Antibacterial activity of the crude extract and fractions of *Spirostachys africana* against multi-drug resistant bacteria

by

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DECLARATION OF INDEPENDENT WORK

I, Ajmal Antoinette (student number: 11640082), declare that this dissertation for a master's degree in Microbiology at the University of Venda is my original work and has not previously been submitted for a degree at this or any other University or institution. The project titled "Antibacterial activity of the crude extract and fractions of *Spirostachys Africana* against multi-drug resistant bacteria" does not contain other persons writing unless otherwise specifically acknowledged and referenced accordingly.

…… ……

Signature **Date**

DEDICATION

This research is dedicated to my mother Dorothy Ajmal and my siblings, Precious Tshikovhi and Zwiitani Tshikovhi.

ACKNOWLEDGEMENT

To begin with, I would like to give thanks to God, for protecting me throughout my research, giving me the power and determination to complete this research.

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ABSTRACT

Background: The high on-going incidences of infectious diseases, specifically those caused by multi-drug resistant bacteria in the last decade has made it a necessity to investigate a variety of antimicrobial drug sources, such as plants. Medicinal plants have played a significant role in drug discovery for western pharmaceuticals recently and have also been used successfully by traditional healers and herbalists to treat various infectious diseases for centuries. Currently, a few medicinal plants are commercialized, reason being most medicinal plants phytochemicals have not been studied yet, although they have been traditionally used by healers. Due to the constant development of multi-drug resistance of bacteria to antibiotics, *S. africana* extracts can provide an opportunity to finding new antibacterial compounds that can be used as the foundation for formulating new antimicrobial drugs.

Objectives: The aim of this study was to screen antibacterial activity of the crude extract and fractions of *S. africana* against multi-drug resistant bacteria and to also evaluate other biological properties.

Methods: Preliminary screening of phytochemical constituents of *S. africana* and fractions was done using standard qualitative and quantitative methods. Antibacterial activity of the extracts was evaluated using the agar well diffusion method and the microdilution assay against MDR bacterial strains. Antioxidant activities of the MCE and its fractions were measured by DPPH and reducing power assays, and the toxicity of the MCE and its fractions was tested on Vero cells using Cell-based high content screening assay.

Results: Phytochemical analysis of the MCE and fractions obtained in this study showed the presence of phenolics, flavonoids, alkaloids, steroids, saponins, cardiac glycosides and terpenoids in most of *S. african*'s test samples. Fraction F1 and F2 both lacked alkaloids and saponins. The micro-plate dilution assay demonstrated that the MCE and all its fractions can inhibit the growth of all selected MDR bacterial strains tested against at different concentrations (0.1mg/ml to >12.5mg/ml), wherein the lowest MIC averages were obtained from fractions F3 and F6, with 0.59 mg/ml and 0.71 mg/ml MIC averages respectively. Contrary to the micro-plate dilution assay, the well diffusion assay demonstrated that MCE and all its fractions were not active against all the selected MDR bacterial strains tested against, as no inhibition was shown against the growth of *K. pneumonia* by any of *S. african*'s test samples. For DPPH assay, the IC_{50} of *S. african*'s test samples ranged between 0.01 ±0.34 mg/ml to 0.62 ± 0.05 mg/ml, whiles for the reducing power assay, EC_{50} measured ranged between 0.61 ± 0.01 mg/ml and 11.30 ± 0.04 mg/ml. The MCE and fraction F2 exhibited the highest toxicity to Vero cells.

Conclusion: The MCE and fractions of the plant *S. africana* have antibacterial activity against MDR bacterial strains, beneficial biological properties and contains potential antibacterial compounds that may be valuable in the discovery of new potential drugs for treatment of infectious diseases

Keywords: Antibacterial activity; Multi-drug resistant bacteria; *Spirostachys africana.*

LIST OF ABBREVIATIONS

µl	:	Microliter
AA	:	Ascorbic acid
AB's	:	Antibiotics
AGE	:	Acute gastroenteritis
ATCC	:	American Type Culture Collection
BEA	:	Benzene: ethanol: ammonium hydroxide (90:10:1)
CEF	:	Chloroform: Ethyl acetate: Formic acid (5:4:1)
CFU	:	Colony Forming Units
CO_2	:	Carbon dioxide
CTE	:	Catechin equivalent
DMSO	:	Dimethyl sulfoxide
EC_{50}	:	Half maximal Effective Concentration
EE	:	Ethyl-acetate
EMW	:	Ethyl acetate: Methanol: Water (40:5.4:4).
ESKAPE	:	*Enterococcus faecium, Staphylococcus aureus Klebsiella pneumonie, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter species*
Fe^{3+}	:	Potassium ferricyanide
Fe^{2+}	:	Potassium ferrocyanide
g	:	grams
GAE	:	Gallic acid equivalent
I%	:	Percentage of Inhibition

IC50	:	Half maximal Inhibitory Concentration
INT	:	p - iodonitrotetrazolium
MCE	:	Methanolic Crude Extract
MDR	:	Multi-drug resistance/t
mg/ml	:	milligram per milliliter
MIC	:	Minimum Inhibitory Concentration
mm	:	millimeters
MRSA	:	Methicillin-resistant staphylococcus aureus
NB	:	Nutrient broth
nm	:	nanometers
No.	:	Number
PBS	:	Phosphate Buffered Saline
QE	:	Quercetin equivalent
S. africana	:	*Spirostachys africana*
TLC	:	Thin Layer Chromatography
TFC	:	Total flavonoid content
TPC	:	Total phenols content
UV	:	Ultraviolet
WHO	:	World Health Organization

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Chapter 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Since their discovery in the 20th century, antibiotics have played a significant role in transforming the field of medicine through treating and successfully preventing infectious diseases (Ventola et al., 2015). However, the excessive and irresponsible use of antibiotics has contributed significantly to the dawn of the antimicrobial resistant era (Hocquet et al., 2016). The spread of antibiotic-resistant bacterial infections is the main cause of the high rates of morbidity and mortality amongst the overall populations worldwide, making this a global health crisis along with other consequences socially and economically (Tacconelli et al., 2018; Zaman et al., 2017).

The diverse groups of bacteria that display multidrug resistance (MDR) have been associated with the pathogenesis of different infections, such as those of the respiratory, skin, gastrointestinal and urogenital diseases, along with the contamination of the wound, that are the main reasons behind the frequent hospital visits, resulting in higher hospital charges and prolonged hospitalization (Elisha et al., 2017).

A group of MDR bacterial strains associated with high resistance rates are known as the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*) pathogens, and have been of interest lately due to the fact that they are responsible for two third of all nosocomial infections, whereby their treatment is severely limited, thus necessitating the need for the development of new preventative methods and therapeutics (Santajit and Indrawattana, 2016).

The high on-going incidences of MDR bacteria in hospitals in the last decade has made it essential to investigate other possible antimicrobial drug sources, such as

plants. Plants and plant products are considered therapeutic/medicinal due to their ability to produce a vast collection of natural secondary metabolites that consist of antimicrobial effects, making them a possible alternative treatment for MDR bacterial infections (Ahmad and Wajid, 2013). The use of plants by traditional healers to treat various infections and the plants very own defence mechanisms against pathogens and infections has led to the interest of researching more about the biological properties of compounds produced by plants (Mazid et al.,2011; Muthu et al., 2006; Van Wyk et al., 1997). These secondary metabolites can be extracted from different parts of the plant such as the root, stem, leaf, flower, fruit and twig exudates using suitable solvents of varying polarities (Sanadhya and Durve, 2014).

Medicinal plants have over the centuries been successfully used traditionally by healers to treat various infectious diseases and recently have had a substantial role in drug discovery for western pharmaceuticals (Rosakutty and Roslin, 2012). The principle of gathering, processing and using plant materials traditionally has been passed down verbally from generation to generation, and to date, this culture is still practiced. In most African countries, 80% of people living in rural communities still depend entirely on medicinal plants for their primary health care needs, due to the obtainability and affordability of this type of health care system (Sigidi et al., 2016).

Spirostachys africana Sond (*S. africana*), commonly known as the tamboti tree, is a medicinal plant that is found to grow in the southern and central parts of Africa and belongs to the family of flowering plants known as Euphorbiaceae, which is a family that consists of a diverse group of plants known to be phytochemically rich in terpenoids and alkaloids (Munkombwe et al., 2006).

Typically known for its numerous heartwood uses to create artefacts such as necklaces, gunstocks and walking sticks, *S. africana* nonetheless is a tree also acknowledged for its medicinal practices in rural based communities in Southern Africa, such as the following: the roots decoctions are used for treating venereal diseases, malaria, constipation, headache and diarrhoea while the stem bark preparations (decoctions or infusions) are used to treat stomach pains, constipation,

skin infections, stomach ulcers, diarrhoea, oral opportunistic infections and many more (Akhalwaya et al., 2018; Seukep et al., 2014; Mathabe et al., 2008; Mathabe et al., 2006; Munkombwe et al., 1997)

Although the successful uses of the plant *S. africana* to treat various ailments by traditional healers has been reported, it is still of much importance to study this plant to obtain detailed information on its safety, quality and dosage requirements for optimal therapeutic effects (Munkombwe et al., 1997). Biological activities of *S. africana* have been studied, and it has been shown by researchers that this indigenous African plant has antibacterial and insecticidal properties, that originate from the presence of different types of compounds found in its various parts (Mulaudzi et al., 2012; Mathabe et al., 2008; Mathabe et al., 2006; Munkombwe et al., 1997).

1.2 STUDY RATIONALE

Bacterial infections caused by bacteria have developed resistance to a wide variety of antibiotics used for treatment, and as a result, only a rare number of effective antibiotic choices are still available for treatment (Miyasaki et al., 2013). The need to discover new active antibacterial drug alternatives to eradicate the problem of multidrug resistance by bacteria has made it a necessity to investigate medicinal plants for their biological activities, with the purpose of developing more potent antibacterial drugs compared to the current ones (Fischbach and Walsh, 2009).

Currently, a few numbers of medicinal plants are commercialized, reason being most medicinal plants' phytochemicals are yet to be studied completely, although they are being administered by traditional healers without scientific validation (Rosakutty and Roslin, 2012). Previous studies on *S. africana* have shown the presence of secondary metabolites contained in this plant, whereby compounds such as diphenols, α ketols, stachynones and acid metabolites have been isolated from the plant's latex, and terpenoids from the stem bark, and also antibacterial properties of this plant against diarrhea causing bacteria has been documented, making this medicinal plant a

potential source for new antibacterial compounds that can be utilised as the foundation for formulating new antimicrobial drugs (Chitemerere and Mukanganyama, 2014).

Although some data has been reported about the biological and phytochemical properties of *S. africana* found in different locations, it is known that the plant's number of secondary metabolites within and its medicinal uses differs according to the location, weather conditions and the type of soil the plant is found to be growing at (Liu et al., 2015). Currently, the medicinal plant *S. africana* located at Matsa village in Nzhelele lacks documented data on its biological properties. Locally, at Matsa village, this plant is overly harvested, where after oral investigation (interviews with locals and traditional healers), the plant was found to be used as herbal medicine to treat various ailments such as flu, diarrhea, dysentery, venereal diseases, wound etc. Previous studies on *S. africana* have reported the presence of saponins, tannins, flavonoids, glycosides, alkaloids, and that the presence of these phytochemicals majorly contribute to the plant's biological properties (Mulaudzi et al., 2012; Mathabe et al., 2008).

Limited studies have been done on the antibacterial properties of *S. africana* against MDR bacteria (ESKAPE Pathogens). This study aimed to investigate the antibacterial activities of the crude extract and fractions of *S. africana* stem barks (located at Matsa village) against selected MDR bacteria.

1.3 RESEARCH QUESTIONS

- Could *S. africana* extracts be a potential source of antibacterial compounds that can inhibit the growth /kill multidrug resistant bacteria?
- Will fractionation increase the biological activities of *S. africana*?

1.4 OBJECTIVES

1.4.1 Main objective

- To evaluate the antibacterial activity of the crude extract and fractions of *Spirostachys africana* against MDR bacteria

1.4.2 Specific objectives

- To extract bioactive compounds from *S. africana* with methanol using maceration
- To fractionate the crude extract using column chromatography
- To screen phytochemicals of the crude extract and fractions using qualitative and quantitative methods
- To determine the antioxidant activities using the DPPH and Reducing power assay
- To evaluate the antibacterial activity of the crude extract and fractions of *S. africana* against the selected MDR bacteria (ESKAPE pathogens) using agar well diffusion assay and micro-plate dilution assay
- To evaluate the toxicity of the crude extract and fractions on Vero cells using Cell-based high content screening assay (cytotoxicity assay).

Chapter 2

LITERATURE REVIEW

2.1 MULTIDRUG RESISTANCE (MDR) IN BACTERIA

For long, antibiotics were the ideal form of antimicrobial agents used successfully to treat bacterial infections since their discovery in 1928, however, their overuse and misuse have led to their major impact on the rise and spread (Figure 2.1) of MDR bacterial strains (Fair and Tor, 2014; Levy, 2013). The bacterial strains that are classified as MDR have developed resistance to multiple types of antibiotics, wherein the progress of their resistance has been mainly due to their extensive use in clinical medicine, agriculture, and veterinary sectors (Tacconelli et al., 2018; Doyle et al., 2013).

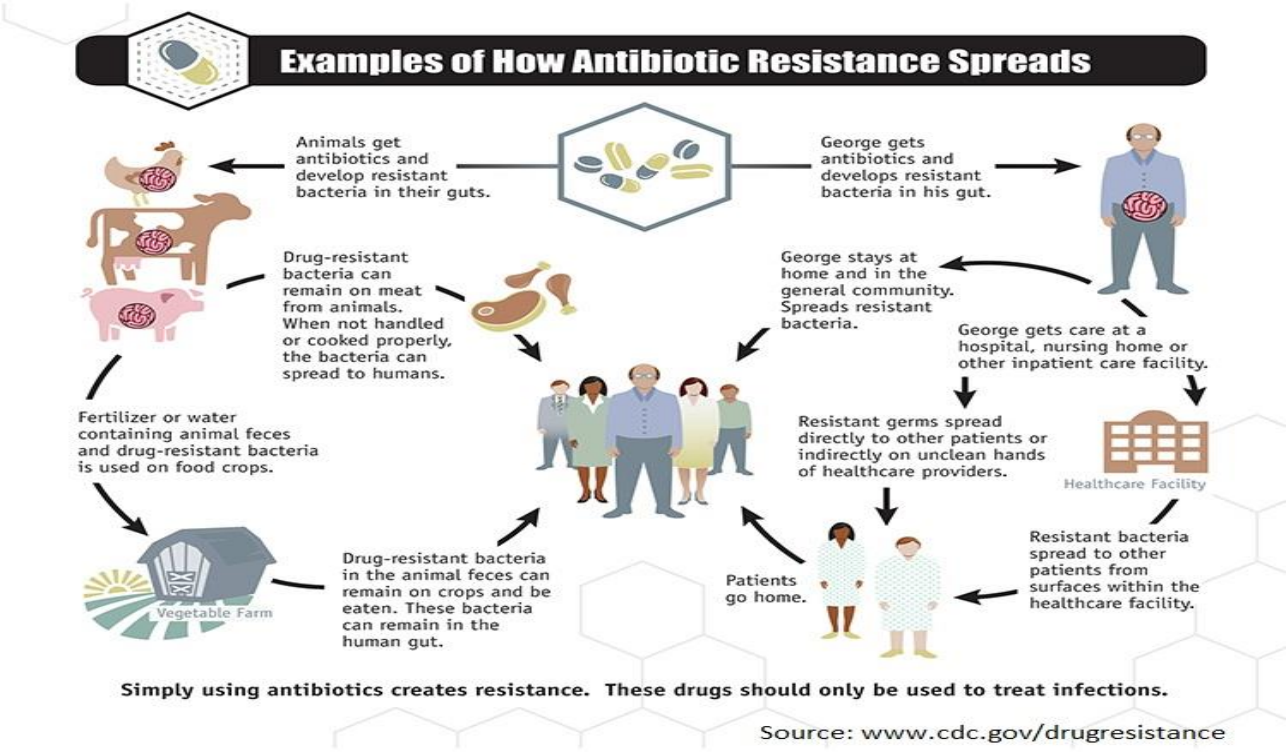

Figure 2.1: The mechanisms of antibiotic resistance spread (www.cdc.gov/drugresistance).

Globally, the rise and spread of MDR bacterial strains have become a major public health threat, as treatment for these strains become highly impossible, since not only are they resistant to the first line of therapy provided, but also, they show resistance to the other forms of therapy provided which are the extra expensive second and third-line antibiotics therapy (Prestinaci et al., 2015). Due to the failure of treatment, MDR bacterial strains are associated with higher hospital cost, prolonged hospitalization, and mortality, which negatively affects the social and economic standards.

2.1.1 ESKAPE pathogens

A group of MDR bacterial strains that are widely known to be allied with high virulence and the presence of several mechanisms of antimicrobial resistance are nicknamed under the acronym ESKAPE (Pendleton et al., 2013). The name “ESKAPE” arose to the fact that the six identified members of this group: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species can successfully “escape” the effects of antibiotics (Boucher et al., 2009). This group of pathogens was first reported by Rice in 2008 and they have since been known worldwide to be the main reason behind the severe hospital infections in all countries, thus contributing to the high mortality rates (Fair and Tor, 2014).

The ESKAPE pathogens have been linked to being the main cause of nosocomial infections, causing serious infections amongst individuals that have a weakened immune system and patients that are critically sick, leading to the high rates of morbidity and mortality worldwide, making this group of bacterial pathogens one of the greatest challenges in clinical practice, wherein their future available treatment choices and medical outcomes raises an alarm of concern as also these MDR strains are forever evolving (Penes et al., 2017; Santajit and Indrawattana, 2016).

a. *Acinetobacter baumannii*

Gram-negative *A. baumannii* is a fast-emerging opportunistic pathogen that is responsible for causing nosocomial infections (Navidinia, 2016). A patient that is already colonized with this bacterial strain upon arrival at the hospital usually introduces the strain in the hospital setting, wherein new patients at the hospital can get infected as *A. baumannii* can survive on the inanimate surface and is also able to resist desiccation (Karlowsky et al., 2003).

Acinetobacter baumannii (Figure 2.2) is well known to cause several infections including bacteremia, pneumonia, meningitis, urinary tract infection, and wound infection, in which it is assumed that *A. baumannii*'s growth favors nosocomial surroundings due to the continuous use of antibiotics by patients in the hospital. Drug-resistant *A. baumannii* strains are recognized to be MDR to numerous groups of antibiotics such as those of carbapenems, quinolones, aminoglycosides, and penicillin (www.atcc.org/superbugs).

