ISOLATION, STRUCTURE ELUCIDATION, ANTIMICROBIAL ACTIVITY AND EFFECTS OF PLANT EXTRACTS USED FOR THE TREATMENT OF "U WELA"

BY

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THESIS

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DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo, for the degree of Doctor of Philosophy in Botany has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

_____ _____

Ramavhale, TT (Ms) **Date**

DEDICATION

I dedicate this work to my dearest parents Ramavhale Edward and Ramavhale Maureen. Thanks to you both, my study journey seemed so easy and full of great opportunities. I have achieved so much thanks to your support and love.

“Vho kondelela zwe zwa balela vhabebi vhanzhi, a vho ngo mphelela mbilu, vho vha hone musi zwi tshi konda, musi ndi tshi kou pfa ndo neta, vho neya nungo”, being the people you are has shown me the love the Lord has for me because both of you can raise a woman of my caliber, no words could ever thank you enough, I love and appreciate you so much!!

To my beloved siblings: Ramavhale Pfariso and Mulisa “ Have patience with all things, but first of all, with yourself” – Swiss Clergyman.

To my fellow African brothers: “A people without the knowledge of their history, origin, and culture is like a tree without roots” – Marcus Garvey

‘U wela” is deadly yet curable. Do not shy away from your culture and traditional way of doing things, you are and have always been an African first.

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ABSTRACT

Medicinal plants still account for a substantial significant portion of daily medication in South Africa and across the globe. In the Vhembe District, traditional health practitioners and local people use medicinal plants to combat various ailments such as “u wela”, sexually transmitted diseases, diabetes, and tuberculosis in humans. “U wela" also known as “divhu” in “Tshivenda” is a sexually transmitted disease that affects males due to unprotected sexual encounters with a woman who had an abortion or miscarriage. The study aimed to investigate medicinal plants used to treat “u wela” and isolate the active compounds from the most promising plant species.

Eight plant species (*Elaeodendron transvaalense* (Burtt Davy) R. H. Archer, *Albizia versicolor* Welw. ex Oliv, *Xanthocercis zambesiaca* Baker, *Cassia abbreviata* subsp. beareana (Holmes) Brenan, *Anthocleista grandiflora* Gilg, *Myrothamnus flabellifolius* Welw. *Mimusops zeyheri* Sond and *Capparis tomentosa* Lam.) used to combat “u wela” were selected from the Ethnomedicinal plant's database of over 300 medicinal plants used for medicinal purposes in humans. These plant species are used by the local community and traditional health practitioners in the Vhembe District to combat “u wela”. The plant materials were extracted with solvents of various polarities such as acetone, hexane, methanol, dichloromethane, ethyl-acetate, and water. Methanol extracted a large quantity of plant materials (15.6%), followed by acetone (6.2%), and DCM (0.2%).

Thin-layer chromatography (TLC) was used to determine the chemical components of different plant extracts. The TLC plates were developed using different eluent solvent systems such as Benzene: ethanol: ammonia hydroxide (BEA), Chloroform: ethyl-acetate: formic acid (CEF), and Ethyl-acetate: methanol: water (EMW). The separated compounds were visualised under ultraviolet light at the wavelength of 365 nm and 254 nm before being sprayed with the vanillin-sulphuric acid spray reagent. More compounds were observed in TLC chromatograms separated with BEA (54%), followed by the CEF (33%) and EMW (13%) solvent system, indicating that the majority of compounds were found to be non-polar.

Serial dilution assay was used to determine the antifungal activity of the plant extracts against the fungal pathogen, *Candida albicans*. The plant extracts of *A. versicolor, C. abbreviata* had excellent activity with a low MIC value ranging between 0.02, and 0.03 mg/ml. Noteworthy, aqueous extracts of *E. transvaalense, A. versicolor, X. zambesiaca, M. zeyheri,* and *C. abbreviata* were active against the tested fungal pathogen with MIC values of 0.02 mg/ml. Furthermore, the plant extracts were screened for antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Neisseria gonorrhoeae*. The acetone and methanol extracts of *E. transvaalense, X. zambesiaca, C. abbreviata, A. grandiflora, C. tomentosa, M. zeyheri,* and *M. flabellifolius* had excellent activity against the bacterial pathogens with MIC values ranging between 0.02-0.08 mg/ml. In bioautograms developed in BEA, active compounds were visible in the acetone, DCM, and ethyl-acetate extracts of *A. versicolor* with R_f values of 0.24. A similar active compound was observed in the DCM extract of *E. transvaalense* with the same R_f value of 0.24 against *E. coli.*

The serial exhaustive extraction method was used to extract plant materials using solvents of different polarities such as hexane, chloroform, acetone, and methanol. The acetone extract was selected based on good antifungal activity against the tested microorganisms and the presence of active compounds was observed in different plant extracts. Column chromatography of the acetone fractions led to the isolation of three compounds. The antimicrobial activity of the isolated compounds was determined against the fungi and bacteria. All compounds were active against the tested microorganisms with MIC values of 0.02-0.08 mg/ml. In bioautography assay, compounds with similar R_f values (0.31) were observed against *N. gonorrhoeae*.

Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) were used for the identification of the isolated compounds. Compound 1 was identified as 10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol and compound 2 as a 1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethen-one. The cytotoxicity of acetone extracts and isolated compounds were investigated using the (3-(4,5-dimethylthiazol) -2,5-diphenyltetrazoliumbromide) (MTT) assay. The crude extract was less cytotoxic with LC_{50} = 1.06 mg/ml. Compound 2 was not toxic at the highest concentration with LC_{50} values greater than 200 µg/ml against the Vero kidney monkey cells. Compound 1 was less toxic towards the Vero cells with LC_{50}

0.179 mg/ml. The study supports the traditional use of the selected plant species to combat “u wela” by the local people and traditional health practitioners

ABBREVIATIONS

A	Acetone
Af	*Aspergillus fumigatus*
ATCC	American Type Culture Collection
Amp	Amphotericin B
BEA	Benzene: ethanol: ammonia hydroxide (90:10:1)
Ca	*Candida albicans*
CEF	Chloroform: Ethyl-acetate: formic acid (5:4:1)
Cn	*Cryptococcus neoformans*
D/DCM	Dichloromethane
D H_20	Distilled water
DPPH	1,1-diphenyl-2-picrylhydrazyl
E	Ethyl-acetate
EMW	Ethyl-acetate: methanol: water (40:5.4:4)
Gn	*Neisseria gonorrhoeae*
H	Hexane
H_2O	Water
HIV/AIDS	Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome
INT	*p*-iodonitrotetrazolium violet
LATS	Limpopo Agro-Food Technology Station
LC_{50}	Lethal Concentration 50
M	Methanol
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
NMR	Nuclear Magnetic Resonance
R_f	Retention factor
ROS	Reactive Oxygen Species
Rpm	Revolutions per minute
STD	Sexually Transmitted Diseases
STI	Sexually Transmitted Infection
TH_2O	Decoction extract
TLC	Thin-Layer Chromatography

TREC	Turfloop Research Ethics Committee
UV- light	Ultraviolet light
WHO	World Health Organization

SYMBOLS AND UNITS

m/z	Mass-to-charge ratio
nm	Nanometre
°C	Degree Celsius
µl	Microliter
ml	Millilitre
mg/ml	Milligrams per millilitre
%	Percentage
min	Minutes
Hrs	Hours
µg/ml	Micrograms per millilitres

Table of Contents

CONFERENCE PRESENTATIONS

Ramavhale, T.T., Mahlo, S.M., Eloff, J.N., 2021. Phytochemical analysis and antifungal potential of plant extracts used traditionally for the treatment of oral candidiasis. University of Limpopo Faculty of Science and Agriculture Research Day (Online).

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Ramavhale, T.T., Mahlo, S.M., Eloff, J.N., 2023. South African traditional medicine as an alternative for the treatment of sexually transmitted diseases. South African Association of Botanists (SAAB) Conference (The Ranch Protea Hotel, Limpopo Province).

Ramavhale, T.T., Mahlo, S.M., Eloff, J.N., 2023. *In-vitro* antifungal activity of fractions from the bark of *Albizia versicolor* welw. Ex oliv. Indigenous Plant Use Forum Congress (Kruger National Park, Mpumalanga Province).

Ramavhale, T.T., Mahlo, S.M., Eloff, J.N., 2023. Traditional medicinal plants used for the treatment of gonorrhea in Vhembe District, Limpopo Province. University of Limpopo Faculty of Science and Agriculture Research Day (Bolivia Lodge, Limpopo Province).

List of Figures

List of Tables

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Infectious diseases caused by antimicrobial-resistant microbes are a major concern worldwide (Gyles, 2011; Srivastava et al., 2014). Amongst the various diseases, sexually transmitted infections (STIs) are pervasive. Sexually transmitted infections are caused by microorganisms that reside on the skin or close to the mucous membrane of the genital region. Furthermore, these infections are quite expensive to treat and cause health complications (Cavanaugh et al., 2011; Li and Webster, 2018). Amongst the STIs, “u wela” also known as “makgoma” in “Sepedi” is a common disease that has been documented to infect males (Mulaudzi, 2001; Ramavhale and Mahlo, 2019), but does not exclude the possibility that females might also be affected after engaging in unprotected sex. “U wela” has been reported mostly in Vhembe District, and it causes morbidity in males. However, it is difficult to treat with conventional western medicine (Shirindi, 2015; Muswede et al., 2021). The symptoms of “u wela” include weight loss, dry mouth, a distended vein appearing on the forehead, inflammation, and swelling of the genital parts (Mulaudzi and Makhubela-Nkondo, 2006). However, information on the therapy of “u wela” is not well documented and little has been reported on medicinal plants used to combat the disease in the Vhembe District, Limpopo Province.

Indigenous knowledge of the origin and usage of medicinal plants has been shared mainly by traditional health practitioners and elderly people. Furthermore, the outcome of failure to convey and document indigenous knowledge may result in the loss of cultural heritage (Turner et al., 2000). Knowledge and expertise related to the traditional use of ethnomedicinal plants may offer a significant alternative to the costly western medicine used by many rural communities (Phumthum et al., 2018).

