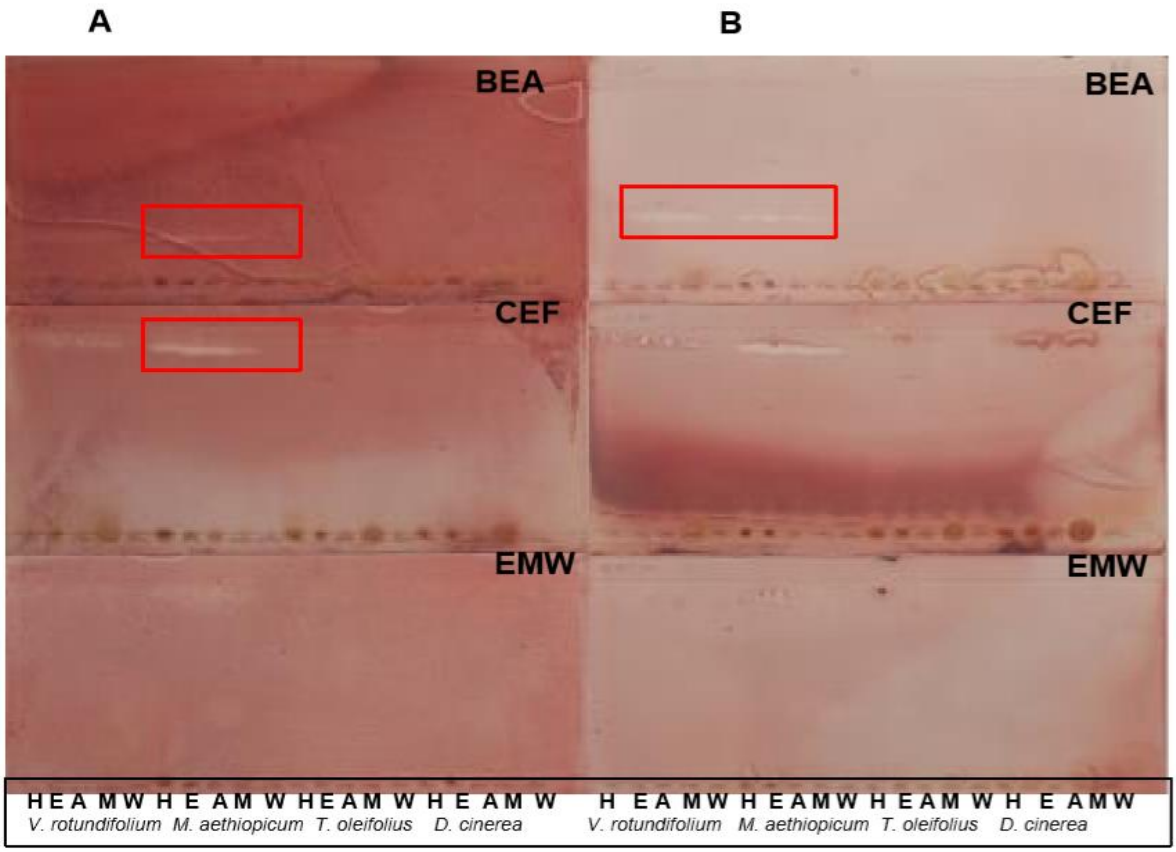

Figure 5.2: The chromatograms of *V. rotundifolium, M. aethiopicum, T. oleifolius* and *D. cinerea* plant extracts separated in BEA, CEF and EMW and sprayed with Gram-positive *S. aureus* (**A**) and *E. faecalis* (**B**). White areas indicate zones of inhibition.

Key: H= n-Hexane, E= Ethyl acetate, A= Acetone, M= Methanol and W= Water.

5.3.2. Serial broth microdilution assay

The quantitative microdilution method in table 5.1 confirms the antibacterial activity of the plant extracts where the MIC values ranged from 0.04-2.5 mg/mL. The lowest average MIC of the plant extracts was against *E. faecalis* (0.80 mg/mL) followed by *P. aeruginosa* (0.88 mg/mL) and lastly, *E. coli* (1.11 mg/mL) and *S. aureus* (1.18 mg/ML). The *T. oleifolius* and *D. cinerea* extracts showed significant antibacterial activity against all tested bacteria. The methanol extract amongst all extracts in both *T. oleifolius* and *D. cinerea* plants showed remarkable MIC values. The methanol extract of *D. cinerea* had highest activity against *S. aureus* (0.16 mg/mL) and *P. aeruginosa* (0.04 mg/mL) while the methanol extract of *T. oleifolius* had highest

activity against *E. faecalis* (0.04 mg/mL) and both *T. oleifolius* and *D. cinerea* methanol extract had highest activity against *E. coli* (0.08 mg/mL. The ethyl acetate, acetone and methanol extract of *M. aethiopicum* and ethyl acetate and water extracts of *V. rotundifolium* had highest activity against *S. aureus* (1.25 mg/mL), while the ethyl acetate extract of *M. aethiopicum* had highest activity against *E. faecalis* (0.16 mg/mL) and the n-hexane and ethyl acetate extracts of *M. aethiopicum* and n-hexane extract of *V. rotundifolium* had highest activity against *P. aeruginosa* (0.31 mg/mL) whereas the n-hexane, ethyl acetate and methanol extracts of *M. aethiopicum* and n-hexane and ethyl acetate extracts of *V. rotundifolium* had highest activity against *E. coli*.

Considering that the MIC value is inversely proportional to the quantity of antibacterial compounds present in plant extracts, the total activity was calculated by dividing the quantity extracted (in mg) from 1 g of plant material by the MIC (in mg/mL) (Table 5.2). The highest total activities were observed with the methanol extract of *D. cinerea* against *P. aeruginosa* (7750 mL/g), *E. faecalis* (3875 mL/g) and *E. coli* (3875 mL/g) respectively, whereas the methanol extract of *T. oleifolius* had the highest total activity against *S. aureus* (4625 mL/g). This means that the quantity extracted from 1 g of the *D. cinerea* material with acetone can be diluted to 7750 mL and still have the ability to inhibit the growth of *P. aeruginosa*.

Table 5.1: The MIC values of the selected plant extracts (mg/mL) against the test microorganisms.

Microorganisms	*V. rotundifolium*					*M. aethiopicum*					*T. oleifolius*					*D. cinerea*						
	H	E	A	M	W	H	E	A	M	W	H	E	A	M	W	H	E	A	M	W	Avg	PC
S. aureus	2.5	1.25	2.5	2.5	1.25	0.63	1.25	1.25	1.25	>2.5	0.31	0.31	0.63	0.08	1.25	0.63	0.31	0.63	0.16	2.5	**1.18**	0.02
E. faecalis	0.63	0.31	0.63	>2.5	>2.5	0.31	0.16	0.63	1.25	>2.5	0.31	0.31	0.63	0.04	0.63	0.31	0.31	0.63	0.08	1.25	**0.80**	0.02
P. aeruginosa	0.31	0.63	1.25	2.5	1.25	0.31	0.31	1.25	2.5	>2.5	0.16	0.16	0.63	0.08	1.25	0.63	0.31	0.31	0.04	1.25	**0.88**	1.25
E. coli	0.63	0.63	>2.5	>2.5	>2.5	0.63	0.63	0.63	2.5	>2.5	0.16	0.63	0.63	0.08	0.63	0.63	0.63	0.63	0.08	2.5	**1.11**	2.5
Average	**1.02**	**0.71**	**1.72**	**2.5**	**1.88**	**0.47**	**0.59**	**0.94**	**2.19**	**2.19**	**0.24**	**0.35**	**0.63**	**0.07**	**0.94**	**0.55**	**0.39**	**0.55**	**0.09**	**1.88**		

Key: H= n-Hexane, E= Ethyl acetate, A= Acetone, M= Methanol, W= Water, Avg= Average, PC= positive control (Chloramphenicol)

Table 5.2: Total activity of the selected plant extracts (mL/g).

Microorganisms	*V. rotundifolium*					*M. aethiopicum*					*T. oleifolius*					*D. cinerea*					
	H	E	A	M	W	H	E	A	M	W	H	E	A	M	W	H	E	A	M	W	Avg
S. aureus	76	112	48	124	208	238	64	104	216	72	710	484	286	4625	216	365	645	302	1938	68	**545**
E. faecalis	302	452	190	124	104	484	500	206	216	72	710	484	286	9250	429	742	645	317	3875	136	**976**
P. aeruginosa	594	222	96	124	208	484	258	104	108	72	1375	938	286	4625	216	365	645	613	7750	136	**961**
E. coli	302	222	48	124	104	238	127	206	108	108	1375	238	286	4625	429	365	317	302	3875	68	**673**
Average	**318**	**252**	**96**	**124**	**156**	**361**	**237**	**155**	**162**	**81**	**1042**	**536**	**286**	**5781**	**322**	**459**	**563**	**383**	**4359**	**102**	

Key: H= n-Hexane, E= Ethyl acetate, A= Acetone, M= Methanol, W= Water, Avg= Average

5.3.3. Antibacterial interaction activity

The n-hexane, ethyl acetate, acetone and methanol extracts of all the plants exhibited the highest activity against the tested bacteria, therefore; they were further studied in combination to determine the antibacterial interaction. The n-hexane, ethyl acetate, acetone and methanol extract of the host and parasite were combined to determine the effect of the combination on the antibacterial activity whether the activity will increase or decrease. The ratios are represented as parasite: host. The n-hexane, ethyl acetate, acetone and methanol extracts of all the plants exhibited the highest activity against the tested bacteria, therefore; they were selected for synergistic and antagonistic study. The n-hexane, ethyl acetate, acetone and methanol extract of the host and parasite were combined to determine the effect of the combination on the antibacterial activity. The ratios are represented as parasite: host.

The 2:1 combination of n-hexane and ethyl acetate extracts of *V. rotundifolium* and *M. aethiopicum* in table 5.3 had notable antibacterial activity against all tested microorganisms with the average MIC of 2.35 mg/mL and 0.39 mg/mL, respectively. The 1:1 combination of acetone extracts and 1:2 combination of ethyl acetate extracts also had the potent antibacterial activity against all tested microorganisms with the average MIC of 0.52 mg/mL and 0.47 mg/mL, respectively.

The 1:1 and 2:1 combination of n-hexane extracts of *T. oleifolius* and *D. cinerea* (Table 5.4) had high antibacterial activity against all tested microorganisms with the average MIC of 0.24 mg/mL and 0.25 mg/mL, respectively. The 1:2 combination of n-hexane and ethyl acetate extracts had notable activity against all tested microorganisms with the average MIC of 0.35 mg/mL.

Table 5.3: The MIC (mg/mL) values of *V. rotundifolium* and *M. aethiopicum* plant extracts in combination

Microorganisms	VR + MA (1:1)				VR + MA (1:2)				VR + MA (2:1)				
	H	E	A	M	H	E	A	M	H	E	A	M	Avg
S. aureus	0.16	0.16	0.31	0.63	0.16	0.16	0.31	0.63	0.16	0.31	0.31	0.63	**0.85**
E. faecalis	0.31	0.31	0.63	1.25	0.63	0.31	0.63	1.25	0.31	0.31	0.63	1.25	**0.71**
P. aeruginosa	0.63	0.63	0.63	0.63	0.16	0.16	0.31	1.25	0.31	0.31	0.63	0.63	**0.77**
E. coli	2.5	1.25	2,5	1.25	1.25	1.25	2.5	2.5	0.63	0.63	1.25	1.25	**1.47**
Average	**0.9**	**0.59**	**0.52**	**0.94**	**0.55**	**0.47**	**0.94**	**1.41**	**0.35**	**0.39**	**0.71**	**0.94**	

Key: H= n-Hexane, E= Ethyl acetate, A= Acetone, M= Methanol, W= Water, Avg= Average, VR=*Viscum rotundifolium*, MA= *Mystroxylon aethiopicum*

Table 5.4: The MIC (mg/mL) values of the *T. oleifolius* and *D. cinerea* plant extracts in combination

Microorganisms	TO + DC (1:1)				TO + DC (1:2)				TO + DC (2:1)				
	H	E	A	M	H	E	A	M	H	E	A	M	Avg
S. aureus	0.08	0.31	0.08	0.31	0.16	0.31	0.08	0.63	0.16	0.31	0.08	0.63	**0.31**
E. faecalis	0.31	0.31	0.16	0.63	0.31	0.16	0.16	1.25	0.16	0.31	0.31	0.63	**0.37**
P. aeruginosa	0.31	0.31	0.63	0.63	0.31	0.31	0.31	0.63	0.31	0.31	0.63	0.63	**0.38**
E. coli	0.31	0.63	0.63	1.25	0.63	0.63	1.25	0.63	0.31	0.63	1.25	0.63	**0.61**
Average	**0.25**	**0.39**	**0.38**	**0.71**	**0.35**	**0.35**	**0.45**	**0.79**	**0.24**	**0.39**	**0.57**	**0.63**	

Key: H= n-Hexane, E= Ethyl acetate, A= Acetone, M= Methanol, W= Water, Avg= Average, TO=*Tapinanthus oleifolius*, DC= *Dichrostachys cinerea*

The Fractional Inhibitory Concentration index (FIC_i) was used to evaluate the synergistic or antagonistic effects of the combinations of plant extracts on the antibacterial activity (Table 5.5 and 5.6). The combination of n-hexane, ethyl acetate and acetone extracts of *V. rotundifolium* and *M. aethiopicum* showed synergistic effects in inhibiting the growth of *S. aureus* with the FIC index of 0.32, 0.26 and 0.37, respectively. In addition, the n-hexane and acetone extracts of *T. oleifolius* and *D. cinerea* also showed synergistic effects in inhibiting the growth of *S. aureus* with the FIC index of 0.39 and 0.25, respectively; whereas the methanol extract of *T. oleifolius* and *D. cinerea* showed antagonistic effects in inhibiting the growth of all tested bacteria with the FIC index of 5.81, 23.63, 23.63 and 31.25.

Table 5.5: The Fractional Inhibitory Concentration of *V. rotundifolium* and *M. aethiopicum*.

Microorganisms	Combination	MIC (mg/mL)	FIC values		FIC index	Outcome
			VR	MA		
S. aureus	n-Hexane	0.16	0.06	0.25	0.32	Synergistic
	Ethyl acetate	0.16	0.13	0.13	0.26	Synergistic
	Acetone	0.31	0.12	0.25	0.37	Synergistic
	Methanol	0.63	0.25	0.5	0.76	Additive
E. faecalis	n-Hexane	0.31	0.49	1	1.49	Indifferent
	Ethyl acetate	0.31	1	1.94	2.94	Indifferent
	Acetone	0.63	1	1	2.00	Indifferent
	Methanol	1.25	0.5	1	1.50	Indifferent
P. aeruginosa	n-Hexane	0.63	1.97	2.03	4.00	Indifferent
	Ethyl acetate	0.63	1	2.03	3.03	Indifferent
	Acetone	0.63	0.5	0.5	1.01	Indifferent
	Methanol	0.63	0.25	0.25	0.50	Synergistic
E. coli	n-Hexane	2.5	3.97	3.97	7.94	Antagonistic
	Ethyl acetate	1.25	1.98	1.98	3.97	Indifferent
	Acetone	2.5	1	3.97	4.97	Antagonistic
	Methanol	1.25	0.5	0.5	1.00	Additive

Key: FIC= Fractional Inhibitory Concentration

Table 5.6: The Fractional Inhibitory Concentration of *T. oleifolius* and *D. cinerea*.

Microorganisms	Combination	MIC (mg/mL)	FIC values		FIC index	Outcome
			TO	DC		
S. aureus	n-Hexane	0.08	0.26	0.13	0.39	Synergistic
	Ethyl acetate	0.31	1	1	2.00	Indifferent
	Acetone	0.08	0.13	0.13	0.25	Synergistic
	Methanol	0.31	3.88	1.94	5.81	Antagonistic
E. faecalis	n-Hexane	0.31	1	1	2.00	Indifferent
	Ethyl acetate	0.31	1	1	2.00	Indifferent
	Acetone	0.16	0.25	0.25	0.51	Additive
	Methanol	0.63	18.8	7.88	23.63	Antagonistic
P. aeruginosa	n-Hexane	0.31	1.94	0.49	2.43	Indifferent
	Ethyl acetate	0.31	1.94	1	2.94	Indifferent
	Acetone	0.63	1	2.03	3.03	Indifferent
	Methanol	0.63	7.88	15.8	23.63	Antagonistic
E. coli	n-Hexane	0.31	1.94	0.49	2.43	Indifferent
	Ethyl acetate	0.63	1	1	2.00	Indifferent
	Acetone	0.63	1	1	2.00	Indifferent
	Methanol	1.25	15.6	15.6	31.25	Antagonistic

Key: FIC= Fractional Inhibitory Concentration

5.4. Discussion

The increased momentum on the research of medicinal plants is mainly influenced by the resistance of pathogenic organisms. These plants are known to exhibit biological and pharmacological activities. The investigation of plant extracts for antibacterial activity is the first step towards the discovery of novel therapeutic agents against the resistant pathogenic organisms (Eloff, 2019). Therefore, the antibacterial activity of the plants (*M. aethiopicum, V. rotundifolium*, *T. oleifolius* and *D. cinerea*.) was tested against both the Gram-negative (*E. coli* and *P. aeruginosa)* and Gram-positive (*S. aureus* and *E. faecalis*) bacteria. These pathogenic bacterial strains are commonly associated with nosocomial infections. To test for the antibacterial activity of the plants, two assays were used, namely; bioautography assay and broth microdilution assay. The p-iodonitrotetrazolium (INT) violet was used as an indicator of growth. INT is a tetrazolium salt that is used in most

colorimetric assays. During the active growth of microorganisms, an electron is transferred from the NADH (a product of the threonine dehydrogenase catalysed reaction) to INT resulting in the purple formazan dye (Masoko, 2007). The clear bands on the chromatograms or the clear wells on the microtitre plate indicated antibacterial activities of the plant extracts, which inhibited the growth of bacteria.

