IN VITRO ANTIBACTERIAL ACTIVITY OF *GUNNERA PERPENSA* AND *ELEPHANTORRHIZA ELEPHANTINA* AGAINST HUMAN PATHOGENIC BACTERIA

TLOTLO RADEBE
(220049598)

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Supervisor: Dr P.H. Mfengwana (DHSc: Biomedical Technology)
Co-supervisor: Dr D. Olivier (D. Tech: Biomedical Technology)

BLOEMFONTEIN
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DECLARATION OF INDEPENDENT WORK

DECLARATION WITH REGARD TO INDEPENDENT WORK

I, Tlotlo Radebe, identity number _____ and student number _____, do hereby declare that this research project, submitted to the Central University of Technology, Free State, for the Master of Health Science in Biomedical Technology degree, is my own independent work; complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules, and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

21/02/2023

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LIST OF ABBREVIATIONS

µg	Microgram(s)
µL	Microlitre(s)
ABS	Absorbance
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
BPH	Benign prostate hyperplasia
C_2H_5OH	Ethanol
C_3H_6O	Acetone
$C_4H_8O_2$	Ethyl acetate
C_6H_{14}	n-hexane
CFU	Colony-forming unit(s)
CH_2Cl_2	Dichloromethane
CH_3COOH	Acetic acid
$CHCl_3$	Chloroform
CSIR	Council for Scientific and Industrial Research
CWS	Cell wall swelling
DCW	Damage to cell walls
DH_2O	Distilled water
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
$FeCl_3$	Ferric chloride
g	Gram(s)
GAE	Gallic acid equivalent
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
ICD	Incomplete cell division
IMP	Increased membrane permeability
INT	p-iodonitrotetrazolium chloride
IUCN	International Union for Conservation of Nature
LCC	Loss of cellular contents
LIM	Loss of intracellular material
M	Molar

MATE	Multidrug and toxic efflux
MDR	Multidrug-resistant [bacteria]
MeOH	Methanol
MF	Major facilitator
mg	Milligram(s)
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
mL	Millilitre(s)
mm	Millimetre(s)
MRSA	Methicillin-resistant *Staphylococcus aureus*
Na_2CO_3	Sodium carbonate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NH_4OH	Ammonia solution
nm	Nanometre(s)
OsO_4	Osmium tetroxide
QE	Quercetin equivalent
RND	Resistance-nodulation-division
SC	Shrinkage of the cell
SEM	Scanning electron microscopy/microscope
SMR	Small multidrug resistance
STD	Sexually transmitted disease
TEM	Transmission electron microscopy/microscope
TLC	Thin-layer chromatography
USA	United States of America
UTI	Urinary tract infection
UV	Ultraviolet
XDR	Extensive drug-resistant [bacteria]

PUBLICATIONS AND CONFERENCE PRESENTATIONS

Published article

- Tlotlo, R., Mfengwana, P.M.A.H. & Olivier, D. (2022). Review on literature of the plant *Elephantorrhiza elephantina* on its healing properties and recent acquired knowledge of its medicinal activities (2000-2020). *Pharmacognosy Journal*, *14*(3), 715-721. (See Appendix.)

Conference presentations

- Tlotlo, R. & Mfengwana, P.M.A.H. (2022). Presentation of antibacterial activity results (national): Antibacterial screening of *Elephantorrhiza elephantina* and *Gunnera perpensa* on human pathogenic bacteria. Indigenous Plant Use Forum (IPUF) 2022 Conference, 04 July – 07 July 2022.
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ABSTRACT

INTRODUCTION: In the history of the African population, medicinal plants are a heritage that has been able to be preserved and used in modern times. The use of medicinal plants is still regarded as a treatment option in the current healthcare sector and among rural communities. Traditional medicinal plants have been used by African communities for many years as they are more affordable, easily accessible, less toxic, and more trustworthy in African cultures because the knowledge of remedies practised by traditional healers has been passed down by the ancestors through cultural practices in current and past generations. In recent years, even with the modernisation of the healthcare system, people still seek help of traditional healers. Multidrug-resistant (MDR) bacteria have increased at an alarming rate over the recent decades and have caused a substantial health burden. MDR bacteria are pathogenic bacteria that are naturally harmful and cause severe infection/disease among humans. These illnesses/conditions may include abdominal pain, bladder problems, cancer, colds, earaches, endometritis, gastrointestinal parasites, gonorrhoea, heart diseases, hypertension, impotence, infertility, kidney problems, poor appetite, rheumatic pains, scabies, syphilis, and urinary infections. This study sought to assess *Elephantorrhiza elephantina* and *Gunnera perpensa* as antibacterial agents used to treat infections associated with MDR bacteria.

METHODS: Medicinal plant extracts (using roots/rhizome) were prepared by maceration with methanol and water. Secondary metabolites were qualitatively and quantitatively assessed, while antibacterial activities were determined using the disk diffusion assay and the *p*-Iodonitrotetrazolium chloride assay on the following Gram-positive bacteria: *Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus*, and *Bacillus subtilis*, and the following Gram-negative bacteria: *Klebsiella pneumoniae* and *Escherichia coli*. Further investigation of the antibacterial activity and the effects of *E. elephantina* methanol extract on the bacterial morphology of *S. aureus* was performed. This was investigated using a scanning electron microscope (SEM) and transmission electron microscope (TEM).

RESULTS: The phytochemical analysis of the plants confirmed that the following phytochemical compounds were present in both the methanol extract of *G. perpensa* and *E. elephantina*: alkaloids, glycosides, flavonoids, tannins, phytosterols, and

phenols. Gallic acid equivalents (GAEs) of the estimated phenolic concentrations ranged from 0.140 ± 0.0076 to 0.068 ± 0.0025 mg (GAE). The estimated flavonoid concentrations ranged from 0.905 ± 0.0190 to 0.375 ± 0.0073 mg (QE/g). The antibacterial activity results of the *E. elephantina* methanol extracts showed strong antibacterial activity against *S. aureus*, *B. subtilis*, and *K. pneumoniae* and indicated the minimum inhibitory concentration at concentrations of 60 µg/mL, 125 µg/mL, and 60 µg/mL respectively. The SEM and TEM evaluation of *S. aureus* showed major structural changes, including damage to cell walls, which was evident from holes in the cell surface. The loss of cellular or cytoplasmic contents resulted in shrinkage of the cell, which was seen by the wrinkled and indented surface of the cell.

CONCLUSION: The antibacterial activity of *G. perpensa* demonstrated strong activity against Gram-positive bacteria *S. aureus* and *B. subtilis*; however, poor activity was demonstrated by the methanol extract and no activity was seen from the aqueous extract against the Gram-negative bacteria *E. coli* and *K. pneumoniae*. The effects of *E. elephantina* on the bacterial cell wall supported the antibacterial properties of the plant, as the results of the SEM and TEM show how the plant extract caused morphological damage and eventually inhibited *S. aureus* growth. *E. elephantina* managed to inhibit the growth of bacteria that are prone to drug resistance, which are eventually classified as MDR. The results obtained from this study indicate that the plant has potential as an antibacterial agent.

KEYWORDS: multidrug-resistant bacteria; traditional medicinal plants; *Elephantorrhiza elephantina*; *Gunnera perpensa*; *Staphylococcus aureus*.

CHAPTER 1:
BACKGROUND

1.1 INTRODUCTION

This chapter provides background to the study. The following are discussed briefly in this chapter: bacteria, antibiotics, medicinal plants, phytochemical properties of plants, plants investigated in this study, problem statement, research aims and objectives, study layout, and study overview.

1.1.1 Bacteria

Human pathogenic bacteria are bacteria that cause disease within humans (Hornef, 2015). The bacteria cause disease through different structures; for example, toxins, buds, and bacterial deoxyribonucleic acid (DNA), which cause alterations in the host cells (Hornef, 2015). When the host (human) becomes sick due to a pathogenic bacterium, they seek medical interventions through clinics or hospitals, depending on the severity of the disease. The infection/disease caused by pathogenic bacteria is commonly treated with antibiotics. Antibiotics are the most widely used drugs for the treatment of diseases and infections caused by pathogenic bacteria (Khan et al., 2009). The discovery and use of antibiotics were believed to help eradicate diseases as the general function of antibiotics is to inhibit/stop the growth of bacteria, which will stop the cause of the disease (Hornef, 2015). However, overuse and incompletion of antibiotic treatment by patients have resulted in certain groups of pathogenic bacteria developing resistance to antibiotics (Khan et al., 2009).

Resistance is acquired naturally or through genetic processes. Selective pressure is the natural way of developing resistance. This occurs when only part of the population are susceptible to the antibiotic used to treat the infection and the surviving bacteria continue to multiply. This creates a bacterial population that is resistant to the antibiotic to which the bacteria were exposed. Selective pressure is a process that can be slowed but not stopped (Courvalin, 2008). Genetically acquired resistance occurs when genetic material is passed between different bacteria. This can occur through the transfer of plasmids. Plasmids are circular DNA molecules found mostly in bacteria that can be transferred between bacteria. This allows for the spread of resistance

easily and quickly among bacteria. When a bacterium's genetic material spontaneously changes or mutates, those changes can create resistance (Courvalin, 2008).

Bacterial resistance to antibiotics can be divided into two groups: intrinsic or innate resistance and acquired resistance (Hancock & Brinkman, 2002; Khameneh et al., 2016). The biological structural membranes of the bacteria are composed of lipids, proteins, and lipoproteins. The cytoplasmic membrane acts as a diffusion barrier for water, ions, nutrients, and transport systems (Courvalin, 2008). Intrinsic resistance includes low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell, and the production of antibiotic-inactivating enzymes (Pang et al., 2019). Certain antimicrobial agents can cause membrane disorganisation. Acquired resistance results from one of the following causes: acquisition of resistant genes by bacteria, mutation of chromosomal DNA, and/or the combination of both mechanisms (Courvalin, 2008). The acquisition of resistant genes can be mediated by transferable genetic elements such as plasmids, transposons, and integrons; notably, transmissible plasmids are a highly efficient means of horizontal gene transfer through a process called conjugation (Khameneh et al., 2016).

During conjugation, the cell surface of both donor (resistant) and recipient (naïve) bacteria comes into contact to form a bridge to transfer conjugative plasmids from the donor bacteria to the recipient bacteria. According to Wang et al. (2020) and Khameneh et al. (2016), the other possible resistance mechanism to antibiotics involves the production of antibiotic-modifying enzymes such as aminoglycoside-modifying enzymes or chloramphenicol acetyltransferase. Regulatory genes are important productions for bacterial resistance and mediate the innate or intrinsic resistance. These genes are also involved in multidrug resistant (MDR) strains that hyper express the efflux systems (Hobson et al., 2021). Efflux pumps are groups of transporter proteins that are involved in pumping out the drug or toxic substrates from within cells to the external environment. Therefore, the intracellular concentration of antibiotics decreases and bacterial cells survive (Dai et al., 2020).

MDR bacteria are bacteria that acquire resistance to antibiotics, which causes the drug to become ineffective against them (Hornef, 2015). MDR bacteria have increased at an alarming rate over recent decades and cause substantial health burden (Khameneh

et al., 2016). MDR bacteria are pathogenic bacteria that contribute to the development of severe infections/diseases among humans. These illnesses/conditions may include abdominal pain, bladder problems, cancer, colds, earache, endometritis, gastrointestinal complaints, gonorrhoea, heart diseases, hypertension, impotence, infertility, kidney problems, poor appetite, rheumatic pains, scabies, syphilis, and urinary infections (Mpofu et al., 2014a; Maroyi, 2016a, 2017b).

Antibiotics have been shown to pose a risk of being ineffective in treating infection/disease caused by bacteria due to the bacteria's ability to acquire resistance to antibiotics. This limits the treatment options available to individuals faced with medical conditions and complications caused by antibiotic-resistant pathogenic bacteria. Antibiotics are the recommended treatment for infections caused by pathogenic bacteria (Khan et al., 2009). Over the years, bacteria have acquired resistance to numerous antibiotics. These bacteria are known as MDR bacteria. This resistance can present itself in multiple ways, by bacteria (1) having the ability to encode on plasmids or on the chromosome, (2) having resistance by inhibiting or decreasing the amount of entry of a drug, (3) changing the receptor (target point of entry) of the drug, or (4) metabolically inactivating the drug (Hutchings et al., 2019; Hobson et al., 2021).

1.1.2 Antibiotics

The discovery of antibiotics dates back 200 years (Hutchings et al., 2019). The reported timeline extends from the 1900s to 2010, when Salvarsan was the first synthetic antibiotic used in 1910, to new drugs on the market such as lipiarmycin and diarylquinoline that were discovered in 2010. In 1985, Farnsworth and Soejarto (1985) reported that in the period of 1959 to 1980, 25% of drugs dispensed in the United States of America (USA) were plant derived. They later identified 119 secondary plant metabolites that were used as drugs. Antibiotics were discovered through clinical, pharmacological, and chemical studies of chemical substances from bioactive compounds that were derived primarily from plants (Farnsworth & Soejarto, 1985). These secondary metabolites were the natural bioactive compounds.

These bioactive natural compounds became the focus of research activities of emerging pharmaceutical companies after the discovery of antibiotics (Duong et al.,

2010). Antibiotics use diverse modes or mechanisms to penetrate bacteria and cause the death of the bacteria. Various antimicrobial agents have different modes of action that act by interfering with cell wall synthesis, plasma membrane integrity, nucleic acid synthesis, ribosomal function, and folate synthesis. Antimicrobial agents may be either bactericidal, killing the target bacterium or fungus, or bacteriostatic, inhibiting its growth. Bactericidal agents are more effective, but bacteriostatic agents can be extremely beneficial since they permit the normal defences of the host to destroy the bacteria (Neu & Gootz, 1996).

Considering the evidence or the rapid global spread of resistant bacteria, the need to find new antimicrobial agents is of paramount importance. Antibiotics are the most widely used drugs for the treatment of illnesses and infections caused by bacteria (Khan et al., 2009). The discovery and use of antibiotics were believed to assist with the eradication of some diseases. The resistance of bacteria to antibiotics has a negative impact on the patient's livelihood, hospitals, and the socio-economic and healthcare systems. There has been an increasing incidence of multiple drug resistant pathogenic bacteria in recent years; with tuberculosis being the most recent MDR strains, extensive drug-resistant (XDR) *Mycobacterium tuberculosis* (Jones et al., 2008). Biological activity investigations in traditional plants guided by isolation have been the most reliable method for the discovery of important novel drugs from medicinal plants. This method is the basis of most early medicines such as aspirin, digitoxin, morphine, quinine, and penicillin (Lahlou, 2013). Biologically active compounds isolated from plant species used in herbal medicines have received significant attention as an alternative treatment due to the side effects of antibiotics in some individuals and the serious burden placed on the healthcare system as a result of the development of antibiotic-resistant bacteria (Khan et al., 2009).

1.1.3 Medicinal plants

Traditional medicinal plants have been used to treat infections/diseases associated with MDR bacteria (Hornef, 2015). With the burdened and poor management of the healthcare system, expensive and inaccessible medical care in most regions of African countries have led to the African population relying more on medicinal plants based on their traditional knowledge of them and for the survival and healthcare of their communities.

In the history of the African population, medicinal plants are a heritage that has been able to be preserved and used in modern times. It is still regarded as a treatment option in the current healthcare sector among rural communities. Traditional medicinal plants have been used by African communities for many years as they are more affordable, easily accessible, less toxic, and more trustworthy in African cultures because the knowledge of remedies practiced by traditional healers has been passed down by the ancestors through cultural practices in current and past generations. Despite the modernisation of the healthcare system, people still seek help from traditional healers. They provide alternative healthcare services, including the use of medicines derived from plants, because they are easily available, more affordable than modern medicine, and accessible in rural, remote areas (Otshudi et al., 2000; Mathabe et al., 2006).

Plants are known to be the earliest sources used to treat sick people when they needed medical attention (Pelletier et al., 1983; Winston & Beck, 1999). It was of interest for scientists to gather knowledge about plants to understand which properties of plants have the ability to provide relief to the individual and cure the infection/illness. Plants have bioactive compounds such as primary metabolites and secondary phytochemicals, which possess healing properties (Kahn & Díaz-Hernández, 2000). Phytochemicals are secondary metabolites plant survival through temporary or continuous threats posed against the plant to thrive in their environment, while also regulating essential functions for plant growth and reproduction of the plant (Molyneux et al., 2007). The rich primary and secondary metabolites found in plants are sugars, proteins, alkaloids, flavonoids, tannins, glycosides, anthraquinones, and steroids, but are not limited to these (Hoste et al., 2008). Through research, it was discovered that plants that possess primary metabolites and secondary phytochemicals that are biologically active and possess healing properties extend to the benefits of patient health. Recommendations throughout the years have encouraged humans to eat more plant-based diets, as this can assist in providing health benefits from phytochemicals in plants that possess health-protective benefits (Dillard & German, 2000; Meskin et al., 2002; Rochfort & Panozzo, 2007; Holopainen et al., 2018).

The medicinal plants in this study were selected because they are commonly prescribed by traditional healers for common infections/diseases including, but not limited to, colds, fever, and abdominal pain, and to offer relief to sick individuals.

Infections treated with these medicinal plants are often caused by pathogenic bacteria. Furthermore, selected bacteria are prone to multidrug resistance and were reported to be commonly related to infections. Plant use against these illnesses were investigated by in vitro analysis of the common bacteria related to those illnesses. Therefore, research and development of alternative medicine turned to plants as they have biologically active compounds (Oladunjoye et al., 2022).

1.1.4 Phytochemical properties of plants

Phytochemicals are simple and complex chemical compounds based on chemical-structural constituents. They are divided into primary and secondary compounds and are produced by plants, fruits, vegetables, seeds, and nuts through natural synthesis (Molyneux et al., 2007). The natural synthesis of these phytochemicals undergoes different metabolism pathways that result in the biochemical structures of the phytochemical compounds. There are primary compounds such as chlorophyll, proteins, and common sugars, and secondary compounds such as terpenoids, alkaloids, flavonoids, reducing sugars, tannins, phenols, and many more. These compounds help to protect the plant against harsh environmental conditions and factors such as pesticides, insects, or fungi (Barbieri et al., 2017). The bioactive secondary metabolites of plants possess different medicinal properties, which are discussed below (Holopainen et al., 2018).

Plants that possess anthelmintic activity are used worldwide in various communities to treat human and livestock diseases (Githiori et al., 2004; Tandon et al., 2011). These plants are known to be rich in tannins and alkaloids, which are extensively mentioned and highlighted to be responsible for providing anthelmintic properties to humans and animals (Athanasiadou et al., 2001; Paolini et al., 2003; Singh et al., 2003; Brunet & Hoste, 2006). Alkaloids were one of the pure compounds isolated in earlier years that exhibits biological activities (antimicrobial properties) (Holstege et al., 1995).

According to Iwu et al. (1999), antimicrobial properties were displayed through activity against Gram-negative bacteria and yeast (*Candida*), which was exhibited by indoquinoline alkaloids from *Cryptolepsis sanguinolenta*. Studies by Kayser et al. (2003) exhibited the antiprotozoal activity of alkaloid quinine against the malaria parasite. Through different plant-based anthelmintic principles and the comparison of

chemical anthelmintics, plants pose the potential of being non-resistible considering the vast fundamental and structural genetic changes that will have to drive such resistance (Stepek et al., 2005). Therefore, medicinal plants are important for research and development of alternative medicine worldwide.

Plants rich in flavonoids have been demonstrated to display antiproliferative effects on various cancer lines and antimicrobial properties (Manthey & Guthrie, 2002; Chahar et al., 2011; Karak, 2019). Tannins, which are water-soluble polyphenols commonly found in higher herbaceous and woody plants, have been reported to have antibacterial properties against *Staphylococcus aureus* (Kaczmarek, 2020; Heidari et al., 2020). Essential oils derived from medicinal plants also pose antimicrobial activity that has been demonstrated against pathogenic bacteria such as *Escherichia coli*, *Enterococcus faecalis*, *S. aureus* (including methicillin resistant *S. aureus*), *Salmonella* spp., and *Vibrio parahaemolyticus*, where cinnamaldehyde was the main antibacterial component of volatile oil and fungi (Tullio et al., 2007; Sienkiewicz et al., 2012).

Due to economic constraints and a lack of medical facilities and commercial antibiotics in rural and developing regions, traditional remedies are widely used to treat diseases. Several of these medicinal plant-based medicines have exceptionally good efficacy, which has resulted in numerous commercial drugs being developed from medicinal plants. Examples of such developed drugs include two of the classes of antimalaria drugs (quinine and artemether/artemisinin derivatives) derived from traditional medicines, and anti-inflammatory agents such as aescin (Beg et al., 2011; Recio et al., 2012; Efferth et al., 2016). Certain aspects may contribute to the efficacy of the medicinal remedy. Traditional healers prescribe medicines based on the symptoms presented by the patient, rather than specifically to treat a disease or specific pathogen that causes the disease. The traditional medicines that have been reported to treat fever, fatigue, anaemia, or any symptom presented by the individual may therefore be interpreted as treating the condition and disease of the patient. The plant kingdom has been one of the alternative routes taken by scientists for the research and development of novel and alternative medicines to counter drug resistance. The undesirable side effects of certain antibiotics led to the search for new antimicrobial agents by finding leads with unique chemical structures that may present a way

forward to unexploited modes of action of plant extracts and healing properties (Oladunjoye et al., 2022).

1.1.5 Plants investigated in this study

Elephantorrhiza elephantina (Burch or Skeels) is commonly referred to as *elandsboontjie* in Afrikaans in South Africa and eland's bean and eland's wattle in English in Namibia and South Africa because elands feed on the species' foliage and pods (Maroyi, 2016b). The medical uses of the plant include the treatment of sexually transmitted diseases (STDs), ear infections, urinary tract infections (UTIs), and bloody diarrhoea in children (Van Vuuren & Frank, 2020). *Gunnera perpensa* is known as the river pumpkin in English, *qobo* in Sesotho, *gobho* in Siswati, *iphuzilomlambo* in isiXhosa and *ugobho* in isiZulu (as *ughobo* refers to the flow of fluids). Its use in traditional medicines is to remove excess fluid from the body, as believed by the Zulu people. *G. perpensa* belongs to the Gunneraceae family and is the only African member of this genus that is widely distributed in the tropical regions of Africa (Chigor, 2014). Traditional uses of *G. perpensa* are used alone in mono-therapeutic applications in the treatment of urinary complaints, kidney problems, sexually transmitted infections, easing of childbirth, and post-natal medication (Maroyi, 2016a). The selected medicinal plants used in this study have been reported to treat similar diseases (such as STDs, ear infections, kidney problems, UTIs, cancer, infertility complications, and pre- and post-natal treatment), which are commonly caused by pathogenic bacteria such as *E. coli*, *K. pneumoniae, S. aureus*, *B. subtills*, and *S. epidermidis* (Aaku et al., 1998; Nkomo & Kambizi, 2009; Maroyi, 2016b, 2017b; Mfengwana et al., 2019).

Multidrug resistance is increasingly common among bacteria such as *S. aureus* and *S. epidermidis*. Multidrug resistant *S. aureus* is referred to as methicillin-resistant *S. aureus* (MRSA) because of its methicillin resistance character and is therefore considered as MDR. MRSA causes outbreaks in hospitals and can be epidemic as *S. aureus* is reported to be the major causative agent of nosocomial and community-acquired infections in communities (Foster, 2002). Antibiotics help with the treatment of infections caused by these bacteria. They assist in the neutralisation of staphylococcal toxins and enzymes because there are no vaccines available. Although they showed activity in previous studies when exposed to the selected plants used in

this study, their effects on the bacteria's morphology was not explored, which led to the purpose of this study.

The aim of this study is to screen selected plants for phytochemical compounds, study the antibacterial activity of plant extracts, and evaluate the bacterial cell morphology using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Bacteria that show sensitivity to the strong active plant extract will be evaluated by SEM and TEM. This will assist in evaluating and understanding the mechanism of action that the plant extract subjects the bacteria to through the morphology.

