BIOASSAY-GUIDED DETECTION OF ANTIMICROBIAL COMPONENTS FROM *SUTHERLANDIA FRUTESCENS* AND *SALVIA AFRICANA-LUTEA*

By

CHIDIMMA CYNTHIA ESTHER OBIDIGBO

Thesis submitted in fulfilment of the requirements for the degree
Master of Science: Biomedical Technology

in the
Faculty of Health and Wellness Sciences

at the
Cape Peninsula University of Technology

Supervisor: Dr Samantha Meyer
Co-Supervisor: Dr Lulama Mciteka

Bellville
2024

DECLARATION

I, Chidimma Obidigbo, declare that the contents of this thesis represent my own unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed:  Date: 26 September 2024

ABSTRACT

Plants remain beneficial to humans and have been used as a source of medicine for various ailments including infections. *Salvia africana-lutea* and *Sutherlandia frutescens* are endemic to South Africa and have been traditionally used to treat and manage a wide variety of infections including respiratory, urinary tract and skin infections, Human Immunodeficiency Virus, coughs and cancer. Many scientific studies have confirmed some of these medicinal activities.

The current study sought to investigate antibacterial properties of *Salvia africana-lutea* and *Sutherlandia frutescens*. To determine the respective minimum inhibitory concentrations, a thorough investigation of antibacterial activity against bacteria such as *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* was carried out using agar well-diffusion and microdilution assays.

Results indicate that *Salvia africana-lutea* extracts have better antibacterial activity in comparison to *Sutherlandia frutescens* extracts. The minimum inhibitory concentrations of *Salvia africana-lutea* extract ranged from 4 to 10mg/ml. When compared to *Sutherlandia frutescens,* which had very poor antibacterial activity; the aqueous, ethanol, and acetone extracts from *Salvia africana-lutea* showed good antibacterial activity against *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Salvia *africana-lutea extracts however* had little to no antibacterial activity against *Pseudomonas aeruginosa*.

Phytochemical techniques, such as Thin-Layer Chromatography, Bioautography, Nuclear Magnetic Resonance Spectrometry, and Liquid-Chromatography Mass Spectrometry were employed to detect and possibly identify the active fractions/compounds. A few compounds (relative to rosmarinic acid) were identified in acetone and ethanol extracts of *Salvia africana-lutea.* By identifying active compounds against medically significant microbes, this study adds a layer of gravitas to the already existing knowledge on medicinal properties of *Salvia africana-lutea*. Outputs of this research may provide leads to possible new drug formulations and preparations that might help control and reduce the burden of infections.

KEYWORDS: Antimicrobial, Bioactive Compounds, Bioautography, Medicinal Plants, Phytochemical*, Salvia africana-lutea*, *Sutherlandia frutescens*

ACNOWLEDGEMENTS

I thank:

- The Almighty God for this privilege, directions, and guidance all through my studies.
- Foremost, I would like to thank my noble supervisors Dr Samantha Meyer and Dr Lulama Mciteka, for their guidance and support throughout my time as their student.
- This work would not have been completed without the expertise of those who gave their support by giving me access to their laboratories at UWC Life Science Building and Chemistry Building: Prof Mervin Meyer, Prof Martin Onani, and Prof Wilfred Mabusela. And I also appreciate the patience of all the staff members and students there.
- Thanks to Mr. Emmanuel Okoye (my husband) for his support and Mrs. Franca Obidigbo (my beloved mother) who always encourages me.
- My father - Late Mr. Paul Obidigbo whose promise kept me going though he was not alive to witness this achievement.
- My senior colleagues, postgraduate students, and lab mates from CPUT and UWC for all their assistance and teaching: Dr Phumuzile Dube, Dr Mediline Goboza, Dr Caroline Tyavambiza, Ms Zanele Mganto-Khuselo, Mr Ndikho Nako, and Ms Prisca Kachepe.
- The financial assistance of the Cape Peninsula University of Technology towards my bursary is also well acknowledged.

DEDICATION

To my late father, who assured me that he would support me throughout my academic career to the farthest extent.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
ANOVA	Analysis of Variance
ATCC	American Types Culture Collection
CPUT	Cape Peninsula University of Technology
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EtOH	Ethanol
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MDR-TB	Multidrug-resistant tuberculosis
MH	Muller Hinton
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometer
MSE	Mass Spectrometry Electron Ionisation
NMR	Nuclear Magnetic Resonance
P. aeruginosa	*Pseudomonas aeruginosa*
PDA	Photodiode Array
QTOF	Quadrupole time-of-flight
RA	Rosmarinic Acid
Rf	Retention Factor
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SEM	Scanning Electron Microscope
TLC	Thin Layer Chromatography
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet
UWC	University of the Western Cape
XDR-TB	Extensively drug-resistant tuberculosis

CLARIFICATION OF TERMS

Antimicrobial agent: a substance that can kill, inhibit or slow the growth of microorganisms.

Antimicrobial resistance: the ability of microorganisms to grow and thrive in the presence of drugs that would normally restrict their natural growth or kill them

Antibacterial: substance that kills bacteria or stops them from growing and causing disease

Bactericidal: an agent that can kill bacteria.

Bacteriostatic: a bacterial growth-inhibiting agent that does not kill the bacteria

Commensal: an organism that causes no harm to the host.

Immunocompromised: an impaired or weakened immune system.

Infectious disease: disorders caused by microorganisms or their products; that can be spread from person to person.

Nosocomial infection: infection or disease acquired whilst in a hospital where the patient was admitted for reasons other than the infection.

Pseudomonas aeruginosa: Gram-negative rod-shaped bacteria that can cause a wide range of infections in immunocompromised individuals.

Retention factor (Rf): The Rf value of a compound (in thin layer chromatography) is equal to the distance travelled by the compound divided by the distance travelled by the solvent front (both measured from the origin).

Staphylococcus aureus: Gram-positive cocci bacteria that produces toxins and enzymes capable of causing a variety of infections.

***Staphylococcus epidermidis*:** Gram positive cocci that is normally a commensal but can cause infections in immunocompromised individuals.

Salvia africana-lutea: a medicinal plant traditionally used in the treatment of a variety of ailments such as skin disorders and wounds

***Sutherlandia frutescens*:** South African medicinal plant traditionally used in the treatment of cancer, tuberculosis, diabetes, anxiety, clinical depression, and as an immune system booster for people living with Acquired Immune Deficiency Syndrome.

Thin layer Chromatography: a chromatography technique that separates components in non-volatile mixtures.

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1.1. Introduction

Antimicrobial resistance (AMR) has become one of the most serious global health threats (Prestinaci *et al*., 2015). AMR is defined as the ability of microorganisms to grow and thrive in the presence of drugs that would normally restrict their natural growth or kill them (Antoñanzas and Goossens, 2019). Infections caused by AMR-pathogens are difficult to treat, thereby increasing chances of severe illness and death in affected patients (Salam *et al*., 2023). AMR causes significant challenges such as increased morbidity and mortality, extended hospital stays as well as increased need for intensive care therapy that may be accompanied by excessive surgery and the use of invasive devices (Friedman *et al*., 2016).

Antimicrobial agents have significantly reduced the impact of infectious diseases, however; the emergence of AMR bacterial strains now threatens the efficacy of available antibiotics. Inappropriate antibiotic consumption is a significant contributing factor to the problem of AMR by common pathogens (Michael *et al*., 2014). Methicillin-resistant *Staphylococcus aureus* is one of the most common AMR-bacterial strains. This *Staphylococcus aureus* strain is known to be resistant to a variety of β-lactam antibiotics such as penicillin and cephalosporin (Ali Alghamdi *et al*., 2023).

Given the severity of the AMR problem, finding alternative prevention and treatment strategies has become an urgent priority (Gellatly and Hancock, 2013). Plants are very beneficial to humans and can be used to develop therapeutic products. Wachtel-Galor and Benzie (2011) reported that close to 25% of active drug constituents are derived from medicinal plants. An estimated 80% of people living in developing countries use traditional medicines (World Health Organisation, 2008). As an example, South Africa has around 30 000 flowering plant species, most of which have medicinal benefits and are used for the treatment of various ailments. To date, many countries have become involved in medicinal plant research (Li and Xing, 2016). As a result, there is an active search for antimicrobial formulations from a variety of plant extracts; with the primary aim of reducing morbidity and mortality from diseases caused by AMR pathogens (Arip *et al*., 2022).

1.2. Problem Statement

The emergence of AMR pathogens has become a challenge in modern medicine. The high prevalence of Human Immunodeficiency Virus (HIV) and the Acquired Immunodeficiency Syndrome (AIDS) in Africa and South Africa in particular, result in high morbidity, high mortality and increased cost of treating infectious diseases. Many antibiotics have become ineffective in the treatment of common infectious diseases. At the same time, these AMR pathogens continue to spread uncontrollably. AMR has caused a significant increase in morbidity and mortality from infections that were previously treatable before the emergence of the AMR problem. Because of the failure of most currently prescribed antimicrobial drugs against AMR bacteria, there is an urgent need to find alternative means to treat diseases caused by these bacteria.

1.3. Aim

The aim of this study was to identify antibacterial fractions/compounds present in *Sutherlandia frutescens* and *Salvia africana-lutea* extracts.

1.4. Objectives

- Prepare aqueous, ethanol, and acetone extracts of *Sutherlandia frutescens and Salvia africana-lutea.*
- Determine the antibacterial activity of *Sutherlandia frutescens* and *Salvia africana-lutea* extracts against *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*
- Phytochemically detect antibacterially active plant extracts by means of Thin Layer Chromatography and Bioautography
- Chemically identify and characterize active fractions/compounds via Nuclear Magnetic Resonance Spectroscopy and Liquid-Chromatography Mass Spectrometry analysis.

1.5. Research Questions

- Will aqueous, ethanolic and acetone extracts of *Sutherlandia frutescens* and *Salvia africana-lutea* have antibacterial activity against *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*?
- What are the antibacterially-active fractions/compounds present in tested plant extracts?

CHAPTER 2
LITERATURE REVIEW

2.1. Background of Research

Many plants have been proven to possess medicinal properties such as antimicrobial and wound healing properties, among others. *Sutherlandia frutescens* and *Salvia africana-lutea* are known to have good antimicrobial activities in addition to other medicinal benefits. Because of their broad-spectrum antimicrobial activity, they may possess active compounds that can be used in drug discovery, specifically in combating the problem of AMR. AMR continues to threaten the lives of many individuals worldwide and has rendered many standard treatments against infections ineffective, making infections harder and even impossible to control.

Medicinal plant research has become popular due to the increased search for new, effective, and affordable drugs. Many plants, such as *Sutherlandia frutescens* and *Salvia africana-lutea* have been proven to possess medicinal properties, including antimicrobial, antioxidant, anti-inflammatory and wound healing activities (van Wyk & Abrecht, 2008; Ezema et al., 2024). The aim of this study was to identify antimicrobial fractions/compounds present in *Sutherlandia frutescens* and *Salvia africana-lutea*. These plants are used traditionally to treat various ailments, including infections. This study is essential as it provides new information on medicinal properties of *Sutherlandia frutescens* and *Salvia africana-lutea* by identifying active compounds against infectious bacteria. This could potentially lead to new drug formulations and preparations that might help control and reduce the burden of infections.

2.2. Antimicrobial Resistance

The injudicious use of antibiotics is a major contributing factor to the worldwide problem of AMR (Sabtu *et al*., 2015). The large-scale use of antibiotics in clinical practice has prompted microorganisms to develop corresponding multi-resistant mechanisms to protect themselves against antibiotics designed to kill them (Salam *et al*., 2023). Because of this, microorganisms may continue to survive even in the presence of drugs intended to kill them, causing a phenomenon known as AMR. In this way, AMR hinders effective prevention and treatment of the ever-increasing range of infections caused by microorganisms.

Once resistance has emerged, AMR strains can be spread by failure to adhere to infection control measures, which can occur within healthcare settings as well as in communities (Sabtu and Enoch, 2015). 'ESKAPE' pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella Pneumonia, Acinetobacter* baumanii, *Pseudomonas aeruginosa,* and *Enterobacter species*) are emerging threats associated with AMR. 'ESKAPE' pathogens can "escape" the effects of antimicrobial agents and cause many hospital-acquired infections (Peterson, 2009). These bacteria are common causes of life-threatening nosocomial infections amongst critically ill and immunocompromised individuals and are characterized by potential drug resistance mechanisms (Rice, 2010). Methicillin-resistant *Staphylococcus aureus is* a type of *Staphylococcus aureus* bacteria that has become resistant to β-lactam antibiotics like penicillin (methicillin and oxacillin) and cephalosporin (Ali Alghamdi *et al*., 2023). Among Gram-negative bacteria; *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter Baumannii* have become highly resistant to prescribed antibiotics (Sabtu and Enoch, 2015).

Mycobacterium tuberculosis strains, which are resistant to currently prescribed drugs, have also emerged. Multi-Drug-Resistant Tuberculosis (MDR-TB) resistant to at least rifampicin plus isoniazid emerged in the 1990s. Extensively Drug-Resistant Tuberculosis (XDR-TB) caused by mycobacterium resistant to isoniazid plus rifampicin, any fluoroquinolone and at least one of the three injectable second-line drugs (kanamycin, amikacin or capreomycin) has also emerged (Jassal and Bishai, 2009; Seung *et al*., 2015).

2.2.1. Mechanisms of Antimicrobial Resistance

Microorganisms can naturally be resistant to an antibiotic, or they may develop resistance to antibiotics after being exposed to them, a phenomenon known as acquired resistance (Sabtu and Enoch, 2015; Jian *et al.,* 2021). There are many ways by which microorganisms can develop antibacterial resistance against antibiotics (**Figure 1**). These include elimination of antibiotics from the bacterial cell through efflux pumps and modification of genes that code for proteins targeted by antibiotics (Lin *et al.,* 2015). In addition to this, many pathogenic bacteria can form dense biofilms, which further protect the community of bacteria from antibiotics, making the bacteria highly- resistant (Sharma *et al*., 2023).

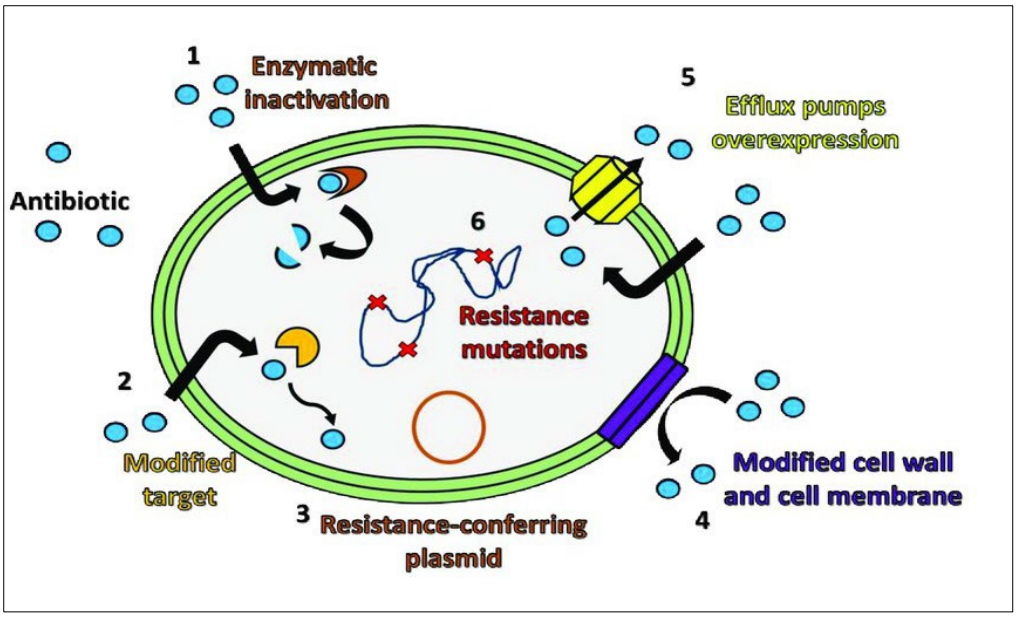


Figure 1. Mechanisms of antimicrobial resistance (De Gaetano *et al*., 2023).

β-lactam antibiotics are the most widely available antibiotics for treating various bacterial infections. Examples of β-lactam antibiotics include penicillin, carbapenems, monobactams and cephalosporins. β-lactam antibiotics contain a 4-membered lactam ring in their structure and act by inhibiting growth of bacteria by inactivating penicillin-binding proteins which are crucial in the biosynthesis of the cell wall (Waxman and Strominger, 1983; De Angelis *et al*., 2020; Pandey and Cascella, 2023). Some bacteria have evolved mechanisms to produce the enzyme β-lactamase, which hydrolyses the β-lactam ring of antibiotics, rendering these antibiotics ineffective in the fight against targeted bacteria. Extended-spectrum β-lactamase renders bacteria resistant to extended-spectrum penicillin and cephalosporins and has been detected in *Enterobacteriaceae*, including *Escherichia coli* (Seo *et al*., 2018; Song *et al*., 2020; Clemente *et al*., 2021).

Multidrug efflux systems are another way by which bacteria become resistant to antibiotics (Huang *et al.,* 2022). Under normal circumstances, antibiotics must enter the bacterial cell through the microorganism's cell membrane to attack specific targets within the cell. Multidrug efflux pumps, synthesised by bacteria, act by reducing the concentration of drugs within the cell. These pumps are found in the bacterial cell membrane and periplasm, where they remove various compounds, such as antimicrobials, toxic heavy metals and organic solvents from bacterial cells (Piddock, 2006). Because of the effect of efflux pumps, the rate at which drugs are excreted is usually faster than the drug penetration rate. This causes the overall amount of antibiotics entering bacterial cells to be reduced to a non-sensitive level (Nikaido and Pages, 2012).

Aminoglycosides are another class of antibiotics used for the treatment of bacterial infections. Examples of aminoglycosides include kanamycin, amikacin, gentamycin, neomycin and dibekacin Ahmed *et al*., 2020). Aminoglycosides inhibit bacterial protein synthesis. As a result, bacteria produce proteins with incorrect amino acids, affecting the integrity of the bacterial cell membrane. Once bacteria start to synthesize aminoglycoside-modifying enzymes, they become resistant to effects of aminoglycosides (Kong *et al*., 2020).

Bacteria may become resistant to antibiotics by modifying genes that encode for proteins targeted by antibiotics (Reygaert, 2018). Because the bacteria are no-longer able to synthesize the usual proteins targeted by the drug, antibiotics fail to exert their effects on bacterial cells. By modifying target sites, antibiotics cannot effectively bind to the bacteria; hence the antibiotics cannot exert any impact on the bacteria. In this way, bacteria continue to thrive even in the presence of antibiotics they have developed resistance against (Lambert, 2005).

2.3. Common Skin Pathogens

A wide variety of pathogens like bacteria, fungi and viruses can cause skin infections. The most common bacteria that cause skin and wound infections are *Staphylococcus* species, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Acinetobacter baumannii* (Puca *et al*., 2021). Bacteria can find a suitable environment within the wound and proliferate in deeper tissues of the skin (Khan *et al*., 2017; Negut *et al*., 2018).

2.3.1. *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) (**Figure 2**) belongs to the Gram-positive bacteria family and has catalase and coagulase-positive enzymatic reactions. This bacterium can live as a commensal in the nose, throat and skin; however, it tends to be pathogenic and induce illnesses by directly invading tissues and producing toxins and enzymes (Michael, 2022). Infections by *S. aureus* have increased proportionally to the number of immunocompromised individuals and in-line with the bacteria's evolving antibiotic resistance through mutation and uptake of mobile genetic elements. *S. aureus* can cause local skin infections such as dermatitis, impetigo and cellulitis. It can also cause deep-seated infections such as bacteraemia and endocarditis (Otto, 2014). Additionally, *S. aureus* is one of the primary causes of nosocomial infections, which in some cases can be fatal (Murray *et al*., 2019).

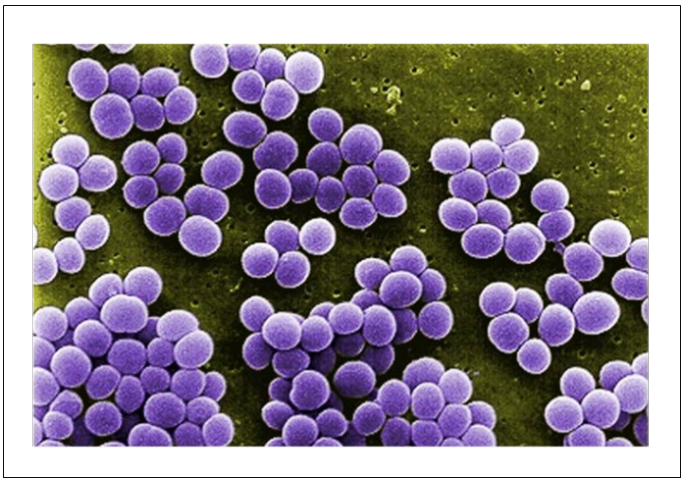


Figure 2. *Staphylococcus aureus* (SEM image) (Arduino, 2001).

2.3.2. Methicillin-resistant *Staphylococcus aureus*

It has also been shown that *S. aureus* can acquire antimicrobial resistance. As an example, its acquisition of the *mecA* gene enables *S. aureus* to resist methicillin, hence the name Methicillin-resistant *Staphylococcus aureus* (MRSA) **(Figure 3)** (Marín *et al*., 2015). The spread of MRSA can be reduced by adhering to proper personal hygiene procedures, environmental sanitation and wearing of protective gear like gloves, especially by healthcare workers. Having a separate apartment for infected patients and adequate cleaning of hospital equipment, such as stethoscopes can also prevent the spread of MRSA (Tong *et al*., 2015).

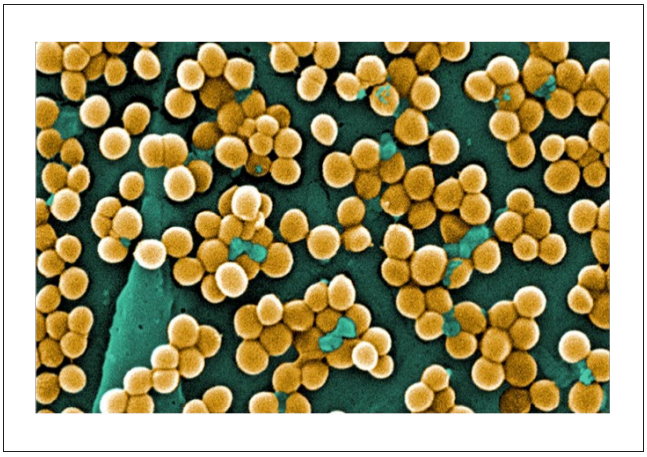


Figure 3. Methicillin-resistant *Staphylococcus aureus* SEM image (CDC, 2005)

2.3.3. *Staphylococcus epidermidis*

Staphylococcus epidermidis (*S. epidermidis*) **(Figure 4)** is an example of an opportunistic pathogen. This Gram-positive bacterium is also able to produce the enzyme coagulase (Pinheiro *et al.*, 2015). *S. epidermidis* is the most common skin and mucous membrane commensal. It is however capable of causing disease in immunocompromised patients (Buttner *et al.*, 2015). *S. epidermidis* is the main cause of infections from medical devices such as central or peripheral intravenous catheters. Infections caused by medical devices are a major problem in regions where medical resources are very limited, such as most South African rural areas. Infections caused by catheters increase the mortality rate, hospital stays and healthcare bills. Infection control and prevention is the best approach in dealing with *S. epidermidis* and *S. aureus* (Chessa *et al*., 2015).

S. epidermidis is also considered a noble cause of nosocomial infections. Its infections are difficult to treat because this bacteria is shielded from host immune response and antibiotics by biofilms (Khodaparast *et al.*, 2016). Innate host defences, mainly phagocytes, monocytes and neutrophils have a critical responsibility in fighting cellular debris and microorganisms, such as *S. epidermidis* (Cheung *et al*., 2010). Investigations have been done and found that several molecular determinants potentiate chronic disease and allow *S. epidermidis* to escape immune response (Sabaté Brescó *et al*., 2017).

Figure 4. *Staphylococcus epidermidis* SEM image (Science Photo Library, 2010).

2.3.4. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (P. aeruginosa) (**Figure 5**) is a Gram-negative, non-sporing, rod-shaped bacterium. It is classified under proteobacteria and is the second most common pathogen in hospitalised patients in Europe (Koehnke and Friedrich, 2015). This opportunistic bacterium can thrive in a wide range of natural and artificial settings, including medical facilities (Gellatly and Hancock, 2013). As such, *P. aeruginosa* is one of the leading causes of nosocomial infections. Raman *et al.* (2018) reported that multidrug-resistant *P. aeruginosa* is a major public health concern whose treatment requires increased hospital resource utilisation and is associated with increased morbidity and mortality. Most *P. aeruginosa* infections manifest in immunocompromised individuals such as those with cystic fibrosis, neutropenia, and severe burns (Mowat *et al*., 2011). Life-threatening nosocomial infections associated with *P. aeruginosa* include pneumonia, urinary tract infections, bacteraemia, cystic fibrosis, bone and joint infections, gastrointestinal infections and other systemic infections (Gellatly and Hancock, 2013).

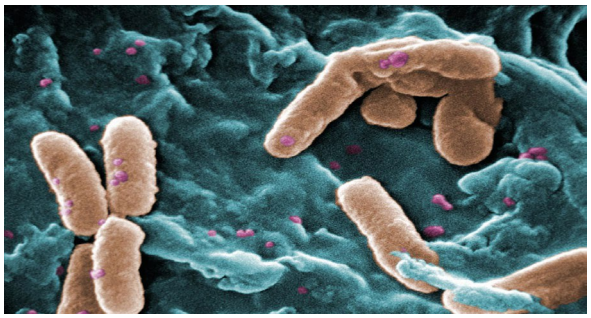


Figure 5. *Pseudomonas aeruginosa* SEM image (CDC, 2012).