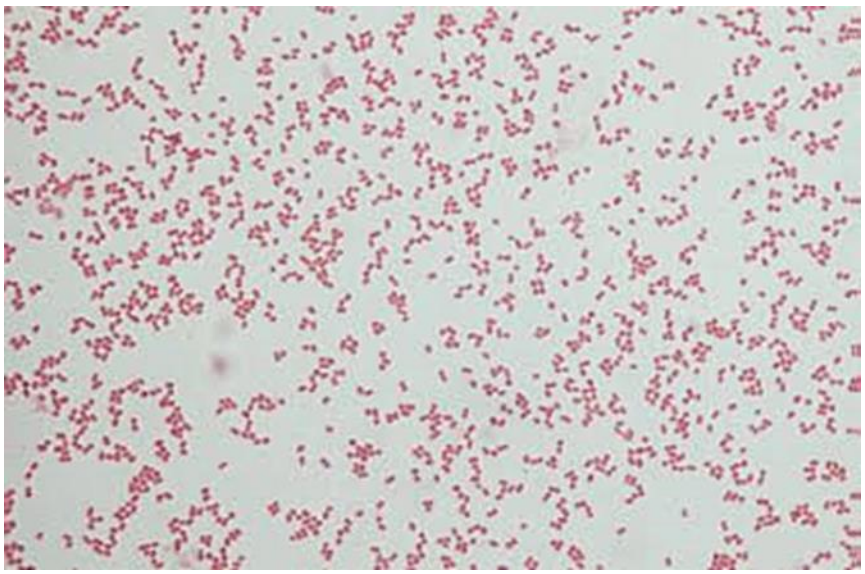

Figure 2.2: *Acinetobacter baumannii* under the microscope (www.uaz.edu.mx).

b. Enterobacter species

Belonging to the coliform group of bacteria, the Gram-negative Enterobacter spp. (Figure 2.3) are responsible for causing vital nosocomial infections (Ku et al., 2012). The species *cloacae* and *aerogenes* under the Enterobacter genus are the main etiological agents of nosocomial infections, being responsible for several infections including bacteremia, lower respiratory tract infections, skin and soft tissue infections (Karlowsky et al., 2003). *Enterobacter spp.* are known to being MDR to the following antibiotics: ampicillin, amoxicillin-clavulanate, first-cephalosporin and cefoxitin (Bouz and Cercenado, 2002)

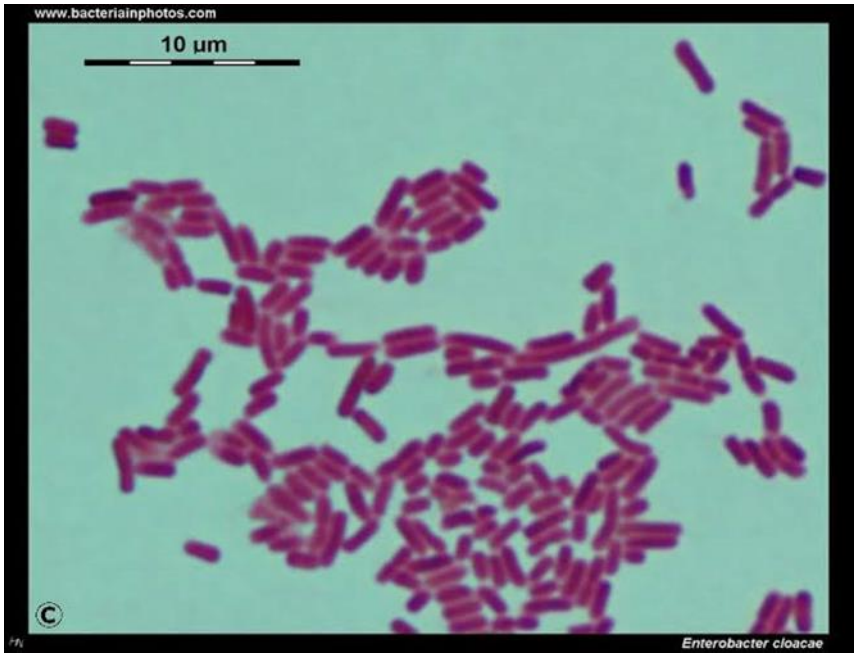

Figure 2.3: *Enterobacter* species under the microscope (https://www.mrsa-today.com/enterobacter-species/).

c. *Enterococcus faecium*

Enterococci are Gram-positive cocci that dispense broadly in the environment such as on the grounds, water, plants, and food, and are also part of the human and animal enteric tract normal flora (Byappanahalli et al., 2012). Under this genus, Gram-positive *E. faecium*, which previously was known as *Streptococcus faecium* (Figure 2.4) is a human pathogen that causes hospital-acquired bacteremia, surgical wound infection, endocarditis, and urinary tract infections (Arias and Murray, 2009).

This strain of bacteria can endure for a long period of time exclusively in hospitals on multiple inanimate objects, and they can also survive in the soil and sewage. *E. faecium* can grow between 10 to 45°C and have an incubation period of 2 days (Navidinia, 2016). In a hospital setting, *E. faecium* can be transmitted through contact with contaminated hands and medical devices. The genus Enterococci has acquired resistance to several antibiotic classes, such as those of erythromycin, glycopeptides, tetracycline, vancomycin, aminoglycosides, gentamicin and streptomycin. The clinical strains of enterococci that are commonly isolated are mostly the strains which are vancomycin-resistant (www.atcc.org/superbugs).

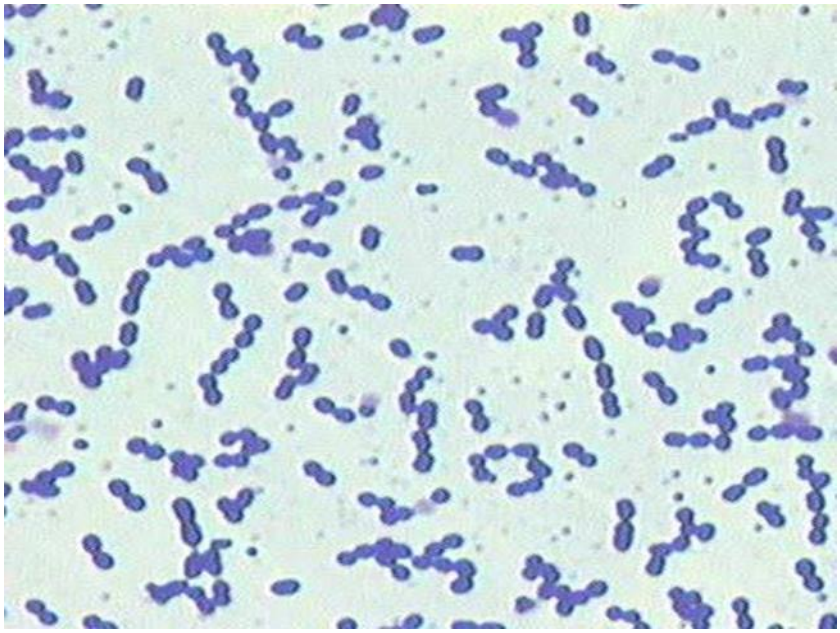

Figure 2.4: *Enterococcus faecium* under the microscope (https://www.mrsa-today.com/enterococcu/).

d. *Klebsiella pneumoniae*

The Gram-negative *K. pneumoniae* bacteria belong to the enterobacteriaceae family and are associated mostly with the lower respiratory tract and urinary tract infections (Petrosillo et al., 2013). This strain of bacterium (Figure 2.5) can be spread through person-to-person contact or by contamination of the environment. Patients in healthcare settings may be infected with *Klebsiella spp*. when they are on ventilators or when they have peripheral venous catheter wounds (Bratu et al., 2005).

K. pneumonia is gradually resistant to penicillin and ampicillin because of its beta-lactamases. In addition, the bacterium belongs to the extended-spectrum beta-lactamase or ESBL strains and is progressively MDR to a broad-spectrum group of antibiotics such as cephalosporin or ceftazidime (www.atcc.orgsuperbugs). The emergence of *K. pneumonia* isolates producing carbapenemases have become the main problem in the last 5 years. Carbapenems can destroy the carbapenems and cause resistance against a wide spectrum of antibiotics (Hirsch and Tam, 2010).

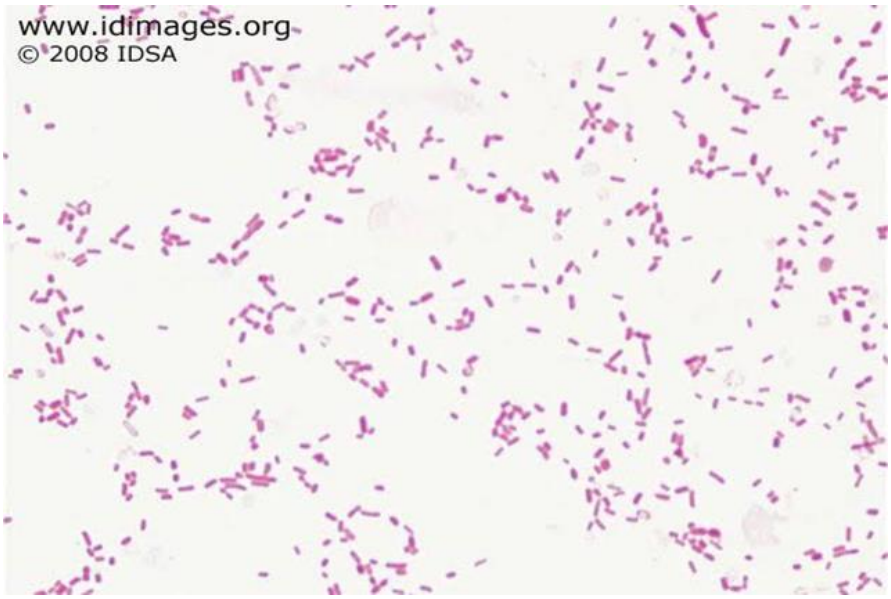

Figure 2.5: *Klebsiella pneumoniae* under the microscope (https://za.pinterest.com/pin/278589926927184967/).

e. *Pseudomonas aeruginosa*

The Gram-negative *P. aeruginosa* (Figure 2.6) is an aerobic, rod-shaped bacterium with unipolar motility. This strain of bacterium is free-living, normally found in soil and water, and occupies the surfaces of plants and animals. *P. aeruginosa* is an emerging opportunistic pathogen of clinical relevance that is known to cause nosocomial infections (Jafari et al., 2013). The following infections are known to be associated with this bacterium: urinary tract infections, respiratory system infections, dermatitis, soft tissue infections and bacteremia (Aloush et al., 2006).

Patients in hospitals that are on breathing machines or those that have wounds from surgery, and babies born prematurely are highly vulnerable and likely to be colonized by *P. aeruginosa*. Overall, individuals with a compromised immune system are highly susceptible to this bacterial strain and healthy people can also develop mild illnesses due to *P. aeruginosa*, especially if they have been exposed to contaminated water. The strains of *P. aeruginosa* show drug-resistance to as many as 15 antibiotics that belong to the following classes of antibiotics: penicillin, cephalosporins, carbapenems, quinols and aminoglycosides (www.atcc.org/superbugs).

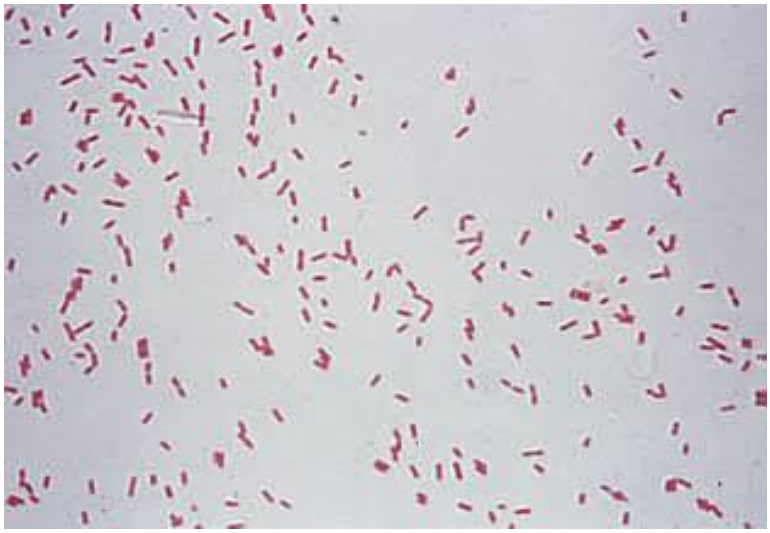

Figure 2.6: *Pseudomonas aeruginosa* under the microscope (textbookofbacteriology.net/pseudomonas.html).

f. *Staphylococcus aureus*

The Gram-positive *S. aureus* is the main cause of nosocomial infections, specifically the strains that are methicillin-resistant (Muto et al., 2003). *Staphylococcus aureus* (Figure 2.7) is commonly found to colonize the nose or the skin of healthy individuals, and when they cause infections in most cases they are asymptomatic. The variety of Staphylococcus infections can range from skin abscess to life-threatening infections such as septicemia or endocarditis (Mukhiya et al., 2012).

Strains of *S. aureus* that are methicillin resistant have been isolated from both hospital and community acquired sources, and these strains are known to contain the genes that confer resistance to methicillin (Ansari et al., 2014). The rise of Methicillin-resistant *Staphylococcus aureus* (MRSA) incidences can be due to possible inclining factors such as the following: intake of antibiotics without medical prescription, lack of awareness, receipt of antibiotics before coming to the hospital, long duration of hospitalization etc. (Mukhiya et al., 2012).

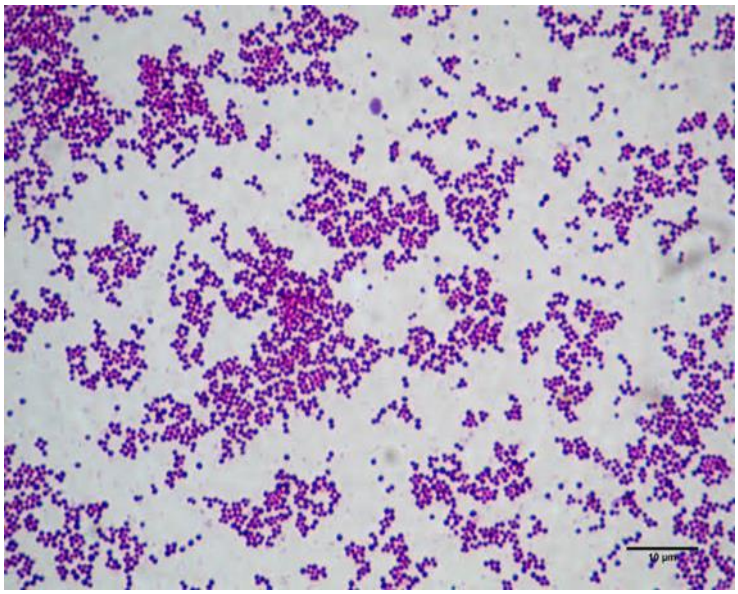

Figure 2.7: *Staphylococcus aureus* under the microscope (https://za.pinterest.com/pin/381680137149195959/).

2.2 MEDICINAL PLANTS

For thousands of years in Africa, plants and plant products have been utilized for medicinal purposes as traditional medicine is more acceptable and affordable compared to the westernized health care systems (Steenkamp et al., 2004). The World Health Organization (WHO) has indicated that 80% of people living in developing countries, especially those based in rural communities still depend entirely on local medicinal plants to cater for their primary health care needs (WHO, 2002). In South Africa, more than 4 000 different medicinal plants have been identified that are used therapeutically by populations of different cultures (Fokunang et al., 2011).

Therapeutic properties of most plants are typically known by traditional healers together with native people of the place that the plant originates from, and only a little number of those plants are scientifically studied (Dhama et al., 2014). The entities in plants responsible for their therapeutic properties against human infections caused by pathogenic microorganism are chemical compounds referred to as phytochemicals, these compounds are biologically active and are documented to benefit human health (Barbieri et al., 2016). Plants utilize these secondary metabolites (phytochemicals) they produce to fight of several diseases they can infected with and to shield themselves against ecological dangers such as drought, UV exposure etc. Different plant parts (root, stem, leaf, flower, and fruit) produce different kinds of phytochemicals, hence different parts of the plant can be used to treat different diseases (Mazid et al., 2011).

2.2.1 Bioactive phytochemicals in medicinal plants

Since historical times, it has been reported that plant species have been used more for therapeutic purposes than as food sources as they comprise of a variety of different phytochemicals with antimicrobial and other biological properties (antioxidant activity, inflection of detoxification enzymes, immune system stimulation, decrease of platelet aggregation and modulation of hormone metabolism and anticancer action) (Saxena et al., 2013; Cowan, 1999). The following are major groups of important phytochemicals that are known to have antimicrobial properties:

a. Alkaloids

Alkaloids are a group of phytochemicals that are characterized by their organic nitrogenous bases in a heterocyclic ring (Figure 2.8) and recognized for their noticeable physiological effects on humans (Barbieri et al., 2017). Morphine was the first therapeutically useful alkaloid to be isolated in the year 1805 from the opium (from the plant *Papaver somniferum*) (Cowan, 1999). The following are families of alkaloids grouped according to type of heterocyclic ring system contained in the alkaloid molecule: pyrrolidine alkaloids, pyridine alkaloids, pyrrolidine-pyridine alkaloids, pyridine-piperidine alkaloids, quinoline alkaloids and isoquinole alkaloids (Koche et al., 2016).

The significance of the naturally-occurring alkaloid group is their pharmacological properties which include but not limited to: antimalarial action, antiasthma effects, anticancer activity, antiarrhythmic effect and antihypertensive effects (Saxena et al., 2013; Karou et al., 2006; Omulokoli et al., 1997). Alkaloids are also well known for their antimicrobial effects, which include their ability to be antibacterial and anti-virulence, hence many studies have reported that alkaloids could be useful as treatment for several infectious diseases.

Sanguinarine, Berberine, Tomatidine, Piperine and Cinchona alkaloids are isolated alkaloids shown by invitro studies to have antibacterial activity against both Gram-positive and Gram-negative bacteria (Barbieri et al., 2017). Some of the antibacterial mechanisms of action of alkaloid phytochemicals include intercalation of DNA and inhibiting DNA synthesis through topoisomerase inhibition (Karou et al., 2006; Cowan, 1999)

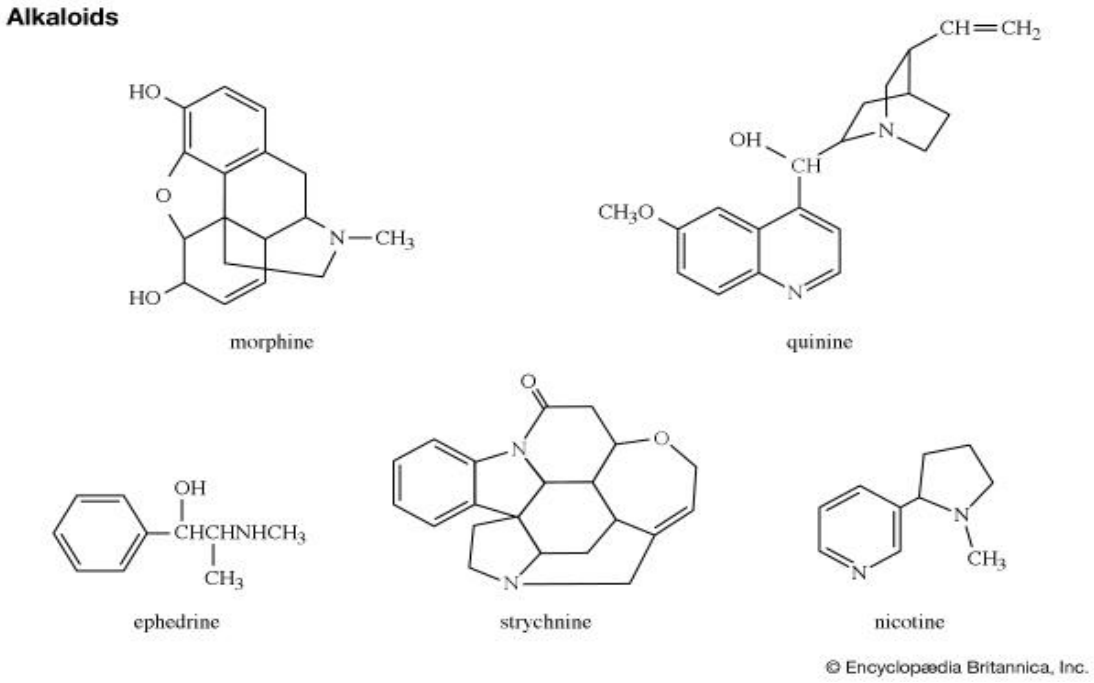

Figure 2.8: Structures of Alkaloids (www.britannica.com).

b. Terpenoids

Naturally occurring terpenoids are a large class of phytochemicals derived from the common five-carbon isoprene units and are characterized by their antimicrobial properties (Koche et al., 2016). Majority of these compounds are multi cyclic structured and their functional groups and basic carbon skeleton differentiates them (Figure 2.9) (Saxena et al., 2013). While terpenoids are characterized by their antimicrobial properties, their use as flavours and fragrances in foods and cosmetics respectively make them to have a significant influence on the yield of agronomical essential crops (Caputi and Aprea, 2011).

The number of the hydrocarbon isoprene, which is the building block of terpenoids with the molecular formula C_5H_8 is used to categorize terpenoids into the following groups:

- Hemiterpenes: Contain one isoprene unit
- Monoterpenes: Comprises of two isoprene units
- Sesquiterpenes: Has three isoprene units
- Diterpenes: Contains four isoprene units

- Triterpenes: Comprise of Six isoprene units
- Tetraterpenes: Composed of eight isoprene units

Terpenoids have documented therapeutic properties such as anticancer, antimalarial, anti-ulcer, anti-inflammatory antimicrobial and diuretic activity to mention a few. For antibacterial activity, thymol and carvacrol are two most studied terpenoids that inhibit the growth of bacteria (Barbieri et al., 2017). Most terpenoids have antibacterial activity against both Gram-positive and Gram-negative bacteria and their mode of action involves disrupting the plasma membrane of the bacteria, which leads to modifications of membrane permeability and leakage of intracellular materials (Barbieri et al., 2017; Saxena et al., 2013; Koche et al., 2016; Cowan, 1999).