1.2 PROBLEM STATEMENT

Over the years, bacteria have become more resistant to a variety of antibiotics. This makes the management and treatment of infections caused by MDR bacteria exceedingly difficult (Khameneh et al., 2016). Solutions are required to reduce this burden on the healthcare sector. This could be done through controlling antibiotics use, developing research to better understand the natural and genetic mechanisms of resistance, and continuing studies on traditional medicinal plants in the development of new drugs, either in their natural or synthetic form, which are effective in sensitising MDR bacteria. In an attempt to discover novel drugs that can combat MDR bacteria, recent studies have explored and demonstrated the in vitro antibacterial activity of *G. perpensa* (Nkomo & Kambizi, 2009; Muleya et al., 2014; Mfengwana et al., 2019) and that of *E. elephantina* (Maroyi, 2017a; Olaokun et al., 2020). However, the mode of action and the effects of the plant extract on the morphology of the bacteria have not been investigated. Therefore, this study focused on further investigating the antibacterial activity of *G. perpensa* and *E. elephantina* to understand the mode of action against MDR bacteria. The treated bacteria were analysed via electron microscopy by using SEM and TEM to elucidate the cellular changes the plant extracts cause to the morphology of bacteria.

1.3 RESEARCH AIM AND OBJECTIVES OF THIS STUDY

1.3.1 Aim of the study

The aim of this study was to investigate the antibacterial activities of *G. perpensa* and *E. elephantina* against human pathogenic bacteria in vitro.

1.3.2 Objectives of the study

The objectives of the study were as follows:

- To screen the phytochemicals from *G. perpensa* and *E. elephantina*;
- To investigate the antibacterial activities of *G. perpensa* and *E. elephantina* by disc diffusion and microdilution methods using pathogenic bacteria; and
- To examine the morphological changes of the microbial cell walls induced by *G. perpensa* and *E. elephantina* using SEM and TEM.

1.4 STUDY LAYOUT

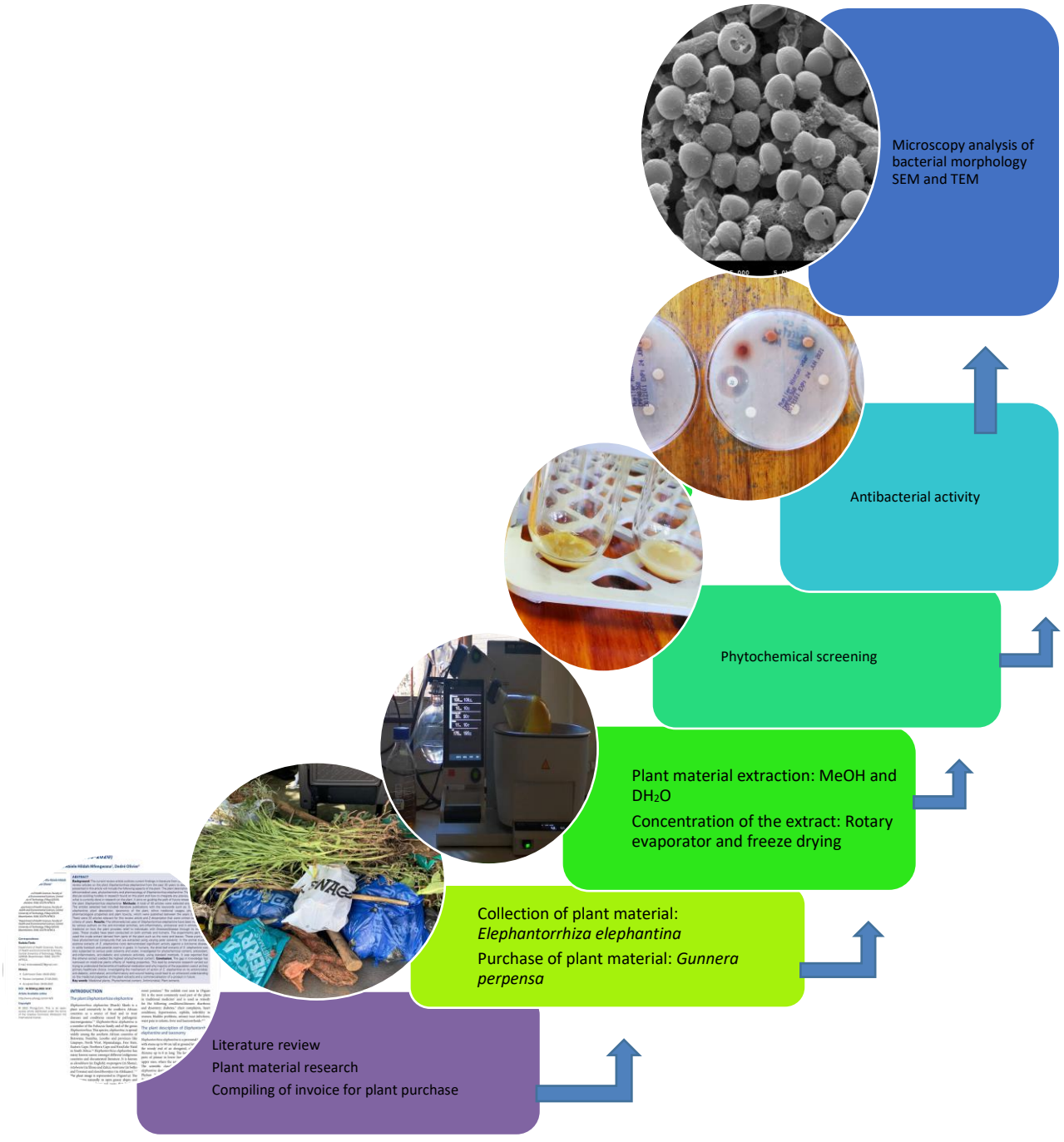


Figure 1.1: A diagrammatic summary of steps in the study of the investigation of the antibacterial activity of *G. perpensa* and *E. elephantina* plant extract

1.5 STUDY OVERVIEW

Chapter 1 introduces the concept of the value and medicinal benefits of traditional medicinal plants in developing countries. It highlights the potential of traditional medicinal plants as a source of novel drug development. The evidence of a large population using traditional medicine emphasises the development of drugs derived purely from plants. The chosen plants are traditionally used for the treatment of various conditions/diseases, but their effectiveness against pathogenic bacteria with the potential of being MDR pathogens and their mechanism of action against the bacteria have not been explored. The study objectives were set to report the selection of plants used for this study, as well as their antibacterial activities, with emphasis on the effects that medicinal plants have on bacterial cell morphology. The results are reported in Chapter 4. The ethnobotanical uses of these selected medicinal plants focused on the bacteria *S. aureus*, *E. coli*, and *K. pneumoniae*, which are more susceptible to drug resistance.

Chapter 2 provides a comprehensive review of medicinal plants and the MDR bacteria investigated and reported on in this study. This chapter discusses the morphological, phytochemical, and pharmacological activities of the selected medicinal plants used for infections caused by pathogenic bacteria and the MDR bacteria's mechanism of action to acquire the resistance. Gaps in the existing body of knowledge regarding these selected plants are highlighted, which are addressed in the subsequent chapters.

Chapter 3 reports on the methodology utilised to conduct this study.

Chapter 4 reports on the phytochemical screening of *G. perpensa* and *E. elephantina* as they have been proven to possess promising pharmacological properties. Furthermore, this chapter reports on phytochemical screening and the in vitro antibacterial properties of selected medicinal plant extracts, with focuses on microscopy analysis of the bacterial morphology present via SEM and TEM.

Deductions made from the antibacterial analysis are discussed in **Chapter 5**, while **Chapter 6** provides the conclusion and recommendations of this study.

CHAPTER 2:
LITERATURE REVIEW

2.1 BACTERIA

Bacteria were first discovered in the second half of the 19th century when scientists desired to understand the emerging devastating epidemic of communicable diseases (Hornef, 2015). Microorganisms can be bacteria, fungi, archaea, or protists, and can be classified as normal body bacteria, opportunistic bacteria and pathogenic bacteria. This is according to the environmental habitat, behavioural patterns, and health condition of the host. Normal body bacteria are a diverse microbial group that can be found on the skin and mucous membranes of every human being from birth until their death (Davis, 1996). The human body harbours approximately 10^{14} bacteria that may help the host to fight off pathogenic bacteria or overgrowth/overpopulation of fungus on the skin and live as commensals, but they may also harm the host by acting as opportunistic bacteria when the host becomes immune compromised (Davis, 1996). Opportunistic bacteria are bacteria that exist as normal body bacteria but when the host's health is compromised, they become a threat to the host and may cause illness/infections (Hornef, 2015).

Pathogenic bacteria are naturally harmful and cause severe infections/diseases among humans, such as the *Salmonella* species. They possess toxins and are not commonly found in human environments. Bacteria are classified in two classes, namely Gram positive and Gram negative (Ehrlich, 1923, cited by Hucker & Conn, 1923). The composition of the bacteria's cell wall and the ability to retain either the primary or counter stain during the Gram staining procedure are the basis for this differentiation. The first known bacteriocin was identified in 1925, which helped in the development of a wide research community focused on discovering new antimicrobial agents (Simons et al., 2020). With the discovery of these antimicrobial agents, the first line of treating pathogenic bacteria was antibiotics (Worthington & Melander, 2013). Antibiotics are the first line of defence administered to patients with bacterial infections. However, over the years there has been a pattern of overuse and misuse of antibiotics prescribed for the prevention and treatment of these infections (Hutchings et al., 2019). This ultimately led to bacteria acquiring resistance against certain antibiotics.

This resistance against antimicrobial agents is seen in both humans and animals (Oladunjoye et al., 2022).

Physicians have resorted to administering combinations of antibiotics, which may have a synergistic effect by producing an effect stronger than the simple sum of the effects of two separate drugs administrated alone on their antagonist; in the case that one agent inhibits the effect of the other (Courvalin, 2008; Oladunjoye et al., 2022). According to Worthington and Melander (2013), the increasing prevalence of infections caused by MDR bacteria is a global health problem that is worsened by the scarcity of novel classes of antibiotics over the past 40 years. This is a serious problem facing the health sector currently, with declining private investment and lack of innovation in the development of new antibiotics. These concerns undermine efforts to combat drug-resistant pathogenic bacteria (Jernigan et al., 2020). Resistant bacteria that cause diseases also place a burden on the health and livelihoods of individuals. The illnesses caused by these resistant pathogenic bacteria lead to longer hospital stays, frequent visits to the doctor, longer recovery time, and expensive medical expenses (Jernigan et al., 2020). Gram-positive and Gram-negative bacteria such as *S. aureus* and *E. coli* respectively have acquired resistance against certain antibiotics such as methicillin and beta-lactamase, fluoroquinolones, third- and fourth-generation cephalosporins, and carbapenems. Some of these antibiotics are the most frequently prescribed medications for bacterial infections (Oladunjoye et al., 2022).

Considering the evidence of the rapid global spread of resistant bacteria, the need to find new antimicrobial agents is of the utmost importance. Antibiotics are the most widely used drugs for the treatment of infections and illnesses caused by bacteria (Khan et al., 2009). The discovery and use of antibiotics were believed to assist with the eradication of some diseases. However, the overuse and incompletion of antibiotic treatment by patients have caused several groups of pathogenic bacteria to become resistant to antibiotics (Khan et al., 2009). The basic mechanisms by which a bacterium can resist an antimicrobial agent are (1) to alter the receptor for the drug (the molecule on which it exerts its effect), (2) to decrease the amount of drug that reaches the receptor by altering entry or increasing removal of the drug, (3) to destroy or inactivate the drug, and (4) to develop resistant metabolic pathways. Bacteria can possess one or all of these mechanisms simultaneously (Hobson et al., 2021).

Bacterial resistance to antibiotics can be divided into two groups: intrinsic or innate resistance and acquired resistance (Hancock & Brinkman, 2002; Khameneh et al., 2016). Intrinsic resistance includes low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell, and the production of antibiotic-inactivating enzymes (Pang et al., 2019). Acquired resistance results from one of the following causes: acquisition of resistant genes by bacteria, mutation of chromosomal DNA, and/or the combination of both mechanisms (Courvalin, 2008; Khameneh et al., 2016). The acquisition of resistant genes can be mediated by transferable genetic elements such as plasmids, transposons, and integrons. Notably, transmissible plasmids are a highly efficient means for horizontal gene transfer through a process called conjugation. During the conjugation procedure, the cell surface of both donor (resistant) and recipient (naïve) bacteria come into contact to form a bridge to transfer conjugative plasmids from the donor bacteria to the recipient bacteria (Hobson et al., 2021). According to Wang et al. (2020) and Khameneh et al. (2016), the other possible resistance mechanism to antibiotics involves the production of antibiotic-modifying enzymes, such as aminoglycoside-modifying enzymes or chloramphenicol acetyltransferase.

Regulatory genes are important productions for bacterial resistance and mediate the innate or intrinsic resistance. These genes are also involved in MDR strains that hyper express the efflux systems (Wang et al., 2020). Efflux pumps are groups of transporter proteins that are implicated in pumping out the drug or toxic substrates from within cells to the external environment. The intracellular concentration of antibiotics thus decreases, and bacterial cells survive (Dai et al., 2020). The pumps can transport one substrate specifically and/or a range of structurally dissimilar antibacterial agents (including multiple classes of antibiotics); such pumps can be associated with multidrug resistance. MDR phenotypes of bacteria can be developed through the mutation of these genes (Hancock & Brinkman, 2002; Hobson et al., 2021). According to Khameneh et al. (2016), in bacteria cells there are five major families of efflux transporters recognised: major facilitator (MF), multidrug and toxic efflux (MATE), resistance nodulation-division (RND), small multidrug resistance (SMR), and ATP-binding cassette. Among them, the MF and RND pumps are the most abundant.

The MF pumps are usually found in both Gram-positive and Gram-negative bacteria and are characterised by a narrow spectrum of activity. They usually pump one, or

sometimes a few, antibiotic classes. However, the RND pumps are found extensively in Gram-negative bacteria and display a wide spectrum of substrates (poly-selectivity), including several classes of antibiotics, antiseptic compounds, dyes, or detergents (Hobson et al., 2021). All these mechanisms of action explain how bacteria acquire resistance against antibiotics (see Figure 2.1).

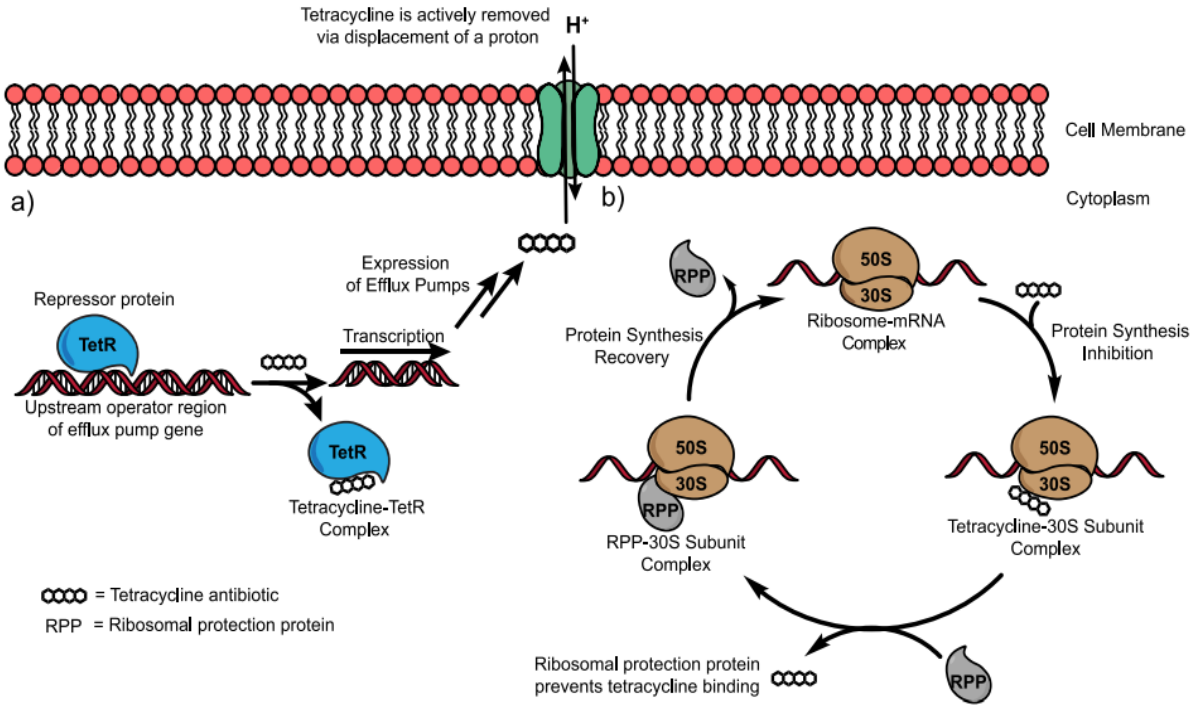


Figure 2.1: Acquired resistance tetracycline resistance mechanisms. (a) Tetracyclines bind to repressor protein Tet(R), which results in its dissociation from mRNA and facilitates the expression of tetracycline-specific efflux pumps. (b) Ribosomal protection proteins (RPPs) can prevent the binding of tetracyclines to the ribosome, allowing translation to proceed.

Source: Hobson et al. (2021)

MDR bacteria are known as one of the most threatening crises faced by the health sector (Hutchings et al., 2019). Oladunjoye et al. (2022) projected that MDR bacteria account for 700 000 deaths per annum worldwide and the number may rise to 10 million by 2050 if adequate care is not taken. In 2016, the World Health Organization released a list of MDR bacteria that are known as emerging and re-emerging superbugs. This list is called the Global Priority Pathogens List. It helps to guide researchers in the exploration and development of novel and active antibiotics for effective treatment of resistant pathogens (Oladunjoye et al., 2022). The most common pathogens on this list are the ESKAPE pathogens, which are the common culprits for the highest mortalities from nosocomial infections, which are associated

with expensive healthcare due to extended periods of hospitalisation (Mulani et al., 2019).

The acronym ESKAPE comprises the six most highly virulent and MDR pathogens *E. faecium*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species that are responsible for the global health crisis caused by the misuse and abuse of antimicrobial agents (Oladunjoye et al., 2022). These bacteria acquire resistance through the following mechanisms. Acquired resistance happens when many plasmid-encoded determinants have recently become inserted into the chromosome. There may be an advantage to the organism having resistance determinants in the chromosome because they will be more stable and cause enzymatic inactivation of the drug. Intrinsic or innate resistance (see Figure 2.2) to antibiotics in bacteria are through alterations to the drug target to prevent binding, accelerated drug efflux to prevent toxic concentrations accumulating in the cell, and a by-pass mechanism whereby an alternative drug-resistant version of the target is expressed (Hobson et al., 2021).

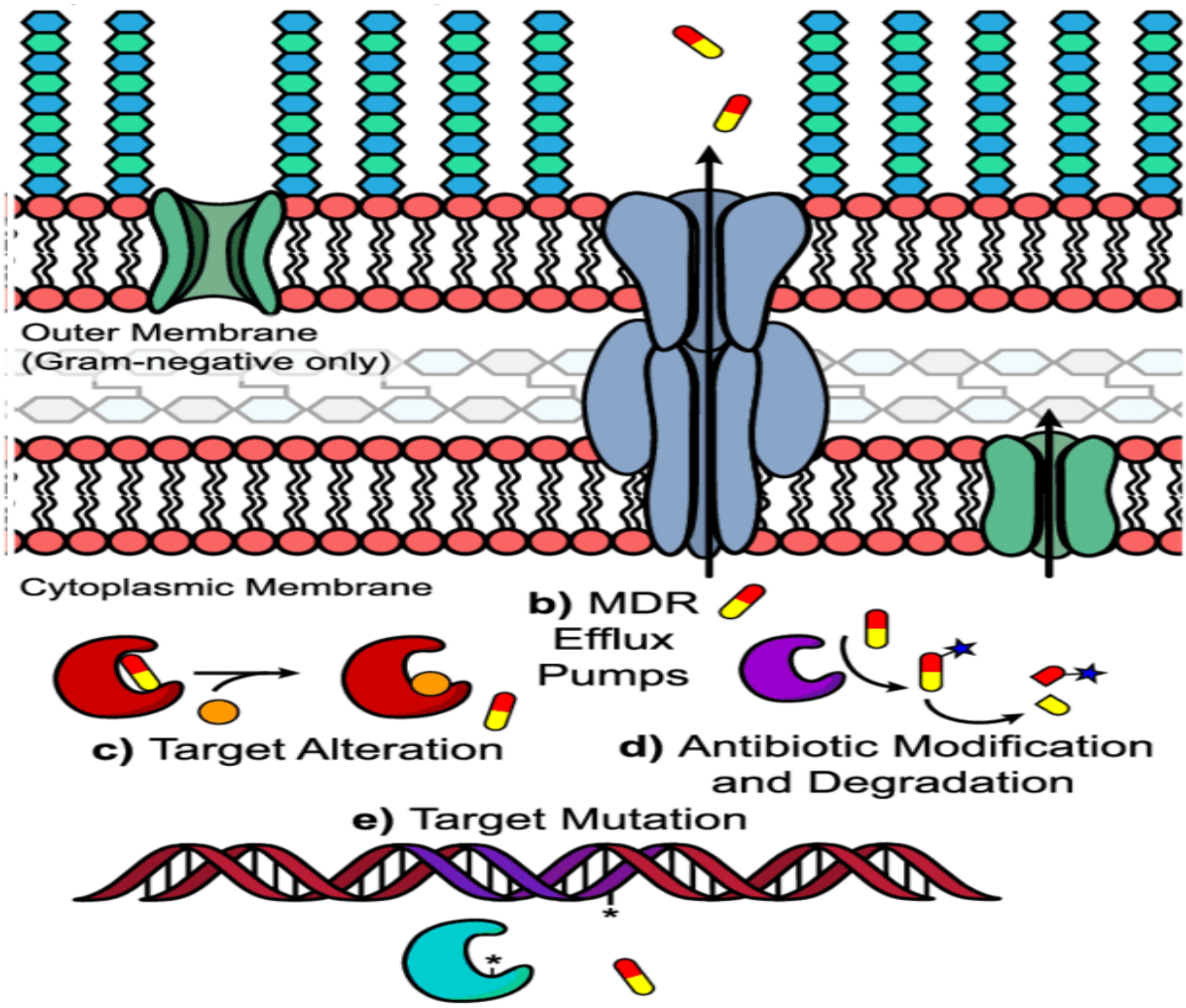


Figure 2.2: Intrinsic and acquired antibiotic resistance in bacteria. (a) Barriers to entry include the outer membrane of Gram-negative bacteria and associated protein pores; (b) efflux pumps; (c) target alteration; (d) antibiotic modification and degradation; (e) antibiotic target mutation.
Source: Hobson et al. (2021)

2.1.1 Gram-positive bacteria: *Staphylococcus* species

Staphylococci are pathogens found in humans and animals that can cause many forms of infection (Foster, 2002). Staphylococci are Gram-positive bacteria that are cocci form and appear in groups of clusters and cause infections. Commonly, antibiotic treatment and surgical drainage are often necessary to cure abscesses, large boils, and wound infections associated with pathogenic bacteria. Some *S. aureus* strains that infect hospitalised patients are habitually resistant to many different antibiotics, apart from the glycopeptides, vancomycin, and teicoplanin that have been reported (Foster, 2002; Oladunjoye et al., 2022). Plasmid-associated vancomycin resistance has been detected in some enterococci and the resistance determinant has been

transferred from enterococci to *S. aureus* in the laboratory and may occur naturally in the environment (Foster, 2002).

2.1.1.1 S. aureus

According to Foster (2002), *S. aureus* is a cocci bacterium that forms clumps and stains positive via Gram's stain. *S. aureus* is responsible for superficial skin lesions (boils, styes) and localised abscesses in other sites, but not limited to these. It may cause more serious infections, particularly in persons weakened by chronic illness, traumatic injury, burns, or immunosuppression. *S. aureus* is one of the major causes of hospital-acquired (nosocomial) infections of surgical wounds often associated with hospitalised patients rather than healthy individuals in the community. It may also cause food poisoning by releasing enterotoxins into food, which may lead to toxic shock syndrome (Foster, 2002).

S. aureus is seen as normal flora in healthy hosts and their natural habitat where it colonises the nasal passage and axillae. It has defences to avoid the host's defence mechanisms to protect itself. These defences are virulence factors, which are the surface proteins that promote colonisation of the host tissues, factors like capsules, and binding protein A that inhibits phagocytosis and toxins that damage host tissues and cause disease symptoms. The capsule and protein A may interfere with phagocytosis to prevent the death of the bacteria. Virulence factors are the main causative agents of symptoms expressed by an individual during infection (Foster, 2002).