1.2 Motivation

“U wela" is a sexually transmitted disease that affects males, following unsafe sexual encounters with a woman who had an abortion or miscarriage (Ramavhale and Mahlo, 2019). Sotho-Tswana-speaking traditionalists refer to it as “Go khutla” (Makgahlela

and Sodi, 2016). Males infected with the disease are required to perform a cleansing ritual to remove contaminants in their bodies (Niehaus, 2013). The symptoms of "u wela" are well-known among traditional health practitioners. Some of the symptoms have previously been reported to be comparable to those associated with HIV/AIDS (Ramavhale and Mahlo, 2019). Traditional health practitioners prefer medicinal remedies for the treatment of "u wela." This is because plants possess secondary metabolites, some of which have antifungal and antibacterial properties (Gunatilaka, 2006). Therefore, screening of medicinal plants could result in the development and detection of novel drugs that are cheap, effective, and less toxic.

Escherichia coli is associated with *Chlamydia trachomatis*, and *Trichomonas vaginalis* infections (Chiu et al., 2021) and causes diarrhoeal as well as urinary tract infections (Nycz et al., 2020). *Candida albicans, Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis,* and *Treponema pallidum* have been reported by the World Health Organisation (WHO, 2002) as the pathogens causing sexually transmitted diseases. *N. gonorrhoeae*, the Gram-negative bacteria causing sexually transmitted infections remains one of the most harmful microorganisms. Infections that are not medicated can result in major complications such as pelvic inflammatory disease (PID) and ectopic pregnancy (Rizzo et al., 2015). Azithromycin is used to treat genital infections caused by *C. trachomatis* such as cervicitis, urethritis, and proctitis (Ruddock et al., 2011). *C. trachomatis* is a human-adapted microorganism with over fourteen different serovars that cause trachoma, which is the main cause of sexually transmitted infections (Chiarelli et al., 2020). *C. albicans* causes candidiasis in humans. This fungal pathogen is resistant to antifungal agents such as caspofungin, micafungin, and anidulafungin (Tóth et al., 2019). Antifungal drugs such as itraconazole econazole, amphotericin B, fluconazole, and ketoconazole are ineffective against some fungal pathogens (Homa et al., 2018). However, some of these drugs such as ciprofloxacin and fluconazole are expensive and are not freely available (Sanguinetti et al., 2015).

Validation and acceptance of therapeutic plants for disease therapy induced by pathogens can be of considerable benefit to all citizens in rural areas who have minimal exposure to essential, lifesaving, and sometimes expensive modern medicine (Patwardhan and Patwardhan, 2005). More than 100,000 licensed traditional health

practitioners in South Africa are serving more than 20 million patients (Street, 2016). Therefore, ethnobotanical surveys, as well as screening studies are essential for recording valuable plant species that may contribute to new antimicrobial compounds in the development of novel drugs.

1.3 Aim

The study aims to investigate plant species used for the treatment of “u wela”, evaluate the activity of plant extracts, isolate and characterize antimicrobial compounds, which could be used to develop new antifungal agents to combat “u wela”.

1.4 Objectives

The objectives of the study were to:

i. Select eight plant species used for the treatment of “u wela” from the database of Ethnomedicinal plant species for further phytochemical analysis.
ii. Determine the antifungal activity of the selected plant species against *Candida albicans*.
iii. Determine the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* for comparative purposes.
iv. Determine the antibacterial activity of the plant extracts against *Neisseria gonorrhoeae*.
v. Isolate antimicrobial compounds from the selected plant species with good activity and determine their chemical structure.
vi. Determine the antioxidant activity of selected plant extracts using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay.
vii. Determine the cytotoxicity of the crude extracts and isolated compounds against Vero monkey kidney cells.

1.5 Hypothesis

Plant secondary metabolites contain active compounds that are not toxic against the bacterial and fungal pathogens that cause sexually transmitted diseases.

1.6 Outline of study

Chapter 1 entails a general introduction to the importance of medicinal plants, “u wela”, rationale, aim, and objectives of the study.

Chapter 2 focuses on the literature reviewed on “u wela”, secondary metabolites, sexually transmitted infections, and diseases, and the toxicity of medicinal plants. The botanical descriptions and literature review of ethnomedicinal uses of selected plants are discussed, and the conclusions are drawn.

Chapter 3 deals with the methods employed during plant extraction, phytochemical analysis, and antimicrobial activity of the plant extracts against the tested microorganisms. The methodologies for serial micro-dilution and bioautography assays are outlined followed by the results, discussion, and conclusions.

Chapter 4 focuses on the antifungal activities of different plant extracts. The methodologies for serial micro-dilution and bioautography assays are outlined followed by the results, discussion, and conclusions.

Chapter 5 dealt with the isolation of antifungal compounds using column chromatography. The methods and results are described in depth.

Chapter 6 is concerned with the structure elucidation of isolated compounds. The methodology is described, and results are given.

Chapter 7 deals with the qualitative and quantitative antioxidant activity of the plant species. The materials and methods used to determine antioxidant activity are given. The results for antioxidant activity are also given.

Chapter 8 deals with antifungal activity and cytotoxicity of the crude extracts and isolated compounds. The methods and results are discussed in depth.

Chapter 9 provides a summary of the study, and the conclusions based on the results obtained.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter deals with a literature review based on “u wela”, indigenous knowledge, conservation of medicinal plant species, plant secondary metabolites, and sexually transmitted infections. Literature reviews were conducted in-depth and compared with the previous and current findings on the uses of different plant species to combat “u wela”.