2.4. Medicinal Plants

With the diminishing efficacy of synthetic drugs and the increasing number of contraindications associated with their usage, the relevance of natural remedies is once again in the spotlight. Because medicinal plants are natural, they are relatively safer and have fewer side effects compared to synthetic antimicrobials (Parekh and Chanda, 2007). Plants produce diverse phytochemicals, many of which are effective against pathogenic microbes and can be explored for developing novel antimicrobials (Vaou *et al.,* 2021). Systematic screening of

phytochemical constituents in medicinal plants with antimicrobial properties can lead to the identification of novel antimicrobials with unique mechanisms of action that can fight AMR pathogens (Ashraf *et al.,* 2023).

Plant-based compounds have been used for the treatment and management of various microbial diseases, such as wounds and inflammatory diseases (Phukan *et al.* 2023). Bacterial infections in wounds are known to hinder effective and timely wound-healing. The ability of pathogenic species to form biofilms cause difficult-to-treat infections and high mortality rates (Di Domenico *et al.,* 2022). Medicinal plants have been used by humans and for animals to treat various external wounds such as chronic, deep, open, suppurative, incised, lacerated and ulcerated wounds (Adetutu, 2011; Mummed *et al*., 2018).

Numerous *in-vitro* and *in-vivo* studies have been conducted on the efficacy of plant phytochemicals as antimicrobial agents (Patra, 2012). Plant extracts and essential oils can work against bacterial strains in various ways. Plant extracts with antimicrobial activity can disrupt the phospholipid bilayer of cell membranes increasing permeability and causing loss of cellular components. Enzymes responsible for producing energy and synthesizing structural components in microorganisms can be damaged when exposed to plant extracts with antimicrobial properties (Barbieri *et al.,* 2017). Some antimicrobial plant extracts act by destroying or inactivating genetic material of invading microorganisms. This usually disturbs the cytoplasmic membrane, impairing active transport, proton-motive force, electron flow and coagulating pathogen cell contents (Kotzekidou *et al*., 2008).

2.5. Phytochemicals

Plants are rich in a wide variety of secondary metabolites such as alkaloids, tannins, terpenoids and flavonoids. These phytochemicals have been found to have antimicrobial properties *in-vitro* (Yadav & Upadhyay, 2022). Phytochemicals are natural chemicals present in plants (Iyer *et al*., 2023). They can be divided into many major classes depending on their chemical structures, biosynthesis pathways, biological properties and botanical origins (Patra, 2012). The most common phytochemical classification is based on their chemical structures, where phytochemicals are classified as alkaloids, phenolics, saponins, terpenoids and many more (Behl *et al.,* 2021). Phytochemicals are enriched in different parts of the plant and can be concentrated using various extraction solvents to yield crude extracts. Through ongoing scientific advances, protocols have been developed for selective enrichment or purification of individual plant metabolites with known antimicrobial functions (Atanasov *et al.* 2015, 2021).

2.5.1. Alkaloids

Alkaloids are nitrogen-containing heterocyclic compounds commonly isolated from plants (Wansi *et al.,* 2013). Because of their large numbers and structural diversity, alkaloids are one of the most important types of natural products. They can be classified based on their chemical core structures into quinolines, isoquinolines, indoles, piperidine alkaloids, *etc*. (Yan *et al.,* 2021). Alkaloids comprise approximately 20% of known secondary metabolites in plants (Kaur & Arora, 2015). They protect plants from predators and regulate plant growth (Chik *et al*., 2013). Therapeutically, alkaloids are well-known for their cardioprotective, anaesthetic and anti-inflammatory properties. Morphine, nicotine, ephedrine, strychnine and quinine are examples of alkaloids used in clinical practice **(Figure 6)** (Kurek, 2019).

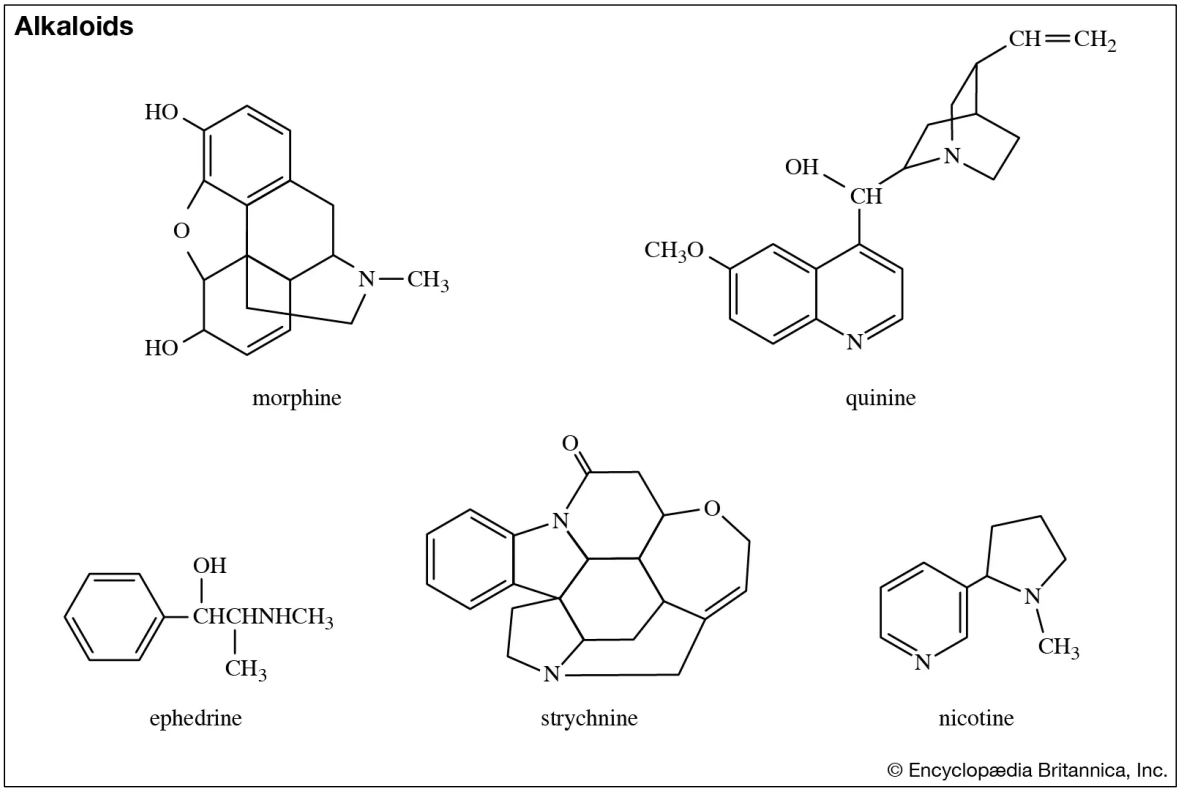


Figure 6. Chemical structures of well-known alkaloids: morphine, quinine, nicotine, strychnine and ephedrine (Halstead *et al.,* 2024).

Alkaloids can also exert antimicrobial effects on pathogens. They can inhibit bacterial growth through various mechanisms such as preventing synthesis of bacterial nucleic acids and proteins, modifying bacterial cell membrane permeability, damaging bacterial cell membrane and cell wall, inhibiting bacterial metabolism and inhibiting bacterial efflux pumps (Yan *et al.,* 2021).

2.5.2. Phenolics

Natural phenolic compounds are secondary metabolites produced by plants for various functions, including for antimicrobial defence. They are a group of naturally occurring phytochemicals that include phenolic acids, flavonoids, lignans and stilbenes **(Figure 7)** (Rudrapal *et al*., 2022). Phenol is the basic skeleton in polyphenols. Polyphenols are characterised by poly-hydroxy substitution of benzene rings and in many cases, they are combined with monosaccharides or polysaccharides in the form of glycosides. They can also be combined with organic acids to produce certain derivatives such as esters or methyl esters (Chen *et al.,* 2024).

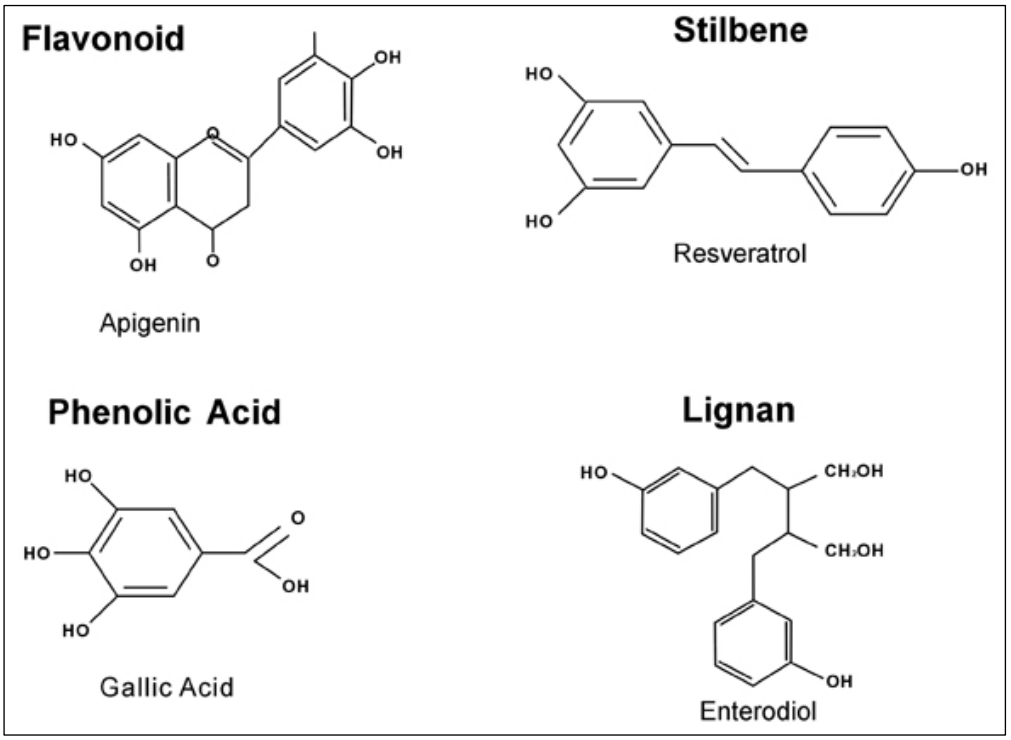


Figure 7. Examples of polyphenolic compounds (Yoon and Baek. 2005)

Plant-derived polyphenolic compounds such as flavonoids and phenolic acids have been shown to demonstrate antimicrobial properties against a broad spectrum of microbes, including multidrug-resistant strains (Miklasińska-Majdanik et al., 2018). As such, polyphenolic compounds are promising weapons in the fight against microorganisms including AMR-bacterial strains (Zacchino *et al.,* 2017). Miklasińska-Majdanik *et al. (*2018) reported that phenolic compounds inhibit bacteria by damaging bacterial membranes, inhibiting virulent factors such as enzymes and toxins as well as suppressing bacterial biofilm formation and exerting synergistic effects when combined with common synthetic drugs (Miklasińska-Majdanik *et al.,* 2018).

2.5.3. Terpenes and their Derivatives

Terpenes are a large group of naturally occurring phytochemicals commonly derived from essential oils (Yadav and Upadhyay, 2022; Iyer *et al.,* 2023). They have characteristic aroma, taste and colour. These features help them repel parasites and act as pesticides. Terpenes also have distinct antimicrobial properties that help plants build immunity against bacteria, fungi, viruses and plasmodium species (Cox-Georgian *et al.,* 2019). They are reported to exhibit bacteriostatic and bactericidal effects against common pathogens (Mahizan *et al.,* 2019). In humans, plant terpenes can exert a wide variety of therapeutic effects such as antimicrobial, antidiabetic, antioxidant, anti-inflammatory and immunomodulatory activities (Yadav and Upadhyay, 2022).

Terpenoids are derivatives of terpenes made by adding or removing functional groups on Terpenes (Barbieri *et al.,* 2017). Examples of terpenoids include mono-terpenoids sesqui-terpenoids and diterpenoids are shown in **Figure 8**.

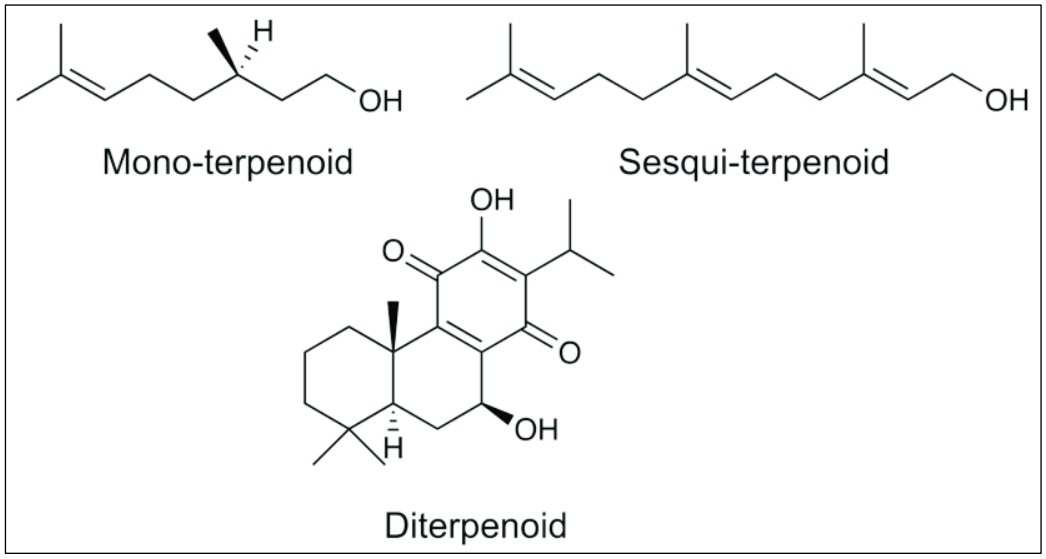


Figure 8. Examples of Terpenoids (Lozada-Ramírez *et al*., 2021).

The antimicrobial activity of terpenoids is determined by their functional groups. Griffin *et al.* (1999) reported in their study that most terpenoids work by inhibiting oxygen uptake and oxidative phosphorylation. (Mahizan *et al.,* (2019) reported that terpenoids have antiseptic potential according to their solubility in water. Their antibacterial action therefore, will depend on factors such as lipophilicity, hydrophobicity and the presence of hydroxyl groups (Zengin Baysal, 2014).

2.6. Medicinal Plants of Interest for the Current Study

2.6.1. *Sutherlandia frutescens*

Sutherlandia frutescens (tribe Galegeae, Fabaceae) is a popular plant in traditional medicine. This leguminous plant (**Figure 9**) is native to South Africa, Lesotho, southern Namibia and southeastern Botswana (van Wyk and Albrecht, 2008). *Sutherlandia frutescens* is drought-tolerant and grows widely in the Western, Eastern and Northern Cape provinces of South Africa as well as in certain parts of KwaZulu-Natal (Aboyade *et al*. 2014). *Sutherlandia frutescens* is locally known as cancer bush, *kankerbos* (Afrikaans) and *unwele* (Zulu) (Ntuli *et al.,* 2018).

Figure 9. *Sutherlandia frutescens* flowering plants (Intelezi African Herbs, 2024)

2.6.1.1. Taxonomy of *Sutherlandia frutescens*

Sutherlandia frutescens, also known as *Lessertia frutescens,* is a prostrate to erect short-lived shrub that varies in height from 0.2 to 2.5 m. Stems have numerous leaves borne mainly towards the tips. The leaves are shortly petiolate, stipulate and pinnate, with ±8 pairs of opposite leaflets and a terminal leaflet. The flowers are red and borne in few-flowered axillary racemes (van Wyk & Albrecht, 2008). Each pod bears many pale brown to dark brown, laterally compressed and kidney-shaped, smooth to markedly wrinkled seeds (van Wyk and Albrecht, 2008; Aboyade *et al.,* 2014).

2.6.1.2. Ethnomedicinal uses of Sutherl*andia frutescens*

Sutherlandia frutescens has a long history of use in traditional medicine for the treatment of cancer, topical wounds, gastritis, dysentery, asthma, fever, diabetes, gonorrhoea, syphilis, stress, depression, arthritis, HIV and epilepsy (van Wyk & Albrecht, 2008; Ntuli *et al.,* 2018 & Hlongwane *et al*., 2023). It is also commonly included in polyherbal formulations administered by traditional healers who claim that it can boost the immune system to fight disease (Chen *et al.,* 2018).

2.6.1.3. Phytochemical compounds in *Sutherlandia frutescens*

The biological activities of *Sutherlandia frutescens* such as antibacterial, antiviral, anticancer and antioxidant properties depend on the type and concentrations of phytochemicals present (van Wyk & Prinsloo, 2020). Phytochemical compounds identified in *Sutherlandia frutescens* include arginine, canavanine, asparagine, flavonoids, gamma amino butyric acid, triterpenoids and pinitol (Hlongwane *et al.,* 2023).

2.6.1.4. Antimicrobial properties of *Sutherlandia frutescens*

Extracts of *Sutherlandia Frutescens* leaves have been reported to have moderate antibacterial activity against a range of pathogenic microorganisms including *S. aureus, Enterococcus faecalis and Escherichia coli* (Katerere & Eloff, 2005; Hübsch, *et al.* 2014). In a study by Nosov *et al.* (2023), extracts of *Sutherlandia frutescens* cell biomass suspension culture demonstrated some inhibitory effect against Gram-positive *S. aureus* but not against Gram-negative *P. aeruginosa*.

2.6.2. *Salvia africana-lutea*

Salvia africana-lutea **(Figure 10)** of the *Lamiaceae* family is a well-known South African medicinal plant, locally known in Afrikaans as *geelblom-salie*. This drought-tolerant plant is indigenous to the Western Cape province and has been utilised for decades to treat various human diseases, such as colds, flu, coughs and body sores (Van Wyk, 2004; Van Jaarsveld, 2013).).

(i) *SAL* flowering plant

(ii) *SAL* plants at the UWC Campus

Figure 10. *Salvia africana-lutea* plant images (San Marcos Growers, 2024.)

2.6.2.1. *Taxonomy of Salvia africana-lutea*

Salvia africana-lutea is an evergreen shrub that belongs to the Lamiaceae (Labiatae) family, commonly referred to as the sage family. It is native to South Africa, although it may be found in other African and Asian countries (Rattray & Van Wyk, 2021). *Salvia africana-lutea* grows to a height of up to 2m. It grows in coastal and sandy areas and is resistant to drought. *Salvia africana-lutea* plants have aromatic grey-greenish leaves and unique clusters of brown funnel-shaped flowers (Wester & Claßen-Bockhof, 2006; Wester, 2013).

2.6.2.2. Traditional uses of *S. africana-lutea*

Salvia africana-lutea is an essential plant species used in traditional medicine. The leaf decoction is used for treating coughs, colds and female reproductive illnesses (Scott *et al*., 2004; Van Wyk & Gorelik, 2017). The first European settlers used *Salvia africana-lutea* leaf aqueous extracts to treat colds, tuberculosis, and chronic bronchitis. Traditional indigenous healers, on the other hand, use ethanol extracts to treat respiratory illness, influenza,

gynaecological problems, fever, headache and digestive disorders (Kamatou *et al*., 2008; Arief *et al.,* 2010; Rattray & Van Wyk, 2021). *Salvia africana-lutea* has also been used by Africans to flavour food (Afonso *et al*., 2019*)*.

2.6.2.3. Phytochemical constituents of *Salvia africana-lutea*

Phytochemical investigations on *Salvia africana-lutea* have shown the presence of many bioactive constituents. Through research, bioactive constituents have been isolated and characterised from aerial parts of *Salvia africana-lutea* and systematically evaluated for various pharmacological activities. Bioactive constituents, including non-volatile and volatile metabolites from essential oils obtained from *Salvia africana-lutea*, were analysed by gas chromatography-mass spectrometry and gas chromatography with a fame ionization detector, and results showed that monoterpenes, diterpenes, triterpenes and sesquiterpene were the primary active metabolites (Bisio *et al.,* 1998a, b; Najar *et al.* 2021).

Phytochemicals from *Salvia africana-lutea* can be used as starting blocks for designing new drugs (Calderón- Oropeza *et al.* 2021). Previous studies have reported interesting pharmacological activities of *Salvia africana-lutea* extracts and phytochemicals such as its ability to suppress microbial infection as well as reduce inflammation, oxidative and related disorders (Ezema *et al.,* 2024).

2.6.2.4. Antibacterial properties of *Salvia africana-lutea*

Experimental research has established that *Salvia africana-lutea* has significant antibacterial activity against the following bacterial strains (*S. epidermidis, P. aeruginosa, S. aureus* and *Escherichia coli (*Ezema *et al.,* 2024*)*. Afonso *et al.* (2019) reported that 100 µl of *Salvia africana-lutea* aqueous extract had significant inhibition of *S. aureus* and *S. epidermidis* at 1:400 dilution, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) estimated to be 0.63 and 1.25 mg/ml, respectively. Furthermore, a study by Dube *et al.* (2020) established that *Salvia africana-lutea* silver nanoparticles improved antibacterial activity against *P. aeruginosa* and *S. epidermidis* with MIC values of 0.375 mg/ml and 0.1875 mg/ml, respectively.

2.7. Phytochemistry

Phytochemistry is the study of chemicals produced by plants particularly secondary metabolites. It is a scientific discipline that focuses on the isolation, analysis, purification and characterisation of chemical structures and biological activities of phytochemical compounds (Egbuna *et al.,* 2018). Phytochemicals produced by plants are responsible for preventing diseases and promoting good health. Extensive research is being done to establish the efficacy of phytochemicals as medicine and understand their mechanisms of their action (Saxena *et al.,* 2013).

Through phytochemistry, it is possible to establish structural compositions of plant metabolites as well as analyse their biosynthetic pathways, medicinal and commercial applications. Knowledge derived from phytochemistry is essential in the search for new drugs, repurposing of existing drugs, characterisation and standardisation of traditional herbal drugs, assessment of plant toxicity, biotechnology and phytoremediation of toxic substances such as poisons and heavy metals (Egbuna *et al.,* 2018). The most used techniques in photochemistry are extraction, isolation, chromatographic techniques, mass spectrometry and NMR (Gallo, 2022). Examples of techniques used in phytochemistry include Thin-Layer Chromatography, Bioautography, Mass Spectrometry and Nuclear Magnetic Resonance and these are discussed below.

2.7.1. Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a quick, affordable and sensitive analytical technique used to determine the number of components in a mixture as well as verify the purity of a compound (Cai, 2014). This technique has been successfully integrated with other techniques for precise and quality detection of potential drug compounds. TLC works on a solubility rule and the procedure is comprised of stationary and mobile phases. The solubility rule *"like dissolves like"* is followed. The more similar the physical properties of the compound is to the mobile phase, the longer it will stay in the mobile phase. As such, the mobile phase will carry the most soluble compounds furthest up the TLC plate (Bele and Khale, 2011). TLC is performed on a sheet of solid surface such as glass, plastic or aluminium foil coated with adsorbent material, which is the solid/stationary phase (Scott, 2004). The mobile phase is a solvent chosen according to the properties of the components in the mixture to be separated (Kumar *et al.,* 2013). During the procedure, the mobile phase is drawn up through the stationary phase by capillary action allowing various compounds within the mixture to separate based on their solubility and retardation in stationary and mobile phases. Silica gel, which is polar in nature, is the most common stationary phase used in research. Highly polar compounds in the

mixture will have strong interaction with silica gel and get separated first, while non-polar compounds will separate last (Lade *et al.,* 2014).

2.7.2. Preparative TLC

Preparative TLC is a chromatographic technique used to separate and isolate amounts of material larger than those that can be separated using analytical TLC. This technique can be used to isolate amounts of 10 – 1000mg for structure elucidation (mass spectrometry), infrared, UV and Nuclear Magnetic Resonance (NMR) (Sherma & Fried, 1987; Primdahl *et al.,* 2022). Compounds to be separated are applied as long streaks rather than spots in the sample application zone. The compounds separate as bands rather than spots. Each band is then scraped off and extracted with a suitable solvent from the desired layer. Compounds recovered from the desired layer may be purified further by TLC or other methods (Sherma & Fried, 1987). Preparative TLC is more efficient in terms of speed and cost compared to flash column chromatography. Additionally, the separated compounds can easily be detected and isolated from the chromatogram (Wing & Bemiller, 1972).

2.7.3. TLC Bioautography

Bioautography is a microbiological screening method used for detecting antimicrobial properties of various substances (Choma & Grzelak, 2011). It integrates chromatographic separation with biological activity detection technology (Wang *et al.,* 2021). Bioautography has enabled rapid progress in the detection of new antimicrobial compounds from natural compounds such as plant extracts (Suleiman *et al.,* 2010). When performing direct TLC bioautography, the developed TLC plate is dipped into a bacterial suspension (Dewanjee *et al.,* 2015). Inhibition zones are formed in places where antimicrobial components are located. These compounds can then be further identified using spectroscopic techniques (Jesionek *et al.,* 2015). Bioautography avoids the time-consuming isolation of inactive compounds. This method is fast and permits bioassay-directed fractionation of bioactive compounds (Suleiman *et al.,* 2010).