From the time when antibiotics were introduced, *S. aureus* has responded to the introduction of these new drugs by rapidly acquiring resistance by genetic mechanisms such as (1) acquisition of extrachromosomal plasmids and (2) by mutations in chromosomal genes (Hobson et al., 2021). The use of combinations of antibiotics to achieve synergy can be a solution but may be seen to cause complications.

2.1.1.2 S. saprophyticus

S. saprophyticus are cocci form bacteria that appear in packages of two to four in clumps and chains and are identified as Gram-positive bacteria. The cocci are usually

seen as a commensal of the skin. It poses the potential to causes UTIs, especially in girls (Foster, 2002).

2.1.1.3 S. epidermidis

S. epidermidis are cocci bacteria that form clumps and are identified as Gram-positive bacteria. The bacteria are associated with infections of indwelling medical devices such as prosthetic devices and catheters (Foster, 2002). It is commonly seen as a human skin commensal in healthy individuals but poses a threat/harm to immunocompromised individuals. It causes infections/diseases such as peritonitis and endocarditis. These infections are not usually nosocomial acquired (Foster, 2002).

Both *S. aureus* and *S. epidermidis* are common causes of infections associated with indwelling devices such as joint prostheses, cardiovascular devices, and artificial heart valves. They are also the two common bacteria seen in infections that are non-responsive to treatment of the first line of antibiotics such as penicillin and methicillin. *S. epidermidis* nosocomial isolates are also often seen to be resistant to several antibiotics, including methicillin (Hobson et al., 2021).

2.1.2 Gram-negative bacteria: *E. coli*

According to Guentzel (1996), *E. coli* is a coliform bacillus that is often referred to as an opportunistic, enteric pathogen. It is identified as a Gram-negative bacterium. Coliform bacilli are the cause of some nosocomial infections. The common sites of nosocomial infection are the urinary tract, surgical sites, bloodstream, and pneumonias (Bereket et al., 2012). They are therefore seen as the premier nosocomial pathogen. Regarding community-acquired infections, it is also noted that *E. coli* is the major cause of UTIs and bloody diarrhoea in children and adults (Evans & Evans, 1996; Maroyi, 2016b).

The disease caused by these pathogenic bacteria is treated mainly through diverse groups of antibiotics that inhibit their growth. The bacteria cannot colonise the host or area of concern, which leads to the death of the bacteria. Over the years, these bacteria have managed to acquire resistance to some antibiotics, namely methicillin and beta-lactamase classes of antibiotics (Khameneh et al., 2016). This resistance can present itself by the ability of the bacteria to encode on plasmids or on the

chromosome. Resistance may inhibit or decrease the amount of drug entry into the cells of the bacteria, changes in the receptor (target point of entry) of the drug, or metabolic inactivation of the drug (Hobson et al., 2021).

Antibiotics may be administrated in combination or through a single dose to treat a case of infection (Oladunjoye et al., 2022). Synergy occurs when a combination of two drugs is administrated, which causes inhibition or killing of the bacteria (Oladunjoye et al., 2022). This mode of treatment is usually used to treat a life-threatening infection, to prevent the development of bacterial resistance, to treat mixed infections of aerobic and anaerobic bacteria, to enhance antibacterial activity (synergy), and to use lower doses of a toxic drug (Oladunjoye et al., 2022). This type of combined treatment is sensible when the precise agents of a serious infection are unknown. The use of two or more drugs to prevent the emergence of resistance is effective for most of the pathogenic bacteria and for therapy of some chronic infections. This type of treatment has also caused its own problems as the bacteria have managed to develop multidrug resistance. Antibiotic resistance is often plasmid mediated and the spread of plasmids between different strains is facilitated by transducing phages (Hobson et al., 2021).

2.2 SCANNING ELECTRON MICROSCOPE (SEM) AND TRANSMISSION ELECTRON MICROSCOPE (TEM)

Membranes define the borders of living cells and of their organelles. They permit cells to distinguish the self from the non-self (Baron, 1996). There has been significant development in understanding the physical properties of cellular membranes in regulating the cells' biological activities through the introduction of light microscopy and furthermore the innovation of SEM and TEM (Williams & Carter, 1996; Zhang et al., 2002). The basic principle of these microscopes is that the resolving power is the most important feature of the optical system. It influences the ability to distinguish between the fine details of a specimen. The key innovations are the scanning of a light beam over the specimen and a high magnification at the level of the detector, through the use of a macroscopic iris. These were followed by an achromatic all-reflective relay system, a non-confocal transmission detector, and novel software for control and basic image processing (Xi et al., 2011).

The design principles were put forward by Marvin Minsky in 1955 (Minsky, 1988), but the experimental demonstration was not accomplished until 20 years later by Cremer

and Cremer (1978, cited in Xi et al., 2011) and Brakenhoff et al. (1979). It was commercialised successfully and has been produced and developed over 17 years, surviving challenges from alternative technologies, including solid-state scanning systems (Minsky, 1988; Xi et al., 2011). Utilising the SEM and TEM for evaluating bacteria's cellular membranes presents great advantages for studying biological samples. These advantages include efficient background rejection, low photodamage, and improved depth discrimination. However, it does have disadvantages such as the cost and complexity of confocal scanning laser microscopy, which hinders its wide application in industries and education (Inoué & Osatake, 1988; Xi et al., 2011).

TEM and SEM analyses in this study aimed to evaluate the cellular membrane morphology of the bacteria when exposed to plant extracts. The observation would provide evidence of any cell membrane alterations through cellular damage/changes the bacteria undergo. This will provide understanding of the mode of action of the plant extract. The mode of actions of the plant extract may present by shrinkage of the cell, swelling of the cell, or causing holes in or breakage of the cell surface of the cell, or alternatively all the above. This information could provide knowledge on the mechanism of action. Comparisons can be made between plant extracts and conventional antibiotics as to how these antibiotics lead to the death of the bacteria by causing the same effects on the bacteria as the plant extract. When observing the damage, the plant extract causes to the bacteria and comparing it to the mode of action of the antibiotics used, they both could be using similar mechanisms to achieve similar results.

2.3 PHYTOCHEMICAL COMPOUNDS

Phytochemically bioactive compounds have been found to act as antimicrobial agents against human pathogens (Barbieri et al., 2017). Phytochemicals are bioactive chemical compounds that are naturally found in plants (Dillard & German, 2000; Holopainen et al., 2018). These compounds have been investigated and developed based on their evolution of antimicrobial properties against viruses, bacteria, and fungi. They are widely found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs, and spices and in plant-based beverages such as wine and tea (Barbieri et al., 2017). Phytochemicals can be classified into numerous major groups based on their

chemical structure, such as alkaloids, sulphur-containing phytochemicals, nitrogen-containing phytochemicals terpenoids, and polyphenols.

2.3.1 Alkaloids

“An alkaloid is a cyclic compound containing nitrogen in a negative oxidation state which is of limited distribution in living organisms” (Pelletier et al., 1983). This definition includes both alkaloids with nitrogen as part of a heterocyclic system, as well as the many exceptions with extra cyclic bound nitrogen such as colchicine or capsaicin (Pelletier et al., 1983). Alkaloids are described as a diverse group of secondary metabolites found in plants and have a selection of structure type, biosynthetic pathways, and pharmacological activities. In recent research, alkaloids have also been isolated from various organisms such as animals, insects, and marine invertebrates, unlike only in traditional focuses such as plants (Pelletier et al., 1983; Roberts, 2013). They are widely used in medicine and prominent in drug therapy. Between the period 1817 and 1820, the laboratory of Pelletier and Caventou Faculty of Pharmacy in Paris identified many active principles from alkaloids that no other laboratory had identified, such as strychnine, morphine, emetine, caffeine, and quinine, among others (Pelletier et al., 1983). Alkaloids have a history of pharmacological activities in analgesics, antimalarial medication, anticancer medication, and products for the treatment of hypertension, mental disorders, and tumours (Duri et al., 1994; Hutchings et al., 1996; Rajnikant, 2005; Roberts, 2013). Humans have used alkaloids from plants for medicinal uses such as pain relievers, stimulants, and muscle paralysers among other characteristics. They have shown medicinal activity against various bacteria and viruses in humans and in animals, such as pesticides and herbicides (Ntsoelinyane, 2014). Figure 2.3 shows the chemical structure of a typical alkaloid with the basic unit of nitrogen from the amino acid.

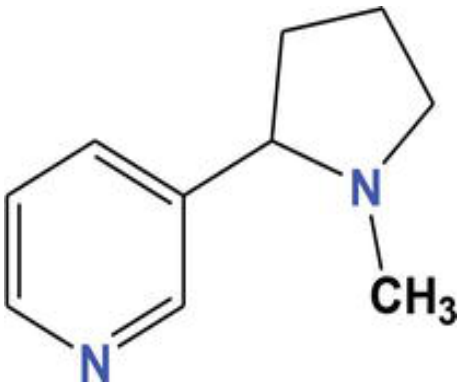


Figure 2.3: Alkaloid structure

Source: New World Encyclopedia (2019a)

2.3.2 Glycosides

Glycosides comprise a large group of secondary metabolites and are widely found in plants (Harborne & Grayer, 1988; Harborne, 1991; Francisco & Pinotti, 2000). They are structurally diverse compounds and are composed of recognised bioactivities that have been used historically up to current times for their traditional uses in treating infections/diseases. Glycosides are of importance regarding their pharmacological and ethnomedical properties, although their management remains to be clarified in terms of the roles of their properties for traditional uses. Glycosides are a structure that consists of two parts: an aglycone (genin) unit, which is identified as the lipophilic part, and a glycone unit, which is identified as the hydrophilic part and which is further composed of one or more sugar components (Bartnik & Facey, 2017). The natural plant diversity of glycosides consists mainly of phenolics, flavonoids, chromones, anthraquinones, cardiacs, and thioglycosides, which have been characterised from plants by their biochemistry and structure-activity relationships, plant sources, and extraction methods (Francisco & Pinotti, 2000; Stobiecki, 2000; Bartnik & Facey, 2017). Researchers have been interested in the traditional and therapeutic uses, mechanisms of action, possible adverse effects, and the toxicity of these metabolites, which led to investigations of the above-mentioned segments, and the prospects and trends in evaluating natural glycosides as new effective therapeutics to combat the threatening antibiotic-resistant bacteria that burden the healthcare system. Figure 2.4 shows the chemical structure of a typical glycoside.

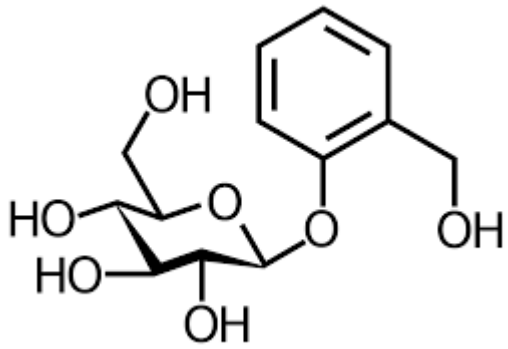


Figure 2.4: Glycoside structure

Source: New World Encyclopedia (2019b)

2.3.3 Flavonoids

Most flavonoids are derived from glycosides (Bartnik & Facey, 2017) and have wide-ranging biological properties. They assist in the reduction of risks related to diseases as they help promote human health (Craig, 1997). Flavonoid aglycones are lipophilic, external flavonoids with a structurally diverse group of natural products that are often highly methylated. A second type of flavonoids are polar flavonoid glycosides (Grayer et al., 2002). The most important variations in their structure arise from the level of oxygenation (hydroxyl or methoxyl groups). The sugar component may consist of hexoses, deoxyhexoses or pentoses and in some cases glucuronic acids with the added possibility of O- or C-glycosidation. Differences in the configuration at the anomeric carbon(s) of the glycosidic units are also possible (Stobiecki, 2000). The health benefits and protection of flavonoids have been reported in antioxidants and anticancer, antibacterial, anti-inflammatory, and cardioprotective agents. Immune system promoting studies thus support their medicinal properties (Tungmunnithum et al., 2018). Figure 2.5 shows the chemical structure of a typical flavonoid.

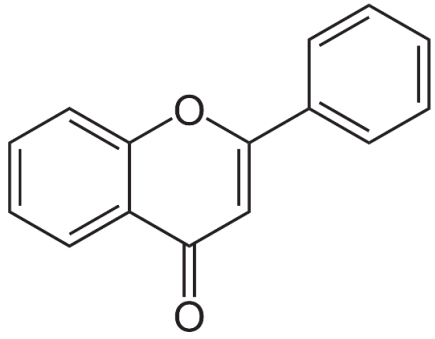


Figure 2.5: Flavonoid structure

Source: Scholarly Community Encyclopedia (2020)

2.3.4 Anthraquinones

Anthraquinones are a class of abundant compounds obtained from natural sources and are widely used as dyes and bioactive molecules (Duval et al., 2016). Plants are the source of approximately 200 compounds that belong to this class, and anthraquinones are found in the roots, rhizomes, leaves, seeds, fruits, and flowers of the plant. Anthraquinones are known for their remarkable biological activities, such as anticancer, anti-inflammatory, diuretic, antiarthritic, antifungal, antibacterial, and antimalarial activities (Duval et al., 2016; Diaz-Munoz et al., 2018; Li & Jiang, 2018).

Anthraquinone compounds with the anthraquinone ring structure are widely found in traditional medicines and they are attracting attention due to their researched pharmacological activity. The diversity of anthraquinones depends on their chemical structures, such as the number of anthraquinone rings and substituents. The difference in chemical structure determines the difference in physiological activity (Duval et al., 2016). Figure 2.6 shows the chemical structure of a typical anthraquinone.

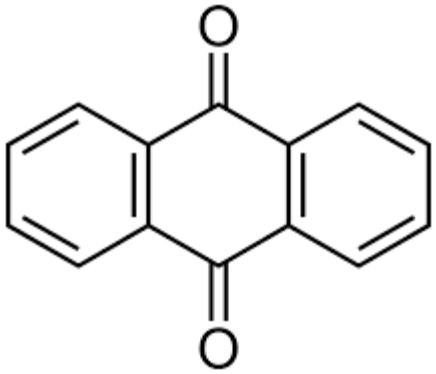


Figure 2.6: Anthraquinone structure

Source: ChemSrc (2022)

2.3.5 Phenols

Phenolic compounds can be divided into simple or complex phenolic compounds, based on their chemical structures (Alu'datt et al., 2017). Plant phenolics and polyphenols are secondary natural metabolites arising biogenetically from the following pathways: either the shikimate/phenylpropanoid pathway that directly provides phenylpropanoids or the polyketide acetate/malonate pathway, which can produce simple phenols, or both; thus, producing monomeric phenols, polymeric phenols, and polyphenols, which play a broad range of physiological roles in plants (Lattanzio, 2013). Plant phenolics have been considered to have a significant role as defence compounds when the plant is subjected to environmental stresses (e.g., high light, low temperature, pathogen infection, herbivores, and nutrient deficiency) and can lead to an increased production of free radicals and other oxidative species in plants for their survival (Lattanzio, 2013). Figure 2.7 shows the chemical structure of a typical phenol compound.

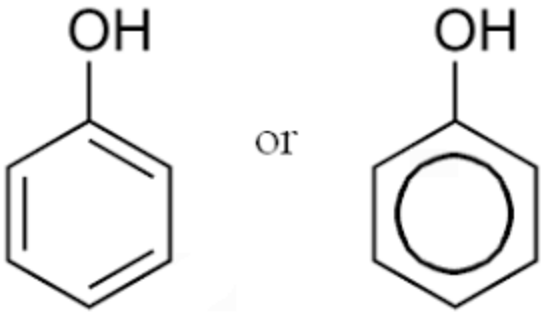


Figure 2.7: Phenol structure

Source: New World Encyclopedia (2019c)

2.3.6 Tannins

Tannins are defined as phenolic compounds of high molecular weight, which may be found in the plant's leaves, bark, and rhizomes, and bound to proteins that form condensed or hydrolysable tannin-protein complexes, which divide them into two main groups in accordance with the chemical structures and properties (Hassanpour et al., 2011). The condensed tannins are polymers of catechins or epicatechins, which are found mainly in fruit and accumulate in the outer layers of plants, whereas hydrolysable tannins are polymers of gallic or ellagic acid and are found in berries and nuts (King & Young, 1999). The increased production of tannins is often associated with a particular pathological condition caused by an insect attack in the plant galls and the levels of vegetable tannins found in parts of the plant, such as fruit and leaves, range between 2% and 5% of the fresh weight (Haslam, 2007). Figure 2.8 shows the chemical structures of the two types of tannins.

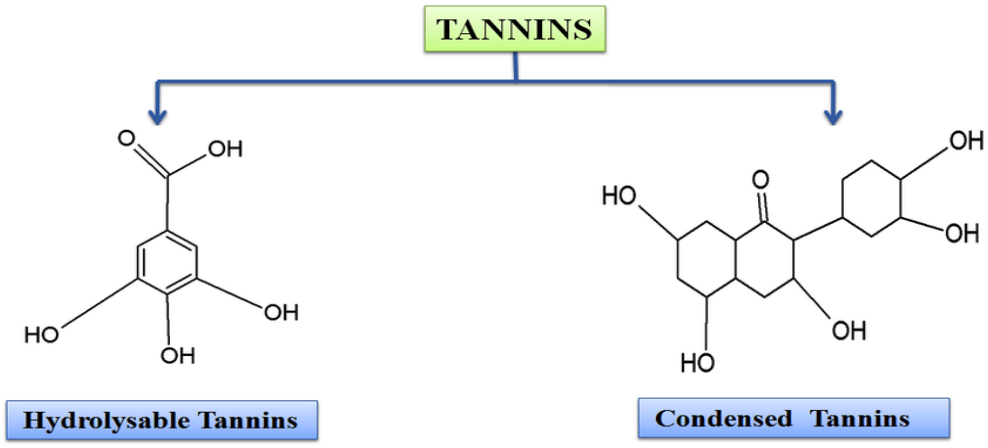


Figure 2.8: Tannins structure

Source: Ghosh (2015)

The benefits and medicinal properties of phytochemicals through pharmacological uses encourage the need to investigate traditional medicinal plants as a possible alternative solution in the development of novel agents to combat MDR bacteria. Traditional medicinal plants provide medical benefits that are important for the good health of individuals.

2.4 TRADITIONAL MEDICINAL PLANTS

This section discusses *E. elephantina* and *G. perpensa* in terms of their plant description and taxonomy, ethnomedical uses, the phytochemicals of the different parts of the plant, and pharmacological studies. The usage of *G. perpensa* by different ethnic groups is also discussed.

2.4.1 *E. elephantina*

E. elephantina (Burch) Skeels is a plant used extensively in Southern African countries as a source of food and to treat diseases and conditions caused by pathogenic bacteria (Mpofu et al., 2014a; Maroyi, 2016b; Makhafola et al., 2019; Van Vuuren & Frank, 2020). The plant was discovered and identified by Dr L.M. Turton in Botswana in March 1995 (Aaku et al., 1998). *E. elephantina* is a member of a small and purely African genus in the *Fabaceae* family and is represented by nine species on the continent. This species is spread widely among the Southern African countries of Botswana, Namibia, and Lesotho, and provinces in South Africa such as Limpopo, North West, Mpumalanga, Free State, Eastern Cape, Northern Cape, and KwaZulu-Natal (McGaw & Eloff, 2008; Olaokun et al., 2020). The generic name *Elephantorrhiza* means “elephant root” and is based, most descriptively, on the large underground stem common to most members of the genus (Aaku et al., 1998; Maroyi, 2017b). *E. elephantina* has many known names among different indigenous people and documented literature. It is known as elands bean in English, *mupangara* in Shona, *intolwane* in isiXhosa and isiZulu, *mositsane* in Sesotho and Setswana, and *elandsboontjies* in Afrikaans (Phillips, 1917; Guillarmod, 1971; Msimanga et al., 2012; Maroyi, 2014; Mpofu et al., 2014a).

The leaves of *E. elephantina* are shown in Figure 2.9A. The plant grows naturally in open grassy slopes and hillsides and produces red roots that look like sweet potatoes

(Msimanga et al., 2012). The reddish root seen in Figure 2.9B is the most used part of the plant in traditional medicine (Maroyi, 2016b) and is used as a remedy for the following conditions/diseases: diarrhoea and dysentery, diabetes (Balogun et al., 2016), chest problems, heart conditions, hypertension, syphilis, infertility in women, bladder problems, UTIs, abdominal pain in infants, fever, and haemorrhoids (Maroyi, 2017b; Van Vuuren & Frank, 2020).

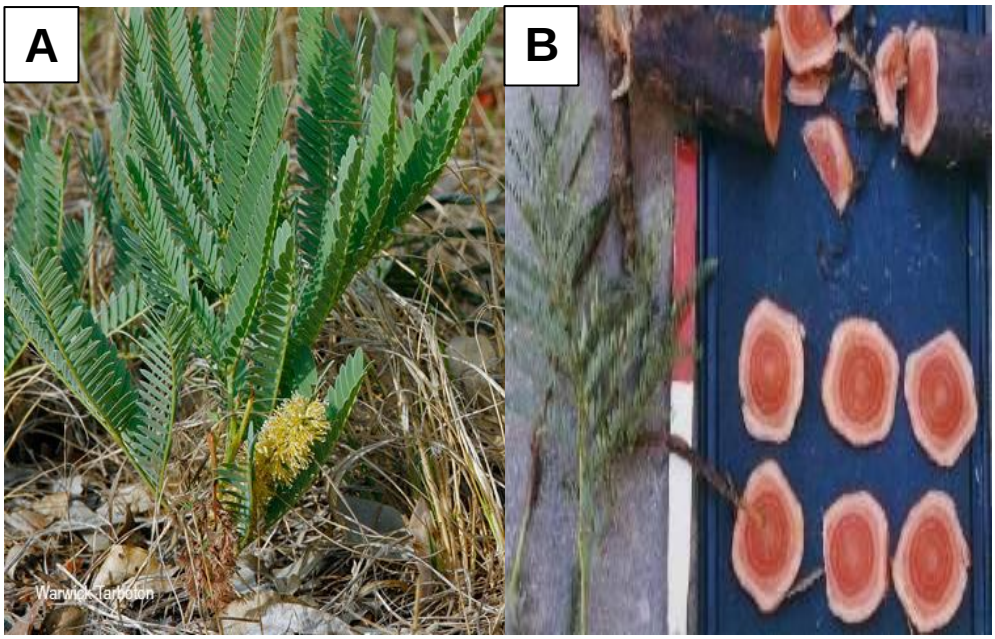


Figure 2.9: (A) The leaves of *E. elephantina* (B); the reddish roots of *E. elephantina*
Source: Grobler (2010); Plant Resources of the World (n.d.)

2.4.1.1 Plant description and taxonomy

E. elephantina is usually widespread, often gregarious, and forms huge patches in hot and dry areas in grasslands and open scrubland. The plant is a perennial low shrub with stems up to 90 cm tall at ground level, and from the woody end of an elongated, often thickened, rhizome up to 8 m long. The leaves consist of two to four pairs of pinnae in the lower leaves and seven to 17 pairs in the upper ones, where the axis is up to 10 cm long (Maroyi, 2017b; Makhafola et al., 2019).

The scientific classification of *E. elephantina* derives from the kingdom Plantae, the phylum Tracheophyta, the class Magnoliopsida, the order Fabales, the family Fabaceae, the genus *Elephantorrhiza*, and the species *elephantina* (Van der Walt & Le Riche, 1999; Van Rooyen et al., 2001).

2.4.1.2 Ethnomedical uses

E. elephantina's healing properties have been explored among both humans and animals. In animals, according to McGaw et al. (2020), studies conducted between 2000 and 2020 have reported that traditional plants are commonly used in ethnoveterinary medicine for South African rural livestock, which is a common feature that is still seen. Ethnoveterinary medicine may play an important role in relieving conditions such as wounds, skin diseases, mild diarrhoea and intestinal worms, and tick-borne disease in livestock, but not limited to these mentioned. *E. elephantina* was one of the plants reported in the survey. It was one of the many other plants used to conduct the study. The plants included were used for a variety of commonly encountered animal diseases and afflictions. This study supports that greater interest has been shown recently in documenting ethnoveterinary medicine, as with ethnobotanical medicine used to treat humans and animals (McGaw et al., 2020).