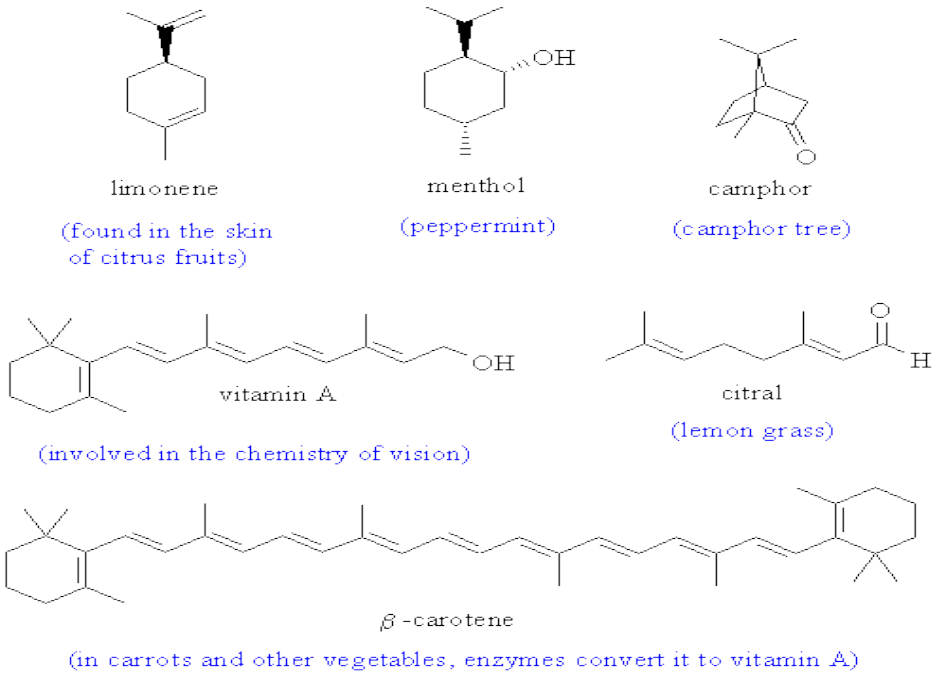

Figure 2.9: Structures of terpenes (www.chem.ucalgary.ca/courses/351/Carey5th/Ch26/ch26-4-1.html).

c. Phenolics and polyphenols

Phenolic and polyphenolics are compounds that signifies the largest group of phytochemicals that are extensively spread in plant species (Cowan, 1999). The naturally occurring phenols are characterized by having hydroxylated aromatic rings and can vary from simple and single aromatic-ringed compounds to highly polymerized compounds (Figure 2.10) (Lin et al., 2016; Cowan, 1999). Phenolics can be categorized into two groups: flavonoids and non-flavonoids (tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others) (Barbieri et al., 2017).

Naturally occurring phenolic compounds consist of several therapeutic properties beneficial to humans such as; antioxidant activity, anticarcinogenic action, anti-inflammatory effects and antimicrobial activity (Lin et al., 2016; Koche et al., 2016). Phenolics and polyphenols exhibit good antibacterial activity against Gram-positive bacteria and less successful activity against Gram-negative bacteria, and their level of inhibition of bacteria is speculated to be linked to the location and number of hydroxyl groups on the phenol group, wherein it is stated that the increased hydroxylation results in increased inhibition (Cowan, 1999). The antibacterial mode of actions of phenols include penetration of the cytoplasmic membrane, disrupting the external membrane of the bacterial wall and by acting on bacterial spores by destabilizing structures of the spores to mention a few (Barbieri et al., 2017; Sabbineni, 2016).

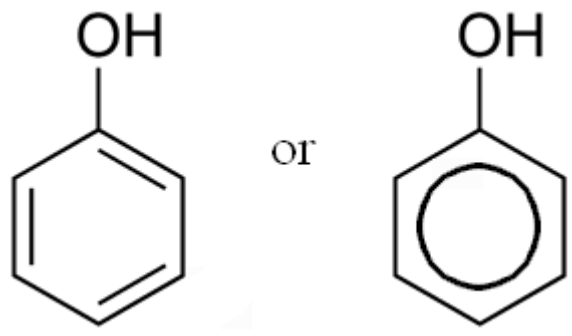

Figure 2.10: Structures of basic phenolics (www.britannica.com/science/phenol).

d. Tannins

Tannins are a diverse group of naturally occurring phenolic polymers that are found practically in every portion of the plant (bark, wood, leaves, fruits and roots) (Khanbabaee and van Ree, 2001; Cowan, 1999). This group of phytochemicals consist of compounds with high molecular weights and compounds that have high affinities for proteins, which results mostly in formation of complexes (Frutos et al., 2004).

The division of tannins can be done in two ways, division based on their chemical nature or division based on their structural characteristics. Based on their chemical nature, tannins can be divided into two groups; hydrolysable tannins (Figure 2.11) and condensed tannins, then based on their structural characteristics, tannins can be categorized into four major groups; Gallotannins, ellagitannins, complex tannins, and condensed tannins (Saxena et al., 2013; Okuda, and Ito, 2011).

The intake of beverages that contain tannins such as green teas and red wines by humans has been suggested that it can cure or prevent a variety of sicknesses, in turn, this has drove a wide interest on tannins and attracted scientific interest (Cowan, 1999). In traditional medicine, plants that contain tannins are administered as external anti-inflammatory, antioxidant and antimicrobial drugs (Barbieri et al., 2017; Saxena et al., 2013). Tannins have successful antibacterial activity against both Gram-positive and Gram-negative bacteria, and their antibacterial mechanism of action involves their ability to inactivate the following: microbial adhesions, microbial enzyme and microbial cell envelope transport proteins (Barbieri et al., 2017; Cowan, 1999).

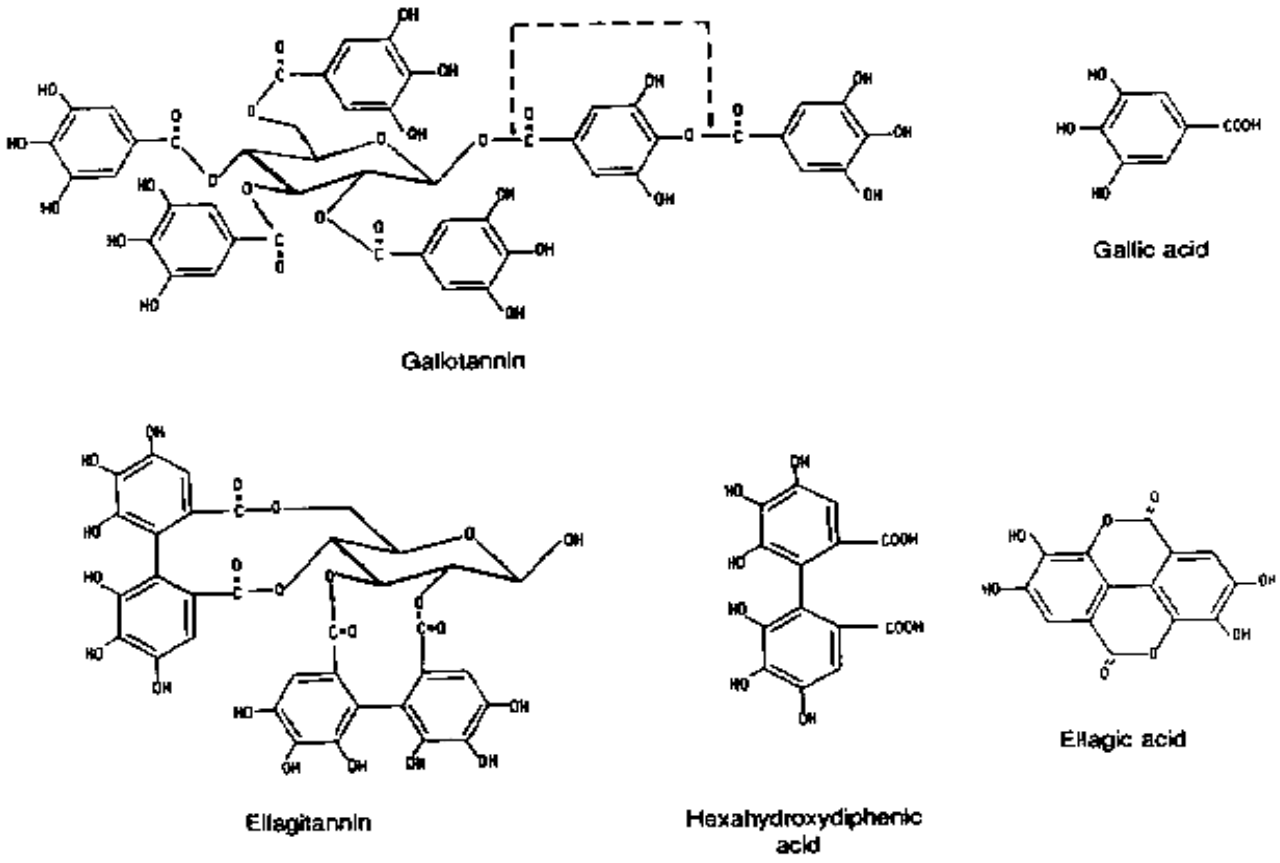

Figure 2.11: Structures of hydrolysable tannins (en.wikipedia.org/wiki/Tannin).

e. Flavonoids

Flavonoids are the largest and diverse group of polyphenolic compounds that are broadly distributed in the plant kingdom. This group of phenolic compounds is known to have several pharmacological properties and it has and still plays a key role in successful therapeutic treatments (Koche et al., 2016; Panche et al., 2016). In some plants, flavonoids occur as aglycones, glucoside and methylated by-products, and they are involved in a variety of biological processes such as: colour and aroma of flowers, growth and development and resistance to pathogenic microorganisms (Panche et al., 2016).

The basic structure of all flavonoids is a typical 15-carbon skeleton (C6-C3-C6) which consists of two aromatic rings connected by a unit of three carbon atoms. Flavonoids can be grouped as flavanols, flavanones, flavonols, isoflavones, flavones and anthocyanins (Figure 2.12), based on the differences in their chemical structures. The

phytochemicals belonging to these groups of flavonoids portray different biological and chemical properties (Brodowska, 2017).

The ability of plants to synthesize flavonoids in response to microbial infections makes it no wonder that when tested in vitro, they have effective antimicrobial activity against many different microorganisms. The flavonoids are the most studied and the most interested in phytochemicals because of their biological and pharmacological activities, including antioxidant, antitumor, anticancer, antiviral, antibacterial, anti-inflammatory, antiallergic, antithrombotic, cardioprotective, hepatoprotective, neuroprotective, antimalarial, antileishmanial, antitrypanosomal and anti-amebial properties (Brodowska, 2017; Koche et al., 2016; Saxena et al., 2013; Cowan, 1999). It has been continuously reported that flavonoids display good antibacterial activity against both Gram-positive and Gram-negative bacterial strains, and their antibacterial mechanism of action involves their ability to inhibit DNA gyrase, cell membrane function and bacterial energy metabolism (Barbieri et al., 2017; Brodowska, 2017; Cushnie and Lamb, 2005).

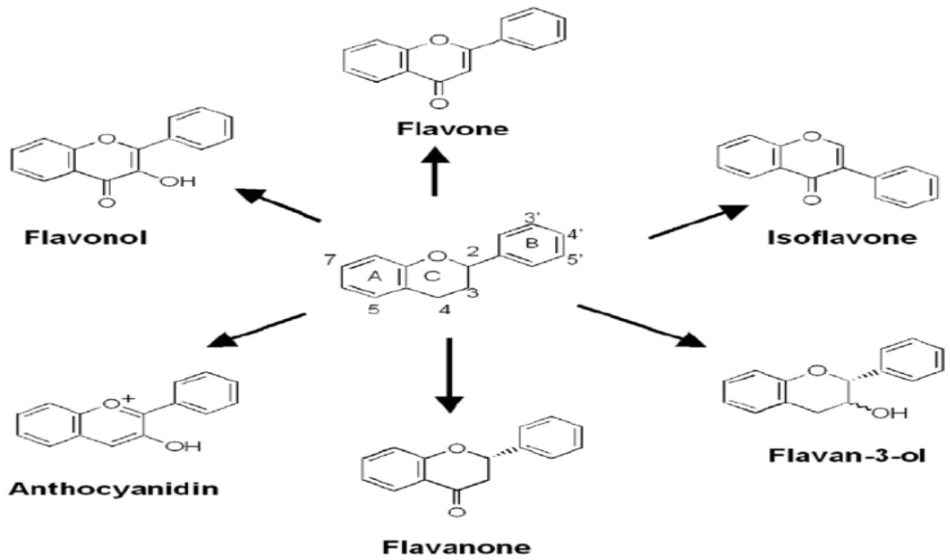

Figure 2.12: structures of different groups of Flavonoids (www.tuscany-diet.net/2014/01/22/flavonoids-definition-structure-classification/).

f. Saponins

Saponins are a group of naturally occurring amphipathic glycosides with foaming characteristics and are abundantly distributed in the plant kingdom (Figure 2.13) (Sparg et al., 2004). Their role in decreasing surface tension because of the presence of both polar and non-polar groups in their structures distinguishes them from other glycosides. Saponins are added to shampoos, liquid detergents, makeup products, beverages and toothpaste due to their chemical, biological and mostly foaming properties (Güçlü-Üstündağ and Mazza, 2007)

Several publications have reported that saponins have a variety of beneficial biological activities such as antioxidant, antigenotoxic, anti-inflammatory, antimicrobial, antimutagenic, antiobesity, Immunostimulatory effects, neuroprotective, hepaprotective, antiulcer, effect on cognitive behaviour, effect on ethanol induced amnesia and many more (Saxena et al., 2013; Arabski et al., 2012; Güçlü-Üstündağ and Mazza, 2007). Although most saponins are known to show good antibacterial activity on both Gram-positive and Gram-negative bacteria, some saponins are unable to penetrate cell membranes of bacterial strains, which makes it impossible for them to be effective against Gram-negative bacteria (Desai et al., 2009). The antibacterial mechanisms of action of saponins include their ability to alter the permeability of cell walls and alteration of cell membrane function by directly disrupting and destabilising bacterial cells (Omojate Godstime et al., 2014)

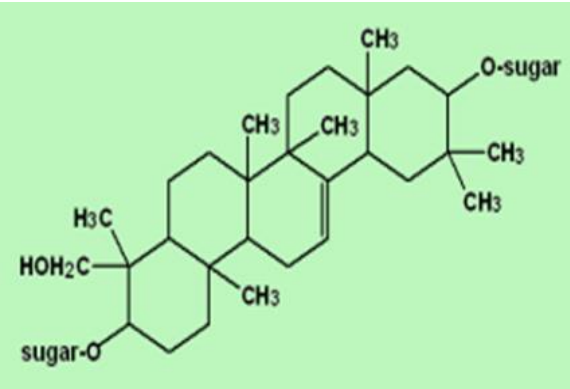

Figure 2.13: Basic structure of Saponins.
(www.phytochemicals.info/phytochemicals/saponins.php).

2.3 MEDICINAL PLANT SELECTED FOR THIS STUDY.

S. africana Sond was chosen as the medicinal plant species to be investigated in this study because it was found to be overly harvested at Matsa village compared to other plants species found in this village. Locally, the residents of Matsa village are a Venda speaking population, and therefore *S. africana* is known as Muonze. Other common names of this plant in South Africa are as follows:

Table 2.1: Common names for *Spirostachys africana* in South Africa.

South African language	**Name of *S. Africana***
Afrikaans	Tambotie
English	Tamboti
Northern Sotho	Modiba
Tsonga	Ndzopfori
Tswana	Morukuru
Swati	UmThombotsi
Zulu	UmThombothi

2.3.1 Description of the plant

S. africana is a deciduous tree that is medium sized and can grow up to a height of between 10 to 20 m (Figure 2.15). Generally known as the tamboti tree, *S. africana* is characterized by the green fruits it bears called “the jumping beans” (Figure 2.16), which move about unpredictably and strongly from it when the larvae inside them move due to high temperatures (plantinfo.co.za/plant/Spirostachys-africana/).

The tree’s distinctive bark is dark brown to black, thick, rough and neatly fractured into rectangular blocks that are arranged in longitudinal rows. While the under bark of the tamboti heartwood (Figure 2.17) is said to be dangerous as it projects a white, poisonous latex when freshly cut. *S. africana* remains one of the protected species in South Africa because of its heartwood uses in the furniture industry and its many uses in making artefacts (Munkombwe et al., 2006).

Figure: 2.14: *Spirostachys africana* tree.

Figure 2.15: Fruits of *Spirostachys africana* (https://pza.sanbi.org/spirostachys-africana).

Figure 2.16: Bark of *Spirostachys Africana*.

2.3.2 Origin and geographic distribution

S. africana originates from Southern Africa. Its generic name “Spirostachys” signifies the spiral arrangement of its flowers on the thorn whereas “africana” represents Africa, where this plant species originates. *S. africana* occurs mostly in low altitude bushvelds, woodlands, watercourses and savannas of the Southern African countries except Lesotho. The trees of this plant species are usually found in groups along rivers and streambanks.

2.3.3 Classification of the plant

Scientifically, *S. africana* is classified as below:

Kingdom: Plantae
Order: Malpighiales
Family: Euphorbiaceae
Subfamily: Euphorbioideae
Tribe: Hippomaneae
Subtribe: Hippomaninae
Genus: Spirostachys
Species: *S. africana*
Binomial name: *Spirostachys africana* Sond.

2.3.4 Medicinal uses and research done on the selected plant

For many years, *S. africana* has been used for different medicinal purposes by indigenous people of the African continent, and it has been known that traditional healers collect and prepare different parts of this tree to treat various infectious diseases (Akhalwaya et al., 2018; Mathabe et al., 2008). Initially, *S. africana* was recognized only for its anti-feedant properties, however, over the years it has been documented that this plant has been proven to have antimicrobial properties, hence further studies are still being done on the plant to date, and more precisely on its biological properties (Elgorashi et al., 2002; Munkombwe et al., 1998).

Traditional healers / herbalists prepare and administer different parts of *S. africana* diversely to treat a variety of ailments. The bark is used medicinally to treat fever, colds, headache, papules, stomach ulcers, diarrhoea, acute gastritis, renal ailments and dysentery, the leaves are most commonly used to treat oral infections and skin infections, and the roots are known to treat venereal disease (Akhalwaya et al., 2018; Seukep et al., 2014; Munkombwe et al., 1998; Duri et al., 1992). Although not well documented, the sap of the tree is also used medicinally, wherein traditional healers/ herbalist uses it to treat toothache and wound myiasis (Seukep et al., 2014; Mukandiwa et al., 2012).

Several researchers have studied *S. africana* found around South Africa and other African countries for their antimicrobial activities and for the isolation and identification of phytochemicals the tree contains. *S. africana* has been reported to have antibacterial activities (Akhalwaya et al., 2018; Mulaudzi et al., 2012; Mathabe et al., 2008; McGaw et al., 2000), antifungal activities (Mulaudzi et al., 2012; Hamza et al., 2006) and potential antioxidant activities (Mathabe et al.,2008).

A number of compounds have been isolated from the latex and the stem bark of *S. africana,* such as stachenol, three diterpenes (diosphenol 2, Ent-3β-hydroxy-beyer-15-ene-2-one and Ent-3β-hydroxy-19-nor-beyer15-ene-2,12-dione) and two triterpenoids (D-Friedoolean-14-en-oic acid and Lupeol) (Mathabe at el., 2008; Munkombwe et al., 1997; Duri et al., 1992; Baarschers et al., 1962).

2.4 SUMMARY OF LITERATURE REVIEW

Antibiotics have been the preferred source of antimicrobial drugs for a period of time in treating infections since the beginning of their discovery, saving more than a million lives, however, the following reasons have led to bacterial strains being multi-drug resistant to antibiotics: the excessive and irresponsible use of antibiotics by patients who are administered the antibiotics, their use in prevention of diseases in livestock, the inability to control infections in health care centres, and also their use in food production(Khan et al., 2013).

The six common bacterial strains that are MDR to antibiotics are known as the ESKAPE pathogens. This group of bacterial pathogens consist of both Gram-negative and Gram-positive strains and are known to be accountable for causing serious infections, leading to the high rates of morbidity and mortality worldwide (Santajit and Indrawattana, 2016). Due to these high incidences of MDR bacterial strains, research for other antimicrobial drug sources other than antibiotics such as plants has become vital in the process of eradicating this public health problem.

Spirostachys africana is a medicinal plant used variously by traditional healers / herbalist to treat different diseases. Studies have also reported that this tree has antimicrobial properties, which supports its uses traditionally (Akhalwaya et al., 2018; Mathabe et al., 2008). The biological properties of *S. africana* makes it a potential source for antibacterial compounds that may useful in the innovation of new potential drugs for treatment of infectious diseases caused by MDR bacterial strains, however, more studies are yet to be done on the plant.