2.7.4. Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic resonance (NMR) spectroscopy has been recognised as a powerful platform for obtaining the metabolite profile of plant extracts (Selegato *et al.,* 2019). It allows the molecular structure of plant compounds to be analysed by observing and measuring the

interaction of nuclear spins when placed in a powerful magnetic field (Emwas *et al.,* 2020). NMR experiments can be carried out under physiological temperature and atmospheric pressure (Baldwin & Kay, 2009; Takeuchi *et al.,* 2019). NMR goes a long way in studying how drugs interact with their targets. This quality is critical in drug design because it is important to understand the interaction between an enzyme of interest and its ligands. One- dimensional NMR spectrometry is by far the most common NMR experiment used for drug studies. It has simple hardware requirements and requires less running time, making it attractive for NMR high-throughput studies. NMR can also be extended to multi-dimensional methods such as 2D, 3D and 4D (Emwas *et al.,* 2020).

2.7.5. Liquid-Chromatography Mass Spectrometry

Liquid-chromatography mass spectrometry (LC-MS) is an analytical technique that combines physical separation capabilities of liquid chromatography with mass analysing capabilities of mass spectrometry. It couples high-resolution chromatographic separation with sensitive and specific mass spectrum detection (Subramani *et al*., 2015). The method involves separation of components by liquid chromatography. The separated sample components are then sprayed into an atmospheric pressure ion source where they are converted into ions in gas. The ions are then converted and sorted according to their mass-to-charge ratio. The detector will count ions emerging from the mass analyser and amplify signal generated from each ion. This create a mass spectrum, which is then used to determine the isotopic nature of the sample, masses of particles/molecules and elucidate chemical structures of molecules (Kofmacher, 2005; Lim & Lord, 2002). LC-MS is useful for analysing small molecules and elucidating structures and chemical properties of different molecules in substances with multi-components (Mukherjee, 2019)

CHAPTER 3
RESEARCH DESIGN AND METHODOLOGY

3.1. Type of Study

This research project was a quantitative, analytical, experimental laboratory study.

3.2. Study Sites

Experimental work was done in research laboratories at the Department of Biomedical Sciences at the Cape Peninsula University of Technology (CPUT) and at Departments of Biotechnology and Chemistry at the University of the Western Cape (UWC).

3.3. Ethics Approval

This study was granted ethics approval by the Health and Wellness Sciences Research Ethics Committee at CPUT, in response to the minimal/negligible risk application submitted.

3.4. Plant Material

Fresh whole plants of *Salvia africana-lutea* were collected at UWC in Autumn, while *Sutherlandia frutescens* plant material was purchased from a commercial nursery in Cape Town, South Africa.

3.5. Preparation of Plant Extracts

Salvia africana-lutea leaves and stems were washed thoroughly with tap water to remove dirt, then rinsed using distilled water and air-dried. The plant material was then chopped and finely ground using a blender (**Figures 11 and 12**). Aqueous extracts were made by adding 4000ml of boiling distilled Millipore water to 400g of plant material. Preparation of ethanol and acetone extracts were done by adding 4000ml of the respective solvent to 400g of plant material.

(i) SAL plants cut and separated into stems and leaves

(ii) SAL blended plant material

Figure 11. Preparation of *Salvia africana-lutea* plant material

Figure 12. *Sutherlandia frutescens* plant material

Mixtures were left on a magnetic stirrer for 24 hours at 25°C, after which they were filtered through glass wool to entrap residual plant material (**Figure 13**). The aqueous extract was further filtered using Whatman no.4 filter paper and lastly dried and concentrated using a freeze drier. In contrast, ethanol and acetone extracts were concentrated at reduced pressure using the rotary evaporator and finally dried under a laminar fume hood, yielding crude extracts. All the powdered dried extracts were weighed and stored at 4°C in dark sealed containers for further experimental use.

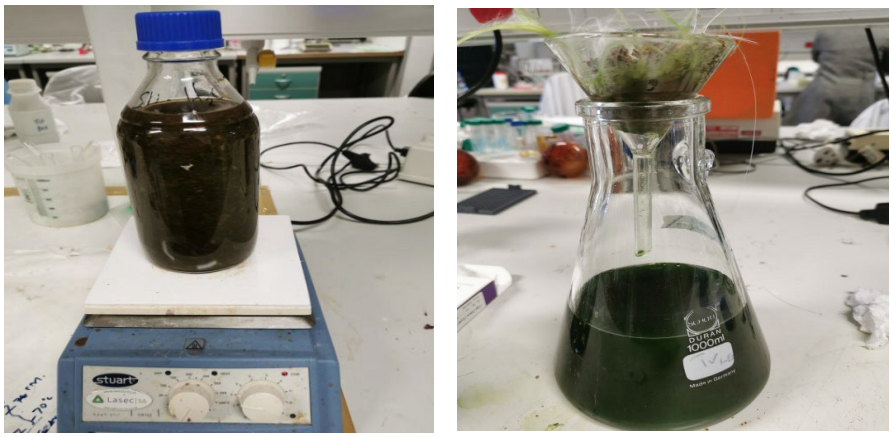


Figure 13. Plant extraction

3.6. Microorganisms

Microorganisms associated with severe infections affecting the skin and wound healing were selected for antibacterial investigation. These bacterial strains were obtained from the American Type Culture Collection (ATCC). Gram-positive bacteria namely, *S. aureus* (ATCC 25923), MRSA (ATCC 33591), *S. epidermidis* (ATCC 12228) and Gram-negative bacterium *P. aeruginosa* (ATCC 27853) were used for this study.

3.7. Agar Well Diffusion Assay

The well diffusion method is a common assay for evaluating the antibacterial activity of substances, such as antibiotics or plant extracts. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested (Rivero-Pino *et al*.; 2023). The agar well diffusion method was used to evaluate antibacterial activity of *Sutherlandia frutescens and Salvia africana-lutea* plant extracts. Bacterial suspensions with an inoculum size of 1 x 10^8 CFU/ml (0.5 McFarland standard) of bacteria were inoculated and spread evenly on Mueller Hinton (MH) agar plates. A hole with a diameter of 6 mm was aseptically punched with a sterile cork borer and 20µl of antibacterial agent or extract at desired concentrations (6.25; 12.5; 25; 50mg/ml) were introduced into wells. Ciprofloxacin (15 µg/ml); a broad-spectrum antibiotic was used as a positive control, while the solvents (water, ethanol and acetone) served as negative controls. The agar plates were incubated at 37^{o}C for 24 hours. The resulting zones of inhibition were measured using a ruler calibrated in millimetres. All samples were tested in triplicate. A graphical representation of the assay (as conducted) is provided in **Figure 14**.

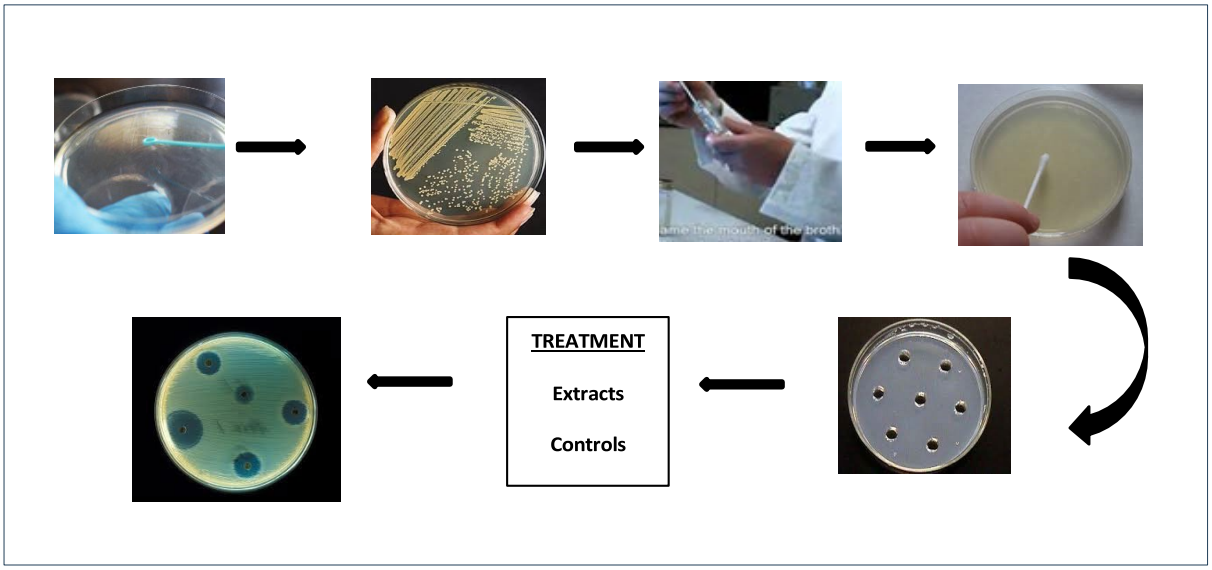


Figure 14. Agar well diffusion assay (graphical representation)

3.8. Minimum Inhibitory Concentration (broth micro-dilution assay)

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that will completely inhibit visible growth of the organism. The MIC of each plant extract was obtained using the broth micro-dilution assay (**Figure 15**). Assays for MIC were run in duplicates. Bacteria were suspended in Mueller Hinton broth (MHB) adjusted to 0.5 McFarland standard, which is 1×10^8 CFU/ml. Serial dilutions of plant extracts were prepared by adding 100 µl of 100mg/ml extract into wells in Row A of the micro- titre plate while 50ul of MH broth was added in Rows B – E wells. Thereafter, 50ul of the extract from Row A was pipetted into Row B and thoroughly mixed, then 50ul from Row B to Row C. These dilutions were done till a concentration of 6.26mg/ml was achieved in Row E. Finally, 50ul of diluted bacteria (1:150) was then added into each well, making a total volume of 100ul (Baris *et al.*, 2006). The same procedure was performed using positive and negative controls. The microplates were covered with a sterile film and incubated at 37°C for 24 hours. The effect of extracts was detected after addition of 10µl of Alamar blue dye and incubation at 37°C for 30 minutes.

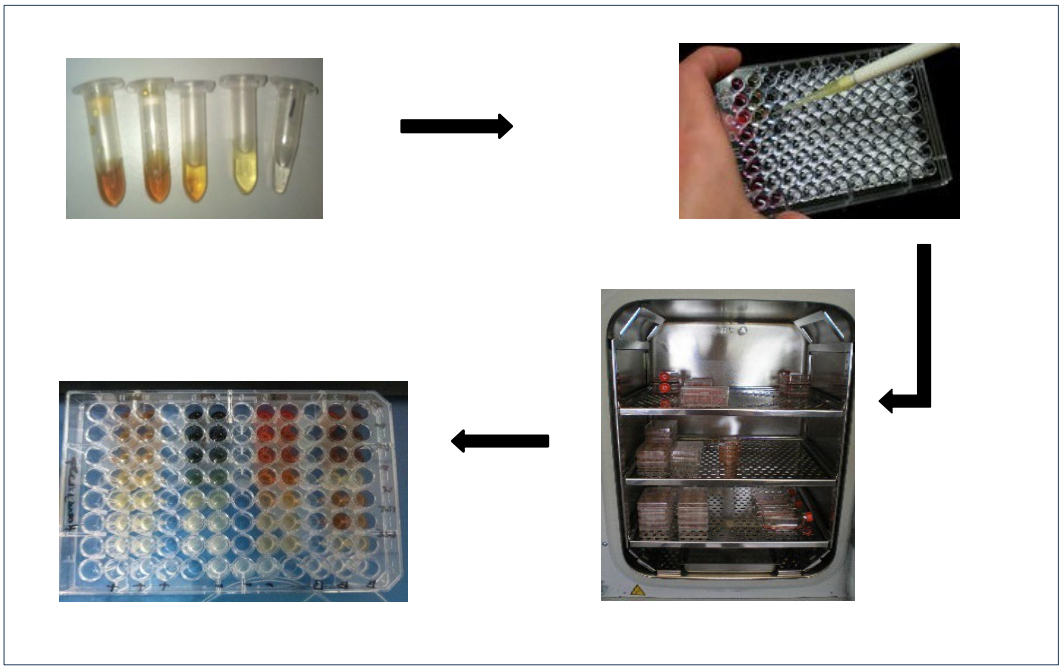


Figure 15. Determination of MIC (broth microdilution method)

3.9. TLC-Bioautography

For direct bioassay on TLC plates, 30µl aliquots of plant extract were applied on 5 x 20cm silica F254 (Merck) glass-backed plates in duplicate for fingerprinting. All plates were developed in hexane: ethyl acetate (8:2 v/v) (Wagner and Bladt, 1996). The developed TLC plates were dried overnight and then viewed under ultraviolet (UV) light (366 nm). Thereafter, one set of plates (of each extract) was sprayed with vanillin-sulphuric acid, made up by dissolving 3g vanillin in 30ml ethanol, to which 5ml concentrated sulphuric acid was added. Some compounds that were not visible under UV light became visible upon spraying and heating (for approximately 20 min) of the developed plates.

Bioautography is a screening method used to determine the presence or absence of active compounds in a sample. It can be combined with chromatography techniques, such as TLC (Choma and Grzelak, 2011). Duplicate TLC plates were used for bioautographic assays. MH agar inoculated with bacterial cultures of *S. aureus,* MRSA*, S. epidermidis* and *P. aeruginosa* were poured onto developed plates under aseptic conditions. These plates were placed on damp paper towels in a plastic tray, covered with cling wrap and incubated at 37 °C for 24 hours. To detect antibacterial activity on plates, they were sprayed with 0.2mg/ml solution of 2,5-diphenyltetrazolium bromide (Sigma Chemicals Co.) and re-incubated at 37ºC for 2 hours. Clear zones on the chromatogram indicated antibacterial (inhibitory) activity of extract component.

3.10. Preparative TLC

After antibacterial compounds were identified by means of Rf values, the TLC assay was repeated on preparative TLC plates. Compounds of interest were marked on the preparative TLC plates. These were then scraped off the plates with a spatula, added into a flask and stirred with ethyl acetate solvent for 1 hour. Thereafter, the silica was filtered off and washed three times with solvent to rinse off compounds of interest. The filtrate was then concentrated *in vacuo* to furnish compounds of interest.

3.11. NMR Spectroscopy

Isolated compounds were analysed using NMR spectroscopy. ^{1}H NMR and ^{13}C NMR spectra were acquired on a Bruker 400 MHz Avance IIIHD Nanobay spectrometer equipped with a 5 mm BBO probe at 333K using standard 1D and 2D NMR pulse sequences. All spectra were referenced to residual undeuterated solvent peaks.

3.12. LC-MS Analysis

LC-MS techniques were employed for further chemical analysis. A Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) (Waters, Milford, MA, USA) was used for high-resolution UPLC-mass analysis. The column eluate was allowed to pass through a Photodiode Array (PDA) detector just before sailing to the respective mass spectrometer, thus enabling simultaneous collection of UV as well as MS spectra of non-volatile profiles of extracts prepared from *Salvia africana lutea*

Electrospray ionisation was used in negative mode with a cone voltage of 15 V, desolvation temperature of $275^{0}C$, desolvation gas at 650 L/h and the rest of the MS settings which were optimized for best resolution and sensitivity.

Data was obtained by scanning from MS settings which were optimised for best resolution and sensitivity. Data was obtained by scanning from m/z 150 to 1500 m/z in resolution mode as well as in MSE mode. In MSE mode, two channels of MS Data were acquired, that is, one was obtained at a low collision energy (4 V) and the other using a collision energy ramp (40-100 V) to obtain fragmentation data as well. Leucine enkephalin was used as a lock mass (reference mass) for accurate mass determination, while the instrument itself was calibrated using sodium formate. Separation was then achieved using a Waters HSS T3, 2.1 x 100 mm,

1.7 $\mu\mu$m column. An injection volume of 2 $\mu\mu$L was used, and the mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid as solvent B. The gradient started at 98% solvent A for 0.5 min and changed to 22% B over 4 min in a linear way. It then went to 44% B after 9 minutes and 100% B after 13 minutes with a wash step of 1 minute, followed by re-equilibration to initial conditions for 2 minutes. The flow rate was 0.3 $\mu\mu$L/min and column temperature was maintained at 55 ^{0}C.

3.13. Statistical Analysis

GraphPad Prism software package (V8) was used for statistical analysis. antibacterial effects were statistically compared by conducting a two-way Analysis of Variance (ANOVA) using the Tukey's Multiple Comparisons Test. All analyses were performed in triplicate. *P*-values (comparing means ± standard error of the mean) were considered significant at $P < 0.05$.

CHAPTER 4
RESULTS and DISCUSSION

4.1. The Antibacterial Activity of the Plant Extracts

4.1.1. Agar Well Diffusion Assay

The antibacterial activity of plant extracts was tested against *S. aureus*, MRSA, *P. aeruginosa* and *S. epidermidis* using the agar well diffusion assay. This assay allowed for the identification of microbes that were sensitive to plant extracts.

To ensure that the antimicrobial susceptibility test method had worked well, a positive control of a standard antibiotic must always be included (Eloff, 2019). Ciprofloxacin, a broad-spectrum antibiotic was used as a positive control. The positive control, Ciprofloxacin (15 µg/ml) inhibited growth of all the tested microorganisms. The zones of inhibition observed were as follows *S. aureus*: 26mm, MRSA: 28mm, *S. epidermidis*: 20mm and *P. aeruginosa*: 24mm (**Table 1**; **Figures 16** and **17**).

A negative control, in most cases the solvent used to dissolve the extract; must be included to ensure that any antimicrobial activity is not caused by the solvent but rather by the plant extract (Eloff, 2019). For this study negative controls (extraction solvents; water, ethanol and acetone) did not have any effect on all microorganisms. No zones of inhibition were observed around wells of all negative controls (**Table 1**; **Figures 16** and **17**).

All extracts of *Sutherlandia frutescens* at different concentrations did not inhibit growth of tested bacteria microbes (**Figure 16**). In literature, extracts of *Sutherlandia frutescens* leaves were reported to possess mild to moderate antimicrobial activity against a range of pathogenic microorganisms, including *S. aureus*, *Enterococcus faecalis* and *Escherichia coli*; but no activity against *P. aeruginosa* (Katerere and Eloff, 2005; Hübsch *et al*., 2014; Nosov *et al.*, 2023). There could be many reasons why the *S.frutescens* extracts did not show any antimicrobial activity in the current study. Many of the antimicrobial compounds in plant extracts are relatively non-polar, so these compounds do not diffuse well in the aqueous agar matrix used in agar diffusion studies (Eloff, 2019).

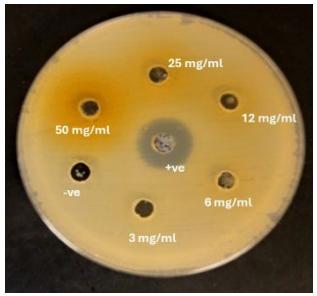
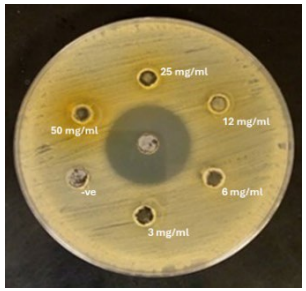
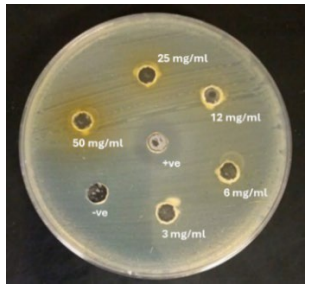
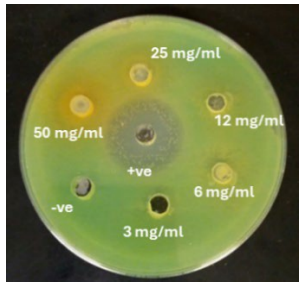
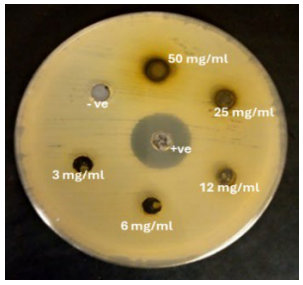
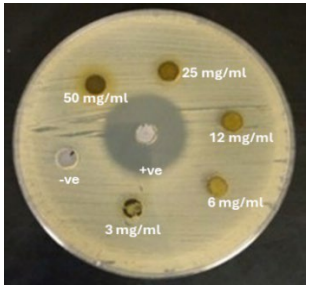
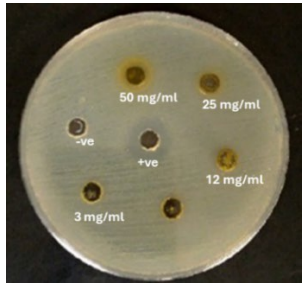
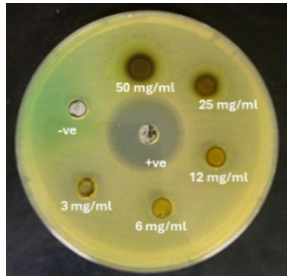
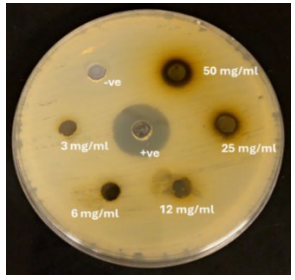
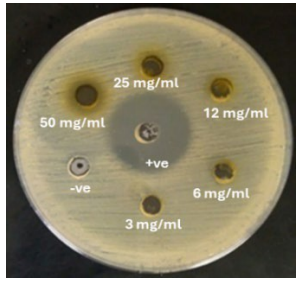
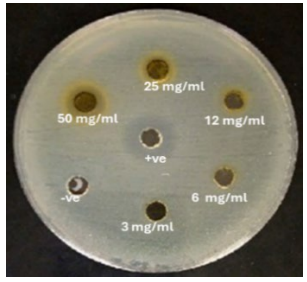
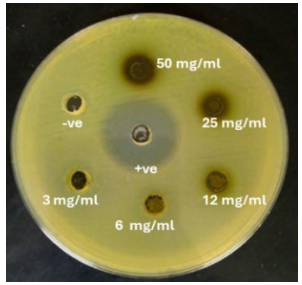
	Staphylococcus aureus	Methicillin-resistant *Staphylococcus aureus*	*Staphylococcus epidermidis*	*Pseudomonas aeruginosa*
Water				
Acetone				
Ethanol				

Figure 16. Agar plates showing the non-inhibition of *S. aureus*, MRSA, *S. epidermidis* and *P. aeruginosa* by varying concentrations of *Sutherlandia frutescens* extracts

The time of collection could have contributed to lack of antibacterial activity of *Sutherlandia frutescens* extracts in the current study. Climate has direct impact on plant ecosystem processes and structures such as photosynthesis, nutrient cycling, transpiration as well as production of both primary and secondary metabolites (Goyal *et al*., 2012). These processes have direct impact on plant phytochemical compositions. Literature is filled with studies on effects of seasonal variation and climatic factors on biological activities of plants (Adeosun *et al*., 2022). It may be possible that our batch of *Sutherlandia frutescens* plant material was collected at an inappropriate time of year and hence, did not possess any secondary metabolites with antibacterial activity.

Due to lack of inhibitory activity of *Sutherlandia frutescens* extracts against tested microbes in this study, it was decided not to continue with the phytochemical investigation of the *Sutherlandia frutescens* extracts. Only *Salvia africana-lutea* extracts were subjected to further antibacterial and phytochemical investigations.

Agar plates in **Figure 17** show effects of varying concentrations of *Salvia africana-lutea* extracts on growth of *S. aureus,* MRSA*, S. epidermidis* and *P. aeruginosa.* Zones of inhibition caused by different extracts of *Salvia africana-lutea* were statistically compared by conducting a two-way ANOVA (**Table 1**). It was shown that different concentrations of *Salvia africana-lutea* extracts had different activities against tested microorganisms. The activity of *Salvia africana-lutea* extracts increased with increasing concentrations from 6.25 to 50 mg/ml is shown in **Table 1**. The zone of Inhibition diameter against MRSA increased from 3.3 to 20 mm at concentrations 6.25 and 50 mg/ml, respectively. All *Salvia africana-lutea* extracts had the highest activity against MRSA and the lowest activity against *P. aeruginosa*. Extracts did not have any activity against *P. aeruginosa*. This is due to the ability of *P. aeruginosa* to resist antibiotics. Strains of *P. aeruginosa* are known to counter antibiotics through intrinsic and acquired resistance mechanisms (Pang *et al*., 2019).

Gram-positive bacteria are generally more sensitive to drug action than Gram-negative bacteria mainly owing to differences in their cell wall composition (Cos *et al*., 2006; McGaw *et al*., 2013). This was shown in the case of *P. aeruginosa*, which was resistant to *Salvia africana-lutea* extracts. All solvents used for *Salvia africana-lutea in this study* had their highest activity against MRSA at a concentration of 50 mg/ ml. The activity of solvent extracts, especially acetone, was generally higher than that of water extracts for most of the microorganisms (and this was statistically significant with *P*-values less than 0.05 (**Table 1**). This is in accordance with published literature which generally states that “organic plant extracts provide more powerful antimicrobial activity as compared to aqueous extracts.” Cowan (1999) reported that most antibiotic compounds identified in plants are saturated organic molecules that can easily solubilise in organic solvents. Preethi *et al.* (2014) and Seyydnejad *et al*. (2010) had similar observations where the alcoholic extract had the best antimicrobial activity.