The n-hexane, ethyl acetate, acetone and methanol extracts of *M. aethiopicum* exhibited significant activity against both *P. aeruginosa* and *E. coli* in the BEA mobile phase (Figure 5.1). The *V. rotundifolium* leaf extracts did not exhibit any activity. Moreover, all Gram-negative bacteria were not susceptible to the treatment of *T. oleifolius* and *D. cinerea* extracts. Furthermore, n-hexane, ethyl acetate, acetone and methanol extracts of *M. aethiopicum* also showed activity against all tested Gram-positive bacteria. While the n-hexane, ethyl acetate, acetone and methanol of *V. rotundifolium* had activity against *E. faecalis*. *T. oleifolius* and *D. cinerea*, leaf extracts did not have antibacterial activity on the tested bacteria.

It is reported that the Gram-negative bacteria are more resistant to antibacterial treatment than Gram-positive bacteria mainly due to their cell wall composition. The Gram-negative bacterial cell wall contains an outer membrane that makes it less sensitive to antibacterial treatment (Cos *et al*., 2006). This was shown in the case of *E. coli* and *P. aeruginosa* which were resistant to *V. rotundifolium*, *T. oleifolius* and *D. cinerea*. The bioautography assay is only limited to the number of active compounds separated by TLC in an extract but it is not a quantitative measure of activity (Suleiman *et al*., 2010), therefore, the microdilution method was used.

The quantitative microdilution method in table 5.1 confirms the antibacterial activity of the plant extracts where the MIC values ranged from 0.04-2.5 mg/mL. The lowest average MIC of the plant extracts was against *E. faecalis* (0.80 mg/mL) followed by *P. aeruginosa* (0.88 mg/mL), *E. coli* (1.11 mg/mL) and *S. aureus* (1.18 mg/mL). The *T. oleifolius* and *D. cinerea* extracts showed significant antibacterial activity against all tested bacteria although they did not show any activity in the bioautography assay. This suggests that the compounds present in *T. oleifolius* and *D. cinerea* extracts may have synergistic or additive effects with one another in inhibiting bacterial growth (Muraina *et al*., 2008). The methanol extract amongst all extracts in both *T. oleifolius* and *D. cinerea* plants showed remarkable MIC values ranging from

0.04 to 0.16 mg/mL against *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli*. The MIC values obtained on the *D. cinerea* crude extracts support the study conducted by Shandukani *et al*. (2018), Eisa *et al*. (2000) and Hurinanthan (2009). The tested bacteria were least susceptible to the treatment of *V. rotundifolium* and *M. aethiopicum* extract where the MIC values ranged from 0.31 to 2.5 mg/mL. The antibacterial activity of these plants may be due to the presence of various bioactive compounds that include tannins, flavonoids and terpenoids which were reported to exhibit various pharmacological activities and antibacterial activity (McGaw and Eloff, 2008).

Considering that the MIC value is inversely proportional to the quantity of antibacterial compounds present in plant extracts, the total activity was calculated by dividing the quantity extracted (in mg) from 1 g of plant material by the MIC (in mg/mL) (Table 5.2). The highest total activities were observed with the methanol extract of *D. cinerea* against *P. aeruginosa* (7750 mL/g), *E. faecalis* (3875 mL/g) and *E. coli* (3875 mL/g) respectively, whereas the methanol extract of *T. oleifolius* had the highest total activity against *S. aureus* (4625 mL/g). This means that the quantity extracted from 1 g of the *D. cinerea* material with acetone can be diluted to 7750 mL and still have the ability to inhibit the growth of *P. aeruginosa*.

The n-hexane, ethyl acetate, acetone and methanol extracts of all the plants exhibited the highest activity against the tested bacteria, therefore; they were selected for synergistic and antagonistic study. The n-hexane, ethyl acetate, acetone and methanol extract of the host and parasite were combined to determine the effect of the combination on the antibacterial activity. The ratios are represented as parasite: host. The 2:1 combination of n-hexane and ethyl acetate extracts of *V. rotundifolium* and *M. aethiopicum* in table 5.3 had notable antibacterial activity against all tested microorganisms with the average MIC of 2.35 mg/mL and 0.39 mg/mL, respectively. The 1:1 combination of acetone extracts and 1:2 combination of ethyl acetate extracts also had the potent antibacterial activity against all tested microorganisms with the average MIC of 0.52 mg/mL and 0.47 mg/mL, respectively. The 1:1 and 2:1 combination of n-hexane extracts of *T. oleifolius* and *D. cinerea* (Table 5.4) had high antibacterial activity against all tested microorganisms with the average MIC of 0.24 mg/mL and 0.25 mg/mL, respectively. The 1:2 combination of

n-hexane and ethyl acetate extracts had notable activity against all tested microorganisms with the average MIC of 0.35 mg/mL.

The Fractional Inhibitory Concentration index (FIC_i) was used to evaluate the synergistic or antagonistic effects of the combinations of plant extracts on the antibacterial activity (Table 5.5). Synergy occurs as a result of the interaction between two or more compounds in ways that mutually enhance each other's effect more significantly than individual contributions (Williamson, 2001). The combination of n-hexane, ethyl acetate and acetone extracts of *V. rotundifolium* and *M. aethiopicum* showed synergistic effects in inhibiting the growth of *S. aureus.* In addition, the n-hexane and acetone extracts of *T. oleifolius* and *D. cinerea* also showed synergistic effects in inhibiting the growth of *S. aureus* whereas the methanol extract showed antagonistic effects in inhibiting the growth of all tested bacteria. Therefore, because the presence and distribution of the phytochemicals on the parasite plants are dependent on the host plant (Deeni and Sadiq, 2002), there is no significant difference in the overall antibacterial activity of the combination of the parasite and host extracts relative to the individual activities.

5.5. Conclusion

The results support the use of the tested plants in traditional medicine. This chapter shows that the plants exhibit antibacterial activity of natural origin and various mechanisms of antibacterial activity. Although most plants are believed to be more active against Gram-positive bacteria than Gram-negative, *M. aethiopicum* had activity against all tested bacteria. These results provide evidence that some medicinal plants might be a potential source of novel antibacterial agents even against some resistant strains of microorganisms.

5.6. References

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Chapter 6: Cell viability assay

6.1. Introduction

The use of medicinal plants has enormously increased over the past decades. Today, over 80% of the people worldwide depend on the herbal traditional medicine as their primary healthcare (Ekor, 2014). Despite the continuous growth on the use of medicinal plants and products, there are emerging concerns related to their safety and public health issues (WHO, 2004). In most countries, medicinal plants and their products are supplied in markets and lack safety and toxicological evaluation. Furthermore, they are continuously provided to consumers without prescription (Tyler, 2000).

People believe that extreme consumption of medicinal plants does not cause harmful effects and as a result, they consume as much as they want (Philoma, 2011; Rey, 2018). This is influenced by the fact that plants have been used in their tradition for long periods of time, therefore, they are assumed to be safe (Edziri *et al*., 2011). In addition, people also believe that because medicinal plants come from nature, they are safe. However, although medicinal plants come from nature, it does not mean that they are safe. Reports have indicated that plants produce toxic reactions, mutagenic effects and allergic reactions (Senior, 1998). For example, *Ginkgo boloba* is one of the medicinal plants that are widely used to treat various diseases such as memory impairment, Alzheimer's disease and concentration difficulties. However, studies have reported that *Ginkgo biloba* can cause hypertension, leucopoenia, thrombocytopenia and hallucinations (WHO, 1999), epileptic seizures (Granger, 2001) and induce liver cancer (Dunnick and Nyska, 2013).

Traditional remedies are prepared in different forms including infusions, decoctions and tinctures; in most cases, the different plants or plant parts are used. As a result, the effect of the interactions between the various compounds within the preparations may be toxic (Olowa and Numeza, 2013; Ikegami *et al*., 2016). Since medicinal plants are used in traditional medicines around the world, it is important to evaluate the cytotoxicity of *V. rotundifolium*, *M. aethiopicum*, *T. oleifolius* and *D. cinerea* leaves because they are used by traditional health practitioners to treat infections.

This will help to determine whether the plant crude extracts are toxic to the host cells or not, as well as to identify the level of cell toxicity.

Cytotoxicity assay is a cell viability test used to analyse the safety of plant extracts/ new drugs on the host cell. The methods used to test cytotoxicity or cell viability are based on cellular functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production and nucleotide uptake activity (Bahuguna *et al*., 2017). The MTT tetrazolium assay is one of the most frequently used methods to evaluate the cell viability and cytotoxicity of plant extracts and new drugs. MTT is a yellow tetrazolium salt that is converted to an insoluble purple formazan colour by the mitochondrial dehydrogenase enzyme in the viable cells with active metabolism. Therefore, the increase or decrease in the number of viable cells is proportional to the mitochondrial activity. The concentration of the formazan is detected using a micro plate reader and compared to the concentration of the untreated cells (Mosman, 1983; van Meerloo *et al*., 2011). The study conducted by Eloff (1998) showed that acetone (amongst other solvents) have low toxicity hence, acetone was selected for cytotoxicity test. In this chapter, the cytotoxic effects of the acetone extracts of the selected plants was tested on the human liver (C3A) cells (derived from the hepG2 cell line) for 24 hours using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay.

6.2. Materials and methods

6.2.1. Cell viability assay

The effects of the plant extracts on the cell viability were assessed on the human liver (C3A) cells using the MTT (3-[4.5-dimethylthiazol- 2-yl]-2.5 diphenyl tetrazolium bromide) reduction assay previously described by Mosmann (1983). The human liver (C3A) cells were cultured in a flask until they were 80% confluent. The cells were then counted using the countless counter (Thermo Scientific) and 0.96 mL cells/ well were seeded into the 96-well plates. The plates were incubated at 37°C in a 5% carbon dioxide (CO_2) incubator to allow the cells to attach. After 24 hours of incubation, test samples (100 µL) at varying final concentrations (100-1000 µg/mL) were added to the wells containing cells. The plates were incubated further for 24 hours with the untreated cells as a negative control. After incubation, the cells were

washed with Phosphate Buffered Saline (PBS) and 10 µL of MTT (5 mg/mL in PBS) was added to each well and the plates were incubated further for 4 hours. Following incubation, 100 µL of dimethyl sulfoxide (DMSO) was added and incubated for an additional 1 hour to dissolve the MTT. After incubation, the lack of purple formazan colour or clear appearance in the wells indicated cytotoxicity of the tested samples on the cells. The absorbance was measured on a BioTek Synergy microplate reader at 560 nm. The percentage of cell growth was calculated based on the comparison with the untreated cells using the formula: (total viability of treated target cells /total viability of untreated target cells) *100.

6.3. Results

The effects of the acetone extracts on the cell viability of the human liver (C3A) cells was determined using the MTT (3-[4.5-dimethylthiazol- 2-yl]-2.5 diphenyl tetrazolium bromide) reduction assay (Figure 6.1 and 6.2). The cytotoxicity was represented as the percentage cell viability. The cell viability of the human liver (C3A) cells on a concentration dependent manner showed that there is no significant difference between the viability of the untreated cells and the cells treated with the acetone extract of all the tested plants (*M. aethiopicum*, *V. rotundifolium, D. cinerea* and *T. oleifolius)*. The acetone extract of *T. oleifolius* had a high percentage of cell viability at the concentration of 100 µg/mL, followed by *D. cinerea* at the concentrations 500 and 600 µg/mL and *M. aethiopicum* at the concentration of 600 µg/mL and lastly, *V. rotundifolium* at the concentration of 1000 µg/mL.

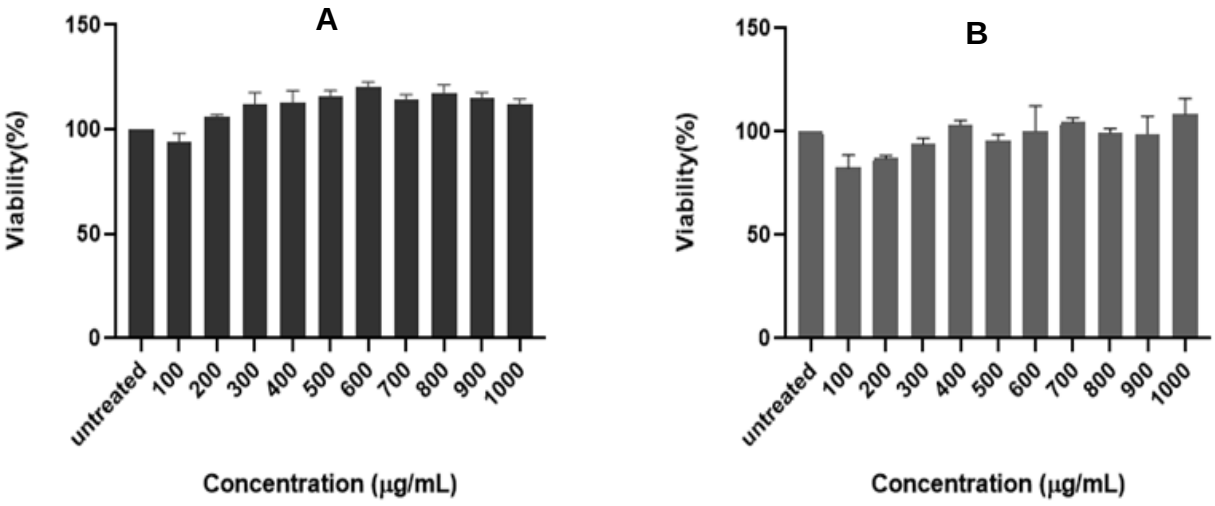

Figure 6.1: The cytotoxicity of *M. aethiopicum* (**A**) and *V. rotundifolium* (**B**) acetone extracts on the human liver (C3A) cells.

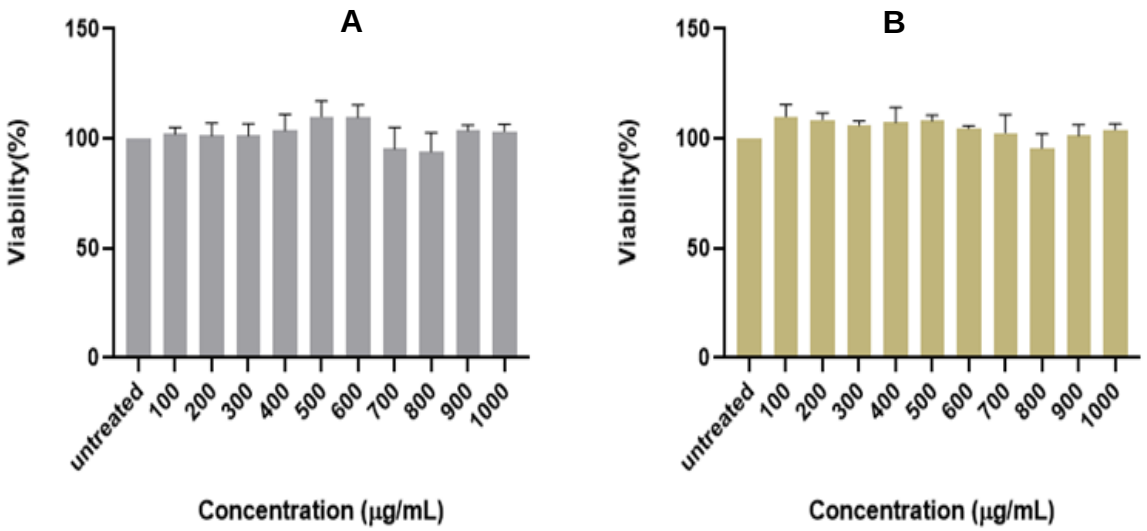

Figure 6.2: The cytotoxicity *D. cinerea* (**A**) and *T. oleifolius* (**B**) acetone extracts on the human liver (C3A) cells.

6.4. Discussion

The herbal medicinal plants are continuously used as treatment for various ailments. However, there is lack of scientific information which includes the safety of the plants on the human cells to support their medicinal use (Kilonzo *et al*., 2016). Therefore, in this study, the effects of the acetone extracts of the selected plants on the cell viability of the human liver (C3A) cells was determined using the MTT (3-[4.5-dimethylthiazol- 2-yl]-2.5 diphenyl tetrazolium bromide) reduction assay (Figure 6.1 and 6.2). This test was performed at different concentrations of the acetone extract since medicinal plants are regarded to be safe in small doses, but may be dangerous in higher doses.

MTT is a tetrazolium salt that is converted from a yellow colour to an insoluble purple formazan colour by the mitochondrial dehydrogenase enzyme in the viable cells with active metabolism. The quantity of the formazan is directly proportional to the number of the viable cells. When the cells die, they lose their ability to convert the MTT into the formazan purple colour. Therefore, the purple colour serves as an indicator of only the viable cells (Diaz *et al*., 2007; Storket *et al*., 2012).

There was no significant difference between the viability of the untreated cells and the cells treated with the acetone extract of all plants (*M. aethiopicum*, *V. rotundifolium, D. cinerea* and *T. oleifolius)* for 24 hours. The cytotoxicity results of *D. cinerea* support the study conducted by other researchers where the plant crude extracts showed no effect on the brine shrimp (Neondo *et al*., 2012) and *Wistar albino* mice (Susithra and Jayakumari., 2018) after 24 hours of treatment. Although all parts of the mistletoes (parasitic plants) are known to be potentially harmful, the two mistletoes (*V. rotundifolium* and *T. oleifolius*) were found to be non-toxic to the human cells.

The level of toxicity of mistletoe varies with the type of host tree on which they grow. In addition, although the cardiac glycosides were found to be present in both the host plants and the parasites, their amount was not enough to make the plants toxic (Hall *et al*., 1986). Medicinal plants, including food, contain compounds that can be regarded as potentially toxic to humans, e.g. cyanogenic glycosides found in many fruit seeds, alpha gliadin produced in wheat oats and rye. Nonetheless, these food sources are regarded as safe. Similarly, both water and oxygen can be harmful or fatal in excessive amounts, so quantity is often an important consideration (Haq, 2008).

6.5. Conclusion

The results obtained in this study suggest that the tested plants may be non-toxic to human cells, hence there was no effect on the cell viability. However, *in vitro* assays only provide a foundation that the results could be potential candidates to be used as antibacterial agents. Further studies including *in vivo* assays are needed in order to support their use in traditional medicine.

