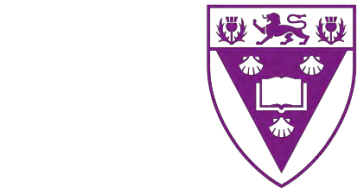

PHARMACOLOGICAL CHARACTERISATION OF MEDICINAL PLANTS USED ETHNOBOTANICALLY FOR THE TREATMENT OF SEXUALLY TRANSMITTED INFECTIONS

A thesis submitted by

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Isaiah 40:31 (NKJV): ***"But those who wait on the Lord shall renew their strength; they shall mount up with wings like eagles, they shall run and not be weary, they shall walk and not faint."***

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Abbreviations

%	Percent
℃	Degrees Celsius
µg	Microgram
µL	Microlitre
Abs	Absorbance
ABTS	2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AMR	Antimicrobial resistance
CYP450	Cytochrome P450
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH-H	Diphenylpicrylhydrazine
E. coli	*Escherichia coli*
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
g	Gram
GAE	Gallic acid equivalent
H. hemerocallidea	*Hypoxis hemerocallidea*
H. africana	*Hydnora africana*

HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
HPV	Human papillomavirus infection
KI	Potassium iodide
LB	Luria Broth
ml	Millilitre
mg	Milligram
mg/ml	Milligram per millilitre
mM	Millimolar
MRSA	Methicillin-resistant staphylococcus aureus
nm	Nanometre
NaOH	Sodium hydroxide
P. aeruginosa	*Pseudomonas aeruginosa*
Rf	Retention factor
rpm	Revolutions per minute
STI	Sexually transmitted infection
TCM	Traditional Chinese Medicine
TFC	Total flavonoid content

TLC	Thin layer chromatography
TM	Traditional medicine
TMS	Traditional Medicinal Systems
TPC	Total phenolic content
TSB	Tryptic Soy Broth (TSB)
S. aureus	*Staphylococcus aureus*
QE	Quercetin equivalent
WHO	World Health Organisation

Abstract

Introduction

Sexually transmitted infections have become a major public health issue, affecting millions of people. These infections can cause serious consequences such as infertility in women, urethritis and prostatitis in men, chronic pain, or an increase in HIV transmission. Antimicrobial resistance is a major challenge in the treatment of diseases, including sexually transmitted infections (STIs). In recent years, research has focused on developing new potential therapeutic agents to prevent and treat infections using medicinal plants, which have been analysed for their bioactive compounds that possess therapeutic properties.

Aim of the study

This study primarily focuses on investigating the antimicrobial activity *in vitro* of *Hypoxis hemerocallidea* and *Hydnora africana* against common bacterial pathogens and the effect of liver drug metabolizing enzymes, as well as determining the antioxidant potential of the plants.

Methods

The corms of *H. hemerocallidea* and *H. africana* were collected from a South African professional indigenous health practitioner, in the Eastern Cape, Makhanda (Grahamstown). Plant extraction was performed by maceration with the solvents: hexane, dichloromethane, methanol, and water.

The resulting extracts were subjected to qualitative phytochemical analysis and a quantitative total phenolic content and total flavonoid content tests were conducted using gallic acid and quercetin as the standards.

The antimicrobial activity of the plant extracts was assessed using a percentage viability antimicrobial assay against four bacterial strains: *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*.

The antioxidant activity was determined qualitatively and quantitatively using the DPPH radical scavenging assay, using ascorbic acid as the standard and absorbance measured at 517 nm.

The liver enzyme inhibition potential of the plant extracts was evaluated using commercial recombinant cytochrome P450.

Results

The qualitative phytochemical screening of *H. hemerocallidea* and *H. africana* in different solvent extracts revealed the presence of phenols, flavonoids, tannins, and alkaloids, especially in the methanol and water extracts. The results of the total phenolic test for *H. hemerocallidea* and *H. africana* revealed that the plant extracts contain phenolic compounds, and the quantity of the phenolic compounds generally increased with increasing extract concentration. The same applied for the total flavonoid content in both plants, which showed a similar trend to the phenolic content, with the methanol extract having the most flavonoids. The results of the qualitative DPPH dot plot showed that the extracts of *H. hemerocallidea* and *H. africana* have compounds in them that possess some level of antioxidant activity. The quantitative DPPH radical scavenging assay demonstrated that the extracts had moderate antioxidant activity, compared to ascorbic acid. The methanol extract of both plants demonstrated the most significant radical scavenging activity showing a concentration-dependent increase. The plant extracts demonstrated different levels of antimicrobial activity, with the most notable antibacterial activity for *H. hemerocallidea* and *H. africana* being against *Staphylococcus aureus*. The results revealed that the extracts exhibited some CYP3A4 enzyme inhibitory activity, notably at high concentrations, with the methanol extract exhibiting inhibitory effect much higher than ketoconazole.

Conclusion

The corms of *H. hemerocallidea* and *H. africana* contain different phytochemicals such as phenols, flavonoids, tannins, alkaloids that possess antioxidant and antibacterial properties, as well as some CYP3A4 enzyme inhibitory activity, notably at high concentrations. Further studies are needed to expand on the mechanism of action of these plant extracts and compounds.

Chapter 1: Introduction

1.1 Introduction and Background of study

Health is increasingly becoming threatened by significant health crises, such as the increasing burden of non-communicable diseases (NCDs), infectious diseases such as the COVID-19 pandemic, sexually transmitted infections, human immunodeficiency virus/acquired immunodeficiency syndrome, tuberculosis, and the alarming rise of antimicrobial resistance (AMR) (1).

Sexually transmitted infections (STIs) are a global burden and have become a significant public health concern, according to the World Health Organization (WHO), there are 340 million new cases annually among individuals under 25 years old (2) and there are more than one million curable STIs acquired daily, with the majority of infections being asymptomatic, with drug resistance being a significant threat to curbing the burden of STIs (3). In 2020, the WHO estimated that 374 million new STI cases would occur that year (3).

STIs are transmitted via unprotected sexual intercourse, i.e., oral, anal, or vaginal sex. Despite there being numerous efforts to prevent and treat STIs, their prevalence and incidence remain high (4). Common STIs include human immunodeficiency virus (HIV), human papillomavirus infection (HPV), chancroid, syphilis, chlamydia, gonorrhoea, and genital herpes (2). Fortunately, some of these STIs can be cured and they respond well to antibiotic treatment if sought promptly. However, if left untreated, they can have severe consequences, leading to serious health complications such as chronic pain, infertility, disseminated infection, and an increased risk of HIV transmission (3,5).

The conventional treatment for STIs is primarily antibiotic therapy. However, the emergence of antibiotic-resistant strains of pathogens, such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, is a growing concern (6). In the recent years, gonorrhoea, a bacterial STI, in particular has had increased antimicrobial resistance and therefore reduced treatment options (7). According to the Gonococcal AMR Surveillance Programme (GASP), *N. gonorrhoeae* has shown high rates of resistance to many antibiotics, including azithromycin, quinolone, and broad-spectrum cephalosporins (8).

While antibiotics are the most common treatment for many STIs, the emergence of antibiotic-resistant strains has complicated the prevention and treatment of these infections, hence the need for alternative therapies (6,9). Antimicrobial resistance is one of the top global public health threats, and it was estimated that 1.27 million deaths in 2019 were due to AMR (1). Antibiotic resistance results from the overuse and misuse of antibiotics in healthcare settings as well as agriculture. The burden of microbial infections extends beyond individual health; they pose significant public health challenges. In addition, the stigma associated with STIs has also contributed to the problem, as many individuals are discouraged from seeking treatment or testing, particularly in areas where conventional antibiotic treatments are limited (2).

According to the WHO, traditional medicine is the knowledge and practices based on indigenous beliefs and experiences used to maintain health and prevent, diagnose, and treat illness (10). Traditional medicine encompasses beliefs, natural remedies, and practices based on cultural traditions rather than scientific evidence (11). Herbal medicine is the most commonly used traditional medicine, and according to WHO, several countries in Africa, Asia, and Latin America use herbal medicines as the primary treatment for diseases (12). WHO recommends the use of traditional medicine in the maintenance of public health, prevention, and treatment of disease, especially for chronic diseases, as they are generally considered safer due to fewer side effects as compared to conventional medicine.

1.2 Problem statement

Sexually transmitted infections are a global public health issue, requiring attention. The burden of these infections contributes to reduced quality of life, morbidity, and in the worst cases, mortality (13). The strategies currently in place to control and prevent the spread of STIs face challenges such as limited access to healthcare services, discrimination and stigma, and the increase in AMR in STI-causing pathogens (14). The increase of resistance to conventional antibiotics renders many first-line treatments ineffective, limiting treatment options, and has led to an urgent need to develop alternative antimicrobial agents (15).

For a long time, many communities worldwide have relied on medicinal plants as a primary source of healthcare, particularly for infectious diseases (16). Investigating the antimicrobial potential of traditionally used medicinal plants is a critical area of research, as a result of increased AMR prevalence and the urgent need for new treatment options (17). This proposed study addresses the

concern by investigating the potential antimicrobial activity of two selected medicinal plants, *Hypoxis hemerocallidea* and *Hydnora africana*, which were traditionally prepared and used by a local herbalist. While these specific plants are not explicitly indicated for treating STIs, they have a history of use for various illnesses.

Medicinal plants offer a potentially rich source of phytochemicals, the bioactive compounds found in plants, which often possess potent antimicrobial properties. This knowledge gap highlights the need to identify new potential sources of antimicrobial agents to tackle the growing problem of antibiotic resistance.

1.3 Research question

Do extracts of *Hypoxis hemerocallidea* and *Hydnora africana* exhibit i*n vitro* antimicrobial activity against common bacterial microorganisms, providing preliminary evidence for their potential efficacy against sexually transmitted infection-causing organisms such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*?

1.4 Aim of the study

The study aimed to conduct a preliminary *in vitro* pharmacological characterization of *Hypoxis hemerocallidea* and *Hydnora africana*, focusing on their *in vitro* antimicrobial activity against common bacterial pathogens and liver enzyme inhibition potential against the cytochrome P450 isoform, CYP3A4.

1.5 Objectives of the study

The objectives were as follows:

- To conduct qualitative and quantitative phytochemical analysis of *H. hemerocallidea* and *H. africana* extracts
- To determine the antioxidant activity of *H. hemerocallidea* and *H. africana* extracts using DPPH assay
- To determine the *in vitro* antimicrobial activity of *H. hemerocallidea* and *H. africana* extracts against common bacterial microorganisms
- To evaluate the potential *in vitro* inhibitory activity of *H. hemerocallidea* and *H. africana* extracts against cytochrome P450 isozyme, CYP3A4

1.6 Importance of the study

The importance of this study is underscored by the need for discovering novel antimicrobials in response to the crisis of antimicrobial resistance. The potential of *H. hemerocallidea* and *H. africana*, plants with a history of use in traditional medicine can be explored, and this could contribute to discovering new therapeutic agents for bacterial infections. Additionally, the scientific basis of traditional medicine practices are explored, supporting the use of these plants for treating infections. This phase of study primarily focuses on common bacterial pathogens, that is *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus*. There is not enough well-documented anecdotal evidence linking *H. hemerocallidea* and *H. africana* directly to the treatment of STIs, however both plants are broadly used in traditional medicine practices, so more research is required to clarify any claims to treating STIs. The findings could lay the groundwork for future investigations on prevention and treatment strategies for STIs.

Declaration

The aim of the study was to focus on pathogens causative of STIs, however due to challenges acquiring the known STI pathogens, and delays, the decision was made to proceed with the study on the following ESKAPE pathogens, *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus* due to their importance in antimicrobial resistance.

Chapter 2: Literature review

2.1 Introduction

For decades, Southern Africa has used different plant-based remedies, also known as phytomedicines for treating, curing, and mitigating ailments or long-term public health problems (16). Medicinal plants or traditional therapies are often the only choice in areas where modern medicine is hard to get to, or inaccessible (18). Ethnobotanists, people who study plants, have found many phytomedicines with phytochemicals (bioactive compounds) that destroy bacteria that cause infections and diseases (19,20). Many people use these plants as medicine, without knowledge or understanding of how these plants work as drugs.

Hypoxis hemerocallidea, also known as the African potato, is a plant that possesses medicinal properties and has been used for a very long time. Its roots contain compounds such as phytosterols, glycosides, and antioxidants that help reduce swelling and destroy microbes (21). Phytochemicals such as tannins and flavonoids in the plant *Hydnora africana* are known to destroy bacteria (22). More research needs to be done on the pharmacology and pharmacotherapy of these plants, as their use traditionally suggests therapeutic potential, however additional scientific validation is required to determine their safety, efficacy and optimal dosage (23).

Ethnobotany assesses the traditional knowledge of plants and their uses (24). In ethnobotany, all plants are used according to their traditional application for medicine, combining the cultural past with biodiversity. The study by Ozioma et al. (2019) asserts that many Southern African traditional healers keep their understanding of plants that can be used as medicine (25).

Advanced technologies, such as High Performance Liquid Chromatography (HPLC), can identify bioactive compounds and assess their potential effectiveness as medicinal agents (26). To make new plant-based medicines, such advanced technologies use already existing data and known drug research. This mix of ethnobotany and pharmacology helps global health attempts to use traditional medicine in health care (27).

2.2 Traditional Medicine and Ethnobotany

Areas and communities without proper medical and healthcare services often rely quite a lot on traditional medicine. Traditional medicine (TM) uses plants with historical knowledge and ethnobotany studies the human-plant relationships for medicine and all these methods use traditional knowledge systems to deal with health problems (28). TM, also known as complementary and alternative medicine (CAM) is known to be the oldest form of healthcare globally making use of natural products (11). In 2002, severe acute respiratory syndrome (SARS) became a global pandemic, first appearing in China, and many treatment options were tried, with several conventional drugs re-purposed, but there was no specific effective treatment. WHO reported then, that TM played a role in the strategy to eradicate SARS, and by late July 2003, no new cases of SARS were being reported (29).

TM comprises of inherited knowledge, skills, and practices that use natural resources to treat illness (30). The WHO defines traditional medicines as the knowledge and practices based on indigenous beliefs and experiences, used in the maintenance of health and the prevention, diagnosis, and treatment of illness (30). TM encompasses beliefs, natural remedies, and practices rooted in cultural traditions rather than scientific evidence (12). In Southern Africa, traditional healers and herbalists play a crucial role in providing treatment for ailments to people, especially in rural communities since contemporary medicine may be too expensive or in most cases unavailable. Traditional healers are generally considered cultural historians since they preserve indigenous plant medicine recipes (31).

Although indigenous medicinal plants are widely used, there is lack of scientific support and regulation around their use. Also, the publicizing of TM presents ethical and environmental concerns (32). Therefore, there is a need for pharmacological investigations to be done to ensure that introduction of TM to modern healthcare systems is safe and effective. For instance, in Southern Africa, several plants such as *Pelargonium sidoides* commonly known as African geranium, traditionally used for respiratory complaints, and some Aloe species such as *Aloe ferox*, have healing properties that have not been properly researched. Traditional knowledge holders need intellectual property rights and benefit-sharing arrangements to avoid exploitation (33). Patwardhan et al. (2023) suggested that traditional medicine has valuable information and tools that can impact global health (34).

Herbal medicine, a widely adopted form of traditional medicine, is especially prevalent in several African and Asian countries (35). WHO recommends the use of traditional medicine in the maintenance of public health, prevention, and treatment of disease, particularly for chronic diseases as they are generally considered safer due to fewer side effects as compared to conventional medicine (36).

In the study by Watanabe et al. (2001), the researchers discovered that certain Japanese physicians preferentially used Kampo medicines, the traditional medicine of Japan, in their daily practice over allopathic medicines (37). Other than the healthcare role that medicinal plants serve, they also contribute significantly to the economic growth of rural communities, specifically in the Hindu Kush-Himalayan countries where the estimated number of medicinal plant species ranges from 7500 to 10,000 (38). As technology and science rapidly advance, there is an increasing demand for natural remedies in healthcare and as such, research institutions have shifted their focus to traditional medicine, especially plant-based sources, to develop new drugs (39).

Ethnobotany is the study of traditional knowledge associated with plants, specifically those that are used for medicinal purposes by indigenous communities. It involves identifying and analysing the traditional use and cultural significance of indigenous plants, looking at people and plants for their cultural and medical value (40). Ethnobotanical information guides researchers to identify which plants are ideal candidates for further screening and chemical analysis (41). A study in Gemad District, Northern Ethiopia highlighted the importance of this kind of research, particularly in preserving and promoting the traditional knowledge of indigenous communities and contributing to the development of modern medicine (42). About 25% of pharmaceutical drugs originate from plants that are used in TM, for example, quinine, and artemisinin, two antimalarial drugs, which resulted from indigenous plants (43).

2.2.1 Historical background and cultural significance of Traditional medicine

Traditional medicines, deeply rooted in ancient healing practices, have a rich historical background across cultures and centuries (44). They continue to be integral to healthcare in many societies, offering alternatives to conventional treatments and preserving cultural heritage (45). Many countries across Africa, Asia, and Latin America use traditional medicines to meet some of their primary healthcare needs (44).

African TM has been widely accessible for centuries by several African populations or communities and about 80% of the continent's population relies on traditional medicine for medical needs (46). TM is commonly practiced in South Africa (SA) and is especially significant in remote areas with limited healthcare resources, where medicinal plants play a vital role in primary healthcare (47). *Sangomas* or *inyangas*, as they are commonly known in South Africa, are highly regarded members of communities, involved in diagnosing, prescribing, and performing rituals to heal a person either physically, mentally, or spiritually using traditional medicinal remedies although some people are sceptical about their abilities (48).

For many years, indigenous plants have been used in traditional medicine to treat a wide range of conditions, primarily because it is believed that they contain bioactive compounds with antimicrobial properties that could be effective against infections including STIs. Traditional healers take advantage of the rich biodiversity of plants in their surroundings. With limited scientific information and knowledge, as well as lack of documentation on the TMs, there is a need to investigate the therapeutic potential of medicinal plants as a source of new treatments for STIs (49).

2.2.2 Traditional Medicinal Systems

Humans have always depended on nature for food and health, shaping the development of Traditional Medicinal Systems (TMS), which represent diverse practices, beliefs, and knowledge systems passed down through generations. TMS have served as the primary healthcare system for many communities throughout history, offering a rich tapestry of therapeutic approaches that have been shaped by centuries of observation, experience, and cultural transmission (50).

TMS continues to play a vital role in healthcare delivery, particularly in rural and underserved communities where access to modern healthcare is limited. They offer a valuable resource for primary healthcare, especially for chronic diseases and conditions for which conventional medicine may have limited options. Moreover, TMS often provide culturally appropriate and affordable healthcare solutions that are accessible to a wide range of populations.

2.2.2.1 Ayurveda

Ayurveda, originating in India, is one of the oldest systems of traditional medicinal systems (51). Ayurveda includes a holistic approach to health, treating the whole person, not just focusing on specific symptoms or illnesses. It considers physical, mental, emotional, and spiritual well-being as interconnected and equally important. Ayurveda healers view health as a state of dynamic equilibrium between the mind, soul, and body. A wide range of natural therapies are used, including herbal remedies, dietary modifications, yoga, and meditation (51).

2.2.2.2 Traditional Chinese Medicine

Traditional Chinese Medicine (TCM) dates back thousands of years and has a rich history. The study by Ma et al. (2021), emphasises that TCM adopts several therapeutic approaches, such as acupuncture, herbal medicine, and massage therapy (52). TCM is one of the advanced TM practices, with hospitals, clinics and community health centres being established. The state encourages exchanges between traditional Chinese medicine and Western medicine and creates opportunities for conventional medical practitioners to learn from their traditional Chinese medicine counterparts (36).

2.2.2.3 Indigenous Healing Practices in Africa

Africa is a diverse continent with lots of indigenous healing practices, that involve the use of medicinal plants, traditional healers, and spiritual rituals. In many African communities and societies, traditional healers play a role in providing healthcare to their communities (53). They possess knowledge of natural products and a deep understanding of local plant resources and their therapeutic uses. By 2018, only a few African countries had taken steps to develop national policies and regulatory frameworks for TCM practice, practitioners and products (36)

2.2.3 Traditional Medicine in Southern Africa

Around 80% of the South African population uses traditional medicines for primary healthcare needs (53). South Africa is known to have a strong history of traditional healing, accommodating around 22,000 plant species, which account for about 10% of the world's plant species. Many medicinal plants have been extensively used in many parts of South Africa, including the provinces

of Limpopo, Eastern Cape, Mpumalanga, and Kwa-Zulu Natal. The scarcity of medical facilities, high costs of medicines and the areas' fauna have contributed to this dependence (54).