Table 1. Inhibition of *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus, Staphylococcus*

epidermidis and *Pseudomonas aeruginosa* by varying concentrations of *Salvia africana-lutea* extracts

Microorganism	Extract Concentration (mg/ml)	Zone of Inhibition (mm) [average ± SEM]			Statistical Significance*		
		Water[a]	Acetone[b]	Ethanol[c]	ab	ac	bc
Staphylococcus aureus	**50.00**	17.7 ± 0.4	20.0 ± 0.1	13.0 ± 0.3	*	****	****
	25.00	15.3 ± 0.1	16.3 ± 0.1	10.3 ± 0.2	ns	****	****
	12.50	11.0 ± 0.3	13.0 ± 0.3	6.7 ± 0.2	*	****	****
	6.25	3.3 ± 0.3	5.0 ± 0.3	4.0 ± 0.5	*	ns	ns
Positive Control: Ciprofloxacin (15 µg/ml)		26 ± 0.1					
Methicillin-resistant *Staphylococcus aureus*	**50.00**	19.7 ± 0.2	23.0 ± 0.3	19.7 ± 0.1	****	****	ns
	25.00	19.3 ± 0.3	20.3 ± 0.1	18.3 ± 0.1	ns	ns	**
	12.50	5.0 ± 0.4	17.3 ± 0.3	14.3 ± 0.4	****	****	****
	6.25	0.0	9.3 ± 0.4	5.7 ± 0.2	****	****	****
Positive Control: Ciprofloxacin (15 µg/ml)		28 ± 0.1					
Staphylococcus epidermidis	**50.00**	13.3 ± 0.3	13.7 ± 0.2	7.0 ± 0.4	ns	****	****
	25.00	6.3 ± 0.2	9.3 ± 0.4	5.3 ± 0.5	****	ns	****
	12.50	5.3 ± 0.3	6.7 ± 0	0.0	ns	****	****
	6.25	0.0	3.3 ± 0	0.0	****	ns	****
Positive Control: Ciprofloxacin (15 µg/ml)		20 ± 0.0					
Pseudomonas aeruginosa	**50.00**	0	0	0	0	0	0

	25.00	0	0	0	0	0	0
	12.50	0	0	0	0	0	0
	6.25	0	0	0	0	0	0
Positive Control: Ciprofloxacin (15 µg/ml)		24 ± 0.0					

Abbreviations: mg/ml: milligram per millilitre; mm: millimetre; µg/ml: micrograms per millilitre; SEM: standard error of the mean (for n=3);

**P* values for comparing main effects: ab = water and acetone; ac = water and ethanol; bc = acetone and ethanol; * $P<0.05$; ** $P<0.01$; **** $P<0.0001$; ns: $P>0.05$

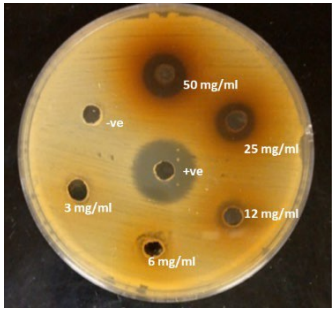
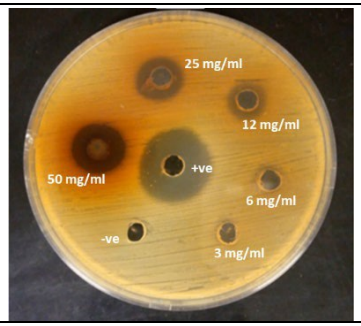
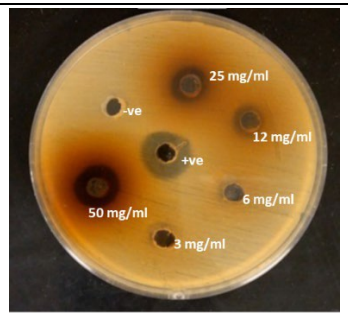
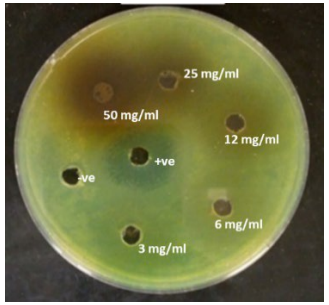
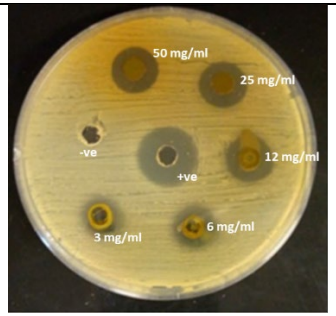
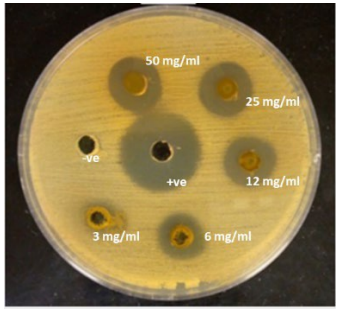
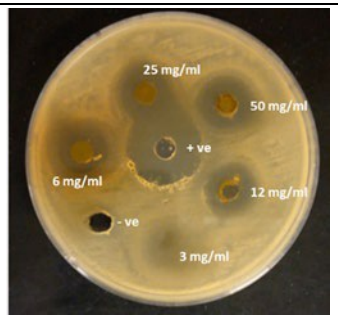
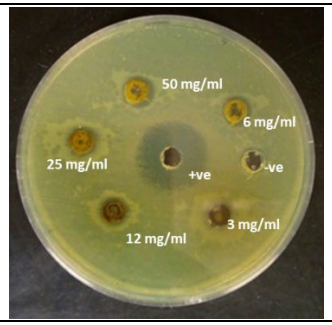
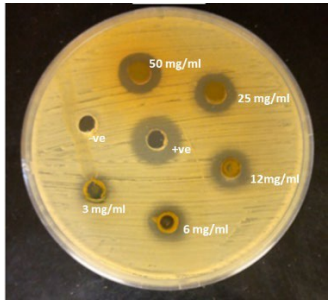
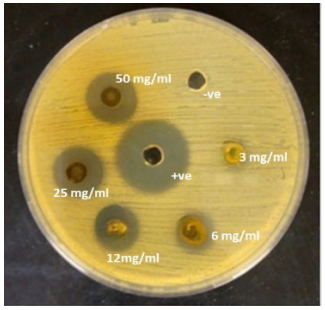
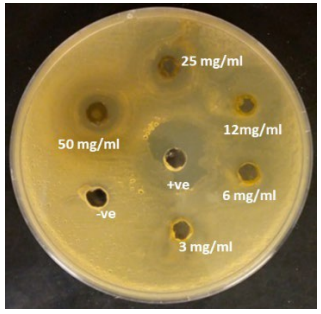
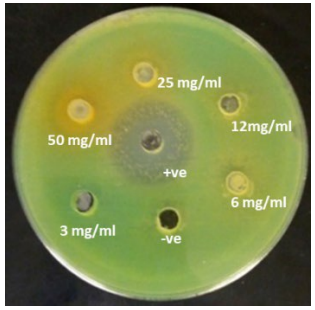
	Staphylococcus Aureus	**Methicillin-resistant *Staphylococcus aureus***	***Staphylococcus epidermidis***	***Pseudomonas aeruginosa***
Water				
Acetone				
Ethanol				

Figure 17. Agar plates showing the inhibition of *S. aureus,* MRSA*, S. epidermidis* and *P. aeruginosa* by varying concentrations of *Salvia africana-lutea* extracts

4.1.2. MIC of *Salvia africana-lutea* extracts

Aqueous, acetone and ethanol extracts of *Salvia africana-lutea* were tested in separate 96-well plates to determine their MICs against initially tested bacterial strains (i.e., *S. aureus*, *S. epidermidis*, *MRSA* and *P. aeruginosa*) whose growths were inhibited in the agar well-diffusion assay. MIC is the lowest concentration of an antimicrobial agent or plant extract that can inhibit growth of a microbial pathogen after incubation (Rekha *et al*., 2018). **Figure 18** shows an example of a completed plate layout used for the MIC assay determination.

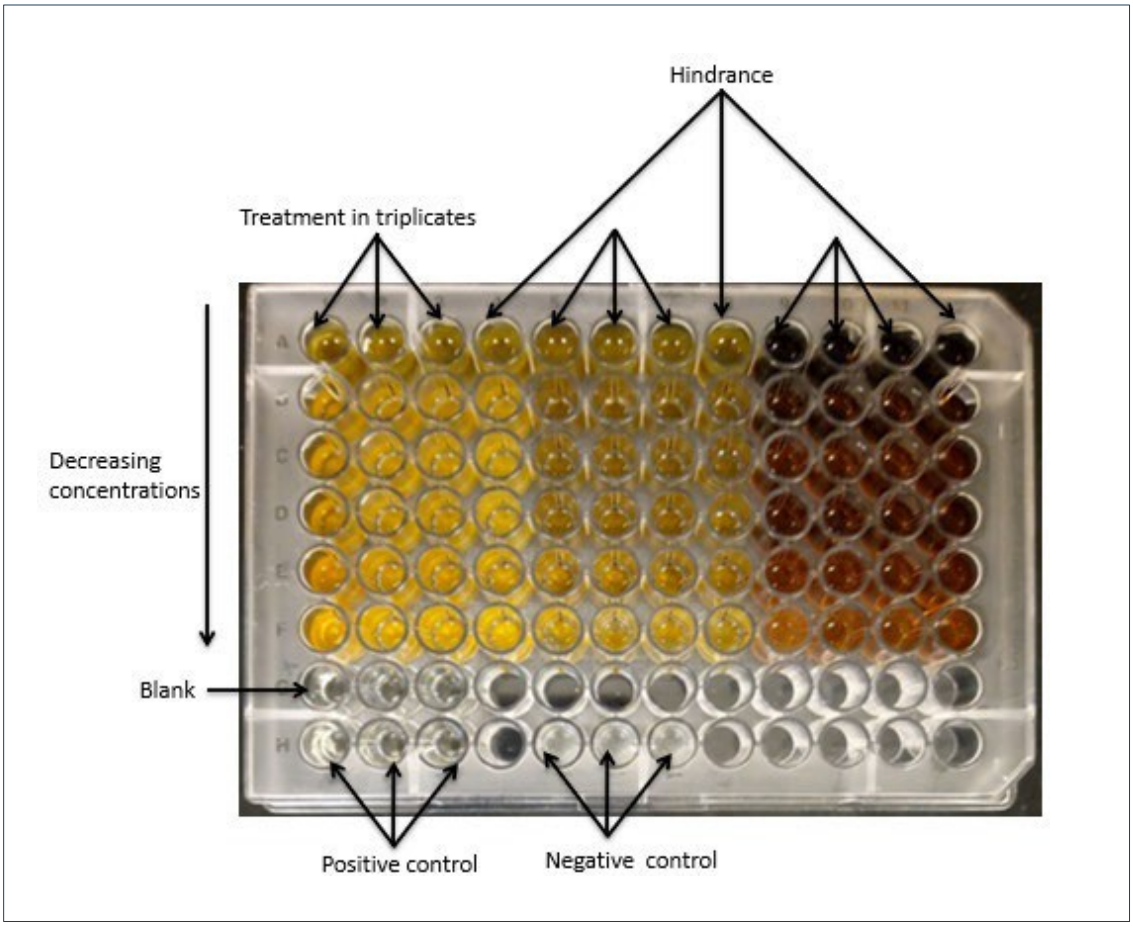


Figure 18. Completed MIC assay plate showing decreasing concentrations of *Salvia africana-lutea extract* treatments in triplicates, with the fourth well as hindrance (no bacterial cells in the hindrance).

The aqueous extract had an MIC ranging between 5 to 10 mg/ml, while acetone and ethanol extracts had MICs ranging between 4 to 8 mg/ml (**Table 2; Figures 19 - 21**). All three extracts had low MIC values against *S. aureus*; the aqueous and acetone extracts were 5mg/ml, whereas the ethanol extract had an MIC of 4mg/ml. For the more resistant MRSA species, the aqueous extract had a higher MIC of 10mg/ml, while acetone and ethanol extracts showed MIC of 4mg/ml each. The aqueous extract again had an MIC of 10mg/ml against *S. epidermidis*, followed by the ethanolic extract at 8mg/ml and the acetone extract showing MIC of 4mg/ml.

Table 2. The minimum inhibitory concentration of *Salvia africana-lutea* extracts against *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*

Microorganism	**MIC (plant extract concentration: mg/ml)**		
	Water	**Acetone**	**Ethanol**
Staphylococcus aureus	5	5	4
Methicillin-resistant *Staphylococcus aureus*	10	4	4
Staphylococcus epidermidis	10	4	8
Pseudomonas aeruginosa	0	0	0

Aligiannis *et al.* (2001) proposed a classification system based on MIC results obtained for plant materials, which was consequently described and implemented by Duarte *et al*. (2005). All plant species with MIC values of up to 8 mg/ml are considered to possess at least some degree of inhibitory effect (Mogana *et al*., 2020). Based on these above-mentioned published theories, it was decided to pursue phytochemical analyses on organic extracts only (i.e., acetone and ethanol extracts).

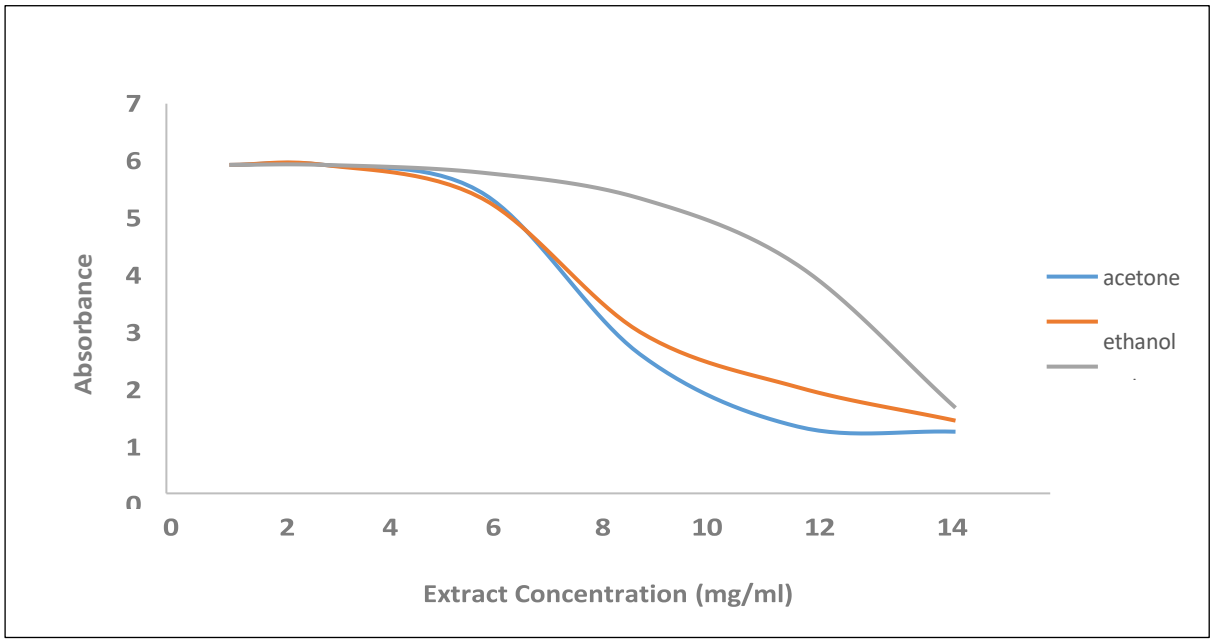


Figure 19. The MIC of *Salvia africana-lutea* extracts against *S. aureus*

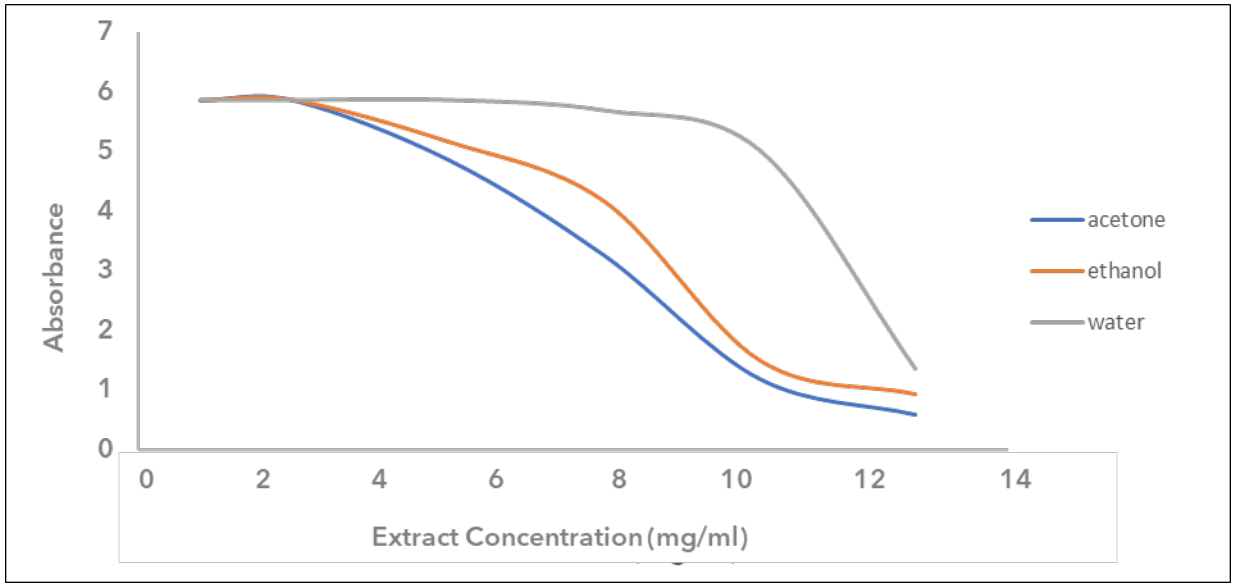


Figure 20. The MIC of *Salvia africana-lutea* extracts against MRSA

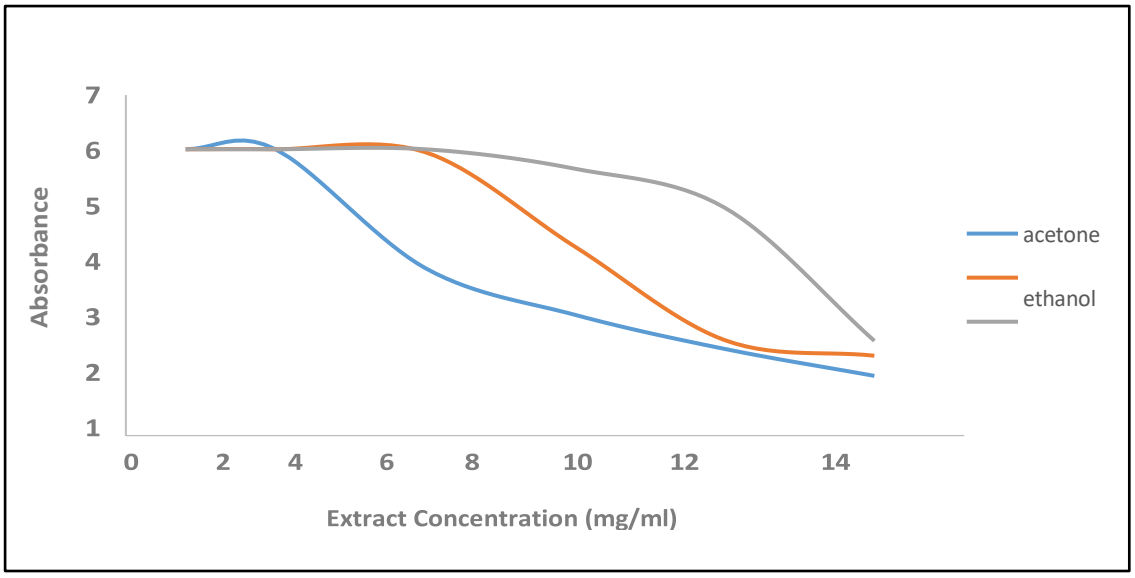


Figure 21. The MIC of *Salvia africana-lutea* extracts against *S. epidermidis*

4.1.3. TLC-Bioautography

TLC is a versatile technique that allows separation of plant extract and is convenient for searching for plant constituents with biological activity (Choma *et al.*, 2015 & Kowalska *et al.*, 2022). Thin-layer chromatograms of *Salvia africana-lutea* acetone and ethanol extracts yielded very similar results, as defined by the Rf values of the separated compounds (image **i** in **Figures 22** and **23**).

The antibacterial compounds had the following Rf values: MRSA – acetone: 0.02; 0.08 (image **ii** in **Figure 20**) and ethanol: 0.01; 0.04 (image **ii** in **Figure 21**); *S.aureus* – acetone: 0.02; 0.05; 0.11 (image **v** in **Figure 20**) and ethanol: 0.10; 0.29 (image **v** in **Figure 21**); *S.epidermidis* - acetone: 0.03; 0.5 (image **iv** in **Figure 20**) and ethanol: 0.01 (image **iv** in **Figure 21**).

The TLC assay was repeated on preparative TLC plates, and after development of plates, compounds of interest were retrieved for further analysis. Due to limited quantities of each compound, all compounds per extract were combined for spectroscopy. Phytochemical results presented hereafter are depicted as per extract (acetone or ethanol).

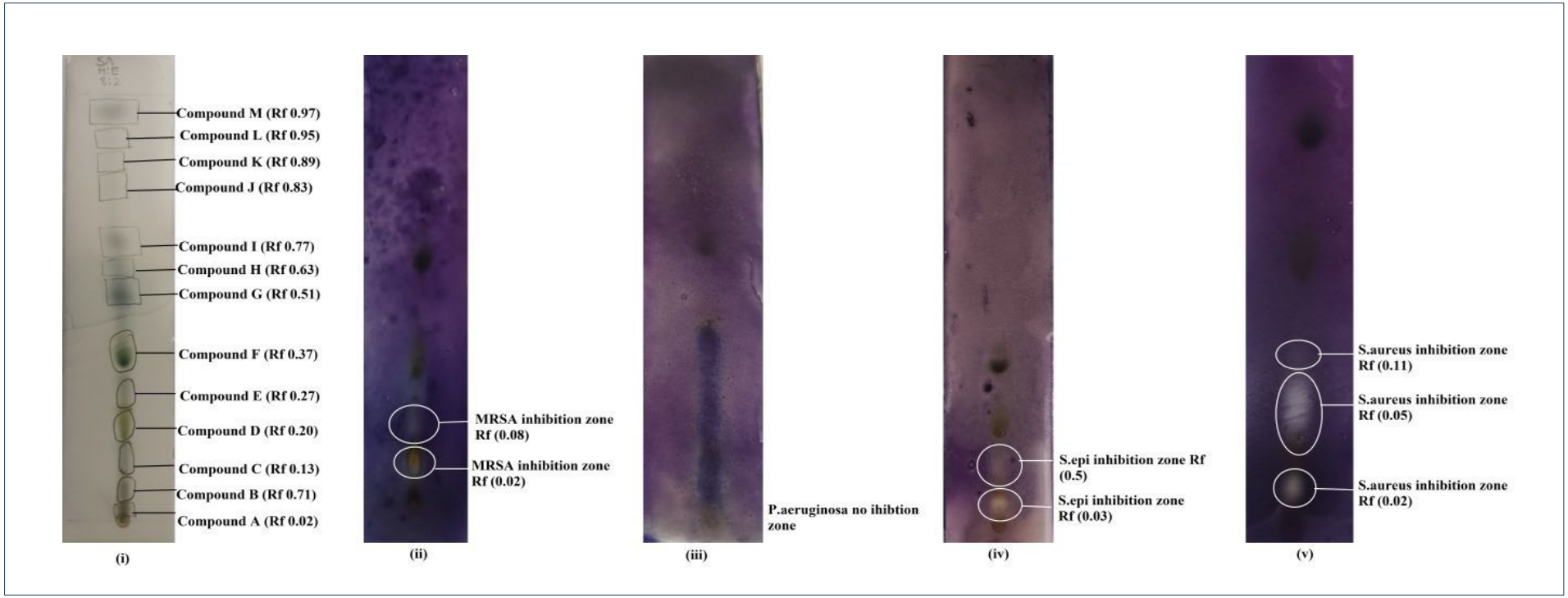


Figure 22. TLC-Bioautography of the *Salvia africana-lutea* acetone extract.

Image (i) is the developed TLC plate of *Salvia africana-lutea* ethanol extract with corresponding bioautograms - (ii) *MRSA*; (iii) *P. aeruginosa*; (iv) *S. epidermidis* and (v) *S. aureus.*

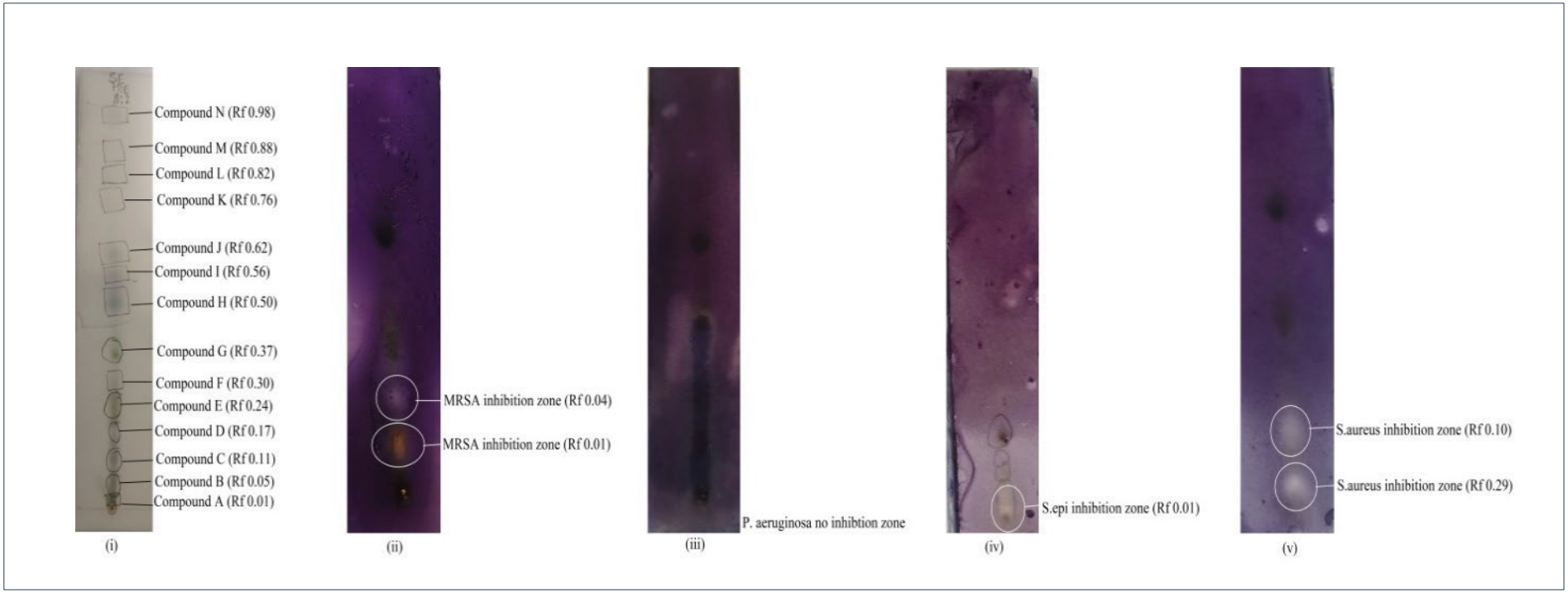


Figure 23. TLC-Bioautography of the *Salvia africana-lutea* ethanol extract.