6.6. References

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Chapter 7: Isolation and purification of antioxidant and antimicrobial compounds

7.1. Introduction

The plant secondary metabolites demonstrate many biological activities and are recently the main focus in drug discovery. These biological activities include antimicrobial, antifungal, anticancer and anti-inflammatory (Vaghasiya *et al*., 2011). The isolation of plant active compounds is the first step in the development of natural drugs. Plant extracts contain various compounds some of which are biologically active and also have different polarities. The purification of plant samples to pure compounds involves various steps known as bioassay guided fractionation (Gericke, 2011). This includes the extraction of the plant powdered material with organic solvents, pre-purification and purification by Thin-Layer Chromatography (TLC) or High-Performance Liquid Chromatography (HPLC) (Claeson *et al*., 1998).

Extraction is the first step in the isolation of bioactive compounds from plant materials that uses solvents of varying polarity. During this process, the solvents diffuse into the plant material and makes the compounds with similar polarity soluble for further analysis. The different methods of extraction depend on the type of substance to be isolated and this includes plant tissue homogenisation, serial exhaustive extraction, soxhlet extraction, maceration, decoction, infusion, digestion, sonication and percolation (Ncube *et al*., 2008). Serial exhaustive extraction was selected to extract the bioactive compounds from the powdered leaves of *Mystroxylon aethiopicum*. Serial exhaustive extraction is a common method of extraction that successively extracts the plant material using solvents of increasing polarity from non-polar to more polar. This ensures that a wide polarity range of compounds could be extracted for purification purpose (Das *et al*., 2010).

Column chromatography is used for separation, identification, and purification of compounds from a mixture based on interaction between a stationary and mobile phase (Enyoh *at al*., 2019). The stationary phase is composed of a solid phase or layer of liquid that is absorbed on the surface while the mobile phase is composed of a liquid or gaseous component. The separation of compound occurs either by partitioning (liquid-solid), adsorption (liquid-solid), affinity (ion exchange) and size

exclusion. In this study, the column chromatography was used. Moreover, the silica gel was used as a stationary phase and solvents of increasing polarities were used as mobile phase (eluents). Because of the difference in the polarities of the compounds, less polar compounds travelled faster through the gel and were collected first while more polar compounds travelled slowly and collected last (Coskum, 2016; Enyoh *at al*., 2019). The compounds were collected as fractions, analysed further using TLC and tested for biological activity. The pure compound was then analysed using NMR for structure elucidation. The aim of this chapter was to isolate bioactive compounds from *Mystroxylon aethiopicum* using bioassay guided fractionation.

7.2. Methods and materials

7.2.1. Serial exhaustive extraction

Serial exhaustive extraction was used to extract bioactive compounds from the *Mystroxylon aethiopicum* leaf material since it showed promising antibacterial activity. The mass of 700 g of ground leaf material was weighed and extracted with 3.5 L of each solvent (n-hexane, dichloromethane, acetone and methanol) in increasing polarity in a bottle. The mixture was vigorously shaken using a shaker (Thermo Scientific MaxQ 3000) at 200 rpm. Shaking was done three times (overnight and two times in 3 hours intervals). The supernatant was filtered, concentrated using rotary evaporator (Buchi R-114) at 50°C and transferred into pre-weighed labelled beakers (250 mL). The remaining solvents were evaporated from the extracts under a stream of cold air at room temperature and the masses of the crude extracts were determined.

7.2.2. Phytochemical analysis

The chemical profiles of the *Mystroxylon aethiopicum* extracts were analysed on aluminium-backed Thin Layer Chromatography (TLC) plates (ALUGRAM® SILg/ UV 254- MACHEREY- NAGEL, Merck) using a method developed by Kotze and Eloff (2002) as described in section 3.2.3.

7.2.3. TLC-DPPH assay

Qualitative 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was done using Thin Layer Chromatography according to the method described by Braca *et al*. (2002) as explained in section 4.2.1.

7.2.4. Bioautography assay

Bioautography was done according to the method described by Begue and Kline (1972) as discussed in section 5.2.2.1

7.2.5. Serial broth micro-dilution assay

A serial broth micro-dilution method described by Eloff (1998) was used to determine the Minimum Inhibitory Concentrations (MIC) values of the extracts against four tested bacterial species as explained in section 5.2.3.1.

7.2.6. Isolation of antibacterial and antioxidant compounds

7.2.6.1. Open column chromatography

The dichloromethane (D1-3) and acetone (A1) extracts resulting from the serial exhaustive extraction were selected to be subjected to open column chromatography, as they had high antibacterial activity. An open column (35 cm height × 4 cm radius) was packed with silica gel 60 (particles size 0.063 - 0.200 mm) (Fluka) using 100% n-hexane. The extracts (130.17 g) were mixed with small amounts of silica gel and subjected to column chromatography. The chemical constituents of the extracts were eluted through an open column using 2 L of the solvent systems illustrated in table 7.1. The fractions were collected and concentrated using a rotary evaporator (Buchi R-114). The solvents were completely evaporated under a stream of cold air in pre-weighed beakers at room temperature and the masses of the crude extracts were determined. The fractions were then tested for antioxidant and antibacterial activity using TLC-DPPH (Section 7.2.3), bioautography (Section 7.2.4) and serial broth micro-dilution (Section 7.2.5).

Table 7.1: Solvent systems used in the first column

Solvent systems	Percentages (%)
n-Hexane	100
n-Hexane: Ethyl acetate	90
	80
	70
	50
	30
	10
Ethyl acetate	100
Ethyl acetate: Methanol	90
	80
	70
	50
	30
	10
Methanol	100

7.2.6.3. Determination of solvent system for second column chromatography

The results from the first open column chromatography showed that the compound of interest is present in the BEA mobile phase (from fractions 4-9). Since the compound is non-polar, various combinations of non-polar solvents were used as mobile phases (Table 7.1). This was done in order to determine the combination of

solvent system that will separate the target compound from the other compounds. The TLC plates were loaded with 10 µL of 10 mg/mL of biologically active fraction and developed in the different mobile phases. The bioautography assay, according to section 7.2.4, was performed to determine the mobile phase that best separates the target biologically active compound. The *E. faecalis* bacterium was used in further tests to determine the antibacterial activity of the fractions since it was susceptible to *M. aethiopicum* and grows fast.

Table 7.2: Solvent systems used as mobile phases to determine the eluent for second column chromatography

Numbering	Solvent systems	Percentages (%)
1	n-Hexane: Acetone	80
2		70
3		60
4		50
5	Acetone: n-Hexane	80
6		70
7		60
8		50
9	Chloroform: Ethyl acetate	80
10		70
11		60
12		50
13	Ethyl acetate: Chloroform	80
14		70
15		60
16		50

7.2.6.4. Second open column chromatography

The results of the biological activities of the fractions obtained from the first column (7.3.2.1) revealed that the fractions 4-9 [n-hexane (70, 50, 30 and 10%) and ethyl acetate (100 and 90%)] exhibit high antibacterial activities and showed similar profiles. Therefore, they were combined for further separation and purification of the

bioactive compounds. The combined fractions were subjected to an open column chromatography (63 x 4 cm) packed with silica gel 60. The column was eluted with 80% chloroform in ethyl acetate (Table 7.2). The eluents were collected in small test tubes and placed under a stream of air to concentrate. The phytochemical profile of the fractions was analysed on the Thin Layer Chromatography (Section 7.2.2) and those with similar chemical profiles were combined. The concentrated fractions were then tested for antioxidant activity using TLC-DPPH (Section 7.2.3) and antibacterial activity using bioautography (Section 7.2.4) and serial broth micro-dilution (Section 7.2.5).

7.3. Results

7.3.1. Serial exhaustive extraction

7.3.1.1. The quantity of plant material extracted from *M. aethiopicum*

Serial exhaustive extraction of *Mystroxylon aethiopicum* (700 g) with different solvents (n-hexane, dichloromethane, ethyl acetate, acetone, and methanol) resulted in a total mass of 331.63 g (Table 7.3). Methanol was the best extractant, extracting a total mass of 267.44 g, followed by n-hexane (35.11 g) and dichloromethane (21.5 g). Meanwhile acetone extracted the least (7.58 g).

Table 7.3: The masses (g) extracted from *M. aethiopicum*

Extracts		Mass residue (g)	
		Mass	**Total**
n-Hexane	H1	23.71	
	H2	9.55	35.11
	H3	1.85	
Dichloromethane	D1	7.74	
	D2	12.02	21.5
	D3	1.74	
Acetone	A1	2.78	
	A2	2.67	7.58
	A3	2.13	
Methanol	M1	204.92	
	M2	43.78	267.44
	M3	18.74	
	Total		**331.63**

7.3.1.2. Phytochemical analysis by TLC

The extracts (n-hexane, dichloromethane, ethyl acetate, acetone, and methanol) extracts of *M. aethiopicum* were separated on the TLC plates using the BEA, CEF, and EMW solvent systems. The separated compounds were visualised under UV light and sprayed with vanillin-sulphuric acid reagent (Figure 7.1). The different colours observed on the chromatograms indicated the different chemical compounds present in the plant material. The distinct compounds were observed on the BEA mobile phase at 254 nm and on the plate sprayed with vanillin-sulphuric acid reagent. This shows that *M. aethiopicum* mostly contain non-polar compounds.

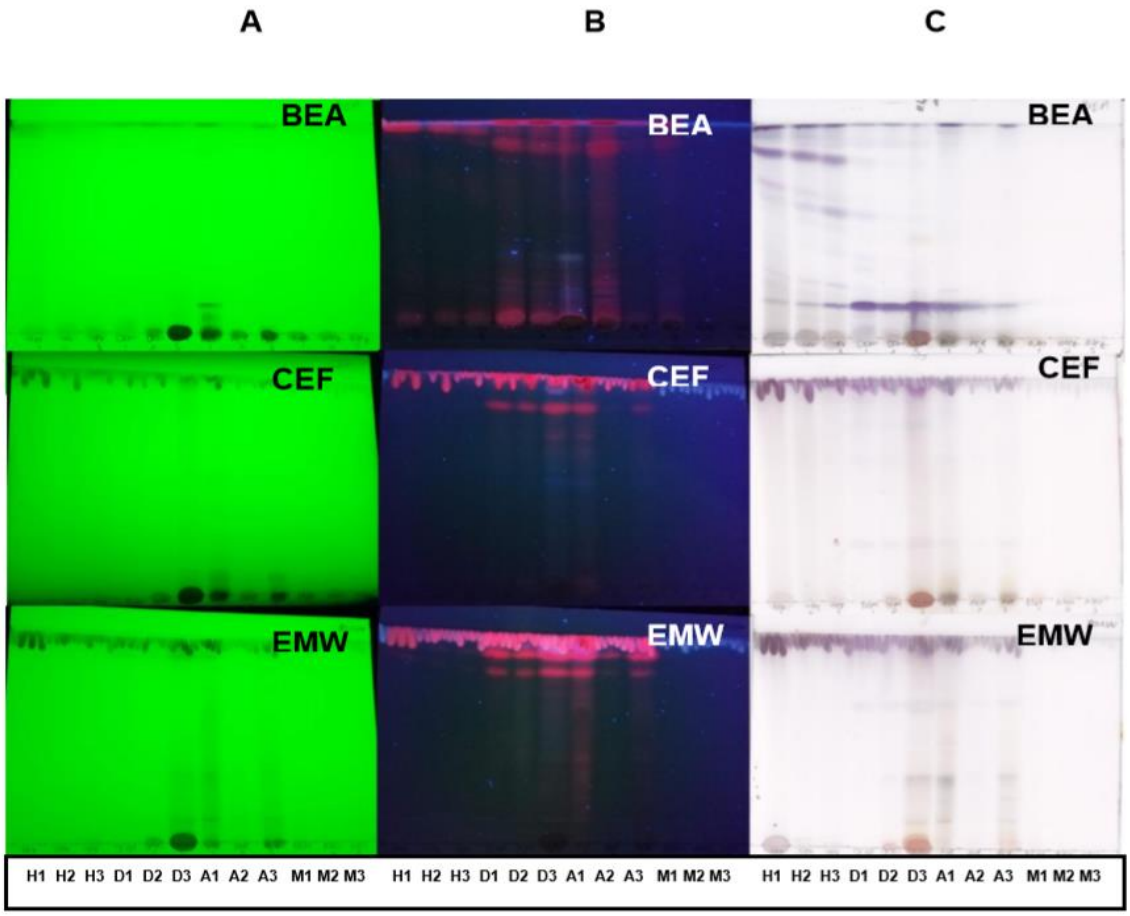

Figure 7.1: The chromatograms of *M. aethiopicum* dried leaves extracted with different solvents and developed in BEA, CEF and EMW mobile systems then visualised under UV light at 365 nm(**A**) and 254 nm(**B**) and then sprayed with vanillin-sulphuric acid reagent (**C**).

Key: H= n-Hexane, D= Dichloromethane, A= Acetone, M= Methanol.

7.3.1.3. TLC-DPPH assay

The antioxidant activities of the crude extracts was determined by spraying the developed TLC plates with 0.2% DPPH in methanol. The antioxidant activity of the plant extracts was observed by the presence of yellow bands on the chromatogram resulting from the reduction of the purple DPPH (Figure 7.2). The results indicated that the acetone extracts have compounds that exhibit antioxidant activity as observed on the BEA, CEF and EMW mobile phase but the compounds did not separate well.

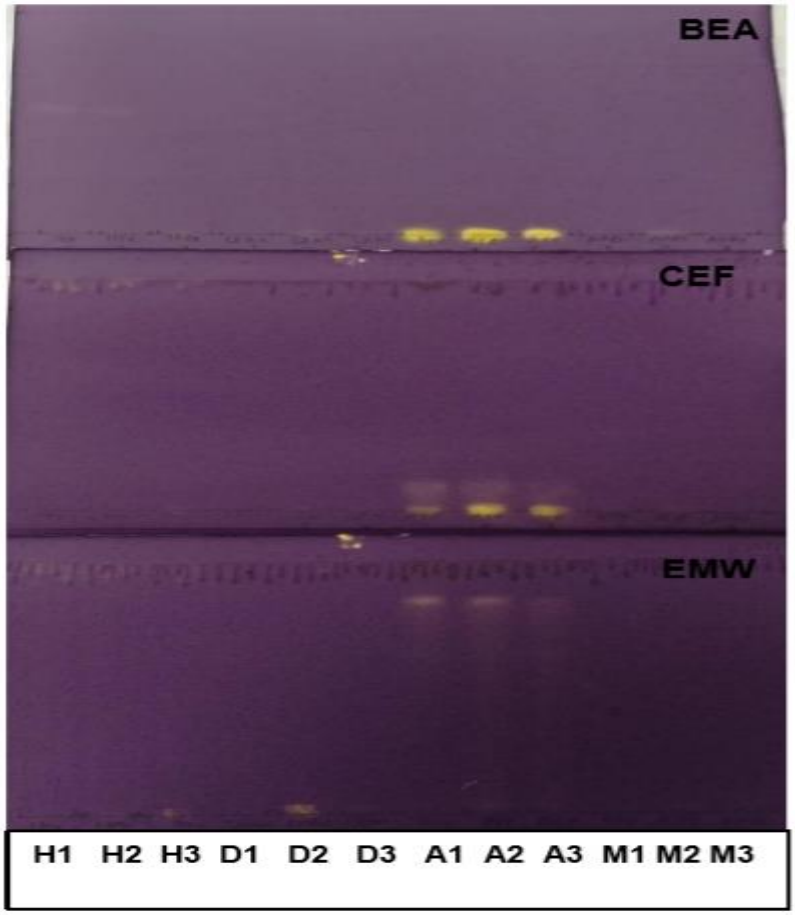

Figure 7.2: The chromatograms of *M. aethiopicum* dried leaves extracted with different solvents and developed in BEA, CEF and EMW mobile systems and sprayed with 0.2% DPPH in methanol. The yellow zones indicate the antioxidant activity.

H= n-Hexane, D= Dichloromethane, A= Acetone, M= Methanol.

7.3.1.4. Bioautography assay

The qualitative antibacterial activity assay of the crude extracts was determined using the bioautography assay where the TLC plates were developed in three mobile phases (BEA, CEF and EMW) and sprayed with *P. aeruginosa*, *E. coli, S. aureus* and *E. faecalis*. Clear bands on the chromatograms indicated antibacterial activities of the plant extracts. The antibacterial activity of *M. aethiopicum* against all tested microorganisms was only observed on the BEA mobile system (Figure 7.3 and 7.4). The n-hexane (H3), dichloromethane (D1-3) and acetone (A1-3) extracts had antibacterial activity against *E. coli* and *P. aeruginosa* while the n-hexane (H1-3),

dichloromethane (D1-3) and acetone (A1-3) extracts had activity against *S. aureus* and n-hexane (H3), dichloromethane (D1-3) and acetone (A1) extracts had activity against *E. faecalis*. The methanol extracts did not exhibit antibacterial activity against all the tested microorganisms.