The current study aimed to screen the antibacterial activity of the crude extract and fractions of *S. africana* found locally (Matsa village) against multi-drug resistant bacteria and to evaluate its other biological properties. This study was done because *S. africana* was overly harvested at Matsa village and only limited data is available about the antibacterial properties of this plant.

Chapter 3

MATERIALS AND METHODS

3.1 PLANT COLLECTION AND STORAGE

Stem barks of *S. africana* were collected in the winter months of 2017 from the Matsa village (Figure 3.1) in Nzhelele, Vhembe district (Limpopo province; South Africa). The plant's identity was confirmed by the Department of Botany, University of Venda in Thohoyandou (Limpopo, South Africa). The harvested stem barks were air dried for 2 weeks at room temperature (20-22°C) to minimize chemical changes. After the plant materials were dried, they were grounded using an electric grinder into a fine powder (700 g) and stored in the dark at room temperature (20-22°C) in closed containers until usage.

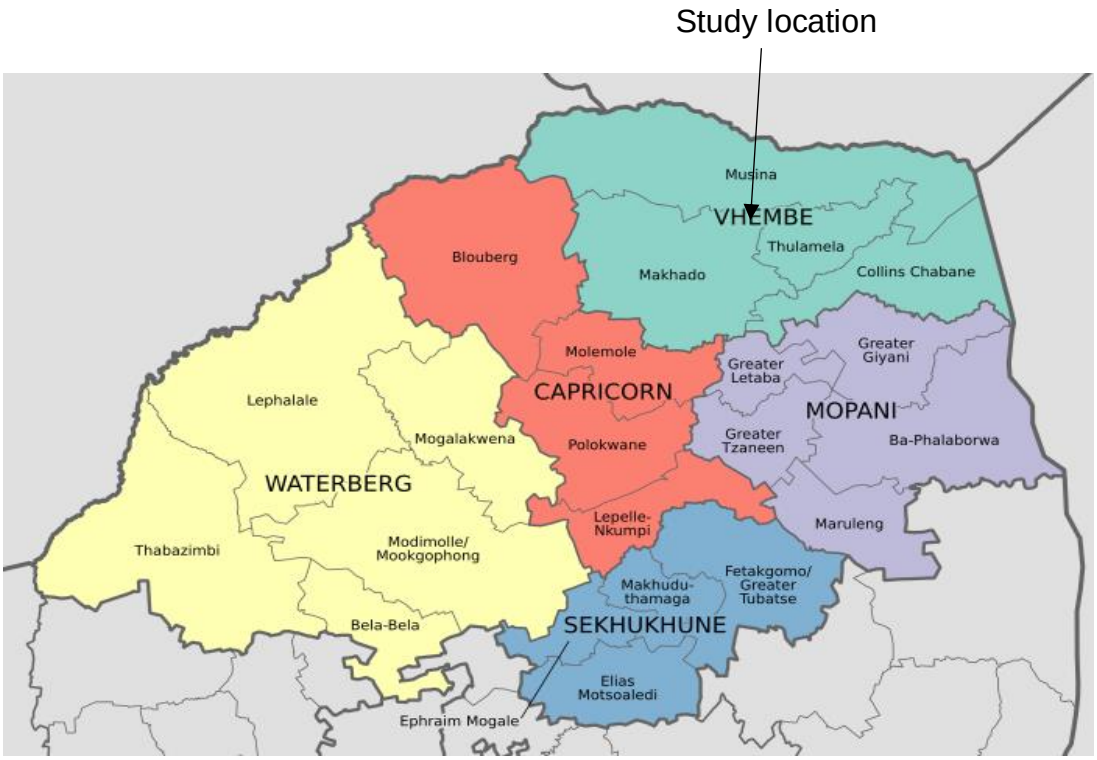

Figure 3.1: Map showing the study location (https://en.wikipedia.org/wiki/List_of_municipalities_in_Limpopo).

3.2 CHEMICALS AND REAGENTS

Most chemicals were obtained from Sigma-Aldrich (Saint Louis, MO, USA) such as quercetin, Folin-Ciocalteu, 2-2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, Aluminium chloride, DMSO and Sodium carbonate. Potassium ferricyanide was purchased from Rochelle chemicals (Gauteng, RSA). Trichloroacetic acid, disodium hydrogen phosphate anhydrous, sodium dihydrogen phosphate and ethanol were obtained from Merck chemical (Kenilworth, NJ; USA). Ferric chloride hexahydrate and ascorbic acid were bought from Associated Chemical Enterprise (Gauteng, RSA).

3.3 EXTRACTION AND FRACTIONATION

To extract the plant's phytochemicals, the air-dried powdered stem bark sample of *S. africana* was macerated three times for 48 hours at room temperature using 95% methanol as the solvent of choice (Figure 3.2). Afterward, the resulting extract was filtered using a Buchner funnel with Whatman filter paper No. 4 (Sigma Aldrich; St Louis, MO; USA). After filtration, the solvent used for maceration was removed using a rotary evaporator (Büchi Labortechnik AG; Flawil, Switzerland) under reduced pressure at low temperature. All crucial precautions were followed to avoid cross-contamination.

The Methanolic Crude Extract (MCE) of *S. africana* was then subjected to fractionation via Gel (silica) column chromatography (Figure 3.3) using ethyl acetate /methanol mixtures of increasing polarity (from 0 to 100%). Thin layer chromatographic profiles were used to combine similar fractions. The following are calculations used to calculate the percentage yield of the methanol crude extract and fractions of *S. africana*:

1. **Percentage yield of methanol crude extract = wgeo/wgpm × 100**
2. **Percentage yield of the fraction with reference to methanol extract = wgfo/wgme×100**

Where wgeo represented weight in grams of extract obtained, wgpm represented weight in grams of plant material used, wgfo represented weight in grams of fraction obtained and wgme represented weight in grams of methanol extract used**.**

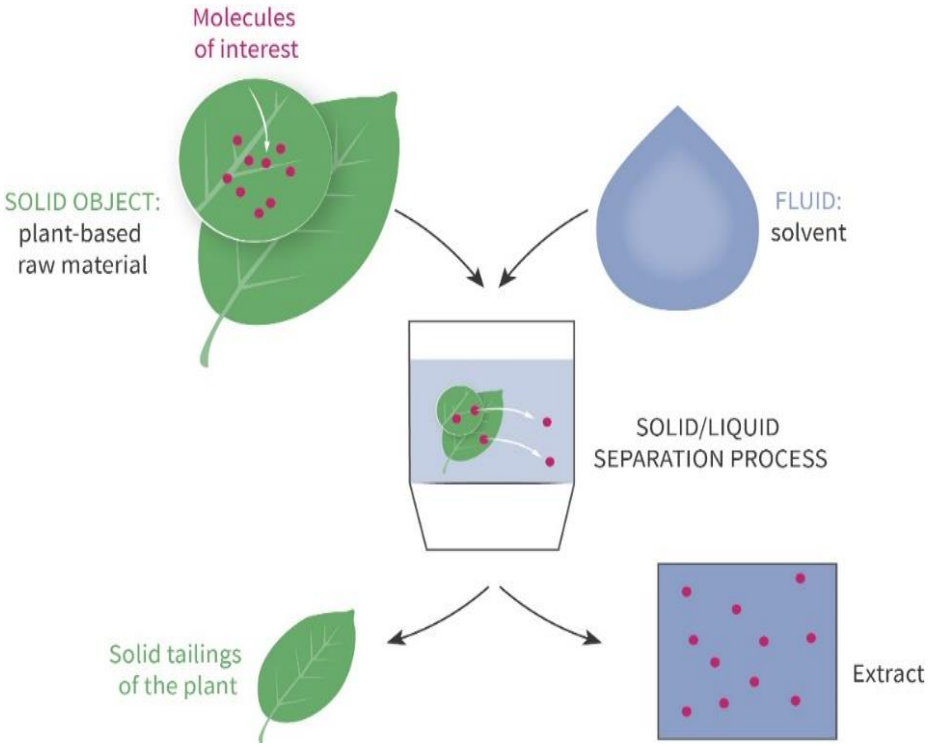

Figure 3.2: Extraction process (https://www.berkem.com).

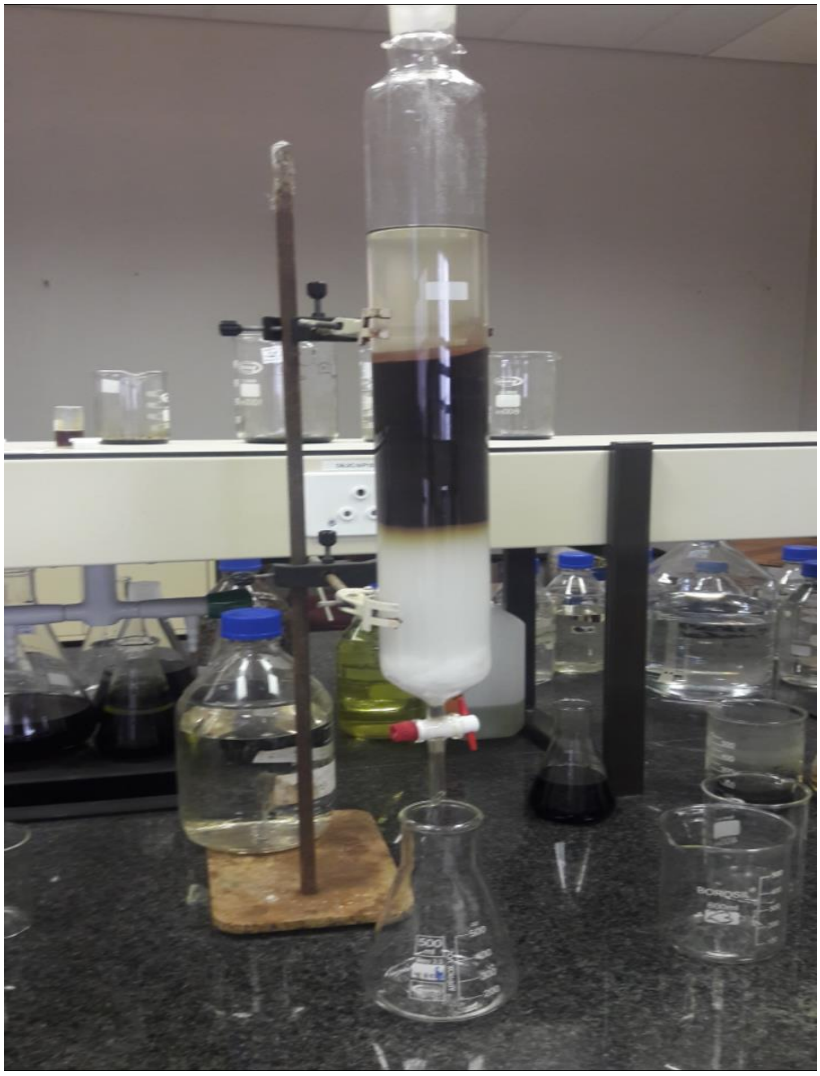

Figure 3.3: Column chromatography set up.

3.4 PHYTOCHEMICAL SCREENING OF THE CRUDE EXTRACT AND FRACTIONS

Phytochemical analysis of the crude extract and fractions were carried out using both the qualitative and quantitative methods. Qualitative phytochemical screening was done using biochemical tests to detect the presence of different secondary metabolites, and Thin Layer Chromatography (TLC) to determine the number and the variety of the secondary metabolites in the CE and fractions. For quantitative analysis, the Total Flavonoid content (TFC) and Total Phenolic content (TPC) in the samples were determined.

3.4.1 Qualitative analysis

a. Biochemical tests

The MCE and fractions of *S. africana* were examined for the presence of the following phytochemicals; Alkaloids, phenols and tannins, saponins, steroids, terpenes, flavonoids, and cardiac glycosides.

- **Alkaloids**

Wagner's method (Jha et al., 2012) was used to test for the presence of alkaloids. Firstly, Wagner's reagent was prepared by mixing 2 g of iodine, 6 g of potassium iodide and 100 ml of distilled water. Few drops of this reagent were then added to each test tubes containing 1 ml of the plant's crude extract or fractions. The presence of alkaloids was indicated by precipitation of the final reaction (Figure 3.4).

Figure 3.4: Test tube showing the presence of alkaloids.

- **Cardiac glycosides**

The Keller – Kilani test (Jaradat et al., 2015) was used to detect cardiac glycosides. Briefly, 0.5 ml of glacial acetic acid, followed by addition of few drops of 2 % Ferric chloride, then 1 ml of sulphuric acid were mixed with 1ml of the plant's crude extract or fractions in test tubes. A brown ring at the interphase or a brown colouration (Figure 3.5) indicated the presence of cardiac glycosides.

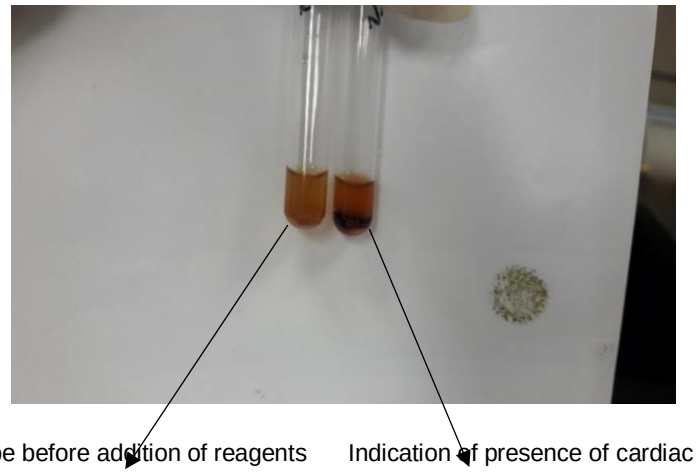

Figure 3.5: Test tube showing the presence of cardiac glycosides.

- **Flavonoids**

To detect flavonoids, few drops of a 4 mg/ml sodium hydroxide solution were added to test tubes containing 1 ml of the crude extract or fractions, then followed by the addition of few drops of 32% hydrochloric acid solution. The presence of flavonoids was indicated by a yellowish colouration or a change of the mixture's colour to colourless (Figure 3.6) (Surya and Hari, 2017).

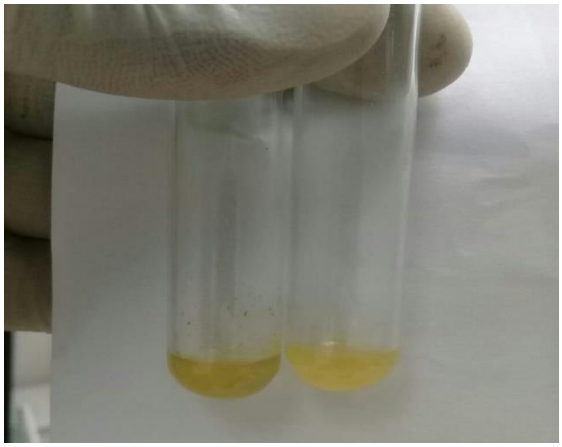

Figure 3.6: Test tube showing the presence of flavonoids.

- **Phenols and Tannins**

To detect phenols and tannins, as described by Jaradat et al (2015), few drops of 2% ferric chloride were added to test tubes containing 1 ml of the plant's crude extract or fractions. A change to black, green or dark blue (Figure 3.7) showed the presence of these phytochemicals.

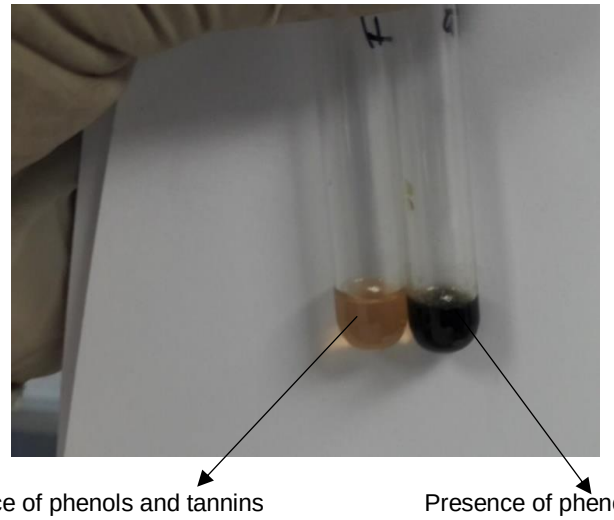

Figure 3.7: Test tube showing the presence of phenols and tannins.

- **Saponins**

One millilitre of the plant's test samples was added to 5 ml of distilled water in test tubes to test for the presence of saponins, as described by Jaradat et al (2015) with slight modifications. The test tubes were then shaken vigorously for 5 minutes and the formation of persistent foam (Figure 3.8) indicated the presence of saponins.

Figure 3.8: Test tube showing the presence of saponins.

- **Steroids**

For detection of steroids, few drops of concentrated acetic acid and few drops of concentrated sulphuric acid were mixed with 1 ml of the plant's MCE or fractions in test tubes. Colour change of the mixture to violet or green (Figure 3.9) indicated the presence of steroids (Ismail et al., 2016).

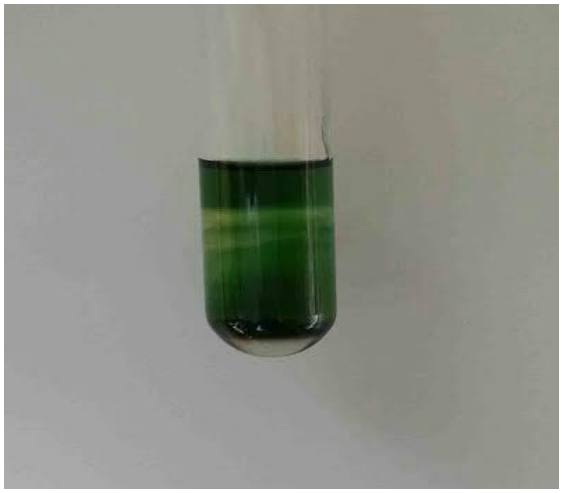

Figure 3.9: Test tube showing the presence of steroids.

- **Terpenoids**

To identify for the presence of terpenoids, a method described by Ismail et al (2016) with minor changes was used. 1 ml of the plant's crude MCE or fractions were dissolved in 0.5 ml of chloroform and 1 ml of concentrated sulphuric acid. Brownish, reddish or dark colouration colour change indicated the presence of terpenoids.

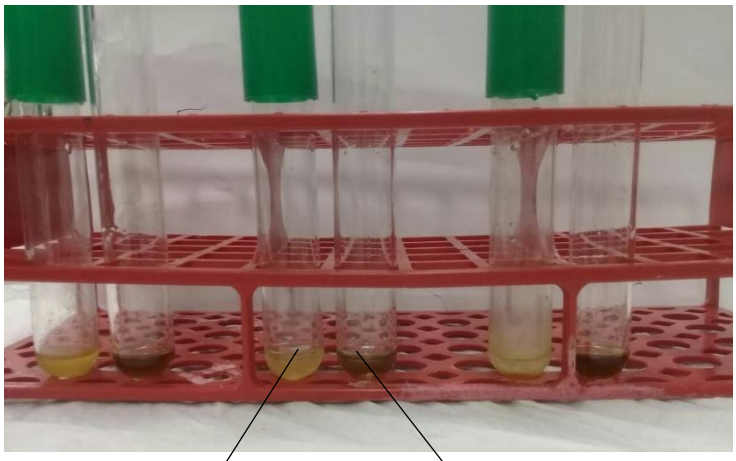

Figure 3.10: Test tube showing the presence of terpenoids.

b. Thin layer chromatography (TLC)

Thin Layer Chromatography TLC was used qualitatively to separate the components of *S. africana*'s methanolic crude extract and its various fractions using silica gel plates (Silica gel 60F-254 TLC aluminium sheet 20x20 cm) as described by Biradar and Rachetti (2013) with slight modifications. Methanolic Crude Extract (MEC) and all its fractions were dissolved in methanol at a concentration of 1 mg/ml, then about 10 µl of each sample was applied manually with the help of the capillary tube on the plate.

For the screening process (separation of components), three eluents solvent systems of different polarities were used, namely BEA (benzene/ethanol/ammonium hydroxide (90:10:1), CEF (chloroform/ethyl acetate/formic acid (5:4:1) and EMW (ethyl acetate/methanol/water (40:5.4:4).

During the screening process, the samples on the plate were kept in TLC glass chamber (solvent saturated), then the mobile phase was allowed to migrate through adsorbent phase up to 3/4th of the plate, where then they were removed and left to dry. All plates were visualized directly after drying by spraying the plates with the reagent vanillin-sulphuric acid. Components of the samples were visible as bands of different colors.