A study revealed that 77% of the local population in Lesotho, live in rural areas that are too far from healthcare facilities to access them by foot (55). Additionally, more than 60% of the country's terrain is mountainous, making it difficult to travel by road and limiting accessibility. Consequently, many local inhabitants resort to alternative medical remedies like medicinal plants (56). Similarly, in resource-poor communities in south-central Zimbabwe, TM is an important source of primary healthcare due to limited access to formal healthcare facilities (57). Community members in Botswana were asked about their perceptions of safety and efficacy of traditional medicines, and 84% them in the study felt that traditional medicines were safe and effective, though some felt that traditional healers were better equipped to treat certain diseases while biomedical doctors were more efficient in treating others (58).

Mongalo and Raletsena (2022) documented an inventory of South African medicinal plants, which is important in assisting researchers access a list of plant species to evaluate for potential phytochemicals (59). There are about 335 medicinal plants from 103 families documented, and the most represented families are Fabaceae (11.64%) and Asteraceae (6.27%). Herbs constitute 36.53%, trees 32.34%, shrubs 29.04%, climbers 1.80%, and parasites 0.30% (59). Common medicinal plants such as chamomile, mint, sage, *Echinacea purpurea* help treat respiratory ailments, including flu, cough, asthma, bronchitis, and pneumonia (60,61). Other medicinal plants also help in managing diabetes mellitus with fewer side effects (62). These plants have phytochemicals such as alkaloids, flavonoids, and tannins, contributing to their therapeutics properties (60). Although there is reasonable activity in vitro of some plant species validating the relevance of use, there is still a need to explore the mechanism of action of such plant species.

Bacterial, viral, and parasitic diseases cause a lot of sickness and death, especially in places with few resources (63). WHO reports that STIs are gaining significant importance at present because their transmission is quite fast, treatment is expensive, and the emergence of drug resistance is a major threat to reducing the burden of STIs (3). Moreover, STIs increase susceptibility to more complicated diseases such as HIV/AIDS, hence it is important that alternative systems, such as indigenous plants which have been used for a long time without any scientific evidence but have proved to work in some settings, are sought in the management of STIs (64).

2.3 Sexually Transmitted Infections

STIs are becoming a significant public health burden (64). The WHO in 2020 estimated 374 million new infections among individuals under 25 years old with one of the four STIs: chlamydia (129 million), gonorrhoea (82 million), syphilis (7.1 million), and trichomoniasis (156 million) (3). These infections are transmitted through unprotected sexual intercourse, including oral, anal, and vaginal sex, as well as from mother to child during pregnancy or childbirth. Despite numerous efforts to prevent and treat them, their incidence and prevalence remain high. Common STIs include gonorrhoea, chlamydia, syphilis, genital herpes, human papillomavirus infection, and HIV (13). Symptoms of these infections include genital discharge, genital sores, itching, and pain during urination (13). STIs that are not treated adequately can lead to infertility, pelvic inflammatory disease, chronic pelvic pain, and the spread of HIV (65).

STIs are caused by several pathogens including bacteria such as *Neisseria gonorrhoea, Treponema pallidum, Gardnerella vaginalis, Chlamydia trachomatis*, viruses such as HIV, herpes simplex virus, HPV, and parasites such as *Trichomonas vaginalis. Neisseria gonorrhoea* is a gram-negative bacterium that causes gonorrhoea, one of the most common STIs worldwide. This bacterium can infect the urethra, cervix, and rectum. *Chlamydia trachomatis* is an obligate intracellular bacterium that causes chlamydia (66).

The current treatment guidelines for STIs focus on early diagnosis and management to prevent complications and reduce the transmission (67). Antibiotics such as ceftriaxone, doxycycline, penicillin, azithromycin, are commonly used treatments for gonorrhoea, chlamydia, syphilis etc. (68).

These antibiotics target bacterial pathogens and exert their pharmacological effect by primarily inhibiting cell wall synthesis and protein synthesis (69). While effective in many cases, antibiotic resistance is proving these drugs ineffective, necessitating the development of potential alternative therapeutics.

The study by Semenya & Potgieter (2013) in the Limpopo province of South Africa sought to investigate the role of Bapedi traditional healers in sexually transmitted infections. The study found that due to limited access to laboratory services, many traditional healers diagnosed and treated STIs based on the symptoms presented by the individual (70).

2.3.1 Challenges in STI Treatment

STIs place a burden on the healthcare systems worldwide. They affect millions of people every year impacting them directly and their communities. STIs predominantly affect women and teens in sub Saharan Africa and are often difficult to diagnose and treat (71). Screenings and public health measures that promote safe sexual behaviours are crucial for preventing all infections (72). Treating STIs presents challenges such as treating one partner and not treating the other which may lead to re-infection, discrimination and stigma, lack of awareness, as well as limited access to testing and treatment (20).

2.3.1.1 Discrimination and stigma

The stigma around STIs often makes it take longer to get diagnosed and treated. In conservative communities, talking about sexual health is frowned upon, which creates problems as people are less likely to embrace STI education and prevention programs since they would be ashamed. The negative thoughts make it harder for individuals to seek help and receive adequate care in time (20). Stigma also can contribute to the rapid spread of STIs because individuals are discouraged from getting tested and treated in time because they are scared that they will be judged. According to a study conducted in the United Kingdom, stigma associated with STIs is a major barrier to accessing healthcare services and it can have a significant impact on mental health and well-being (73).

2.3.1.2 Lack of awareness

Another major challenge associated with dealing with STIs is a lack of awareness. A study done by Balán et al. (2019), found out that several people do not understand the risks associated with having unprotected sexual intercourse or know how to protect themselves from STIs and this can result in the spread of infections (74). In a study conducted in Nigeria, the researchers found that poor knowledge of STIs among young people was a major factor contributing to the high prevalence of STIs (75).

2.3.1.3 Limited access to testing and treatment

Limited access to testing services and treatment also presents a crucial challenge in dealing with STIs. Chesson et al. (2017) reports that STIs affect society and the economy, especially in low-

and middle-income nations with few healthcare facilities (76). Many people may not have access to healthcare facilities or may not be able to afford testing and treatment. This can result in untreated infections, which can lead to complications and the spread of infections. A study conducted in South Africa found that limited access to testing and treatment was a significant challenge in dealing with STIs (77). Treatment for STIs is harder to acquire when financial resources are limited.

2.3.1.4 Antimicrobial resistance

Antimicrobial resistance (AMR) is one of the biggest challenges in the treatment of STIs, especially gonorrhoea, which resists the current therapies (78). According to WHO, antimicrobial resistance results when microorganisms do not respond to the antimicrobial medicines. AMR makes infections increasingly difficult to treat, which in turn leads to higher risks of spread of disease, severe illness, and in worse cases, deaths (79). It is becoming a global pandemic, and it was estimated by WHO in 2019 that bacterial AMR was directly responsible for 1.27 million global deaths (79). According to Kim et al. (2024), AMR makes treatment plans more difficult, and healthcare escalate since it requires different or combined medicines (63). This problem gets worsened when antibiotics are used irrationally in healthcare settings (80).

The pathogens of interest in this study are ESKAPE pathogens, i.e., *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*. These pathogens are regarded as highly resistant to antibiotics, contributing majorly to AMR. They are of importance in AMR testing because they are known to develop resistance to multiple antibiotics , making it difficult to treat infections (81). WHO has categorized these organisms into priority levels in the global priority list of antibiotic-resistant bacteria, as either critical-priority or high-priority organisms (82). Critical-priority organisms are those that are resistant to last-resort antibiotics such as carbapenems, while high-priority organisms still have treatment options available, but the rise in resistance is of concern (83).

A. baumannii is a gram-negative bacterium, commonly associated with hospital-acquired infections and is highly resistant to antibiotics, and according to WHO, it is classified as a critical-priority organism. *E. coli* is a gram-negative bacterium, a major cause of UTIs, gastrointestinal infections among others, and the extended-spectrum beta-lactamase (ESBL) producing *E. coli*

strain is highly resistant to antibiotics. According to WHO, it is classified as a critical-priority organism. *S. aureus* is a gram-positive bacterium, contributing to skin infections, pneumonia and bloodstream infections, and is classified as a high-priority organism. *P. aeruginosa* is a gram-negative, opportunistic organism affecting immuno-compromised persons and is classified as a critical-priority organism (84,85).

The treatment has become more difficult, as these pathogens have developed ways to escape the body's defence mechanisms and cause illness. Some antimicrobial resistance mechanisms include enzymatic drug degradation, whereby bacteria produce enzymes which break down the antibiotic e.g. beta-lactamase enzymes produced by *S. aureus* or *E. coli* can break down beta-lactam antibiotics such as penicillin. The structure of the target site on the bacteria where the antibiotic binds can be altered e.g. methicillin-resistant staphylococcus aureus (MRSA), bacterial target for methicillin (penicillin-binding protein) is changed, preventing the drug from binding (86). The drug can also be eliminated from the cell through efflux pumps which lowers the amount of drug inside the pathogen e.g. *P. aeruginosa* and *E. coli* have pumps which remove drugs such as fluoroquinolones, beta-lactams and tetracycline. The formation of a biofilm covering protects the bacteria such as *S. aureus* from antibiotics as the drug cannot penetrate the pathogen... Reduced drug permeability through reduced number of porin channels on a cell reduces the drug's ability to reach its target, for example, *P. aeruginosa* reduces the permeability of its outer membrane to antibiotics such as beta-lactams and fluoroquinolones (87).

2.3.1.5 Drug and food interactions

The concurrent use of traditional medicine together with conventional medicine, such as antibiotics, can pose challenges due to the potential for herb-drug interactions. Traditional medicines also contain active compounds that can influence the same metabolic pathways as conventional drugs, leading to increased or decreased drug levels in the body (88). For example, a common herbal remedy, St. John's Wort may interact with a CYP3A4 inhibitor such as tetracycline, and increase the metabolism of the drug in the body and lower the drug's plasma concentration, therefore reducing its effectiveness and potentially increasing side effects (89). Certain herbs may induce or inhibit the enzymes that play a role in drug metabolism, and this may impact the absorption, distribution, metabolism, and elimination of the antibiotics in the body (90).

These interactions may affect the effectiveness of the antibiotic therapy, resulting in treatment failure and contributing to the development of antibiotic resistance (91). Additionally, herbal remedies may also have antimicrobial properties of their own similar to those of antibiotics which means they have overlapping effects on the body (92).

These interactions between traditional herbal remedies and CYP3A4 are of interest because this isoform plays a role in drug metabolism, as it metabolises majority of conventional drugs (93). Once the activity of CYP3A4 is altered by a phytochemical, the level of drug in the blood will lead to therapeutic failure, therefore it helps in optimising therapy (94). According to WHO, populations that face difficulties accessing healthcare services have the highest incidence of STIs (56).

A different study carried out in the KwaZulu-Natal province in South Africa, sought to explore the possibility of cooperation between traditional healers and nurses within biomedical healthcare systems to treat STIs. The results showed that there were conflicting opinions among the two groups. Traditional healers believed that the nurses did not appreciate their work and thus did not refer patients to them, while the nurses were of the view that traditional healers lacked expertise and prescribed expired medications and that traditional medicine was not considered evidence-based (95). Traditional healers and biomedical experts can collaborate to make STI management easier and more effective, especially in low-resource areas.

2.4 Medicinal Plants and the role of Plant-Based Antimicrobials

The alarming increase in AMR poses a challenge to modern medicine (96). Conventional antimicrobials have been overused and misused, leading to drug-resistant pathogens and, hence many ineffective treatments (96,97). Herbal remedies for STIs have emerged as an alternative to standard treatments. Plant-based antimicrobials are promising yet difficult to standardize, administer dosage, and ensure safety. Adding plant-based treatments to national health policy could make them more popular and accessible, especially in cultures that value traditional medicine (98).

For many years, people have used medicinal plants with known beneficial compounds to treat several illnesses. *H. hemerocallidea* and *H. africana* are examples of medicinal plants that have been used for many years. Studies have shown that these plants have antibacterial, anti-

inflammatory, and immunomodulatory properties that can be used in medicine (99,100). Medicinal plants are prepared by traditional healers and herbalists into different dosage forms which include mixtures, extracts, decoctions, tinctures, teas, and pastes. Different plant parts contain different bioactive compounds that are used for medicinal purposes (101). However, some parts are toxic and therefore are not used, while others are used in medicine. Examples of plant parts used are shown in the figure 2.1 and the proportions in figure 2.2. Table 2 shows common medicinal plants used for the treatment of STIs in South Africa. These include *Blepharis diversispina, Carica papaya, Arctopus monacanthus, Albizia adianthifolia, Bidens pilosa.* Different plant parts are used to make decoctions which are taken orally (101).

Figure 2.1: Plant parts used medicinally (101)

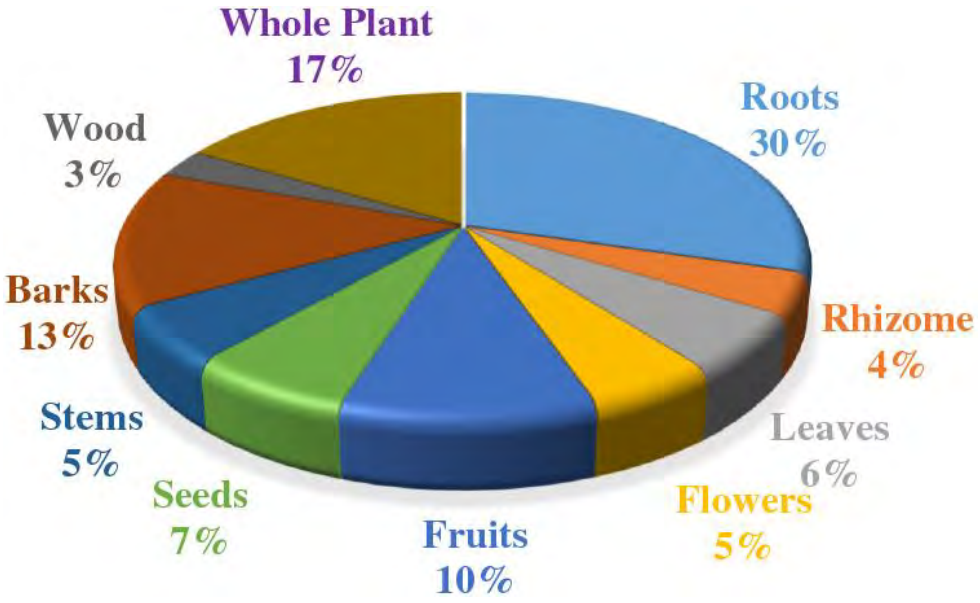

Figure 2.2: Proportions of plant parts used medicinally (101)

Table 2: Common medicinal plants used for the treatment of STIs in SA (102)

Botanical name	**Family**	**Vernacular name**	**Parts used**	**Mode of preparation & administration**	**STIs treated**
Blepharis diversispina	Acanthaceae	Mooka pitsi (Sepedi)	Root	Decoction and taken orally	Chlamydia
Carica papaya	Caricaceae	Mophopho (Sepedi)	Root	Decoction and taken orally	Gonorrhoea
Arctopus monacanthus	Apiaceae	Sieketroos (Afrikaans)	Root	Pound, decoction and taken orally	Gonorrhoea Syphilis
Albizia adianthifolia	Fabaceae	Igowane (Zulu)	Leaves	Bark is boiled with *T. dregeana* bark in 2 L of water and taken as an enema	Gonorrhoea Syphilis
Bidens pilosa	Asteraceae	Uqandolo	Whole plant	Stem and leaves are combined with *C. brachiate*, *R. multifidus*, and *S. sanguinea* stem and boiled in water and then taken orally	Syphilis Genital sores

2.4.1 Phytochemicals found in medicinal plants

Plants produce secondary metabolites that have therapeutic properties such as antioxidant, anti-inflammatory, antifungal, antimalarial, anticancer, and antibacterial properties (103). Medicinal plants have phytochemicals, which are bioactive compounds produced by the plants with therapeutic benefits. These phytochemicals include flavonoids, phenols, alkaloids, saponins, tannins and others, and are classified based on their chemical structures (104). Figure 2.3 shows the major phytochemical groups found in medicinal plants, with phenols, alkaloids, and terpenoids representing the most.

Phenolic compounds are characterized by a phenyl ring bearing one or more hydroxyl substituents. They serve to protect plants from disease or damage and also contribute to the plant's colour, aroma and flavour (102,105). Within this class of compounds, flavonoids, phenolic acids, and tannins are considered the main groups (106,107). Some polyphenolic compounds serve as scavengers that remove reactive oxygen species (ROS) molecules generated during oxidative bursts (106–108).

Flavonoids are polyphenolic molecules containing 15 carbon atoms with two aromatic rings and are the largest group of polyphenols (106). They are further composed of several subclasses including flavonols, flavan-3-ols, isoflavones, flavones, anthocyanidins, and flavanones (106,108) Health benefits attributable to the flavonoids include prevention of oxidative cell damage (antioxidant and free radical scavengers), anti-inflammatory, anti-carcinogenic and antibacterial effects (109).

Phenolic acids protect plants against environmental stressors such as heat, infections, and wounding (110). They also have been shown to have biologically important activities, including antioxidant, anti-inflammatory, antimutagenic, and anticancer properties (107–109).

Saponins are a group of compounds belonging to glycoside derivatives of steroids found in many plant species and are known to form colloidal suspension upon shaking in water. They are also known for their haemolytic, antimicrobial and antioxidant activities (107,109).

Alkaloids are a class of nitrogen-bearing, cyclic organic compounds, and some well-known drugs that are alkaloids include atropine, morphine and quinine (107,109,111).

Terpenes and terpenoids are large, structurally diverse secondary metabolites found in plants and are comprised of five-carbon isoprene units that are arranged in various combinations. They have been shown to possesses antimicrobial, antifungal, antiparasitic, antiviral, antiallergenic, antispasmodic, antihyperglycemic, anti-inflammatory and immunomodulatory properties (109).

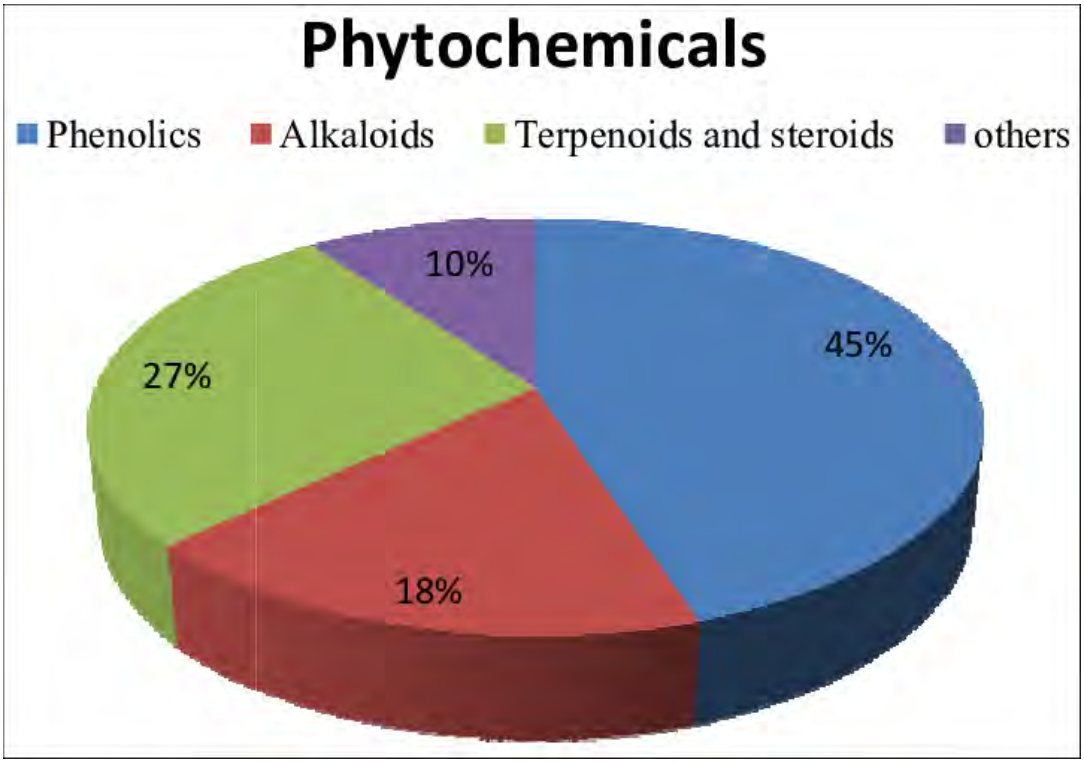

Figure 2.3: Major phytochemical groups found in medicinal plants (104)

2.4.2 *Hypoxis hemerocallidea*

H. hemerocallidea is a herbaceous plant with star-shaped leaves and tuberous roots as shown in figure 2.4, that belongs to the Hypoxidaceae family (112). It is also known as the African potato, star lily, yellow star, afrika-patat (Afrikaans), ilabatheka (Zulu), inongwe (isiXhosa), lotsane (Sesotho). It is easily identified by its yellow star-shaped flowers and strap-like leaves (112). *H. hemerocallidea* is predominantly found in Southern Africa, mainly Lesotho, South Africa, Mozambique, and Zimbabwe and grows in fertile, well-draining fields (113). Its use in Africa is

widespread and it is one of the few medicinal plants of scientific interest. The extracts of its tuberous roots, also known as the corm, are used to make decoctions, taken orally which are used to treat a number of ailments such as enhance the immune system, treat infections and inflammation, digestive issues, boosting immunity, and wound healing etc (114).