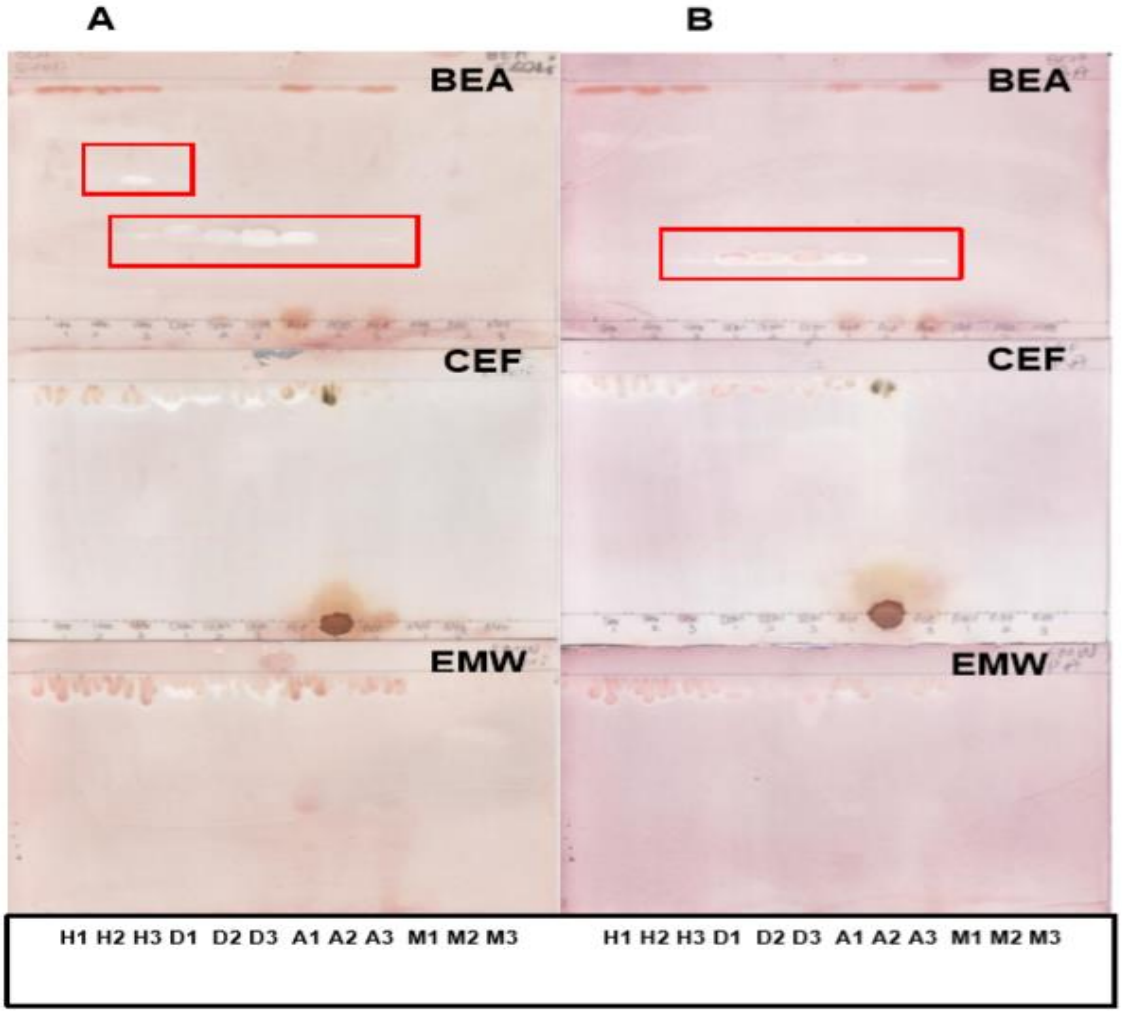

Figure 7.3: The chromatograms of *M. aethiopicum* plant extracts separated in BEA, CEF and EMW and sprayed with *E.* coli (**A**) and *P. aeruginosa* (**B**). White areas indicate zones of inhibition.

Key: H= n-Hexane, D= Dichloromethane, A= Acetone, M= Methanol.

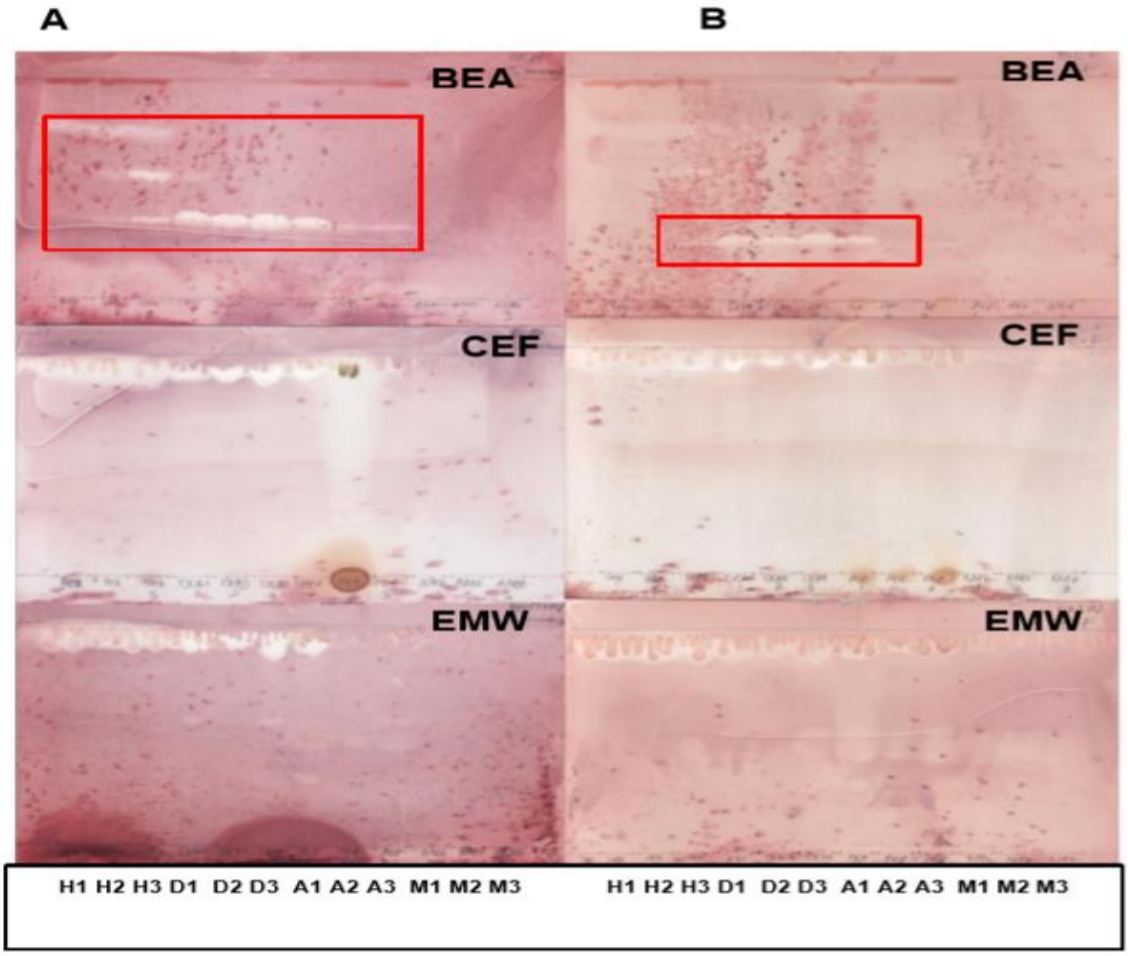

Figure 7.4: The chromatograms of *M. aethiopicum* plant extracts separated in BEA, CEF and EMW and sprayed with *S. aureus* (**A**) and *E. faecalis* (**B**). White areas indicate zones of inhibition.

Key: H= n-Hexane, D= Dichloromethane, A= Acetone, M= Methanol.

7.3.1.5. Serial broth micro-dilution assay

The quantitative micro-dilution method was used to determine the Minimum Inhibitory Concentration of the crude extracts against *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli*. The MIC values ranged from 0.08-2.5 mg/mL. The n-hexane (H1) extract had the lowest average MIC of 0.08 mg/mL, followed by acetone (A1) (0.71 mg/mL) and dichloromethane (D1) (0.82 mg/mL). The n-hexane, dichloromethane and acetone extracts had high activity against all tested microorganisms whereas the methanol extracts had the least activity.

Table 7.4: The MIC values of *M. aethiopicum* plant extracts (mg/mL) against the test microorganisms

Microorganisms	H1	H2	H3	D1	D2	D3	A1	A2	A3	M1	M2	M3	Avg	PC
S. aureus	0.08	0.31	0.31	0.16	0.16	0.31	0.31	2.5	0.63	1.25	2.5	2.5	**0.92**	0.02
E. faecalis	1.25	2.5	2.5	0.63	0.63	0.63	0.63	2.5	1.25	2.5	2.5	2.5	**1.67**	0.02
P. aeruginosa	0.63	0.63	0.63	1.25	1.25	2.5	1.25	1.25	2.5	2.5	2.5	2.5	**1.62**	1.25
E. coli	1.25	2.5	2.5	1.25	0.63	1.25	0.63	2.5	0.63	2.5	2.5	2.5	**1.72**	2.5
Average	**0.80**	**1.49**	**1.49**	**0.82**	**0.67**	**1.17**	**0.71**	**2.19**	**1.25**	**2.19**	**2.5**	**2.5**		

Key words: H= n-Hexane, D= Dichloromethane, A= Acetone, M= Methanol, Avg= Average, PC= Positive control (Chloramphenicol)

7.3.2. Isolation of active compounds

7.3.2.1. First open column chromatography

7.3.2.1.1. The masses of the collected fractions

The dichloromethane (D1-3) and acetone (A1) extracts resulting from the serial exhaustive extraction exhibited high antibacterial activity and were therefore selected for isolation of antibacterial compounds. The combined extracts (130.17 g) were subjected to first open column chromatography where they were eluted with solvents of varying percentages (Table 7.1). The fractions were collected, dried and masses were recorded in table 7.5 below. The total mass of 20.74 g was obtained from the fractions. The highest amount of plant extract was eluted with 50% n-hexane in ethyl acetate (4.76 g) followed by 30% n-hexane in ethyl acetate (3.84 g) and 100% methanol (2.2 g) whereas 100% ethyl acetate (0.22 g) and 100% methanol (0.24 g) eluted the least.

Table 7.5: The masses (g) of the fractions from fractionation of a combination of dichloromethane (D1-3) and acetone (A1).

Numbering	Elution solvent	Percentages (%)	Mass (g)
1	n-Hexane	100	0.5
2	n-Hexane: Ethyl acetate	90	1.58
3		80	1.11
4		70	1.93
5		50	4.76
6		30	3.84
7		10	1.49
8	Ethyl acetate	100	0.22
9	Ethyl acetate: Methanol	90	0.33
10		80	0.4
11		70	0.68
12		50	0.59
13		30	0.65
14		10	0.24
15	Methanol	100	2.2
		Total	**20.74**

7.3.2.1.2. Phytochemical analysis by TLC

The TLC profile of the collected fractions was analysed on the TLC plates. The TLC plates were developed in the BEA, CEF and EMW mobile phase and visualised under the UV light (Figure 7.5) and sprayed with vanillin-sulphuric acid (Figure 7.6). Most fluorescent compounds were observed at 254 nm in all the mobile phases whereas most the vanillin reactive compounds were observed on the BEA mobile phase. The 70%-10% n-hexane in ethyl acetate, 100% ethyl acetate and 90% ethyl acetate in methanol extracts had similar compounds on the BEA mobile phase (Figure 7.6).

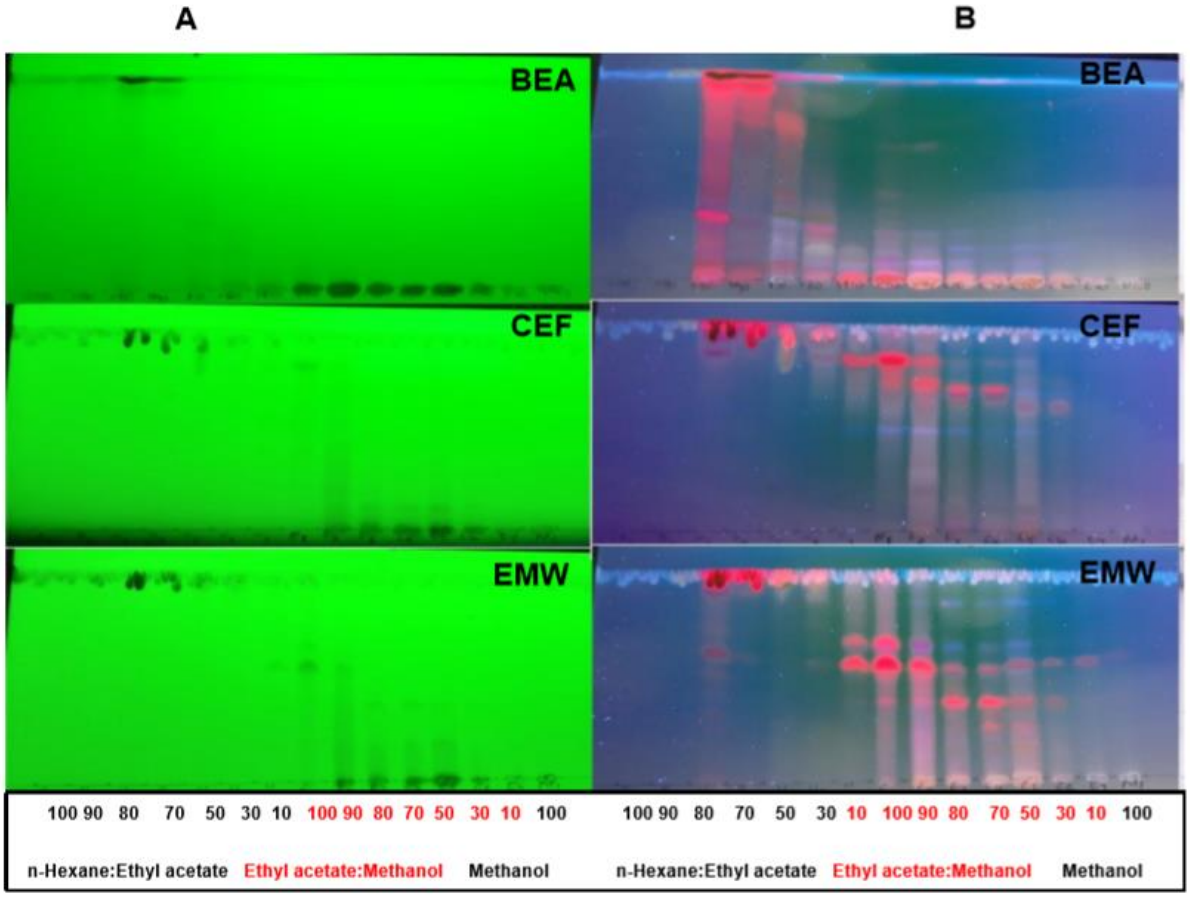

Figure 7.5: The chromatograms of *M. aethiopicum* fractions separated in BEA, CEF and EMW mobile systems then visualised under UV light at 365 nm (**A**) and 254 nm (**B**).

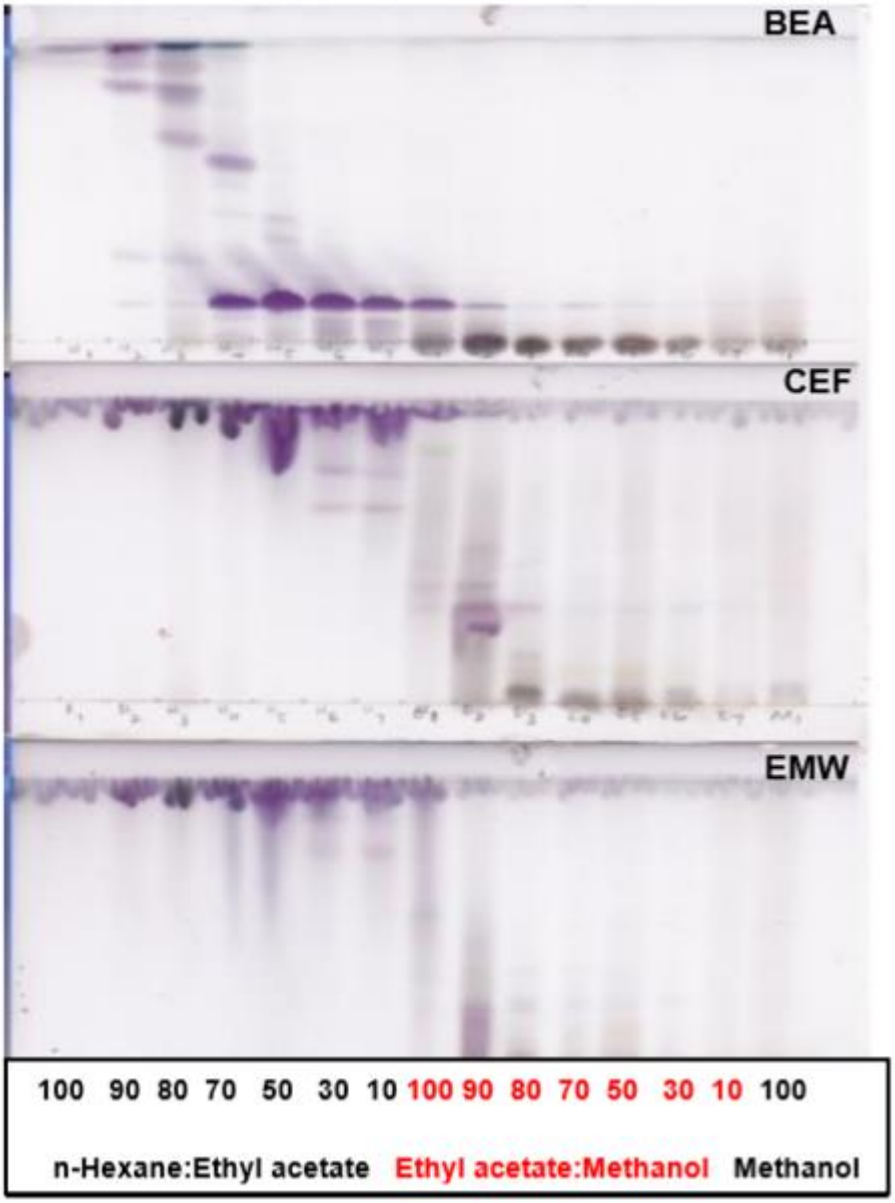

Figure 7.6: The chromatograms of *M. aethiopicum* fractions separated in BEA, CEF and EMW mobile systems then sprayed with vanillin- sulphuric acid.

7.3.2.1.3. TLC-DPPH

The developed TLC plates were sprayed with 0.2% DPPH in methanol in order to determine the antioxidant activity of the fractions. The results indicated that the fractions do not exhibit distinct antioxidant compounds as observed on the BEA, CEF and EMW mobile phase where the compounds did not separate.