3.4.2 Quantitative phytochemical screening

For quantitative phytochemical screening of the plant's crude extract and fractions, total phenolic content (TPC) and total flavonoids contents (TFC) were determined.

a. Total flavonoid content

To determine the TFC of the MCE and fractions of *S. africana*, the aluminum chloride colorimetric method was used, where quercetin was used as a standard as described by Anokwuru et al (2015) with slight modification. Approximately 80 µl of distilled water was mixed with 100 µl of CE and fractions (1 mg/ml) in each well of 96 well plates in triplicates. A volume of 100 µl of Aluminium chloride ($AlCl_3$) solution was placed into each well and the plates incubated for 30 minutes at room temperature (20-22 °C). The absorbance was measured using VersaMax^{TM} tunable microplate reader at 420

nm. The total flavonoids were then assessed by comparing with quercetin as a standard and expressed as quercetin equivalents per gram (mg QE/g).

b. Total phenolic content

To determine the TPC of the MCE and fractions of *S. africana*, Folin-Ciocalteu colorimetric method was used as described by Anokwuru et al (2015) with slight modification. About 80 µl of distilled water was placed into each well of a 96 micro-well plate and 20 µl of extracts, as well as 2 fold serial dilutions of Gallic acid which was used as a control, were all placed into wells in triplicates. Then, the 60 µl of 7% Sodium Carbonate (3.5 g dissolved in 50 ml distilled water) and 60 µl of the Folin-Ciocalten were added to each well. The absorbance was measured using a VersaMax^{TM} tunable microplate reader. The total phenols were then evaluated by comparing with gallic acid as a standard and then expressed as Gallic acid equivalents per gram (mg GAE/g).

3.5 ANTIOXIDANT ANALYSIS

For this study, the antioxidant activities of the MCE and its fractions were measured by DPPH (1, 1-diphenyl-2-picrylhydrazyl radical) scavenging activity and reducing power assays.

3.5.1 DPPH free radical scavenging assay

For the determination of the free radical scavenging activity of the MCE and its fractions, DPPH assay was run according to Li et al (2009) with few modifications. Concisely, in the first row of 96 micro-well plates, 100 µl distilled water and 100 µl of each sample and the controls were mixed. Then, the 2-fold serial dilutions of samples were done and gave the following concentrations in mg/ml: 1, 0.5, 0.25, 0.125, 0.0625, 0.0314, 0.0156 and 0.0078. A volume of 200 µl of DPPH/ethanol (125 mM) solutions was added into each well and the plates were incubated for 30 minutes in the dark. After incubation, the absorbance of each well was read by the microplate reader (Versa max) at 517 nm. The percentage of inhibition (I%) of DPPH was calculated using the formula:

Percentage of inhibition = $A_0 - A_{sample} / A_0 \times 100$

Where A_0 represented the absorbance of the negative control and A_{sample} represented the absorbance of the test samples.

To analyse the antioxidant capacity of the test samples using I% calculations, the inhibitory concentration (IC_{50}) of each sample was determined graphically using the regression line (Y = ax + b), from the plot of I% (y-axis) against the concentration of the sample (x-axis). IC_{50} is determined as the concentration of sample that causes 50% reduction in the DPPH colour.

The results are expressed as mean ± standard deviation. Ascorbic acid was used as a standard, whereas water mixed with DPPH was considered as a negative control. This was done in three replications.

3.5.2 Reducing power assay

To determine the reducing power of the test samples, the reducing power assay was done according to Li et al (2009) with few modifications. Briefly after adding 50 µl of the sodium phosphate buffer (0.2M, 6.6 pH) in the 96 micro-well plates, 50 µl from 1 mg/ml of the test samples and the positive control (ascorbic acid) in triplicates were added in the first row and followed by 2-fold serial dilutions. Afterward, 50 µl of 1% potassium hexacyanoferrate aqueous solution was added to each well. The 96 micro-well plates were then placed in an incubator for 20 minutes at 50˚C.

After incubation, 50 µl of 10% trichloroacetic acid was added to each well. 80 µl of each mixture was transferred into a new 96 micro-well plate and then followed by the addition of 80 µl of distilled water and 16 µl of ferric chloride (0.1%). The reading was done by VersaMax™ tunable microplate reader at 690 nm. Colour change from yellow to different shades of green was an indication of the presence of reducers in the test samples.

To analyse the antioxidant capacity of the test samples, the effective concentration (EC_{50}) of each sample was determined graphically using the regression line (Y = ax +

b), from the plot of absorbance of the samples (y-axis), against the concentration of the sample (x-axis). EC_{50} is determined as the effective concentration of the antioxidant necessary to decrease the radical concentration by 50%.

3.6 ANTIBACTERIAL ASSAY

3.6.1 Culture media

Nutrient agar media and Muller-Hinton agar media were purchased from Davis Diagnosis (Johannesburg, South Africa). Gentamycin (10 mg/ml) antibiotic was bought from Sigma Aldrich (Saint Louis, MO, USA).

3.6.2 Bacterial strains

The following multidrug-resistant pathogenic bacteria were chosen for this study (*Enterococcus faecium* ATCC 700221, *Staphylococcus aureus* ATCC BAA 2312, *Klebsiella pneumoniae* ATCC BAA 1705, *Acinetobacter baumanii* ATCC BAA1605, *Pseudomonas aeruginosa* ATCC BAA 1744 and *Enterobacter cloacae* ATCC BAA 2341) were purchased from Anatech culture collection (Johannesburg, South Africa). Bacteria were cultured in Nutrient agar and used for assays.

3.6.3 Agar well diffusion method

The agar well diffusion assay (Balouiri et al., 2016) was used to evaluate the antibacterial activity of the MCE and its various fractions. The MCE and fractions were dissolved in 20% dimethyl sulfoxide (DMSO) at 3 different concentrations; 200 mg/ml, 100 mg/ml and 50 mg/ml. Colonies of each selected MDR bacterial strains were picked off from a fresh 24 hrs stock plate and suspended in sterile water to match the turbidity standard of 0.5 McFarland Standard. The resulting bacterial culture suspension was then uniformly spread on the surface of a Mueller Hinton agar plate.

At this point, wells were punctured aseptically with a cork borer (6 mm) in the agar in each plate (cork borer was sterilized after each puncture), and about 100 µl of the MCE and the fractions were poured in the different wells and allowed to stand at

room temperature (20-22°C) for about an hour. Gentamycin (10 mg/ml) was used as a positive control and 20% DMSO was used as a negative control. The plates were then incubated at 37°C. After 24 hours of incubation, the zones of inhibition appeared as the clear areas around the wells and the diameter of the growth of inhibition around each well was measured with a ruler in millimeters (mm).

The inhibition zone (in mm) results were then used to calculate the percentage growth inhibition of the samples with reference to the positive control (10 mg gentamycin) with the following formula:

Percentage inhibition = (TS – SC) / PC × 100

Where TS represents the test sample, SC represents negative control and PC represents positive control (Bibi et al., 2011).

3.6.4 Micro-plate dilution assay (Determination of the minimum inhibitory concentrations (MICs))

Micro-plate dilution assay (Balouiri et al., 2016) was used to determine the minimal inhibitory concentrations for the MCE and its various fractions. For each sample, the assay was done in triplicates, where two rows were used for the assay, and one row was used as a sterility check (no bacterial suspension poured). MDR bacterial strains were cultured in nutrient agar overnight before being used in the assay.

The assay was performed by aseptically adding 100 µl of the nutrient broth into each well of a 96 micro-well plate, then 100 µl of the MCE and its various fractions at starting stock concentrations of 50 mg/ml were added in the first row of the 96 microwell plates, followed by two-fold serial dilutions in the rest of the rows. After serial dilution and the addition of the inoculum, the final concentrations ranged between 12.5 mg/ml to 0.1 mg/ml. successively, the overnight bacterial cultures were prepared as standardised bacterial inoculums (0.5 McFarland) and 100 µl was added into each well of the 96 microwell plate.

The plates were then incubated at 37°C for 24 hrs. Gentamycin (10 mg/ml) was used as a positive control and distilled water was used the negative control. After incubation, 0.4 mg/ml of p - iodonitrotetrazolium (INT) chloride was added into each well as an indicator of bacterial activity and returned to the incubator to be incubated at 37°C for an hour. After the incubation, plates were observed with the colour change and the MIC was recorded as the lowest concentration of the methanolic crude extract and fractions that inhibited the bacterial growth.

3.7 CYTOTOXICITY ASSAY

For evaluation of the toxicity of the MCE and fractions on cells, Cell-based high content screening assay was used. This assay involves the use of the machine “Image Xpress Micro XLS”, which analyses the number of total, live and dead cells in each well of the micro-well plate seeded with cells that are treated with either extract/fraction/compounds (Pringle et al., 2018).

3.7.1 Preparation and treatment of cell line

For this study, Vero cells were chosen as the cell line of choice and were cultured in DMEM (low in glucose) and 10% fetal bovine serum. To avoid contamination of the cells, 10% penicillin–streptomycin antibiotics was added to the culture medium. For the assay, each well of the 96 well micro-plate was seeded with 4000 cells and left overnight for attachment.

For the treatment process, Vero cells were treated using 100 µl of the MCE and fractions (100 mg/ml) for 48 hours at 37°C with 5% CO_2. The three fractions that had good antibacterial activity were tested at 5 concentrations (12.5, 25, 50 100 and 200 µg/ml), whereas the MCE and the remaining two fractions with low antibacterial activity were tested at 2 concentrations (25 and 100 µg/ml) for the cytotoxicity assay. All samples were tested in triplicates. Melphalan (100 mM stock) was used as a positive control at the following concentrations: 30, 15, 7.5, 3.75 and 1.875 µM.

3.7.2 Biochemical analysis of treated cells

For analysis of treated cells after incubation, the medium and treatment were aspirated from the wells of the micro-plates, then followed by the addition of 50 µL of Hoechst 33342 staining solution to each well, and further incubated for 10 minutes at room temperature. Right before image acquisition of the micro-well plates, 50 µL of Propidium Iodide (PI) which is another staining solution was added to the wells. The results were acquired using the Image Xpress Micro XLS machine (using MetaXpress 6.1 Software) with the DAPI and Texas red filters, where Cell viability was expressed as nuclei per site of the cells treated with the MCE/fractions compared to the untreated control (Pringle et al., 2018).

The dual stains mentioned above are stains used to check cell viability (percentage of live and dead cells) and were prepared in the following manner (Yurdakök and Baydan, 2013):

- Hoechst (5 µg/ml) was put in 10 ml of Phosphate Buffered Saline (PBS)
- PI solution (100 µg/ml) was put in 10 ml PBS

3.8 DATA ANALYSIS AND IMAGING

The data was analysed using Microsoft Excel and results obtained are reported as mean ± standard deviation (for experiments done in triplicates). For cytotoxicity assay, the molecular device ImageXpress Micro XLS was used to acquire the fluorescent micrographs of the micro-well plates for analysis.

Chapter 4

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Extraction and fractionation

After extraction of the air-dried plant material, the final MCE weighed 61.27 g, with a percentage yield of 9%. The MCE was then fractionated using solvent-solvent fractionation producing a total of 20 fractions. From the 20 fractions obtained, TLC was used to combine similar fractions, wherein after analysis, 20 fractions resulted into six fractions which were named; F1, F2, F3, F4, F5, and F6. The following table summarizes the solvents used, masses, and the percentage yield of the fractions obtained in relation to the MCE.

Table 4.1: Fractions obtained from the methanolic crude extract and their respective percentage yields.

Fraction name	Solvent used	Mass in grams (g)	Percentage yield with reference to the methanolic crude extract (%)
F1	EE 100%	2.7	4.4
F2	EE 100%	2.3	3.8
F3	EE 90: 10 M	3.06	5
F4	EE 70: 30 M	11.96	18
F5	EE 30: 70 M	3.68	6
F6	EE 30: 70 M	0.81	1.32

Key: EE = Ethyl Acetate, M= Methanol

4.1.2 Phytochemical composition

a. Thin Layer Chromatography (TLC)

Thin Layer Chromatography was used to efficiently screen the breakdown as well as the quality evaluation of the plant *S. africana* and its phytochemical by-products. Three solvents systems were used to run TLC, which were BEA, CEF and EMW [BEA (benzene/ethanol/ammonium hydroxide (90:10:1), CEF (chloroform/ethyl acetate/formic acid (5:4:1) and EMW (ethyl acetate/methanol/water (40:5.4:4)], wherein the different bands on the plates (Figure 4.2 to 4.4) were observed after spraying the plates with the reagent vanillin-sulphuric acid.

After the running of the plates, more bands were observed in the TLC plates run in EMW solvent system, followed by the TLC plates run in the CEF solvent system which showed few bands for some samples, whiles plates run in the BEA solvent system had the least number of bands.

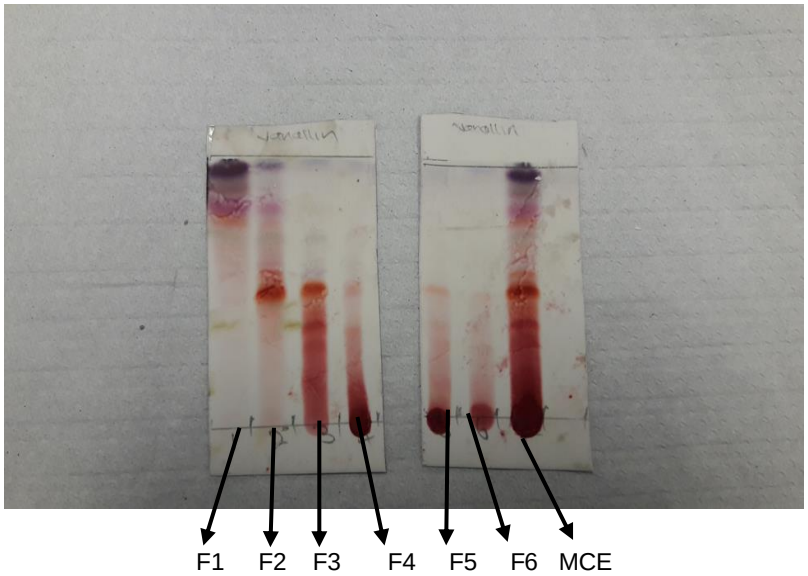

Figure 4.1: Chromatograms of *Spirostachys africana*'s methanolic crude extract and fractions developed in EMW solvent system.

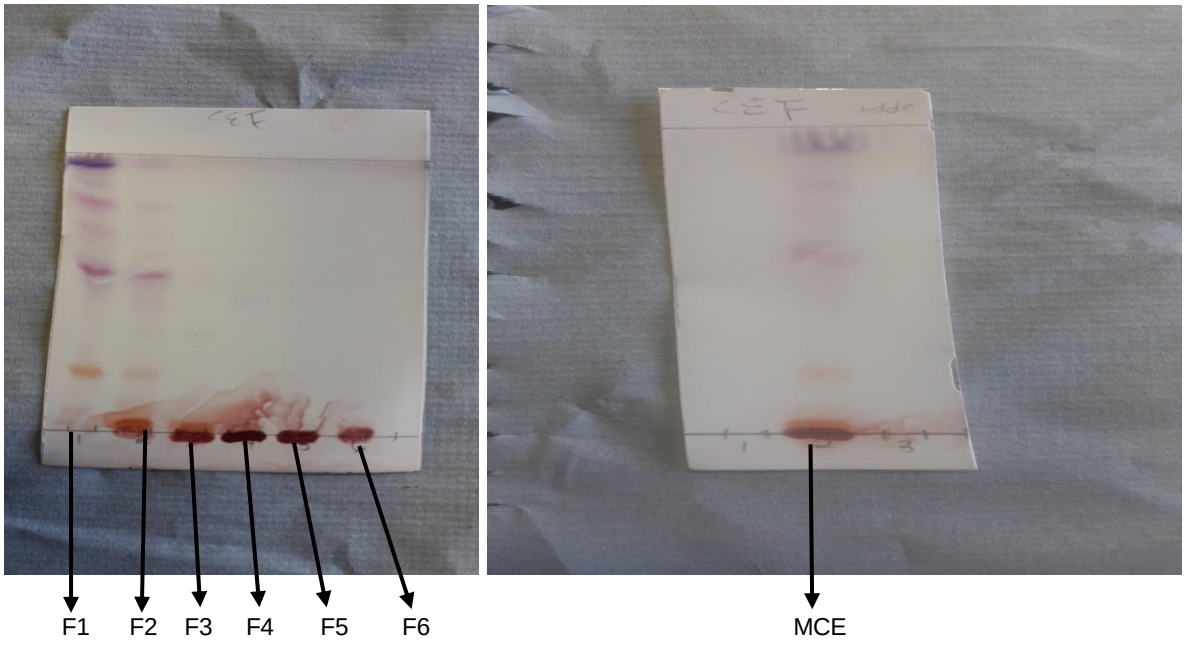

Figure 4.2: Chromatograms of *Spirostachys africana*'s methanolic crude extract and fractions developed in CEF solvent system.

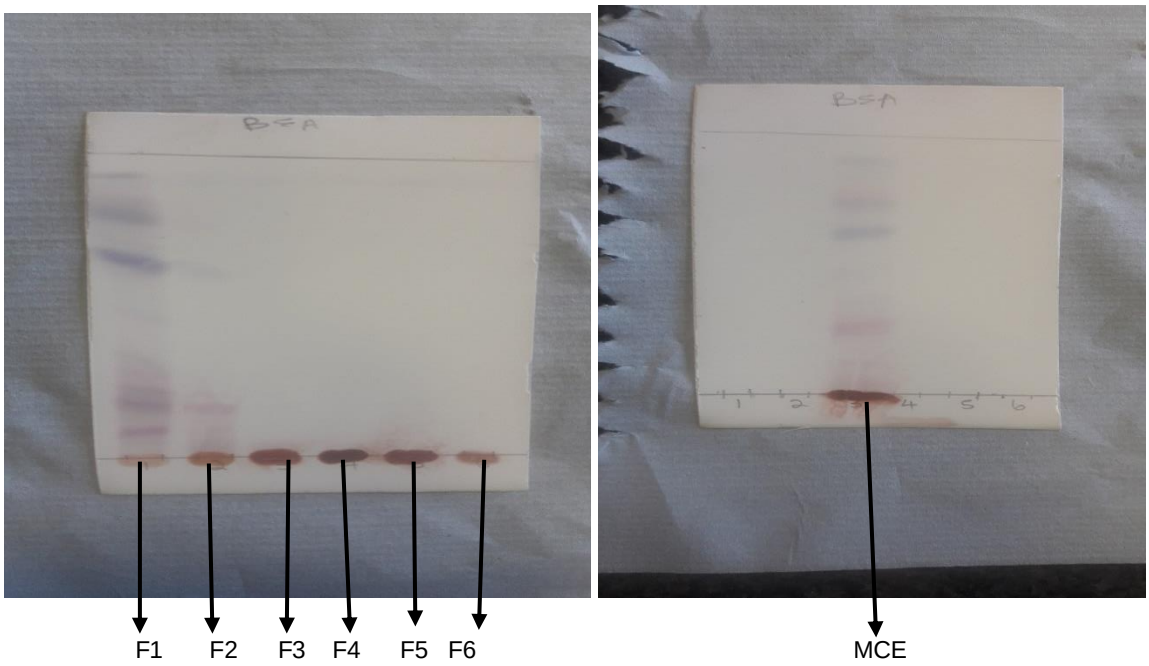

Figure 4.3: Chromatograms of *Spirostachys africana*'s methanolic crude extract and fractions developed in BEA solvent system.

b. Biochemical tests

Phytochemical analysis of the methanolic crude extract and fractions obtained in this study showed the presence of different groups of secondary metabolites. All secondary metabolites tested in this study (Phenolics, flavonoids, alkaloids, steroids, saponins, cardiac glycosides, and terpenoids) were present in F3, F4, F5, F6 and MCE, however, saponins and alkaloids were absent in F1 and F2 (Table 4.2).

Table 4.2: Secondary metabolites found in the methanolic crude extract and its fractions.