In the past two decades, efforts have been made to publish the varying uses of medicinal plants in South African ethnoveterinary medicine in greater detail, as were their biological activity investigations relating directly to their ethnoveterinary use by McGaw and Eloff (2008). McGaw and Eloff (2008) identified a downfall in the survey, where Martin et al. (2001, cited in McGaw & Eloff, 2008) mentioned that the shortcomings of employing such a system included the inefficacy or toxicity of remedies, there was uncertainty regarding overdosing regimens and lack of standardisation, as well as the unavailability of plant material during certain seasons of the year (Martin et al., 2001). The study conducted surveys in some of the provinces in South Africa, such as Limpopo and the North West province. This means that not all provinces were analysed, and provinces that were not mentioned were the Eastern Cape, the Free State, and KwaZulu-Natal. Further investigation in the mentioned provinces could be included in future studies as they have a high rate of breeding rural livestock, and it could yield significant results for medicinal research in ethnoveterinary medicine as the plant is known to grow within these provinces (Grobler, 2010). In animal studies, acetone (C_3H_6O) extracts of *E. elephantina* roots demonstrated significant activity against a tick-borne disease in cattle (Naidoo et al., 2005) and parasite worms in goats (Maphosa & Masika, 2012).

In humans, *E. elephantina* has been reported as being used against conditions such as diarrhoea, ulcers, skin wounds, infertility of women, UTIs in both men and women, and the relief of fevers caused by illnesses associated with pathogenic bacteria in humans, as well as blood purifying and cleansing of the womb (Mpofu et al., 2014a; Maroyi, 2016b, 2017b). Plant extracts using different polar solvents such as ethanol (C_2H_5OH), methanol, and aqueous solution have demonstrated relief to diarrhoea, wound infection, fever, and fertility problems in women (Dold & Cocks, 2001; Akter et al., 2012; Msimanga et al., 2012; Maroyi, 2017b; Olaokun et al., 2020; McGaw et al., 2020). The plant's roots are widely reported to be used in mixtures or solely in the treatment of conditions. The other parts, such as the leaves, are seldom used to treat conditions (Maroyi, 2017b; Olaokun et al., 2020).

The traditional uses of *E. elephantina* extract in the treatment of diarrhoea are widely researched and reported (Mathabe et al., 2006; Msimanga et al., 2012; Mpofu et al., 2014a; Maroyi, 2016b, 2017b). Researchers have subjected bacteria associated with gut flora such as *E. coli* and *Streptococcus* species to mainly the plant's roots and recently the leaf extract for further investigation aimed to understand and isolate phytochemical compounds such as phenol, flavonoids, and tannins that are reported to have antidiarrheal and antibacterial properties (Maroyi, 2016b, 2017b; Kudumela & Masoko, 2018). Further investigation of how the mechanism of action of the plant extract's antibacterial properties could be assessed and to establish their safety and efficacy is needed. Gut flora have the potential to become pathogenic and cause diarrhoea (Mathabe et al., 2006; Maroyi, 2016b, 2017b) and that is the reason why the above-mentioned pathogens are commonly used in antidiarrheal and antibacterial studies of other authors as they are known to be opportunistic pathogens and part of the gut flora.

Since the plant's roots are reported to be used more, the overexploitation of the roots of *E. elephantina* has put the plant on the Red Data List of medicinal plants in the Southern African regions since the beginning of the 2000s (Talukdar, 2002; Maroyi, 2017b; Olaokun et al., 2020). When a population of *E. elephantina* is destroyed, the chance of regeneration is limited as they are known to grow in relatively natural areas, which are in rural areas or areas with open grass on land that is surrounded by fewer homes or buildings. The plant shows evidence of an unusual type of germination where the seedling buries its plumule (the bud within the embryo from which the stem

and leaves develop) and shoots and thus arising from well below the ground surface (Davy, 1922; Grobler, 2010). They are not weedy in character, even though a large colony of these plants may seem that way. Attempting to preserve the plant has led to scientists exploring other parts of the plant and focusing on the leaves.

Recent research by Olaokun et al. (2020) explored the phytochemical content and antidiabetic, anti-inflammatory, antioxidant, and cytotoxic activity of leaf extracts of *E. elephantina.* It is reported that there is a lack of information on the leaves' biological activity and few reports on the plants' antidiabetic activity in humans and animals (Martin et al., 2001; McGaw & Eloff, 2008; Kudumela & Masoko, 2018; McGaw et al., 2020; Olaokun et al., 2020). The dried leaf extract of *E. elephantina* was subjected to the following solvents: acetone, ethanol, cold water, and hot water, and investigated for polyphenolic, tannin, and flavonoid content and antioxidant, anti-inflammatory, antidiabetic, and cytotoxic activities, using standard methods. Olaokun et al. (2020) reported that the phenolic and flavonoid contents of the ethanol extract were the highest to acetone. In addition, the inhibition of α-amylase activity by the ethanol extracts was the strongest to acetone and water. Furthermore, the ethanol extract was the least cytotoxic, which was tested on mouse cells against H4IIE liver and differentiated C2C12 muscle cells (myotubules). However, for the other assays, the hot water extract was more active than the ethanol and acetone. The hot water extract in a concentration-dependent manner stimulated the highest C2C12 glucose utilisation activity, in addition to exhibiting the strongest antioxidant and anti-inflammatory activities (Olaokun et al., 2020).

Water and alcohol are used in preparing herbal remedies in traditional practice, which highlights the significance of the results. Olaokun et al. (2020) recently studied the biological activities of *E. elephantina* leaf extracts. This indicates that there are still opportunities to conduct further investigations on the leaves of this plant where the extract is subjected to other solvents of various polarity such as hexane, ethanol, and acetone. The analysis of the study showed that the leaves can be used as an alternative to the roots, which can ensure the long-term usage and preservation of the plant. However, further investigation should be conducted to gain more scientific evidence on the plant's leaf extract. This will prevent the plant from being dug up, which would cause it to die; instead, only the leaves could be harvested, which would extend the plant's lifetime and allow the leaves to bloom again. As a result, if the plant

is dug up, this method of self-preservation would not be possible. Plant preservation may help to ensure the plant's existence and may even prevent extinction (Grobler, 2010).

2.4.1.3 Phytochemicals of the different parts of the plant

The plants' secondary metabolites, known as phytochemicals, play an important role in reducing the occurrences of many diseases such as diabetes, syphilis, dysentery, fever, hypertension, and haemorrhoids (Olaokun et al., 2013; Olaokun et al., 2020). The phytochemical compounds such as glycosides, phenols, saponins, and tannins that are detected in the plant extract help in the relief of illnesses or healing of wounds through their rich medicinal activity as mentioned in the literature (Aaku et al., 1998; Mpofu et al., 2014b; Kudumela & Masoko, 2018; Makhafola et al., 2019) and therefore support the ethno-pharmacological uses of the plant. Phytochemicals are plant-derived molecules known to be a rich source of diverse compounds found in different parts of a plant when extracted using a solvent that could serve as the basis for rational drug design (Pathania et al., 2015). Research has led to investigating the phytochemical properties found in the roots and rhizomes and leaves of *E. elephantina*. In these parts of the plant, investigations were conducted on which phytochemicals are commonly found in the plant's extracts. The phytochemicals of *E. elephantina* include gallic acid, glucoside, flavonoid, phenols, tannins, and phytosterols (Mathabe et al., 2006; Mthembu, 2007; Kudumela & Masoko, 2018; Van Vuuren & Frank, 2020).

According to Mthembu (2007), the findings from the aqueous and methanol extracts of the rhizomes include flavonoids and indicated the presence of 5.8% to 22.3% tannins found in the rhizome of the plant. Tannins are the main compound found in the roots of the plant (Aaku et al., 1998; Mpofu et al., 2014b; Makhafola et al., 2019). An additional finding from the study of Mthembu (2007) indicated that 16.8% of sugars were detected in the rhizomes of the plant. The sugars found in the rhizome of *E. elephantina* were b-sitosterol, gallic acid, methyl gallate, catechin, and pentahydroxyflavone, among other compounds. The compounds detected were mainly phenolic compounds with a flavonoid skeleton (Mthembu, 2007). The paper reported on the plant's good antioxidant activity when tested against and compared to

the antioxidant activity of green tea. These results were a significant indication of scientific evidence of *E. elephantina*'s antioxidant properties (Mthembu, 2007).

Kudumela and Masoko (2018) reported secondary metabolites that were detected in the leaves to be flavonoids, cardiac glycosides, alkaloids, steroids, and tannins. Thin-layer chromatography (TLC) fingerprint profiling of the plant extracts and spectrophotometric methods for quantitative determination were used to support the phytochemistry detections. The findings highlighted the absence of terpenoids and saponins from *E. elephantina.* However, the phenolic, tannin, and flavonoid content of *E. elephantina* were reported to be present in high quantities when compared to other plants (Kudumela & Masoko, 2018). The phytochemicals that could be responsible for medicinal activity in medicinal plants are commonly steroids, tannins, flavonoids, and glycosides, which are present in *E. elephantina*, as found by Kudumela and Masoko (2018). Terpenoids and saponins are seen as the compounds responsible for antibacterial activity (Mthembu, 2007; Kudumela & Masoko, 2018), which could be an indication that *E. elephantina* could be a potential antibacterial agent.

2.4.1.4 Pharmacological studies

Investigations of *E. elephantina*'s antimicrobial activity and toxicity have been performed in the past two decades (Mpofu et al., 2014b; McGaw & Eloff, 2008; Olaokun et al., 2020) using extracts isolated from different solvents with ranging polarisations. Minimum inhibitory concentration (MIC) and disc diffusion methods were used to investigate the antimicrobial activity. Assays such as TLC and spectrophotometry, among others, were used as qualitative and quantitative assays to investigate which phytochemical compounds are present in this plant (Kudumela & Masoko, 2018).

The toxicity of *E. elephantina* was investigated by subjecting the extract to mouse cells (H4IIE liver cells and differentiated C2C12 muscle cells [myotubules]) (Olaokun et al., 2020). The results from previous studies (Talukdar, 2002; Mthembu, 2007; Maroyi, 2017b; Kudumela & Masoko, 2018; Olaokun et al., 2020) indicated that the phenolic and flavonoid compounds extracted from *E. elephantina's* roots and leaves have antimicrobial activity that may inhibit bacterial infections, which ultimately leads to the healing of infections caused by bacteria. *E. elephantina*'s phytochemical compounds

may use different phytochemicals such as anthraquinones, steroids, and flavonoids for antimicrobial activity/healing properties. The explanation of the mechanisms/mode of actions of the plant having stronger affinity when compared to conventional antibiotics to inhibit bacteria may be caused by the solvents used to extract compounds. The active phytochemical compounds found in medicinal plants differ and may sometimes be ineffective since bacteria have the ability to develop resistance against the mechanism of inhibition. Investigation of new plant species could therefore be of clinical value in the discovery and development of novel drugs for the treatment of infectious diseases caused by pathogenic bacteria (Maroyi, 2016b).

Maharaj et al. (2019) expressed that as much as research has been conducted on *E. elephantina* for evidence of antimicrobial, anti-inflammatory, antimalaria, and antidiabetic activities and wound healing (Dold & Cocks, 2001; Akter et al., 2012; Msimanga et al., 2012; Balogun et al., 2016), no confirmation has been made by researchers of any further development of products and commercialisation. Maharaj et al. (2019) further highlighted that past literature evidence should have already led to the commercialisation of pharmaceutical products in respect of antimicrobial products. However, the lack of products to show indicates that more research is needed, with more corresponding data of past research to strengthen the knowledge before pharmaceutical companies would invest in the commercialisation of products (Maharaj et al., 2019). This poses questions for research as to whether scientists are collecting information as part of the natural drug discovery paradigm or whether they are interpreting the traditional uses correctly and undertaking biological assays using correct screening and appropriate models and understanding mechanisms of action and safety fully. This highlights that there are gaps within the scientific studies that should lead to more investigations performed on *E. elephantina* sampled and found in other provinces of the country where the plant is prominent, as geographic factors influence the secondary metabolites produced by plants.

There are several scientific reports on *E. elephantina*'s antibacterial, anti-inflammatory, and antidiabetic activities (Mthembu, 2007; Maroyi, 2017b; Kudumela & Masoko, 2018; Olaokun et al., 2020) but not much research has been conducted on its safety. Numerous studies have provided scientific evidence that validates the traditional uses of *E. elephantina*. A documented commercialisation of a product from the plant *E. elephantina* was made by the Council for Scientific and Industrial

Research (CSIR) on the plant's extract from the roots for the management and treatment of benign prostate hyperplasia (BPH) (Mthembu, 2007) and placed on the commercial market. This is a singular case where a product was developed from *E. elephantina* extract especially for BPH and male pattern baldness since the plant has not been documented to be traditionally used for these conditions (Fouché et al., 2015). This could be linked to some of the traditional uses of the plant by individuals with bladder or urinary problems due to its antibacterial properties. This indicates that research that produces satisfying results with enough scientific evidence does lead to product commercialisation. This leads to the question regarding what more can be done to close the knowledge gaps and produce more supporting evidence for more product development of *E. elephantina*'s active ingredients (Maharaj et al., 2019).

2.4.2 *G. perpensa*

G. perpensa belongs to the Gunneraceae family and is recorded to be the only species of the genus *Gunnera* found in Africa (Mariotti et al., 2014; Maroyi, 2016a; Patel et al., 2020). In 1767, Linnaeus was first to describe *G. perpensa* L. as the first species of the genus to exist in African countries such as Sudan, Ethiopia, the Democratic Republic of the Congo, Burundi, Madagascar, Rwanda, Uganda, Kenya, Tanzania, Botswana, Namibia, Zimbabwe, Mozambique, Lesotho, South Africa, and Swaziland (Simelane et al., 2010; Maroyi, 2016a). In South Africa, it is most prominently found in KwaZulu-Natal, the Eastern Cape, Limpopo, Free State, and the Western Cape (Mammo et al., 2017; Balogun et al., 2016). *G. perpensa* is used as a source of food, apart from its medicinal uses, by some ethnic groups in South Africa, such as the Venda and Zulu people. Other ethnic groups in South Africa mainly use it as a medicinal plant to treat common symptoms of illness such as colds, inflammatory conditions, fevers, ear infections, and urinary complaints/UTIs and diseases such as cancer (Maroyi, 2016a; Manduna et al., 2019).

G. perpensa is commonly referred to as the "river pumpkin" in English because of its morphology and the geographic location of the plant. It has many vernacular names such as *gobho* in Siswati, *iphuzilomlambo* in isiXhosa, *qobo* in Sesotho, and *ugobho* or *uklenza* in isiZulu (Khan et al., 2004; Maroyi, 2016a; Mathibe et al., 2016). The plant image is represented in Figure 2.10, which shows the leaves and flowers of this plant. The plant mainly grows in moist habitats, wet areas, and along riverbanks, as seen in

Figure 2.10a, and cannot endure cold conditions or frosty weather. *G. perpensa* roots are generally used as medication for the treatment of pre- and post-natal complications, kidney and bladder complaints, UTIs, and inflammatory conditions, while the leaves are used for treating/dressing/healing of wounds (Nkomo & Kambizi, 2009; Mfengwana et al., 2019). This implies that *G. perpensa* may possess medicinal and antibacterial healing properties (Buwa & Van Staden, 2006; Mariotti et al., 2014; Webb, 2017).

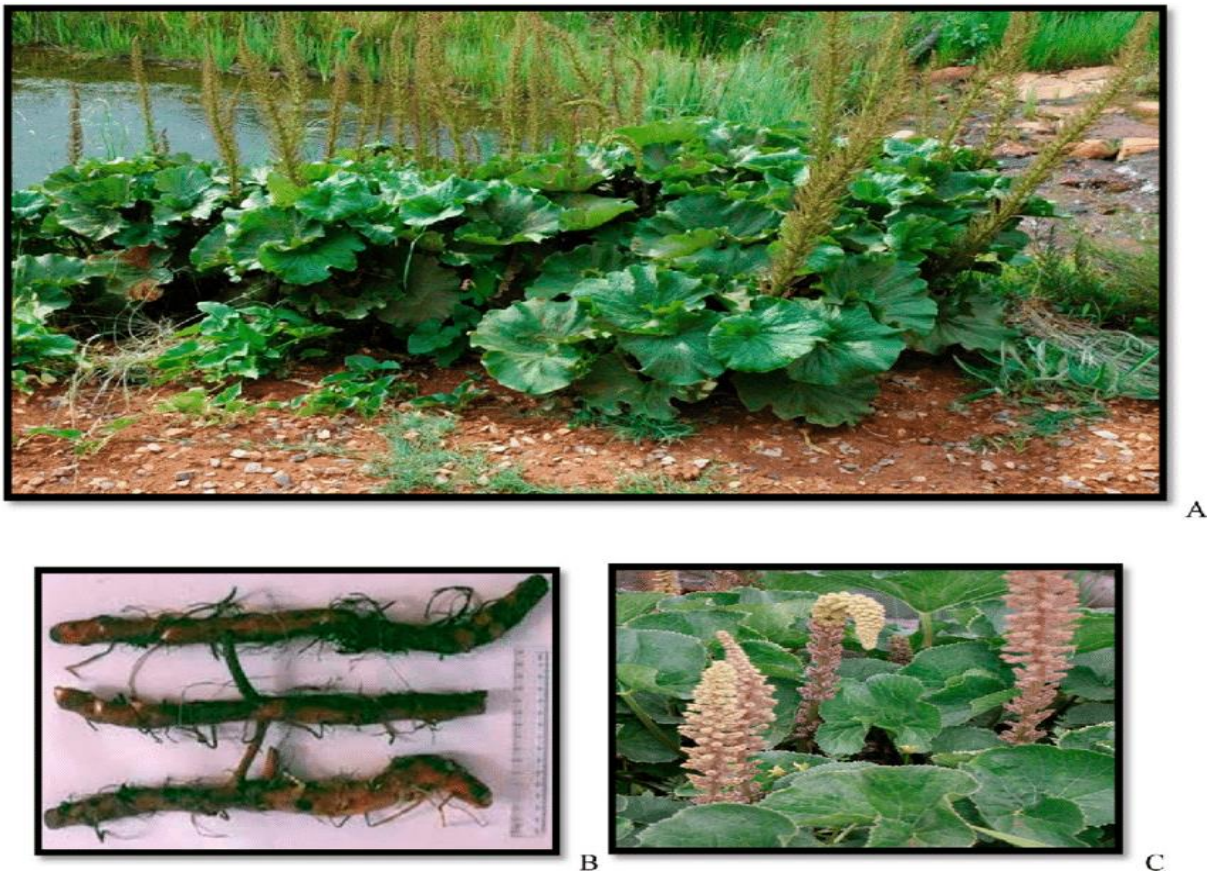


Figure 2.10: *G. perpensa*: (A) whole plant, (B) rhizomes and roots, and (C) flowers and leaves
Source: Mendes (1978, cited in Simelane et al., 2010)

2.4.2.1 Plant description and taxonomy

G. perpensa is a full-bodied perennial herb that may grow up to 1 m tall, with approximately 30 cm thickset roots that are dark brown or blackish in colour on the outside but yellow or pinkish-red inside (Simelane et al., 2010; Maroyi, 2016a; Mammo et al., 2017). All the leaves arise from the centre tuft near the top of the apex, directly above the soil level. The leaves are bulky, dark bluish-green, kidney shaped, and covered with hair projectiles on both surfaces, as well as along the veins of early

developing leaves. The margins of the leaves are irregularly toothed. The veins are very noticeable on the lower surface of the leaf, radiating from the point where the petiole joins the leaf, which is referred to as palmate radiation (Filippich et al., 1991; McGaw et al., 2005; Brookes & Dutton, 2007). The flowers are numerous, small and not very noticeable, pinkish or reddish brown, and protrude from a long spike that is taller than the leaves. The female flowers are at the base, male flowers at the top, and bisexual flowers in the middle of each spike (Ronse de Craene & Wanntorp, 2006).

G. perpensa derives from the kingdom Plantae, the phylum Tracheophyta, the clade Angiospermae, the class Eudicotidae, the order Gunnerales, the family Gunneraceae, the genus *Gunnera*, and the species *perpensa* L. It is part of a group of herbaceous flowering plants. Among six subgenera, *Gunnera* is the only one of its family with roughly other 50 species (Brookes & Dutton, 2007; Simelane et al., 2010; Mammo et al., 2017).

2.4.2.2 Ethnomedical uses

G. perpensa is a diverse plant as all the parts of this plant are used for traditional decoctions due to their medicinal healing properties (Nkomo & Kambizi, 2009; Maroyi, 2016a). This includes the plant's roots, rhizome, stem, and leaves. It is used mostly within rural populations where traditional healers advise that aqueous extractions be used for the treatment of dysmenorrhea, to relieve rheumatoid pain, to assist in childbirth, and to treat female infertility (Muleya et al., 2014; Mfengwana et al., 2019; Ramulondi et al., 2022). According to Maroyi (2016a), *G. perpensa* is one of the important ingredients of at least three commercialised traditional concoctions in South Africa, known as *imbiza ephuzwato*, *inembe*, and *isihlambezo,* which are used for childbirth and the treatment of pre- and post-partum complications, and *imbiza ephuzwato*, which is used as a multi-purpose medication.

According to Dold and Cocks (2001), *G. perpensa* is reported to be heavily traded in Swaziland, the Eastern Cape, and KwaZulu-Natal (Maroyi, 2016a; 2017a). *G. perpensa* has been categorised as a declining plant species in South Africa using the modified International Union for Conservation of Nature (IUCN) Red List Categories and Criteria version 3.1 of threatened species (Williams et al., 2013; Raimondo, 2009; Chigor, 2014; Maroyi, 2017a). Other reasons for the significant

decline of *G. perpensa* could also be due to the destruction of its wetland habitat caused by development and agriculture and overexploitation of its rhizomes and roots, which are sold in the medicinal *muthi* markets throughout South Africa. This leads to unsustainable harvesting of *G. perpensa* for herbal medicine, which continues to threaten its existence (Maroyi, 2016a).

2.4.2.3 Usage by different ethnical groups

G. perpensa is best recognised in the province of KwaZulu-Natal, where it has been used traditionally as a *muthi* (medicinal) plant by the indigenous Zulu people for a long time (Simelane et al., 2012; Ramulondi et al., 2022). The common uses of the decoctions or infusions of the root or rhizome are as treatment for the following condition/diseases: abdominal pain, bladder problems, cancer, colds, earache, endometritis, gastrointestinal parasites, gonorrhoea, heart disease, hypertension, impotence, infertility, kidney problems, poor appetite, rheumatic pain, scabies, syphilis, and urinary infections. It is also used for body cleansing (Nkomo & Kambizi, 2009; Mathibe et al., 2016; Mfengwana et al., 2019). The Zulu ethnic group's traditional healers use *G. perpensa* root decoction to stimulate milk production, among many other uses (Simelane et al., 2012). According to both Drewes et al. (2005) and Maroyi (2016a), a decoction of the rhizomes of *G. perpensa* can be used for the treatment of psoriasis or applied as a dressing for wounds. However, Drewes et al. (2005) placed more focus on the leaves and stems of *G. perpensa* as having more potential for wound-healing properties, but from observations had noted that contact with the fresh stems and leaves could cause slight skin irritation.

Furthermore, the leaves of *G. perpensa* are used in the Eastern Cape province as a dressing for wounds by the rural inhabitants (Drewes et al., 2005; Asong et al., 2019). In the Eastern Cape in the ethnoveterinary industry, resource-limited farmers use *G. perpensa* as an alternative control for gastrointestinal parasites in village chickens (Chulayo et al., 2011; Mwale et al., 2014; Maroyi, 2016a). For humans, the plant's stem is boiled with water and a glass of decoction is taken as a remedy for constipation by the Xhosa ethnical group in the Eastern Cape (Maroyi, 2016a). This indicates that the decoction has different uses for humans and animals in one province, which further confirms that traditional healers prescribe and use the plant differently for animals and humans (Mwale et al., 2014; Maroyi, 2016a).

In the KwaZulu-Natal province, *G. perpensa* leaves are collected from the wild as a vegetable with the local name of *imifino* in isiZulu and are used as a source of food (Maroyi, 2016a; 2017a). This is also witnessed among the Venda population in the Limpopo province. They collect fresh *G. perpensa* leaves and cook them with other indigenous or traditional leafy vegetable species. Similarly, in Swaziland, the roots, stalks, and stems of *G. perpensa* are a source of edible leafy vegetables and are also used as ingredients for traditional beer (Maroyi, 2016a). In Lesotho, *G. perpensa* leaves are used for dressing for wounds and boils and as heating pads (Moteetee & Kose, 2017; Asong et al., 2019; Mfengwana et al., 2019). A decoction of roots is used for most female reproductive problems such as to relieve menstrual pain (Maroyi, 2016a; Moteetee & Kose, 2017), cramping/contractions in pregnant women (Moteetee & Kose, 2016; Ramulondi et al., 2022), expulsion of the placenta in both women and animals (Khan et al., 2004; Moteetee & Kose, 2016; Maroyi, 2016a, 2017a), and for vermifuge in both humans and animals (Mwale et al., 2014; Maroyi, 2016a). The leaves are burned, crushed, and smoked by humans to treat headaches (Maroyi, 2016a).