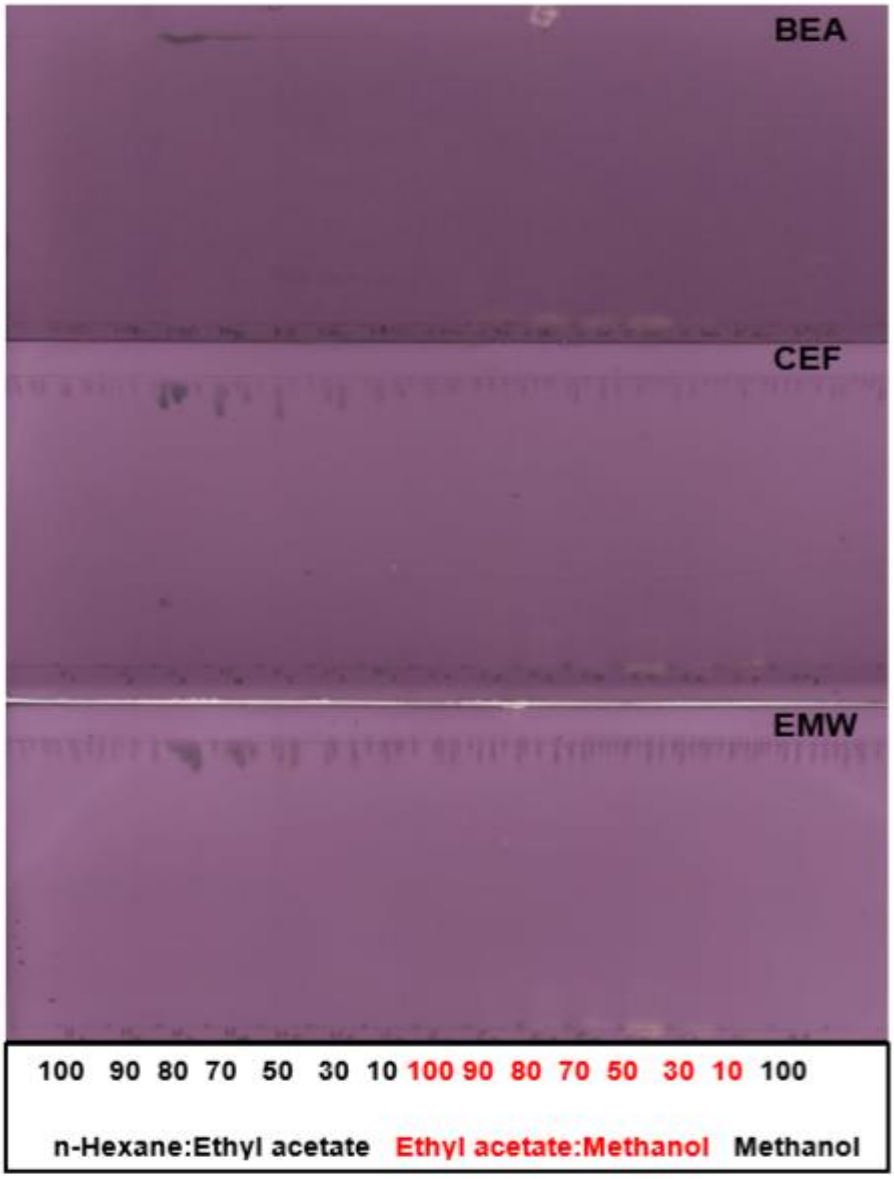

Figure 7.7: The chromatograms of *M. aethiopicum* fractions developed in BEA, CEF and EMW mobile systems and sprayed with 0.2% DPPH in methanol. The yellow zones indicate the antioxidant activity.

7.3.2.1.4. Bioautography assay

The antibacterial activity of the fractions was determined using the bioautography method. The TLC plates were developed in BEA, CEF and EMW mobile phases and sprayed with *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli* (Figure 7.8 and 7.9). The 70%-10% n-hexane in ethyl acetate, 100% ethyl acetate and 90% ethyl acetate in methanol extracts showed activity against all the tested microorganisms on the BEA mobile phase.

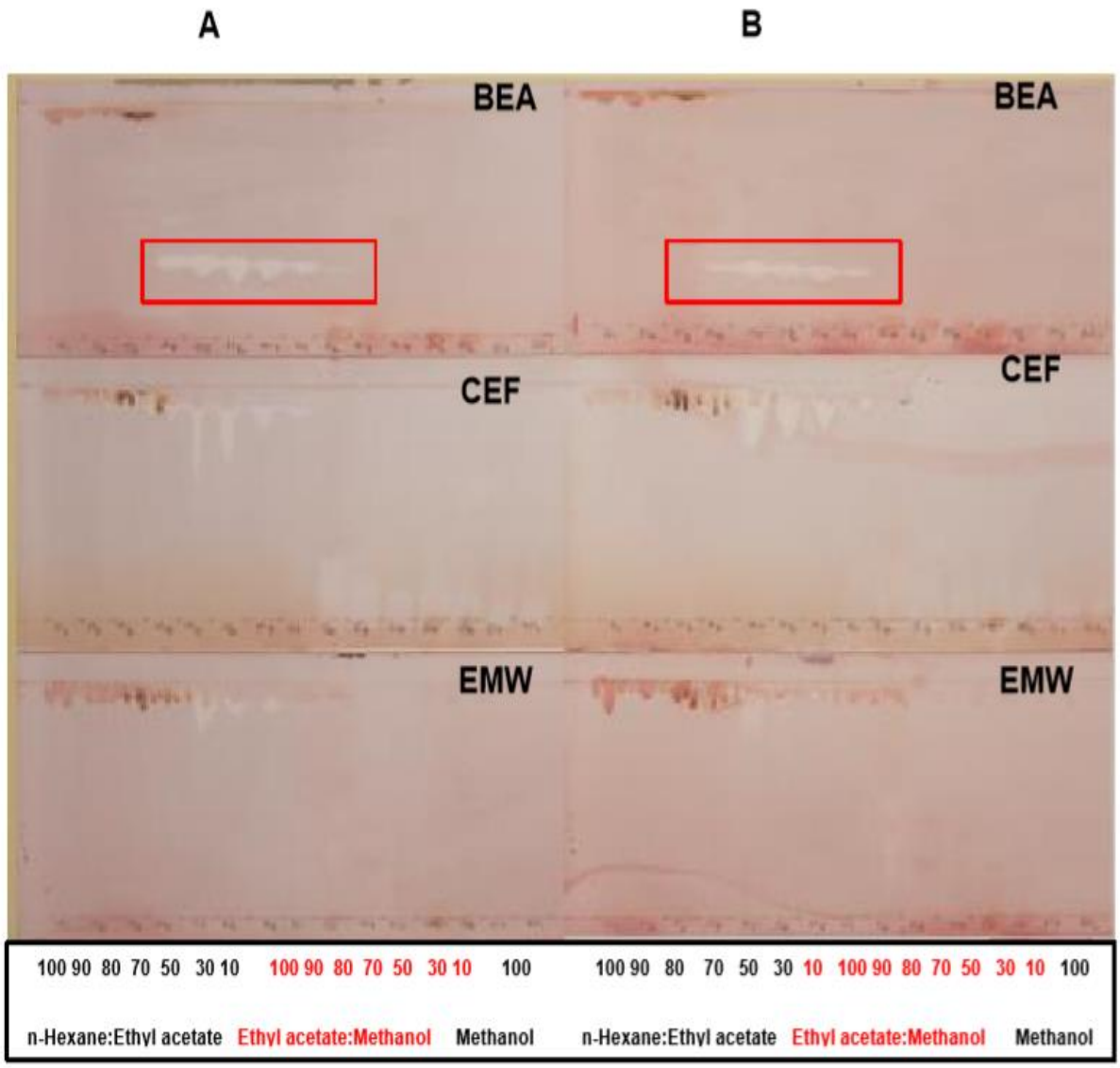

Figure 7.8: The chromatograms of *M. aethiopicum* fractions separated in BEA, CEF and EMW and sprayed with *E. faecalis* (**A**) and *S. aureus* (**B**). White areas indicate zones of inhibition.

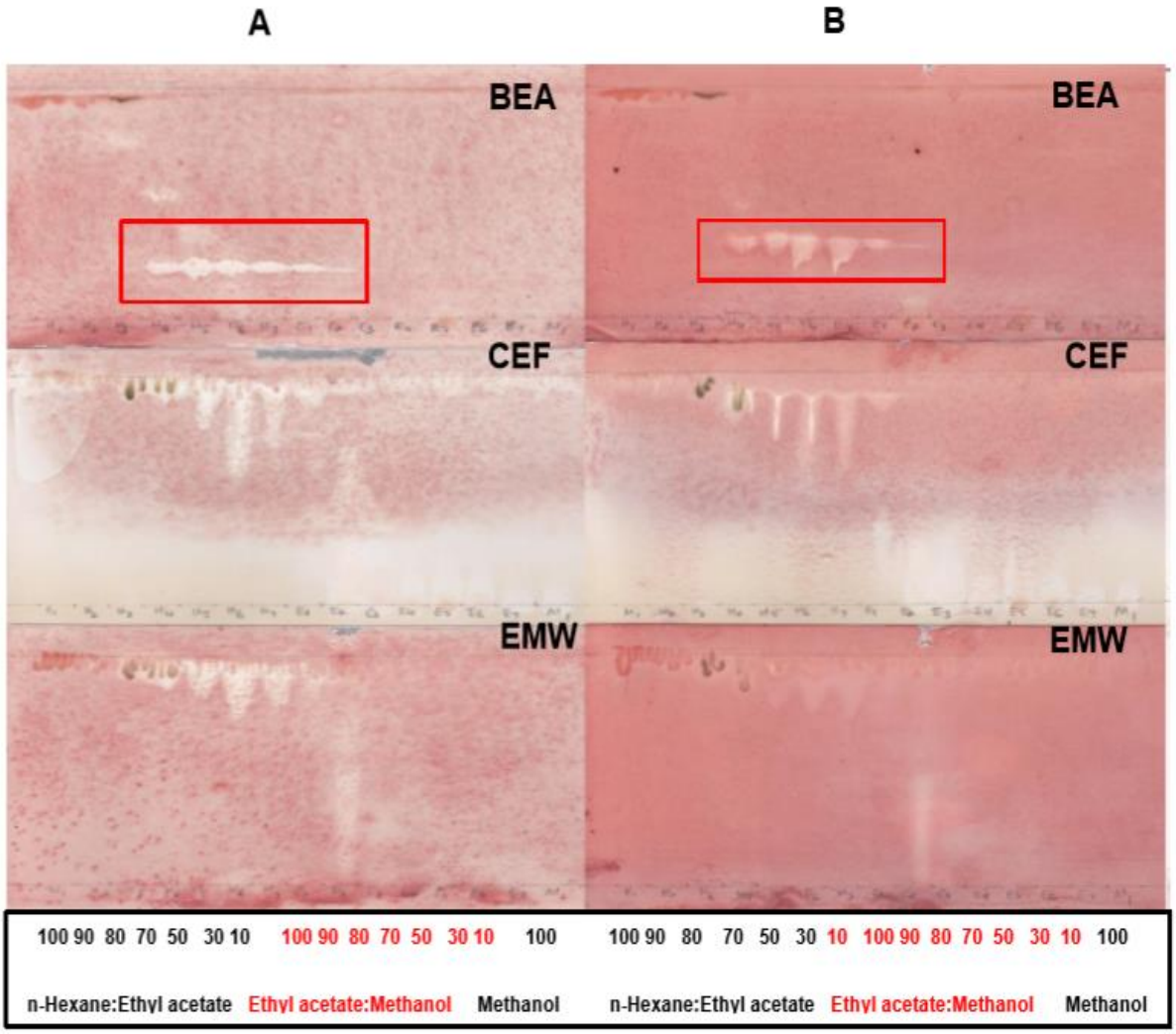

Figure 7.9: The chromatograms of *M. aethiopicum* fractions separated in BEA, CEF and EMW and sprayed with *E.* coli (**A**) and *P. aeruginosa* (**B**). White areas indicate zones of inhibition.

7.3.2.1.5. Serial broth micro-dilution assay

The serial broth micro-dilution method was performed to quantify the antibacterial activity of the fractions. The Minimum Inhibitory Concentration of the fractions was determined against *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli*. Fraction 4 had the lowest average MIC against all tested microorganisms (0.65 mg/mL) followed by fraction 8 (0.94 mg/mL) and fraction 9 (1.56 mg/mL). The lowest MIC value was found on the fraction 4 against *E. faecalis* (0.08 mg/mL).

Table 7.6: The MIC (mg/mL) of fractions from the first column chromatography against test microorganisms

Microorganisms	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Avg	PC
S. aureus	>2.5	>2.5	2.5	0.63	2.5	2.5	>2.5	1.25	2.5	2.5	2.5	2.5	>2.5	>2.5	2.5	**2.29**	0.02
E. faecalis	>2.5	2.5	2.5	0.08	2.5	2.5	2.5	0.63	1.25	2.5	>2.5	0.63	2.5	>2.5	>2.5	**2.01**	0.02
P. aeruginosa	>2.5	>2.5	>2.5	1.25	2.5	2.5	>2.5	1.25	1.25	2.5	>2.5	2.5	2.5	2.5	>2.5	**2.25**	1.25
E. coli	>2.5	>2.5	>2.5	0.63	>2.5	2.5	2.5	0.63	1.25	2.5	2.5	1.25	>2.5	>2.5	>2.5	**2.08**	2.5
Average	**2.5**	**2.5**	**2.5**	**0.65**	**2.5**	**2.5**	**2.5**	**0.94**	**1.56**	**2.5**	**2.5**	**1.72**	**2.5**	**2.5**	**2.5**		

Key: Avg= Average, PC= Positive control (Chloramphenicol), 1= 100% n-Hexane, 2= 90% n-Hexane: Ethyl acetate, 3= 80% n-Hexane: Ethyl acetate, 4= 70% n-Hexane: Ethyl acetate, 5= 50% n-Hexane: Ethyl acetate, 6= 30% n-Hexane: Ethyl acetate, 7= 10% n-Hexane: Ethyl acetate, 8= 100% Ethyl acetate, 9= 90% Ethyl acetate:Methanol, 10= 80% Ethyl acetate: Methanol,11= 70% Ethyl acetate: Methanol, 12= 50% Ethyl acetate: Methanol, 13= 30% Ethyl acetate: Methanol, 14= 10% Ethyl acetate: Methanol and 15= 100% Methanol

7.3.2.2. Second column chromatography

7.3.2.2.1. Determination of solvent system for second column chromatography

The results from the first open column chromatography showed that the targeted biologically active compound is non-polar. Therefore, various combinations of non-polar solvents were used as mobile phases (Table 7.1) to identify the combination that best separates the target compound from the other compounds. The developed TLC plates were sprayed with *E. faecalis* and from the results in figure 7.10 below, it can be seen that the 80% chloroform in ethyl acetate separated the compound of interest the best. Therefore, this combination was chosen as the eluent system for the second column chromatography.

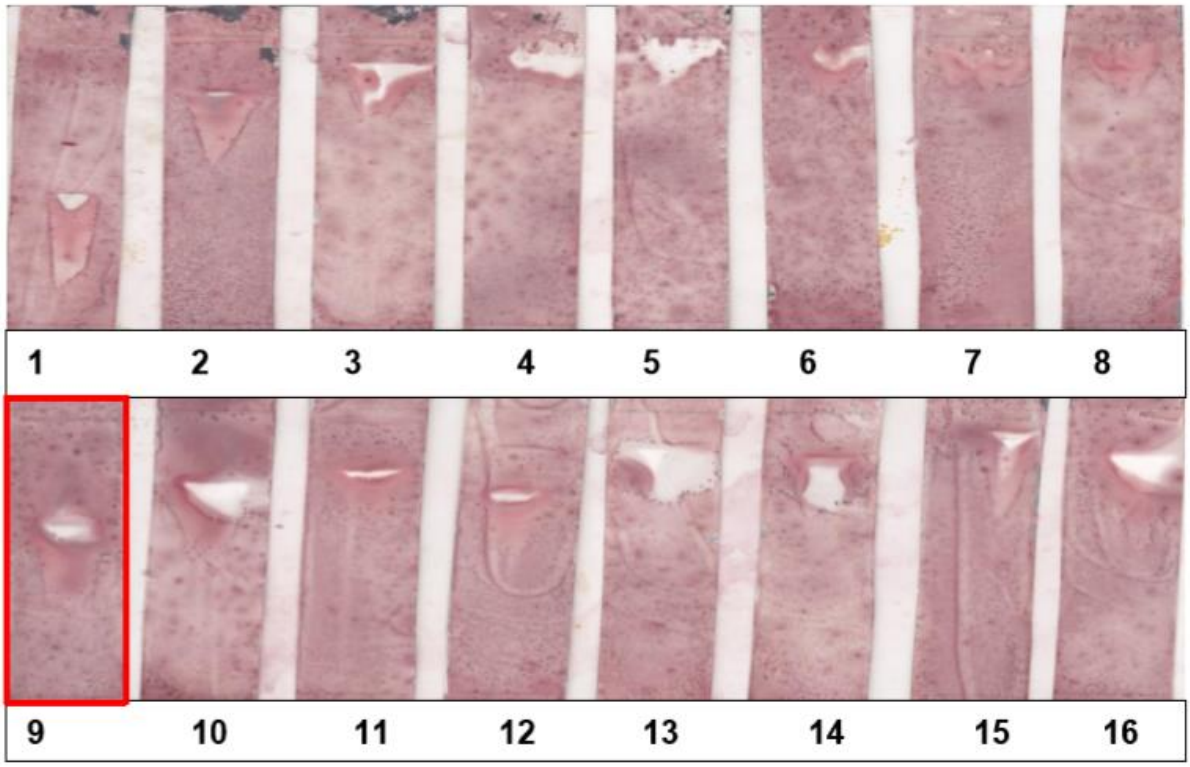

Figure 7.10: The antibacterial activity of the different pools against *E. faecalis.*

Key: 1= 80% n-Hexane, 2= 70% n-Hexane, 3= 60% n-Hexane, 4= 50% n-Hexane, 5= 80% Acetone, 6= 70% Acetone, 7= 60% Acetone, 8= 50% Acetone, 9= 80% Chloroform, 10= 70% Chloroform, 11= 60% Chloroform, 12= 50% Chloroform, 13= 80% Ethyl acetate, 14= 70% Ethyl acetate, 15= 60% Ethyl acetate, 16= 50% Ethyl acetate.

7.3.2.2.2. Phytochemical analysis of the fractions

The second open column chromatography of fractions 4-9 [n-hexane (70, 50, 30 and 10%) and ethyl acetate (100 and 90%)] was carried out using 80% chloroform in ethyl acetate as the eluent system. A total of 440 fractions were collected in test tubes and spotted on TLC plates to determine their TLC profile. The TLC profile was visualised under a UV light at 365 nm (**A**) and 254 nm (**B**) (Figure 7.11) and sprayed with vanillin-sulphuric acid (Figure 7.12). Most of the phytochemicals were observed on the chromatograms visualised at 254 nm and on the chromatograms sprayed with vanillin sulphuric acid reagent.