The main phytochemical found in *H. hemerocallidea* is a glycoside called hypoxoside, which undergoes hydrolysis by β-glucosidase to form rooperol (113) as shown in figure 2.5. Hypoxoside has no biological activity, but gets converted to rooperol, which is biologically active, and is known to exhibit strong antioxidant, anti-inflammatory, and anticancer properties (99).

Figure 2.4: Hypoxis hemerocallidea (African potato) (112)

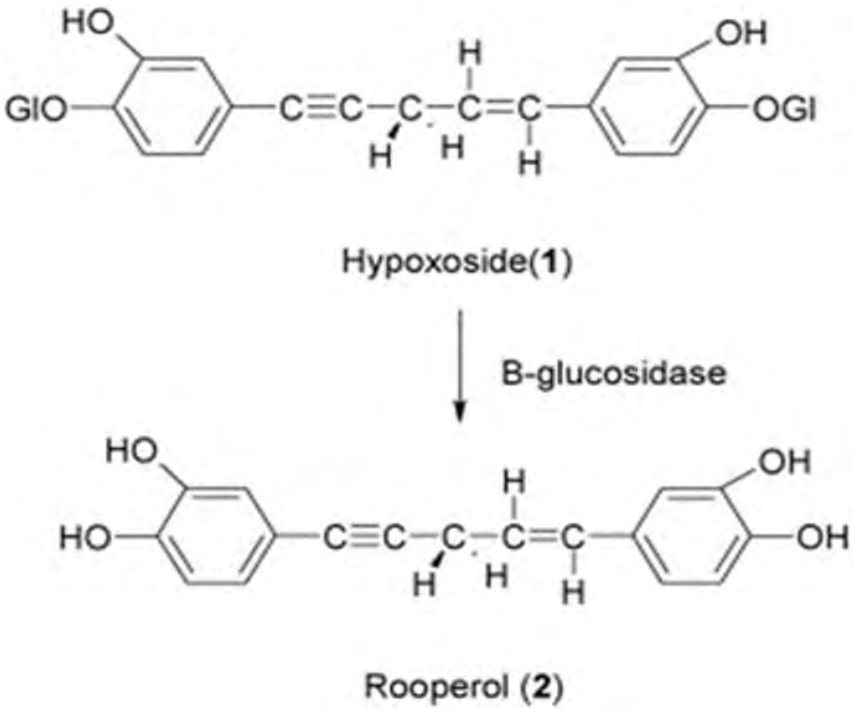

Figure 2.5: Structure of hypoxoside and rooperol (99)

A study conducted by Viallard et al. (2006) showed that *H. hemerocallidea* shows potential pharmacological properties (115). A methanolic extract of this plant was administered to patients who had been living with HIV for over 2 years in the mid-1990s and, it was noted that the CD4+ count remained stable, and the serum p24 HIV antigen decreased (115). From these observations, it was concluded that rooperol, a phytochemical in *H. hemerocallidea* has potent pharmacological properties relevant to cancer, inflammation and HIV (115).

A cytotoxicity study done in 2011 on *Hypoxis* species using cancer cell lines, revealed that the extracts induced cell cycle arrest and apoptosis of the cells (116). Another study also performed cytotoxicity tests on *H. hemerocallidea* using the brine shrimp lethality assay and concluded that the plant is genetically non-toxic (117). Another study assessed the genotoxicity of water extracts of different *Hypoxis* species, including *H. hemerocallidea*, and the study revealed that no genotoxic potential was found in human hepatoma cells (118). However further research needs to be done to understand the mechanism of action and identify any potential drug interactions of the plant. The selection of this plant for the study is based on its use in traditional medicine by an indigenous

health practitioner, and it is believed that it has a long history of use for different therapeutic purposes, making it of significant interest for scientific investigation.

2.4.3 *Hydnora africana*

H. africana is a parasitic plant as shown in Figure 2.7, that belongs to the Hydnoraceae family. It can be found in dry and semi-dry southern Africa countries, such as South Africa, Namibia, and Botswana (119). It is also known as jackal food, mavumbuka (isiZulu), ubuklunga, bobbejaankos (Afrikaans). It feeds on host plants through their roots since it lacks chlorophyll. It has a fibrous stem that develops underground and bright orange to red-flowers that emerge from the ground (120).

Decoctions and infusions of *H. africana* have been used to treat dysentery, diarrhoea, kidney and bladder complaints, gastrointestinal disorders, and skin cuts (121). In the Amathole district municipality, Eastern Cape, South Africa, it was noted in an ethnobotanical survey that *H. africana* was the most frequently prescribed medicinal plant for managing dysentery among traditional medical practitioners, herb-sellers and rural elders (121). In Sudan, this plant (*H. africana*) decoction is also used as a remedy for inflammation and tonsillitis (122).

H. africana is rich in phytochemicals such as flavonoids, alkaloids, phenolics, tannins, and antioxidants. *H. africana* extracts have shown efficacy against bacterial and fungal pathogens, supporting its traditional use in treating infections. Studies indicate that the plant's bioactive compounds may inhibit bacterial strains such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (123). The presence of phenolic and flavonoid compounds contributes to the plant's antioxidant activity, and antioxidant activity is important because it supports and strengthens the immune system, protects cells from damage, and prevents chronic illnesses. By investigating the mechanism of action and identifying any potential interactions of this plant with other conventional drugs, this could contribute insight into its bioactive properties.

Figure 2.6: Hydnora africana (122)

2.5 Liver enzyme metabolism studies

The liver is an organ located in the upper right portion of the abdomen and is involved in many metabolic and physiological processes that occur in the body (124). These processes are performed by a complex network of enzymes known as the cytochrome P450 (CYP) enzymes. CYP enzymes are involved in the biochemical reactions, responsible for drug clearance, detoxification, metabolism of substances and drugs, toxins and endogenous compounds (125). Understanding liver enzyme metabolism, particularly the CYP system is crucial in understanding pharmacological processes and their implications for drug discovery (126).

There are different isoforms of CYP450 enzymes such as CYP2D6, CYP3A4, CYP3A5, CYP2C9 which have different substrate specificities. Figure 2.8 shows two pie charts of different CYP450 isoforms and their abundance. A study showed how CYP2D6 played a role in the metabolism of the drug, tramadol, and different models predicted its clearance with notable discrepancies in CYP2D6 contribution, underscoring the complexity of drug metabolism (127).

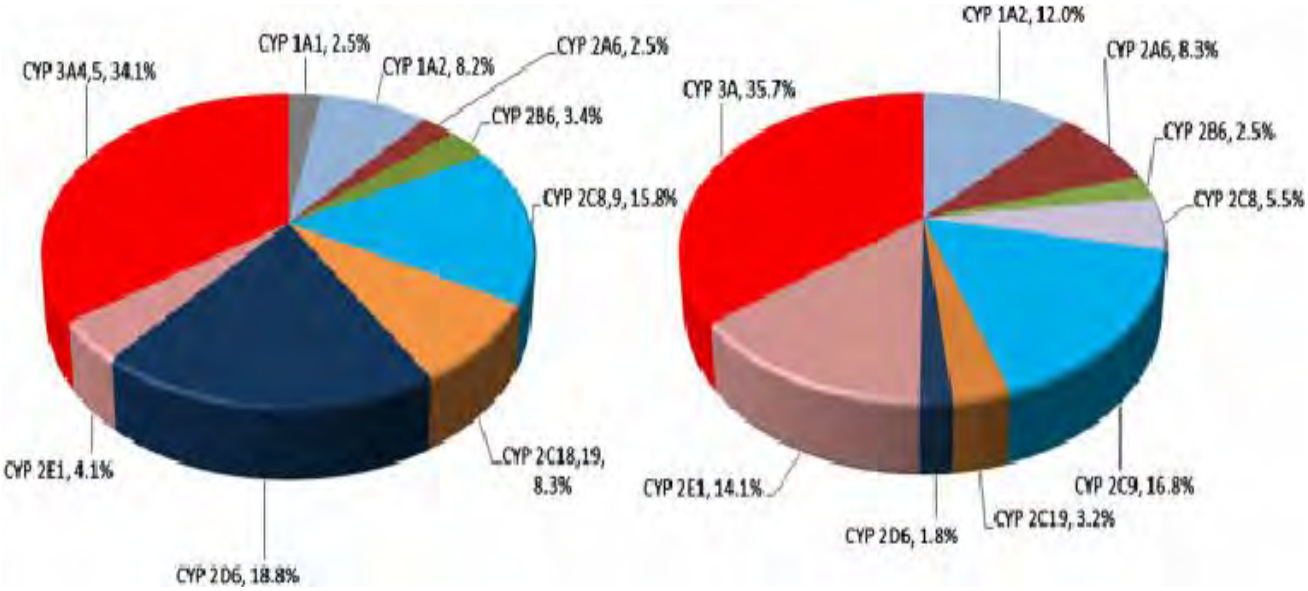

Figure 2.7: The left chart represents the proportions of drugs metabolised by CYP450 enzymes, while the right chart represents the CYP450 enzyme abundance in the liver (128)

When natural products and CYP450 enzymes interact, potential risks could arise, because natural products may not have undergone rigorous safety evaluations, thus increasing the chances of metabolic bioactivation and side effects (127,129). Enzyme inhibition studies are important for drug development, as they guide on the mechanism of enzymatic action and help to identify metabolic pathways (130). These studies provide guidance on how to understand potential drug-drug interactions, which may lead to toxicity of co-administered drugs (131).

The enzyme inhibitors can either be reversible or irreversible and affect enzyme activity, which may have serious implications for drug therapy. Reversible enzyme inhibitors bind non-covalently to the active site, allowing for the potential recovery of enzyme function upon their removal, while irreversible enzyme inhibitors form covalent bonds with the enzyme, leading to permanent inactivation. For example, the pharmacokinetics of tramadol highlight the importance of recognizing specific CYP isoforms involved in drug metabolism; variations in CYP2D6 activity significantly affect tramadol clearance and overall therapeutic efficacy, as demonstrated in metabolic studies using human liver microsomes (127).

An area of concern is the potential interaction between medicinal plants and antimicrobial drugs, because different plants may induce or inhibit the effectiveness of the dug. The compounds in the plants may inhibit the effectiveness of the drug by affecting absorption or metabolism of the drug reducing efficacy such as St. John's Wort. Therefore, this is important evidence to justify that potential drug interactions is important for this study because growing issues such as antibiotic resistance can be tackled, some plants may interfere with antibiotic effectiveness or cause side effects, so it is important to know potential risks to avoid treatment failure or harm.

2.6 Chapter summary

This literature review suggests that traditional medicine and ethnobotany could have the potential to find alternative treatments for infections and tackle the crisis of AMR. When AMR spreads and healthcare is scarce in low-resource places, *H. hemerocallidea* and *H. africana* may be innovative plant-based remedies.

Chapter 3: Qualitative screening and quantitative analysis of different solvent extracts of *H. hemerocallidea* and *H. africana* samples

3.1 Introduction

Many infectious diseases have been treated with plant extracts (132). Medicinal properties of plants are attributed to the different active compounds that they produce. The phytochemicals (secondary metabolites) have potential health benefits that help alleviate illness. The phytochemicals are classified according to their chemical structures and include phenolic compounds, flavonoids, alkaloids, terpenoids, saponins etc (103). Maceration is an extraction technique used to obtain plant extracts, whereby the plant material is soaked in solvent, with occasional agitation, at a particular temperature. The solvent dissolves the active compounds from the plant material over time (133).

H. hemerocallidea and *H. africana* are both indigenous to Southern Africa and have been studied for their medicinal properties. Traditionally, extracts of *H. hemerocallidea* are made into decoctions and taken orally to treat ailments such as infections and inflammation, digestive issues, boosting immunity, and wound healing (114). *H. africana* is used to treat dysentery, diarrhoea, kidney and bladder complaints, gastrointestinal disorders, and skin cuts (121). This chapter outlines the method used for extraction, qualitative identification, and quantitative analysis of four solvent extracts of the *H. hemerocallidea* and *H. africana* plant samples.

3.2 Methods and materials

3.2.1 Sample collection and preparation

The corms of *H. hemerocallidea* and *H. Africana*, as shown in Appendix A, were collected from a South African professional indigenous health practitioner, Makhosi Thandokazi May, who runs her indigenous heath practice and is based in the Eastern Cape, Makhanda (Grahamstown). The corms were cleaned and allowed to air dry at room temperature, as seen in Appendix B. Once completely dry, the corms were ground into fine powders using an electric grinder and stored in glass vials at room temperature.

3.2.2 Extraction of plant material

The procedure used for extraction of the plant material was maceration. Four solvents of increasing polarity, namely; hexane, dichloromethane (DCM), methanol, and water, were used for the process. The powdered material was transferred into a conical flask with hexane solvent. The conical flask was placed into the sonicator (Mrc, Professional ultrasonic cleaner) at 37℃ for one hour, as shown in Appendix B and C. After one hour, the filtrate was filtered to separate liquid and solid fractions using filter paper discs (Filter Discs Qual, three hw, 125 mm, Germany). Hexane solvent was added to the conical flask and the process was repeated for another hour, and the filtrate was combined with the first one. Hexane solvent was added again to the conical flask, which was transferred to a rotator orbital shaker overnight (Appendix C), for 18 hours, set to 180 revolutions per minute (rpm). The following morning, the supernatant was filtered and combined with the previous two batches. The extract was then concentrated under reduced pressure at 40℃ using a rotary evaporator (Appendix C) and a speed of 120 rpm. The extraction process was repeated for the other three solvents: DCM, methanol, and water. The crude extracts were stored at room temperature for further use.

3.2.3 Qualitative phytochemical analysis

Qualitative phytochemical screening was performed to identify the presence of different phytochemicals (bioactive compounds) in the plant extracts. All plant extracts initially underwent preliminary phytochemical screening following the procedure described by Alemu et al. (2024), with slight modifications (134). This screening identifies phytochemicals using specific reagents that react and produce specific colours with variable intensities.

3.2.3.1 Phenols

A few drops of ferric chloride solution were added to a one millilitre (ml) of plant extract. The development of a dark green or bluish-green colour indicated the presence of phenolic compounds. The colour results due to the formation of a complex between the phenolic group and the iron ion in ferric chloride solution.

3.2.3.2 Flavonoids

A volume of 2 ml of the plant extract was mixed with two ml of dilute NaOH solution, followed by adding one ml of dilute HCl. The formation of a yellow colour, which turned colourless upon the addition of dilute HCl, confirmed the presence of flavonoids.

3.2.3.3 Saponins

A volume of 2 ml of the plant extract was diluted with one ml of distilled water and shaken vigorously for approximately 30 seconds. The formation of a stable foam layer persisting on the surface for at least 10 minutes indicated the presence of saponins, and this is due to surfactant properties of saponins.

3.2.3.4 Alkaloids

Wagner's reagent (iodine in potassium iodide solution) was added to one ml of the plant extract. The formation of a reddish-brown precipitate was considered a positive test for alkaloids.

3.2.3.5 Quinones

A few drops of NaOH solution were added to two ml of plant extract. The development of a pink or red colour indicated the presence of quinones. This is due to quinones ability to undergo oxidation reactions.

3.2.3.6 Terpenoids

Salkowski's test - a few drops of concentrated H_2SO_4 were added to one ml of the plant extract. The appearance of a reddish-brown colour at the interface of the two layers was considered a positive test for terpenoids.

3.2.3.7 Anthraquinones

A few drops of NaOH solution were added to two ml of plant extract. The development of a pink or red colour indicated the presence of anthraquinones.

3.2.3.8 Steroids

A volume of two ml of the plant extract was mixed with two ml of chloroform, and one ml of concentrated H_2SO_4 was then carefully layered beneath the chloroform layer. The formation of a green or blue-green colour at the chloroform-acid interface was considered a positive test for steroids.

3.2.3.9 Tannins

The plant extract (2 ml) was diluted with five ml of distilled water, and 2-3 drops of ferric chloride solution were added. The development of a black or blue-green colour indicated the presence of tannins. The resulting colour was due to the formation of a complex with the iron ion of ferric chloride.

3.2.3.10 Coumarins

The plant extract (2 ml) was diluted with five ml of distilled water, and two ml of NaOH solution was added. The formation of a yellow colour indicated the presence of coumarins.

3.2.3.11 Phlobatannins

The plant extract (1 ml) was treated with one ml of 1% HCl. The formation of a red precipitate was considered a positive test for phlobatannins.

3.2.4 Quantitative phytochemical analysis

3.2.4.1 Total phenolic content

The total phenolic content (TPC) in *H. hemerocallidea* and *H. africana* plant extract was measured using the Folin-Ciocalteu (FC) spectrophotometric method with slight modifications (135). FC allows the quantification of the phenolic content in plant extracts using a colorimetric principle, by measuring the absorbance of the blue complex formed when the extract reacts with the phenolic compounds in FC reagent, and this is quantified using a calibration curve with a known standard (136), as shown in Appendix D.

Gallic acid was used as the positive standard reference for calibration with concentrations ranging from 5 µg/ml to 1000 µg/ml in 10 mL ethanol. The plant extracts were prepared with dimethyl

sulfoxide (DMSO) solution with concentrations of 5 µg/ml, 50 µg/ml, and 500 µg/ml. A volume of 25 uL of extract was pipetted into each well in triplicate and combined with 125 uL of FC reagent (diluted 1:1 with distilled water). After 5 minutes, 100 uL of 7.5% anhydrous sodium carbonate was added to each well, and the mixture was incubated in the dark at room temperature for two hours. Absorbance was measured at 765 nm on a microplate spectrophotometer reader (Epoch 2 Microplate Reader, BioTek Instruments, Winooski, VT, USA). The standard gallic acid calibration curve was used to calculate the TPC using the equation y = mx + c, and TPC was expressed as mg/g gallic acid equivalents (GAE) using the formula:

$C = xV / m$

where:

C: TPC in mg/g gallic acid equivalents

x: concentration from the standard curve

V: volume of extract in mL

m: mass of plant extract in grams

3.2.4.2 Total flavonoid content

The total flavonoid content (TFC) in each plant extract of *H. hemerocallidea* and *H. africana* was measured following the procedure described by Matic et al. (2017) with modifications (137). Quercetin was used as the standard reference for calibration with concentrations ranging from 5 µg/ml to 1000 µg/ml in 10 mL ethanol, as shown in Appendix E. Plant extracts were prepared with DMSO with concentrations of 5 µg/ml, 50 µg/ml, and 500 µg/ml. A volume of 12.5 uL of plant extract was pipetted into each well in triplicate and combined with 12.5 uL of HCl (0.1% v/v in 95% ethanol). Then 225 uL of HCl was added (2% v/v in distilled water) to each well. The reaction mixture was incubated at room temperature for 30 minutes, and absorbance was measured at 360 nm on a microplate spectrophotometer reader (Epoch 2 Microplate Reader, BioTek Instruments, Winooski, VT, USA). The quercetin calibration curve was used to calculate the TFC using the equation y = mx + c, and TFC was expressed as mg/g quercetin equivalents (QE) using the formula:

$C = xV / m$

where:

C: TFC in mg/g quercetin equivalents

x: concentration from the standard curve

V: volume of extract in mL

m: mass of plant extract in grams

3.2.4.3 Data analysis

Once the absorbance readings for TPC and TFC were obtained and recorded, the readings were exported to Microsoft Excel. Calibration curves using known concentrations of the standards, gallic acid for TPC and quercetin for TFC, were generated. The phenolic content and flavonoid content were then calculated based on the calibration curves. No statistical comparison was done for the data. The results were presented as the mean of the triplicate measurements.