In the Khoisan (Nama) group, the plant's only known use is in relation with alcoholism (Nortjé & Van Wyk, 2015), whereas in the Basotho and Zulu population, *G. perpensa* has various medical uses such as for maternal reproductive aliments (dysmenorrhea) and galactagogue and is alternatively used as a source of food (Maroyi, 2016a). The common uses of *G. perpensa,* which is the most renowned plant in traditional medicines in Southern Africa, is for the treatment of, among others, bladder problems, cancer, headaches, hypertension, fever, sores, stomach bleeding, menstrual pain relief in women and infertility in females, and inflammatory conditions, and for blood purifying (Drewes et al., 2005; Moteetee & Kose, 2016; Van Vuuren & Frank, 2020). This could indicate that the plant is not commonly used in the Nama population who reside in the Limpopo plains of South Africa. They use alternative medicinal plants such as *Gomphocarpus cancellatus* and *G. fruticosus* for stomach ailments, *Helichrysum odoratissimum* for fever, and *Drosera capensis* for cancer, for example (Nortjé & Van Wyk, 2015; Moteetee & Kose, 2016).

2.4.2.4 Phytochemicals of the different parts of the plant

Phytochemicals are plants' secondary metabolites that assist in the plant's survival against harsh environments, pathogens, and insects (Mfengwana et al., 2019). Phytochemicals are also seen as guardians of human health (Craig, 1997) as consuming a diet rich in plant foods will provide the body with many antioxidants that contain health-protective benefits. The phytochemical investigation of a plant may be because of subsequent interest to authenticate the extraction of the plant material, the separation and isolation of the constituents of interest, but also characterising the isolated compounds via quantitative evaluation (Gupta et al., 2012). *G. perpensa* contains an abundance of phytochemical compounds such as phenols, flavonoids, and terpenoids, which are detected in the plant extracts that have been associated with protection from and/or treatment of chronic disease such as heart disease, cancer, diabetes, hypertension, rheumatoid arthritis, bladder problems, and reproductive problems in women (Craig, 1997; Khan et al., 2004; Ramulondi et al., 2022). These phytochemicals are extracted from the different parts of the plant, such as the roots, rhizomes, leaves, and stem using polar and non-polar solvents in in vitro studies, which serve as the basis of rational drug design.

Previous research has led to more investigations into the phytochemical properties/compounds found in the roots/rhizomes, stems, and leaves of *G. perpensa.* The investigations focused on which phytochemical compounds are commonly found and further identification of the exact compound composition in the different parts of the plant. The phytochemicals of *G. perpensa* (roots/rhizome) in water extracts showed only the presence of flavonoids, terpenoids, and tannins; however, its methanolic and dichloromethane extracts revealed the presence of flavonoids, tannins, saponins, terpenoids, and alkaloids (Mfengwana et al., 2019). According to Mfengwana et al. (2019), it could be indicative of the possibility that the active ingredients of this plant are hydrophobic and are thus extracted better with solvents that are non-polar.

Phytochemical screening of the rhizomes showed the presence of alkaloids, flavonoids, steroids, saponins, tannins, and glycosides (Simelane et al., 2010). Furthermore, Chigor (2014) reported on the isolation of alkaloids, flavonoids, phenols, proanthocyanidins, and tannins from aqueous and methanol rhizome extracts of

G. perpensa, which supports the plants' secondary metabolites as reported in the literature; however, findings on the presence or absence of steroids and anthraquinones are contradictory. This could be due to the variation in the plant's geological location, soil content, and extraction method used in different studies. According to Mtunzi et al. (2012), *G. perpensa* did not cause heavy metal toxicity in humans but rather has inorganic elements that are beneficial to persons with micronutrient deficiencies (Maroyi, 2016a). The identification and characterisation of the compound constituents resulted in the isolation of 3,3',4'-tri-Omethyl ellagic acid lactone and Z-methyl lespedezate from methanol extracts of *G. perpensa* roots (Khan et al., 2004; Brookes & Dutton, 2007). The ellagic acid lactone, 1,1'-biphenyl-4,4'-diacetic acid, p-hydroxybenzaldehyde, and glucose were released by acid hydrolysis of *various G. perpensa* extracts. Further investigations led to the isolation of minor component trimethyl ether of ellagic acid glucoside from aqueous extracts of the dry *G. perpensa* rhizomes (Mathibe et al., 2016). The major component discovered was Z-venusol, a phenylpropanoid glycoside, which causes a "state of spontaneous" uterine muscle contractility in rat muscles (Khan et al., 2004).

According to Drewes et al. (2005), 1,4-benzoquinone isolated from the leaves and stems of *G. perpensa* inhibited the in vitro growth of human pathogens such as *S. epidermidis* and *S. aureus*, which supports the plant's medicinal properties. Muleya et al. (2014) reported significant activity against *E. faecalis, E. coli, P. aeruginosa, S. aureus, Aspergillus fumigatus,* and *Candida albicans* (Moteetee & Kose, 2017).

2.4.2.5 Pharmacological studies

Phytochemicals are known to possess antioxidant (Peleyeju, 2018), antibacterial (Moteetee & Kose, 2017), antifungal (Buwa & Van Staden, 2006), antidiabetic (Balogun et al., 2016), anti-inflammatory (Van Vuuren & Frank; 2020), anticancer (Khan et al., 2004, Mathibe et al., 2016) properties, and due to these properties, they are largely used for medicinal purposes. *G. perpensa* has been reported as one of the medicinal plants used for the treatment of cancer in the Eastern Cape province. The major component Z-venusol was subjected to human breast cancer cells (MCF-7 cell line) to investigate the effects of the extract on the cells, which resulted in positive outcomes (Mathibe et al., 2016). The component inhibited the cells, caused 34%

elevation of cAMP levels, and caused cell death via apoptosis (Mathibe et al., 2016). This supports that *G. perpensa* possesses anticancer properties (Muleya et al., 2014).

The plant's rhizomes have been proven to have analgesic and anti-inflammatory properties (Hutchings et al., 1996; Nkomo & Kambizi, 2009, cited in Dube, 2014; Olaokun et al., 2020). According to Iwalewa et al. (2007), *G. perpensa*, among other herbs, was reported to be used for the management of joint pain and rheumatism. This correlated with findings reported by Hutchings et al. (1996). More than a decade later, Nkomo and Kambizi (2009, cited in Dube, 2014) investigated these claims and their findings suggested that *G. perpensa* possesses both antinociceptive and anti-inflammatory activity. This further supports its traditional use for pain management in anti-inflammatory studies, as reported by Hutchings et al. (1996).

Plants have been extensively researched to find solutions to a problem that has existed over the past years. Bacteria have become more resistant to a variety of antibiotics. This makes the management and treatment of infections/illnesses caused by MDR bacteria exceedingly difficult (Khameneh et al., 2016). Solutions are required to reduce this burden on the healthcare sector. The management of the use of antibiotics, developing research to better understand the natural and genetic mechanisms of resistance, and continuing studies on traditional medicinal plants into the development of new drugs, either in their natural or synthetic form, are highly important. The development of new antibiotics from plants should focus on being effective in sensitising MDR bacteria (Oladunjoye et al., 2022).

The purpose of this study is to screen the selected plants for phytochemical compounds and the antibacterial activity of the plant extracts and to evaluate the bacterial cell morphology using SEM and TEM. The MDR bacteria likely to show greater sensitivity to the plant's extract will be evaluated through SEM and TEM. This will assist to evaluate and understand the mechanism of action the plant extract subjects the bacteria to through the morphology.

CHAPTER 3:
METHODOLOGY

3.1 INTRODUCTION

Medicinal plants are important for the discovery and identification of new therapeutic compounds. For thousands of years, humankind has been using plant sources to alleviate or cure illnesses. Plants constitute a source of novel chemical compounds that are of potential use in medicine and other applications. For the isolation of biological components, extraction from plants is one of the more sustainable approaches (Jadhav et al., 2009). Sample preparation is the crucial first step in the analysis of plants/herbs, because it is necessary to extract the desired bioactive chemical components from the herbal material for further separation and characterisation (Huie, 2002). For obtaining better quality and higher efficiency of extraction from herbs, one must optimise the methods. The choice of an appropriate solvent is of essential importance, along with the application of a compatible extraction method. For the extraction of therapeutically desired active constituents, various solvents such as water, ethanol, chloroform, ethyl acetate, methanol, and others are commonly used in experiments.

For the selection of solvents, the “like dissolves like” principle is applicable. Polar solvents will thus extract polar substances and non-polar material will be extracted by non-polar solvents (Farnsworth & Soejarto, 1985). Mixtures of the solvents are also occasionally used to achieve better extraction efficiency. The most problematic hurdle in drug development from plants is answering the simple question of what kind of solvent should be used for the extraction of the chemical compounds. A variety of different solvents have been used to extract secondary metabolites from plants/herbs. The choice of solvent also depends on the intended use of the extract. Different considerations, which will lead to the type of solvent to use, will be determined by the following, for example: If the aim is antimicrobial component screening, then the effect of the solvent on subsequent separation procedures is not important, but it should not inhibit the bioassay procedure (Farnsworth & Soejarto, 1985; Nasir et al., 2015).

3.2 METHODOLOGY

The method carefully chosen for this study was solvent extraction. Solvent extraction is the most popular extraction method and was deemed the most suitable for this study. It replicates what the literature has reported on the preparation and use of traditional plants to treat aliments. Solvent extraction has also been standardised to achieve a satisfactory measure of reproducibility (Nasir et al., 2015). The purpose of standardising extraction procedures for the production of crude drugs is to obtain the therapeutically desired portion and to eliminate inactive material by treatment with selective solvents and methods. Standardisation of extraction procedures contributes significantly to the final quality of the herbal drug. The researchers aim to have a complete idea of the bioactivity of crude extracts, by optimising the extraction methodology to achieve the broadest possible range of phytochemicals. A typical extraction process may be performed following the steps of Handa et al. (2008), as discussed in the following subsections.

3.2.1 Collection and authentication of plant material and drying

Plant material can be used both in fresh and dry form. The most preferable is the dried material because of beneficial outcomes such as stable isolation of secondary metabolite components. These metabolites are useful in antimicrobial assays as they yield satisfactory results, which, as previously mentioned by Nasir et al. (2015), it is how traditional healers advise they should be used.

3.2.2 Extraction

Water is a widely used solvent for the extraction of plant material throughout the world and is recommended by traditional healers. Organic solvents used for extraction in experiments have been reported to give more reliable antimicrobial activity compared to water-based extracts (Parekh et al., 2005). The solvents that are most used for the preliminary investigations in antimicrobial activity experiments in plants are methanol, ethanol, and water, as they yield satisfactory results. Methanol and ethanol have less polar characteristics and most phytochemicals are known to be non-polar. With the principle of “like dissolves like”, the two solvents will produce more satisfactory results than water (Lapornik et al., 2005).

3.2.3 Filtration

Filtration of the extracts is important to eliminate any artefacts and debris from the bark, the fruit, or any large particles such as crystallisation caused by evaporation. The use of Whatmann 10 mm filter paper is the most reported method in these experiments as it yields suitable results (Nasir et al., 2015; Mfengwana et al., 2019) .

3.2.4 Concentration

Aqueous solution are mostly concentrated through freeze drying and the organic extracts are commonly concentrated with a rota-evaporator.

The extraction method plays a vital role in the separation and characterisation of different phytochemicals from plants or herbs, and screening plant extracts leads to novel drug discovery (Nasir et al., 2015).

3.3 MATERIAL

3.3.1 Plant material collection

E. elephantina was collected from a farm in Thaba 'Nchu (coordinates 29°03'21.0" S, 26°53'18.9" E), Free State, South Africa, and was authenticated at the Free State National Botanical Garden by Professor Zietsman, with specimen voucher GP0020. *G. perpensa* was purchased from Random Harvest Nursery's South African indigenous plants section and authenticated by their botanists.

3.3.2 Medium used

Mueller-Hinton (MH) blood agar, Sabouraud Dextrose Agar, MH broth, and MH agar were purchased from National Health Laboratory Services in a ready-to-use format.

3.3.3 Control drugs

Discs (6 mm filter paper discs) dipped in saline were used as negative controls. Commercial (Sigma) Gentamicin discs (10 µg) and Ampicillin discs (10 µg) were used as positive controls against gram-negative and gram-positive bacteria respectively.

3.3.4 Sterilisation

Laboratory materials such as cylinders, beakers, McCartney bottles, pipettes, test tubes, filter papers, and other metal apparatus such as spatulas and forceps were sterilised using a hot air oven at a temperature of 160 °C for one hour prior to using them. The wire loops were sterilised by heating them in the blue flame of a Bunsen burner until red hot and allowing them to cool, disposable loops were also used and disposed after one individual use to avoid contamination, and 70% alcohol was used to swab and clean the workbench area to prevent contamination prior to and after the procedure. The process was conducted aseptically to avoid contamination.

3.4 METHODS

3.4.1 Extraction method

Plant materials were washed, air dried at room temperature away from sunlight, and ground to a fine powder. The 20 g fine powder was extracted using methanol and distilled water at room temperature for 48 hours while shaking. The extracts were filtered using Whatmann 10 mm filter paper, and new solvents were added again to the plant for more extracts until the solvents remained clear. The organic extracts were concentrated with a rota-evaporator, while the aqueous extract was lyophilised using a freeze dryer.

3.4.2 Qualitative phytochemical analysis of *E. elephantina* and *G. perpensa*

3.4.2.1 Qualitative phytochemical analysis

The phytochemical analysis was performed by adopting the methods and procedures of previous studies (Harborne, 1973; Sofowora, 2006; Prashant et al., 2011; Al Ghasham et al., 2017), with minor modifications. The methanol and aqueous crude extracts of *G. perpensa* and *E. elephantina* were subjected to qualitative phytochemical screening. Nine active compounds underwent screening. The qualitative results were expressed as (+) for the presence and (-) for the absence of phytochemicals.

(a) Detection of alkaloids

In this screening, 0.2 g of crude extract was dissolved in 2 mL of 1% hydrochloric acid (HCl) and then filtered with 6nm filter paper. Then after, 1 mL of Meyer's reagent and 1 mL of Drangendorff reagent were added to the filtrate obtained. The formation of orange precipitate confirms the presence of alkaloids.

(b) Detection of anthraquinones

Two millilitres of chloroform ($CHCl_3$) was added to 0.2 g of the extract and the resulting mixture was vigorously shaken for five minutes prior to filtration. Equal volumes of the filtrate obtained, and 10% ammonia solution (NH_4OH) were thoroughly mixed, and the formation of bright pink colouration in the aqueous layer of the mixture confirmed the presence of anthraquinones.

(c) Detection of glycosides

One milliliter acetic acid (CH_3COOH) was added to 0.25 g crude extract of plant material. One drop of 0.1% ferric chloride ($FeCl_3$) was added to the mixture, and 1 mL sulphuric acid (H_2SO_4) was added to the mixture. The formation of brown-ring precipitation indicated a positive test for glycosides.

(d) Detection of flavonoids

Ethyl acetate ($C_4H_8O_2$; 2.5mL) was added to 0.125 g crude extract of plant material. The mixture was heated for three minutes, allowed to cool, and then filtered, after which 0.25 mL of ammonia solution (NH_4OH) was added to the filtrate and the mixture was shaken. The formation of intense yellow precipitation indicated the presence of flavonoids.

(e) Detection of phenols

For this screening, 0.25 g of plant aqueous extract was treated with two to three drops of 10% ferric chloride ($FeCl_3$) solution. The formation of dark blue or bluish black precipitate confirmed the presence of phenols.

(f) Detection of saponins

The crude extract (0.5 g) was boiled in 5 mL of distilled water for five minutes and filtered. The filtrate (5 mL) was mixed with 3 mL of distilled water in a graduated

cylinder and shaken vigorously and left for five minutes for persistent frothing. The froth was thereafter mixed with three to four drops of olive oil and shaken again for observation of the emulsion layer, which signifies the presence of saponins.

(g) Detection of tannins

The crude extract (0.125 g) was added to 2.5 mL of distilled water. The mixture was boiled. It was filtered while hot and one drop of ferric chloride ($FeCl_3$) was added. The formation of black-blue precipitate confirmed the presence of tannins.

(h) Detection of triterpenes

To detect triterpenes, 0.2 g extract was treated with 1 mL chloroform ($CHCl_3$) and filtered. The filtrate was then treated with 3 mL H_2SO_4, shaken, and allowed to stand. The appearance of a golden/brownish colour was an indication of triterpenes.

(i) Detection of phytosterols

To detect phytosterols, 0.125 g of the crude extract was treated with 2.5 mL chloroform ($CHCl_3$) and filtered. One milliliter of H_2SO_4 was added to the filtrate with caution to the side of the test tube. The formation of a brown ring at the layer junction indicated the presence of phytosterols.

3.4.3 Quantitative phytochemical analysis of *E. elephantina* and *G. perpensa*

3.4.3.1 Total phenolic content determination

The concentration of phenolic content in 1 mg/mL methanol crude extracts of the selected plants was determined using the spectrophotometric method described by Hussain et al. (2011) and Singleton et al. (1999), with modifications. The determination of the total phenol content employed the Folin-Ciocalteau method, where 0.1 mL of extract and 0.9 mL of distilled water were mixed in a 25 mL volumetric flask. To this mixture, 0.1 mL of Folin-Ciocalteau phenol reagent was added and the mixture was shaken well, whereafter 1 mL of 7% sodium carbonate (Na_2CO_3) solution was added to the mixture after five minutes and filled to 2.5 mL with distilled water. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared according to the Folin-Ciocalteau method. The mixtures were incubated for 30 minutes at room temperature and the absorbance for test and standard solutions

was determined against the reagent blank at 760 nm with an ultraviolet (UV)/visible spectrophotometer. A graph was constructed using the absorbance of the standards and used to calculate the concentrations of the phenols. The total phenol content was expressed as µg/mg of GAE/g of extract as calculated from the graph equation y = 0.001x + 0.0464, R^2 = 0.9927, where y is the absorbance at 760 nm and x is the amount of GAE (µg/mL) (Arif & Fareed, 2011; Tambe & Bhambar, 2014).

3.4.3.2 Total flavonoid content determination

The total flavonoid content was determined by the aluminium chloride colorimetric assay. One millilitre of 1 mg/mL methanol extracts of the selected plants was mixed with 4 mL of distilled water in a 25 mL volumetric flask. To the flask, 0.30 mL of 5% sodium nitrite ($NaNO_2$) was added. About 0.3 mL of 10% aluminium chloride ($AlCl_3$) was added to the mixture after five minutes and this was mixed. After five minutes, 2 mL of 1 M sodium hydroxide (NaOH) was added, and this was filled to 10 mL with distilled water. A set of reference standard solutions of quercetin (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared according to the aluminium chloride colorimetric assay method. The absorbance for test and standard solutions was determined against the reagent blank at 510 nm with a UV/visible spectrophotometer. A graph was constructed using the absorbance of the standards and used to calculate the concentrations of the flavonoids. The total flavonoid content was expressed as QE/mg of extract, as calculated from the equation y = 0.0002x + 0.0957; R^2 = 0.9952, where y is the absorbance at 510 nm and x is the amount of QE (mg/mL) (Hussain et al., 2011; Tambe & Bhambar, 2014).

3.4.3.3 Microorganisms collected for testing

The pathogenic organisms that were used in the study were donated by National Health Laboratory Services in Universitas in Bloemfontein, namely *E. coli*, *K. pneumoniae*, *S. aureus*, *S. saprophyticus, S. epidermidis*, and *B. subtilis*. The bacteria were inoculated onto MH agar and MH blood agar to enhance growth, incubated aerobically at 37 °C, and later used for this study. To ensure the pure isolation of microorganisms, Gram's stains were performed on the respective bacteria according to Bartholomew and Mittwer's (1952) method, with minor modifications

using crystal violet instead of gentian violet as the primary stain, decolourising with 95% alcohol instead of absolute alcohol and safranin as the counterstain.

To produce bacterial counts that are within an expected range for this study, all bacterial suspensions were prepared using the MH broth to obtain an optical density comparable to the density of 0.5 McFarland barium sulphate standard (turbidity =108 colony-forming units [CFU]/mL).

3.5 IN VITRO ANTIBACTERIAL ASSAY

3.5.1 Antibacterial activity

The antibacterial activities of different extract concentrations (60, 125, 250, 500, and 1 000 µg/mL) of *G. perpensa* and *E. elephantina* were assessed against selected bacteria mentioned above as part of human pathogenic bacteria where the zone of inhibition and the MIC of different extracts were compared with those of different standards such as Ampicillin and Gentamicin for antibacterial activity.

3.5.1.1 Disk diffusion assay

The antibacterial activity was conducted using the disc diffusion method as adopted from Su et al. (2015) and Balouiri et al. (2016). The agar plates were inoculated with 0.5 M McFarland (i.e., 1.5 x 108 CFU/mL) of the bacteria and spread with the aid of a sterile cotton swab. Sterile Whatman No. 1 (6 mm) discs impregnated with each diluted sample of *G. perpensa* and *E. elephantina* extract were placed on a plate with ranging concentrations. A disc soaked with sterile saline was used as a negative control and Ampicillin and Gentamicin were used as positive controls. The plates were incubated aerobically for 24 hours at 37 °C and the zone of inhibition was measured thereafter. This experiment was done in triplicate and the mean was calculated and reported.

3.5.1.2 Microdilution assay

The MIC for each plant extract against a range of bacteria was determined by a modification of Eloff's (1998) method. The assay was performed in 96-wells µL plates by adding 50 µL of MH broth to all wells. In Row A, 50 µL of 1 mg/mL sample extract was added with a micropipette. Two wells were used as controls: one containing only the broth, and the other a growth control containing both broth and test organisms,

i.e., broth with bacteria and solvent. Ampicillin and Gentamicin (0.1 mg/mL) were used as positive control antibiotics. After adding 50 µL of the bacterial suspension to each row (except for the sterility control), the microplate was covered and incubated at 37 °C with 100% relative humidity for 24 hours. After 24 hours of incubation, 40 µL of a 2 mg/mL solution of p-iodonitrotetrazolium chloride (INT) was added to each row and the plate was further incubated for 30 minutes to ensure adequate colour development. INT is a dehydrogenase activity-detecting reagent, which is converted into an intense red-purple formazan by metabolically active microorganisms. The inhibition of growth will be indicated by clear solution with a yellowish colour or a definite decrease in colour reaction (lighter pinkish/purplish colour). The inhibition of growth indicated by a clear solution, or a yellowish colour will be taken as value of the MIC of the extracts.

3.6 CELL INTEGRITY DESTRUCTION INVESTIGATION

Cell integrity destruction was investigated through SEM and TEM.

3.6.1 SEM

SEM was performed according to Chew et al.'s (2018) method. During bioassay preparation, the selected bacteria cells were exposed to *G. perpensa* extract, *E. elephantina* extract, and control samples as untreated bacteria. Materials for SEM examination were fixed in 0.1 M (pH 7.0) sodium phosphate-buffered glutardialdehyde (3%) for at least three hours, which was followed by one hour fixation in similarly buffered osmium tetroxide (OsO_4) (1%). The materials were dehydrated in a graded ethanol (C_2H_5OH) series (50%, 70%, and 95% for 20 minutes in each phase, followed by two changes of 100% for one hour in each phase). The materials were dried using a critical point dryer (Tousimis, Maryland, USA). After drying, the material was mounted on stubs (Cambridge pin type 10 mm) with epoxy glue and gold coated (± 60 nm) with a Bio-Rad sputter coater (United Kingdom). The specimens were examined with a JSM-7800F Extreme-Resolution Analytical Field Emission SEM (Tokyo, Japan).