Sample	Test						
	Phenols and tannins	Flavonoids	Alkaloids	Cardiac Glycosides	Steroids	Terpenoids	Saponins
F1	+	+	-	+	+	+	-
F2	+	+	-	+	+	+	-
F3	+	+	+	+	+	+	+
F4	+	+	+	+	+	+	+
F5	+	+	+	+	+	+	+
F6	+	+	+	+	+	+	+
MCE	+	+	+	+	+	+	+

Key: "+" = present; "- "= absent

c. Total Flavonoid Content (TFC) and Total Phenolic Content (TPC)

The total flavonoid content (TFC) was reported as quercetin equivalents with reference to the regression equation of the calibration curve ($Y = 3.454x – 0.1318$ and $R^2 = 0.9266$) while the total phenolic content (TPC) was reported as gallic acid equivalents with reference to the regression equation of the calibration curve ($Y = 2.069x + 0.645$

and R^2 = 0.6272). TFC measured ranged between 6.98 ± 0.07 and 26.7 ± 0.17 mg QE/g, while for TPC content measured varied between 26,8 ± 0,08 and 55.36 ± 0.23 mg GAE/g (table 4.3).

Fraction F1 had the highest total flavonoid content (26.7 ± 0.17 mg QE/mg) compared to the rest of the samples, wherein fraction F3 had the least amount of flavonoid content (6.98 ± 0.07 mg QE/mg). The highest total phenolic content was contained in fraction F2 (55.36 ± 0.23 mg GAE/g), where else fraction F1 had the least amount of total phenolic content (26.8 ± 0.08 mg GAE/g).

Table 4.3: TPC and TFC of the methanolic crude extract and its fractions.

SAMPLE	Total flavonoid content (mg QE/g)	Total phenolic content (mg GAE/g)
F1	26.7 ± 0.17	26.8 ± 0.08
F2	9.12 ± 0.1	55.36 ± 0.23
F3	6.98 ± 0.07	46.94 ± 0.01
F4	7.42 ± 0.02	54.49 ± 0.01
F5	7.7 ± 0.02	34.16 ± 0.01
F6	8.61 ± 0.01	30.66 ± 0.66
MCE	7.50 ± 0.02	29 ± 0.01

All values are expressed as mean ±SD of three replicates;

4.1.3 Antioxidant activity

The IC_{50} of DPPH free radical scavenging activities and the EC_{50} of the reducing power assay were calculated using regression linear equation of calibration curves of each sample to determine the antioxidant activity in *S. africana*'s MCE and its fractions. For the DPPH free radical scavenging activity, the decolourization of the samples from purple to yellow was an indication of antioxidant activity (Figure 4.4), whereas for the reducing power assay, the colour change from yellow to different shades of green was an indication of the presence of reducers in the test samples (Figure 4.5). The lower the value of IC_{50} or EC_{50}, the higher the antioxidant activity. IC_{50} is defined as the concentration that inhibits half of the DPPH, whereas EC_{50} is demarcated as the

effective concentration of the antioxidant necessary to decrease the radical concentration by 50%.

For DPPH free radical scavenging activity, the IC_{50} of the samples ranged between 0.01 ±0.34 mg/ml to 0.62 ± 0.05 mg/ml, whiles for EC_{50} measured for the reducing power ranged between 0.61 ± 0.01 mg/ml and 11.30 ± 0.04 mg/ml (Table 4.4). The positive control Ascorbic acid had an IC_{50} of 0.01 ± 0.34 mg/ml and EC_{50} of 0.61 ± 0.01 mg/ml. Amongst all samples of *S. africana* tested, fraction F3 had both the lowest IC_{50} and EC_{50} Measured, whereby the IC_{50} was 0.02 ± 0.07 mg/ml and the EC_{50} was 2.26 ± 1.01 mg/ml. The second fraction to have the second lowest IC_{50} and EC_{50} was fraction F2. The samples with the least amount of antioxidant activity were fraction F1, F5, and the MCE, which had the highest measurement of both the IC_{50} and EC_{50} values. Fraction F1 had both the lowest IC_{50} and EC_{50}, which were 0.62 ± 0.05 mg/ml and 11.30 ± 0.04 mg/ml respectively.

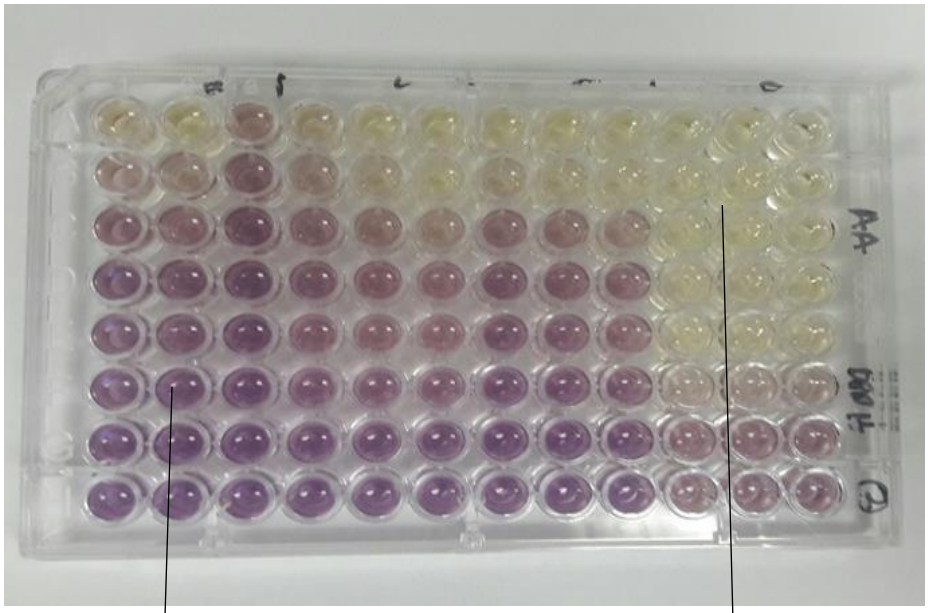

Figure 4.4: Plate showing results of DPPH scavenging assay.

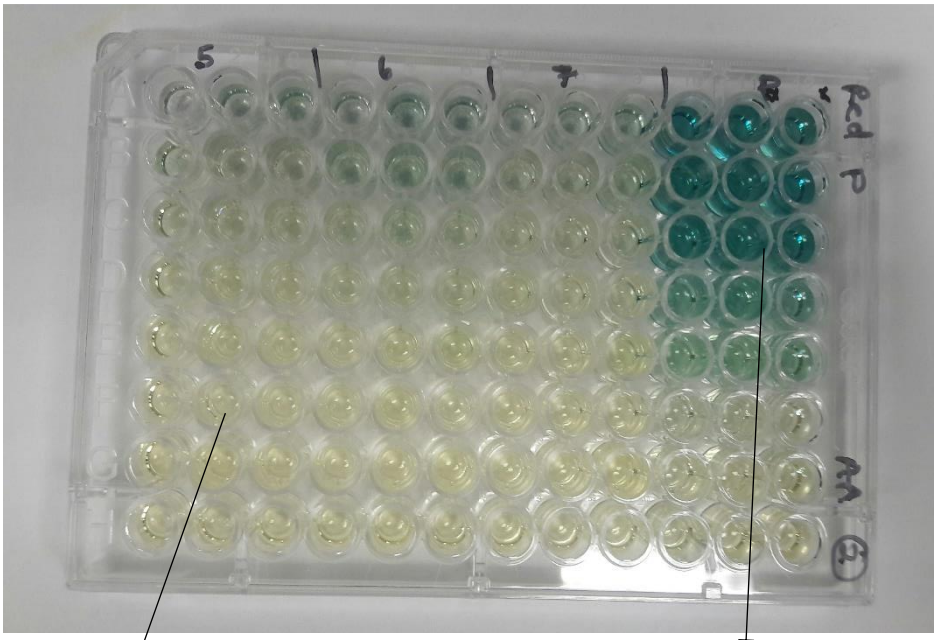

Figure 4.5: Plate showing results of reducing power assay.

Table 4.4: The inhibition of DPPH (IC_{50}) and the reducing power (EC_{50}).

Sample	IC50 (mg/ml)	EC50 (mg/ml)
F1	0.62 ± 0.05	11.30 ± 0.04
F2	0.09 ± 0.21	2.26 ± 1.01
F3	0.02 ± 0.07	1.87 ± 0.00
F4	0.32 ± 0.00	3.50 ± 2.35
F5	0.55 ± 0.23	7.70 ± 0.28
F6	0.31 ± 0.43	4.60 ± 0.00
MCE	0.36 ± 0.71	8.82 ± 0.71
Ascorbic acid	0.01 ± 0.34	0.61 ± 0.01

4.1.4 Antibacterial activity

The MCE and fractions of *S. africana* were tested for their antibacterial activity against MDR bacterial strains using agar well diffusion assay, where zones of inhibition were measured in millimetres (mm) (Figure 4.6) and the microdilution assay, where the lowest concentration inhibiting the bacteria was recorded in mg/ml (Figure 4.10).

ATCC BAA strains of Gram-negative bacteria and Gram-positive bacteria were used as test microorganisms.

a. Agar well diffusion assay

The MCE and fractions of *S. africana* showed antibacterial activity against the bacterial strains tested against, besides the bacterial strain *K. pneumonia* for fraction F1 (Table 4.5). The inhibition zones ranged from 7 to 18 mm. Fractions F2 and F3 showed the most effective antibacterial activity with the largest inhibition zones against most bacterial strains tested against, compared to the other *S. africana*'s test samples. Gentamycin at a concentration of 10 mg/ml was used as a Positive control.

The zone of inhibition (in mm) obtained was then used to calculate the percentage of inhibition with reference to the positive control's (10mg/ml) antibacterial activity. Figures 4.7, 4.8 and 4.9 represent the relative percentage inhibition by the fractions and MCE at concentrations 50 mg/ml, 100 mg/ml and 200 mg/ml respectively.

At a concentration of 50 mg/ml, only the MCE and fraction F2 and F3 inhibited ≥25% of the growth of four bacterial strains. MCE and fraction F3 at 100 mg/ml were the only two samples that managed to inhibit ≥30% of the growth of 5 bacterial strains. The high percentages of inhibition of the MCE and its various fractions (besides F1) were seen at a concentration of 200 mg/ml, were they all had at least inhibited 50% growth of one bacterial strain.

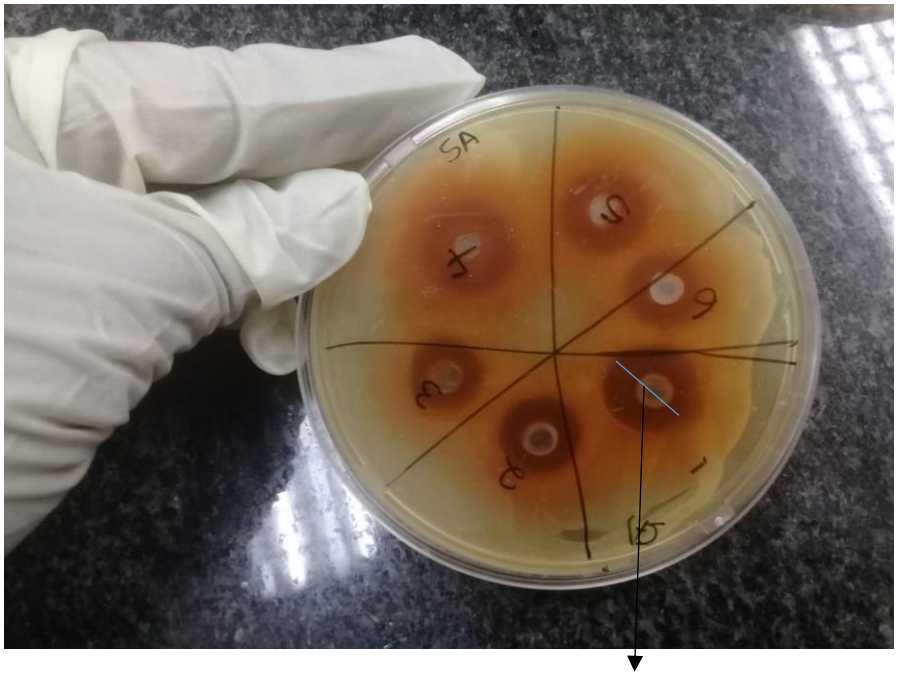

Figure 4.6: Plate showing inhibition zones of bacterial growth by *Spirostachys africana*.

Table 4.5: Antibacterial properties of *S. africana* extract and its fractions.

Samples	**Concentration (mg/ml)**	***A. baumanii***	***E. cloacae***	***E. faecium***	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***
	200	0	0	0	0	0	0
F1	**100**	0	0	0	0	0	0
	50	0	0	0	0	0	0
F2	**200**	14	10	11	0	13	17
	100	12	8	8	0	11	15
	50	10	0	8	0	10	13
F3	**200**	15	14	12	0	14	18
	100	13	8	11	0	11	15
	50	9	0	9	0	10	13
F4	**200**	11	14	0	0	0	14
	100	10	11	0	0	0	13
	50	7	7	0	0	0	12
F5	**200**	9	0	10	0	10	13
	100	7	0	0	0	9	7
	50	0	0	0	0	9	0
F6	**200**	10	0	0	0	9	13
	100	9	0	0	0	8	10

	50	0	0	0	0	0	9
CE	**200**	13	14	11	0	15	14
	100	10	11	9	0	13	12
	50	8	8	7	0	12	11
Positi - ve control	**10**	**20**	**32**	**30**	**0**	**35**	**32**

Positive control = gentamycin (10 mg/ml)

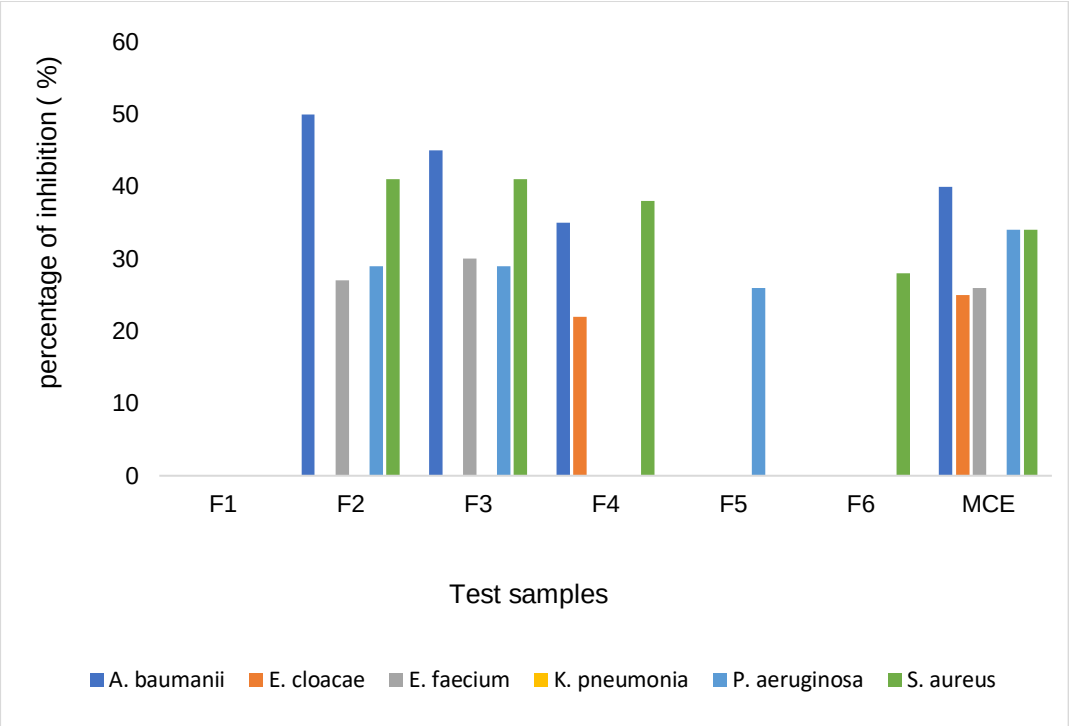

Figure 4.7: Comparative percentage of inhibition of the MCE and fractions at 50 mg/ml against MDR bacteria.

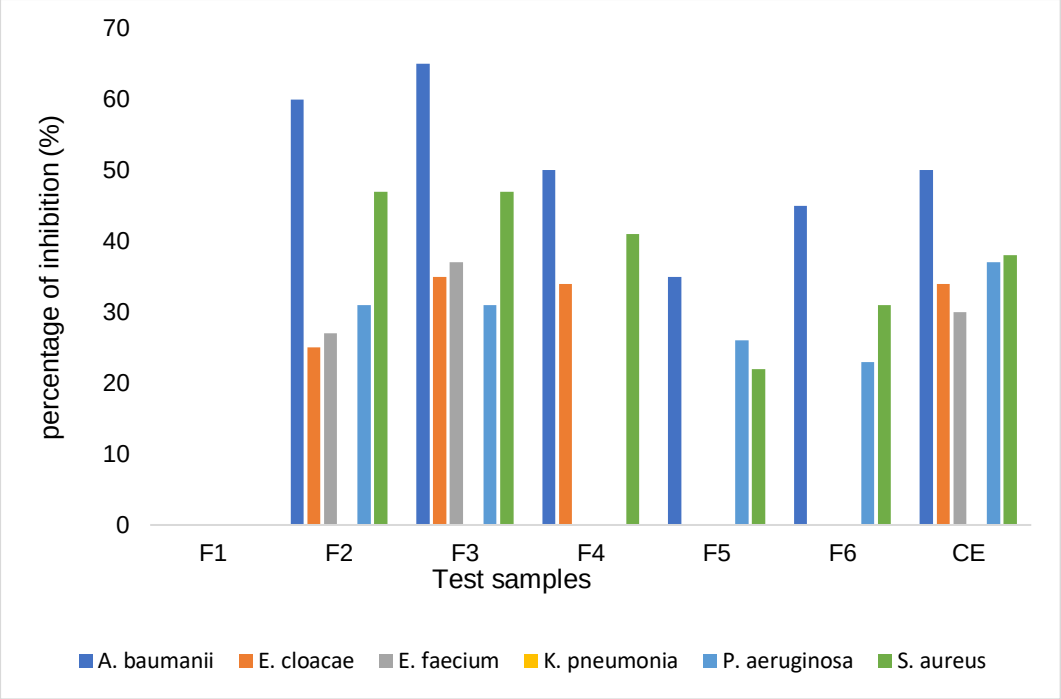

Figure 4.8: Comparative percentage of inhibition of the MCE and fractions at 100 mg/ml against MDR bacteria.

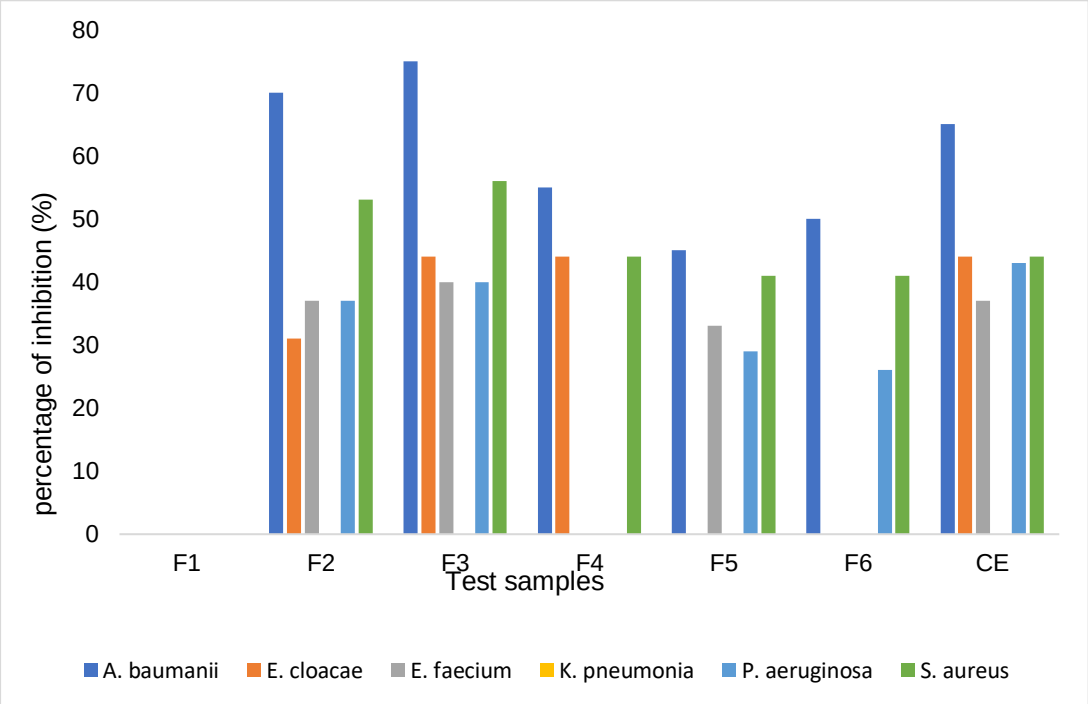

Figure 4.9: Comparative percentage of inhibition of the MCE and fractions at 200 mg/ml against MDR bacteria.

b. Microdilution assay

The minimum inhibitory concentrations (MIC) in mg/ml of the MCE and all fractions (table 4.6) were determined. Contrary to the agar well diffusion assay results, the micro-plate dilution assay results revealed that the MCE and all its fractions can inhibit the growth of all MDR bacterial strains tested against but at different concentrations.