3.3 Results

The qualitative phytochemical screening results of *H. hemerocallidea* and *H. africana* plant extracts are shown in Tables 3.1 and 3.2 respectively. They revealed the presence and absence of different phytochemicals including phenols, flavonoids, saponins, alkaloids, terpenoids, tannins, and coumarins. The amounts differed in the different solvents used for extraction, with the methanol and water solvent extracts containing an appreciable amount of most phytochemicals.

3.3.1 Qualitative phytochemical analysis

Table 3.1: Qualitative phytochemical screening of H. hemerocallidea plant extracts

Phytochemical	**Extract Samples**			
	Hexane	**DCM**	**Methanol**	**Water**
Phenols	+/-	+/-	++	+++
Flavonoids	-	+	++	+++
Saponins	-	-	+/-	+
Alkaloids	+/-	+/-	+	++
Quinones	-	-	+	+
Terpenoids	-	+/-	++	+++
Anthraquinones	-	-	+	+
Steroids	-	-	+++	++
Tannins	+/-	+/-	++	+++
Coumarins	-	+	++	++
Phlobatannins	-	-	-	-

[Impressive presence (+++); Present in appreciable quantity (++); Moderate presence (+); Trace presence (+/-); Not present (-)]

Table 3.2: Qualitative phytochemical screening of H. africana plant extracts

Phytochemical	**Extract Samples**			
	Hexane	**DCM**	**Methanol**	**Water**
Phenols	+/-	+/-	++	+
Flavonoids	-	+	++	-
Saponins	-	-	+/-	-
Alkaloids	+	+	++	++
Quinones	-	-	+/-	-
Terpenoids	-	-	+	-
Anthraquinones	-	-	-	-
Steroids	-	-	+	-
Tannins	+/-	+/-	++	+
Coumarins	-	+	++	+/-
Phlobatannins	-	-	-	-

[Impressive presence (+++); Present in appreciable quantity (++); Moderate presence (+); Trace presence (+/-); Not present (-)]

3.3.2 Quantitative phytochemical analysis

3.3.2.1 Total Phenolic Content

The phenolic content in the *H. hemerocallidea* extracts was measured using the FC method and the results expressed as mg/g GAE. The methanol extract showed the highest phenolic content, followed by the water extract at the highest concentration as shown in Figure 3.1. The hexane and DCM extracts showed lower phenolic content values. The phenolic content of the *H. africana* extracts showed a similar trend to the *H. hemerocallidea* extracts, with methanol yielding the

highest phenolic content, followed by the water extract. The hexane and DCM extracts showed low phenolic content as shown in Figure 3.2.

Table 3.3: Total phenolic content of H. hemerocallidea plant extracts

Total phenolic content of *H. hemerocallidea* extracts in mg/g GAE				
	Hexane	**DCM**	**Methanol**	**Water**
5 µg/ml	0.063 ± 0.001	0.073 ± 0.0006	0.135 ± 0.002	0.080 ± 0.004
50 µg/ml	0.089 ± 0.003	0.126 ± 0.006	0.493 ± 0.003	0.092 ± 0.005
500 µg/ml	0.286 ± 0.006	0.413 ± 0.004	3.486 ± 0.019	0.731 ± 0.023

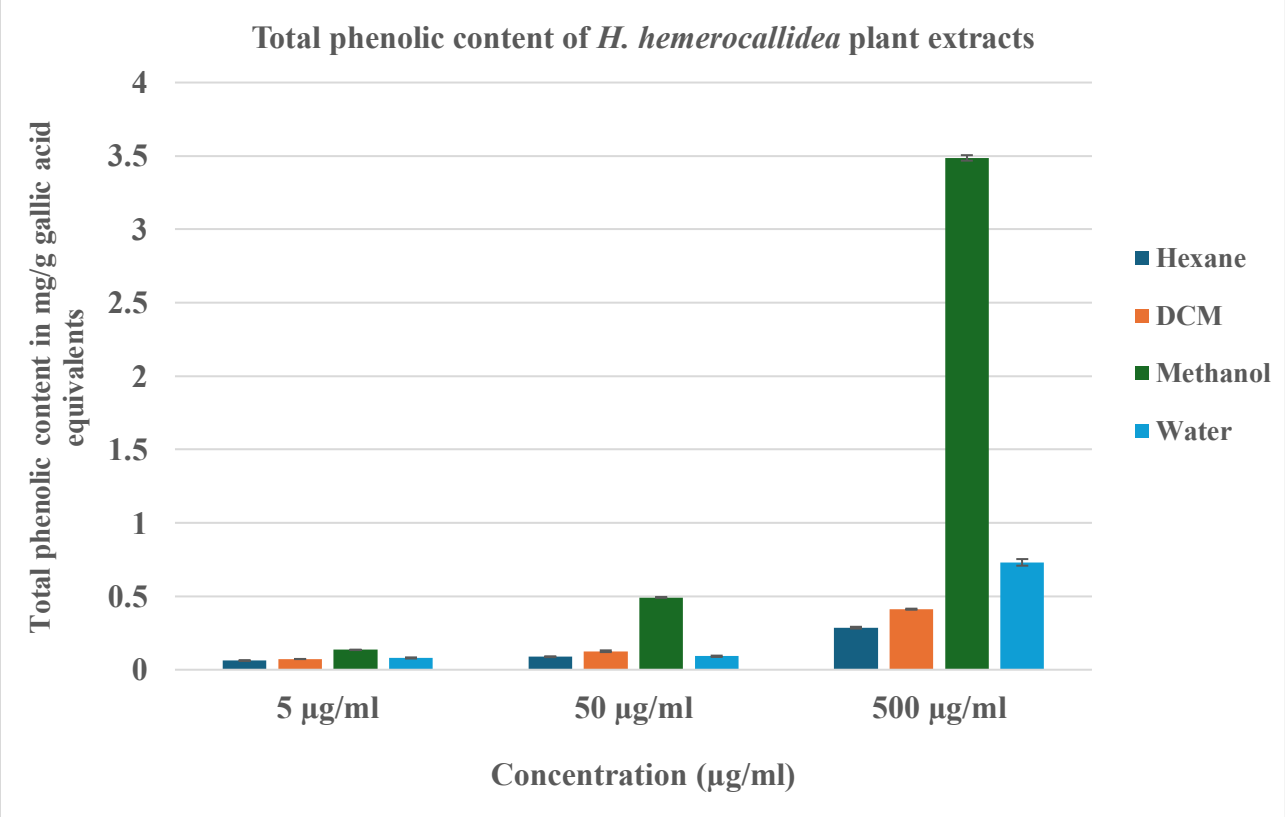

Figure 3.1: Total phenolic content of H. hemerocallidea plant extracts in mg/g gallic acid equivalents. Four different solvent extracts: Hexane, DCM, Methanol, Water at concentrations of 5 µg/ml, 50 µg/ml, and 500 µg/ml.

Table 3.4: Total phenolic content of H. africana plant extracts

Total phenolic content of *H. africana* extracts in mg/g GAE				
	Hexane	**DCM**	**Methanol**	**Water**
5 µg/ml	0.026 ± 0.003	0.142 ± 0.019	0.177 ± 0.010	0.353 ± 0.009
50 µg/ml	0.076 ± 0.006	0.208 ± 0.008	0.762 ± 0.003	1.524 ± 0.001
500 µg/ml	0.233 ± 0.016	1.254 ± 0.014	4.379 ± 0.016	8.758 ± 0.024

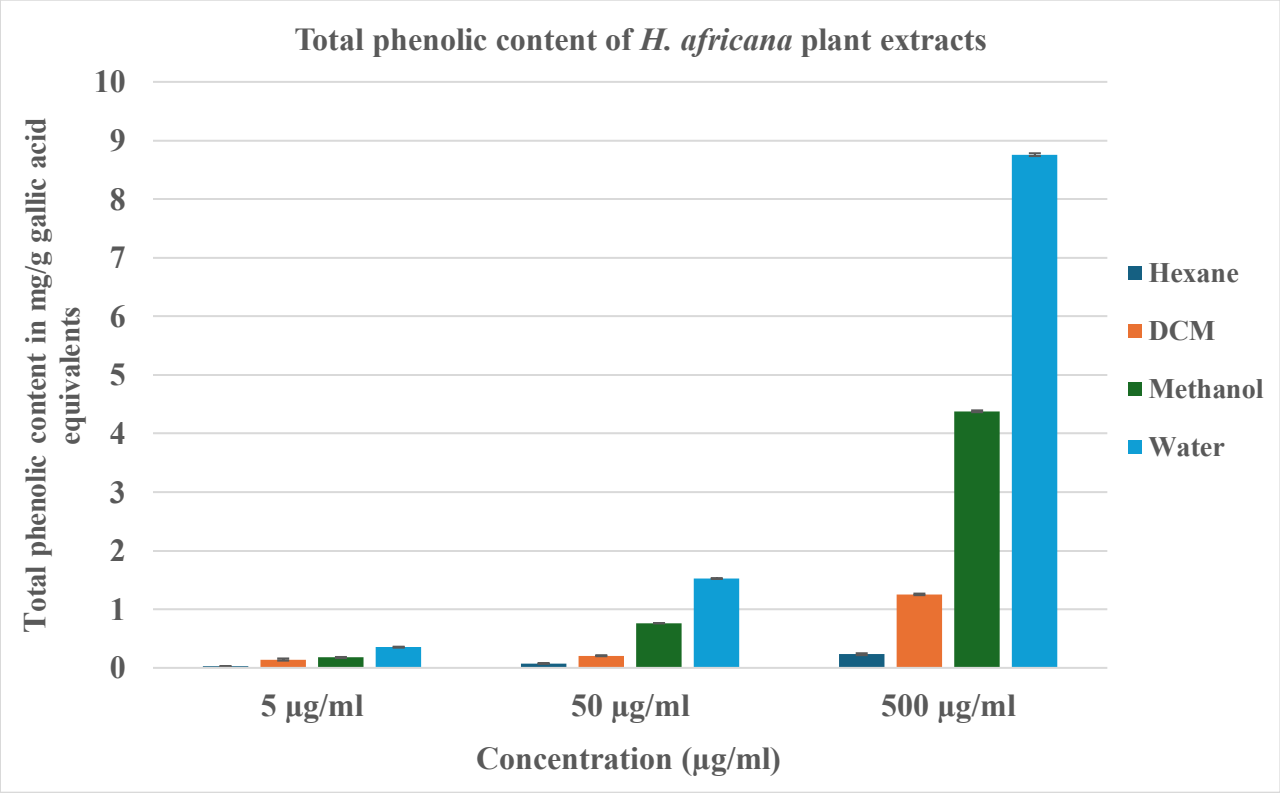

Figure 3.2: Total phenolic content of H. africana plant extracts in mg/g gallic acid equivalents. Four different solvent extracts: Hexane, DCM, Methanol, Water at concentrations of 5 µg/ml, 50 µg/ml, and 500 µg/ml.

3.3.2.2 Total Flavonoid Content (TFC)

The flavonoid content in the *H. hemerocallidea* extracts are expressed as mg/g QE. The hexane extract showed the highest flavonoid content, followed by the DCM extract at the highest concentration as shown in Figure 3.3. This shows a concentration-dependent relationship.

Table 3.5: Total flavonoid content of H. hemerocallidea plant extracts

Total flavonoid content of *H. hemerocallidea* extracts in mg/g QE				
	Hexane	**DCM**	**Methanol**	**Water**
5 µg/ml	0.254 ± 0.008	0.262 ± 0.011	0.135 ± 0.005	0.157 ± 0.006
50 µg/ml	0.385 ± 0.008	0.319 ± 0.003	0.145 ± 0.003	0.164 ± 0.005
500 µg/ml	1.145 ± 0.010	1.043 ± 0.002	0.266 ± 0.011	0.526 ± 0.008

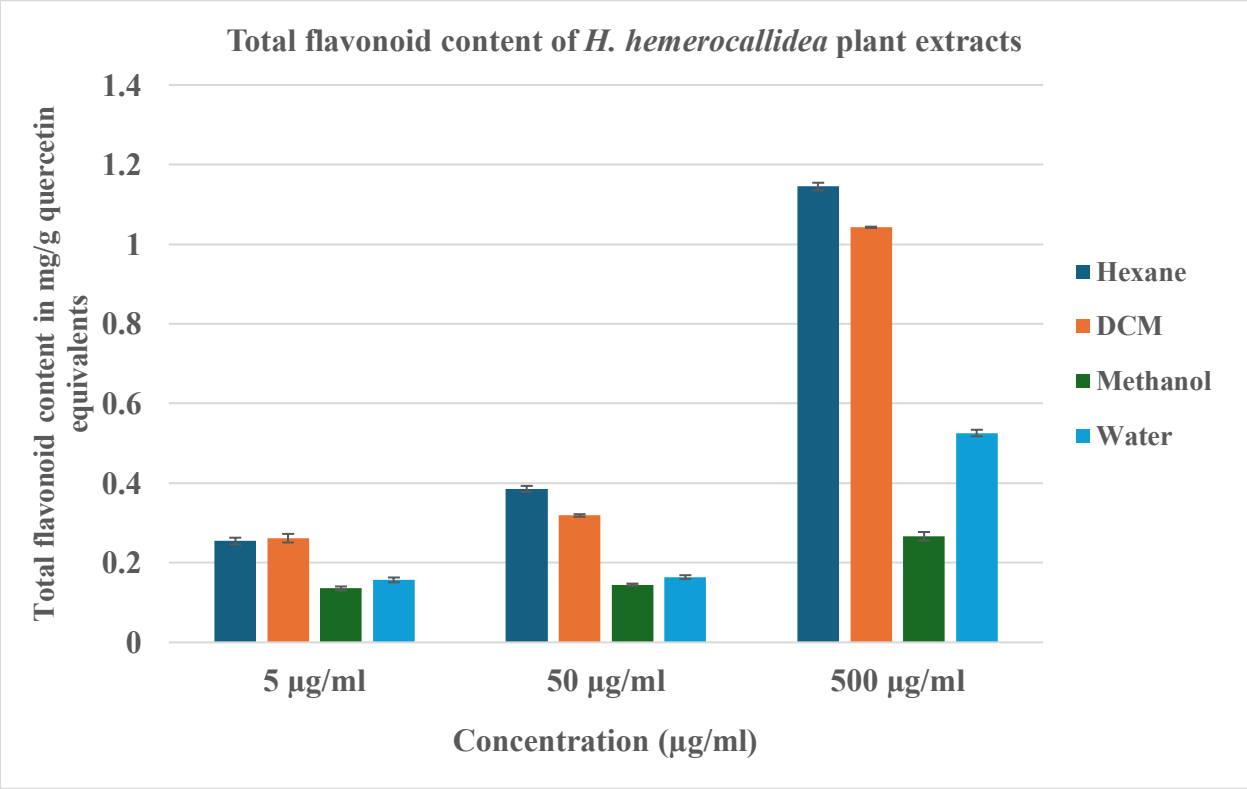

Figure 3.3: Total flavonoid content of H. hemerocallidea plant extracts in mg/g quercetin equivalents. Four different solvent extracts: Hexane, DCM, Methanol, Water at concentrations of 5 µg/ml, 50 µg/ml, and 500 µg/ml.

Table 3.6: Total flavonoid content of H. africana plant extracts

Total flavonoid content of *H. africana* extracts in mg/g QE				
	Hexane	**DCM**	**Methanol**	**Water**
5 µg/ml	0.041 ± 0.006	0.004 ± 0.002	0.104 ± 0.004	0.120 ± 0.020
50 µg/ml	0.0003 ± 0.006	0.037 ± 0.003	0.110 ± 0.019	0.127 ± 0.022
500 µg/ml	1.230 ± 0.020	2.344 ± 0.013	0.400 ± 0.018	0.377 ± 0.006

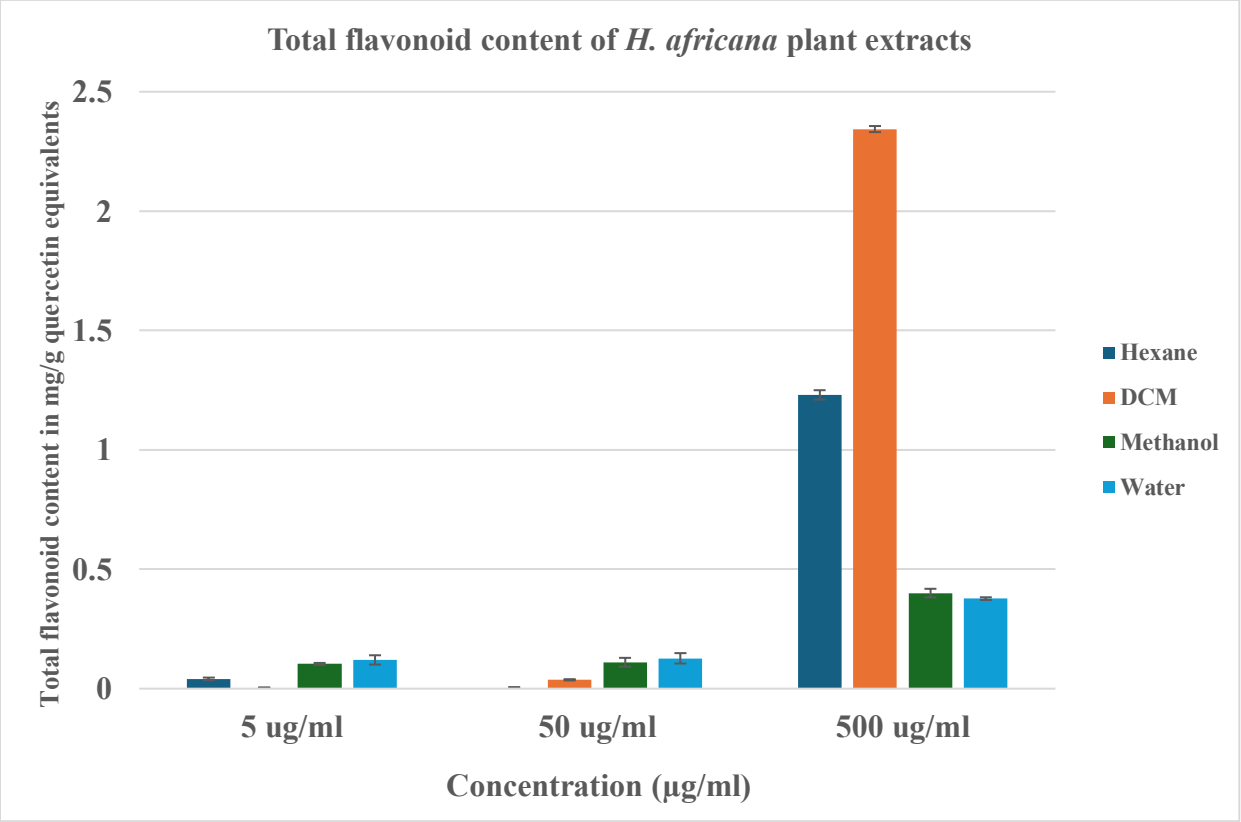

Figure 3.4: Total flavonoid content of H. africana plant extracts in mg/g quercetin equivalents. Four different solvent extracts: Hexane, DCM, Methanol, Water at concentrations of 5 µg/ml, 50 µg/ml, and 500 µg/ml extract.