3.6.2 TEM

The TEM bioassay was performed according to Zajmi et al.'s (2015) method. During bioassay preparation, the selected bacteria cells were exposed to *G. perpensa* extract, *E. elephantina* extract, and control samples as untreated bacteria. Materials for TEM examination were fixed in 0.1 M (pH 7.0) sodium phosphate-buffered glutardialdehyde (3%) for three hours, followed by one hour fixation in similarly buffered osmium tetroxide (OsO_4) (1%). The material was dehydrated in a series of acetone (C_3H_6O) concentrations of 50%, 70%, and 95% (20 minutes for each step) and twice in 100% acetone (C_3H_6O) (one hour for each step). The dehydrated materials were embedded in epoxy resin and polymerised at 70 °C for eight hours in special moulds. A UM7 Leica Ultramicrotome (Vienna, Austria) was used to prepare 60 nm thick sections with a glass knife. A double staining technique was performed using uranyl acetate (Merck, Darmstadt, Germany), followed by lead citrate (Merck, Darmstadt, Germany). Each step was performed for five minutes, and the sections were rinsed after each staining. The sections were viewed using a Philips CM100 TEM (FEI, the Netherlands).

3.7 STATISTICAL ANALYSIS

Tests were carried out in triplicate and the data are reported as means and ± standard deviations. The data obtained from the study were analysed statistically by the two-way analysis of variance (ANOVA) using Microsoft Excel 2019. Data from the test groups from each experiment were compared with the controls and $p<0.05$ was accepted and considered as being statistically significant.

CHAPTER 4:
RESULTS

4.1 INTRODUCTION

Phytochemical screening analysis of *E. elephantina* and *G. perpensa* are reported in Table 4.1. A positive (+) sign is used to indicate the presence of the phytochemical with a colour change, and a negative with a (-) sign to indicate the absence of phytochemicals and no colour change. The total phenol content of *E. elephantina* and *G. perpensa* was estimated using the standard curve created with values of gallic acid (see Figure 4.1). The estimated total flavonoid content in this study was measured using the aluminium chloride colorimetric assay and quercetin to set up a calibration curve, as shown in Figure 4.2. The total phenolic content was estimated using the gallic standard curve and expressed as GAE in mg/g extract (Hussain et al., 2011; Singleton et al., 1999; Tambe & Bhambar, 2014). Table 4.2 displays the estimated total phenol content values of *E. elephantina* and *G. perpensa*, which were analysed at 1 mg/mL against gallic acid. The total flavonoid content was estimated using the quercetin standard curve and expressed as QE in mg/g extract (Hussain et al., 2011; Tambe & Bhambar, 2014). Table 4.3 displays the estimated total flavonoid content values of *E. elephantina* and *G. perpensa*, which were analysed at 1 mg/mL against quercetin.

The antibacterial activities of *E. elephantina* and *G. perpensa* were investigated using extracts (methanol and aqueous) that showed rich phytochemical compounds respectively as observed from the qualitative and quantitative screening test performed and reported in Section 4.2. To determine the antibacterial activity and MIC of each plant extract, selected gram-positive and gram-negative bacteria were treated with different concentrations of plant extracts. The zone of inhibition was measured and the colour change/intensity of the colometry assay was compared to that of the controls used.

4.2 PHYTOCHEMICAL SCREENING OF *E. ELEPHANTINA* AND *G. PERPENSA*

The phytochemical screening results showed that *G. perpensa* had no presence of anthraquinones in either the aqueous or methanol extracts, whereas *E. elephantina*

showed the presence of anthraquinones only in the methanol extracts and not the aqueous extract. *E. elephantina* showed no presence of saponins and triterpenoids but both the aqueous and methanol results of *G. perpensa* showed the presence of the above-mentioned phytochemicals. *G. perpensa* showed the presence of steroids.

Table 4.1: Qualitative phytochemical results

Phytochemical test	*E. elephantina* (MeOH)	*E. elephantina* (DH_2O)	*G. perpensa* (MeOH)	*G. perpensa* (DH_2O)
Alkaloids	+	+	+	-
Glycosides	+	+	+	-
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Phytosterols	+	+	+	+
Phenols	+	+	+	-
Anthraquinones	+	-	-	-
Saponins	-	-	+	+
Triterpenoids	-	-	+	+

Phytochemical screening: (+) means present and (-) means absent.

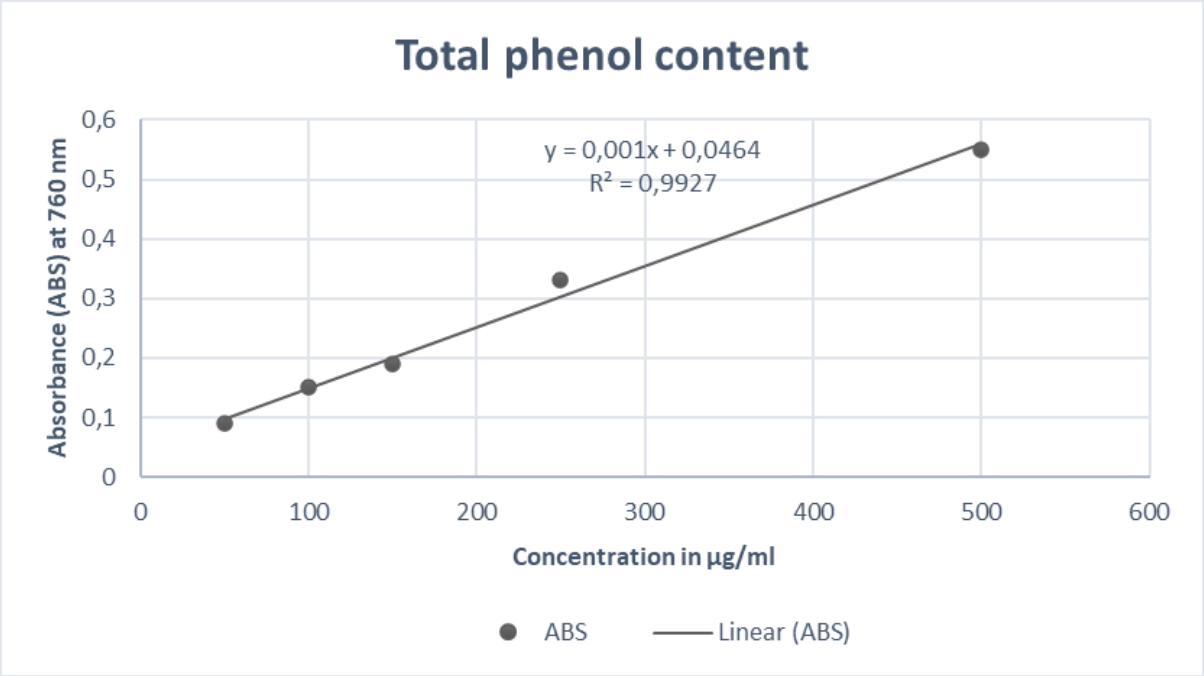
Table 4.2 displays the estimated total phenol content values of *E. elephantina* and *G. perpensa* methanol crude extracts, which were analysed at 1 mg/mL against gallic acid.

Table 4.2: Estimated total phenolic content of *E. elephantina* and *G. perpensa* methanol crude extracts, which were analysed at 1 mg/mL against gallic acid

Sample [1 mg/mL]	Absorbance means	Polyphenol concentration (mg/GAE)
E. elephantina	0.19	0.140 ± 0.0075
G. perpensa	0.12	0.068 ± 0.0025

E. elephantina extracts showed more polyphenols than *G. perpensa*. The GAEs of the estimated phenolic concentrations ranged from 0.140 ± 0.0075 to 0.068 ± 0.0025 mg/GAE.

The estimated total phenolic content in this study was measured using the Folin-Ciocalteau assay and gallic acid (as a phenolic acid) to set up a calibration curve, as shown by Figure 4.1. The total phenolic content was estimated using the gallic standard curve and expressed as GAE in mg/g extract (Hussain et al., 2011; Singleton et al., 1999; Tambe & Bhambar, 2014).



ABS = Absorbance

Figure 4.1: Gallic acid calibration curve for the estimation of *E. elephantina* and *G. perpensa* phenol content

The estimated total flavonoid content in this study was measured using the aluminium chloride colorimetric assay and quercetin to set up a calibration curve, as shown in Figure 4.2. Total flavonoid content was estimated using the quercetin standard curve and expressed as QE in mg/g extract (Hussain et al., 2011; Tambe & Bhambar, 2014). Table 4.3 displays the estimated total flavonoid content values of *E. elephantina* and *G. perpensa* methanol crude extract, which were analysed at 1 mg/mL against quercetin.

Table 4.3: Estimated total flavonoid content of *E. elephantina* and *G. perpensa* methanol crude extract, which were analysed at 1 mg/mL against quercetin

Sample [1 mg/mL]	Absorbance means	Flavonoids concentration (mg/QE)
E. elephantina	0.303	0.905 ± 0.0190
G. perpensa	0.181	0.375 ± 0.0073

E. elephantina extracts showed more polyphenols than *G. perpensa*. The GAEs of the estimated phenolic concentrations ranged from 0.140 ± 0.0075 to 0.068 ± 0.0025 mg/GAE.

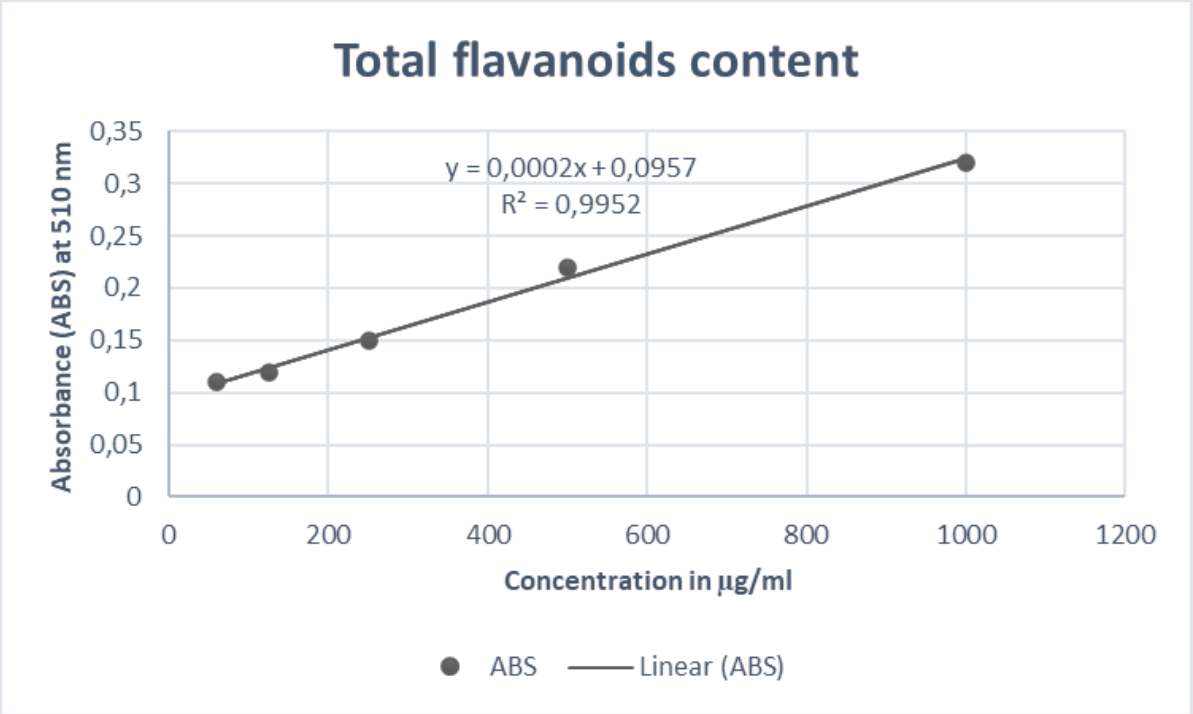


Figure 4.2: Quercetin equivalent (QE) calibration curve for the estimation of *E. elephantina* and *G. perpensa* flavonoid content

4.3 ANTIBACTERIAL ACTIVITY OF *E. ELEPHANTINA* AND *G. PERPENSA*

The MIC for *E. elephantina* and *G. perpensa* methanol extracts to inhibit *S. aureus* was 60 µg/mL, while the aqueous extracts of both plants showed the MIC at 125 µL/mL. *E. elephantina* methanol extracts revealed strong antibacterial activity against *B. subtilis* and *K. pneumoniae* (when compared with the positive controls), with the MIC of 125 µg/mL and 60 µg/mL respectively, as shown in Table 4.4. However, its aqueous extracts showed poor activity against *B. subtilis* and showed no activity against *E. coli.* The MIC of *E. elephantina* methanol extract was 250 µg/mL, and the aqueous extract showed no activity against *E. coli.* Furthermore, *G. perpensa* aqueous extract showed no activity against the selected gram-negative bacteria *E. coli* and *K. pneumoniae*, even at the highest concentration of 500 µg/mL. *G. perpensa* water and methanol extracts' MIC ranged between 60 and 125 µg/mL towards the active gram-positive bacteria *S. aureus* and *B. subtilis*, as shown in Table 4.4.

Table 4.4: Antibacterial analysis results of *E. elephantina and G. perpensa* from both the disc diffusion and microdilution assays

Bacteria	Concentration (µg/mL)	E1	E2	G1	G2	Control A	Control B	Control C
S. aureus	500	+	++	++	++	++	++	-
	250	+	+	++	++			
	125	++	+	+	+			
	60	++	+	+	-			
B. subtilis	500	-	+	++	++	++	++	-
	250	++	-	++	++			
	125	+	-	+	++			
	60	-	-	-	++			
K. pneumoniae	500	++	++	-	-	-	++	-
	250	++	++	-	-			
	125	++	+	-	-			
	60	++	-	-	-			
E. coli	500	+	-	++	-	+	++	-
	250	+	-	++	-			
	125	-	-	++	-			
	60	-	-	+	-			

E1**:** *E. elephantina* methanol extract***; E2*****:** *E. elephantina* aqueous extract***; G1***: *G. perpensa* methanol extract***; G2:*** *G. perpensa* aqueous extract**; [++]**: sensitive with zone of inhibition ≥ 16 mm and bright yellow colour**; [+]**: sensitive with zone of inhibition ≤16 mm and yellow colour; **[-]**: resistant with no zone of inhibition. **Control A**: Ampicillin; **Control B**: Gentamicin; **Control C**: Saline. MIC of each active extract is presented with cross signs (**+**) and yellow colouring.

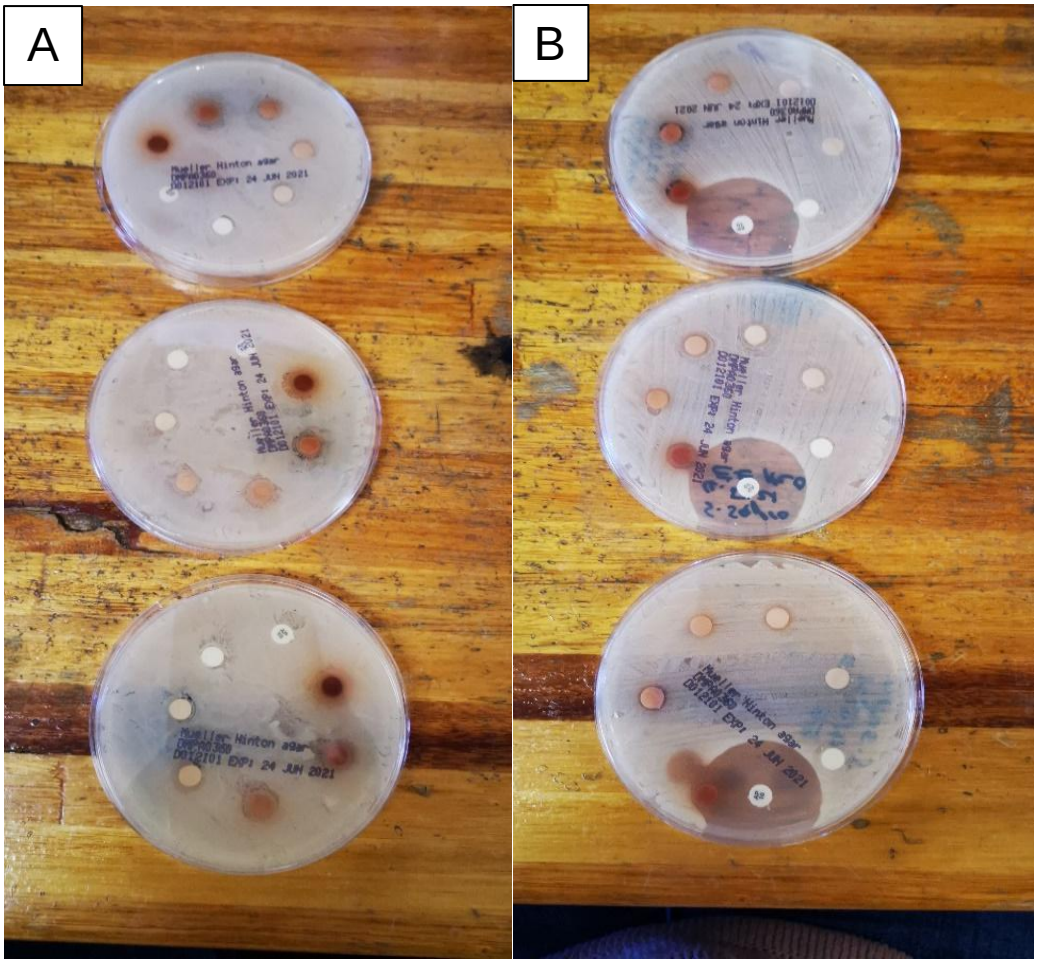


Figure 4.3: (A) Illustration of resistance showing no zone of inhibition, and (B) illustration of sensitivity showing zone of inhibition

4.4 THE EFFECT OF *E. ELEPHANTINA* ON THE CELL MORPHOLOGY OF *S. AUREUS*

The effect of *E. elephantina* extract on the bacterial cell morphology was examined using SEM and TEM. SEM was used to examine the morphology of the cells of *S. aureus* treated with methanol extracts of *E. elephantina*, for 24 hours at 37 °C. The untreated samples of *S. aureus* were considered as a control. Under normal conditions, the cells of *S. aureus* under a microscope appear as clusters of cocci cells (see Figure 4.4A). The cells of *S. aureus* that were treated with the methanol extracts of *E. elephantina* at the MIC concentration of 0.6 mg/mL and higher concentration of 2.5 mg/mL are shown in Figure 4.4 and 4.5.

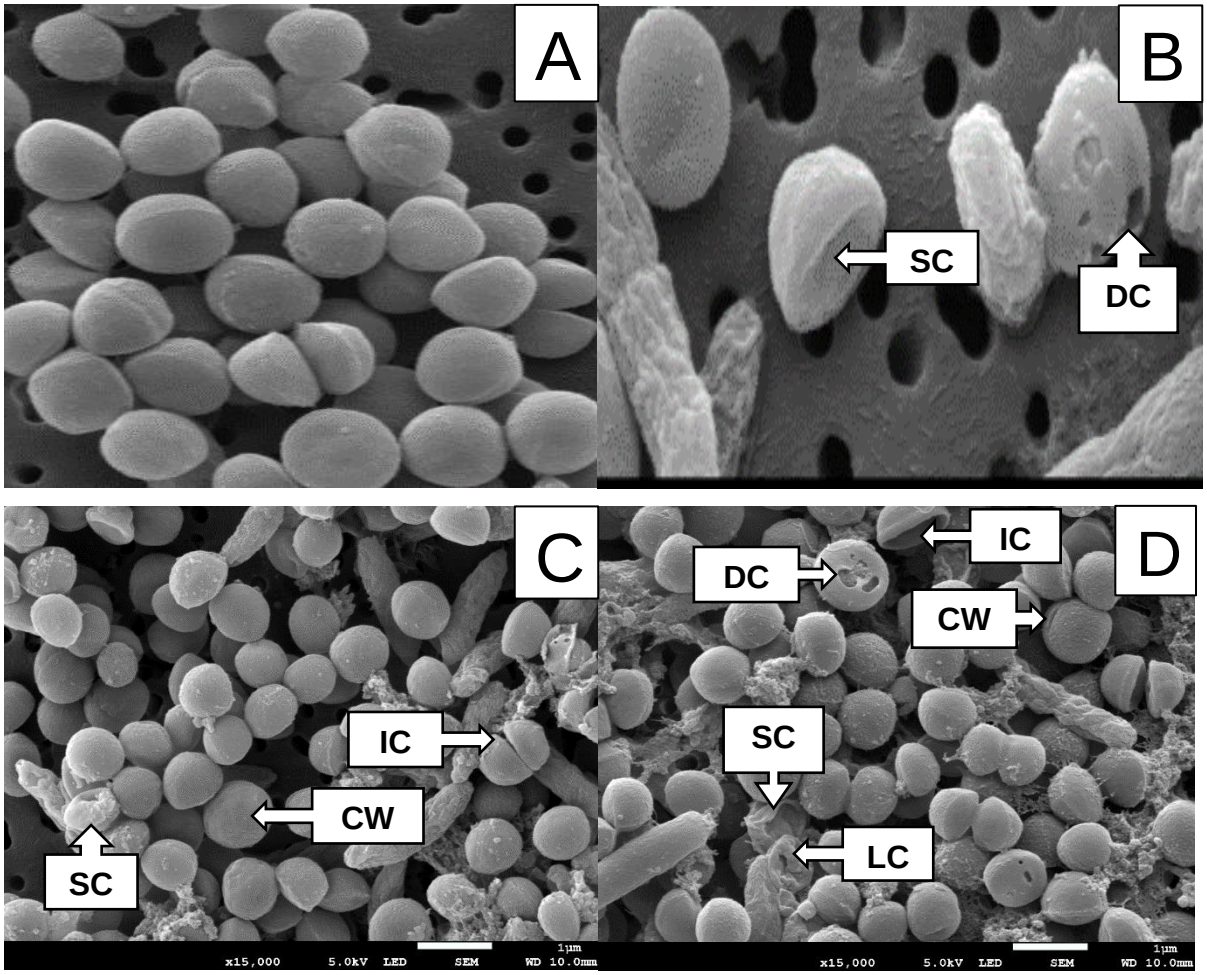


Figure 4.4: SEM images of *S. aureus* treated with *E. elephantina* methanol extract: (A) control cells (untreated); (B) 0.6 mg/mL MIC of *E. elephantina* extract; (C) 0.2 mg/mL lowest concentration of *E. elephantina* extract; (D) 2.5 mg/mL highest concentration of *E. elephantina* extract. (B, C, D) different effects of *E. elephantine* on bacterial cell wall and structure. (B) Damaged cell (DC) with formation of holes on the cell and shrinkage of the cell (SC); (C&D) shrinkage of the cell (SC), incomplete cell (IC) division seen by loss of cellular (LC) contents and swollen cell wall (CW).

Different effects were seen on the bacterial cell walls and structures as shown in Figure 4.4 after subjecting the cells to the methanol extracts of *E. elephantina*. These effects are damaged cell (DC) wall, which is evident through the formation of holes on the surface; loss of cellular (LC) contents, as seen by shrinkage of the cell (SC); and incomplete cell (IC) division, which may result by swelling of cells (CWS). These results shows that *E. elephantina* inhibited the growth of *S. aureus* by changing the morphology of the bacteria.