The MIC values of the MCE and its fractions ranged from 0.1mg/ml to >12.5mg/ml. Fractions F3 and F6 had better activity against all the tested organisms compared to rest as they had lower MIC averages. F6 showed the lowest overall MIC value which was 0.59 mg/ml, whereas fraction F1 overall had the highest MIC's for all the tested bacterial strains. The most sensitive MDR bacterial strain was *P. aeruginosa*, which had the lowest MIC values. All MIC's for Gentamycin (positive control) against all the MDR bacteria were < 0.1 mg/ml.

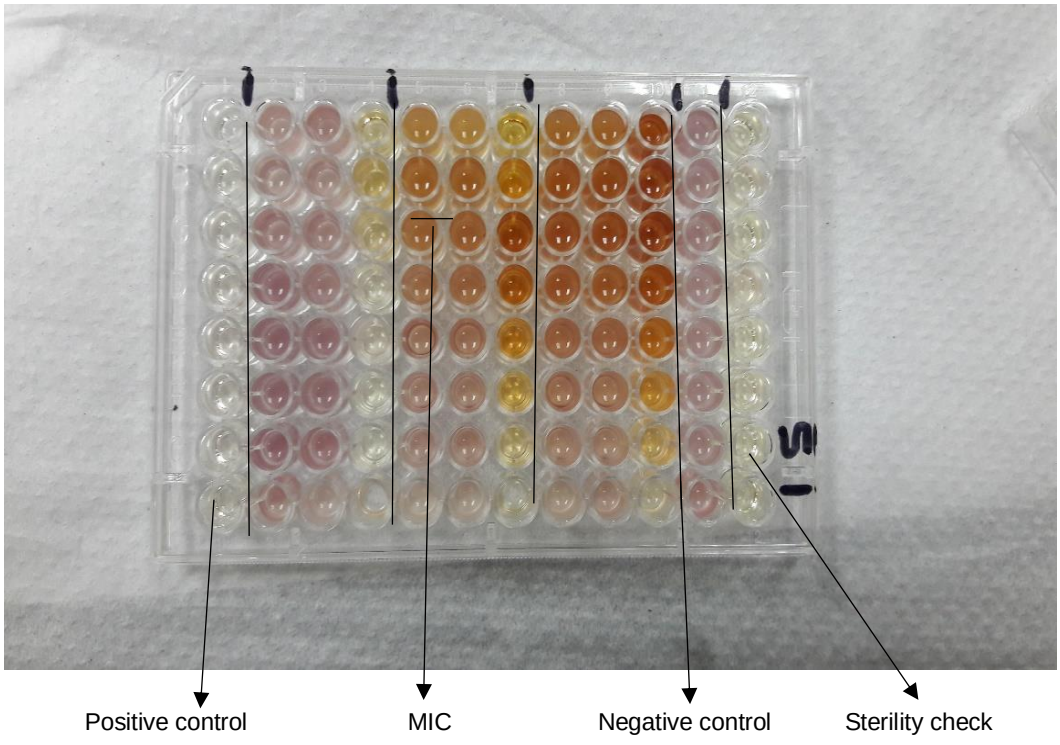

Figure 4.10: Plate showing results of microdilution assay.

Table 4.6: Minimum inhibitory concentrations of MCE and its fractions in mg/ml.

	Plant extract and fractions (mg/ml)						
Bacterial species	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**MCE**
A. baumannii	>12,5	0,78	0,39	1,56	1,56	0,78	0,78
E. cloacae	12,5	0,39	0,2	0,78	0,78	0,2	0,2
E. faecium	12,5	6,25	1,56	6,25	6,25	0,78	6,25
K. pneumonia	>12,5	1,56	0,39	1,56	1,56	0,78	1,56
P. aeruginosa	6,25	0,39	0,2	0,78	1,56	0,2	0,1
S. aureus	6,25	1,56	0,78	3,13	3,13	1,56	1,56
Average MIC's for MEC and fractions	**N/A**	**1,82**	**0,59**	**2,34**	**2,47**	**0,72**	**1,74**

Key: N/A = Not applicable

4.1.5 Cytotoxicity assay

The cytotoxic effects of *S. africana*'s methanolic crude extract and its fractions were evaluated on Vero cell line at different concentrations. The Image Xpress machine with the DAPI and Texas red filters was used to determine the cell viability of cells treated with MCE and its fractions. As mentioned in chapter 3.6, the fractions that had good antibacterial activity were tested at five concentrations (12.5, 25, 50 100 and 200 µg/ml), whereas the MCE and fractions with low antibacterial activity were tested only at 2 concentrations (25 and100 µg/ml).

Fraction F1 was excluded for this assay due to its inability to dissolve in any solution for re-suspension to be used in the assay. From the graphs (figure 4.11 to figure 4.16), it can be noted that the total number of cells per image without any treatment (control noted as 0 concentration) were all above 2000, whereas treatment of cells with Melphalan (toxic compound as positive control) led to a decreased total number of cells per image to as little as under 100. The positive control and untreated cells results were used as a reference to assess the level of toxicity of the test samples.

Amongst all the samples tested for toxicity, MCE and fraction F2 showed the highest toxicity to cells, wherein comparison to the negative control, the total number of cells was 50 % lower. Fraction F3 and F5, however showed to cause cell proliferation (at certain concentrations) as the total number of cells per image were higher than those of the negative control.

a. Fractions tested at five different concentrations (F2, F3, and F6)

The fraction F2 was found to be toxic at all concentrations tested and inhibited cell viability by more than 60% (Figure 4.11). Fraction 3 (Figure 4.12) was found to be toxic at concentration 100 µg/ml (4% inhibition) and 200 µg/ml (45% inhibition). At concentrations below 100 µg/ml, stimulation of cell replication was observed. The maximum stimulation in cell replication was observed at a concentration of 50 µg/ml (13% increase).

Fraction F6 led 1st to a stimulation of cell proliferation (Figure 4.13) at 12.5 µg/ml (5% increase and then was toxic from concentration 25 µg/ml (9% decrease in cell number). The cell numbers are inversely correlated to the concentration of fractions. As the concentration increases, the number of cells decreases.

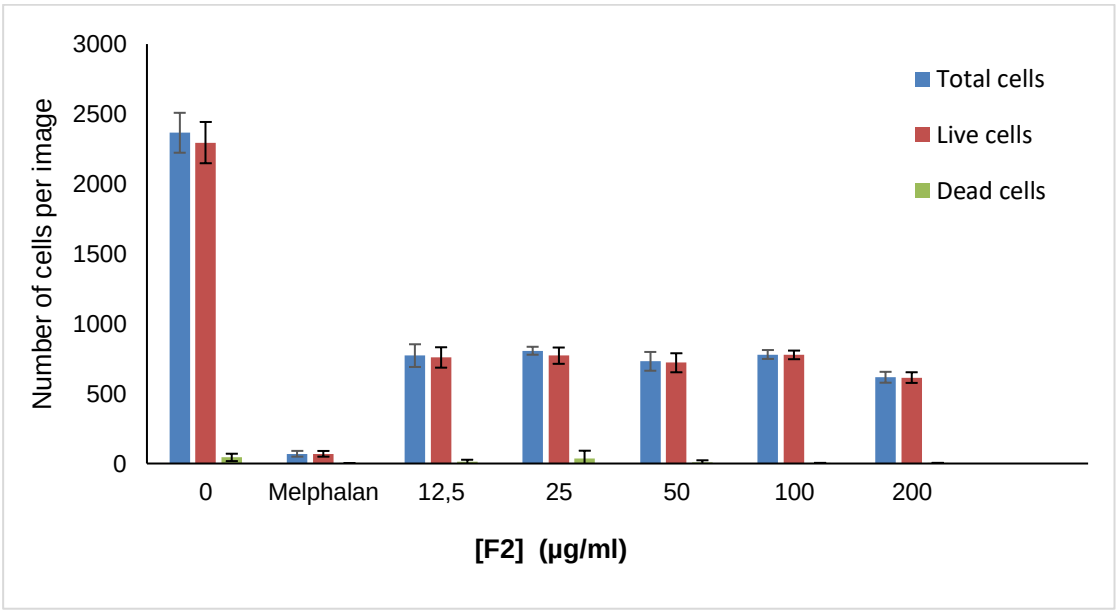

Figure 4.11: Toxicity effects of *Spirostachys africana* fraction F2 against Vero cell line after 48 hours of incubation.

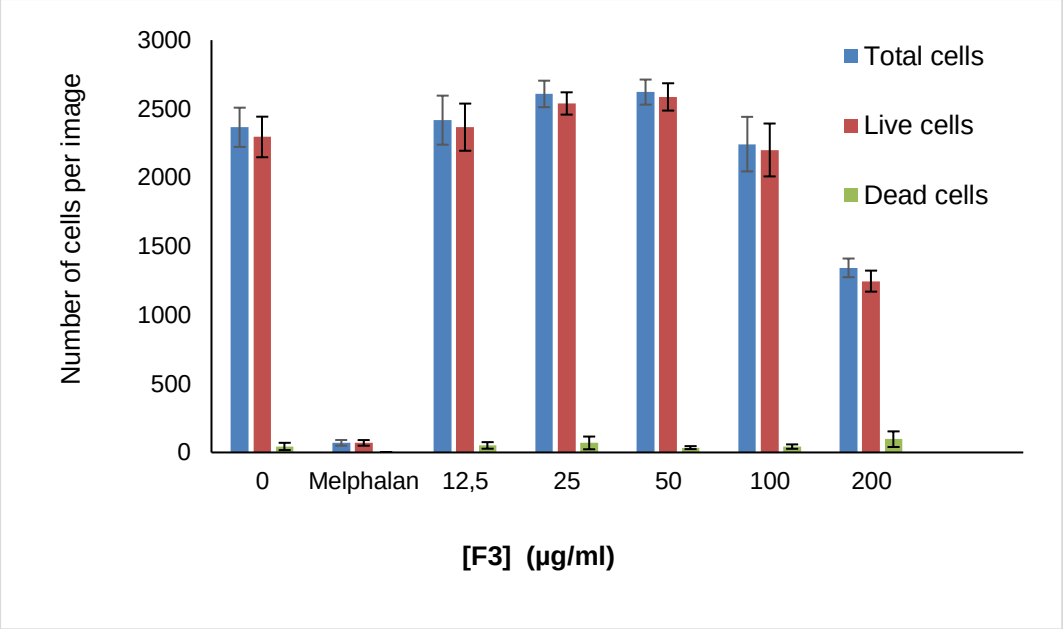

Figure 4.12: Toxicity effects of *Spirostachys africana* fraction F3 against Vero cell line after 48 hours of incubation.

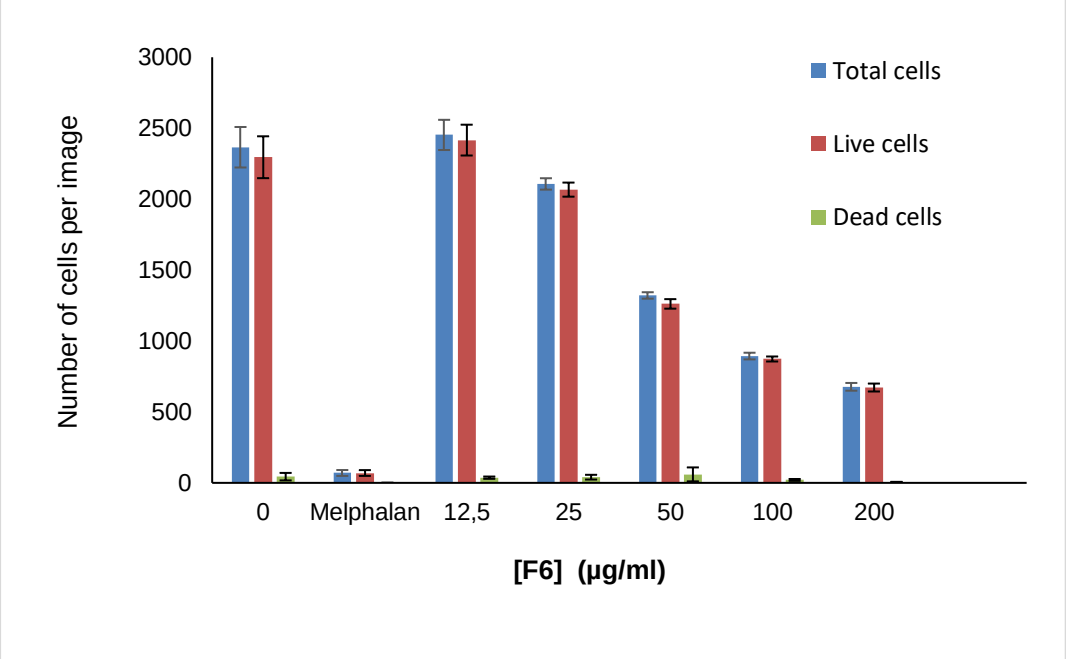

Figure 4.13: Toxicity effects of *Spirostachys africana* fraction F6 against Vero cell line after 48 hours of incubation.

b. Fractions tested at two concentrations (F4, F5, and MCE)

Fraction F4 (Figure 4.14) was found to be toxic at 25 µg/ml (5% inhibition) and at 100 µg/ml (43% inhibition). At both concentrations tested, fraction F5 (Figure 4.15) induced cell numbers (6% increase in cell number). The MCE of the S. africana (figure 4.16) was found to be very toxic at both concentrations tested against (68% decrease in cell numbers. None of the MCE and fractions that were tested and found to be toxic whereas toxic as the positive control used (Melphalan). Melphalan led to 96% inhibition of cell numbers.

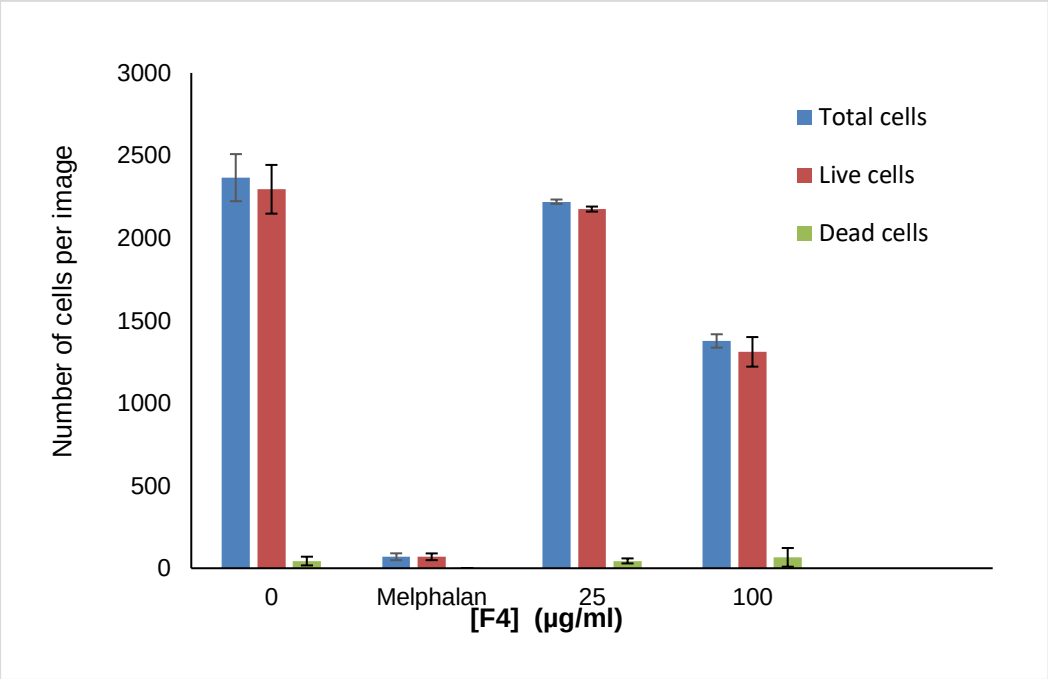

Figure 4.14: Toxicity effects of *Spirostachys africana* fraction F4 against Vero cell line after 48 hours of incubation.

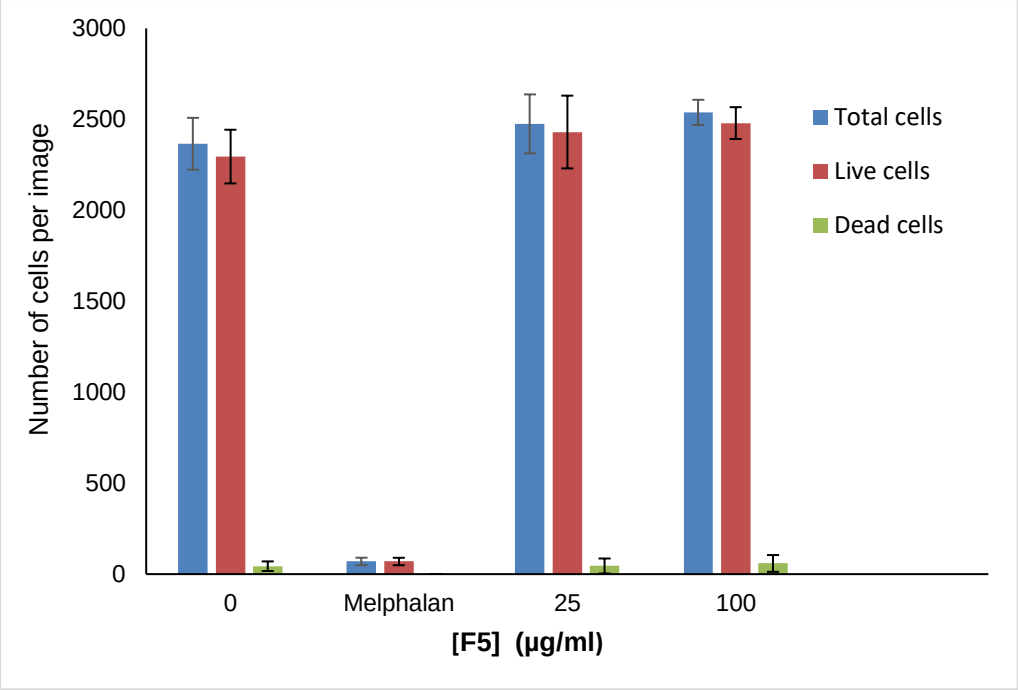

Figure 4.15: Toxicity effects of *Spirostachys africana* fraction F5 against Vero cell line after 48 hours of incubation.

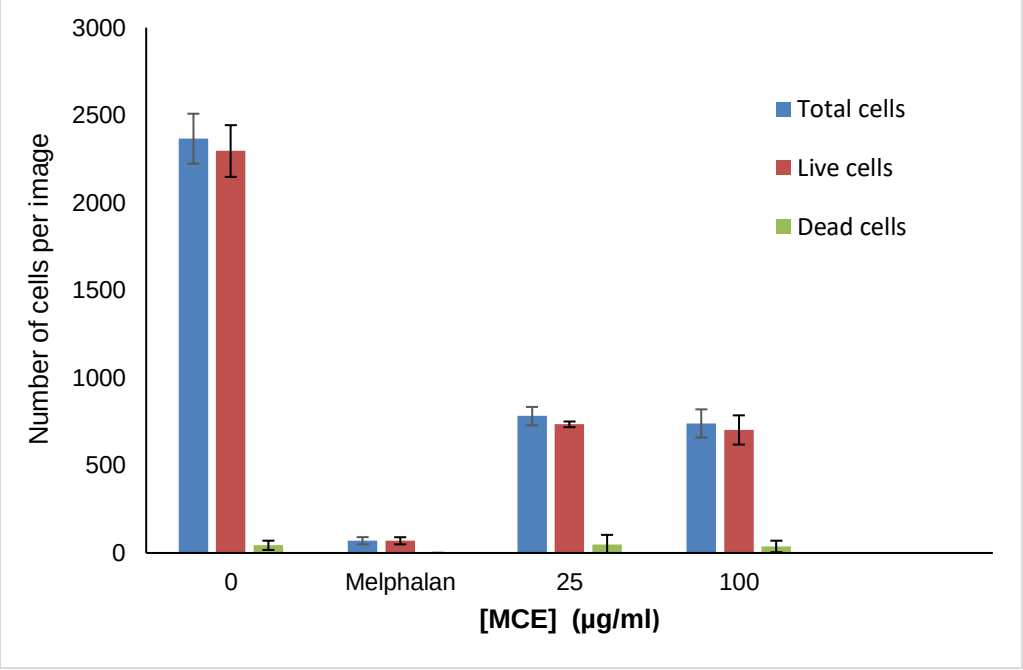

Figure 4.16: Toxicity effects of *Spirostachys africana* MCE against Vero cell line after 48 hours of incubation.