3.4 Discussion

3.4.1 Qualitative phytochemical analysis

The presence of phytochemicals in plants may explain their use in traditional medicine. Phytochemical screening of *H. hemerocallidea* and *H. africana* extracts in different solvent extracts showed the presence of phenols, flavonoids, tannins, and alkaloids, especially in methanol and water extracts. The screening for plant secondary metabolites is essential in understanding the plants' pharmacological properties to support their traditional use in treating infections and other ailments (138). The solvent used for extraction has an influence on the type of phytochemical and therefore amount of phytochemical present in the particular extract (138).

Solvent choice in extraction has an effect on the phytochemicals present in the extracts as well as the amount present (139). The phytochemicals found in the plants such as phenolic compounds, flavonoids, alkaloids, terpenoids have different solubilities in the different solvents (139). Solvent polarity influences the solubility of the compounds, for example the more polar phytochemicals such as phenols and flavonoids dissolve better in polar solvents such as methanol and water, whereas the non-polar phytochemicals such as terpenoids tend to dissolve better in less polar solvents such as hexane and DCM (140).

The polarity of a solvent also determines its ability to interact with specific phytochemicals (140). For instance, a solvent like methanol which is polar are often chosen because they can extract a broad spectrum of compounds, whereas non-polar solvents such as chloroform or hexane are more selective for lipophilic substances (140). Extraction yield also differs with different solvents due to the solvent's ability to break down the plant tissue and dissolve the phytochemicals. Again, polar solvents like methanol and water have higher yields compared to less polar solvents like hexane.

Table 3.1 shows some of the phytochemicals present in *H. hemerocallidea,* which included phenols, flavonoids, saponins, alkaloids, terpenoids, anthraquinones, and steroids. This corresponds with the findings of Bassey et al. (2014) regarding the phytochemicals found in *Hypoxis* species, which included alkaloids, phenols, flavonoids, saponins, alkaloids, terpenoids, and steroids (141). The hexane extract showed the least presence of phytochemicals. It contained trace amounts of phenols, alkaloids, and tannins. The DCM extract showed slightly more trace

amounts of phenols, alkaloids, terpenoids, and tannins, and a moderate presence of flavonoids and coumarins. There was a strong presence of phenols and flavonoids in the methanol and water extracts. Methanol and water are both polar solvents, which are able to efficiently extract polar compounds like phenols and flavonoids, by forming hydrogen bonds with the compounds. Saponins, alkaloids, and terpenoids were present in moderate to high levels in the methanol and water extracts as well, suggesting that polar solvents are more effective in extracting a wider range of phytochemicals from *H. hemerocallidea*. A similar study of *H. hemerocallidea* extracts found phenols, flavonoids, saponins, alkaloids, tannins, and terpenoids particularly in the methanol extracts (117). Tannins were highly present in methanol and water. Other compounds like anthraquinones, coumarins, and steroids were also detected in the extracts. The test used for phlobatannins did not indicate their presence across all extracts.

Table 3.2 shows the phytochemical screening results of *H. africana* which revealed the presence of different phytochemicals in the different extracts, particularly, the methanol extract which showed their presence in high concentrations. Phenols, flavonoids, saponins, alkaloids, quinones, tannins, and coumarins were found. This is similar to a study conducted by Nethate et al. (2011), where alkaloids, tannins, and flavonoids were present in large quantities in the methanol extracts (142). The hexane extract showed trace amounts of phenols and tannins, with a moderate presence of alkaloids. The water extract had fewer phytochemicals, that is phenols, alkaloids, and tannins. Due to the polar nature of methanol, it was able to extract compounds more efficiently (119).

Phenols possess strong antioxidant properties, which neutralise free radicals, and this supports the use of *H. hemerocallidea* and *H. africana* in managing inflammatory conditions and protecting the body (143). Phenols also possess antimicrobial properties (144). Flavonoids are also antioxidants and were moderately present in the DCM extract and strongly present in the methanol and water extracts. They reduce inflammation, neutralise reactive oxygen species and enhance the immune system (145). Flavonoids have been noted to have antidiabetic, anticancer, and neuroprotective properties, therefore their presence in *H. hemerocallidea* may contribute to its traditional use in treating inflammatory conditions and metabolic disorders (145).

Terpenoids were present in the DCM, methanol, and water extracts in different amounts. They are known to possess antimicrobial and anti-inflammatory properties and their presence in the plant

supports its role in enhancing the immune system and fighting off infections (105). Tannins were present in all extracts, with higher concentrations observed in the methanol and water extracts. This is due to the fact that tannins are also polar compounds in nature, possessing hydroxyl groups, and water-soluble, and so are able to form hydrogen bonds with the polar solvents. Tannins have astringent properties and are able to inhibit bacterial and fungal growth. They also have antioxidant and anti-inflammatory effects, which may explain the traditional use of *H. hemerocallidea* in wound healing and gastrointestinal disorders (146). Alkaloids were seen in all extracts. The strong presence of steroids in the methanol and water extracts is significant, as these compounds are known to exhibit anti-inflammatory and immunomodulatory properties.

The strong presence of phytochemicals in the methanol and water extracts shows that polar solvents extract phytochemicals more efficiently, and this is due to the presence of the hydroxyl groups making the compounds more polar. The therapeutic properties of *H. hemerocallidea*, including its antioxidant, antimicrobial, anti-inflammatory, and immunomodulatory effects, can be attributed to the combined actions of phenols, flavonoids, alkaloids, tannins, terpenoids, coumarins, and steroids (147) .

3.4.2 Quantitative phytochemical analysis

The results for *H. hemerocallidea* and *H. africana* show that the plant extracts contain phenolic compounds, and the quantity of the phenolic compounds generally increases with increasing extract concentration. For *H. hemerocallidea*, at the highest concentration of 500 µg/ml, the methanol extract showed the highest TPC content, and this suggests that is could be the most effective solvent for extracting phenolic compounds from this plant. The water extracts also showed a significant increase in TPC at higher concentrations, suggesting that it could also be a good solvent for extracting these compounds. The hexane and DCM extracts showed lower TPC values compared to methanol and water, suggesting they might not be as effective in extracting phenolic compounds from this plant. Phenolic compounds are known for their antioxidant and anti-inflammatory properties (138, 139).

The *H. africana* extracts also contained phenolic compounds, and the quantity of phenolic compounds generally increased with increasing extract concentration. At 500 µg/ml, the water extract showed the highest phenolic content, indicating it might be the most effective solvent for

extracting phenolic compounds in this plant. The DCM also shows a significant increase in TPC at the highest concentration. The hexane and methanol extracts showed lower TPC values compared to the DCM and water extracts, suggesting they might not be as effective in extracting phenolic compounds from this plant. The high TPC in methanol and water extracts of both plants highlights the polar nature of phenolic compounds.

The flavonoid content in *H. hemerocallidea*, showed a similar trend to the phenolic content, with the methanol extract having the most flavonoids. The hexane and DCM extracts contained only trace amounts, suggesting a limited ability to solubilize flavonoids. Overall, the results indicate that the *H. hemerocallidea* contains flavonoids, and the amount increases with increasing concentration. Flavonoids are hydrophilic in nature and dissolve readily in polar solvents such as methanol and water (150), hence the observed results. This supports the potential for the plants to be used as antioxidants, as these phytochemicals can neutralize free radicals that cause oxidative stress (151). These findings from the quantitative phytochemical analysis suggest that *H. hemerocallidea* and *H. africana* could possess some therapeutic potential.

3.5 Chapter summary

The results of the phytochemical analysis of *H. hemerocallidea* and *H. africana* corm extracts confirmed the presence of various phytochemical compounds, including phenols, flavonoids, saponins, tannins, alkaloids, anthraquinones, quinones, terpenoids, steroids, and coumarins. The amount of these phytochemicals varied across different extracts. The quantitative analysis of phenolic content in *H. hemerocallidea* revealed that the methanol extract had the highest TPC, followed by the water extract, and the hexane and DCM extracts had lower TPC amounts. In *H. africana*, the methanol and water extracts had the highest TPC at the highest concentration. The flavonoid content in *H. hemerocallidea* revealed that the hexane and DCM had more flavonoids than the methanol and water extracts, whereas *H. africana* also showed a high flavonoid content in the hexane and DCM extracts at high concentrations.

Chapter 4: Antioxidant activity of different solvent extracts of *H. hemerocallidea* and *H. africana* samples

4.1 Introduction

Antioxidants are substances that inhibit oxidation, which is a chemical reaction that can produce free radicals, that lead to oxidative damage to our body structures. They prevent cell damage that is caused by the free radicals, so they play a role in maintaining cellular health (152).

Free radicals are unstable, highly reactive molecules that cause oxidative damage to cells, DNA, proteins, and other structures in the body (153). Free radicals are molecules that have at least one or more unpaired electrons in their outer shell, making them highly reactive (154). They are produced in metabolic processes, that naturally occur in the body, and high concentrations of these molecules can lead to oxidative stress, which results in damage to cell structures, and in the long run leads to the development of chronic conditions such as cancer, autoimmune disorders, neurodegenerative conditions and inflammation (155). This results when the body has too many free radicals and there not enough antioxidants to neutralize the free radicals (155).

Oxidative stress contributes to the onset and progression of various diseases, including diabetes, obesity, neurological diseases, such as Alzheimer's disease and Parkinson's disease and is also implicated in cardiovascular disease and cancer (156). Oxidative stress arises in bacterial infections, from altered metabolic pathways and has been implicated in organ damage and the development of malignancies (157). The oxidative stress can be initiated by the pathogen's own metabolic activity or in the process of altering host metabolism, and it can initiate damage to tissues resulting in imbalanced regulation processes (158).

Antioxidants are more stable than free radicals and are able to donate electrons to the free radicals and neutralise them (159). Some antioxidants are naturally produced in the body via natural metabolism such as glutathione, superoxide dismutase, catalase (160), while other antioxidants are present in the food consumed as natural products such as vitamins C and E, carotenoids (161). Research has shown that many plants possess antioxidant properties (162). Phytochemicals that possess antioxidant potential include phenols and flavonoids, and these neutralise the free radicals,

hence medicinal plants have been recognised as a natural source of antioxidant compounds (163). This chapter focuses on the determining the antioxidant capacity of *H. hemerocallidea* and *H. africana* samples.

4.2 Methods and materials

4.2.1 Qualitative antioxidant screening

The hexane, DCM, methanol and water extracts of *H. hemerocallidea* and *H. africana* were prepared as described in chapter 3. The dot plot method described by Matseke et al (2025), was followed with slight modifications (164). The plant extracts were spotted in a dot manner on a pre-coated silica TLC plate. The plate was then left to air dry for 5 minutes, and once dry, the TLC plate was spotted with freshly prepared 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. A positive indication of antioxidant activity was taken if there was a white to pale yellow discoloration of the dots.

4.2.2 DPPH antioxidant activity assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is widely used to assess the antioxidant capacity of compounds by measuring their ability to reduce the stable DPPH radical (165). DPPH is a stable free radical that is purple in colour, and when an antioxidant is added to it, it reacts with the DPPH and donates electrons to it (166), making the DPPH more stable, reducing it to diphenylpicrylhydrazine (DPPH-H), as shown in figure 4.1, and as a result, the purple colour fades to pale yellow, and the faster the colour fades, the stronger the antioxidant (165,167). This assay is simple, rapid, and cost-effective. The principle of the DPPH assay is based on the reduction of DPPH by an antioxidant which results in scavenging of the DPPH radical, resulting in a decrease in the intensity of the purple colour (168).

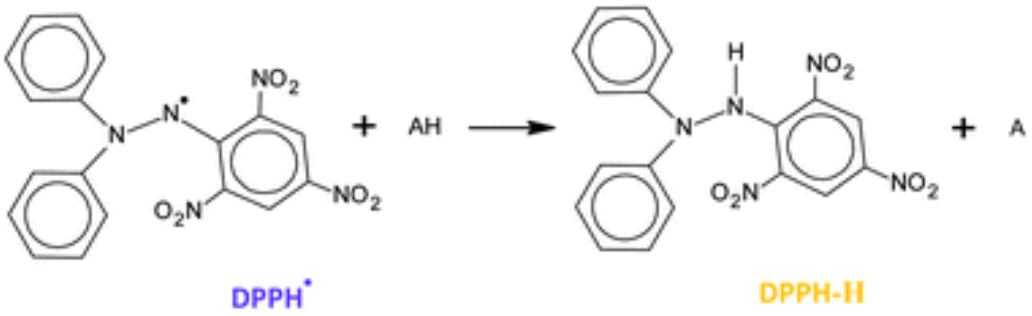

Figure 4.1: Mechanism of reduction of DPPH to DPPH-H (169)

The DPPH radical scavenging activity was determined using a method described by Kwon et al. (2017) with slight modifications (170). Three concentrations of the plant extracts were used i.e., 5 µg/ml, 50 µg/ml, and 500 µg/ml. Freshly prepared DPPH, dissolved in methanol at a concentration of 0.1mM was prepared. 100 µl of DPPH was added to each well, and 200 µl of plant extract sample was added to the same wells in a 96-well plate. The mixture was incubated in the dark at room temperature for 30 minutes, then the absorbance was read at 517 nm using a microplate reader (Epoch 2 Microplate Reader, BioTek Instruments, Winooski, VT, USA). Ascorbic acid was used as the standard antioxidant control at varying concentrations of 5, 10, 50, 100, 250, and 500 µg/ml. All the extracts were prepared in triplicate, and the percentage of radical scavenging activity was calculated using the formula below.

% DPPH radical scavenging activity = $[1 - (Abs_{sample} / Abs_{control})] \times 100$,

Where:

Abs_{sample} - absorbance of the extracts

$Abs_{control}$ - absorbance of the control

4.2.3 Data analysis

The absorbance readings for the DPPH assay were obtained and exported to Microsoft Excel. A calibration curve using known concentrations of ascorbic acid was plotted. The radical scavenging activity (antioxidant potential) was calculated. No statistical comparison was done for the data. The results were presented as the mean of the triplicate measurements.

4.3 Results

The results for the qualitative antioxidant screening (dot plot) and the quantitative antioxidant analysis (DPPH radical scavenging assay) are presented in figures 4.2 – 4.4. The IC_{50} values are shown in Table 4.1. Figure 4.1 visually represents the antioxidant activity of the extracts. Figures 4.3 and 4.4 represent the quantitative scavenging activity of the extracts and ascorbic acid standard. Table 4.1 shows the IC_{50} values indicating the concentration required to scavenge 50% of DPPH radicals. The results suggest that *H. hemerocallidea* and *H. africana* have some radical scavenging ability, though lower than ascorbic acid.

4.3.1 Qualitative antioxidant screening (dot plot method)

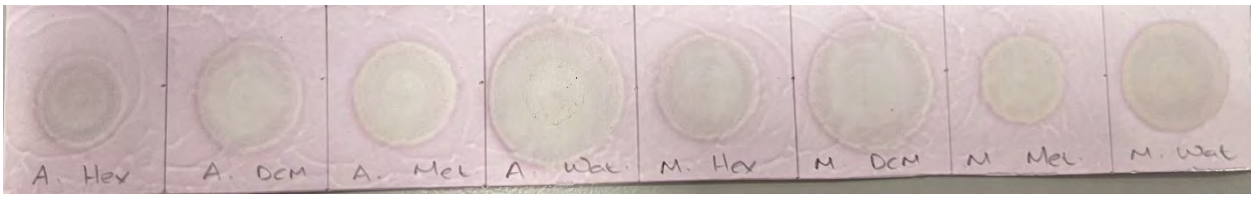

Figure 4.2: Dot plot of H. hemerocallidea and H. africana extracts after spotting with DPPH dissolved in methanol

4.3.2 DPPH radical scavenging assay

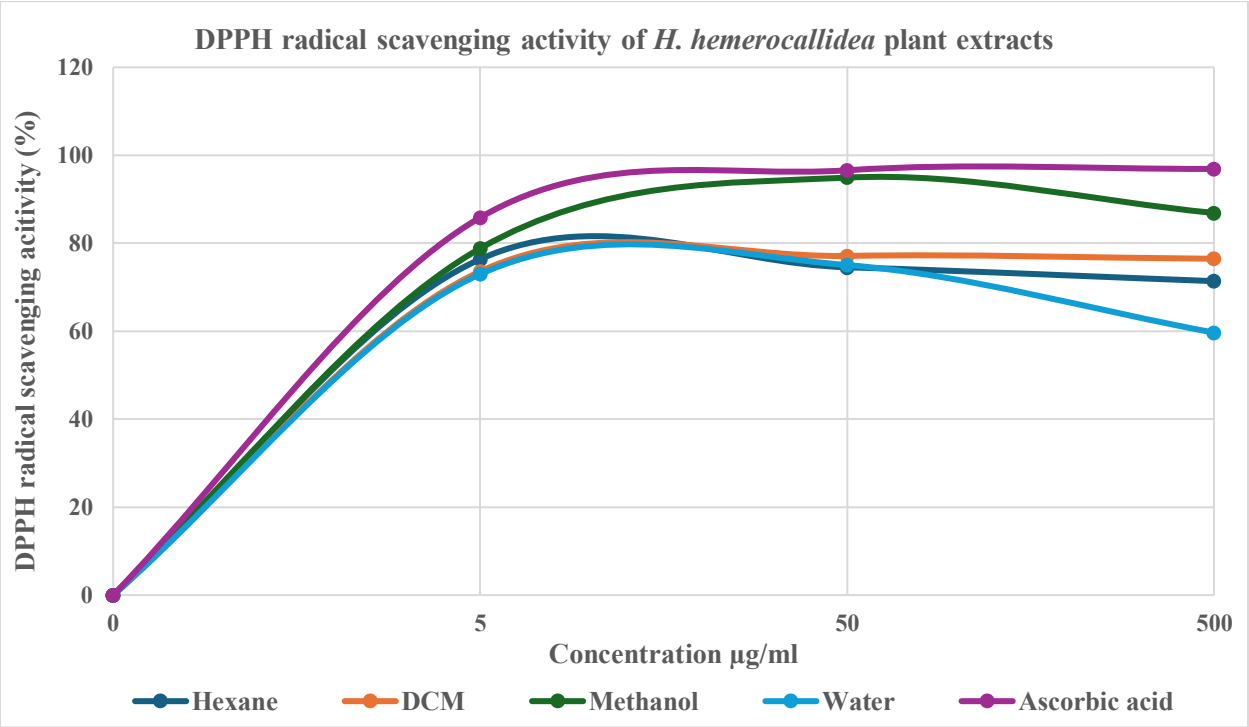

Figure 4.3: DPPH radical scavenging activity (%) of H. hemerocallidea plant extracts and ascorbic acid

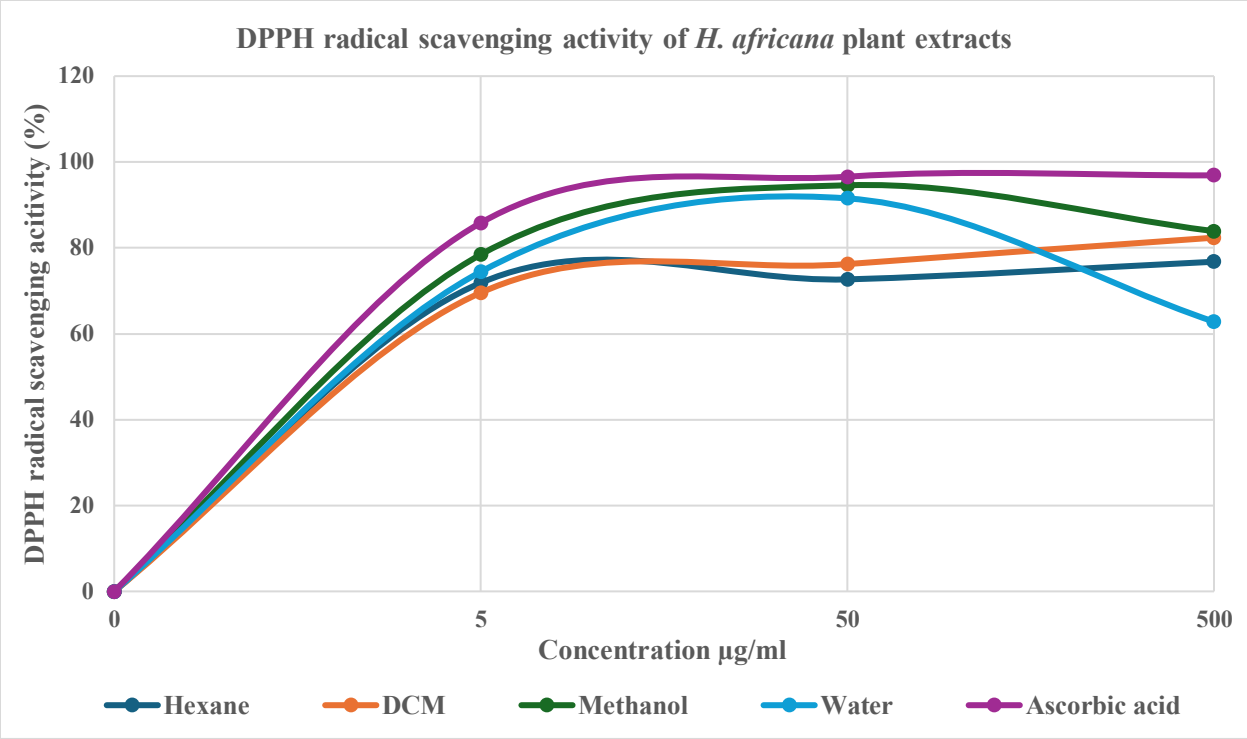

Figure 4.4: DPPH radical scavenging activity (%) of H. africana plant extracts and ascorbic acid

Table 4.1: IC_{50} values of H. hemerocallidea and H. africana plant extracts and ascorbic acid

Extracts and standard	**IC50 value (µg/ml)**		
	H. hemerocallidea	***H. africana***	**Ascorbic acid**
Hexane	2.2378	2.2697	1.2328
Dichloromethane	2.2085	2.2224	1.2328
Methanol	1.9525	1.9687	1.2328
Water	2.3938	2.1499	1.2328

4.4 Discussion

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to evaluate the free radical scavenging activity, which determines the hydrogen-donating capacity of a molecule (171). The qualitative antioxidant screening and quantitative DPPH radical scavenging assay both used DPPH, a stable free radical, to evaluate the antioxidant activity of *H. hemerocallidea* and *H. africana* plant extracts, by measuring their ability to reduce the stable DPPH radical, and the reaction was

monitored through a decrease in absorbance at 517 nm (172). The IC50 values were also determined and compared ascorbic acid. The antioxidant activity varied across the different extracts, with the water and methanol extracts having the strongest scavenging potential.