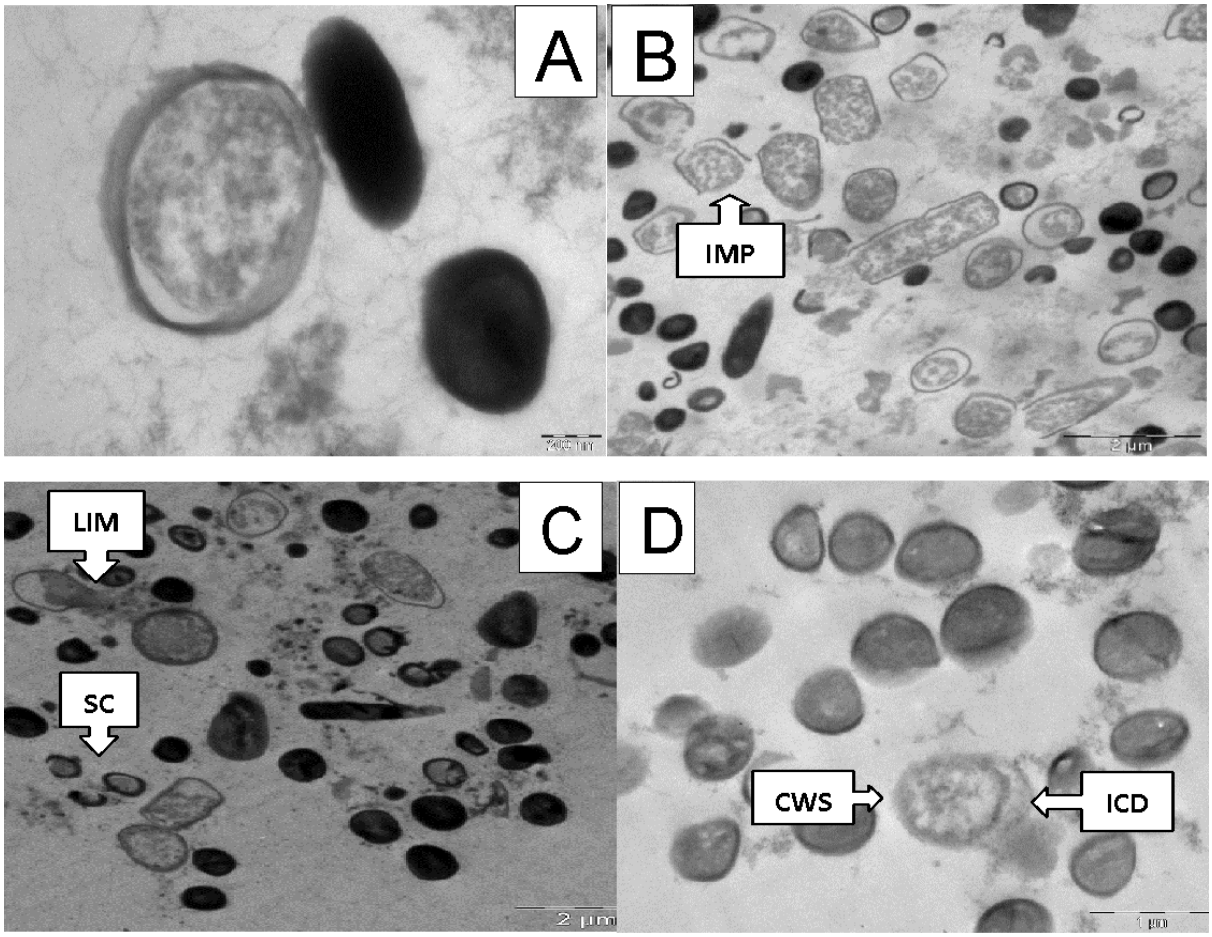


Figure 4.5: TEM images of *S. aureus* treated with *E. elephantina* methanol extract. (A) control cells (untreated) with intact, dense cell membrane; (B) 0.6 mg/mL MIC of *E. elephantina* extract; (C) 0.2 mg/mL lowest concentration of *E. elephantina* extract; (D) 2.5 mg/mL highest concentration of *E. elephantina* extract. (B, C, D) different effects of *E. elephantine* on bacterial cell wall and structure. (B) Increased membrane permeability (IMP) seen by lack of intact cell membrane, (C) loss of intracellular material (LIM) causing shrinkage of cells (SC) and (D) incomplete cell division (ICD) seen by cell wall swollen (CWS).

Different effects were seen on the bacterial cell wall and structure, as shown in Figure 4.5, after subjecting the cells to the methanol extracts of *E. elephantina*. These effects are increased membrane permeability (IMP), which causes loss of intracellular material (LIM); ICD, which may result in CWS; and SC. These results show that *E. elephantina* inhibited the growth of *S. aureus* by changing the morphology of the bacteria.

CHAPTER 5:
DISCUSSION

5.1 DISCUSSION

Phytochemicals are bioactive chemical compounds that are naturally found in plants (Dillard & German, 2000; Holopainen et al., 2018). They are also widely found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs, and spices and in plant-based beverages such as wine and tea (Barbieri et al., 2017). Investigations have found that bioactive compounds act as antimicrobial agents against human pathogens (Barbieri et al., 2017). The bioactive compounds have been investigated to understand the medicinal benefits of plants' healing properties against viruses, bacteria, and fungi. This observation was also made when traditional healers prescribe certain plants or synergics of different plants to bring relief to patients who sought help for certain medical conditions, which prompted interest in the medicinal properties of plants'. This resulted in the discovery of phytochemical compounds in different plants and their parts. Phytochemicals can be classified into numerous major groups based on their chemical structure, such as alkaloids, sulphur-containing phytochemicals, nitrogen-containing phytochemicals, terpenoids, and polyphenols. These compounds have been identified, classified, and subjected to different studies of antimicrobial, anti-inflammatory, anticancer, and many more. Research may support their medicinal properties as phytochemicals, which could result in new drug discoveries.

The findings of the qualitative phytochemical analysis in this chapter correlate with the findings of Mthembu (2007), Simelane et al. (2012), Chigor (2014), and Mammo et al. (2017). Some of the studies mention that *G. perpensa* collected from South Africa has steroids. However, Manduna et al. (2014) and Mfengwana (2019) reported no steroid presence in *G. perpensa* collected from Lesotho. The results of this study confirmed that the plant in South Africa has steroids, which supports the findings of the previous research mentioned above. The presence of steroids is an indication that the plant has antioxidant, anti-inflammatory, anticancer, and antibacterial activity (Raju et al., 2004; Rajasree et al., 2016). Steroids have a special type of component called sterol (phytosterols), which have a significant hypocholesterolemic effect (Kabir et al., 2021).

The presence of anthraquinones was identified, classified, and investigated in studies by Drewes et al. (2005) and Abraham et al. (2011) on their pharmacological use, such as antimalarial, antibiotic, antitumour, and herbicidal activities, in screening the antibacterial potential of isolated compounds from the aerial parts of *G. perpensa*. Anthraquinones were not detected in the investigated *G. perpensa* plant as investigations focused on the rhizome/root parts of the plant, which could indicate that the compound may only be found in the aerial parts of the plant. Anthraquinones were only detected in the methanol extract of *E. elephantina* from the rhizomes/roots. According to Mammo (2018), in most cases, the biosynthesis of secondary metabolites takes place in a specific part of the plant, but only some of them accumulate at the site of synthesis. For example, linamarin (a toxin) is synthesised in the leaves of the cassava plant and stored in the roots (Paiva, 2000). This could explain why it may be found in the aerial part of *G. perpensa* but in *E. elephantina* it is found in the roots/rhizome due to biosynthesis.

The compound may also be hydrophilic, since it was only detected in the methanol extracts of *E. elephantina* in this study and previous studies (Drewes et al., 2005; Abraham et al., 2011). This would explain why the extraction solvent is a critical factor in the isolation of phytochemicals as 'like dissolves like'. Furthermore, the following bioactive compounds were present in both the methanol extracted crude extract of *G. perpensa* and *E. elephantina*: alkaloids, glycosides, flavonoids, tannins, phytosterols, and phenols. *G. perpensa* did not have anthraquinones, while *E. elephantina* showed the presence of anthraquinones. *E. elephantina* did not show the presence of saponins and triterpenoids in this study, which is consistent with the findings of Kudumela and Masoko (2018), who reported that terpenoids and saponins were not detected in *E. elephantina* extracts using TLC in the following solvents: n-hexane (C_6H_{14}), dichloromethane (CH_2Cl_2), acetone and methanol for extraction. An aqueous extract of *G. perpensa* showed the presence of flavonoid, tannins, phytosterols, saponins, and triterpenoids. The active ingredients of both plants prove to be better extracted from the non-polar condition.

Medicinal plants are popular for their multiple uses, affordability, and rich content they possess. They are the primary recommended source of medication in rural areas. This is due to the fact affordable healthcare is not accessible for most of the rural population and is unaffordable. The focus on the secondary metabolites of medicinal plants' is

due to their effects on sick people who find relief and even a cure for most of their medical conditions. Investigating the benefits of phytochemical compounds strengthens previous research that noted that quercetin is the main flavanol compound that possesses anticancer activity (Craig, 1997); that alkaloids have many effects including antimalarial, antiasthma, vasodilator, antihypertensive and antitumor properties; that sulphur-containing phytochemicals have strong antimicrobial properties; and that polyphenols have potential effectiveness in the chemoprevention of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases (Ferrazzano et al., 2011; Heim et al., 2002; Ramassamy, 2006; Barbieri et al., 2017). *E. elephantina* compared to *G. perpensa* is a plant species with a large quantity of quantitative phytochemicals and, therefore, showcasing greater potential as an antibacterial agent against MDR bacteria.

E. elephantina and *G. perpensa* have traditionally been used as medicinal plants to treat bacterial infections and conditions associated with MDR bacteria (Nkomo & Kambizi, 2009; Mathibe et al., 2016; Mfengwana et al., 2019). Pathogenic bacteria that acquire resistance to antibiotics have motivated the need to investigate traditional herbs/plants as an alternative possible solution (Khameneh et al., 2016). Furthermore, investigating medicinal plants to treat infections caused by antibiotic-resistant pathogens could help to combat the arising MDR bacteria hurdle that poses a substantial burden on the healthcare sector. Plant source treatment of aliments caused by pathogenic bacteria has been common throughout human history. The phytochemical compounds of plants are known to have diverse and different healing properties, as the pharmacological uses of certain plants have been explored. Traditional healers often prescribe different medicinal plants and different parts of the plant as a treatment for medical problems. These parts and plants are used in combinations as traditional concoctions in some instances. This is synergised when a combination of two or more plants is administered to produce relief and healing for the individual. There are commercialised products of traditional medications with synergy on the markets, namely *Imbiza* herbal mixture, *isihlambezo*, and *inembe* (Ramulondi et al., 2022). Therefore, conducting research on medicinal plants as alternative ways to treat MDR bacteria is a step in a positive direction.

From the antibacterial analysis, only the methanol extracts of *E. elephantina* inhibited the bacterial growth of all the Gram-positive and Gram-negative bacteria investigated in this study (see Table 4.4). This supports the phytochemical findings in Table 4.2, where *E. elephantina* methanol extracts were the only ones to possess anthraquinones and yielded higher quantities of total phenol and flavonoid content than *G. perpensa*. Furthermore, this finding supports the antibacterial properties of the metabolites, as previous studies have reported the antibacterial, antimalarial, antitumor, and herbicidal activities of anthraquinones, phenols, and flavonoids (Drewes et al., 2005; Abraham et al., 2011).

G. perpensa extracts did not show activity against the Gram-negative bacteria *E. coli* and *K. pneumoniae* investigated in this study. It has been reported that *E. coli* and *K. pneumoniae* are enteric pathogens that could develop resistance as their coliform antigens may interfere with bacterial killing (Foster, 2002). Furthermore, this could also be because, in this study, anthraquinones were not detected from the roots/rhizome of *G. perpensa* and the plant extract yielded low quantities of phenols and flavonoids. However, previous reports showed the presence of anthraquinones in leaf extrats of *G. perpensa* (Drewes et al., 2005). This justifies the different distribution of compounds in the one plant and the location of plant sampling, as different environments may play a role in the presence of compounds in a plant. Several factors might also contribute to these results, such as the preparation of extracts by using different solvents that are compatible with the polarity of the phytochemical compounds for good isolation yields, different extraction methods with the choice of solvent, the duration of extraction and filtration, and the concentration of the quantity of solution to acquire the crude extracts (Nasir et al., 2015).

The ability of bacteria to acquire resistance through additional mechanisms used by bacteria such as the entry of the extract or drug into the cell through the permeability of the cell wall, could have contributed to the result. This is due to the structure of the bacterial cell wall. In Gram-negative bacteria, there is a higher lipid content, and many antimicrobial agents may be either hydrophobic or hydrophilic, making it difficult to penetrate the outer membrane as compared to Gram-positive bacteria, which possess a much thicker peptidoglycan layer. This layer does not act as an effective barrier to permeation, and therefore it can allow antimicrobial agents to pass through more easily (Ehrlich, 1923, cited by Hucker & Conn, 1923; Hobson et al., 2021). The

methanol extract of the plants is hydrophilic; they could therefore be compatible with the structural cell wall of the bacteria. Therefore, increasing the plant extract gives a greater opportunity to penetrate the bacteria. With the strong activity reported from *E. elephantina*, it may confirm that the extract to the cell wall was 'like dissolves like', which resulted in the advantage of penetrate and inhibiting bacteria to cause their death.

The antibacterial activity of *G. perpensa* seen against the Gram-positive bacteria *S. aureus* and *B. subtilis* in this study could have resulted from the rich content of phytochemical compounds that the plant possesses, as reported in Table 4.1. Among these metabolites, the detection of steroids from plant root extracts were crucial as it supported its antibacterial activity. Mthembu (2007), Simelane et al. (2012), Chigor (2014), and Mammo et al. (2017) have also detected steroids from *G. perpensa* collected from South Africa. However, Mfengwana (2019) reported that there was no steroid present in *G. perpensa* L. collected from Lesotho. This is a clear chemical difference between the same plants collected from different locations, which may influence the antibacterial properties of the plant. According to Khan et al. (2008), steriods synthesised derivatives of thiourea and urea are better antibacterial agents than standard drug chloramphenicols where compounds 3 and 5 have 3b-acetoxy that showed strong activity against *S. aureus*. This activity was also shown in this study, which showed the potential of the *G. perpensa* plant to have steroids.

E. elephantina showed stronger antibacterial activity against the bacteria than *G. perpensa*. The plant extract also showed effects on the morphology of the bacteria by causing substantial damage to the morphology of the cell wall. SEM evaluation of *S. aureus* showed significant structural changes compared to untreated cells that led to cell death (see Figure 4.4B). Further evaluation of the lowest concentration (see Figure 4.4C) and the highest concentration (see Figure 4.4D) subjected to the cells also showed structural changes compared to untreated samples (see Figure 4.4A). Figure 4.4B showed some DC, which was evident from holes on the surface of the cell. The loss of cellular or cytoplasmic contents resulted in SC, which was seen by the wrinkled and indent of the cell surface (see Figure 4.4B). Figure 4.4C showed the lowest concentration of 0.2 mg/mL and Figure 4.4D showed the highest concentration of 2.5 mg/mL. The extract also caused IC division, evident by the open shell of the cell

structure and swollenness of the cells caused by the penetration of plant extracts into the cell.

Cell swelling led to damage to the cytoplasmic membrane, seen by the slit on the cell surface, which eventually resulted in LC content. Loss of cellular or cytoplasmic contents also resulted in SC, which was seen in the wrinkled surface of the cell seen in Figures 4.4B, 4.4C, and 4.4D. The effects of *E. elephantina* on the morphology of bacterial cells also demonstrated some damage to the structure of cells because the treated cells examined using TEM showed cell wall distortion, which resulted in IMP (see Figures 4.5B and 4.5C) and therefore caused SC. When cell membrane permeability increased, intracellular or cytoplasmic contents were lost. ICD (see Figure 4.5D) and separation of the cytoplasmic membrane from the cell wall were some of the morphological changes caused by *E. elephantina* extracts in bacterial cells.

Telavancin is an antibiotic that is a multifunctional lipoglycopeptide that disrupts both cell wall synthesis and cell membrane integrity in *S. aureus* (Higgins et al., 2005). Telavancin inhibits late-stage peptidoglycan biosynthesis in a substrate-dependent manner and binds to the cell wall and agitated the potential and permeability of the bacterial cell membrane. It is one of the drugs used to treat infections caused by *S. aureus* (Higgins et al., 2005). Beta-lactamase antimicrobial drugs also target and inhibit bacterial cell wall biosynthesis. Methicillin resistance is usually caused by the acquisition of a non-native gene encoding a penicillin binding protein (PBP2a), with significantly lower affinity for beta-lactamase. Resistance to methicillin is not mediated by a plasmid-borne beta-lactamase. It was referred to as intrinsic resistance in some of the early literature, whereby this resistance allows cell wall biosynthesis, the target of beta-lactamase, to continue even in the presence of typically inhibitory concentrations of antibiotics (Peacock & Paterson, 2015). The phytochemical composition of *E. elephantina* is rich in active compounds. These phytochemicals have complex genetic structures that were recently introduced to bacteria. Alkaloids are also known to cause cell membrane disruption and consequently leakage of cytoplasmic contents. Through different phytochemical plant bases and chemical biochemistry, plants pose the potential of being non-resistible given the vast fundamental and structural genetic changes that will have to drive such resistance (Stepek et al., 2005).

This could explain the disruption of the cell membrane and the loss of cytoplasmic contents (see Figures 4.5B and 4.5C) observed for bacterial cells treated with *E. elephantina*. The effects of the plant extract on the cell membrane are indicative that the biosynthesis of the cell membrane was substantially disrupted. Furthermore, the genetic alterations that the bacteria cell develops to acquire resistance against plant extract will take time; therefore, giving longevity to the newly developed antibacterial agents from plant-based sources. Medicinal plants are an important alternative to the research and development of alternative medicine worldwide.

The results observed in Chapter 4 in terms of the potential of *E. elephantina* possessing antibacterial properties and showing strong activity against *S. aureus* are supported by the results obtained by SEM and TEM. The morphological changes caused by *E. elephantina* therefore, identified the plant extract as the best treatment and may be a strong option for the development of a new drug that can control resistant pathogens that cause infectious diseases. The external and internal cellular damage of *S. aureus* indicates that *E. elephantina* managed to inhibit the growth of bacteria that are prone to resisting drugs, which could eventually be classified as MDR, and will result in the death of bacteria. The CSIR has led research using the study of Mthembu (2007), which has made known that the extract of *E. elephantina* has potent activity in inhibiting the 5-alpha reductase enzyme, which is responsible for converting testosterone to dihydrotestosterone (DHT) (McConnell et al., 1992; Ellis & Sinclair, 2008; Maharaj et al., 2019). *E. elephantina* showed the potential of enzyme-inhibiting properties that could assist in combatting the antibiotic resistance of bacteria via enzymatic metabolism/mechanism. The plant has shown strong qualities of destruction of cell wall synthesis and previously known enzymatic metabolism properties that MDR bacteria will not be able to acquire resistance towards in the next few years, which has great potential for further antibacterial drug discovery not limited to cancer agents. Thus, future inventions regarding *E. elephantina* should be directed towards commercialising it as an antibacterial agent as adequate and extensive research has been conducted on the potential of the plant to possess antibacterial properties, especially toward MDR bacteria. The aim of this study was achieved, as it showed evidence that the mechanism of action of *E. elephantina* does possess strong antibacterial properties towards MDR bacteria.

CHAPTER 6:
CONCLUSION, RECOMMENDATIONS, AND FUTURE RESEARCH

6.1 CONCLUSION

The beneficial medicinal effects of plant phytochemicals typically result from the synergy of secondary products present in the plant. This has resulted in researchers conducting extensive research in order to better understand the benefits of traditional medication and why the majority of the population still uses it as their primary healthcare choice. However, some of the research has been questioned in terms of whether appropriate studies or guidelines that will lead to the authentication of traditional medicines from plant extracts for their formal incorporation into the healthcare system will follow through. There is still much to investigate and understand regarding *E. elephantina* in terms of the mode of action of how the plant extract inhibits the various bacteria subjected to it, as its antidiabetic and anti-inflammatory properties have not been thoroughly investigated, but more literature has been produced on the phytochemistry, toxicity and anticancer properties of plant species. Furthermore, different parts of the plant, such as the leaves, have been explored as an alternative to the root to preserve the plant, as it has been listed in the IUCN Red List database as a “declining” species (Williams et al., 2013). The knowledge gap on medicinal plants has narrowed in the past decades. Further investigating and understanding the mode of action of *E. elephantina* and its mechanism of action in terms of the plant’s antimicrobial, antidiabetic, anticancer, anti-inflammatory, and wound healing potential could lead to a better understanding of the medicinal properties of the plant extracts and the commercialisation of products in the future. The aim of this study was to investigate the antibacterial analysis of *E. elephantina* and *G. perpensa*, with the focus on investigating the mechanism of action of the plant extract that exhibits strong activity against pathogenic bacteria.

The investigated plants in this study were screened for phytochemical constituents and were found to possess various compounds. This supports the literature on the medical benefits of plants, fruits, and vegetables that have an abundance of phenolic compounds such as flavonoid compounds, phytosterols, terpenoids, tannins, pigments, and other natural antioxidants that are important for the good health of individuals. They have the potential to be considered as a source of useful drugs that

can expand the antibiotics spectrum. Traditional medicinal plants have been associated in the literature and research with medical diseases/conditions that are often caused by pathogenic variants such as bacteria, viruses, and carcinogens that cause adverse medical conditions. This is due to the presence of various phytochemical constituents, such as alkaloids, flavonoids, phenol, terpenoids, saponin, steroids, and tannins. It can be concluded that *E. elephantina* and *G. perpensa* possess abundant phytochemicals that are produced as secondary metabolites. These compounds could be useful to combat antibiotic resistant bacteria as they can be used for drug discovery and the development of new antimicrobial drugs in the pharmaceutical industry, as they are known to have pharmacological benefits. The investigated plants are used as medicine for several ailments in traditional medicine practises. Future studies may be conducted to isolate and identify the active compounds of both plant extracts.

The extracts of *E. elephantina* revealed strong antibacterial activity against both Gram-positive and Gram-negative bacteria when compared to *G. perpensa*. Furthermore, the methanol extracts *E. elephantina* were the only extract that possessed anthraquinones, which could explain the strong antibacterial activity of this plant. The presence of other phytochemicals may add to the medicinal benefits of *E. elephantine,* as they are known for their pharmacological properties, as discussed in Chapter 2. *E. elephantina* is a promising novel antibacterial agent for combatting MDR bacteria. Additionally, both plant extracts proved to have good potential as alternative medicines to combat MDR bacteria. These findings support the plant's historical use in African communities to help relieve illnesses caused by pathogenic bacteria. Further analysis of the morphological changes through electron microscopy has shed light on the mode of action of *E. elephantina*. The effects of *E. elephantina* on the bacterial cell wall supported the plant's antibacterial properties as the results of the SEM and TEM showed how the plant extract causes extensive morphological damage and eventually inhibits *S. aureus* growth. The results obtained in this study indicate that the plant has potential as an antibacterial agent against MDR bacteria.

6.2 RECOMMENDATIONS

A recommendation based on this study is the isolation of the compounds from these plants to determine the specific compounds responsible for antibacterial activity. The isolation could be performed using high-performance liquid chromatography and gas chromatography-mass spectrometry.

Fingerprinting and comparative studies on the antibacterial activities of *G. perpensa* from South Africa and Lesotho may be conducted. This will specifically investigate the antibacterial properties of steroids from this plant and provide more information on its antibacterial activity.

E. elephantina has shown good antibacterial properties towards MDR bacteria. With the results of this study on the plant's roots and the combination of other literature on the roots of the plant, in vivo research needs to be conducted to prove the efficacy of the plant's properties, which may lead to a synthetic form of the antibacterial agent.

6.3 FUTURE RESEARCH OPPORTUNITIES

Future opportunities from this study are to evaluate the effects of *G. perpensa* on *S. aureus* through SEM and TEM. *E. elephantina* showed evidence as a potential antibacterial agent. It inhibited the growth of the bacteria and achieved cell death by causing morphological changes to the cells of *S. aureus. G. perpensa* showed similar potential towards *S. aureus* and cell imaging could support the antibacterial properties it displayed.

Further studies on the isolation of the phytochemical compounds may be conducted to identify the specific compound the plants possess to support their antibacterial properties.

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APPENDIX

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Review Article

Review on Literature of the Plant *Elephantorrhiza Elephantine* on its Healing Properties and Recent Acquired Knowledge of its Medicinal Activities (2000-2020)

Radebe Tlotlo[1,*], Polo-Ma-Abiele Hildah Mfengwana[2], Dedré Olivier[3]

Radebe Tlotlo[1,*], Polo-Ma-Abiele Hildah Mfengwana[2], Dedré Olivier[3]

[1]Department of Health Sciences, Faculty of Health and Environmental Sciences, Central University of Technology, P/Bag X20539, Bloemfontein, 9300, SOUTH AFRICA.
[2]Department of Health Sciences, Faculty of Health and Environmental Sciences, Central University of Technology, P/Bag X20539, Bloemfontein, 9300, SOUTH AFRICA.
[3]Department of Health Sciences, Faculty of Health and Environmental Sciences, Central University of Technology, P/Bag X20539, Bloemfontein, 9300, SOUTH AFRICA.