2.2 Background of “u wela”

"U wela also known as “Divhu” in “Tshivenda” is a sexually transmitted ailment disseminated by females who have not been cleansed following the loss of a child. Traditional health practitioners and local people in several parts of Venda use medicinal plants to treat "u wela” (Mulaudzi, 2001). Males contaminated with "u wela" are tainted by the lochia discharged by females, which is composed of carcinogenic chemicals (Mulaudzi, 2007), and distinctive herbs are used for therapy (Masevhe et al., 2015). Traditional health practitioners are conversant of the symptoms of "u wela," which include the malfunctioning of the male’s reproductive system, terrible headaches, fever, a jutting dry tongue, and abnormal isolated social conduct (Mulaudzi and Makhubela-Nkondo, 2006). Previously, it was reported that traditional health practitioners in the Vha-Venda community would make a blend of urine and herbs from both spouses to cure "u wela”. New therapy approaches have been proposed and are being researched instead of a blend of herbs and urine (Mulaudzi, 2001).

2.3 Importance of medicinal plants

Medicinal plants still account for a substantial significant portion of daily medication in South Africa. The use of medicinal plants as a fundamental component of the African traditional medical services framework is possibly the most established and diverse of any beneficial framework (Mahomoodally, 2013). More importantly, these plants are sold at the market to generate income for the local community (Figure 2.1) (Van Wyk

and Prinsloo, 2018). The Limpopo Province has been designated as a national therapeutic plant source region (Williams et al., 2000).

Figure 2.1 (A) Conventional shop with dried medicinal plant materials displayed on shelves, (B) A conventional urbanised herb shop with medicinal plant material, offering commercial herbal preparations, (C) and (D) conventional open herbal market and selling of packaged herbal products (Ndhlala et al., 2011).

In rural regions, local traditional medicine and plant-based medicine are still widely practiced to combat various ailments in humans. Furthermore, the continued loss of biodiversity might have a significant influence on the use of medicinal plants, which is especially widespread in low-income regions (Dzerefos et al., 2017). The traditional health practitioners in the Limpopo Province believe that their traditional healthcare function is in jeopardy as medicinal plants become inadequately scarce (Rasethe et al., 2022). There is limited information on the medicinal usage of plant species to

combat “u wela” particularly in the Vhembe District of Limpopo Province. This necessitates the relevance of ethnobotanical surveys in obtaining indigenous knowledge on plant species used for medicinal purposes, which might lead to the development of novel, safer, and less expensive robust medicines. It is critical to record indigenous medicinal plant knowledge for future generations before it is lost.

2.4 Uses of ethnomedicine

Plant parts such as seeds, roots, leaves, fruits, bark, flowers, or the whole plant may be utilized for medicinal purposes. These plant parts contain active chemicals that have direct or indirect therapeutic impacts and are employed as medicinal treatments (Vyas et al., 2019). Furthermore, active compounds found in plants are generated and stored and also have physiological impacts on living forms (Phillipson, 2001). The plant's active compounds may interact with one another, and this interaction may be helpful and valuable to both or detrimental to either, or it may remove the bad impacts of both (Silva et al., 2010). The capacity of plant components to inhibit the development of certain illnesses is also a distinguishing feature. Plant-inferred constituents can significantly improve difficult-to-treat sicknesses, such as "u wela”.

Despite the pharmaceutical industry's rising progress and expanding advancement, the globe still relies heavily on ethnomedicine to cure diseases (Bhuda and Khazamula, 2022). Ethnomedicinal use of plant species by the local community is highly significant, and it is growing every day in most regions of the world (Charles and Bonareri, 2020). The local community prefers traditional medicine to western medicine due to the lack of primary health care particularly in rural parts of South Africa. More importantly, the local community considers traditional medicine since it is accessible, cheap, and more cost-effective. Furthermore, most impoverished people rely on herbal items and medicines to treat different human and animal ailments (Peerzada et al., 2020).

2.5 Indigenous knowledge

Indigenous knowledge is the information that is passed verbally from generation to generation by the local communities. This information is valuable not only to the indigenous peoples but also to researchers in the area who are working to improve the surrounding local conditions and people's well-being (Gangan et al., 2014).

Furthermore, traditional health practitioners have extensive knowledge of specific locations of plant species used to combat various diseases in humans. Due to this long use for food and medicine by the indigenous people, some plant species, are recognized in local communities through local names. More importantly, this information is passed from generation to generation by the local people and the traditional health practitioners. This local knowledge is extremely beneficial in the field of plant conservation (Bamigboye et al., 2017).

Indigenous plant knowledge, particularly therapeutic plant knowledge, appears to be one of the most endangered. Many factors have been blamed for the loss of plant knowledge, including lumbering, development of business sectors, globalization, and cultural fusions, as well as other human populace pressures (Sabbag, 2022; Sinha and Samad, 2021). Most traditional medicinal plant knowledge is accessible in rural communities and is passed verbally from generation to generation (Zubaidah et al., 2020). The documentation of indigenous and traditional knowledge is critical for subsequent evaluations that may lead to sustainable natural resource use in the future (Jamshid-Kia et al., 2018), thereby educating people about the importance of biodiversity preservation. Documentation on the utilization of medicinal plants is required for future research (Masevhe et al., 2015).