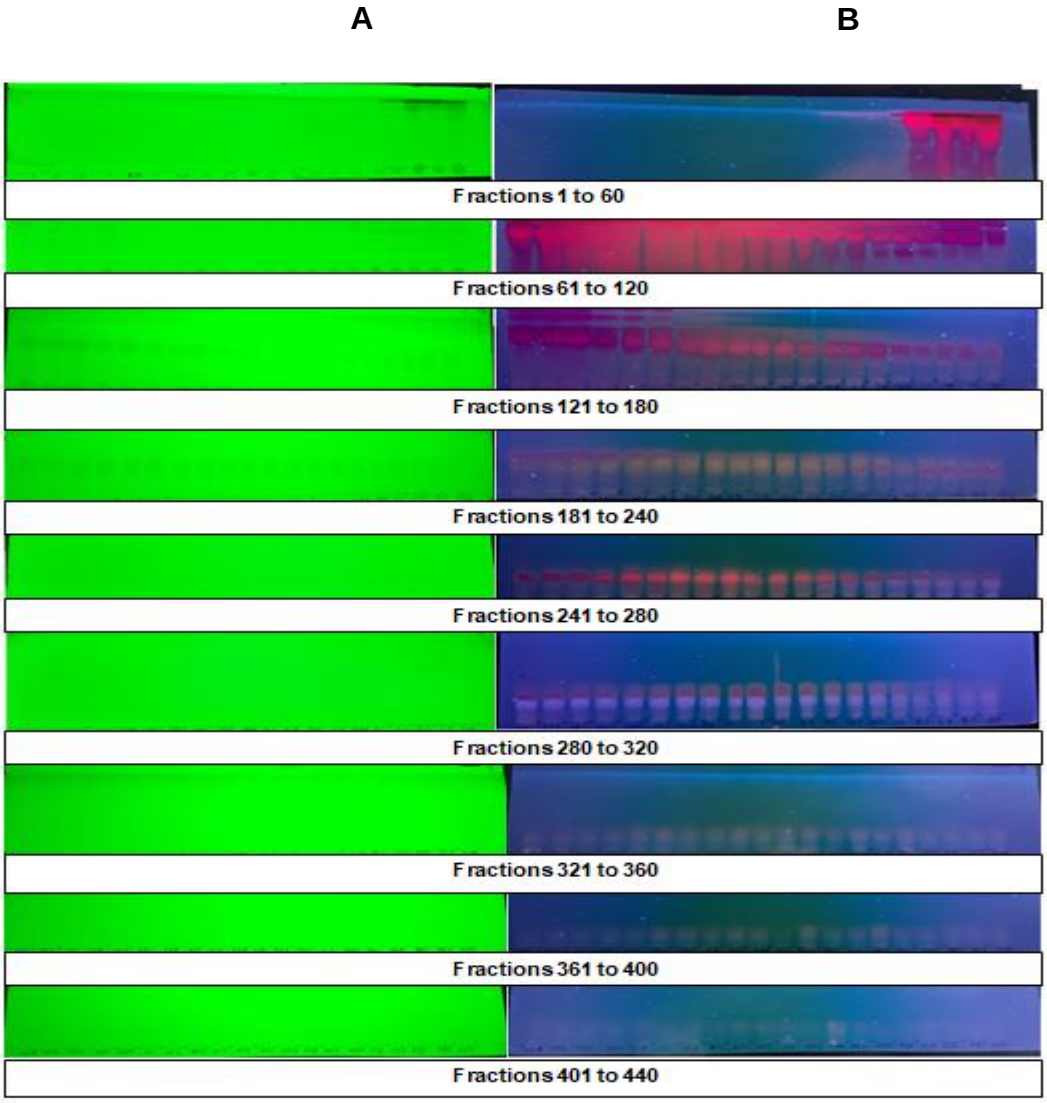

Figure 7.11: Chromatograms of fractions collected from the elution of fractions 4-9 from the first open column by 80% chloroform in ethyl acetate and developed in 80% chloroform in ethyl acetate mobile phase. Then visualised under a UV light at 365 nm (**A**) and 254 nm (**B**).

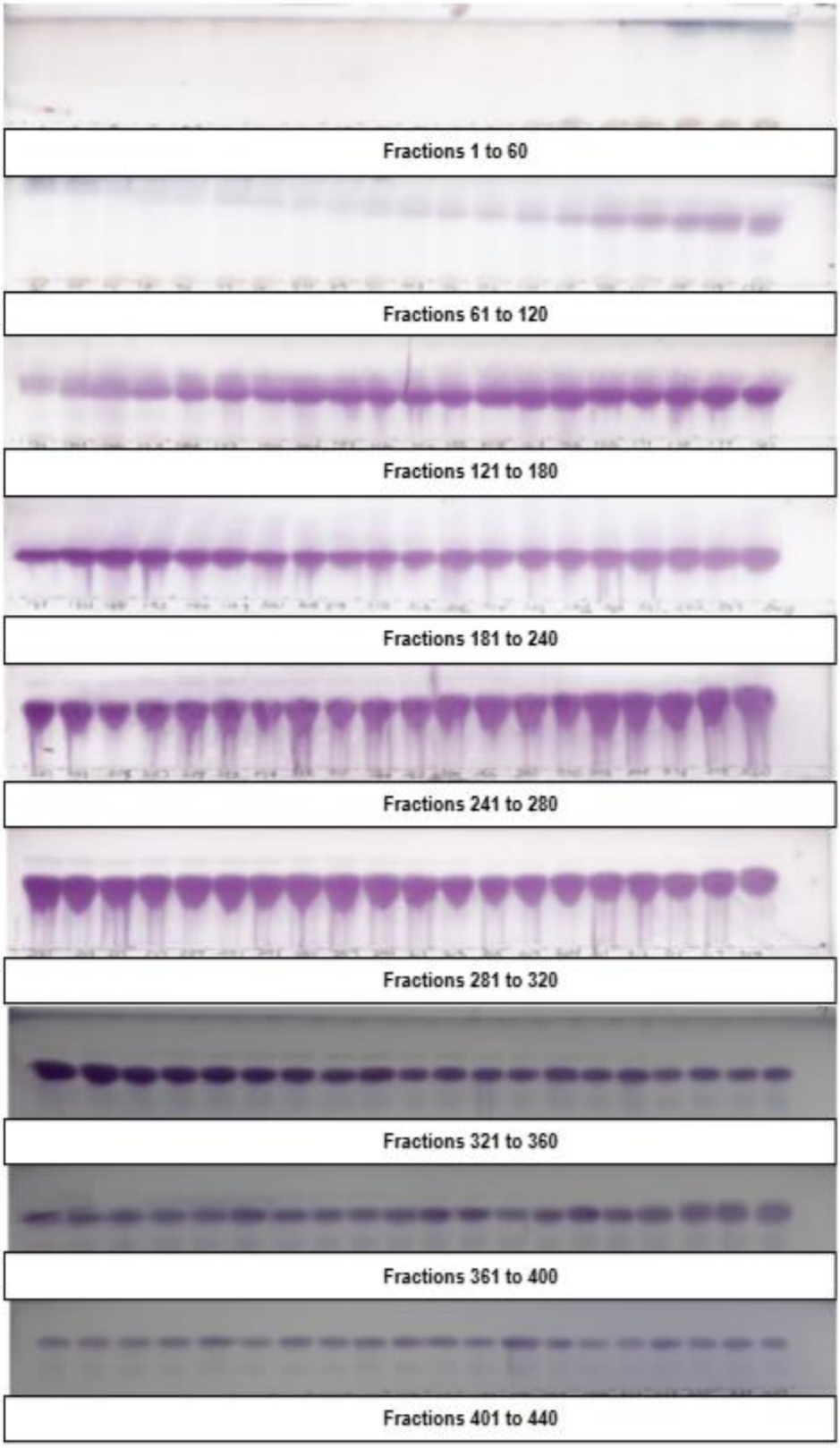

Figure 7.12: Chromatograms of fractions collected from the elution of fractions 4-9 from the first open column by 80% chloroform in ethyl acetate, developed in 80% chloroform in ethyl acetate mobile phase and sprayed with vanillin-sulphuric acid.

The collected fractions were combined into groups based on their TLC profile. Group 1 (124-180), group 2 (181-240), group 3 (241-320), group 4 (321-400) and group 5 (401-440). The fractions were analysed for TLC profile using TLC plates (Figure 7.13). A similar compound was observed in all the groups.

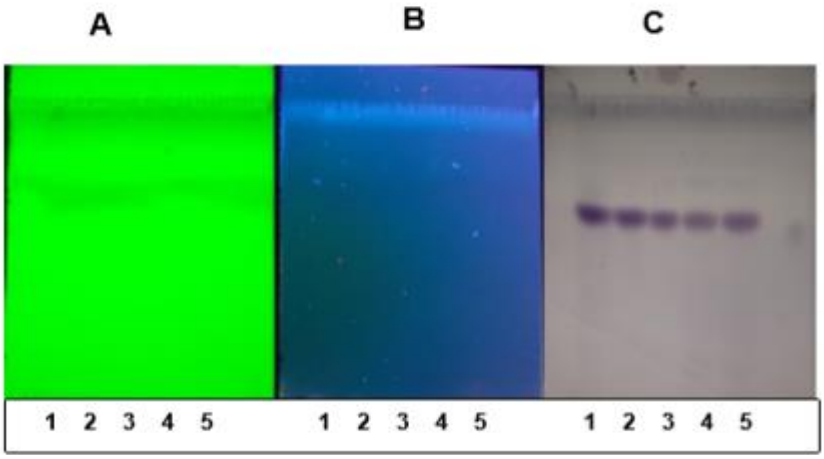

Figure 7.13: The phytochemical analysis of the grouped fractions visualised under a UV light at 365 nm (**A**) and 254 nm (**B**) and sprayed with vanillin-sulphuric acid reagent (**C**).

The groups of fractions were tested for antibacterial activity against *E. faecalis* using bioautography assay (Figure 7.14). The TLC plates were developed in 80% chloroform in ethyl acetate and sprayed with *E. faecalis*. The results in figure 7.14 below indicated that the fractions have activity against *E. faecalis* as observed by the white colour on the chromatogram.

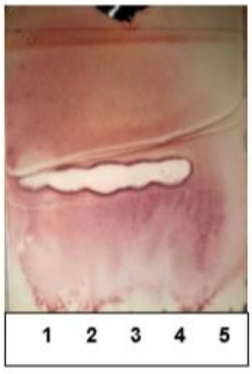

Figure 7.14: The antibacterial activity of the grouped fractions against *E. faecalis*. **1**= Fractions 124-180**, 2**= Fractions 181-240, **3**= Fractions 241-320**, 4**= Fractions 321-400, **5**= Fractions 401-440.

The antibacterial activity of the fractions was quantified using broth micro-dilution assay (Table 7.7). The average MIC against *E. faecalis* was 250 µg/mL. Group 2 and 3 had the highest activity, with the MIC of 125 µg/mL followed by group 4 and 5 with MIC of 250 µg/mL and lastly group 1 with the MIC of 500 µg/mL.

Table 7.7: The MIC values (ug/mL) of the grouped fractions against *E. faecalis*

Groups of fractions	MIC values (µg/mL)
1	500
2	125
3	125
4	250
5	250
Average	250
Positive control	125

Key: 1= fractions 124-180**, 2**= Fractions 181-240, **3**= fractions 241-320**, 4**= fractions 321-400, **5**= fractions 401-440, Positive control (Chloramphenicol)

The five groups of fractions were combined together since they had similar TLC profile and biological activities.

The flow diagram below (Figure 7.13) represents the summary of the whole process of isolation of the antibacterial compound from *M. aethiopicum* leaves. The diagram shows collected fractions, masses and the pure compound obtained. The pure compound was further characterised using Nuclear Magnetic Resonance (NMR) for structural elucidation.

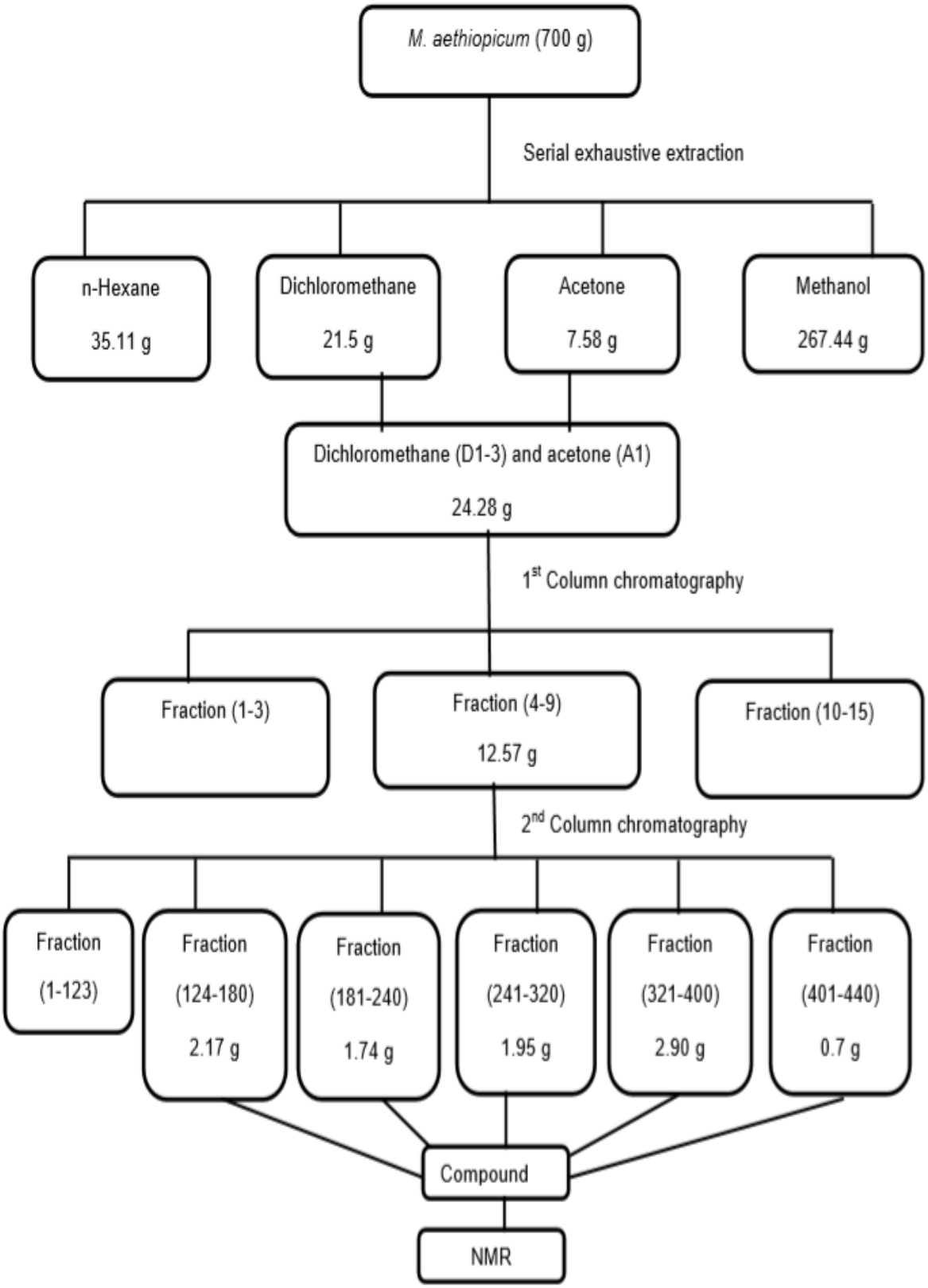

Figure 7.15: Flow diagram of the isolation process of the active compound.

7.4. Discussion

The leaves of *Mystroxylon aethiopicum* showed interesting antibacterial activity against the tested microorganisms during preliminary antibacterial analysis of the leaves of four plants (*Mystroxylon aethiopicum*, *Viscum rotundifolium*, *Dichrostachys cinerea* and *Tapinanthus oleifolius*) in Chapter 5. Therefore, *Mystroxylon aethiopicum* was selected for further isolation and purification of bioactive compounds. To extract the bioactive compounds from the leaves, large scale extraction using serial exhaustive method was carried out with solvents of varying polarity (n-hexane, dichloromethane, acetone and methanol). This is the first step in the isolation process which ensures that a wide range of compounds are obtained for purification purposes (Das *et al*., 2010).

A total mass of 331.63 g was extracted from 700 g of *M. aethiopicum* leaves (Table 7.3). Methanol extracted highest mass of 267.44 g, followed by n-hexane (35.11 g) and dichloromethane (21.5 g) while acetone extracted the least (7.58 g). The results indicated that *M. aethiopicum* leaves contain non-polar to polar compounds since most of the plant materials were extracted from n-hexane and methanol. The extracts from serial exhaustive extraction were spotted on the TLC plates and developed in BEA, CEF and EMW mobile phases for phytochemical analysis (Figure 7.1). Most compounds were observed in the BEA mobile phase than the CEF and EMW. This suggested that *M. aethiopicum* mostly contain non-polar compounds. Similar results were obtained in the preliminary screening (Chapter 3). Furthermore, similar phytochemicals were observed in the dichloromethane and acetone extracts on the chromatogram sprayed with vanillin-sulphuric acid reagent in the BEA mobile phase. Although methanol extracted the highest amount of the plant material, there were no compounds separated in methanol extracts in all mobile phases. Polar compounds are known to have a strong affinity with the polar silica on the TLC plate and as a result, they bind to the polar silica and do not move (Kagan and Flythe, 2014).

The extracts were analysed for free radical scavenging activity using the DPPH assay (Figure 7.2). The results showed that the acetone extracts exhibit antioxidant activity in all the mobile phases but the antioxidant active compounds were separated in the EMW mobile phase. The qualitative antibacterial activity of the

crude extracts determined using the bioautography assay (Figure 7.3 and 7.4) revealed that, the non-polar compounds present in the n-hexane, dichloromethane and acetone extracts have activity against all tested microorganisms while the methanol extracts did not exhibit any antibacterial activity. Furthermore, most of the compounds that were active had similar chemical profile indicating that they could be the same compounds.