Image (i) is the developed TLC plate of *Salvia africana-lutea* ethanol extract with corresponding bioautograms - (ii) *MRSA*; (iii) *P. aeruginosa*; (iv) *S. epidermidis* and (v) *S. aureus.*

4.2. Phytochemical Analysis of the Plant Extracts

4.2.1. NMR Spectroscopy

Unfortunately, due to extremely poor yields of bioactive secondary metabolites, which were between 0.2 - 6.3 mg (from preparative TLC plates), the resulting mixtures could not be resolved using ^{1}H NMR (**Figs 24**, **25** and **26**) and ^{13}C NMR approach (**Fig 27**). It was therefore suggested that LC-MS/HPLC method be used to resolve these mixtures.

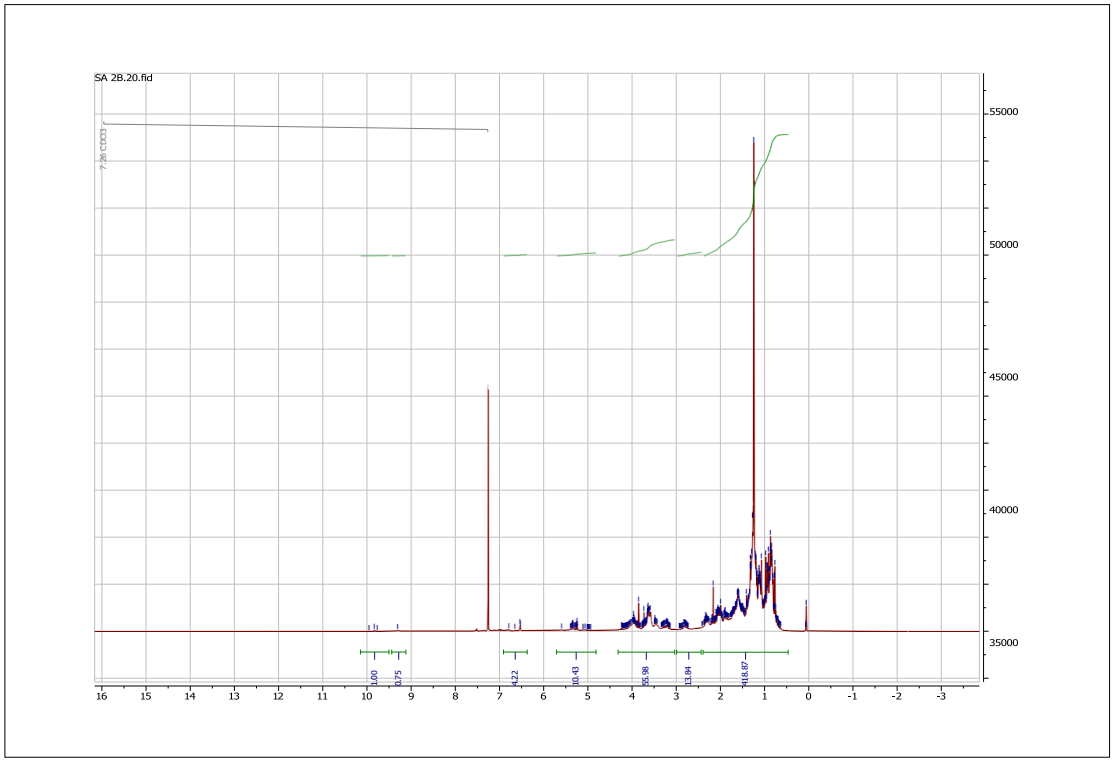


Figure 24. ^{1}H NMR of *Salvia africana-lutea* acetone extract.

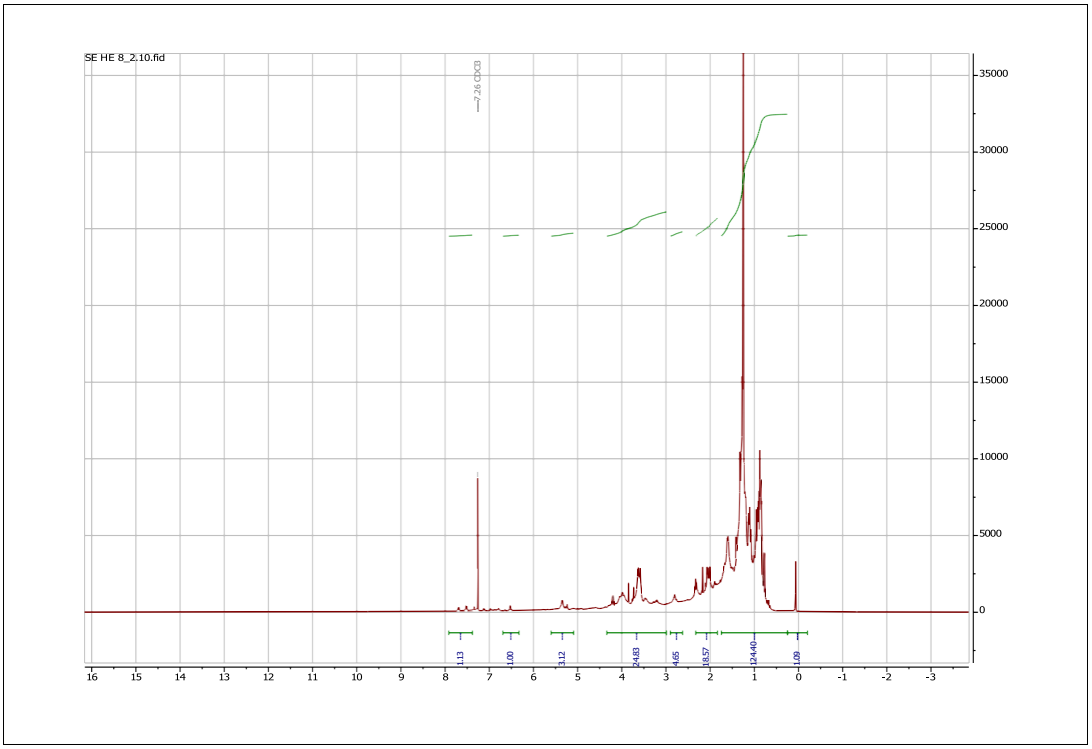


Figure 25. ^{1}H NMR of *Salvia africana-lutea* acetone extract.

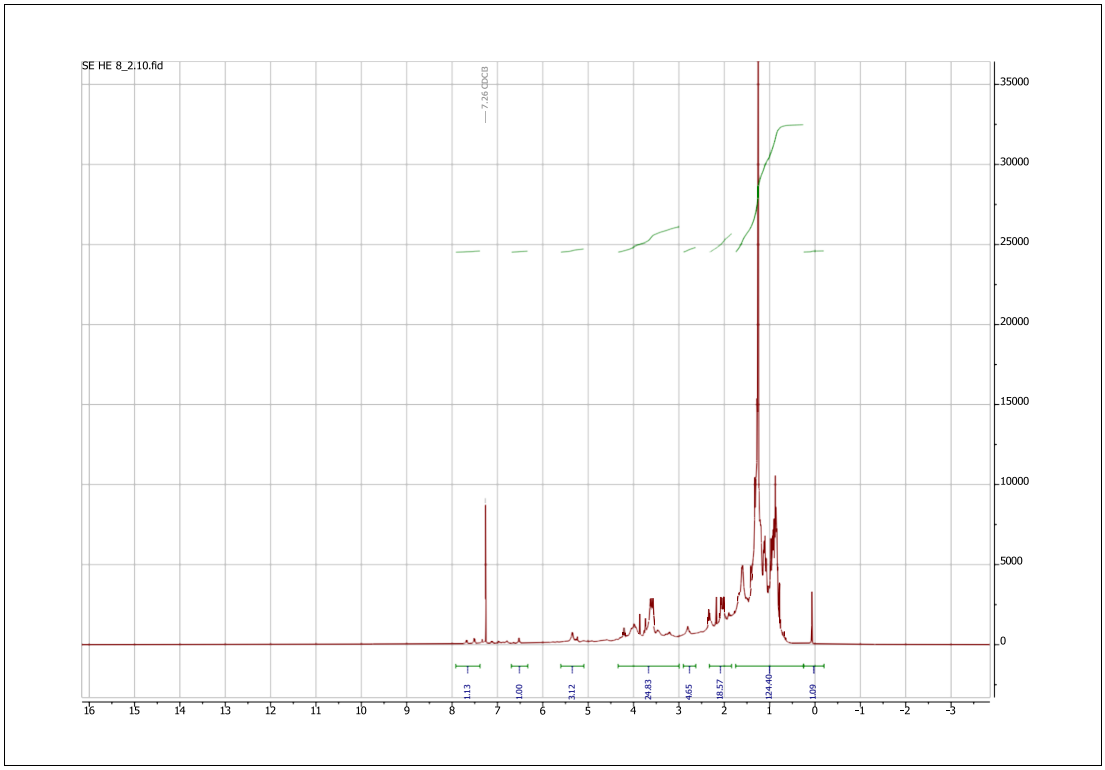


Figure 26. ^{1}H NMR of *Salvia africana-lutea* ethanol extract.

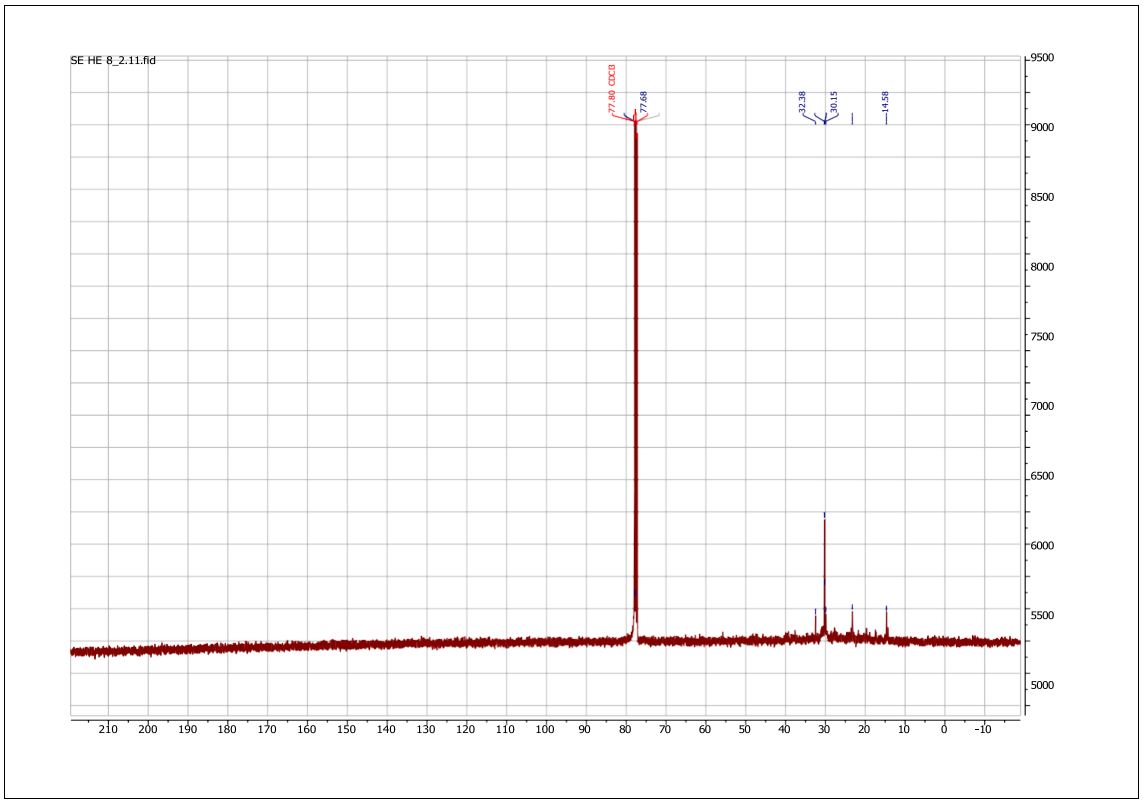


Figure 27. ^{13}C NMR of *Salvia africana-lutea* ethanol extract.

4.2.2. Liquid Chromatography-Mass Spectroscopy Analysis

Upon choosing the optimum solvent mixture for extractions (for LC-MS analysis), both the acetone and ethanol extracts of *Salvia africana-lutea* furnished similar and highly informative chromatograms. A total of 25 identified, tentatively identified and unknown compounds are presented in **Table 3**.

Table 3. Identification of compounds in both the acetone and ethanol extracts of *Salvia africana-lutea*.
Bold text indicates major compounds on the chromatogram.

Compound Number	Average Rt (min)	Average Mz	Tentative ID	Molecular Formula	Class	Relative to Rosmarinic Acid	
						Salvia Acetone Extract	*Salvia* Ethanol Extract
			Structure Rank 1			**mg/L**	**mg/L**
1	6,471	359,1499	(+)-Lariciresinol A	C20H24O6	7,9'-epoxylignans	26,6	33,3
2	6,59	359,1509	(+)-Lariciresinol B	C20H24O6	7,9'-epoxylignans	19,2	24,9
3	6,665	331,1544	Gibberellin A4;GA4	C19H24O5	C19-gibberellin 6-carboxylic acids	21,8	1,2
4	6,68	319,1561	Unknown	C18H24O5		18,9	48,5
5	7,103	403,1757	Rosmarinic acid deriv a	C22H28O7	Germacranolides and derivatives	61,9	75,3
6	**7,163**	**403,176**	**Rosmarinic acid deriv b**	**C22H28O7**	**Germacranolides and derivatives**	**84,8**	**101,3**
7	7,273	403,1763	Rosmarinic acid deriv c	C22H28O7	Macrolides and analogues	42,4	65,8
8	7,434	345,1698	Epirosmanol	C20H26O5	C20-gibberellin 6-carboxylic acids	17,9	32,7
9	7,504	421,1516	Crepidiaside A	C21H26O9	O-glycosyl compounds	17,9	33,8

10	8,084	363,1809	Gibberellin A97	C20H28O6	C20-gibberellin 6-carboxylic acids	11,7	66,4
11	8,47	329,1752	Carnosol	C20H26O4	C19-gibberellin 6-carboxylic acids	69,9	14,0
12	**8,573**	**401,1609**	**Tenellic acid A;(-)-Tenellic acid A**	**C22H26O7**	**Diphenylethers**	**103,0**	**111,4**
13	8,77	315,1598	Gibberellin A9;GA9	C19H24O4	C19-gibberellin 6-carboxylic acids	79,2	31,0
14	9,058	359,1856	Methylrosmanol	C21H28O5	21-hydroxysteroids	24,6	30,3
15	9,287	373,2025	Hydroxybenzoyl)epoxyjaeschkeanadiol;Jaeskeanin; Ferutinin alpha-epoxide	C22H30O5	p-Hydroxybenzoic acid alkyl esters	33,0	16,5
16	9,321	343,1542	Rosmadial	C20H24O5	Benzofurans	23,0	25,1
17	9,467	431,2077	Melleolide B	C24H32O7	Melleolides and analogues	2,7	65,4
18	9,586	329,1762	Carnosol isomer	C20H26O4	C19-gibberellin 6-carboxylic acids	56,5	21,0
19	9,646	431,2076	Schizandrin;(+)-Schizandrin;Schisandrol A;Wuweizichun A	C24H32O7	Hydrolyzable tannins	1,1	31,0
20	9,852	403,2125	Epoxyjaeschkeanadiol 5alpha-vanillate	C23H32O6	M-methoxybenzoic acids and derivatives	25,0	27,1
21	9,972	359,1872	Rosmanol methyl ether	C21H28O5	21-hydroxysteroids	24,9	22,7
22	**10,139**	**359,186**	**Epirosmanol methyl ether**	**C21H28O5**	**21-hydroxysteroids**	**205,2**	**161,5**
23	10,747	373,2013	Ethylrosmanol	C22H30O5	Diterpene lactones	32,8	16,4
24	**10,957**	**403,2117**	**Strophanthidin;Strophanthidin K;Strophanthidine**	**C23H32O6**	**Cardenolides and derivatives**	**90,5**	**85,4**
25	11,468	345,2069	Methoxy-carnosic acid	C21H30O4	21-hydroxysteroids	16,0	18,8

The UPLC- QTOF- MS profiles showed the presence of a variety of similar compounds in both the acetone and the ethanol extracts, respectively (**Fig 28** and **Fig 29**).

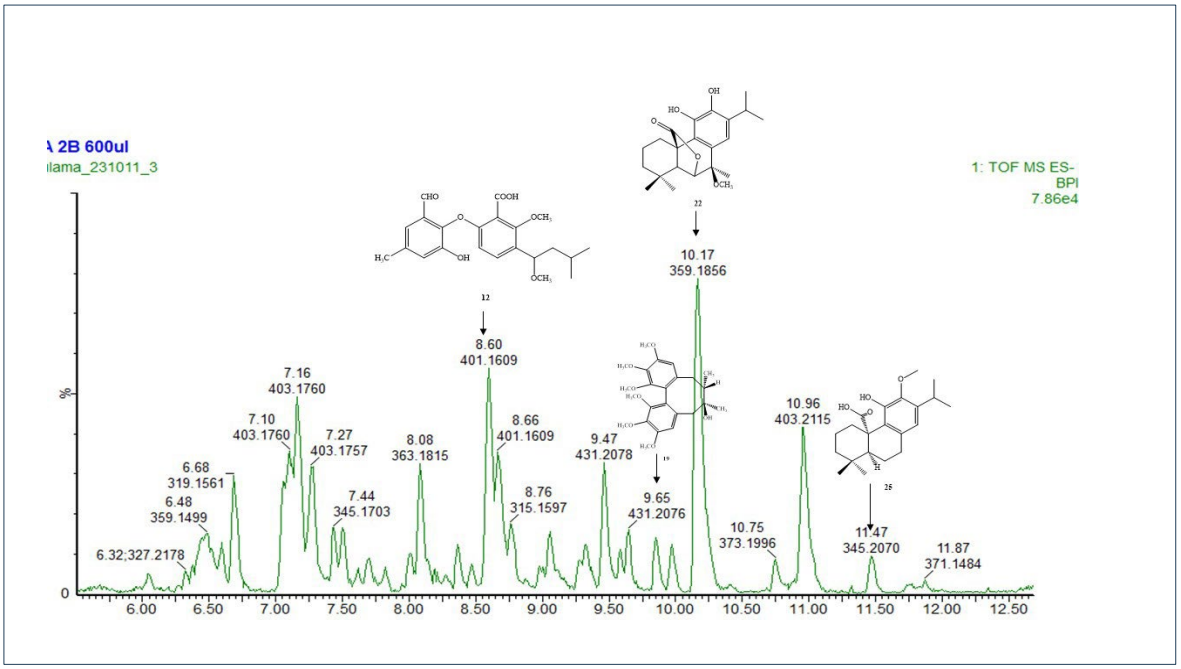


Figure 28. UPLC-MS chromatographic fingerprint for the *Salvia africana-lutea* acetone extract

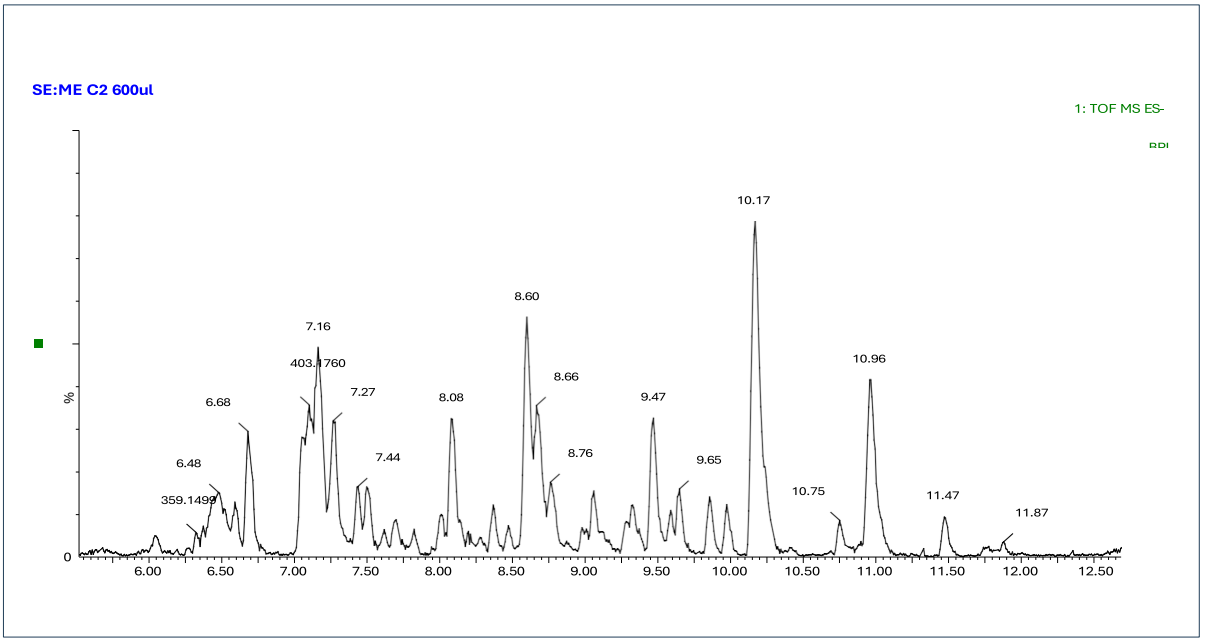


Figure 29. UPLC-MS chromatographic fingerprint for the *Salvia africana-lutea* ethanol extract

UPLC-QTOF-MS-profiles revealed the presence of a variety of compounds in both the acetone and ethanol extracts, which were similar in all respects, except only with regards to retention times (**Fig 28** and **Fig 29**).

The MS peaks, as indicated in the respective chromatograms (**Fig 28** and **Fig 29**), represent compounds that emanate from a variety of secondary metabolite classes such as epoxylignan derivatives **1** and **2**, tetracyclic diterpenoid carboxylic acids/diterpenoids **3**, **10** and **13**, macronalide derivatives **5**, **6** and **7**, 21-hydroxysteroids **8**, **21**, **22**, **23**, and **14**, benzofuran **16**, *O*-glycosyl compound **9**, diterpenoids **11**, **18**, and **25**, melleolide **17**, a diphenyl ether **12**, Epoxy jaeschkeanadiols **15** and **20**. A hydrolysable tannin **19**, a cardenolide **24**, and an unknown compound **4**. These compounds in both the acetone and ethanol extracts were identified by comparing their retention times and their respective mass spectra, as shown in **Figs 28** and **29** against rosmarinic acid as the standard reference.

The chromatogram of the acetone extract (**Fig 2**) revealed that the most prominent peak is epirosmanol methyl ether **22** with the retention time of 10.17 minutes and an average mass of 359.1856 g mol^{-1} followed by tenellic acid **12** with a retention time of 8.60 minutes and average mass of 401.1609 g mol^{-1} then followed by strophanthidin **24**, with an average retention time of 403.2115 $gmol^{-1}$ followed by rosmarinic acid derivative B **6** with retention time of 7.16 minutes and an average mass of 403.1760 g mol^{-1} with gibberellin A9 **13** not so prominent, and with schizandrin **19** being the least prominent (**Table 3**).

Similarly, the chromatogram of the ethanol extract (**Fig 29**) indicates epirosmanol methyl ether **22** as being the most prominent compound, which is then followed by derivative B **6**, followed by strophanthidin **24**, followed by rosmarinic acid derivative A **5**, with gibberellin A4 **3** being the less prominent. In both the acetone and the ethanol extracts epirosamanol methyl ether **22** was identified as the major constituent, furnishing a count 205.2 mg/L and 161.5 mg/L, respectively. Schizandrin **19** with 1.1 mg/L, was identified as the least minor constituent of the acetone extract while gibberellin A4 **3** (1,2 mg/L) was identified as the least minor product of the ethanol extract.