2.6 Conservation of medicinal plant species

The over-exploitation of plant parts such as barks, bulbs, roots, stems, and tubers, as well as whole plants, may lead to the local extinction of the plant species. Despite the fact that the protection of indigenous plant species has been a priority for decades, some plant populations are continuing to deteriorate at a disturbingly rapid rate (Jose, 2012; Monzón et al., 2011). As such, many therapeutic plants become extinct before their use is documented (Van Wyk and Prinsloo, 2018). It is necessary to consider the long-term use of therapeutic plants, which involves the creation and development of good harvesting procedures (Chen et al., 2016). For the growing demand for medicinal plants to be satisfied, it is critical to protect these plant species through cultivation or other preservation measures such as *in-situ* and *ex-situ* conservation (Xego et al., 2016). Each species may have unique biological, economic, health, social, and cultural connections that must be acknowledged (Astutik et al., 2019; Schippmann et al., 2002). According to Malik et al. (2021) and Vermeulen (2009), controlled, experimental

collections and long-term observations are required to increase yield frameworks and best practices for plant collecting.

2.7 Plant secondary metabolites

Plants provide a rich source of secondary metabolites that are employed as medications (Al-Snafi, 2015). These secondary metabolites are produced by plants as part of their usual metabolic processes. Phytochemicals are secondary metabolites produced by plants that have biological functions but are not considered as food. These chemicals have the potential to have a positive impact on health, and their biological importance includes antibacterial, antifungal, antioxidant, anti-inflammatory, anticancer, antiaging, and antiatherosclerosis properties among others (Khan and Javaid, 2019; Lu et al., 2021). However, further research is required to attest and establish the medicinal value(s) and actions of these phytochemicals. Despite our understanding of phytochemicals, a large proportion of them remain unidentified (Defossez et al., 2021). The integration and assembly of secondary metabolites are linked to a variety of environmental conditions in growth environments, including temperature, moisture, and illumination (Dong et al., 2011). This proposes that the synthesis of secondary metabolites in many plant species is influenced by the development of the environment. Secondary metabolites such as alkaloids, sterols, terpenes, flavonoids, saponins, glycosides, cyanogenic, tannins, resins, lactones, quinines, and volatile oils are integrated and assembled by therapeutic plants, according to various investigations (Poddar et al., 2020).

2.8 Sexually transmitted infections and diseases

Sexually transmitted infections (STIs) infect over a million people across the globe (WHO, 2018). The variety and recurrence of sexual infections and disorders resulting from harmful microorganisms have increased (Figure 2.2), making them an extensive cause of illness and death among the youth (Moore et al., 2020). Sexually transmitted infections and diseases (STIs and STDs) are transmitted from one person to the next through sexual activity. However, some of these diseases are spread by other means other than sexual contact (Torrone et al., 2018). Bacteria, viruses, and parasites can cause sexually transmitted diseases. STDs have diverse symptoms, transmission mechanisms, and therapeutic options (Nazer et al., 2019). Practically everyone is prone to STDs, and the weaker their immune system, the more likely they are to

contract them (Geremew et al., 2017). Most STDs can be managed with medicine, however, neglected STDs can lead to infertility, pregnancy problems, cervical cancer, pelvic inflammatory disease, birth abnormalities, and a five-fold greater risk of HIV transmission (Santana et al., 2018).

Several STDs result from viruses, which are far more challenging, if not impossible, to treat. Hepatitis B, hepatitis C, herpes simplex virus (HSV), human immunodeficiency virus (HIV), human papillomavirus (HPV), and genital warts are just a few of the symptoms that can be eradicated (Schranz et al., 2018; Johnson and Jackson, 2021). The human immunodeficiency virus is the causative agent of HIV infection. HIV impairs the body's capability to combat viruses, germs, and fungi that emerge during the disease and can ultimately result in AIDS. AIDS is a potentially fatal disease (Kojima and Klausner, 2019). Since prehistoric times, medicinal plants have been proven to be effective therapeutic agents for STIs and STDs. The medicinal plants that have been investigated are crucial for determining antibacterial, antifungal, and antiviral activity in transmissible illnesses. As such, different medicinal plants may exert their effects through more than one technique (Ghasemi et al., 2013).