4.4.1 Qualitative antioxidant screening

The results of the dot plot as shown in figure 4.1, showed that the extracts of *H. hemerocallidea* and *H. africana* have compounds in them that possess some level of antioxidant activity. This is a visual indication of the radical scavenging potential of the extracts. A colour change from purple to yellow indicates the ability of the extract to neutralize DPPH, and the more the purple colour fades, the higher the antioxidant activity, and the intensity of the yellow colour and the size of the bleached area is proportional to the antioxidant activity of the extract (173).

There were variable levels of antioxidant activity among the different extracts. The dot-plot shown in figure 4.1 is not very clear, but with the naked eye, the methanol and water extracts of both plants demonstrated noticeable antioxidant activity, as they discoloured the DPPH the most. The polarity of the solvents also has an influence on the extraction of antioxidant compounds. Higher antioxidant activity was observed with the methanol and water extracts because they are both polar solvents and more effective in extracting phenolic compounds, which are known for their antioxidant properties (174).

4.4.2 DPPH antioxidant activity assay

The scavenging activity of *H. hemerocallidea* and *H. africana* was compared to the standard antioxidant, ascorbic acid. Ascorbic acid is a potent antioxidant, and it showed the highest DPPH scavenging activity across all concentrations. It is able to readily donate electrons due its strong reducing potential and neutralize the DPPH free radical (175), confirming the validity of the assay and we can compare its activity to that of the plant extracts.

Generally, antioxidant activity increased with increasing concentration which is similar to studies done by Nair et al. (2007), where the radical scavenging activity of the methanol and water extracts showed high activity with higher concentrations (171). For the *H. hemerocallidea* extracts, the methanol extract showed strong scavenging activity across the concentrations, showing that

methanol is effective in extracting compounds from the plant that possess antioxidant potential such as the polar compounds which include the phenols and flavonoids. A study by Mannathoko et al. (2017) , confirmed this, where an in-vitro analysis of the antioxidant properties of the methanol extract of *H. hemerocallidea* corm were conducted and found that the 50 µg/ml concentration had the highest activity (176). The hexane, DCM, and water extracts also showed promising radical scavenging activity. Water is a polar solvent and is able to extract more polar compounds. The increased polarity of methanol is due to the hydroxyl groups. The results for *H. africana* were slightly similar to those of *H. hemerocallidea*. The methanol extract demonstrated the most significant radical scavenging activity showing a concentration-dependent increase. The other extracts also exhibited notable scavenging activity, although lower than the methanol extract.

IC_{50} values were calculated and shown in table 4.1. IC_{50} is the concentration of a substance that is required to inhibit 50% of its target activity, with a lower IC_{50} indicating better scavenging ability and higher potency. Ascorbic acid showed the strongest antioxidant activity (1.2328 µg/ml). For the *H. hemerocallidea* extracts, the methanol extract showed the strongest antioxidant activity closest to ascorbic acid. The hexane, DCM, and water extracts have weaker activity, needing higher concentrations to achieve 50% inhibition. The IC_{50} results for *H. africana* were slightly similar to those of *H. hemerocallidea*. Kokoette and Sekelwa (2020) conducted a study on the evaluation of the antioxidant activitiy of the methanol and water extracts of *H. hemerocallidea*, and reported on the IC_{50} values ranging between 37 and 39 µg/ml, indicating notable free radical scavenging activity (177).

4.5 Chapter summary

Both plants showed concentration-dependent increases in radical scavenging activity across the different solvent extracts, but *H. hemerocallidea* generally showed higher antioxidant activity. The water extract of *H. africana* showed relatively good scavenging potential, suggesting the presence of water-soluble antioxidants. The hexane and DCM extracts showed limited activity. These results indicate both species possess valuable antioxidant potential, but with different profiles.

Chapter 5: Antibacterial activity of different solvent extracts of *H. hemerocallidea* and *H. africana*

5.1 Introduction

When the host body tissue is infected by microbes such as bacteria, viruses, fungi and/or parasites, microbial infections result, and these infections are classified according to the invading micro-organisms, the symptoms of disease they present and the duration of infection (178). Antimicrobials have been and continue to be important in the treatment of these infections. The emergence and spread of antibiotic-resistant microorganisms pose a significant threat to global public health (178). The increasing prevalence of infections caused by drug-resistant bacteria, fungi, and parasites necessitates the urgent development of new antimicrobial agents (1).

The conventional antibiotics used commonly are becoming less effective, highlighting the need for alternative approaches to treat infections. The increasing prevalence of antimicrobial resistance has stimulated an interest in natural products, including plant extracts, as potential sources of new antimicrobial agents (9,179).

Antimicrobial drugs are classified either as antibacterial, antiviral, antifungal or antiparasitic depending on the type of microbe involved, as well as their sources whether natural, semisynthetic or synthetic (180). These drugs may also be classified based on the range of microbes they act against such as narrow spectrum which act on a smaller range of microbes and broad spectrum which act on a wider range of microbes (180). Antimicrobial drugs exert their mechanisms of actions in different ways such as inhibiting nucleic acid synthesis, disrupting metabolic pathways, inhibiting cell wall synthesis, disrupting the cell membrane, or blocking protein synthesis (178). AMR is a natural process that occurs over time when microorganisms undergo genetic changes in their genome. AMR is also accelerated by the misuse and overuse of antimicrobials in healthcare settings and agriculture (79).

Medicinal plants have been used for many years and are becoming a potential source for the discovery of new antimicrobial compounds (181). *H. hemerocallidea* and *H. africana* are two medicinal plants that have been used in various traditional healing practices across Africa (181).

These plants have been reported to possess a wide range of pharmacological activities, including antimicrobial properties. However, the specific antimicrobial potential of different solvent extracts from these plant species has not been extensively explored. This study aims to investigate the antibacterial activities of various solvent extracts derived from *H. hemerocallidea* and *H. africana*.

5.2 Methods and materials

The hexane, DCM, methanol and water extracts of *H. hemerocallidea* and *H. africana* were prepared as described in chapter 3. The following bacterial strains were cultured for the bioassay in the Rhodes University Marine Natural Products Laboratory (RUMNP):

- *Acinetobacter baumannii* ATCC 19606
- *Staphylococcus aureus* ATCC 12600
- *Escherichia coli* ATCC 10536
- *Pseudomonas aeruginosa* ATCC 27853

5.2.1 Preparation of cultures

The bacterial strains were cultured using standard microbiological media, Tryptic Soy Broth (TSB) for *A. baumannii* and Luria Broth (LB) for *S. aureus*, *E. coli*, and *P. aeruginosa.* To prepare TSB, 30 g of dehydrated TSB was dissolved in one litre of purified water. The solution was heated with agitation, boiled for one minute and sterilized for 15 minutes at 121°C. The solution was allowed to cool to between 45-50°C while gently mixing and poured into sterile petri dishes. To prepare LB, 15.5 g of dehydrated LB was dissolved in one litre of purified filtered water. The solution was heated with agitation, boiled for one minute and before sterilization, bacteriological agar was added and sterilized for 15 minutes at 121°C.

5.2.2 Culturing procedure

The bacterial strains were incubated in 5 ml of media broth at 37°C overnight with agitation at 140 rpm. The cultures were mixed with sterile glycerol (25% v/v), and the samples were aliquoted into cryovials and stored at -80°C.

5.2.2.1 Preparation of bacterial cultures

Single colonies from the cultures stored at 4°C were selected using a sterile toothpick and suspended in 5 ml of culture medium. The suspension was incubated overnight at 37°C on a shaker

at 160 rpm. 50 µl of overnight bacterial suspension was transferred to a fresh 5 ml test tube of sterile culture medium. The suspension was incubated under identical conditions until it reached exponential growth phase, measured via spectrophotometry.

5.2.2.2 Dilution of bacterial cultures

The cultures were diluted as follows:

- *A. baumannii*: 50 µl of log-phase culture in 10 mL TS broth
- *E. coli*: 100 µl of log-phase culture in 10 mL LB broth
- *S. aureus*: 100 µl of log-phase culture in 10 mL LB broth
- *P. aeruginosa*: 150 µl of log-phase culture in 10 mL LB broth

In a sterile 96-well plate, 20 µl of the plant extract at a concentration of 0.5 mg/ml, 20 µl of bacterial suspension, and 160 µl of culture media were added. The plates were incubated at 37°C for 6 hours in a sealed plastic container. Once the incubation period elapsed, 20 µl of resazurin working solution was added to each well, and the plates were monitored visually until a clear colour difference appeared between the negative and positive control wells. Fluorescence intensity was measured using a microplate reader at 560/590 nm (excitation/emission). The negative control wells contained bacterial cells, media, and antibiotics (0.6 µM novobiocin for the gram-positive bacteria, *S. aureus* or 1 mM nalidixic acid for the gram-negative bacteria, *A. baumannii, E. coli,* and *P. aeruginosa*). The positive control wells contained bacterial cells and media only.

The two antibiotics in the negative control wells served as controls in the bacterial viability assay to inhibit the growth of gram-positive bacteria and gram-negative bacteria respectively and to validate the accuracy of the assay.

Novobiocin is a DNA gyrase inhibitor, which blocks the replication of bacterial DNA by inhibiting the enzyme DNA gyrase, involved in the supercoiling of bacterial DNA. Gram-positive bacteria have a thick peptidoglycan layer in their cell walls, but no outer layer, which makes it easier for the antibiotic to penetrate the cell wall and inhibit DNA replication. Novobiocin is typically not used today due to its toxicity profile and resistance, but now primarily used in research and laboratory diagnostics (182).

Nalidixic acid is a first-generation quinolone, which also inhibits DNA, preventing DNA replication (183). Gram-negative bacteria have an outer membrane, making it harder for antibiotic penetration, but nalidixic acid is able to pass through the membrane porins and disrupt DNA processes. Nalidixic acid was used to treat UTIs but is no longer used due to resistance (184), and now fluoroquinolones have also been developed which are more effective.

Percentage viability (%) was calculated as follows:

$$\% \text{ Viability} = \frac{(\text{Fluorescence}_{\text{treated sample}} - \text{Fluorescence}_{\text{background}})}{(\text{Fluorescence}_{\text{control}} - \text{Fluorescence}_{\text{background}})} \times 100$$

Where:

Fluorescence $_{\text{treated sample}}$ - fluorescence of bacterial culture exposed to plant extract

Fluorescence $_{\text{control}}$ - fluorescence of the control

5.3 Results

The antibacterial activity of *H. hemerocallidea* against *A. baumannii* was less effective. The DCM and methanol extracts reduced viability to about 42.37% and 33.22% respectively, while the hexane and water extracts showed little to no inhibition. In contrast, *H. africana* showed strong antibacterial activity, particularly in the hexane and DCM extracts, which reduced bacterial viability to about 1.58% and 1.36% respectively. For *E. coli*, neither plant exhibited strong antibacterial effects, as all extracts had high viability values ranging above 100%, indicating minimal bacterial inhibition. Similarly, against *P. aeruginosa*, both plants showed limited antibacterial activity. The hexane extract of *H. hemerocallidea* reduced viability to about 71.23%, and the methanol extract of *H. africana* showed a moderate effect, reducing viability to about 69.16%.

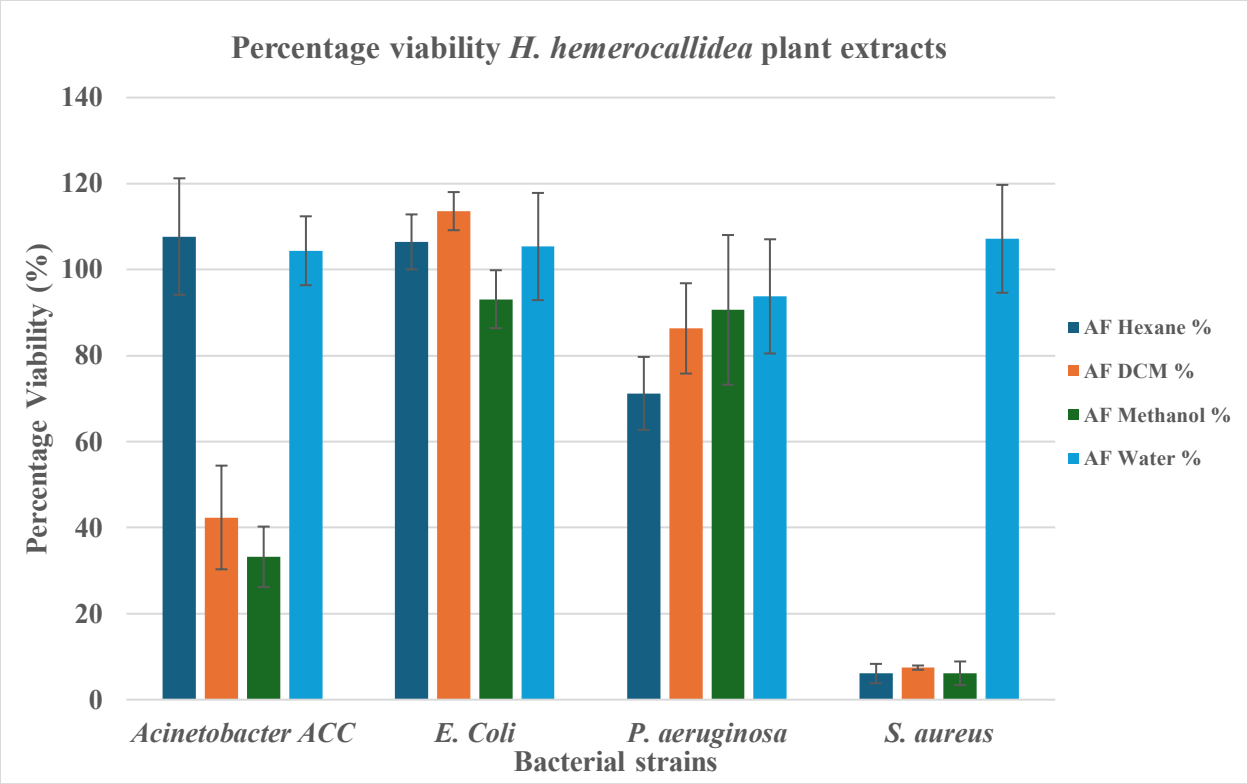

Figure 5.1: Percentage viability of bacterial strains for H. hemerocallidea plant extracts

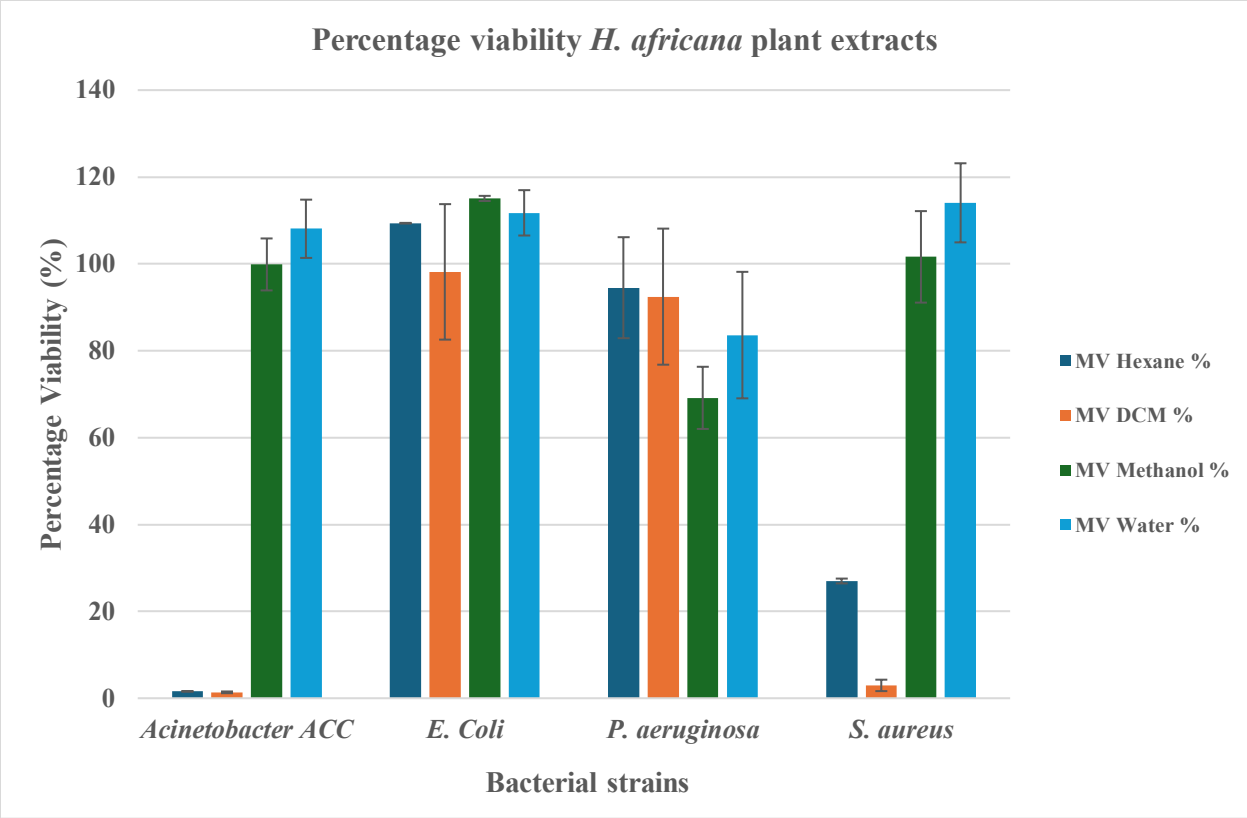

Figure 5.2: Percentage viability of bacterial strains for H. africana plant extracts

5.4 Discussion

Bacterial viability is the ability of bacteria to survive, reproduce, and perform metabolic functions under specific environment conditions (185). The percentage viability of *H. hemerocallidea* and *H. africana* plant extracts was evaluated against four bacterial strains, *A. baumannii*, *E. coli, P. aeruginosa*, and *S. aureus*.