Chemical structures of the detected compounds, accompanied by relevant published biological information from previous studies, is presented hereafter:

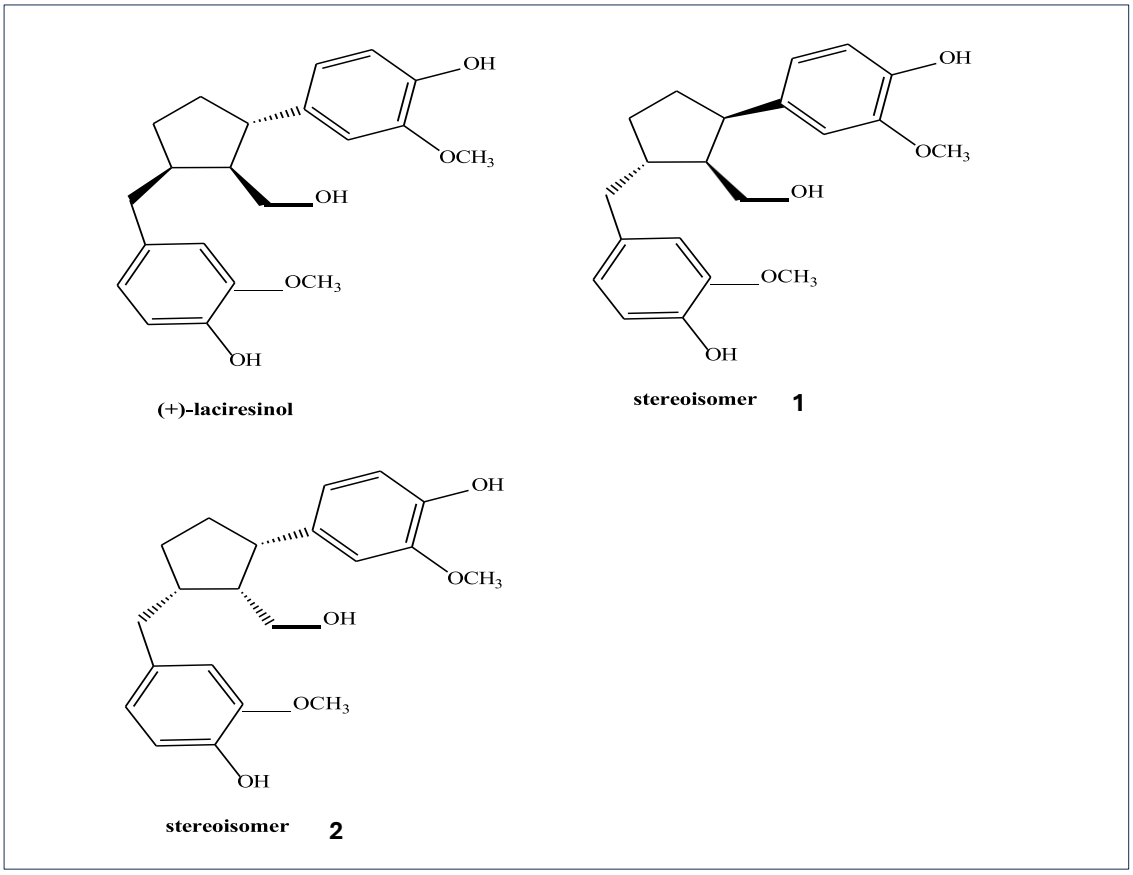


Figure 30. Chemical structures of (+)-Lariciresinol and sterioisomers (Nishiwaki *et al*., 2011).

(+)- Lariciresinols A (**1**) and B (**2**), **(Figure 30)** lignans which belong to a type of phenylpropanoids isolated from *Rubia philippinensis* were found to possess significant antibacterial effect against foodborne pathogens such as *Staphylococcus aureus* KCTC1621 and *Escherichia coli* 0157:H7 (Bajpai *et al*., 2017; Nishiwaki *et al*., 2011).

Gibberellins **3**, **10**, and **13, (Figure 31)** which constitute a wide variety of tetracyclic diterpenoid carboxylic acids, are found within higher plants where they play a pivotal role as endogenous growth regulators, thus, fostering organ expansion as well as responsible for developmental changes (Hedden and Thomas, 2012). These compounds were first encountered as secondary metabolites of the fungus *Gibberella fujikuroi*. They have also been seen in certain species of endophytic bacteria, fungi, and some variety of lower plants where their role in these organisms continue to remain obscure (Hedden and Thomas, 2012). In one study, *Aloe vera* and gibberellin **13** were found to induce similar anti-inflammatory activity in diabetic animals. The activity of gibberellin-like substances was thought to be the ones responsible for wound-healing and anti-inflammatory activity of *Aloe vera* (Davis *et al*., 1989).

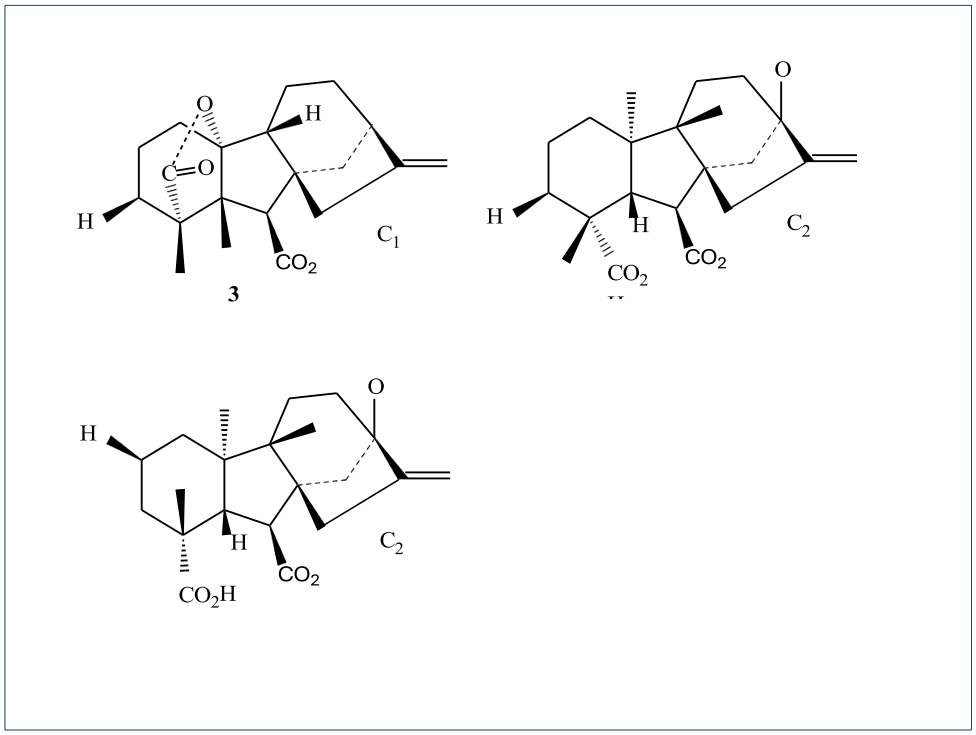


Figure 31. Chemical structures of Gibberellins **3**, **10**, and **13** (Hedden and Thomas, 2012)

The rosmarinic acid (RA) has been identified and isolated from a litany of plant species amounting to 162 plants which include various *Salvia* species. Carpati and Oriente (1958) were the first to determine the chemical structure of RA, which they formally extracted from *Rosmarinus officinalis* (*Lamiaceae)* (Petersen and Simmonds, 2003). There are, however, many substances upon which RA can be derived, such as lithospermic acid isolated from *Lycopus europaceus*, salvianolic acid isolated from *Salvianolic miltiorrhiza*, and others. RA is a C_{18}-type of caffeic acid derivative whilst lithospermic and salvianolic acids are C_{27}-type caffeic acid derivatives (Kernou *et al*., 2023; Ijaz et al., 2023). Compounds **5**, **6**, and **7** are all C_{22}-type rosmarinic acid derivatives **(Figure 32)** (Kernou *et al*., 2023).

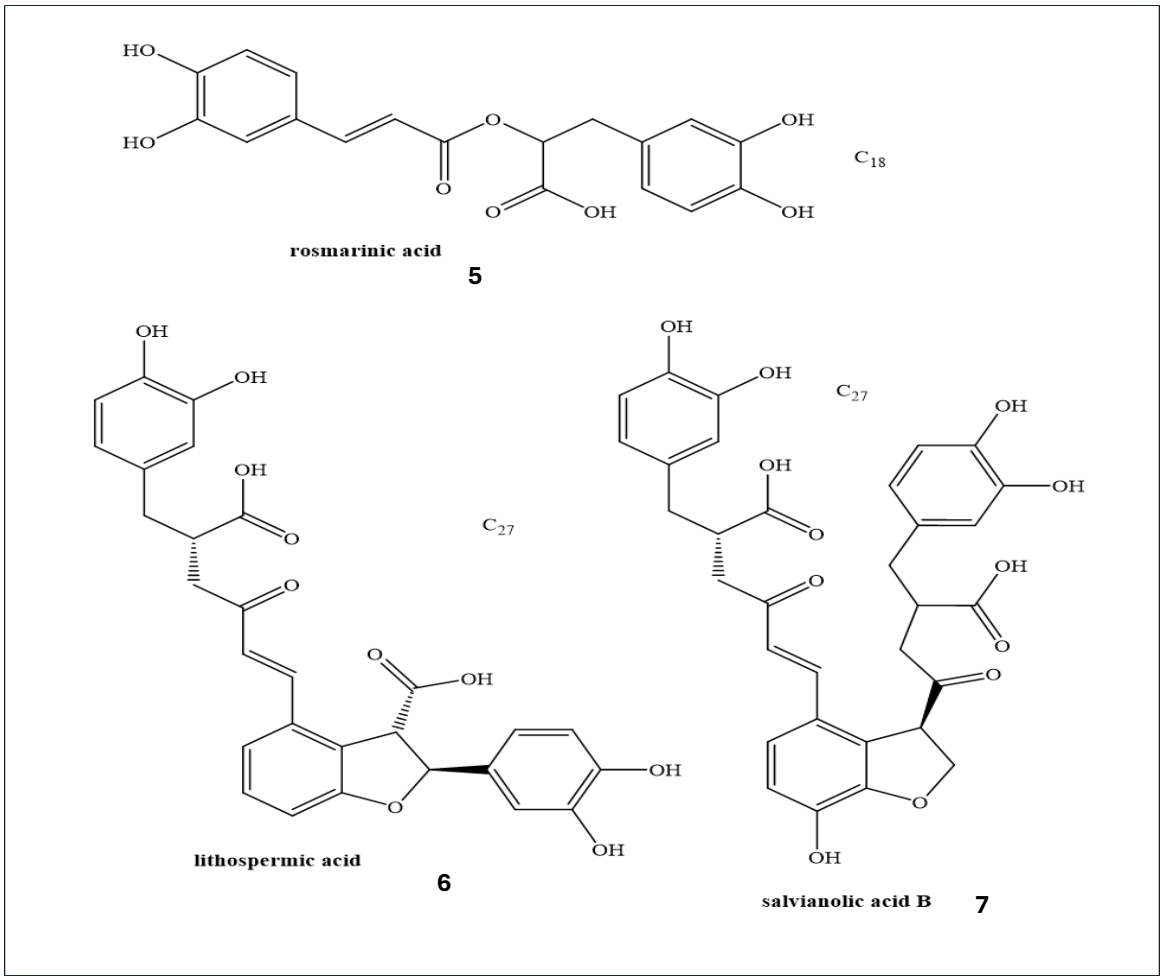


Figure 32. Chemical structures of Rosmarinic acid derivatives (Kernou *et al*., 2023; Ijaz et al., 2023)

It is worth noting that the following diterpenoids **8**, **21**, **11**, and **25 (Figure 33)** have previously been isolated and identified from *Salvia africana-lutea* as well as other *Salvia* species where they are alleged to have medicinal properties (Donthu *et al*., 2021).

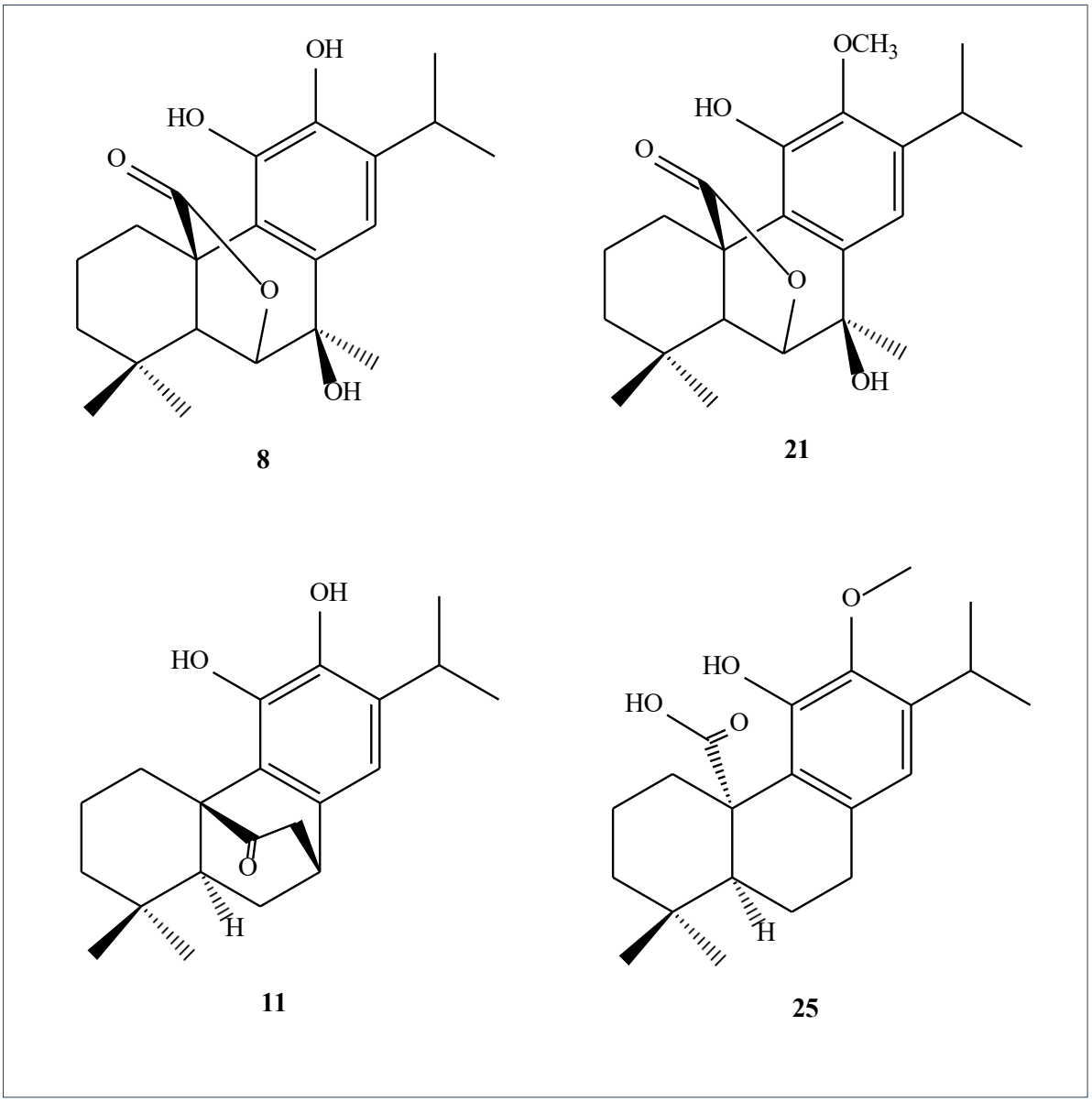
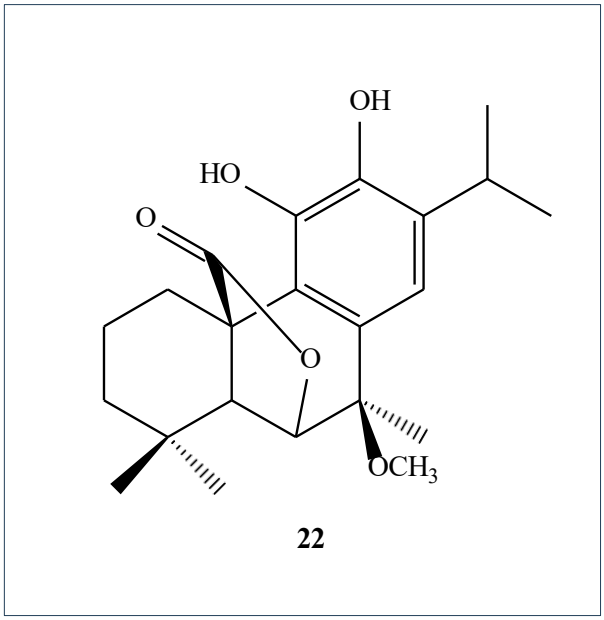


Figure 33. Chemical structures of diterpenoids **8**, **21**, **11**, and **25**

Compounds **22** and **18** (an isomer of **11**) were previously extracted from *Rosmarinus officinalis L* were both were shown to possess dose-dependent increase in vasorelaxant activity (Zhang *et al*., 2019).



Ethylrosmanol **23 (Figure 34)**, a tricyclic diterpene of abietane type was previously isolated from *Salvia officinalis L* and its structure elucidated using X-ray crystallography (Masterova *et al*., 1989; Wardana *et al*., 2021).

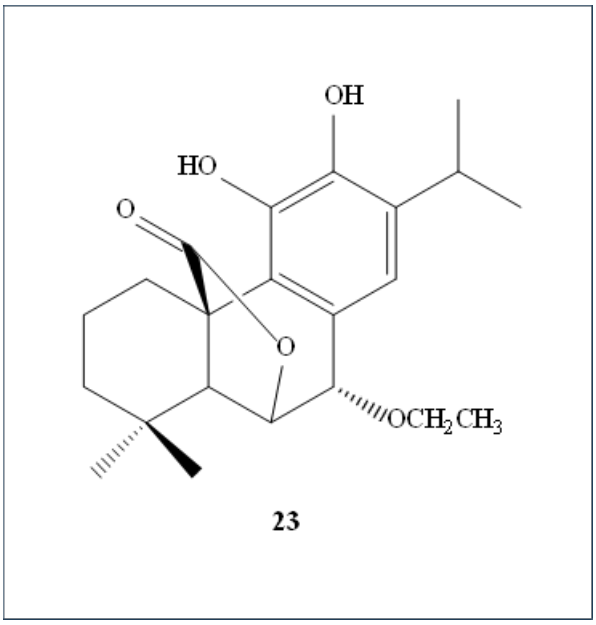


Figure 34. Chemical structure of Ethylrosmanol, a tricyclic diterpene of abietane (Wardana *et al*., 2021)

In a study by Marero and co-workers (2009), methylrosmanol **14**, **(Figure 35)** an abietane-type diterpene was furnished via a two-step reaction scheme, which revealed treatment of carnosol **11** with $NaHCO_3/H_2O$ in acetone gave rise to rosmanol which was then converted to **14** and other competing compounds upon treatment with H_2SO_4 in methanol at room temperature (Marrero *et al*., 2009).

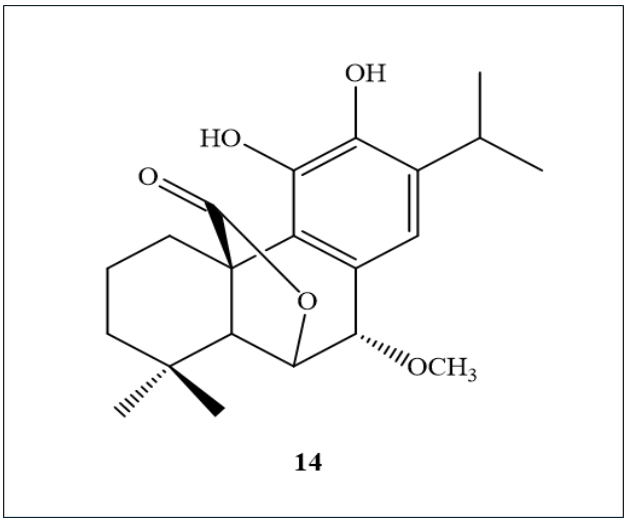


Figure 35. Chemical structure of Methylrosmanol; an abietane-type diterpene (Marrero *et al*., 2009)

Rosmadial **16, (Figure 36)** which is classified as a diterpene lactone was isolated from the leaves of *Rosmarinus officinalis L* a long time ago (Nakatani *et al*., 1983).

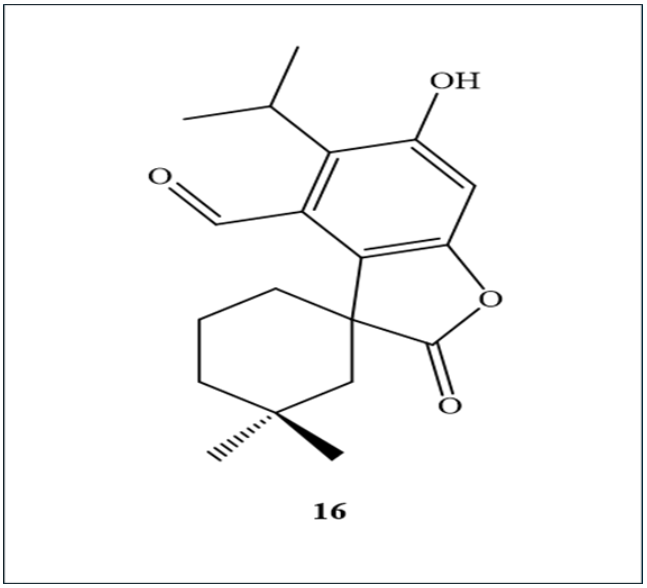


Figure 36. Chemical structure of Rosmadial, a diterpene lactone (Nakatani *et al*., 1983)

Crepidiaside A **9**, **(Figure 37)** a sesquiterpene, together with five hydroxycinnamic acid derivatives which include chlorogenic acid, 5-feruloylquinic acid, 3,5-dicaffeoylquinic acid, and methyl 3,5- dicaffeoylquinate were isolated from the aerial parts of *Leontodon saxatilis Lam.* where they displayed a positive antimyeloma activity. It was shown in this study that crepidiaside A **16** showed moderate activity (Durán *et al*., 2021).

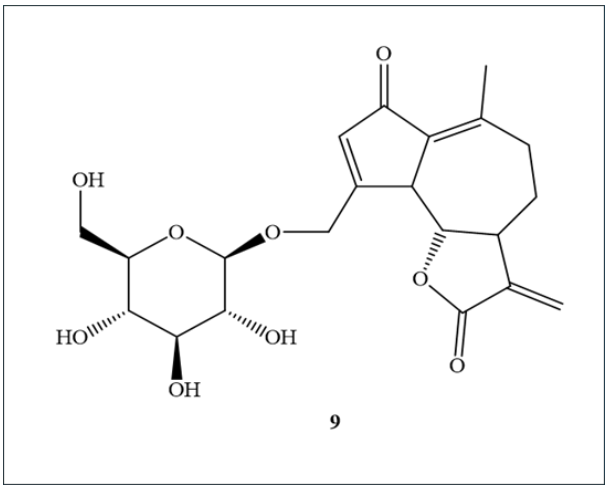


Figure 37. Chemical structure of Crepidiaside A; a sesquiterpene (Durán *et al*., 2021)

Melleolide B **17 (Figure 38)** together with other melleolides such as melleolides C and D in the *Armillaria species* have been found as characteristic secondary metabolites which belong to the class of protoilludane-type sesquiterpenoids. These melleolides have been found to possess astonishing antibacterial activity against *Bacillus cereus*, *Bacillus subtilis*, and *Escherichia coli* (Engels *et al*., 2011). Due to their unique molecular scaffold which is comprised of an orsellinic acid moiety which is esterified to give rise to a tricyclic sesquiterpene alcohol, melleolides display two distinct structure-activity relationships for their cytotoxic as well as antifungal bioactivities (Wick *et al*., 2016).

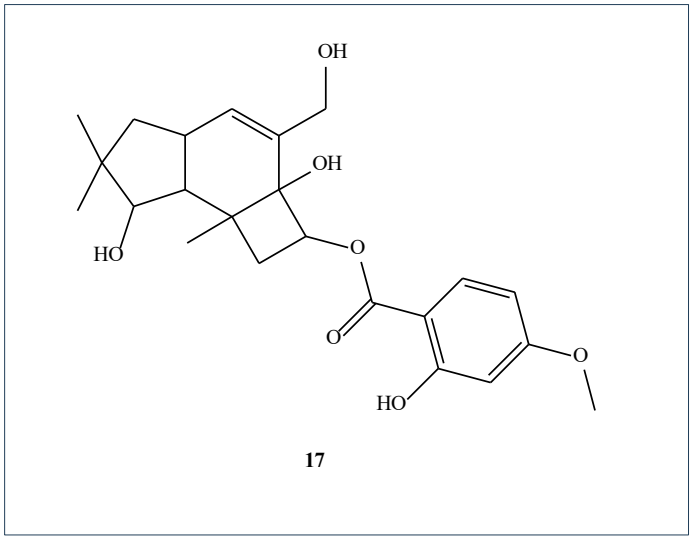


Figure 38. Chemical structure of Melleolide B (Engels *et al*., 2011)

A variety of tenellic acids which include tenellic acid A **12 (Figure 39)** have been isolated from a freshwater fungi, *Dendrospora tenella*, and these were later characterized using NMR and MS techniques. These compounds were found to possess antimicrobial activity against Gram-positive bacteria (Oh *et al*., 1999).