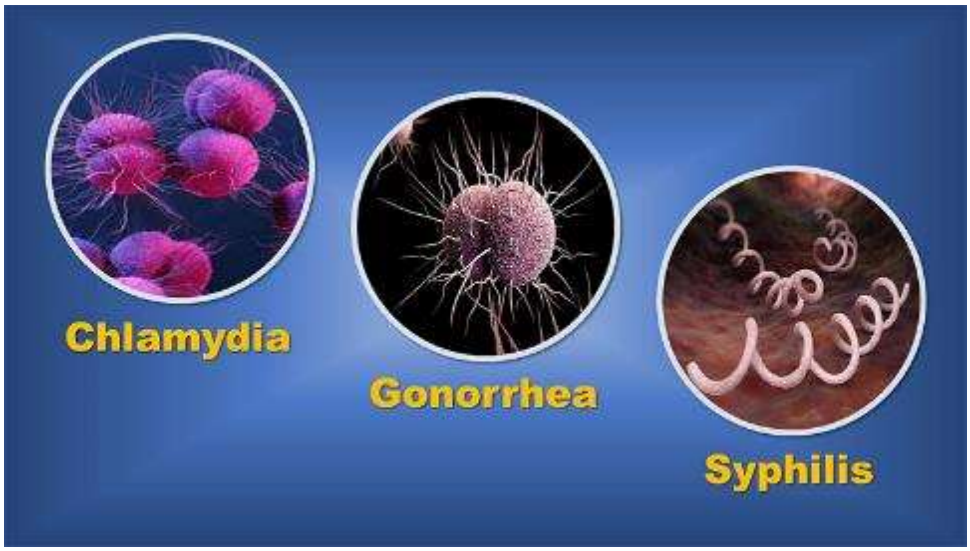


Figure 2.2 Illustrations of three microorganisms that cause sexually transmitted diseases (Crane et al., 2021).

Although protection such as condoms, can help minimize the probability of STD contraction, there are no foolproof or fully safe ways to prevent the spread of these

infections (Leitgeb, 2010). The pathogens that cause the majority of STIs and STDs have been recognized as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponema pallidum*, and *Trichomonas vaginalis* (Barrientos-Durán et al., 2020; Frickmann, 2019). However, in underdeveloped nations, *N. gonorrhoeae* and *T. pallidum* have been identified as the utmost common causes of sexually transmitted infections (Adamson et al., 2020). The contraction of the *N. gonorrhoeae* STIs induces infertility and pelvic inflammatory illness in women (Jennings and Krywko, 2018), whereas *T. pallidum* is the pathogen that causes syphilis (Figure 2.3), which raises the risk of HIV infection and hemolysis-related diseases (Cameron, 2018).

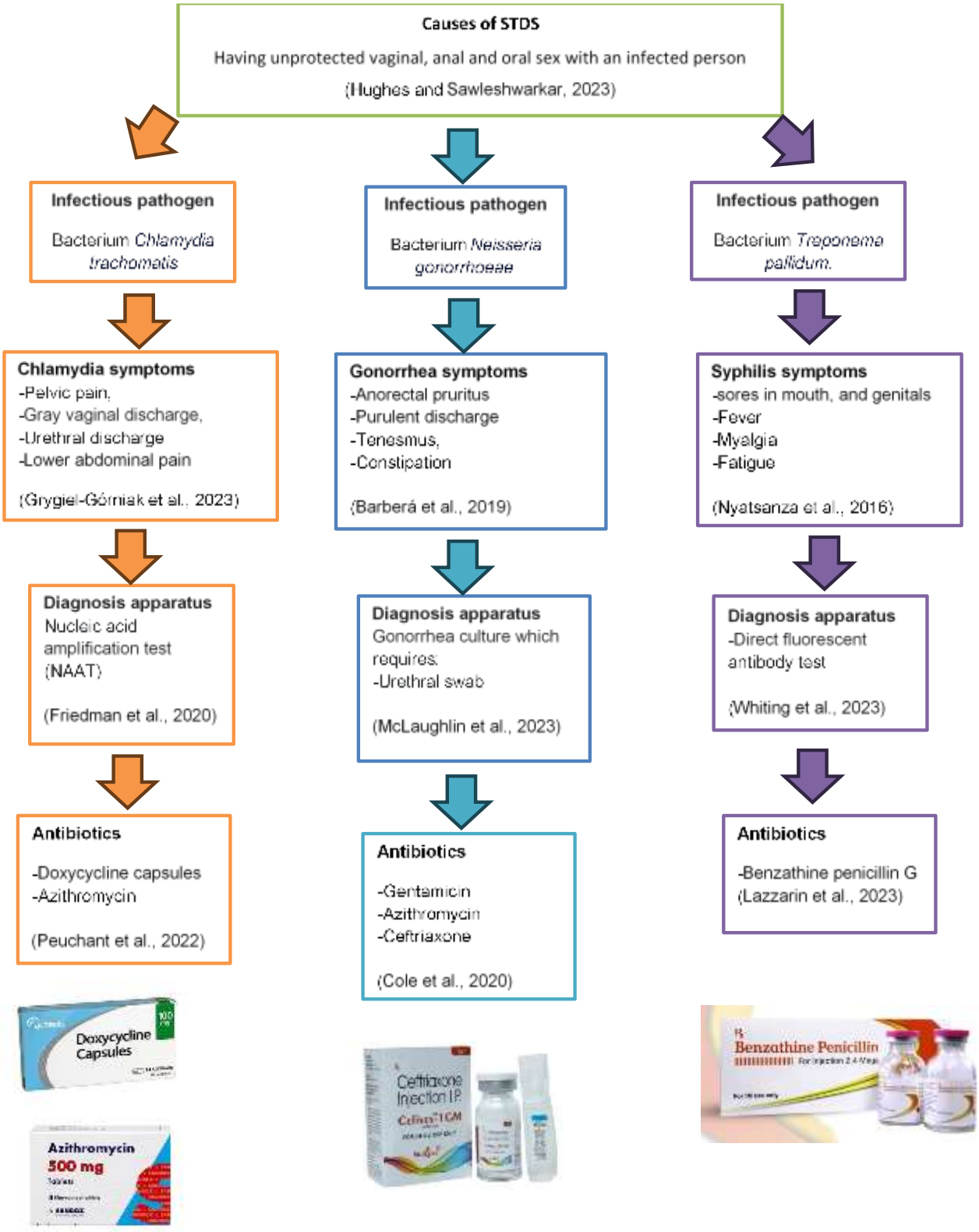


Figure 2.3 Flow chart indicating the manifestation of three bacterial pathogens in a female body.