The hexane, DCM, and methanol extracts of *H. hemerocallidea* showed strong inhibition, with viability values approximately 6-7%, while the water extract was ineffective. The most notable antibacterial activity (lowest viability) was observed against *S. aureus*. For *A. baumannii*, the DCM and methanol extracts showed moderate antibacterial activity, while the hexane and water extracts were ineffective as the percentage viability was above 100%. For *E. coli* and *P. aeruginosa*, all extracts had high viability and poor activity. Therefore, it can be concluded that *H. hemerocallidea* was slightly highly effective against *S. aureus* but limited to no activity against the gram-negative bacteria (*E. coli* and *P. aeruginosa*) and some moderate activity against *A. baumannii*. Based on

the knowledge of the structure of cell walls in gram positive and gram-negative bacteria, possible mechanisms of action of the plant extract can be elucidated. *S. aureus* is gram positive bacterium with a thick peptidoglycan layer in the cell wall but lacks an outer membrane. Since *H. hemerocallidea* shows effectiveness against *S. aureus* it can be hypothesised that it disrupts the bacterial cell wall, similar to B-lactam antibiotics, or it could cause leakage of important cell components through the porin channels of the bacteria.

There was limited to almost no activity against the gram-negative bacteria, *E. Coli* and *P. aeruginosa*, and this could be due to the fact that gram-negative bacteria have outer membranes that contain lipopolysaccharides, making it harder for antimicrobials to enter the cell. This limited activity suggests the extract may not effectively permeate the outer membrane, reducing its ability to reach intracellular components.

Moderate activity was observed against *A. baumannii* bacteria, perhaps suggesting that the extract does not fully disrupt the bacterial cell membrane and interfere with bacterial processes. For *H. hemerocallidea*, the hexane, DCM, and methanol extracts against *S. aureus* could be further investigated as potential alternative treatments and the results could be compared to standard antibiotics. *H. africana* also demonstrated strong inhibition, particularly with its hexane and DCM extracts against *A. baumannii* and *S. aureus*. However, the methanol and water extracts were ineffective, with viability values exceeding 100%. The extracts against *E. coli* and *P. aeruginosa* had high viability, suggesting no significant antibacterial effects.

The phytochemicals extracted by a particular solvent may also contribute to the bacterial viability. The DCM extract of *H. hemerocallidea*, had the highest inhibition, particularly against *S. aureus,* while exhibiting limited activity against *E. coli* and *P. aeruginosa*. This could suggest that lipophilic compounds, such as alkaloids, terpenoids, and some flavonoids, may be responsible for bacterial inhibition.

The gram-negative bacteria, *E. coli* and *P. aeruginosa* have shown to be highly resistant to both plant extracts, as shown by the high percentage viability values. Solvent polarity also influences bacterial viability because it affects the type of phytochemicals extracted, hence their ability to disrupt bacterial cell functions. The non-polar solvents, like hexane and DCM showed better inhibition compared to the more polar solvents, methanol and water, suggesting the phytochemicals might be lipophilic such as the terpenoids and alkaloids, making them more

effective against *S. aureus*, a gram-positive bacterium. Both plants had high viability percentages across all solvent extracts against *E. coli*, indicating that there was little to no inhibition of bacterial growth, whereas with *P. aeruginosa*, there was moderate viability, suggesting weak inhibition of bacterial growth.

A study conducted in 2021 by Aremu et al, reported that silver nanoparticles made from *H. hemerocallidea* plant extracts inhibited the growth of bacteria like *E. coli* and *P. aeruginosa* just like the broad-spectrum antibiotic streptomycin (186). However, in this study, the solvent extracts did not significantly inhibit the growth of *E. coli* or *P. aeruginosa*, opposing the results in the study by Aremu et al. (2021), and perhaps the difference could be due to the type of compounds being tested. In that study, silver nanoparticles were used, which could have enhanced the antibacterial effects by increasing the bioavailability and penetration of the bioactive compounds, whereas in this study, crude plant extracts were used, which potentially could have lacked sufficient concentrations of active compounds. This could potentially explain why Aremu et al. observed stronger activity than was seen in this study.

Another study evaluated the antibacterial activity of *H. africana* against various pathogens, reporting zones of inhibition ranging from 0 to 25 mm, indicating its potential as a natural antimicrobial agent (123). However, in this study, the *H. africana* extracts demonstrated weak inhibition against *E. coli* and *P. aeruginosa*, with high viability percentages, and in the study of Wintola and Afolayan (2015), their results reported stronger inhibition. This difference may be due to variations in the extraction methods and/or solvent choice. The present study used a bacterial viability assay, while the previous study used the disc diffusion method, which could potentially result in different interpretations of antibacterial activity.

5.5 Chapter summary

The results of the bacterial viability assay for the four ESKAPE pathogens showed strong inhibition against S.*aureus.* Particularly, *H. africana* showed strong antibacterial effects, specifically with its hexane and DCM extracts against *A. baumannii* and *S. aureus*. In contrast, the extracts against *E. coli* and *P. aeruginosa* showed minimal antibacterial activity, suggesting no significant antibacterial effects.

Chapter 6: Liver enzyme metabolism studies

6.1 Introduction

Liver enzyme metabolism studies guide in understanding how drugs and other substances are metabolised, which is important for predicting potential drug interactions (187). Drug clearance from the body influences the pharmacokinetics of drugs (188). If the CYP450 enzymes are inhibited, this can result in altered drug efficacy and toxicity. It is therefore important to understand the CYP450 enzyme system, not to just predict how drugs will interact but also to tailor medicine regimens for patients to enhance drug safety (189). The CYP450 enzyme system processes are affected by a number of things, such as age, gender, disease, drug-drug interactions, therefore regulation of the system is crucial to prevent consequences such as adverse drug reactions due to increased plasma concentrations, drug-drug interactions and altered drug efficacy.

Cytochrome P450 3A4 (CYP3A4) is the most abundant CYP450 isoenzyme involved in drug metabolism. It is an important isoform because it is responsible for catalysing multiple oxidative reactions in the body, metabolising majority of the administered drugs (190). Its expression varies among individuals due to a number of factors such as genetics, environmental factors, as well as physiological factors (191). Understanding the interactions between CYP3A4 and other compounds helps to predict drug metabolism and potential drug-drug interactions, which can lead to drug toxicity, because CYP3A4 can be both induced and inhibited (191). For example, St. John's Wort (*Hypericum perforatum*) induces CYP3A4, and this reduces the efficacy of antidepressants such as selective serotonin reuptake inhibitors (SSRIs) (192). Another plant called Ginkgo Biloba induces CYP3A4 increasing the risk of bleeding when taken concurrently with anticoagulants such as warfarin (193). Some plants even inhibit CYP3A4 such as grapefruit leading to increased levels of statins in the body. Therefore, the inhibition of CYP3A4 isoform can affect the drug level in the body and cause detrimental side effects.

6.2 Materials and methods

6.2.1 CYP450 enzyme inhibition

An in vitro cytochrome P450 screening assay (Vivid® CYP450 Screening Kit, Life Technologies™, California, USA) was used to assess the inhibition of human P450 isozymes involved in hepatic drug metabolism, particularly the isoform, CYP3A4 by the plant extracts. The assay is fluorescence based allowing rapid identification of compound-CYP450 interactions. The plant extracts were analysed for their capacity to inhibit the production of a fluorescent signal in reactions using recombinant human CYP450 isozymes and specific substrates. The higher the fluorescence the less the inhibition.

6.2.1.1 Materials

H. hemerocallidea and *H. hypoxis* extracts were prepared at three different concentrations i.e. 5 ug/ml, 50 ug/ml, and 500 ug/ml, as described in chapter 3. Ketoconazole, a potent CYP450 enzyme inhibitor of liver enzymes was used as the standard (positive control). Dimethyl sulfoxide (DMSO) solution was used as the solvent for both the plant extracts and ketoconazole, and a DMSO solution alone served as the blank control.

6.2.1.2 Method

The sample extracts were prepared in DMSO to yield stock solutions of 2.5x the desired test concentrations and were diluted in the buffer to achieve final working concentrations. Ketoconazole solutions were prepared similarly for use as a positive control. Volumes of 50 µL of enzyme solution, 50 µL of substrate, and 40 µL of plant extract or control were placed into each well of a black 96-well plate. The blank wells received DMSO in place of the inhibitor. The reaction mixture was then incubated at 37°C for 30 minutes to allow the reaction to proceed. Fluorescence was measured at one-minute intervals for an hour at excitation and emission wavelengths (415/460 nm). The percent inhibition was calculated using the equation below.

% Inhibition = [1 – (Abs $_{sample}$ / Abs $_{control}$)] x 100,

Where:

Abs $_{sample}$ - absorbance of the extracts

Abs $_{control}$ - absorbance of the control

6.2.1.3 Data analysis

The fluorescence readings for the CYP450 enzyme inhibition test were obtained and exported to Microsoft Excel, where the percentage inhibition was calculated. The results were presented as the mean of the triplicate measurements with standard error of mean. No statistical comparison was done for the data.

6.3 Results

The percentage inhibition of *H. hemerocallidea* is shown in figure 6.1, and these results suggest that the *H. hemerocallidea* extracts show CYP450 enzyme inhibitory activity, particularly at high concentrations. As the concentration of extract increases, the percentage inhibition generally increases, showing a concentration-dependent relationship. At 5 µg/ml, the hexane extract had moderate inhibition, with a negative percentage inhibition at 50 µg/ml, suggesting no effect at this concentration; DCM extract showed almost no inhibition, and the methanol extract had slightly higher inhibition compared to hexane, meaning more polar compounds extracted by methanol contributed to its activity. At the highest concentration of 500 µg/ml, DCM extract shows the highest percentage inhibition, suggesting the presence of inhibitory compounds. At the highest concentration, the methanol and water extract also show significant inhibition.

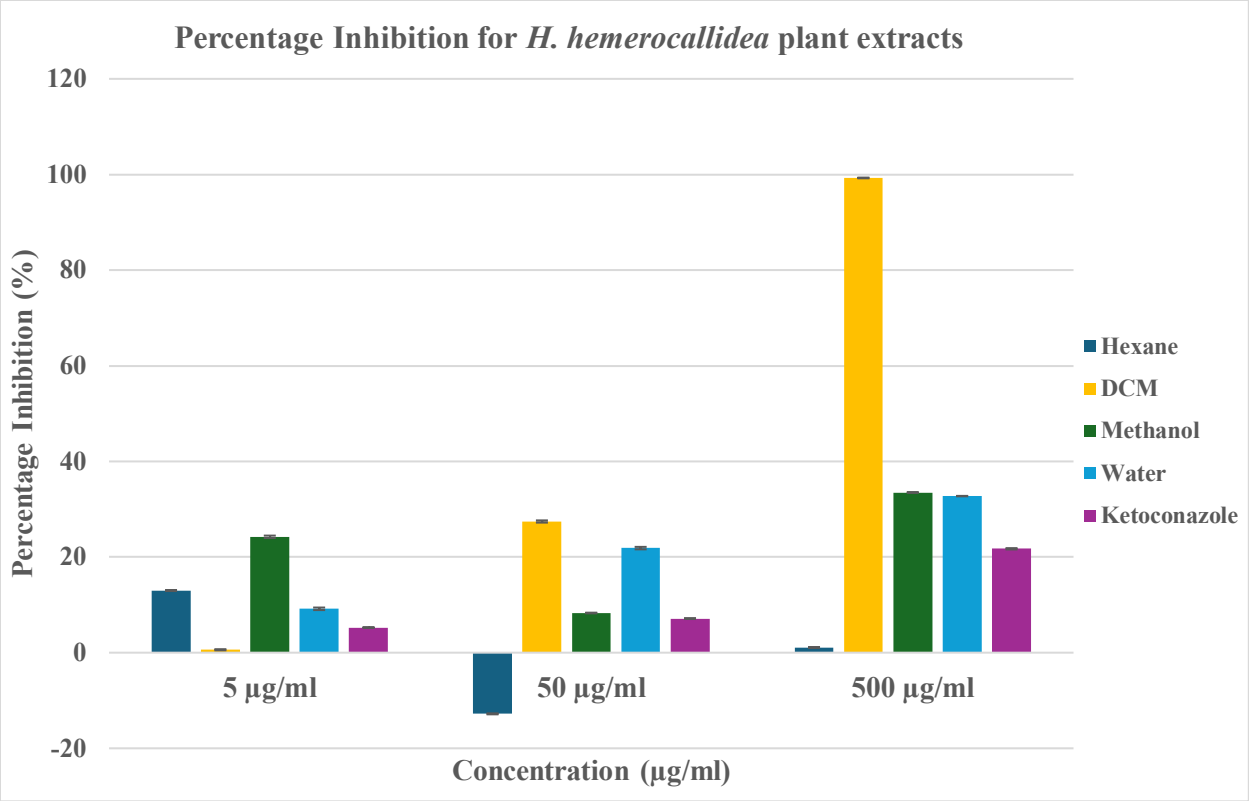

Figure 6.1: Percentage inhibition for H. hemerocallidea plant extracts

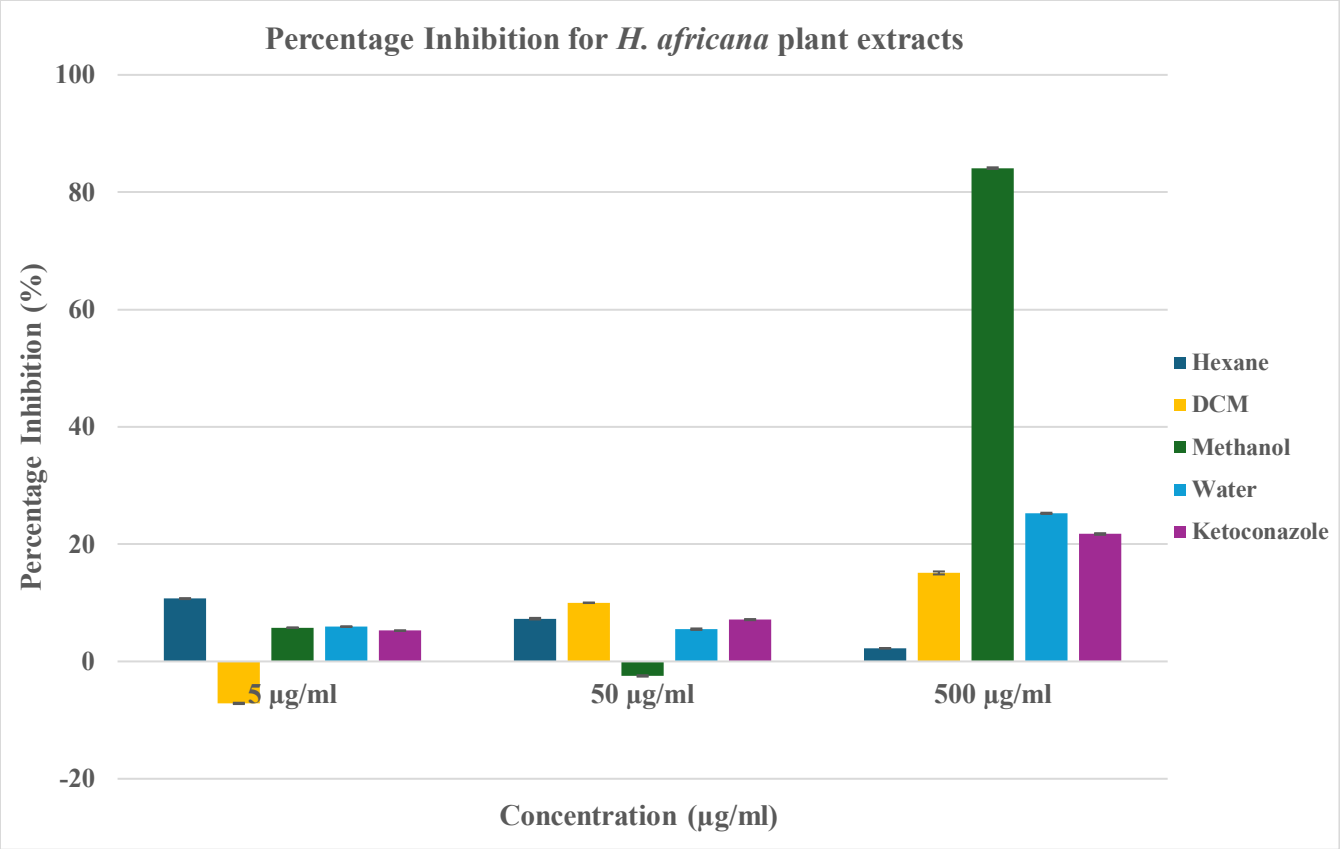

Figure 6.2: Percentage inhibition for H. africana plant extracts

6.4 Discussion

Cytochrome P450 enzymes are involved in the metabolism of many endogenous and exogenous compounds (194). These enzymes are primarily located in the liver but can also be found in the intestines and kidneys as well. The inhibitory effects of *H. hemerocallidea* and *H. africana* extracts on CYP3A4 were evaluated using different solvent extracts. Ketoconazole, a known potent CYP3A4 enzyme inhibitor showed increasing inhibition with higher concentrations.

The strong inhibitory effect of the DCM extract at the highest concentration of 500 µg/mol suggests the presence of highly non-polar compounds that can significantly impact CYP3A4 activity (195). This suggests that the DCM extract is inhibiting the enzyme's metabolising activity, and this could potentially lead to altered plasma concentration of drugs. The clinical implication of this, is that if the extract is taken concurrently with a drug that is metabolised by CYP3A4, its concentration in blood will increase leading to undesirable side effects. A study was conducted by Katerere et al. (2008) on the extracts of *H. Hemerocallidea,* and it was found that the extracts

contained phytochemicals such as sterols and norlignans, which may contribute to enzyme inhibition (196). Antioxidant compounds such as phenolic compounds and flavonoids known to be present in *H. Hemerocallidea*, have been reported to inhibit CYP450 enzymes (197). At the low concentrations of 5 and 50 µg/mol, the extracts have low percentage inhibition values suggesting possible induction of CYP3A4. The findings are similar to the report by Fasinu et al (2013), Nair et al (2007) and a review by Matyanga et al. (2020), who reported that extracts of *H. hemerocallidea* inhibited the activity of various CYP450 isoforms including CYP3A4 (198–200), evidenced by reduced production of the metabolites of the substrates of the enzymes (198,200).

The *H. africana* plant extracts also show some CYP450 enzyme inhibitory activity, particularly at higher concentrations. As the plant extract concentration increases, the percentage inhibition increases, showing a concentration-dependent relationship, and this could suggest that the plant extract components are likely responsible for inhibiting the CYP450 enzyme. The methanol extract also showed the highest percentage inhibition compared to the other solvent extracts at all concentrations, suggesting that methanol may be extracting the most potent inhibitory compounds from the plant. There was a small inhibitory effect with the hexane extract.

The strong inhibitory effect of the methanol extract suggests that polar compounds such as phenols and flavonoids, which are highly soluble in methanol, may be responsible for the observed inhibition. This is similar to the findings by Mkala et al. (2021), who noted that *H. africana* contains a high concentration of phenolic compounds, which are known inhibitors of CYP enzymes (119). This suggests that the methanol extract inhibits CYP3A4 metabolism activity, therefore with the potential of increasing plasma concentration of concurrent drugs metabolised by CYP3A4. The clinical implication of this, is that if the extract is taken concurrently with a drug that is metabolised by CYP3A4, its concentration in blood will increase leading to undesirable side effects and increased risk of toxicity.

The hexane and DCM extracts showed weaker inhibition, suggesting they may induce enzyme activity at low concentrations. The plant extracts tested in this study demonstrated inhibitory effects on CYP3A4, a key enzyme involved in the pharmacokinetics of many commonly used drugs, particularly during their metabolism in the liver. When CYP3A4 activity is inhibited, the metabolism of drugs is slowed down, leading to delayed drug clearance and elevated plasma concentrations, which can increase the risk of adverse effects (190).