4.2 DISCUSSION

Medicinal plants are considered reservoirs of phytochemical constituents, which play a major role in the plants' characteristics of being considered therapeutic (Balandrin et al., 1985). The extraction process is very important as it is the first step where soluble medicinally active constituents of the plant are extracted with the chosen solvent/s (figure 4.1). For this study, methanol was the preferred solvent of choice for extraction, as it is known to extract the maximum number of biologically active compounds from medicinal plants (Dhawan and Gupta, 2017).

The methanolic crude extract of *S. africana* obtained in this study yielded 9% based on the dry weight of the stem bark, however, compared to another study also done on *S. africana* stem bark by Akhalwaya et al (2018), the crude extract they attained was extracted with a combination of dichloromethane and methanol (with a ratio 1:1) and yielded a percentage of 19.67 %, which is more than twice the percentage yielded in this study.

The MCE of *S. africana* contains a concoction of many different secondary metabolites in low concentrations that may co-operate antagonistically or synergistically to exhibit their potential biological activities. Therefore, by fractionating the MCE of the plant, the phytochemical constituents get separated based on their polarity into their respective fractions. Hence, the different fractions will contain particular phytochemicals which will now be found in different concentrations compared to their initial concentrations in the MCE and can exhibit better biological activities compared to that of the MCE (Seelinger et al., 2012).

For fractionation of MCE, ethyl acetate and methanol mixtures of increasing polarity (from 0 to 100%) were used as solvents for running the column chromatography for separations of compounds. This mixture was chosen based on literature, which indicated that when the methanol extract is partitioned with ethyl acetate, fractions that are rich in biologically active phytoconstituents can be eluted (Anokwuru et al., 2017; Parkash et al., 2015).

From all the fractions obtained, fraction F4 extracted the most amount of total extractable compounds with the highest percentage yield of 18%, whereas fraction F6 yielded the least amount (1.32%), extracting the lowest extractable compounds from the MCE (Table 4.1). This can mean that the combination of 70% ethyl acetate and 30% methanol can be a good combination to extract most phytochemical constituents from the plant *S. africana*, but, it is not guaranteed that the phytochemicals extracted will have maximum potential biological activities. These results agreed with the study by Anokwuru et al (2017), which also reported that the fraction eluted with a combination of 70% ethyl acetate and 30% methanol had the highest percentage yield of extractable compounds.

For the quality evaluation of medicinal plants, phytochemical screening is one of the best tools that can be used (Basma et al., 2011). For this study, the presence of phytochemicals in *S. africana*'s MCE and its fractions was investigated using both the qualitative and quantitative methods.

Using the biochemical tests, all different groups of phytochemicals, namely phenols and tannins, flavonoids, alkaloids, steroids, saponins, cardiac glycosides and terpenoids tested in this study were present in some samples (Table 4.2). These secondary metabolites are known to have biological activities which include antimicrobial, antifungal, anticancer and anti-inflammatory activities (Salem et al., 2013).

Fractions F1 and F2 were the only two fractions of MCE that didn't comprise of all the phytochemicals tested for in this study, were they lacked the presence of saponins and alkaloids, whereas the remaining four fractions (F3, F4, F5, and F6) which were eluted with ethyl acetate and methanol mixtures of different polarity ratios had all the phytochemicals that the MCE also consisted of. Both fraction F1 and F2 were fractionated with 100% ethyl acetate, which possibly can indicate that the elution of phytochemicals from *S. africana*'s MCE with only pure ethyl acetate can exclude the extraction of saponins and alkaloids. Other studies also reported the absence of alkaloids from pure ethyl acetate fractions (Abdulmalik et al., 2016; Khanum et al.,

2013; Temidayo, 2013) and the absence of saponins from pure ethyl acetate fractions (Rehman et al., 2018; Temidayo, 2013; Ekam et al., 2010).

For TLC analysis of phytochemicals, the presence of various phytochemical compounds found in the MCE and the fractions of *S. africana* was shown by the distinguished different color bands portrayed by individual phytochemical compounds due to their reaction with the spray reagent used (vanillin-sulphuric acid). Three mobile phase systems namely BEA (benzene/ethanol/ammonium hydroxide (90:10:1)), CEF (chloroform/ethyl acetate/formic acid (5:4:1)) and EMW (ethyl acetate/methanol/water (40:5.4:4)) were used to separate the compounds based on their polarities, and it has been acknowledged that the BEA solvent system is well known to separate non-polar compounds (lignin, wax, lipids, aglycon, sterol, etc.), the CEF solvent system is known best to separate compounds with intermediate polarity, whiles the EMW solvent system is considered the best solvent system for separation of polar compounds (phenolic compounds, flavonoids, terpenoids, saponins, tannins, anthocyanin, amino acids, and glycosides) (Widyawati et al., 2014; Biradar and Rachetti, 2013).

Based on the results obtained in this study (Figure 4.1 to Figure 4.3), *S. africana* contained more polar compounds compared to the non-polar and the intermediate compounds, since the chromatograms run in the EMW solvent system separated more compounds compared to the CEF and BEA. Whiles the findings of this study correlated with those of Nyaberi et al (2017) which reported that the solvent system EMW separated more phytochemicals of plant extracts compared to the CEF and BEA solvent system, more studies (Kabongo et al., 2016; Makhafola and Eloff, 2012; Nyenje and Ndip, 2012; Seanego and Ndip, 2012) however were in contrary with these findings, as they reported that the greatest separation of phytochemicals was obtained using the CEF solvent system. Although studies can report which solvent system is best for separation of phytochemicals, the separation of compounds in plant extracts or fractions depend uniquely on the type of compounds found in that particular plant of interest.

From the TLC plates developed in the EMW solvent system, which separated the compounds well, it can be noted that the MCE contained more compound bands compared to all fractions. Each fraction (F1 to F6) contained phytochemical constituents that were all present in the MCE (Figure 4.3), indicating that the MCE indeed is a mixture of phytochemicals and through fractionation, those phytochemicals get separated into different portions (fractions) according to their polarities and the solvents used.

The quantitative screening of phytochemical constituents in this study indicated that *S. africana*'s MCE and its fractions are rich in flavonoids and phenols as shown in Table 4.3. The contents of both flavonoid and phenolic compounds were investigated since these compounds are known to be the most significant secondary metabolites found in medicinal plants, because of the many health benefits and biological activities they possess. The ascending order of TFC was: F1>F2>F6>F5>MCE>F4>F3. Fraction F1 was the highest phenolics content fraction (26.7 ±0.17 mg QE/g) whereas fraction F3 was the least phenolics content fraction (6.98 ± 0.07 mg QE/g). The ascending order of TPC was: F2>F4>F3>F5>F6>MCE>F1. Fraction F2 had the highest amount of total phenol contents (55.36 ± 0.23 mg GAE/g) whereas fraction F1 had the least amount of phenolic contents (26.8 ± 0.08 GAE/g).

For TFC results, four fractions had higher flavonoid contents than the MCE, whiles for the TPC, five fractions had higher phenolic contents than the MCE. The reasons why fractions had higher contents (flavonoids and phenolics) than the MCE could be the fact that the MCE contained a lot of phytochemicals other than flavonoids and phenolics and that during the fractionation process, the MCE got purified which caused the concentration of flavonoids and phenolics to increase (Yakubu et al., 2014; Sannigrahi et al., 2010).

Fraction F2 which was eluted with 100% ethyl acetate had the highest TPC and the second highest TFC, indicating that flavonoids and phenolics are soluble in ethyl acetate and ethyl acetate can be a good solvent for extraction of these phytochemicals. The high amount of TFC and TPC in samples of *S. africana* can

potentially be an indication that this medicinal plant has antioxidant activity as flavonoids and phenols found in plants are highly operational free radical scavenging and antioxidant contents (Atanassova et al., 2011). The MCE TPC and TFC results of *S. africana*'s stem bark in this study were similar to those of Amoo et al (2012), which reported *S. africana*'s leaves and twigs to having TFC and TPC of 26.7 ± 0.52 mg QE/g and 69.1 ± 2.13 GAE/g respectively, however, a study by Mulaudzi et al (2012) reported a lesser amount of TPC in *S. africana*'s stem bark (0.01± 0.13 mg CTE/g), which could possibly be due to the solvent used during the extraction process.

In the human body during normal conditions, the generation and the reduction of reactive oxygen species (ROS) is controlled by the natural antioxidant defence system, however, due to specific pathological conditions, ROS can be found to be in excess and tend to cause several disorders and diseases by attacking macronutrients (Li et al., 2018). These ROS's are free radicals that contain oxygen and are formed because of oxygen metabolism and have significant roles in cell signalling and homeostasis (Lalhminghlui and Jagetia, 2018.).

To fight the unwanted effects of excess ROS (free radicals) in the human body, discovering external potential antioxidant sources can help to boost the endogenous antioxidant defences of the body, which in return disorders and diseases can be avoided (Kasote et al., 2015). Medicinal plants are known to be potential sources of natural antioxidants that contribute to the neutralisation of ROS for their own survival, therefore, it is of importance to evaluate their antioxidant potential for the wellbeing of humans.

The DPPH free radical scavenging assay determined the antioxidant activity potential of *S. africana*'s extract and its fractions by their ability to scavenge free radicals by hydrogen donation. During the assay, the colour in the DPPH solution changed from the purple to the colour yellow/ lighter than purple (Figure 4.4). For the reducing power assay, the antioxidant activity potential of *S. africana*'s extract and its fractions was determined by their capability to have a reducing power, which was indicated by the

conversion of Fe^{3+} to Fe^{2+}, where the yellow colour of the test solution changed to different shades of green to indicate the conversion (Figure 4.5).

As stated in chapter 4.1.3 in this present study, the lower the value of IC_{50} or EC_{50}, the higher the antioxidant activity. It was observed that for DPPH scavenging activity, fractions F2 and F3 had very strong antioxidant activity as their IC_{50} (0.09 ± 0.21 mg/ml and 0.02 ± 0.070 mg/ml respectively) were as low as the IC_{50} of the standard (0.01 ± 0.34 mg/ml) used, which indicates that this fraction has compounds that have rich antioxidant properties that can neutralize free radical molecules. The MCE and fractions F4 and F5 had medium antioxidant activity, whereas the remaining fractions showed weak antioxidant activity, making them poor antioxidants sources.

For the reducing power assay, also fractions F2 and F3 had very strong antioxidant activity as their EC_{50} (2.26 ± 0.01 mg/ml and 1.87 ± 0.00 mg/ml respectively) were as low as the EC_{50} of the standard (0.61 ± 0.01 mg/ml) used, indicating that these fractions have a high number of compounds that are reducers (antioxidants). Fractions F4 and F6 had medium antioxidant activity. The IC_{50} and EC_{50} of the MCE were higher than most of the fractions, showing that the MCE had weak antioxidant activity compared to the fractions, making it a poor antioxidants source if not purified.

To the best of our knowledge, no study has been published about the antioxidant properties of the stem bark of *S. africana*, however, a study by Amoo et al (2012) indicated that the leaves and twigs of the plant *S. africana* have potent antioxidant sources, wherein their radical scavenging activity percentage was 96.6 ± 0.06 mg/ml. This indicates that the tree (*S. africana*) can be a good source of antioxidant compounds.

Previous studies have reported that the antioxidant capacity of plants is related directly to the phenolics and flavonoid contents of plants, meaning the higher the TPC and TFC, the greater the antioxidant activity of the plant, making the phenolics and flavonoids very noteworthy compounds (Li et al., 2018; Kumar and Jain, 2015; Aghaei

et al., 2014; Shehata et al., 2014; Guo et al., 2011). From the results of this study, fractions F2 and F3 had the highest amounts of TPC and the lowest IC_{50} and EC_{50} values, which evidently agrees with results of other studies that phenolic compounds are indeed rich in antioxidant properties.

The interest and search for new antibacterial agent sources is very important due to the increasing number of multi-drug resistant pathogenic microbes. The secondary metabolites of plant extracts inhibit or kill the growth of microorganisms by different mechanisms, such as their ability to interfere with the phospholipid's bilayer of the cell membrane, leading to the increase of the cell's permeability and cellular constituents being lost (Nazzaro et al., 2013).

Although few studies have been done on the plant *S. africana* in search of its potential antibacterial activities and isolation of compounds, no data is yet available for the plant's bioactivity against the bacterial strains that are multidrug resistant, which are the strains mainly responsible for the high rate cases of morbidity and mortality globally (Tacconelli et al., 2018). Also, no data is documented specifically about the antibacterial properties of the plant *S. africana* that's located in Matsa village, Nzhelele.

The MCE and its various fractions were tested for their inhibitory activity against MDR bacterial strains. In this study, the zone of inhibition of the samples varied from 7 mm to 18 mm (table 4.4). Fraction F1 didn't show any zone of inhibition against any bacterial strain, which however does not necessarily indicate the absence of antibacterial activity. The growth of the MDR bacterial strain *K. pneumoniae* was not inhibited by any sample using the agar well diffusion assay, however, during the micro-plate dilution assay, its growth was inhibited at certain concentrations, this could be due to the fact that when using the agar well diffusion assay to determine antibacterial activity, the antibacterial effect could have been affected by the type of agar used, salt concentration, the incubation temperature or even the molecular size of the antibacterial constituent (Compean and Ynalvez, 2014).

Staphylococcus aureus was found to be the most susceptible bacteria to all the samples in different concentrations tested against, followed by *A. baumannii.* The highest zone of inhibition was exhibited by Fraction F3 against *S. aureus,* which was 18 mm. The MDR bacterial strain *E. faecium* was the least susceptible compared to the other strains.

The zone of inhibitions (in mm) obtained were used to calculate the percentage of inhibition at different concentrations reference to the positive control. Figure 4.7, 4.8 and 4.9 represent the relative percentage of inhibition by the fractions and methanolic crude extract at different concentrations (200 mg/ml, 100 mg/ml and 50 mg/m).

At the concentration of 50 mg/ml, few samples managed to inhibit more than 10% of some of the bacterial strains (*A. baumannii, P. aeruginosa and S. aureus*), wherein the highest % of inhibition was shown by fraction F2, which inhibited close to 50% of the bacterial growth of *A. baumannii*. At both concentrations of 100 mg/ml and 200 mg/ml, there is a significant increase in the percentage of inhibition by most of the samples compared to at 50 mg/ml. Fraction F2 and F3 inhibited more than 7 % growth of *A. baumannii.*

For the determination of minimum inhibitory concentration, microplate dilution assay was used. For this study, the MIC values of the MCE and its fractions ranged from 0.1 mg/ml to 12.5 mg/ml. Fractions F3 and F6 had the lowest average MIC values (0.52 mg/ml and 0.72 mg/ml respectively). The most sensitive MDR bacterial strain was *P. aeruginosa*, which had the lowest MIC values.

Minimum inhibitory concentration results of *S. africana*'s MCE and its fractions obtained in this study aligned with other studies done on the same plant (*S. africana*). Akhalwaya et al (2018) obtained MIC values that ranged between 0.05 to 8 mg/ml, their study was based on determining the antibacterial activity of *S. africana* against bacterial strains that cause oral infections. Mathabe et al (2006) investigated the antibacterial activity of *S. africana* against diarrheal causing agents, were they

obtained MIC valued that ranged between 0.05 mg/ml to 0.6 mg/ml. A study by McGaw et al (2000) also investigated antibacterial activity of *S. africana* against bacterial strains that cause diarrhoea, but however, only one MIC value was obtained from one bacterial strain (*S. aureus*) which was 3.31 mg/ml.

Overall antibacterial results of this study indicated that most fractions have better activity than the MCE. Fraction F3 had the best antibacterial activity compared to the MCE of *S. africana* and its other fractions since it had the overall lowest MIC average against the MDR bacterial strains and the highest overall zone of inhibition average against all tested bacterial strains. This then makes fraction F3 a potential source of antibacterial compounds.

Previous studies on *S. Africana* isolated 5 different types of terpenoids (three diterpenes and two triterpenes) and one phytochemical named stachenol which has not been classified yet (Mathabe at el., 2008; Munkombwe et al., 1997; Duri et al., 1992: Baarschers et al., 1962). Terpenoids isolated from these studies showed antibacterial activity against bacterial strains, therefore, it can be assumed that the antibacterial activity displayed in this study by *S. africana* against MDR bacterial strains could be due to the presence of terpenoids in the MCE and all the fractions. Other studies have also suggested that the antibacterial activity of different medicinal plants is due to the following phytochemicals; alkaloids, flavonoids, and tannins (Abdulhamid et al., 2014; Compean and Ynalvez, 2014; Vaghasiya et al., 2011; Savoia, 2012), and all these phytochemicals were detected in the plant and its different fractions making them the potential reasons behind *S. africana* antibacterial activities. The successful antibacterial activities of the MCE and fractions against the MDR bacterial strains indicate that *S. africana* consist of phytochemicals that can inhibit/kill the growth of MDR bacteria.

The ability of Medicinal plants to produce bioactive compounds that consists of antimicrobial substances can be a great platform in discovering new treatment for MDR bacterial infections, however, to predict that the plant's crude extract and its fractions are safe for use could be deceptive and harmful to the well-being of the

patient receiving treatment (Elisha et al., 2017). For this study, the toxicity of *S. africana* against Vero cells was evaluated using Cell-based high content screening. This assay uses a set of analytical methods (automated microscopy, multi-parameter imaging processors and visualization tools) for quantification of data extraction from cell populations. Vero cells used in this assay were initially exposed to the test samples (MCE /fractions) and then changes in cell morphology were detected using image analysis to detect whether the test samples were toxic or not to the Vero cells based on cell viability. Cell viability was expressed as nuclei per site of the cells treated with the samples compared to the untreated cells.

The toxicity effects of *S. africana*'s test samples varied in this study. Three of the fractions (F3, F5, and F6) at certain concentrations induced cell proliferation, which could potentially be an indication of the samples having beneficial effects if used as the treatment for various disorders or diseases. Fraction F5 can be considered the safer sample amongst all tested samples because, at both concentrations tested against, it was not at all toxic, however, it induced cell proliferation. At high concentrations, fractions F3 and F6 were toxic, though the toxicity was way lesser than that of the positive control, which can still indicate the safety of this samples.

Fraction F2 and MCE were the two test samples that were more toxic to the cells compared to the other samples. Both these fractions were toxic at all concentrations tested, and inhibited cell viability by more than 60%. The results of the MCE which is the crude extract of *S. africana* in this study agreed with those of Mathabe et al (2008), which reported that the crude extract of this plant is more toxic to the Vero cells compared to its fractions and compounds, hence purifying the crude extract can decrease the toxicity of this plant. Overall, all test samples were not as toxic as Melphalan (positive control) which was highly toxic to the cells, with an inhibition of 98%.

Fraction F3 showed the presence of promising biologically active phytochemicals that had good antioxidant properties, no /very low toxicity to cells and good antibacterial activity against MDR bacterial strains, making this fraction a potential source for

development of antibacterial drugs that can fight infections caused by MDR bacteria and justifies the use of *S. africana* for traditional therapy for curing certain infectious diseases.

Chapter 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

This study indicated that the medicinal plant *Spirostachys africana*'s methanolic crude extract and its fractions had antibacterial activity against multidrug-resistant bacteria and that the susceptibility of the MDR bacterial strains varied for each test samples (MCE and fractions) tested against. This study also showed that *S. africana* contained phytochemical constituents that can be good antioxidants and some that can potentially be used for anticancer studies since some test samples were toxic to cells (Vero cells).

This concluded that *S. africana* can be a potential source of antibacterial compounds that can inhibit the growth /kill MDR bacteria and possibly be used as sources for treatment of infections caused by MDR bacterial strain as seen from the results of this study. It also concluded that fractionation of the MCE does increase the biological activities of *S. africana*, since fraction F3 that was fractionated with 70% EE: 30% M had the best overall antibacterial activity, better antioxidant potential and was less cytotoxic compared to the MCE of *S. africana*.

5.2 RECOMMENDATIONS

- More studies on the isolation and characterization of bioactive compounds found in *S. africana* (located at Matsa village) need to be done to determine the exact compounds responsible for its biological activities, such as the antibacterial activity.
- Studies on anticancer potential of *S. africana* need to be partaken to determine if this plant possess compounds that can kill cancerous cells

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