2.9 Plant species used for the treatment of Sexually Transmitted Diseases

The Limpopo Province has the highest STI prevalence rates, followed by KwaZulu-Natal and the Eastern Cape (Maema et al., 2019). Nowadays, research focuses on medicinal plants that are used to combat sexually transmitted diseases in humans (Mongalo and Raletsena, 2022). It is vital to emphasize that such medicinal plants are easily accessible and cheap compared to western medicine (Oladeji et al., 2023). Furthermore, restricted access to healthcare facilities, inadequate number of research facilities, prescription shortages, and bad attitudes among health personnel make traditional medicine more enticing (Ngobeni et al., 2023). Numerous individuals, especially those living in rural regions, are reluctant to seek western medical assistance after being diagnosed with STIs due to the shame associated with disclosing sexual behaviour (Mulaudzi et al., 2011). Previously, it was reported that plant species such as *Senna italica, Helinus intergrifolius, Ximenia caffra*, and *Tinospora fragosa* are used for the treatment of sexually transmitted diseases in the Limpopo Province (Chauke et al., 2015). Furthermore, the roots of *Catharanthus roseus* and the bark of *Senna petersiana* and *Anredera cordifolia* are used to combat gonorrhoe in the Mandiwana village, Vhembe District (Mulaudzi et al., 2015).

2.10 Toxicity of medicinal plants

Herbs are being used for the treatment, curing, and prevention of various diseases in humans. However, the toxic effect of some herbal medicines is a major concern. Toxicology is a branch of science that deals with the adverse effects of chemical constituents on living organisms (Gowda et al., 2014). It is critical to examine the toxicity of medicinal plants since most of the South African population still depends on medicinal plants as a first-line therapy (Street et al., 2008). There is a misconception that natural remedies are safer in comparison to modern-day conventional treatments. However, plants that display beneficial properties may also have cytotoxic properties (Hamidi et al., 2014). Compounds that are found in plants interact with each other or with cells that can cause toxicity (Lin et al., 2020). Therefore, establishing the safety and efficacy of these plant extracts and essential oils is vital.

2.11 Botanical description of eight medicinal plants used in the study

Eight plant species used for the treatment of “u wela” in Vhembe District, Makhado Local Municipality were selected from a database on ethnomedicinal plants of over 300 plant species used for therapeutic purposes obtained from four Districts of Limpopo Province (Capricorn, Mopani, Vhembe, and Waterberg). The plants were selected based on the treatment of “u wela” from ethnobotanical data and the availability of the plant species. Pictures of the selected plant species were taken from Nzhelele (Figure 2.4-Figure 2.12).

Elaeodendron transvaalense (Burtt Davy) R.H.Archer is a small to medium-sized tree of the Celastraceae family. The plant is also known as anthill saffron or bushveld saffron. The bark is grey and often smooth (Figure 2.4). Reduced lateral branches with oblong leaves that are approximately 50 mm long and 20 mm broad are common. The leaf margin is occasionally serrated. Greenish blossoms give way to rectangular, yellow to dark orange berry-like fruits that are delicious (Tshikalange and Lawal, 2020). *E. transvaalense* is found throughout Southern Africa, including Angola, Botswana, Namibia, Mozambique, South Africa, Zambia, Zimbabwe, and Swaziland (Williams et al., 2013). In South Africa, the plant species is found in the eastern and summer rainfall zones spanning from the KwaZulu-Natal coast northwards through eastern Mpumalanga into Limpopo and North-West Provinces (Rasethe and Semenya, 2019). In traditional medicine, *E. transvaalense* is commonly used to cure a variety of disorders such as cough, diarrhoea, stomach ailments, rash, gout, haemorrhoids, menorrhagia, diabetes, swellings, and sexually transmitted infections (Bansal and Priyadarsini, 2021). The stem bark is used to make infusions or decoctions that are either taken orally or used as an enema (Khumalo et al., 2019).

Figure 2.4 *Elaeodendron transvaalense* (Burtt Davy) R. H. Archer (Mulamanamana) (A), Tree (B), Foliage and (C), Leaves.

Albizia versicolor, popularly known as the Governor's plum belongs to the family Fabaceae (Saidi and Tshipala-Ramatshimbila, 2006). It is a deciduous tree with an open, spreading crown that typically grows from 5 to 18 meters tall, but can grow smaller in poor soils or up to 20 meters tall (Figure 2.5). The trunk is normally short and free of branches for up to 5 metres, sometimes up to 12 metres. It is straight and cylindrical, up to 60cm in diameter, sometimes up to 150cm in diameter (Botha and Venter, 2002). The plant parts are used to treat anaemia, swollen glands, coughs, joint aches, tapeworms, fever, diarrhoea, ulcers, and sexual illnesses (Mudau et al., 2020).