The potential implications of these findings are significant, particularly in understanding potential herb-drug interactions. CYP3A4 is responsible for metabolizing a wide range of pharmaceutical drugs, and potent inhibition, such as observed with the DCM extract, may result in altered drug metabolism and potential toxicity.

For example, erythromycin is an antibiotic metabolised by CYP3A4 (186,190), and so if the extracts block CYP3A4 activity, the concentration of erythromycin in the blood will increase, increasing the risk of side effects such as liver toxicity or QT prolongation. Also, if the extract (which inhibits CYP3A4) is taken concurrently with a CYP3A4 inhibitor, such as ketoconazole, there may be a potentiated inhibition on the enzyme, leading to additive increase of drug in blood.

Simvastatin, a cholesterol-lowering agent, is primarily broken down by CYP3A4. Its accumulation in the blood, due to enzyme inhibition can increase the risk of muscle damage (myopathy). Similarly, midazolam, a sedative, also metabolized by CYP3A4, may produce excessive and prolonged sedation or respiratory depression if not properly metabolized (190,191).

These interactions are particularly concerning for drugs with a narrow therapeutic index, where small changes in drug concentration can shift the balance from therapeutic to toxic effects. Therefore, knowing whether medicinal plant extracts inhibit or induce CYP450 enzymes is important to avoid harmful drug interactions.

6.5 Chapter summary

The *in vitro* CYP450 inhibition assay evaluated the potential impact of *H. hemerocallidea* and *H. africana* extracts on herb-drug interaction possibility and drug metabolism. The results revealed that the extracts exhibited some CYP3A4 enzyme inhibitory activity, notably at high concentrations, with the methanol extract exhibiting inhibitory effect much higher than ketoconazole, suggesting that the extracts may interfere with CYP450-mediated drug metabolism.

Chapter 7: Summary of study, conclusions, limitations and future recommendations

7.1 Overall summary

The qualitative phytochemical screening of *H. hemerocallidea* and *H. africana* corm extracts revealed that they contain phytochemical compounds including phenols, flavonoids, saponins, alkaloids, quinones, terpenoids, anthraquinones, steroids, coumarins, and tannins in varying amounts.

The quantitative analysis of phytochemicals present in *H. hemerocallidea* and *H. africana* was carried out by a total phenolic content and a total flavonoid content assay. The results for the total phenolic content in *H. hemerocallidea* revealed that the methanol extract showed the highest phenolic content, followed by the water extract at the highest concentration, and the hexane and DCM extracts showed lower phenolic content values. The results for the total phenolic content in *H. africana* revealed that the methanol and water extracts had the highest phenolic content as well. The total flavonoid content in *H. hemerocallidea* revealed that the hexane and DCM had more flavonoids than the methanol and water extracts, whereas *H. africana* also showed a high flavonoid content in the hexane and DCM extracts at high concentrations.

The results of the qualitative dot plot antioxidant screening test showed that both plants have compounds in them that possess some level of antioxidant activity. With the naked eye, higher antioxidant activity was observed with the methanol and water extracts of both plants, as they discoloured the DPPH the most, and because they are both polar solvents, they are more effective in extracting phenolic compounds.

The quantitative DPPH radical scavenging activity assay for both plants showed a concentration-dependent increase across the different solvent extracts, but *H. hemerocallidea* generally showed higher antioxidant activity. The water extract of *H. africana* showed relatively good scavenging potential, suggesting the presence of water-soluble antioxidants. The hexane and DCM extracts showed limited scavenging activity.

The antibacterial activity investigated by assessing the percentage viability of the four bacterial strains exposed to the extracts demonstrated strong inhibition especially against *S. aureus*. *H.*

africana also demonstrated strong inhibition, particularly with its hexane and DCM extracts against *A. baumannii* and *S. aureus*. The extracts against *E. coli* and *P. aeruginosa* had high viability, suggesting no significant antibacterial effects.

The results of the in vitro CYP450 inhibition assay revealed that the extracts showed some CYP3A4 enzyme inhibitory activity, notably at high concentrations, with the methanol extract exhibiting inhibitory effect much higher than ketoconazole.

7.2 Conclusion

Medicinal plants have been traditionally used for years because they possess various therapeutic properties. Research in this field is now gaining popularity as researchers try to find new drug candidates for the treatment of several diseases.

H. hemerocallidea and *H. africana* are both native plants to Africa and have commonly been used in traditional medicine for various ailments. The results of the study found that both plants contain different phytochemicals and possess some level of antioxidant and antibacterial activity. Both plants demonstrated some degree of radical scavenging activity, though when compared to ascorbic acid, the IC_{50} values suggested weak antioxidant activity. Both plants had various antibacterial activity against certain pathogens suggesting them as potential natural antimicrobial agents. They also showed potential inhibition of CYP3A4 isoform of CYP450. Further studies are required to isolate the compounds responsible for the enzyme inhibition and antibacterial activity.

7.3 Limitations and future recommendations

- The DPPH radical scavenging assay was used to measure the antioxidant potential, but other assays such as the Ferric reducing antioxidant power (FRAP) or 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) could have provided a broader antioxidant profile of the plant extracts.
- The percentage viability antimicrobial assay was performed against four important bacterial strains, i.e., *A. baumannii, E. coli, P. aeruginosa,* and *S. aureus*. More strains could have been added for a full antimicrobial spectrum of the plant extracts, such as

antibiotic-resistant strains like *Methicillin*-resistant *Staphylococcus aureus* (MRSA), and STI-related bacterial strains including *Neisseria gonorrhoea,* and *Chlamydia trachomatis*.

- A cytotoxicity assay such as MTT viability assay could have been performed to assess cytotoxicity.
- Specific bioactive compounds that bring about activity should be identified, isolated and quantified using techniques such as HPLC or GC-MS.

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Appendices

APPENDIX A

Plant preparation and handling

Hypoxis hemerocallidea

Hydnora africana

APPENDIX B

Extraction process

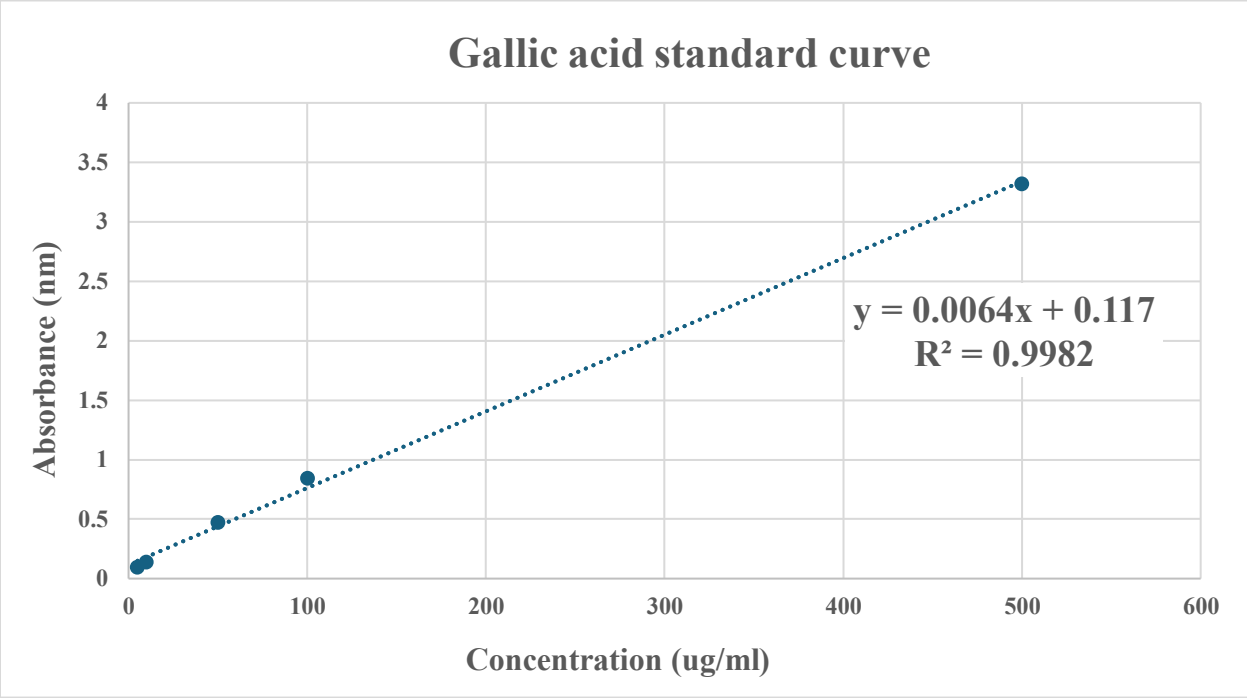
APPENDIX C

Extraction process continued

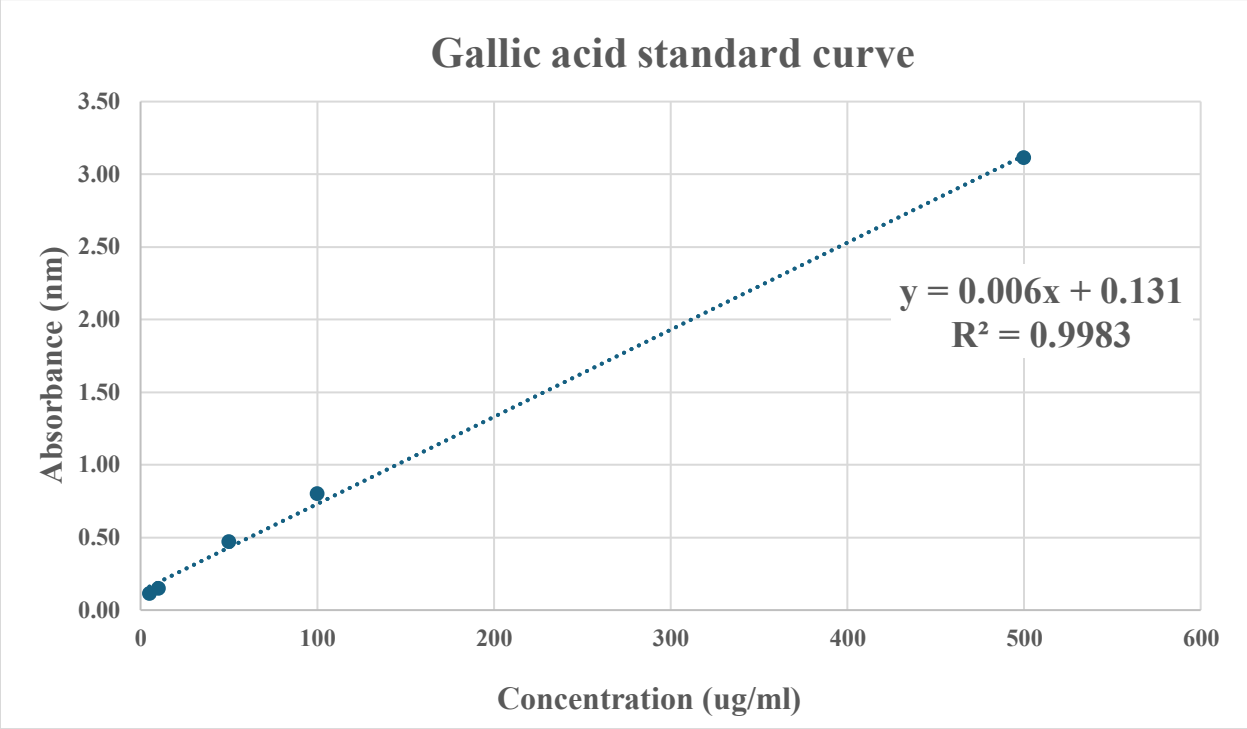

heidolph

APPENDIX D

Gallic acid standard curve used to calculate Total phenolic content of *H. hemerocallidea* plant extracts

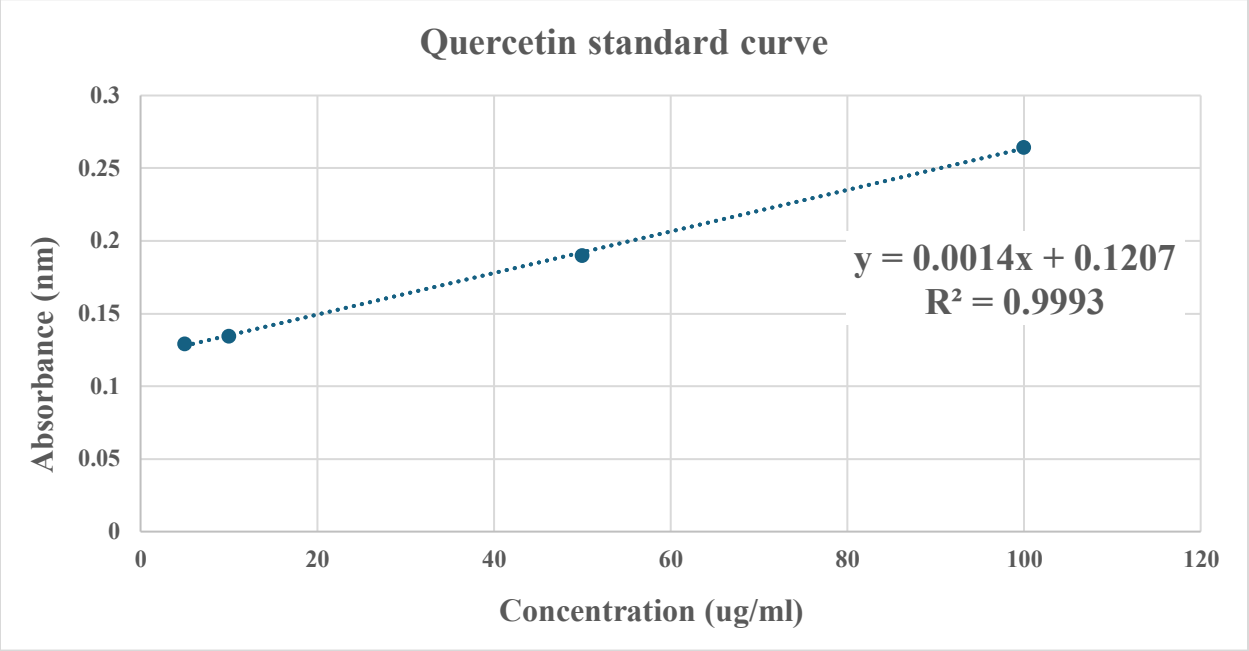


Gallic acid standard curve used to calculate Total phenolic content of *H. africana* plant extracts

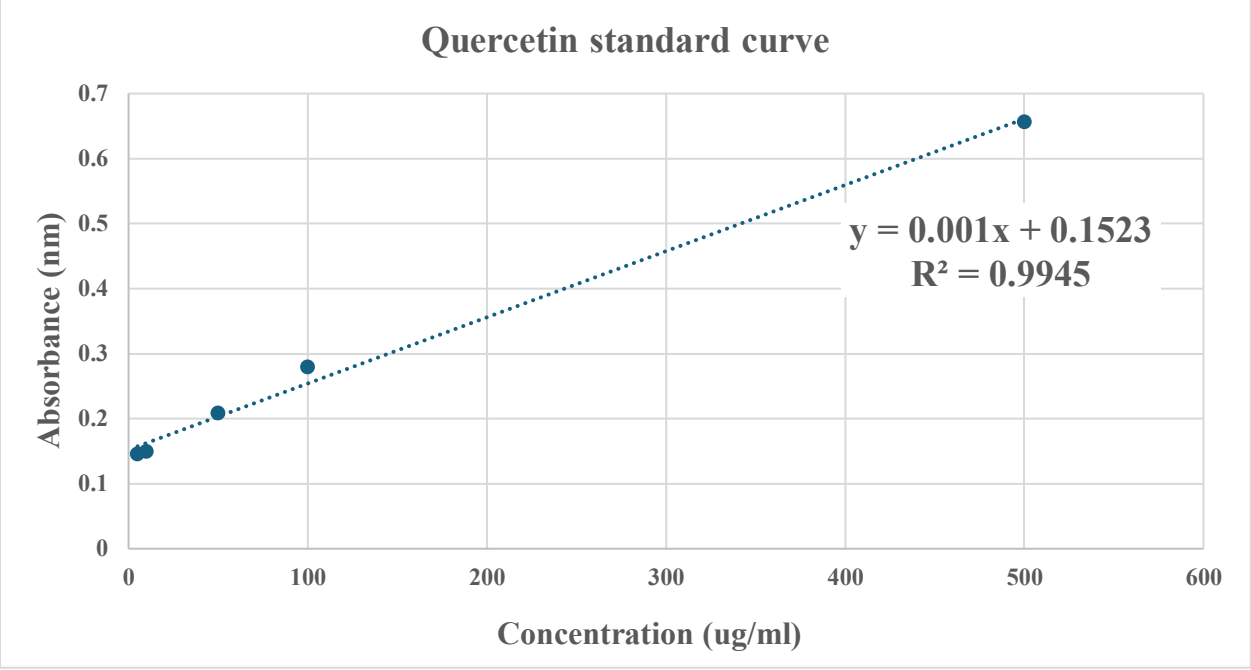


APPENDIX E

Quercetin standard curve used to calculate Total flavonoid content of *H. hemerocallidea* plant extracts



Quercetin standard curve used to calculate Total flavonoid content of *H. africana* plant extracts



APPENDIX F

Abstract from the 2023 Postgraduate Research day, hosted by the Faculty of Pharmacy at Rhodes University

The in-vitro investigation of the antimicrobial activity of medicinal plants used ethnobotanically for the treatment of sexually transmitted infections

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Introduction: Sexually transmitted infections (STIs) are a global burden and have become a significant public health issue, affecting millions of people. These infections can lead to serious consequences such as infertility in women, chronic pain, or an increased risk of HIV transmission. Dealing with them presents challenges such as stigma and discrimination, lack of awareness, as well as limited access to testing services and treatment. It is crucial to provide education, awareness, and access to treatment to decrease the incidence and prevalence of STIs on individuals and society. Research in recent years has focused on developing new approaches to prevent and treat STIs.

Method: This proposed research project aims to examine the efficacy of a mixture of medicinal plants in treating STIs by determining their antimicrobial activity against *Chlamydia trachomatis* and *Neisseria gonorrhoea* and explore the cytotoxicity against cell lines. The plant samples will be collected from a traditional healer as traditional medicines that are used for STIs, and who believes the plant samples are effective for the treatment of STIs. The bioactive compounds will be extracted from the plant material using appropriate solvents. The susceptibility of these strains will be determined using standard microbiological methods i.e., the zone of inhibition and minimum inhibitory concentration (MIC). Cytotoxicity testing will be performed using an MTT assay to evaluate the potential toxicity profiles on human tissues.

Keywords: antimicrobial activity, sexually transmitted infections, traditional medicine, medicinal plants

APPENDIX G

Abstract from the 2024 Research Symposium, hosted by the Faculty of Pharmacy at Rhodes University

Pharmacological Characterization of *Hypoxis hemerocallidea* and *Hydnora africana*: Ethnobotanical Approaches to the Treatment of Sexually Transmitted Infections

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Introduction:
Sexually transmitted infections (STIs) remain a significant global health challenge, with approximately 340 million new cases annually among individuals under 25 years old. These infections can lead to severe health complications such as chronic pain, infertility, and an increased risk of HIV transmission. While conventional treatments exist, the emergence of antibiotic resistance and the socio-economic barriers to accessing healthcare have necessitated the exploration of alternative therapeutic options. Traditional medicine, particularly medicinal plants used ethnobotanically, offer a potential alternative.

Objectives:
The efficacy of *Hypoxis hemerocallidea* and *Hydnora Africana* in treating STIs will be examined by:
1. determining the antimicrobial activity of the plant extracts against STI-causing pathogens.
2. and assessing potential herb-drug interactions.

Methodology:
Plant extracts of *Hypoxis hemerocallidea* and *Hydnora africana* were prepared using hexane, dichloromethane, methanol, and water. Preliminary phytochemical screening included analysis of total phenolic and flavonoid content. Thin Layer Chromatography (TLC) is yet to be conducted, as well as antimicrobial activity which will be assessed through disk diffusion and minimum inhibitory concentration (MIC) assays, and herb-drug interaction potential which will be evaluated through cytochrome P450 enzyme testing.

Conclusion:
The results will offer insights into the potential of *Hypoxis hemerocallidea* and *Hydnora Africana* as treatments for STIs.