The antibacterial activity of the extracts was quantified using the broth micro-dilution method where the Minimum Inhibitory Concentration was determined. The MIC values ranged from 0.08-2.5 mg/mL. The n-hexane, dichloromethane and acetone extracts had high activity against all tested microorganisms whereas the methanol extracts had the least activity. Therefore, dichloromethane (D1-3) and acetone (A1) extracts were selected for isolation of antibacterial compounds. The combined extracts (24.28 g) were subjected to first open column chromatography where they were eluted with solvents of varying percentages. The total mass of 20.74 g was obtained from the fractions (Table 7.5) with highest amount of plant extract eluted with 50% n-hexane in ethyl acetate (4.76 g) whereas 100% ethyl acetate (0.22 g) and 100% methanol (0.24 g) eluted the least.

The phytochemical profile of the collected fractions was analysed on the TLC plates (Figure 7.5 and 7.6). Most fluorescent compounds were observed at 254 nm in all the mobile phases whereas, most of the vanillin reactive compounds were observed in the BEA mobile phase. The 70%-10% n-hexane in ethyl acetate, 100% ethyl acetate and 90% ethyl acetate in methanol extracts had similar compounds in the BEA mobile phase (Figure 7.6). The fractions did not exhibit distinct antioxidant compounds. The antibacterial activity of the fractions determined using the bioautography method (Figure 7.8 and 7.9), indicated that 70%-10% n-hexane in ethyl acetate, 100% ethyl acetate and 90% ethyl acetate in methanol extracts have activity against all the tested microorganisms in the BEA mobile phase. Fraction 4 had the lowest average MIC against all tested microorganisms (0.65 mg/mL) followed by, fraction 8 (0.94 mg/mL) and fraction 9 (1.56 mg/mL).

The results obtained from first open column chromatography showed that the targeted biologically active compound is non-polar. Therefore, bioautography of *M. aethiopicum* fractions was done using different non-polar solvent combinations as

mobile phases as a guidance to determine the combination that best separates the targeted compound from the other compounds. The 80% chloroform in ethyl acetate combination separated the compound of interest the best and it was used as the eluent system for the second column chromatography. The fractions 4-9 [n-hexane (70, 50, 30 and 10%) and ethyl acetate (100 and 90%)] were subjected to second column chromatography using 80% chloroform in ethyl acetate as the eluent system. A total of 440 fractions were collected in test tubes and spotted on TLC plates to determine their TLC profile (Figure 7.11 and 7.12). Most of the phytochemicals were observed on the chromatograms visualised at 254 nm and on the chromatograms sprayed with vanillin-sulphuric acid reagent.

The fractions with similar TLC profile were combined. Five groups [group 1 (124-180), group 2 (181-240), group 3 (241-320), group 4 (321-400) and group 5 (401-440)] were obtained and analysed further on TLC to determine their TLC profile. A similar TLC profile was observed in all the groups (Figure 7.13). The antibacterial activity of the fractions against *E. faecalis* using bioautography assay (Figure 7.14) indicated that, all the groups of fractions have activity against *E. faecalis* as observed by the white colour on the chromatogram. The average MIC of the groups of fractions against *E. faecalis* was 250 µg/mL (Table 7.7). Group 2 and 3 had the highest activity (125 µg/mL) while group 1 had the least (500 µg/mL). The five groups of fractions were combined together to form one compound since they had a similar TLC profile and biological activities, suggesting that they have the same compound. The pure compound was further characterised using Nuclear Magnetic Resonance (NMR) for structural elucidation.

7.5. Conclusion

The bioassay-guided fractionation enabled the isolation of the bioactive compound from *M. aethiopicum* leaves with the use of preparative TLC and column chromatography. This is the first report on the isolation of bioactive compounds from *M. aethiopicum* leaves. The isolated compound has a potential in the development of antibacterial drugs due to the antibacterial activity that was observed throughout the isolation process. The structural elucidation of the isolated compound will be analysed in the following chapter.

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Chapter 8: Structural elucidation

8.1. Introduction

The structural elucidation of compounds involves a combination of analytical techniques and methods such as Nuclear Magnetic Resonance (NMR), ultraviolet (UV), infrared (IR) spectroscopy and mass spectroscopy (MS) (Breton and Reynolds, 2013). In this study, NMR and MS techniques were used as tools for analysis and identification of the compounds isolated from *M. aethiopicum*.

Nuclear Magnetic Resonance (NMR) is a spectroscopic technique that is used to characterise the molecular structures of natural products. NMR spectroscopy detect samples with a mass of 1 mg or less and also samples that have a low concentration of 1-10 µM (Lindon and Nicholson, 2008) or a volume of 600 µL (Bothwell and Griffin, 2011). To completely characterise the chemical structure, NMR combines both 1-dimentional and 2-dimentional NMR experiments. The 1-dimentional NMR experiments include ^{1}H proton NMR, carbon (^{13}C) NMR and distortionless enhancement through polarisation transfer (DEPT) while the 2-dimentional NMR experiments include Heteronuclear Multiple Bond Correlation (HMBC), Correlation Spectroscopy (COSY) and Heteronuclear Single Quantum Correlation (HSQC) (Claridge, 1999).

In the ^{1}H proton NMR, the spectrum provides information about the number of protons present in the sample and in the carbon (^{13}C) NMR, the spectrum is used to determine the type of carbon present in the sample. In the distortionless enhancement through polarisation transfer (DEPT) NMR, the spectrum provides information about the number of hydrogens attached to each carbon. In a HSQC experiment, the spectrum shows heteronuclear correlations which arise as a result of $^{1}J_{CH}$ couplings between ^{13}C nuclei and ^{1}H protons attached to the corresponding atoms. This makes it possible to detect all CH, CH_2 and CH_3 groups with chemical shift assignment. In the COSY experiment, the spectrum shows homonuclear correlations (spin couplings) between vicinal hydrogens separated by three bonds ($^{3}J_{HH}$). This makes it possible to identify the neighbour carbon atoms connected by a chemical bond. In the HMBC experiment, the spectrum shows heteronuclear correlations between ^{1}H proton and ^{13}C (^{15}N) nuclei separated by two, three and

sometimes four chemical bonds, making it possible to detect indefinite fragments around a given C or N atom (Breton and Reynolds, 2013; Elyashberg, 2015).

Mass Spectrometry is an imperative analytical tool in science fields such as chemistry, physics, biochemistry, pharmacy, medicine, and many related fields. The fundamental principle of Mass Spectrometry is to generate ions from inorganic or organic compounds, to separate these ions based on their mass-to-charge ratio (m/z) and to detect them qualitatively and quantitatively by their respective m/z and abundance. The sample is ionised thermally by electric fields or by impacting energetic electrons, ions or photons. The ions can be single ionised atoms, clusters, molecules or their fragments or associates (Gross, 2017). Mass Spectrometry is used for relative molecular weights (Mr) determination, identification of functional groups, structural elucidation of unknown substances, determination of the average number and sequence of constituents of macromolecules and in some instances, it yields their three-dimensional structure (He *et al*., 2001; Harris *et al*., 2007). The aim of this chapter was to elucidate the chemical structure of the isolated compound using Nuclear Magnetic Resonance and Mass Spectroscopy.

8.2. Methods and materials

8.2.1. Structural elucidation of isolated compound

The clean sample of the isolated compound was sent to the Department of Chemistry, University of Limpopo (Turfloop Campus) for characterisation using the NMR. The compound was characterised using 1-dimensional NMR (^{1}H, ^{13}C and DEPT 135) and 2-dimensional NMR (HMBC, HSQC, and COSY). Exactly 20 mg of the compound was dissolved in chloroform and ran using 400 MHz NMR Spectrometer (Bruker) at 400 MHz, chloroform-d as a reference signal solvent and at a temperature of 295.5 K. Professor Ofentse Mazimba, based at the Department of Chemical and Forensic Sciences at the Botswana International University of Science and Technology, assisted with the structural elucidation using the provided spectroscopic data.

8.3. Results

The NMR spectra (^{13}C, ^{1}H Proton, DEPT 135, COSY, HSQC, and HMBC) of the isolated compound are represented in figure 8.1 to 8.6. Table 8.1 shows the summary of the ^{13}C and ^{1}H Proton shift values of the isolated compound compared to the values obtained from literature. The NMR spectra helped in the characterisation of compound (Figure 8.7).

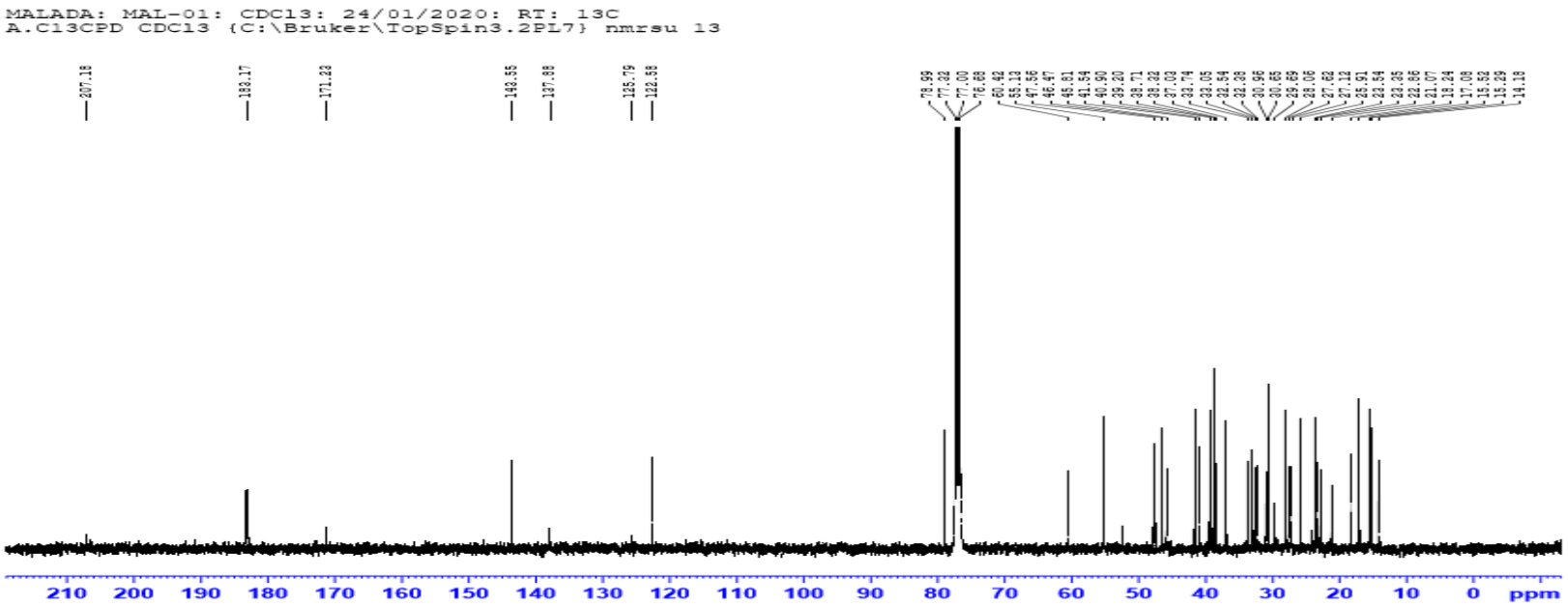

Figure 8.1: ^{13}C NMR spectrum of the isolated compound.

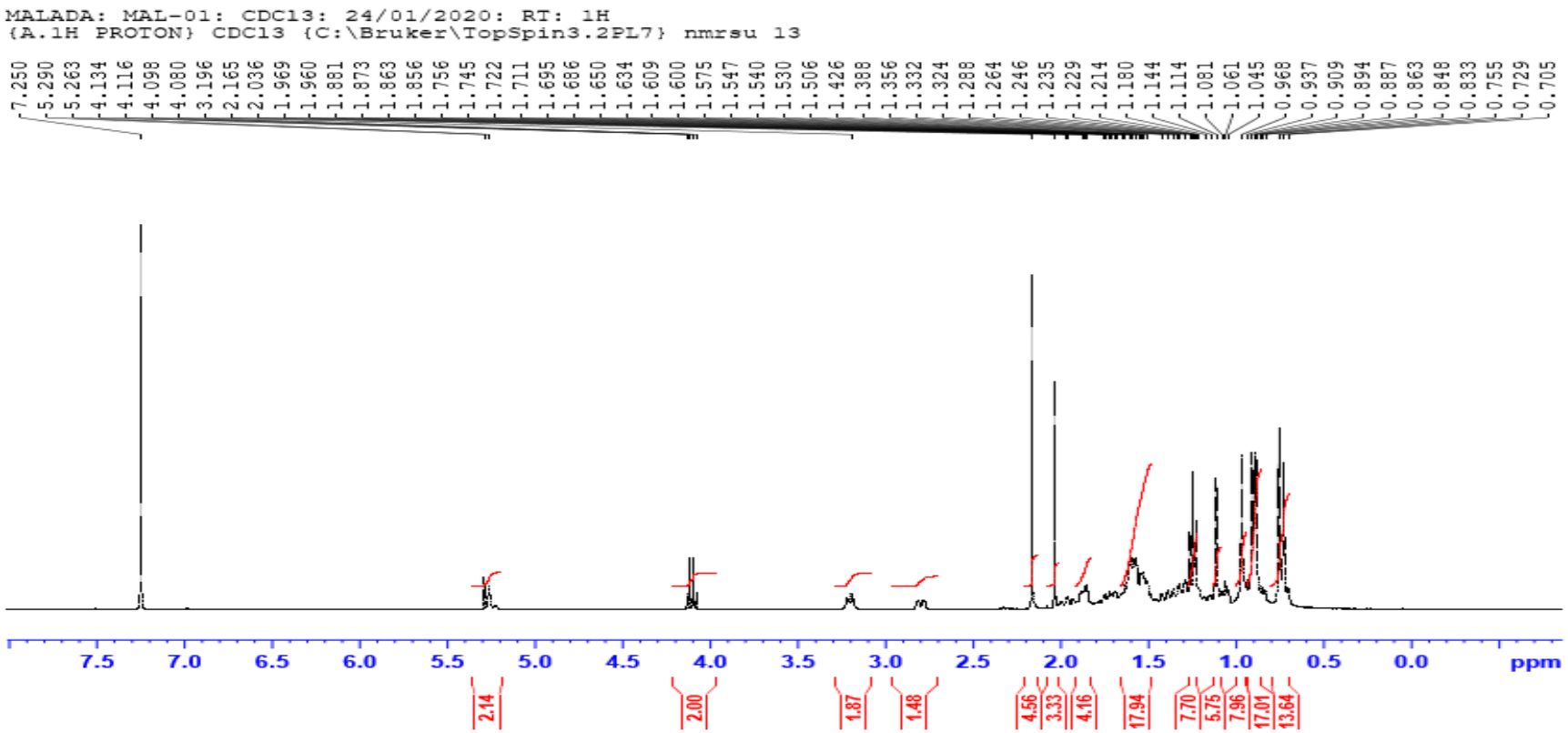

Figure 8.2: ^{1}H proton NMR spectrum of the isolated compound.

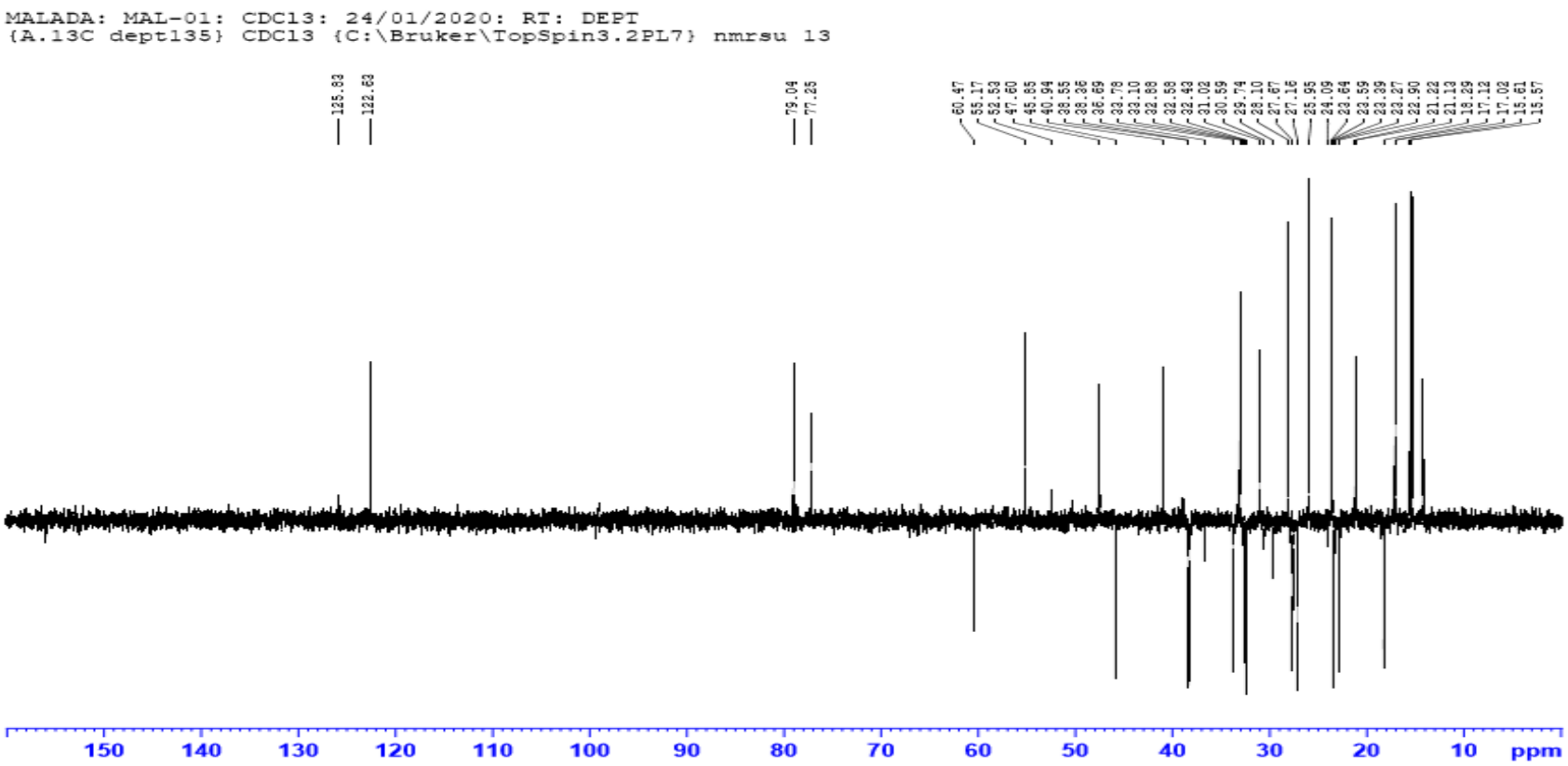

Figure 8.3: DEPT 135 NMR spectrum of the isolated compound.