Correspondence
Radebe Tlotlo
Department of Health Sciences, Faculty of Health and Environmental Sciences, Central University of Technology, P/Bag X20539, Bloemfontein, 9300, SOUTH AFRICA.
E-mail: tlotloradebe027@gmail.com

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ABSTRACT

Background: The current review article outlines current findings in literature from undertaken studies and review articles on the plant *Elephantorrhiza elephantine* from the past 20 years to date. The information presented in this article will include the following aspects of the plant. The plant description and taxonomy, ethnomedical uses, phytochemistry and pharmacology of *Elephantorrhiza elephantine*. The article looks to discuss existing hurdles in research found on this plant and how to integrate any previous research with what is currently done in research on the plant. It aims on guiding the path of future research direction on the plant *Elephantorrhiza elephantine*. **Methods:** A total of 40 articles were selected and read through. The articles selected had included literature publications with the keywords such as; *Elephantorrhiza* elephantine, plant description, taxonomic of the plant, ethno medicinal usages, phytochemicals, pharmacological properties and plant toxicity, which were published between the years 2000 – 2021. There were 32 articles relevant for this review article and 2 dissertation that were written between the criteria of years. **Results:** The ethomedicinal uses of *Elephantorrhiza elephantine* have been investigated by various authors on the anti-microbial activities, anti-inflammatory, anticancer and in ethnoveterinary medicine on how the plant provides relief to individuals with illnesses/disease through its traditional uses. These studies have been conducted on both animals and humans. The experiments performed used the crude extract derived from parts of the plant such as the roots and leaves. These plant parts have phytochemical compounds that are extracted using varying polar solvents. In the animal studies, acetone extracts of *E. elephantine* roots demonstrated significant activity against a tick-borne disease in cattle livestock and parasite worms in goats. In humans, the dried leaf extracts of *E. elephantine* was also subjected to various polar solvents and water, investigated for phytochemical content, antioxidant, anti-inflammatory, anti-diabetic and cytotoxic activities, using standard methods. It was reported that the ethanol extract yielded the highest phytochemical content. **Conclusion:** The gap in knowledge has narrowed on medicinal plants effects of healing properties. This lead by extensive research carried out trying to understand the benefits of traditional medication and why majority of the population uses it as they primary healthcare choice. Investigating the mechanism of action of *E. elephantine* on its antimicrobial, anti-diabetic, antimalarial, anti-inflammatory and wound healing could lead to an enhanced understanding on the medicinal properties of the plant extracts and a commercialisation of a product in future.

Key words: Medicinal plants, Phytochemical content, Antimicrobial, Plant extracts.

INTRODUCTION

The plant *Elephantorrhiza elephantine*

Elephantorrhiza elephantine (Burch) Skeels is a plant used extensively in the southern African countries as a source of food and to treat diseases and conditions caused by pathogenic microorganisms.[1-4] *Elephantorrhiza elephantine* is a member of the *Fabaceae* family and of the genus *Elephantorrhiza*. This species, *elephantine*, is spread widely among the southern African countries of Botswana; Namibia, Lesotho and provinces like Limpopo, North West, Mpumalanga, Free State, Eastern Cape, Northern Cape and KwaZulu-Natal in South Africa.[5,6] *Elephantorrhiza elephantine* has many known names amongst different indigenous countries and documented literature. It is known as *elandsbean* (in English); *mupangara* (in Shona), *intolwane* (in Xhosa and Zulu); *mositsane* (in Sotho and Tswana) and *elandsboontjies* (in Afrikaans).[7-11] The plant image is represented in (Figure1a). The plant grows naturally in open grassy slopes and hillsides, and produces red roots that look like sweet potatoes.[9] The reddish root seen in (Figure 1b) is the most commonly used part of the plant in traditional medicine[3] and is used as remedy for the following conditions/diseases: diarrhoea and dysentery; diabetes,[3] chest complaints, heart conditions, hypertension, syphilis, infertility in women, bladder problems, urinary tract infections, waist pain in infants, fever and haemorrhoids.[4,12]

The plant description of *Elephantorrhiza elephantine* and taxonomy

Elephantorrhiza elephantine is a perennial low shrub with stems up to 90 cm tall at ground level and from the woody end of an elongated, often thickened rhizome up to 8 m long. The leaves consist of 2-4 pairs of pinnae in lower leaves and 7-17 pairs in upper ones, where the axis is up to 10 cm long.[3,12] The scientific classification of *Elephantorrhiza elephantine* derives from the Kingdom *Plante*; the Phylum *Tracheophyta*, the Class *Magnoliopsida*, the Order *Fabales*, the Family *Fabaceae*, the Genus *Elephantorrhiza* and Species *elephantine*.[13,14]

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Figure 1a: The plant's image. (Modimolle, Nov 2014).

Figure 1b: The reddish roots (prota4u.org).

Ethnomedical uses

Elephantorrhiza elephantine's healing properties has been explored respectively in both humans and animals. In animals, according to *McGaw et al.*,[15] studies done in the past decade between 2009 – 2019 has reported that traditional plants are commonly used in ethnoveterinary medicine amongst South African rural livestock which is a common feature still seen. Ethnoveterinary medicine may play an important role in relieving conditions such as wounds, skin diseases, mild diarrhoea and intestinal worms and tick-borne disease in livestock and not limited to these mentioned. *Elephantorrhiza elephantine* was one of the many plants reported in the survey, used to conduct the study amongst the many other plants used for a variety of commonly encountered animal diseases and afflictions. This study supports that greater interest has been shown recently in documenting ethnoveterinary medicine (EVM), as with ethnobotanical medicine used in treating humans and animals.[15] In the past decade, efforts have been made to publish the varying uses of medicinal plants in South African EVM in greater detail, as were their biological activity investigations relating directly to their ethnoveterinary use by *McGaw and Eloff*.[5] In the same article a downfall in the survey was identified, where the author of the article *Martin et al.*,[16] mentioned that the shortcomings of employing such a system included inefficacy or toxicity of remedies, there was uncertainty over dosing regimens and lack of standardisation, as well as unavailability of plant material during certain seasons of the year.[16] The study conducted surveys in some of the provinces in South Africa, in places like Limpopo and the North-west province. This identified that not all provinces were analysed, provinces that were not mentioned were the Eastern Cape, the Free State and KwaZulu-Natal province. Further investigation in the mentioned provinces could be included in future studies as they have a high rate of breeding rural livestock and it could yield significant results towards the medicinal research in EVM and the plant is known to grow within these provinces (*pza.sanbi.org/elephantorrhiza-elephantina*, 2019). In the animal studies, acetone extracts of *E. elephantine* roots demonstrated significant activity against a tick-borne disease in cattle livestock[17] and parasite worms in goats.[18]

In humans, *E. elephatine* has been reported to be used against conditions such as diarrhoea, blood purifying, ulcers, skin wounds, cleansing of the womb, infertility of the women, urinary tract infections in both men and women and the relief of fevers caused by illnesses associated with pathogenic bacteria in humans.[1,2,12] The plant's extract using different polar solvents such as ethanol, methanol and aqueous solution have demonstrated relief to diarrhoea, wound disease, fever and fertility problems in women.[4,8,15,16,19,20] The plant's roots are widely reported to be used in mixtures or solely in treatment of the conditions. The other parts such as the leaves are seldom used when treating conditions.[4,16] The traditional uses of *E. elephantine* extract in the treatment of diarrhoea is much researched and widely reported.[1,3,8,12,21] Researchers have subjected microorganisms associated with gut flora such as *Escherichia coli* and *Streptococcus* species to mainly the plant's roots and recently the leaves extract for further investigation aimed to understand and isolate phytochemical compounds like phenol; flavonoids and tannins reported to be responsible for antidiarrheal and antibacterial activities.[2,12,22] Further investigation of how the mechanism of action of the plant extract works could be assessed and also establish their safety and efficacy. The gut flora has the potential to become pathogenic and causes diarrhoea[7,12,21] and that is the reason why the above mentioned pathogens are commonly used in antidiarrheal and antibacterial studies of other authors as they are known to be opportunistic pathogen and part of the gut flora.

However, since roots are reported to be used more, the overexploitation for the root of the plant *E. elephantine* has put the plant on the Red Data List of medicinal plants in the Southern African regions since the beginning of the 2000s.[6,12,23] When a population of *elephantine* is destroyed, the chance of regeneration is limited as they are known to grow in relatively natural areas. These are in rural areas or areas with an open row(s) of grass on land that is surrounded with fewer homes or buildings. The plant shows evidence of an unusual type of germination where the seedling buries its *plumule* (the bud within the embryo from which the stem and leaves develop) and shoots, thus arising from well below the ground surface (*pza.sanbi.org/elephantorrhiza-elephantina*, 2019).[24] They are not weedy in character even though a large colony of these plants may seem that way. In trying to preserve the livelihood of the plant, this has led to scientist exploring other parts of the plant putting the focus on the leaves. Recent research by *Olaokun et al.*,[8] explored the phytochemical content, anti-diabetes, anti-inflammatory, antioxidant and cytotoxic activity of leaf extracts of *Elephantorrhiza elephantine*. It's reported there is a lack of information on the leaves' biological activity and not much reports on the plants' anti-diabetic

activity in humans and animals.[5,6,15,16,22] The dried leaves extracts of *E. elephantine* were subjected to the following solvents; acetone, ethanol, cold water and hot water and investigated for polyphenolic, tannin and flavonoid content, antioxidant, anti-inflammatory, anti-diabetic and cytotoxic activities, using standard methods. *Olaokun et al.*,[6] reported that the phenolic and flavonoid contents of the ethanol extract were the highest to acetone. In addition, the inhibition of α-amylase activity by the ethanol extracts was the strongest to acetone and water. Furthermore, the ethanol extract was the least cytotoxic which was subjected on mouse cells against H4IIE liver and differentiated C2C12 muscle cells (myotubules). However, for the other assays, the hot water extract was the most active than the ethanol and acetone. The hot water extract in a concentration dependent manner stimulated the highest C2C12 glucose utilisation activity in addition to exhibiting the strongest antioxidant and anti-inflammatory activities.[6] Water and alcohol are used in preparing herbal remedies in traditional practice, indicating the significance of the results. According to a study by,[6] was the most recent study conducted reporting the biological activities of *E. elephantine* leaf extracts. This indicates that they are still opportunity to do further investigate on the leaves of this plant where it is subjected to other solvents of various polarity such as hexane, ethanol and acetone. The analysis of the study shows that the leaves can be used as an alternative, ensuring the long-term usage and preservation of the livelihood of the plant. However, more investigation should be conducted to gain more scientific evidence on the plant's leaf extract. This will prevent the plant from being dug up which would cause it to die; instead, only the leaves would be harvested extending the plant's lifetime and allowing the leaves to bloom once more. As a result, if the plant is dug up, this method of self-preservation would not be possible. Plant self-preservation may help to ensure the plant's existence and may even prevent extinction.

METHODS AND MATERIAL

A review of literature search on the plant species was undertaken by the use of different electronic databases such as Google Scholar, ScienceDirect, Sabinet ePublication, African Journals, PubMed using keywords such as; *Elephantorrhiza elephantine*, plant description, taxonomic of the plant, ethno medicinal usages, phytochemicals, pharmacological properties and plant toxicity. A total of 60 articles were selected and analysed. The articles selected had included literature publications with the keywords mentioned above and these articles were published between the years 2000 – 2021. Although articles that dated back from the 1900s were used as early reference literature. There were 32 articles relevant for this review article and 2 dissertations that were written between the criteria of years. There were eighteen (18) articles that were excluded based on the relevance of information needed for the article. An observation was made on where current research on the plant *Elephantorrhiza elephantine* is established, what was previously researched on the plant and what more could be investigated to expand our knowledge in literature and identify what challenges could be overcome in the years to come research.

Phytochemicals of the different parts of the plant

Plants' secondary metabolites known as phytochemicals play an important role in reducing the occurrences of many diseases such as diabetes, syphilis, dysentery, fever, hypertension and haemorroids.[4,25] The phytochemical compounds like glycosides, phenols, saponins and tannins which are detected in the plants extract help in the relief of illnesses/healing of wounds with the rich medicinal activity as mentioned in previous literature of[1,3,12,26] and therefore supports the ethno pharmacological uses of the plant. Phytochemicals are plant-derived molecules (PDMs) known to be a rich source of diverse compounds found in different parts of a plant when extracted using a solvent that could serve as the basis for rational drug design.[27] Research has lead into investigating the phytochemical properties found in the roots/rhizomes and leaves of the plant *E. elephantine*. In these parts of the plant, investigations on which phytochemicals are commonly found as the plant's extracts. The phytochemicals of *Elephantorrhiza elephantine* ranges and includes namely gallic acid, glucoside, flavonoid, phenols, tannins and phytosterols.[4,21,22,28]

Rhizomes/Roots

According to *Mthembu*,[28] findings from the aqueous and methanol extract of the rhizomes includes flavonoids and have indicated the presence of 5.8-22.3% tannins found in the rhizome of the plant. Tannins is the main compound found in the roots of the plant.[1,11,26] An additional finding from the study of,[28] indicted that 16.8% of sugars were detected in the rhizomes of the plant. The following sugars found in the rhizome of *E. elephantine* were b-sitosterol, gallic acid, methyl gallate, catechin and pentahydroxyflavan, among other compounds. The compounds detected were mainly phenolic compounds with a flavonoid skeleton.[28] The paper reported on the plant's good antioxidant activity when tested against and compared to the antioxidant activity of green tea. These results were significant indication of scientific evidence of *E. Elephantine* supporting the plant's ability of antioxidant properties.[28]

Elephantorrhiza elephantine leaves

Kudumela and Masoko,[22] findings reported using the following solvents; n-hexane, dichloromethane, acetone, and methanol for extraction. The phytochemical compounds that were detected in the leaves were flavonoids, cardiac glycosides, alkaloids, steroids and tannins. The Thin Layer Chromatography (TLC) fingerprint profiling of the plant extracts and spectrophotometric methods for quantitative determination were used to support the phytochemistry detections, it was resulted in the study that terpenoids and saponins were not detected as extracts of *Elephantorrhiza elephantine* using TLC. However, the phenolic, tannins and flavonoid content of *E. elephantine* was reported to be the third highest in the study when compared to the other plants. The phytochemicals which could be responsible for medicinal activity in medicinal plants are commonly steroids, tannins, flavonoids and glycosides which were present in all the plants selected for the study.[21] Terpenoids and saponins are seen as compounds responsible for antibacterial activity[22,28] and this could be an indication that *Elephantorrhiza elephantine's* medicinal activity when using the leaves may result from other phytochemical compounds. *Kudumela and Masoko*,[22] used the plant's dried leaf as they were avoiding problems associated with fresh plant material such as bacterial and fungal contamination since most plants live in a mutualistic relationship with microorganisms. The phytochemical compounds such as phenols, tannins and saponins have therapeutic value and may possess one or more biological activity hence the importance of preliminary screening of phytochemicals when studying medicinal plants.

Pharmacological studies

Investigations on the plant's antimicrobial activity and toxicity has been performed in the past decade[1,5,6] using different solvents with ranging polarisations to extract the phytochemical compounds of *Elephantorrhiza elephantine*. Minimum Inhibitory Concentration (MIC) and disc diffusion methods were used to investigate the antimicrobial activity, assays like TLC and spectrophotometry amongst others were used as qualitative and quantitative assays to investigate which phytochemical compounds are obtainable within the plant.[22]

The toxicity of the plant was investigated by subjecting the extract against mouse cells against H4IIE liver and differentiated C2C12 muscle cells (myotubules). The results in previous studies[6,20,22,28] have indicated that the phenolic and flavonoid compounds extracted from

Elephantorrhiza elephantine's roots and leaves do pose antimicrobial activity which may inhibit bacterial infections which ultimately leads to the healing of infections caused by the microorganism. The plants phytochemical compounds of *E. elephantine* may use different phytochemicals for antimicrobial activity/healing properties. This could explain the different mechanisms/mode of actions which the plant uses or have a stronger affinity to inhibit microorganisms because of the solvents used to extract the compounds than the mechanism used by conventional antibiotics that were also derived from medicinal plants years ago. The active phytochemical compounds found in newly investigated medicinal plants could be different or ineffective since bacteria may have developed resistance against the mechanism of inhibition. Therefore, new plant species investigation could be of clinical value in new development for the treatment of infectious diseases caused by pathogenic microorganisms.[2]

However,[26] expresses that as much as research has been done on the medicinal plant *E. elephantine* for evidence of anti-microbial; anti-inflammatory, anti-malaria, anti-diabetic and wound healing,[9,19,20,30] no confirmation was acknowledged from researchers for any further development of product and commercialised. Maharaj *et al.*[19] further highlights that the past literature evidence complied should have already led to the commercialisation of pharmaceutical products in respect to antimicrobial product. However, due to the lack of product to show, this indicates that more research is needed with more corresponding data of past research to strengthen the knowledge before pharmaceutical companies would invest in commercialisation of products.[19] This poses questions towards research as to whether scientist are collecting information as part of the natural drug discovery paradigm or whether they interpreting the traditional uses correctly and undertaking biological assays using correct screening and appropriate models and also understanding mechanism of action and safety fully. This highlights that there are gaps within the science studies which should lead to more investigation performed on *E. elephantine* sampled and found in other provinces of the country where the plant is prominent, geographic factors do influence secondary metabolites produced by plants. This will be used for comparison of old research to new research and provide a broader sight of knowledge on *E.elephantine* medicinal properties and strengthen the scientific findings. This review corroborates with[26] in that *Elephantorrhiza elephantine* has several scientific reports to prove efficacy through pharmacological studies by the research studies conducted in antibacterial, anti-inflammatory and anti-diabetic but not limited to these mentioned[4,12,22,28] but not much has been done on the safety. These studies illustrated scientific evidence which validates the traditional uses of the plant. A documented commercialization of a product from the plant *Elephantorrhiza elephantine* was made by Council for Scientific and Industrial Research (CSIR) on the plant's extract from the roots for the management and treatment of Benign Prostate Hyperplasia (BPH)[28] and placed on the commercial market. In literature, medicinal uses of the plant *Elephantorrhiza elephantine* have been reported to treat disease such as Urinary tract infections (UTIs) and bladder infection however, not limited to the ones mentioned but fewer research documentation was found regarding its benefits against cancer. *Mthembu*[28] had conducted studies which included the investigation of anticancer using plant extracts from *E. elephantine*. The CSIR led a research using the study of,[28] which has made known that the extract of *E. elephantine* has potent activity in inhibiting the 5-alpha reductase enzyme which is responsible for converting testosterone to dihydrotestosterone (DHT). Dihydrotestosterone is a causative factor in the progression of BPH.[28,31] In addition the mode of action of inhibitors of the 5-alpha reductase enzyme could also be used for the treatment of male pattern baldness.[32] This led the CSIR to developing a synthetic product of the extract of the plant for the management and treatment of male pattern baldness. A product is licenced by the CSIR who filed a patent [33][Patent No US 9,061,023 B2] on the extracts of *E. elephantine* for the management and treatment of benign prostatic hyperplasia (BPH).The discovery was licensed to Afriplex a South African botanical manufacturing company (https://www.csir.co.za/csir-and-afriplexpartners-bringing-health-solutions) and the product is currently traded as Folicin A/T for BPH and male pattern baldness (https://afriplex.co.za/what-we-do/product-manufacturing/active-ingredients/).[28] This is a singular case in which the product developed from *E. elephantine* extract, especially for BPH and male pattern baldness since the plant was not documented to be traditionally used towards such an illness/diseases. This could rather be linked to some of the traditional use of the plant by individuals with "bladder or urinary" problems and based on its anti-bacterial properties. This indicates that research that produces satisfying results with enough scientific evidence in the case of study found in the article,[26] product commercialisation is possible. This leads to question on what more can be done to close the gap of knowledge and produce more supporting evidence for this plant to achieve more health benefiting products manufacturing.

CONCLUSION

The gap of knowledge on medicinal plants has narrowed down in the past decade because of extensive research carried out by different scientist trying to better understand the benefits of traditional medication and why majority of the population still uses it as they primary healthcare choice. However, some of the research has been questioned on whether or not appropriate studies or guidelines that will lead to authentication of traditional medicines from the plant extracts for their formal incorporation into the healthcare system will follow through. There are still gaps in understanding the mode of action on how the plant extract inhibit the various microorganisms subjected to it, its anti-diabetic properties and antimalarial properties has not been thoroughly investigated but more literature has come forth on the phytochemistry, toxicity and anticancer properties of the plant species whereby a BPH product has been commercialized from the root crude extracts *Elephantorrhiza elephantine*.[28] Furthermore, different parts of the plant such as the leaves have been researched as an alternative instead of the root to preserve the plant as it has been listed on the red list database. Further investigating and understanding the mode of action of *E. elephantine* on its mechanism of action on the plants antimicrobial, anti-diabetic, antimalarial, anti-inflammatory and wound healing could lead to a better understanding on the medicinal properties of the plant extracts and a commercialization of a product in future.

LIST OF ABBREVIATIONS

EVM	Ethno veterinary medicine
PDM	Plant-derived molecules
TLC	Thin Layer Chromatography
MIC	Minimum Inhibitory Concentration
CSIR	Council for Scientific and Industrial Research
BPH	Benign Prostate Hyperplasia
UTI	Urinary tract infections
DHT	Dihydrotestosterone

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Radebe T, wrote and made the corrections to the article.

Mfengwana PH and Olivier D read and approved the final manuscript.

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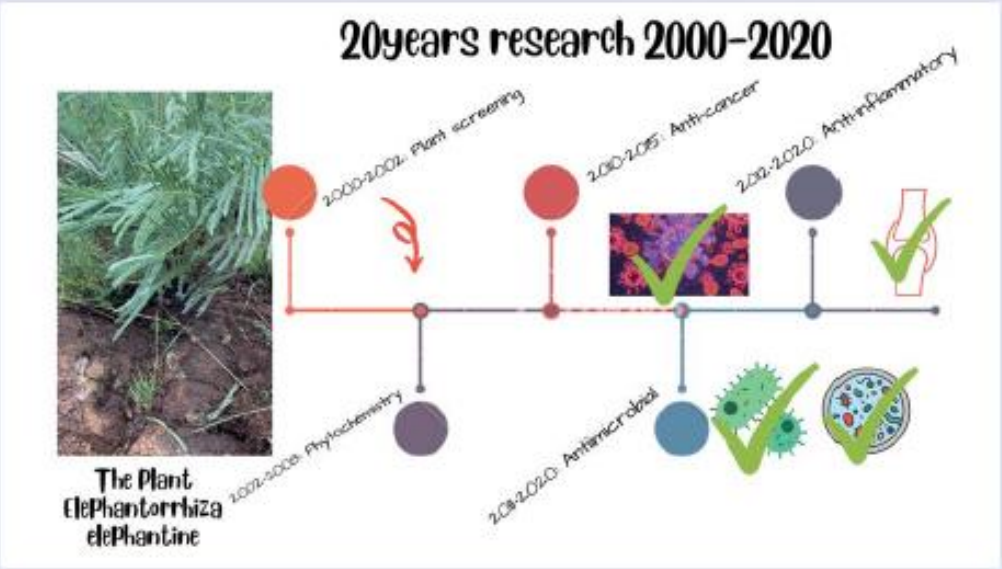
GRAPHICAL ABSTRACT



ABOUT AUTHORS

Tlotlo Radebe is a Haematology Part-Time Lecturer in the Heath Science department, in Biomedical Technology field in the Health and Environmental science faculty at Central University of Technology, Bloemfontein, Free State, South Africa. She holds a BHSc in Medical Laboratory science (honours). Ms Radebe is currently enrolled for the Masters of Health Sciences in Biomedical Technology at the Central University of Technology. Her research interest is in traditional medicines, and the title of her research project is: "In vitro antibacterial activity of *Gunnera perpensa L.* and *Elephantorrhiza elephantina* against human pathogenic bacteria". Her medicinal plant research interest started in 2020.

Dr Polo-Ma-Abiele Hildah Mfengwana is the Head of Health Sciences Department at Central University of Technology, Free State (CUT) and holds a Doctor of Health Sciences in Biomedical Technology degree from CUT. Her research is on the ethnopharmacology, with concentrated focus on the safety and efficacy of traditional medicinal plants used to treat cancer, wounds, and inflammation by traditional healers in African countries. She is supervising more than 10 Masters students and serves as the research article peer reviewer for international journals.

Dr Dedré Olivier is a senior lecturer in the programme Biomedical Technology at Central University of Technology (CUT), Free State. She holds a DTech: Biomedical technology from CUT. She serves on the Scientific Advisory Committee for Microbiology under the auspices of the Society of Medical Laboratory Sciences in South Africa, and is registered with the Health Professions Council of South Africa as Medical Technologist in the Clinical Pathology discipline. Her research areas include tuberculosis, HIV and traditional medicinal plants. In addition to post-graduate supervision, she also supervise undergraduate research projects.

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