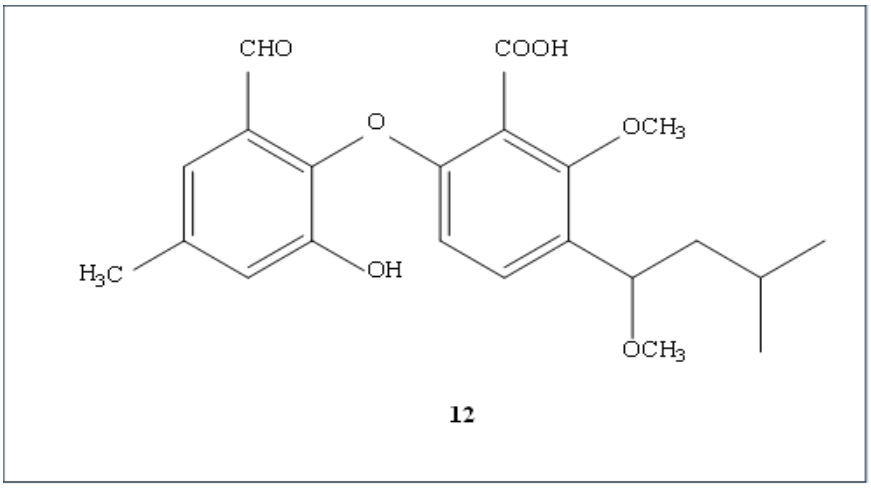


Figure 39. Chemical structure of tenellic acid A (Oh *et al*., 1999)

A few secondary metabolites from the roots of *Ferula hezarlalehzarica*which include the p-hydroxybenzoic acid alkyl ester **15 (Figure 40)** were isolated and evaluated for their preferential cytotoxicity against the PANC-1 human pancreatic cancer cell line (Alilou *et al*., 2020).

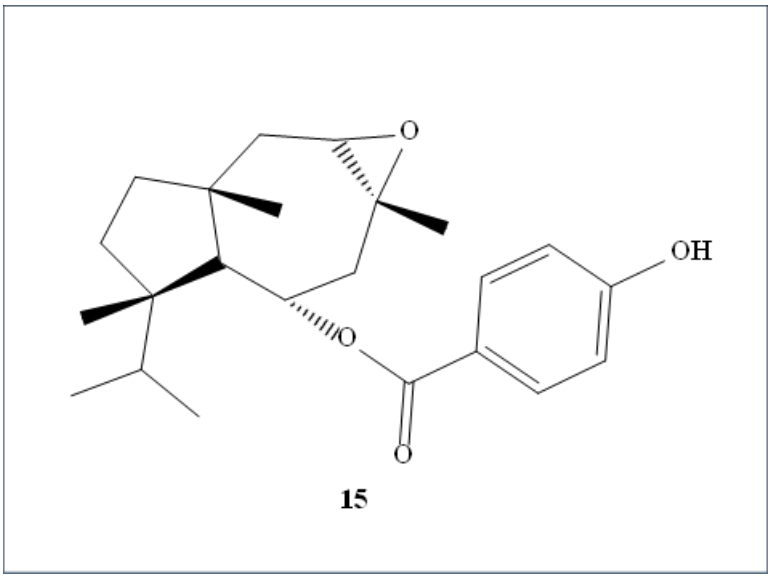


Figure 40. Chemical Structure of the p- hydroxybenzoic acid alkyl ester (Alilou *et al*., 2020)

Extractions of the seeds of *Ferula hermonis*, a plant that has been used in the Middle East as an aphrodisiac as well as for treating frigidity and impotence, furnished duacane sesquiterpenoids, which include the epoxy jaesckeanadiol vanillate **20 (Figure 41)** (Auzi *et al*., 2008).

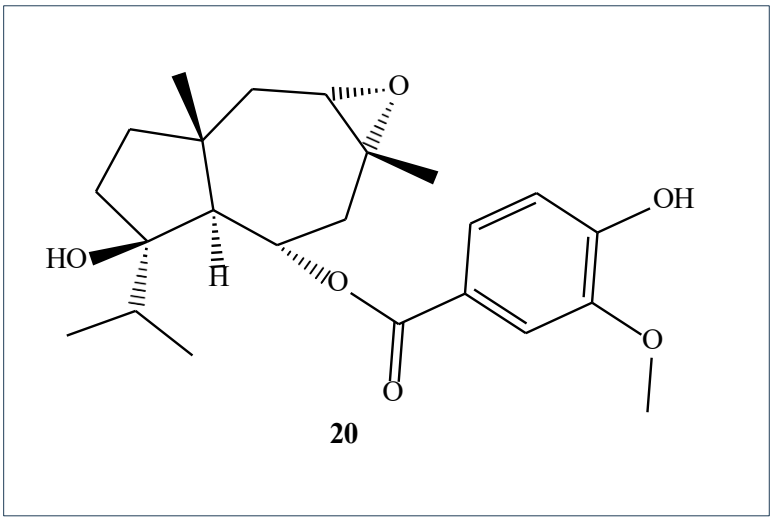


Figure 41. Chemical structure of epoxy jaesckeanadiol vanillate (Auzi *et al*., 2008)

Schisandrin **19 (Figure 42)** isolated from *Schisandra chinensis*was found to be the most active compound of this herb, where it was evaluated for its cognitive-enhancing activity (Hu *et al*., 2012).

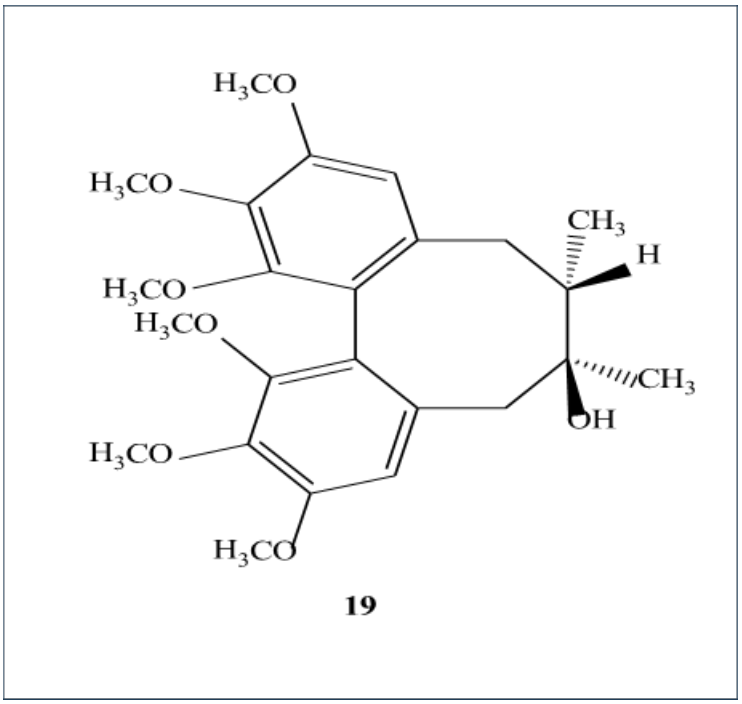


Figure 42. Chemical structure of Schisandrin (Hu *et al*., 2012).

Strophanthidin K **(Figure 43) 24**, a cardenolide, has recently been demonstrated as a hopeful anticancer agent where its role involves modulating various proteins responsible for effecting cell cycle arrest, apoptosis, and autophagic cell death has been demonstrated (Reddy *et al*., 2020).

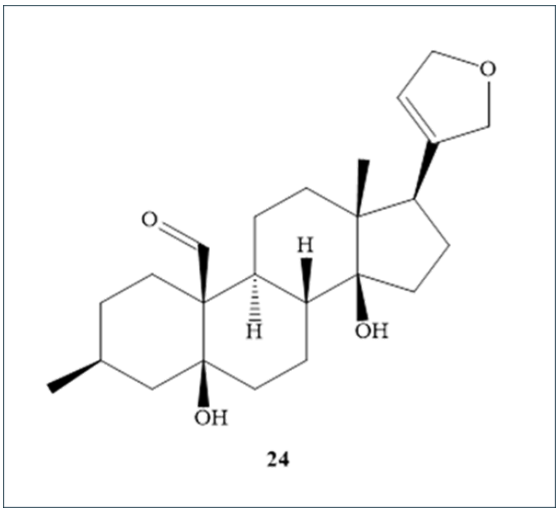


Figure 43. Chemical structure of Strophanthidin K (Reddy *et al*., 2020)

CHAPTER 5

CONCLUSION

5.1. General Conclusions

Due to increasing AMR of infectious pathogens and the widespread use of medicinal plants as an alternative therapy for many ailments, this research study focused on investigating antibacterial potential of two common South African plants, namely S*utherlandia frutescens* and *Salvia africana-lutea.* Aqueous, acetone and ethanol extracts of these two plants were prepared and tested *in-vitro* for antibacterial activity against a range of common skin pathogens i.e. *S. aureus,* MRSA*, S. epidermidis* and *P. aeruginosa*. *Sutherlandia frutescens* extract did not show any antibacterial activity against tested pathogens. *Salvia africana-lutea* extracts, on the other hand, had relatively good antibacterial activity against tested bacteria in the agar well-diffusion assay and noteworthy MICs of between 4 and 10mg/ml. Acetone and ethanol extracts were subjected to phytochemical studies that linked antibacterial activity using the TLC-bioautography assay. These were then further studied using NMR-spectroscopy and LC-MS. Unfortunately, the NMR-spectroscopy could not elucidate the structures, but LC-MS analysis indicated that the organic extracts had similar phytochemical profiles, with similar compounds exhibiting bioactivity against the various microorganisms. From these detailed chemical analyses, several compounds were identified (relative to rosmarinic acid). Rosmarinic acid is a polyphenol compound that naturally occurs in many medicinal plants (Takano *et al*., 2004).

5.2. Significance of Study

Results of the TLC-bioautography screen indicated the presence of several antibacterial phytochemicals in organic extracts of *Salvia africana- lutea*. This provides justification to further study this plant in the search for new antibacterial agents. Results from this study can thus form a foundation for the development of new drug formulations and preparations that might help control and reduce the burden of infections, including those caused by AMR-resistant bacteria.

5.3. Limitations of Study

- Due to the relatively small yield of bioactive compounds (from preparative TLC plates), the resulting mixtures could not be resolved using NMR-spectroscopy.

- *Sutherlandia frutescens* plant extracts did not yield any antibacterial activity in the current study despite some reports of bioactivity in literature. This could be due to seasonal variation i.e. the time the plants were collected, which could result in less secondary metabolites being produced.

- Additionally, many of the antibacterial compounds in plant extracts are relatively non-polar, so these compounds do not diffuse well in the aqueous agar matrix used in agar diffusion studies (Eloff, 2019).

5.4. Recommendations for Future Studies

- Optimisation of the preparative TLC assay to accommodate increased loading of the extracts could possibly result in appropriate yields of compounds necessary for NMR-spectroscopy.

- Future studies should focus on *Sutherlandia frutescens* plant material being collected from a different site and at a different season/time of year.

- Alternative antimicrobial inhibition study methods should be explored for future studies.

BIBLIOGRAPHY

Aboyade, O.M., Styger, G., Gibson, D. & Hughes, G. (2014) '*Sutherlandia frutescens*: the meeting of science and traditional knowledge,' *Journal of alternative and complementary medicine (New York, N.Y.)*, 20(2), pp. 71–76. doi: 10.1089/acm.2012.0343.

Adeosun, W.B., Bodede, O. & Prinsloo G. (2022). 'Effect of Different Climatic Regions and Seasonal Variation on the Antibacterial and Antifungal Activity, and Chemical Profile of *Helichrysum aureonitens Sch. Bip.' Metabolites*, 12(8):758, doi: 10.3390/metabo12080758.

Adetutu, A., Morgan, W. A. & Corcoran, O. (2011). 'Ethnopharmacological survey *and in vitro* evaluation of wound-healing plants used in South-western Nigeria,' *Journal of ethnopharmacology*, 137(1), pp. 50–56. doi: 10.1016/j.jep.2011.03.073.

Afonso, A., Pereira, O., Fernandes, A., Calhelha, R., Silva, A. (2019) 'Phytochemical Composition and Bioactive Effects of *Salvia africana, Salvia officinalis 'Icterina'* and *Salvia mexicana* Aqueous Extracts,' *Molecules (Basel, Switzerland)*, 24(23), p. 4327. doi: 10.3390/molecules24234327.

Ahmed, S., Sony, S.A., Chowdhury, M.B., Ullah, M. *et al.* (2020) 'Retention of antibiotic activity against resistant bacteria harbouring aminoglycoside-N-acetyltransferase enzyme by adjuvants: a combination of in-silico and *in- vitro* study,' *Scientific reports*, 10(1). doi: 10.1038/s41598-020-76355-0.

Akhter, N., Rabaan, A.A. & Alqumber, M.A.A. (2023). 'Antimicrobial Resistance: A Growing Serious Threat for Global Public Health.' *Healthcare (Basel)*,11(13):1946. doi: 10.3390/healthcare11131946.

Ali Alghamdi, B., Al-Johani, I., Al-Shamrani, J.M., Musamed Alshamrani, H. (2023). 'Antimicrobial resistance in methicillin-resistant *staphylococcus aureus*,' *Saudi journal of biological sciences*, 30(4), p. 103604. doi: 10.1016/j.sjbs.2023.103604.

Aligiannis, N., Kalpotzakis, E., Mitaku, S. & Chinou, I.B. (2001). 'Composition and antimicrobial activity of the essential oils of two Origanum species.' *Journal of Agricultural and Food Chemistry*, 40:4168–70.

Alilou, M., Dibwe, D.F., Schwaiger, S., Khodami, M. (2020). 'Antiausterity activity of secondary metabolites from the roots of *Ferula hezarlalehzarica* against the PANC-1 human pancreatic cancer cell line,' *Journal of natural products*, *83*(4), pp.1099-1106.

Antoñanzas, F. & Goossens, H. (2019). 'The economics of antibiotic resistance: a claim for personalised treatments,' *The European journal of health economics: HEPAC: health economics in prevention and care*, 20(4), pp. 483–485. doi: 10.1007/s10198-018-1021-z.

Arief, M.M.F., Fattah, A.A., Mohammed, A. & ElMwafy, H.M. (2010) 'Chemical and Bioactivity Studies on *Salvia africana-lutea* : Cytotoxicity and Apoptosis Induction by Abietane Diterpenes Isolated from *Salvia africana-lutea*,' *Journal of Basic and Environmental Sciences*, 5, pp. 72–79.

Arip, M, Selvaraja, M., R. M., Tan, L.F., Leong, M.Y. *et al.* (2022) 'Review on Plant-Based Management in Combating Antimicrobial Resistance - Mechanistic Perspective,' *Tat Ng Chin , Review on Plant-Based Management in Combating Antimicrobial Resistance - Mechanistic Perspective. Frontiers in Pharmacology,* 13:879495. doi: 10.3389/fphar.2022.879495.

Ashraf, M.V., Pant, S., Khan, M.A.H., Shah, A.A., Siddiqui, S. *et al.* (2023). 'Phytochemicals as Antimicrobials: Prospecting Himalayan Medicinal Plants as Source of Alternate Medicine to Combat Antimicrobial Resistance,' *Pharmaceuticals*, *16*, 881.

Atanasov, A.G., Zotchev, S.B. & Dirsch, V.M. (2021). 'Natural products in drug discovery: advances and opportunities' *Nature Reviews Drug Discovery,* 20, 200–216.

Bahar, M., Ashebr A., Teka, F., Adugna, N. *et al.* (2018) '*In vitro* antibacterial activity of selected medicinal plants in the traditional treatment of skin and wound infections in eastern Ethiopia,' *BioMed research international*, 2018, p. 1862401. doi: 10.1155/2018/1862401.

Bajpai, V.K., Shukla, S., Paek, W.K., Lim, J. *et al.* (2017). 'Antibacterial action of jineol isolated from Scolopendra subspinipes mutilans against selected foodborne pathogens,' *Frontiers in Microbiology*, 8: https://doi.org/10.3389/fmicb.2017.00552

Baldwin, A. J. & Kay, L. E. (2009). 'NMR spectroscopy brings invisible protein states into focus,' *Nature chemical biology*, 5(11), pp. 808–814. doi: 10.1038/nchembio.238.

Barbieri, R., Coppo, E., Marchese, A., Daglia, M. *et al*. (2017). 'Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity,' *Microbiological Research*, 196:44-68. doi: 10.1016/j.micres.2016.12.003.

Behl, T., Kumar, K., Brisc, C., Rus, M. *et al.* (2020). 'Exploring the multifocal role of phytochemicals as immunomodulators,' *Biomedicine & Pharmacotherapy,* 133:110959. doi: 10.1016/j.biopha.2020.110959.

Bele, A. & Khale, A. (2011). 'An overview on thin layer chromatography,' *International Journal of Pharmacy and Pharmaceutical Sciences, 2.*

Bisio, A., Fontana, N., Romussi, G., & Tommasi, N. (1998) 'Diterpenes and triterpenes from Salvia aurea L. Part 2: constituents of labiatae,' *Pharmazie Stellenangebote*, 53. 210-211.

Büttner, H., Mack, D. & Rohde, H. (2015). 'Structural basis of *Staphylococcus epidermidis* biofilm formation: mechanisms and molecular interactions,' *Frontiers in cellular and infection microbiology*, 5, p. 14. doi: 10.3389/fcimb.2015.00014.

Cai, L. (2014). 'Thin layer chromatography,' *Current protocols essential laboratory techniques*, 8(1). doi: 10.1002/9780470089941.et0603s08.

Calderón-Oropeza, M., Ramírez-Briones, E., Rodríguez-García, G., Salvador-Hernández, J. *et al.* (2021) 'Metabolic Correlations of Salvia dugesii Fernald and Salvia gesneriiflora Lindl. & Paxton with Native Salvia Plants from Four Continents Using Essential Oils Compositions,' *Records of Natural Products*, 15(4), pp. 312–323. doi: 10.25135/rnp.223-19.121912.

CDC, (2012). '*Pseudomonas aeruginosa* bacteria, SEM' *Science Photo Library*. https://www.sciencephoto.com/media/186205/view

CDC, (2005). '*MRSA* Fast Facts' *https://edition.cnn.com/2013/06/28/us/mrsa-fast-facts/index.html*

Arduino, M.J. (2001). 'Public Health Image Library (PHIL)' CDC. https://phil.cdc.gov/details.aspx?pid=11155

Chen, L., Xu, M., Gong, Z., Zonyane, S. *et al.* (2018) 'Comparative cardio and developmental toxicity induced by the popular medicinal extract of *Sutherlandia frutescens* (L.) R.Br. detected using a zebrafish Tuebingen embryo model,' *BMC complementary and alternative medicine*, 18(1). doi: 10.1186/s12906- 018-2303-9.

Chen, X., Lan, W. & Xie, J. (2024). 'Natural phenolic compounds: Antimicrobial properties, antimicrobial mechanisms, and potential utilization in the preservation of aquatic products,' *Food chemistry*, 440: p. 138198. doi: 10.1016/j.foodchem.2023.138198.

Chessa, D., Ganau, G. & Mazzarello, V. (2015). 'An overview of *Staphylococcus epidermidis* and *Staphylococcus aureus* with a focus on developing countries,' *Journal of infection in developing countries*, 9(6), pp. 547–550. doi: 10.3855/jidc.6923.

Cheung, G.Y., Rigby, K., Wang, R., Queck, S.Y. *et al.* (2010). *Staphylococcus epidermidis* strategies to avoid killing by human neutrophils. *PLOS Pathogens*, 6(10):e1001133. doi: 10.1371/journal.ppat.1001133.

Chik, S.C.C., Or, T.C.T., Luo, D., Yang, C.L.H. *et al.* (2013). 'Pharmacological effects of active compounds on neurodegenerative disease with gastrodia and uncaria decoction, a commonly used poststroke decoction,' *The Scientific World Journal*, (1), p.896873. doi: 10.1155/2013/896873.

Choma, I. M. & Grzelak, E. M. (2011). 'Bioautography detection in thin-layer chromatography,' *Journal of chromatography A*, 1218(19), pp. 2684–2691. doi: 10.1016/j.chroma.2010.12.069.

Choma, I.M. & Wioleta J. (2015). 'TLC-Direct Bioautography as a High Throughput Method for Detection of Antimicrobials in Plants,' *Chromatography* 2(2): 225-238.

Clemente, L., Leão, C., Moura, L., Albuquerque, T. *et al.* (2021). 'Prevalence and characterization of ESBL/AmpC producing *Escherichia coli* from fresh meat in Portugal,' *Antibiotics (Basel, Switzerland)*, 10(11), p. 1333. doi: 10.3390/antibiotics10111333.

Cos, P., Vlietinck, A.J., Vanden, B.D. & Maes, L. (2006). 'Anti-infective potential of natural products: how to develop a stronger *in vitro* proof-of-concept,' *Journal of Ethnopharmacology,* 106: 290- 302. 10.1016/j.jep.2006.04.003.

Cowan, M. (1999). 'Plant products as antimicrobial agents.' *Journal of Clinical Microbiology, Rev*; 12: 564-582.

Cox-Georgian, D., Ramadoss, N., Dona, C. & Basu, C. (2019). 'Therapeutic and medicinal uses of terpenes.' *Medicinal plants: from farm to pharmacy*, pp.333-359.

Davis, R., & Maro, N. (1989). 'Aloe vera and gibberellin. Anti-inflammatory activity in diabetes,' *Journal of the American Podiatric Medical Association*, *79*(1), 24-26.

De Angelis, G., Del Giacomo, P., Posteraro, B., Sanguinetti, M. *et al.* (2020) 'Molecular mechanisms, epidemiology, and clinical importance of β-lactam resistance in Enterobacteriaceae,' *International journal of molecular sciences*, 21(14), p. 5090. doi: 10.3390/ijms21145090.

De Gaetano, G.V., Lentini, G., Famà, A., Coppolino, F. *et al.* (2023). 'Antimicrobial Resistance: Two-Component Regulatory Systems and Multidrug Efflux Pumps.' *Antibiotics*, 12(6). 965. doi: 10.3390/antibiotics12060965. PMID: 37370284

Dewanjee, S., Gangopadhyay, M., Bhattacharya, N., Khanra, R. (2015) 'Bioautography and its scope in the field of natural product chemistry,' *Journal of pharmaceutical analysis*, 5(2), pp. 75–84. doi: 10.1016/j.jpha.2014.06.002.

Domenico, D., Oliva, E. G. & Guembe, A. (2022). 'The Current Knowledge on the Pathogenesis of Tissue and Medical Device-Related Biofilm Infections,' *Microorganisms*, 10.

Donthu, N., Kumar, S., Mukherjee, D., Pandey, N. *et al.* (2021). 'How to conduct a bibliometric analysis: An overview and guidelines,' *Journal of business research*, *133*, pp.285- 296.

Duarte, M.C.T., Figueira, G.M., Sartoratto, A., Rehder, V.L.G. *et al.* (2005). 'Anti-Candida activity of Brazilian medicinal plants,' *Journal of Ethnopharmacology,* 97:305–11.

Dube, P., Meyer, S., Madiehe, A. & Meyer M. (2020) 'Antibacterial activity of biogenic silver and gold nanoparticles synthesized from *Salvia africana-lutea* and *Sutherlandia frutescens*,' *Nanotechnology*, 31(50), p. 505607. doi: 10.1088/1361-6528/abb6a8.

Durán, A.G., Rial, C., Gutiérrez, M.T., Molinillo, J.M.G. *et al.* (2021). 'Sesquiterpenes in Fresh Food.' In: Xiao, J., Sarker, S.D., Asakawa, Y. (eds) Handbook of Dietary Phytochemicals. Springer, Singapore. https://doi.org/10.1007/978-981-15-4148-3_47

Egbuna, C., Ifemeje, J., Kryeziu, T., Mukherjee, M. *et al.* (2018). 'Introduction to Phytochemistry,' doi: 10.13140/RG.2.2.18812.23687

Eloff, J.N. (2019). 'Avoiding pitfalls in determining antimicrobial activity of plant extracts and publishing the results.' *BMC Complementary Medicine and Therapies,* 19(1):106. doi: 10.1186/s12906-019- 2519-3.

Emwas, A.H., Szczepski, K., Poulson, B.G., Chandra, K. *et al.* (2020) 'NMR as a 'gold standard' method in drug design and discovery,' *Molecules (Basel, Switzerland)*, 25(20), p. 4597. doi: 10.3390/molecules25204597.

Engels, B., Heinig, U., Grothe, T., Stadler, M. *et al.* (2011). 'Cloning and characterization of an Armillaria gallica cDNA encoding protoilludene synthase, which catalyzes the first committed step in the synthesis of antimicrobial melleolides.' *The Journal of biological chemistry*, 286(9), 6871–6878.

Ezema, C., Aguchem, R., Aham, E., Ezeorba, W. *et al.* (2024). '*Salvia africana-lutea* L.: a review of ethnobotany, phytochemistry, pharmacology applications and future prospects,' *Advances in Traditional Medicine*, 24(3), pp. 703–724. doi: 10.1007/s13596-023-00726-x.

Friedman, N.D., Temkin, E. & Carmeli, Y. (2016). 'The negative impact of antibiotic resistance.' *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, 22(5), pp. 416–422. doi: 10.1016/j.cmi.2015.12.002.

Gallo, M. (2022) "Extraction and isolation of natural products," *Separations*, 9(10), p. 287. doi: 10.3390/separations9100287.

Gellatly, S. L. & Hancock, R. E. W. (2013). '*Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses,' *Pathogens and disease*, 67(3), pp.159–173. doi: 10.1111/2049-632X.12033.

Goyal S., Lambert C., Cluzet S., Mérillon J.M. *et al.* (2012). '*Plant Defence: Biological Control.'* Springer; Dordrecht, The Netherlands: Secondary Metabolites and Plant Defence; pp. 109–138.