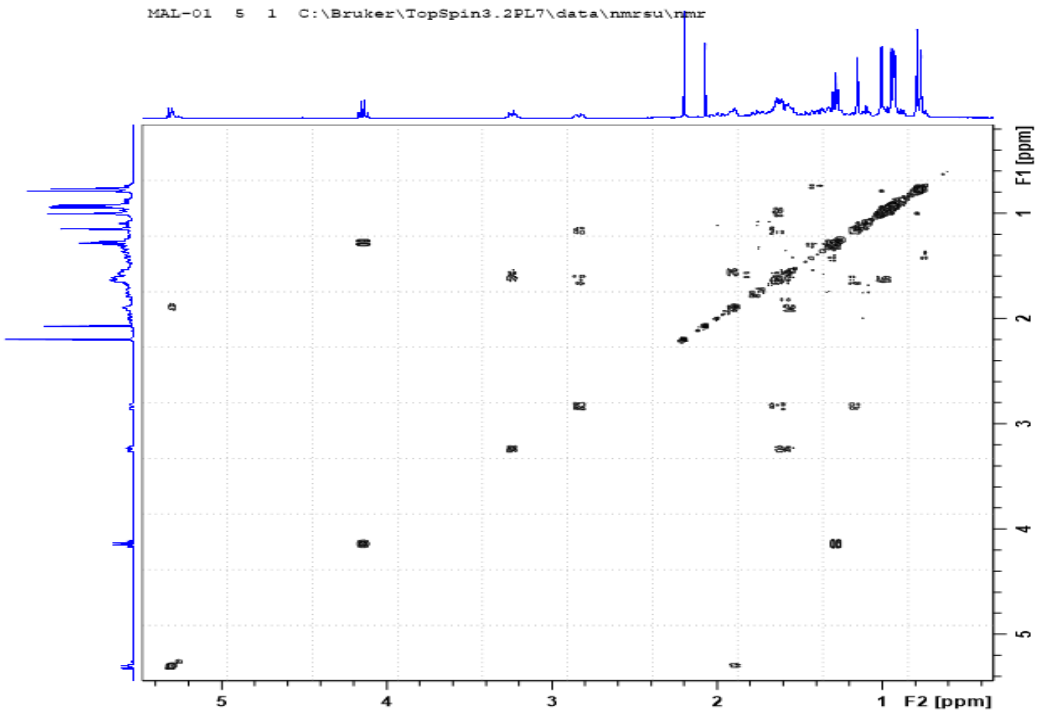

Figure 8.4: COSY NMR spectrum of the isolated compound.

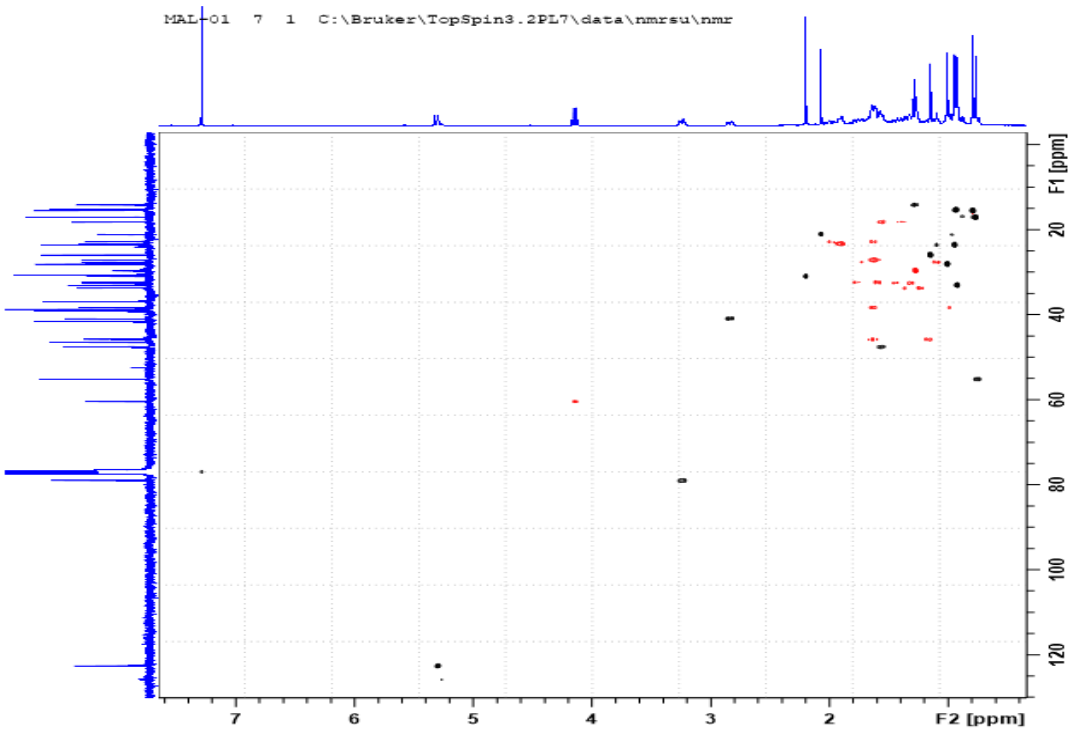

Figure 8.5: HSQC NMR spectrum of the isolated compound.

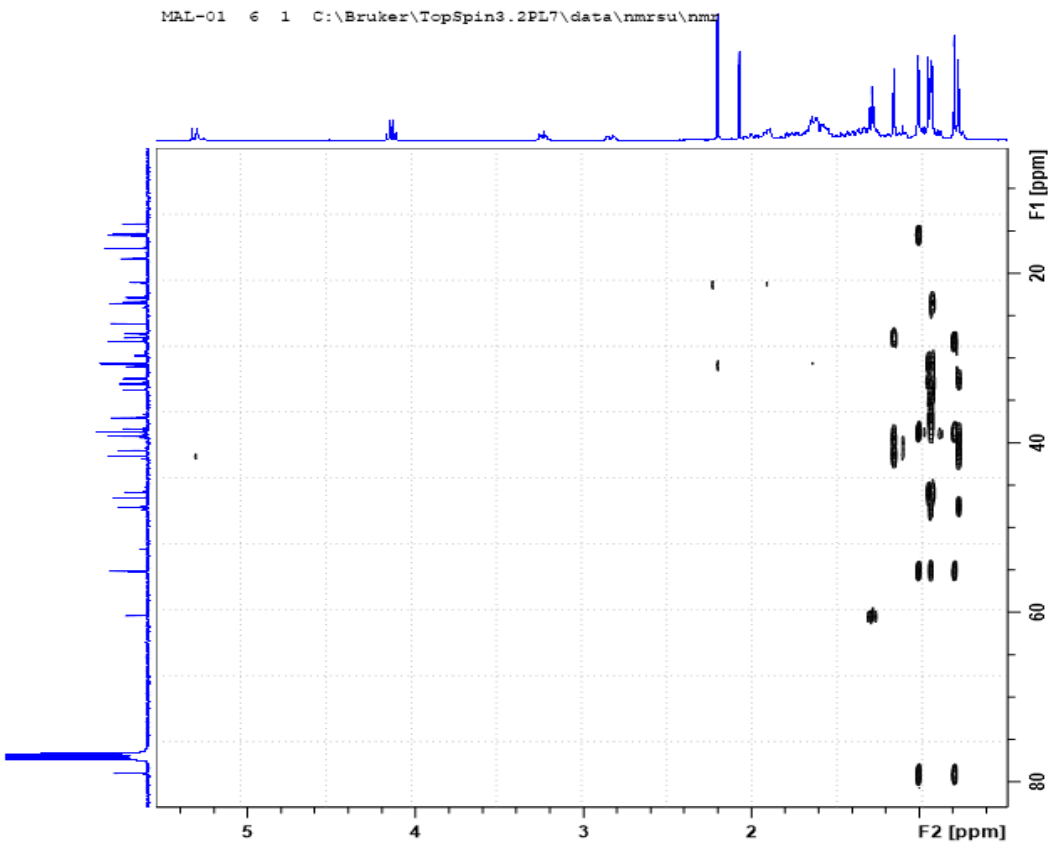

Figure 8.6: HMBC NMR spectrum of the isolated compound.

Table 8.1: ^{1}H and ^{13}C NMR ($CDCl_3$) data of the isolated compound.

Position	^{1}H	$^{1}H^{a}$	^{13}C	$^{13}C^{a}$
1	1.24 m and * m	* and ***	38.3	38.1
2	** m and 1.96 m	*** and 1.87-1.90 m	23.3	23.5
3	3.21 *dd* (*J*= 4, 10 Hz)	4.49 m	78.9	80.9
4	-	-	38.7	37.7
5	0.84 m	0.83-0.88	55.1	55.3
6	1.24 m and **	** and ***	18.2	18.2
7	***	**	32.8	32.6
8	-	-	39.1	39.3
9	*	***	47.5	47.6
10	-	-	37.0	37.0
11	** and *	** and 1.87-1.90 m	22.8	23.4
12	5.26 brs	5.28 t (*J*=3.5 Hz)	122.5	122.6
13	-	-	143.5	143.6
14	-	-	41.5	41.6
15	1.24 m and †	*and***	27.6	27.7
16	*** and 1.96 m	**and 1.99 dt (*J*=13.6, 13.6,3.9 Hz)	22.8	22.9
17	-	-	46.4	46.5
18	2.78 *dd* (*J*=4, 9.6 Hz)	2.82 *dd* (*J*=13.6, 4.1 Hz)	40.9	41.0
19	1.24 m and †	* and ***	45.8	45.9
20	-	-	30.7	30.7
21	** and ***	**	33.7	33.8
22	** and †	***	32.5	32.4
23	0.88 s	0.85 s	15.5	16.7
24	0.89 s	0.87 s	28.1	28.0
25	0.87 s	0.94 s	15.3	15.4
26	0.75 s	0.76 s	17.0	17.1
27	1.11 s	1.13 s	25.9	25.9
28	-	-	183.1	182.7
29	0.97 s	0.93 s	23.5	23.6
30	0.90 s	0.91 s	33.0	33.1
acetyl CH_3	2.16	2.05 s	21.1	21.3
acetyl C=O	-	-	171.2	171.0

[a]Endo *et al*., 2019; *1.00-1.13 ppm; **1.17-1.48ppm; ***1.52-1.81 ppm; *1.85-1.88 m; **1.51-1.64 m; ***1.32-145 m; †1.68-1.78 m

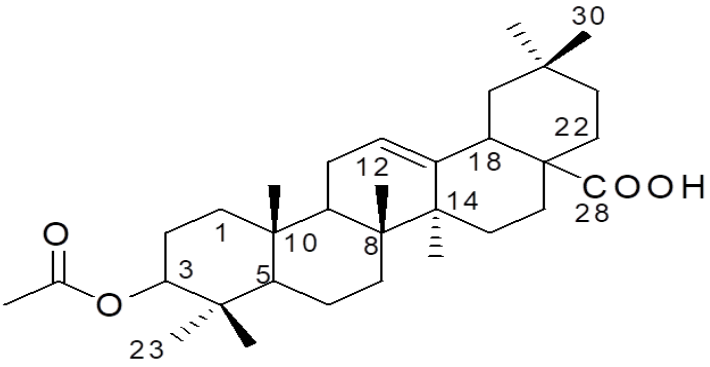

Figure 8.7: Oleanolic acid acetate isolated from *Mystroxylon aethiopicum*.

8.4. Discussion

The structure of the isolated compound from *Mystroxylon aethiopicum* was elucidated using NMR, MS analysis in comparison with the reported information from literature. The structure of the compound was successfully deduced from ^{1}H, ^{13}C, COSY, HSQC and HMBC NMR spectra. The ^{13}C and ^{1}H Proton shift values from the NMR spectra of the isolated compound was in agreement with the values obtained from literature (Table 8.1). The isolated compound was found to be oleanolic acid acetate (OAA), a triterpenoid. This compound has been isolated from various plants including, *Vigna angularis* (Oh *et al*., 2013), *Phaseolus angularis* seeds (Hwang *et al*., 2014) and *Quercus crispula* Blume outer bark (Endo *et al*., 2019). Therefore, this is the first report on the isolation of oleanolic acid acetate from the leaves of *M. aethiopicum*. Many studies have shown the different pharmacological activities of OAA such as, antiosteoporotic, antiallergy activity and anti-inflammatory activity (Mukai and Sato, 2011; Oh *et al*., 2013; Choi *et al*., 2013; Kim *et al*., 2018; Lim *et al*., 2019a; Lim *et al*., 2019b).

8.5. Conclusion

The compound isolated from *M. aethiopicum* was identified as oleanolic acid acetate. The spectroscopic data obtained from NMR compared with the data obtained from literature review confirmed the identity of this compound. This compound has already been isolated from various plants; however, this is the first report on the isolation of oleanolic acid acetate from the leaves of *M. aethiopicum*.

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Chapter 9: General discussion, conclusions and recommendations

9.1. General discussion

The increasing rate of antibiotic resistance caused by the overuse and misuse of antibiotics (including poor patient compliance and improper disposal of antibiotics) has caused a resurgence of dependence on complementary and alternative medicine. Plants represent the greatest source of active substances that can be used in medical therapy. However, the increasing demand for traditional herbal medicine consequently brought challenges with regards to the efficacy, quality, safety, and standardisation of plants (Taylor *et al*., 2001; Chikezie, 2015). Thus, this study was aimed at isolating and characterising the antibacterial and antioxidant compounds from the leaf extracts of the two parasitic plants (*Viscum rotundifolium* and *Tapinanthus oleifolius*) and determining the synergistic antibacterial effects of the parasitic plants with their hosts (*Mystroxylon aethiopicum* and *Dichrostachys cinerea*). The safety evaluation regarding the use of the plants in traditional medicine as treatment for infections was also determined. The four plant species were selected for the study based on the information from literature as they are used traditionally to treat various ailments.

Extraction is one of the primary steps used when determining biologically active compounds from the plants. This process allows the separation of the desired bioactive compounds from the plant materials (Zhang *et al*., 2018). In the current study, leaves of the plants were extracted using solvents of varying polarity from non-polar to polar to allow the extraction of distinct compounds based on their polarity. The terpenoids, flavonoids, phlobatannin, tannins, steroids and cardiac-glycosides were present in all the plants. The Thin Layer Chromatography (TLC) was used to compare the compounds present between the host and the parasite. The chromatograms (CEF and EMW) sprayed with vanallin-sulphuric acid reagent confirmed that similar phytochemicals exist between the parasites and host plants. *T. oleifolius* was significantly high in flavonoid and tannin content while the *M. aethiopicum* was high in phenolic content. However, the overall quantities of the phytoconstituents seemed to be high in the parasites than the host plants.

The qualitative antioxidant activity using the DPPH assay on the TLC showed that the compounds that exhibit antioxidant activity are non-polar to polar. The *T. oleifolius* and *D. cinerea* plant extracts had prominent antioxidant compounds which were seen by the yellow bands against the purple background of DPPH. The antioxidant activity of the plants was confirmed by quantitative tests.

M. aethiopicum showed activity against all tested bacteria while *V. rotundifolium* only had activity against *E. faecalis.* Both *T. oleifolius* and *D. cinerea* did not have activity against all the tested bacteria. The quantitative antibacterial test confirmed the activity of the plant extracts where the MIC values ranged from 0.04-2.5 mg/mL. The *T. oleifolius* and *D. cinerea* extracts had lowest MIC against all tested bacteria although they did not show any activity in the bioautography assay. The methanol extracts in both *T. oleifolius* and *D. cinerea* plants showed the lowest MIC values ranging from 0.04 to 0.16 mg/mL. This may have been a result of the synergistic interaction of compounds within the extracts.

The effect of the parasites on the antibacterial activity of the hosts was determined since there is a close association between the two plants. Therefore, the n-hexane, ethyl acetate, acetone and methanol extracts of parasites and hosts were combined. The combination of *V. rotundifolium* and *M. aethiopicum (*n-hexane, ethyl acetate and acetone extracts) and *T. oleifolius* and *D. cinerea* (n-hexane and acetone extracts) showed synergistic effects in inhibiting the growth of *S. aureus* whereas the methanol extract of *T. oleifolius* and *D. cinerea* showed antagonistic effects in inhibiting the growth of all tested bacteria. However, there was no significant difference in the overall antibacterial activity of the combination of the parasite and host extracts relative to the individual activities. This may be caused by the similar distribution of phytochemicals between the two.

Some traditional health practitioners believe that medicinal plants have no side effects because they come from nature. However, studies have indicated that some plants produce toxic reactions, mutagenic effects and allergic reactions (Senior, 1998). Therefore, safety evaluation of traditional medicinal plants is of great importance. The cell viability assay was used to evaluate the cytotoxic effects of the plants' acetone extracts on human liver (C3A) cells. The plants were found to be non-toxic on the human liver (C3A) cells.

Based on the preliminary antibacterial assays, *M. aethiopicum* had good activity amongst all the four selected plants. Therefore, it was selected for isolation and purification of bioactive compounds. The bioassay-guided fractionation led to the isolation of oleanolic acid acetate. Although oleanolic acid acetate has been isolated from different plants, this was the first time it was isolated from *M. aethiopicum*.

9.2. Conclusions and recommendations

This study has demonstrated that the selected plants have an antibacterial potential that is ascribed to the diverse phytochemicals they exhibit. This study has demonstrated the isolation, characterisation and identification of oleanolic acid acetate from *M. aethiopicum*. This study also demonstrated that the four selected plants are non-toxic to human liver (C3A) cells. Therefore, more *in vitro and in vivo* cytotoxicity assays on different cell lines are recommended in order to support their use in traditional medicine. Furthermore, this study recommends the evaluation of anti-cancer properties of the parasitic plants as reported by other studies.

9.3 References

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