Griffin, S.G., Wyllie, S.G., Markham, J.L. & Leach, D.N. (1999). 'The role of structure and molecular properties of terpenoids in determining their antimicrobial activity,' *Flavour and fragrance journal*, 14(5), pp. 322–332. doi: 10.1002/(sici)1099-1026(199909/10)14:5<322::aid-ffj837>3.0.co;2-4.

Hedden, P. & Thomas, S.G. (2012). 'Gibberellin biosynthesis and its regulation.' *Biochemical Journal,* 444(1):11-25. doi: 10.1042/BJ20120245.

Hlongwane, M.M., Mohammed, M., Mokgalaka, N.S. & Dakora, F.D. (2023) 'The potential of rhizobacteria to mitigate abiotic stress in *Lessertia frutescens*,' *Plants*, 12(1), p. 196. doi: 10.3390/plants12010196.

Hu, D.C., Yunfeng, H., Rongrong, H., Na, L. *et al.* (2012). 'An Antioxidant Lignan from *Schisandra chinensis,* Ameliorates A*β*1–42-Induced Memory Impairment in Mice,' *Oxidative Medicine and Cellular Longevity*, 721721, 7 pages, https://doi.org/10.1155/2012/721721

Huang, L., Wu, C., Gao, H., Xu, C. *et al.* (2022) 'Bacterial multidrug efflux pumps at the frontline of antimicrobial resistance: An overview,' *Antibiotics (Basel, Switzerland)*, 11(4), p. 520. doi: 10.3390/antibiotics11040520.

Hübsch, Z., Van Zyl, R.L., Cock, I.E.*et al.* (2014). 'Interactive antimicrobial and toxicity profiles of conventional antimicrobials with Southern African medicinal plants.' *South African Journal of Botany,* 93:185–197. doi: 10.1016/j.sajb.2014.04.005.

Ijaz, S., Iqbal, J., Abbasi, B.A., Ullah, Z. *et al.* (2023). Rosmarinic acid and its derivatives: Current insights on anticancer potential and other biomedical applications. *Biomedicine & Pharmacotherapy,* 162:114687. doi: 10.1016/j.biopha.2023.114687.

Iyer, M., Pal, K. & Upadhye, V. (2023). 'Phytochemicals and cancer,' in *Recent Frontiers of Phytochemicals*. Elsevier, pp. 295–308.

Jassal, M. & Bishai, W. R. (2009) 'Extensively drug-resistant tuberculosis,' *The Lancet infectious diseases*, 9(1), pp. 19–30. doi: 10.1016/S1473-3099(08)70260-3.

Jesionek, W., Móricz, Á., Alberti, A., Ott, P. *et al.* (2015) 'TLC-direct bioautography as a bioassay guided method for investigation of antibacterial compounds in Hypericum perforatum L,' *Journal of AOAC International*, 98(4), pp. 1013–1020. doi: 10.5740/jaoacint.14-233.

Jian, Z., Zeng, L., Xu, T., Sun, S.*et al.* (2021). ‘ Antibiotic resistance genes in bacteria: occurrence, spread, and control.’ *Journal of Basic Microbiology*, 61: 1049– 1070.

Kamatou, G.P., Makunga, N.P., Ramogola, W.P. & Viljoen, A.M. (2008) ‘South African Salvia species: a review of biological activities and phytochemistry,’ *Journal of ethnopharmacology*, 119(3), pp. 664–672. doi: 10.1016/j.jep.2008.06.030.

Katerere, D. R. & Eloff, J. N. (2005). ‘Antibacterial and antioxidant activity of *Sutherlandia frutescens (Fabaceae)*, a reputed anti-HIV/AIDS phytomedicine,’ *Phytotherapy research: PTR*, 19(9): 779–781. doi: 10.1002/ptr.1719.

Kaur, R. & Arora, S. (2015). ‘Alkaloids-important therapeutic secondary metabolites of plant origin,’ *Journal of critical reviews*, 2, pp. 1–8.

Kernou, O.N., Azzouz, Z., Madani, K. & Rijo, P. (2023). ‘Application of rosmarinic acid with its derivatives in the treatment of microbial pathogens.’ *Molecules*, *28*(10), p.4243.

Khan, H. A., Baig, F. K. & Mehboob, R. (2017). ‘Nosocomial infections: Epidemiology, prevention, control and surveillance,’ *Asian pacific journal of tropical biomedicine*, 7(5), pp. 478–482. doi: 10.1016/j.apjtb.2017.01.019.

Khodaparast, L., Shahrooei, M., Stijlemans, B., Merckx, R. *et al. (*2016). The Possible Role of *Staphylococcus epidermidis* LPxTG Surface Protein SesC in Biofilm Formation. *PLoS One*, 11(1):e0146704. doi: 10.1371/journal.pone.0146704.

Koehnke, A. & Friedrich, R. E. (2015). ‘Antibiotic discovery in the age of structural biology-a comprehensive overview with special reference to development of drugs for the treatment of *Pseudomonas aeruginosa* infection,’ *In vivo*, 29(2): pp. 161–167.

Kong, J., Wu, Z.X,, Wei, L., Chen, Z.S. *et al.* (2020). ‘Exploration of Antibiotic Activity of Aminoglycosides, in Particular Ribostamycin Alone and in Combination With Ethylenediaminetetraacetic Acid Against Pathogenic Bacteria.’ *Frontiers in Microbiology*, 11:1718. doi: 10.3389/fmicb.2020.01718

Kotzekidou, P., Giannakidis, P. & Boulamatsis, A. (2008) ‘Antimicrobial activity of some plant extracts and essential oils against foodborne pathogens *in vitro* and on the fate of inoculated pathogens in chocolate, LWT,’ *Food Science and Technology*, 41, pp. 119–127.

Kowalska, T., & Sajewicz, M. (2022). ‘Thin-Layer Chromatography (TLC) in the Screening of Botanicals-Its Versatile Potential and Selected Applications.’ *Molecules (Basel, Switzerland)*, *27*(19), 6607.

Kumar, S., Jyotirmayee, K. & Sarangi, M. (2013). 'Thin layer chromatography: A tool of biotechnology for isolation of bioactive compounds from medicinal plants,' *International Journal of Pharmaceutical Sciences Review and Research*, 18, pp. 126–132.

Lade, B., Patil, A. & Hariprasad, K.A. (2014). 'A Comprehensive Working, Principles and Applications of Thin Layer Chromatography,' *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 5, pp. 486–503.

Lambert, P. (2005). 'Bacterial resistance to antibiotics: Modified target sites,' *Advanced drug delivery reviews*, 57(10), pp. 1471–1485. doi: 10.1016/j.addr.2005.04.003.

Li, Q. & Xing, B. (2016). 'Vitamin D3-Supplemented Yogurt Drink Improves Insulin Resistance and Lipid Profiles in Women with Gestational Diabetes Mellitus: A Randomized Double Blinded Clinical Trial.' *Annals of Nutrition and Metabolism*, 68, 285-290.

Lim, C.K. & Lord, G. (2002). 'Current developments in LC-MS for pharmaceutical analysis,' *Biological & pharmaceutical bulletin*, 25(5), pp. 547–557. doi: 10.1248/bpb.25.547.

Lin, J., Nishino, K., Roberts, M.C., Tolmasky, M. *et al.* (2015) "Mechanisms of antibiotic resistance," *Frontiers in microbiology*, 6. doi: 10 .3389/fmicb.2015.00034.

Mahizan, N.A., Yang, S.K., Moo, C.L., Song, A.A. *et al.* (2019). Terpene Derivatives as a Potential Agent against Antimicrobial Resistance (AMR) Pathogens. *Molecules*, 24(14):2631. doi: 10.3390/molecules24142631.

Marín, L. F. C., Arciniegas, G. E. & Vivas, M. C. (2015). 'Characterization of Staphylococcus aureus Isolates that colonize medical students in a hospital of the City of Cali, Colombia,' *International Journal of Microbiology*, pp. 1–6.

Marrero, J.G., Moujir, L., Andres, L.S., Montano, N.P. *et al. (*2009). 'Semisynthesis and biological evaluation of abietane-type diterpenes. Revision of the structure of rosmaquinone.' *Journal of natural products*, *72*(8), pp.1385-1389.

McGaw, L.J., Bagla, V.P., Steenkamp, P.A., Fouche, G. *et al.* (2013). 'Antifungal and antibacterial activity and chemical composition of polar and non-polar extracts of *Athrixia phylicoides* determined using bioautography and HPLC.' *BMC Complementary Medicine and Therapies,* 13, 356.

Michael, C. A., Dominey-Howes, D. & Labbate, M. (2014). 'The antimicrobial resistance crisis: Causes, consequences, and management,' *Frontiers in public health*, 2. doi: 10.3389/fpubh.2014.00145.

Miklasińska-Majdanik, M., Kępa, M., Wojtyczka, R.D., Idzik, D. *et al.* (2018) 'Phenolic compounds diminish antibiotic resistance of Staphylococcus aureus clinical strains,' *International journal of environmental research and public health*, 15(10), p. 2321. doi: 10.3390/ijerph15102321.

Mogana, R., Adhikari, A., Tzar, M.N., Ramliza R. *et al.* (2020). 'Antibacterial activities of the extracts, fractions and isolated compounds from *Canarium patentinervium* Miq. against bacterial clinical isolates.' *BMC Complementary Medicine and Therapies,* 20, 55. https://doi.org/10.1186/s12906-020- 2837-5

Mowat, E., Paterson, S., Fothergill, J.L., Wright, E.A. *et al.* (2011). '*Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections.' *American Journal of Respiratory and Critical Care Medicine,* 183(12):1674-9. doi: 10.1164/rccm.201009-1430OC.

Mukherjee, P. (2019). 'LC–MS: A Rapid Technique for Understanding the Plant Metabolite,' *Analysis*. 10.1016/B978-0-12-813374-3.00011-9. "

Murray, C.J.L., Ikuta, K.S., Sharara, F., Swetschinski, L. *et al*. (2019). 'Global burden of bacterial antimicrobial resistance, a systematic analysis.' *The Lancet*, 399 (10325): 629 – 655.

Najar, B., Mecacci, G., Nardi, V., Cervelli, C. *et al.* (2021). 'Volatiles and antifungal-antibacterial-antiviral activity of south African Salvia spp. Essential oils cultivated in uniform conditions,' *Molecules (Basel, Switzerland)*, 26(9), p. 2826. doi: 10.3390/molecules26092826.

Nakatani, R.I. (1983). 'A New Diterpene Lactone, Rosmadial, from Rosemary (*Rosmarinus officinalis* L.),' *Agricultural and Biological Chemistry*, Volume 47, Issue 2, Pages 353–358,

Nikaido, H. & Pagès, J.M. (2012). 'Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria,' *FEMS microbiology reviews*, 36(2), pp. 340–363. doi: 10.1111/j.1574-6976.2011.00290.x.

Nishiwaki, H., Kumamoto, M., Shuto, Y. & Yamauchi, S. (2011). 'Stereoselective syntheses of all stereoisomers of lariciresinol and their plant growth inhibitory activities.' *Journal of Agricultural and Food Chemistry,* 59(24):13089-95. doi: 10.1021/jf203222w.

Nosov, A. V., Titova, M. V. & Fomenkov, A. A. (2023). 'Callus and suspension cell cultures of *Sutherlandia frutescens* and preliminary screening of their phytochemical composition and antimicrobial activity,' *Acta Physiologiae Plantarum*, 45.

Ntuli, S. S. B. N., Gelderblom, W. C. A. & Katerere, D. R. (2018) 'The mutagenic and antimutagenic activity of *Sutherlandia frutescens* extracts and marker compounds,' *BMC complementary and alternative medicine*, 18(1). doi: 10.1186/s12906-018-2159-z.

Oh, H., Kwon, T.O., Gloer, J.B., Marvanová, L. *et al.* (1999). 'Tenellic Acids A− D: New Bioactive Diphenyl Ether Derivatives from the Aquatic Fungus Dendrospora t enella.' *Journal of natural products*, *62*(4), pp.580-583.

Otto, M. (2014). '*Staphylococcus aureus* toxins,' *Current opinion in microbiology*, 17, pp. 32–37. doi: 10.1016/j.mib.2013.11.004.

Pandey, N. & Cascella, M. (2023). '*Beta-Lactam Antibiotics*.' StatPearls Publishing.

Parekh, J. & Chanda, S. (2007). '*In Vitro* Antimicrobial Activity and Phytochemical Analysis of Some Indian Medicinal Plants,' *Turkish Journal of Biology*, 31: pp. 53–58.

Patra, A. K. (2012). 'An overview of antimicrobial properties of different classes of phytochemicals,' in *Dietary Phytochemicals and Microbes*. Dordrecht: Springer Netherlands, pp. 1–32.

Petersen, M. & Simmonds, M.S.J. (2003). 'Rosmarinic acid.' *Phytochemistry 62,* pp 121-125

Peterson, L. R. (2009). 'Bad bugs, no drugs: No ESCAPE revisited,' *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*, 49(6), pp. 992– 993. doi: 10.1086/605539.

Phukan, M.M., Punj, P., Pranay, R.S., Samson, A. *et al.* (2023) 'Chapter 9 Antimicrobial, anti-inflammatory, and wound-healing activities of medicinal plants,' in *Phytochemicals in Medicinal Plants*. De Gruyter, pp. 205– 226.

Piddock, L. (2006). 'Multidrug-resistance efflux pumps ? not just for resistance.' *Nature Reviews Microbiology,* 4: 629–636. https://doi.org/10.1038/nrmicro1464

Pinheiro, L., Brito, C.I., de Oliveira, A., Martins, P.Y. *et al.* (2015). '*Staphylococcus epidermidis* and *Staphylococcus haemolyticus*: Molecular Detection of Cytotoxin and Enterotoxin Genes.' *Toxins (Basel),* 7(9):3688-99. doi: 10.3390/toxins7093688.

Preethi, R., Devanathan, V.V. & Loganathan, M. (2010). 'Antimicrobial and Antioxidant Efficacy of Some Medicinal Plants Against Food Borne Pathogens.' *Advanced Biomedical Research,* 4 (2): 122-125. 31.

Prestinaci, F., Pezzotti, P & Pantosti, A. (2015). 'Antimicrobial resistance: a global multifaceted phenomenon.' *Pathogens and Global Health*, 109(7):309-18. doi: 10.1179/2047773215Y.0000000030.

Puca, V., Marulli, R.Z., Grande, R., Vitale, I. *et al.* (2021) 'Microbial species isolated from infected wounds and antimicrobial resistance analysis: Data emerging from a three-years retrospective study,' *Antibiotics (Basel, Switzerland)*, 10(10), p. 1162. doi: 10.3390/antibiotics10101162.

Raman, G., Avendano, E.E., Chan, J., Merchant, S. *et al.* (2018). 'Risk factors for hospitalized patients with resistant or multidrug-resistant *Pseudomonas aeruginosa* infections: a systematic review and meta-analysis.' *Antimicrobial Resistance and Infection Control*, 7:79. doi:10.1186/s13756-018-0370-9.

Rattray, R. D. & Van Wyk, B.E. (2021). 'The botanical, chemical and ethnobotanical diversity of southern African Lamiaceae,' *Molecules (Basel, Switzerland)*, 26(12), p. 3712. doi: 10.3390/molecules26123712.

Reddy, D., Ghosh, P. & Kumavath, R. (2020). 'Strophanthidin Attenuates MAPK, PI3K/AKT/mTOR, and Wnt/β-Catenin Signaling Pathways in Human Cancers.' *Frontiers in Oncology*, 9:1469. doi: 10.3389/fonc.2019.01469.

Rekha, S. R., Kulandhaivel, M. & Hridhya, K. V. (2018). 'Antibacterial Efficacy and Minimum Inhibitory Concentrations of Medicinal Plants Against Wound Pathogens.' *Biomedical and Pharmacology Journal J*;11(1).

Reygaert, W. C. (2018). 'An overview of the antimicrobial resistance mechanisms of bacteria,' *AIMS microbiology*, 4(3), pp. 482–501. doi: 10.3934/microbiol.2018.3.482.

Rivero-Pino, F., Leon, M.J., Millan-Linares, M.C. and Montserrat-Dela Paz, S., (2023). Antimicrobial plant-derived peptides obtained by enzymatic hydrolysis and fermentation as components to improve current food systems. *Trends in Food Science & Technology*, *135*, pp.32-42

Romussi, G., Fontana, N., Mascolo, N., Capasso, R. *et al.* (1998) 'Chemical composition of essential oils from some Salvia species,' *Phytotherapy research: PTR*, 12(S1), pp. S117–S120.

Rudrapal, M., Khairnar, S.J., Khan, J., Dukhyil, A.B. (2022). 'Dietary Polyphenols and Their Role in Oxidative Stress-Induced Human Diseases: Insights Into Protective Effects, Antioxidant Potentials and Mechanism(s) of Action.' *Frontiers in Pharmacology,* 13:806470. doi: 10.3389/fphar.2022.806470.

Sabaté B.M., Harris, L.G., Thompson, K., Stanic, B. *et al.* (2017). 'Pathogenic Mechanisms and Host Interactions in *Staphylococcus epidermidis* Device-Related Infection.' *Frontiers in Microbiology*, 8:1401. doi: 10.3389/fmicb.2017.01401.

Sabtu, N., Enoch, D. A. & Brown, N. M. (2015). 'Antibiotic resistance: what, why, where, when and how?,' *British medical bulletin*, p. ldv041. doi: 10.1093/bmb/ldv041.

Salam, M.A., Al-Amin, M.Y., Salam, M.T., Pawar, J.S. *et al.* (2023). 'Antimicrobial resistance: A growing serious threat for global public health,' *Healthcare (Basel, Switzerland)*, 11(13), p. 1946. doi: 10.3390/healthcare11131946.

San Marcos Growers, (2024). '*Salvia africana-lutea* at San Marcos Growers' https://www.smgrowers.com/products/plants/plantdisplay.asp?plant_id=1405

Saxena, M., Jyoti, S., Nema, R., Dharmendra, S. *et al.* (2013). Phytochemistry of Medicinal Plants, *Journal of Pharmacognosy and Phytochemistry*, 1:168-182.

Scarpati, M.L. & Oriente, G. (1958). ' Isolamento e costituzione dell'acido rosmarinico (dal *rosmarinus off*.)' *Ric. Sci.*, 28 pp. 2329-2333

Science Photo Library, (2010). '*Staphylococcus epidermidis* bacteria, SEM' https://www.sciencephoto.com/media/129706/view/staphylococcus-epidermidis-bacteria-sem

Scott, G., Springfield, E. P. & Coldrey, N. (2004). 'A pharmacognostical study of 26 south African plant species used as traditional medicines,' *Pharmaceutical biology*, 42(3), pp. 186–213. doi: 10.1080/13880200490514032.

Selegato, D. M., Pilon, A. C. & Carnevale Neto, F. (2019). 'Plant metabolomics using NMR spectroscopy,' in *Methods in Molecular Biology*. New York, NY: Springer New York, pp. 345–362.

Seo, K.W., Kim, B.K., Jeon, H.Y., Suk-Kyung L.S. *et al.* (2018). 'Comparative genetic characterization of third-generation cephalosporin-resistant *Escherichia coli* from chicken meat produced by integrated broiler operations in South Korea,' *Poultry science*, 97(8): pp. 2871–2879. doi: 10.3382/ps/pey127.

Seyydnejad, S.M., Niknejad, M., Darabpoor, I. & Motamedi, H. (2010). 'Antibacterial Activity of Hydroalcoholic Extract of *Callistemon citrinus* and *Albizia lebbeck*.' *American Journal of Applied Sciences*, 7 (1): 13-16. 32.

Sharma, S., Mohler, J., Mahajan, S.D., Schwartz, S.A. *et al.* (2023) 'Microbial biofilm: A review on formation, infection, antibiotic resistance, control measures, and innovative treatment,' *Microorganisms*, 11(6), p. 1614. doi: 10.3390/microorganisms11061614.

Song, J., Oh, S.S., Kim, J., Park, S. & Shin J. (2020) 'Shin Clinically relevant extended-spectrum β-lactamase-producing *Escherichia coli* isolates from food animals in,' *South Korea Frontiers in Microbiology*, 11, pp. 1–9.

Subramani, P., Anish, R., Balamurugan, S., Muralidharan, S. *et al.* (2015). 'An overview of liquid chromatography-mass spectroscopy instrumentation,' *Pharmaceutical Methods*, 5, pp. 47–55.

Suleimana, M.M., McGaw, L.J., Naidoo, V. & Eloff, J.N. (2009). 'Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species.' *African Journal of Traditional, Complementary and Alternative Medicines*, (1):64-78. doi: 10.4314/ajtcam.v7i1.57269.

Takano, H., Osakabe, N., Sanbongi, C., Yanagisawa, R. *et al.* (2004). 'Extract of *Perilla frutescens* enriched for rosmarinic acid, a polyphenolic phytochemical, inhibits seasonal allergic rhinoconjunctivitis in humans.' *Experimental Biology and Medicine,* 229 (3): 247-54. doi: 10.1177/1535370204229

Takeuchi, K., Baskaran, K. & Arthanari, H. (2019). 'Structure determination using solution NMR: Is it worth the effort?,' *Journal of magnetic resonance (San Diego, Calif.: 1997)*, 306, pp.195–201. doi: 10.1016/j.jmr.2019.07.045.

Tong, S.Y., Davis, J.S., Eichenberger, E., Holland, T.L. *et al. (*2015). '*Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management.' *Clinical Microbiology Reviews,* 28(3):603-61. doi: 10.1128/CMR.00134-14. PMID: 26016486

Van Wyk, A. & Prinsloo, G. (2020). 'Health, safety and quality concerns of plant-based traditional medicines and herbal remedies,' *South African Journal of Botany*, 133, pp. 54–62.

van Wyk, B.E. & Albrecht, C. (2008). 'A review of the taxonomy, ethnobotany, chemistry and pharmacology of Sutherlandia frutescens (Fabaceae),' *Journal of ethnopharmacology*, 119(3), pp. 620–629. doi: 10.1016/j.jep.2008.08.003.

Van Wyk, B.-E. & Gorelik, B. (2017). 'The history and ethnobotany of Cape herbal teas,' *Suid-Afrikaanse tydskrif vir plantkunde. South African Journal of Botany*, 110, pp. 18– 38. doi: 10.1016/j.sajb.2016.11.011.

Vaou, N., Stavropoulou, E., Voidarou, C., Tsigalou, C. (2021). 'Towards Advances in Medicinal Plant Antimicrobial Activity: A Review Study on Challenges and Future Perspectives.' *Microorganisms*, *9*, 2041.

Wachtel-Galor, S. & Benzie, I. F. F. (2011). '*Herbal Medicine*.' CRC Press/Taylor & Francis.

Wang, M., Zhang, Y., Wang, R., Wang, Z. *et al.* (2021) 'An evolving technology that integrates classical methods with continuous technological developments: Thin-layer chromatography bioautography,' *Molecules (Basel, Switzerland),* 26(15), p. 4647. doi: 10.3390/molecules26154647.

Wardana, A.P., Aminah, N.S., Rosydab, M., Abdjanb. M.I. *et al.* (2021). 'Potential of diterpene compounds as antivirals, a review.' *Heliyon https://doi.org/10.1016/j.heliyon.2021.e07777*

Waxman, D. J. & Strominger, J. L. (1983). 'Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics,' *Annual review of biochemistry*, 52(1), pp. 825–869. doi: 10.1146/annurev.bi.52.070183.004141.

Wester, P. & Claßen-Bockhoff, R. (2006). 'Bird pollination in South African Salvia species,' *Flora*, 201(5), pp. 396–406. doi: 10.1016/j.flora.2005.07.016.

Wester, P. (2013). 'Sunbirds hover at flowers of *Salvia* and *Lycium*,' *The Ostrich*, 84(1), pp. 27–32. doi: 10.2989/00306525.2013.774300.

Wick, J., Heine, D., Lackner, G., Misiek, M. *et al.* (2016). 'A fivefold parallelized biosynthetic process secures chlorination of Armillaria mellea (honey mushroom) toxins.' *Applied and environmental microbiology*, *82*(4), pp.1196-1204.

World Health Organization, (2008). 'Traditional Medicines,' Available online: http://www.who.int/mediacentre/factsheets/fs134/en/

Yadav, N. & Upadhyay, R.K. (2022). 'Therapeutic and insecticidal potential of plant terpenes: A review,' *International Journal of Green Pharmacy*,16(1):14

Yan, Y., Li, X., Zhang, C., Lv, L. *et al.* (2021). 'Research Progress on Antibacterial Activities and Mechanisms of Natural Alkaloids: *A Review.' Antibiotics (Basel),* 10(3):318. doi: 10.3390/antibiotics10030318.

Zacchino, S.A., Butassi, E., Liberto, M.D., Raimondi, M. *et al.* (2017). 'Plant phenolics and terpenoids as adjuvants of antibacterial and antifungal drugs.' *Phytomedicine: international journal of phytotherapy and phytopharmacology*, 37:27-48. doi:10.1016/j.phymed.2017.10.018.

Zengin, H. & Baysal, A. H. (2014). 'Antibacterial and antioxidant activity of essential oil terpenes against pathogenic and spoilage-forming bacteria and cell structure-activity relationships evaluated by SEM microscopy,' *Molecules (Basel, Switzerland)*, 19(11), pp. 17773–17798. doi: 10.3390/molecules191117773.

Zhang, J., Chen, T., Li, K., Xu, H. *et al.* (2019) Screening active ingredients of rosemary based on spectrum-effect relationships between UPLC fingerprint and vasorelaxant activity using three chemometrics. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* 15;1134-1135. doi: 10.1016/j.jchromb.2