Figure 2.5 Albizia versicolor Welw. ex Oliv (Muvhambangoma), (A), tree and (B), leaves.

Xanthocercis zambesiaca is a member of the Fabaceae family (Figure 2.6). It is frequently referred to as the nyala tree (Nizami and Sayyed, 2018). In Africa, the species may be found in Botswana, Malawi, Mozambique, South Africa, Zambia, and

Zimbabwe (Van Wyk and Smith, 2001). *X. zambesiaca* is an evergreen to semi-deciduous tree with a single short stem, a hefty spherical crown, and slightly falling leaves. It may reach a height of 260 m (Brummitt et al., 2007) and flourishes at low elevations in hot climates, in the rich alluvial soils of major river valleys, on riverbanks and alongside lakes, and occasionally in drier places (Tree, 1916). The white wood is firm, weighty, and has an appealing fine feel. Traditional health practitioners employ the boiled stem and roots of *X. zambesiaca* to cure stomach symptoms and "Nyoko," a gall bladder dysfunction (Ngobeni, 2016). Furthermore, it is used in traditional remedies to treat diabetes and gastro-intestinal problems. The root and bark decoction is used to treat colds and snakebites.

Figure 2.6 *Xanthocercis zambesiaca* Baker (Mutshato), (A) tree, (B) fruit, and (C) leaves.

Cassia abbreviata is a small to medium-sized deciduous tree with unusual cylindrically formed fruits (Sobeh-et-al., 2018). This perennial tree is a member of the Caesalpiniaceae family (Figure 2.7). It is distributed in Gabon, Swaziland, Kenya, Tanzania, South Africa, Botswana, and Zimbabwe (Jobe et al., 2019). *C. abbreviata* is a tree that may reach a height of 10 m. The bark is light brown, the crown is rounded, and the leaves turn yellowish (Mongalo and Mafoko, 2013). *C. abbreviata* has complex leaves with 5 to 12 pairs and is threatened (Sobeh et al., 2018). It is widely used in the communities. Oral root infusions are used as an abortifacient, an aphrodisiac, and a purgative. *C. abbreviata* root extracts have been shown to have direct inhibitory action against HIV-1c replication *in-vitro* (Leteane et al., 2012). Preparations made from stem bark, leaves, and twigs have also been used to cure headaches, toothaches, heavy menstruation, and eye infections (Dangarembizi et al., 2015).

Figure 2.7 *Cassia abbreviata* Oliv. (Muboma), (A) tree, (B) leaves, and (C) Seed pod.

Anthocleista grandiflora, popularly known as the forest fever tree, is a member of the family Gentianaceae (Figure 2.8). It is a tall and thin tree up to 30 m tall that grows in tropical and subtropical forests in East and South Africa. It is also found in Zanzibar and the Comore Islands. It has very huge leaves that may grow up to 100 cm and 50 cm in length and are placed in clusters (Adikwu et al., 2020). The tree is often epiphytic, with auxiliary spines or tendrils, leaves that are opposite, occasionally alternating, rarely verticillate, fasciculate, or in a whorl, and stipules that are normally present but are often reduced to lines linking petiole bases. The flowers occur in cymes, which are frequently clustered into thyrses. The blooms are often bisexual and cream in colour. It is not edible as food, but it contains therapeutic characteristics in the roots, stems, bark, leaves, and flowers (Bensandy et al., 2012). The bark and leaves are used to treat malaria, diarrhea, hypertension, diabetes, and venereal disease (Adikwu et al., 2020). The bark is also used to treat epilepsy, typhoid fever, hepatitis, stomach pains, hemorrhoids, syphilis, diabetes, as a contraceptive, and as a laxative and purgative (Anyanwu et al., 2015).

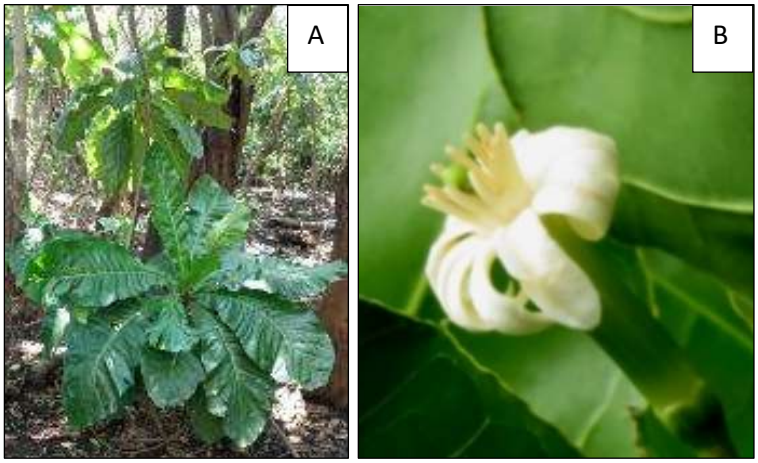


Figure 2.8 *Anthocleista grandiflora* Gilg (Muene-ene) (A), tree and (B) flower.

Myrothamnus flabellifolius, popularly known as the resurrection aromatic bush, is a member of the Myrothamnaceae family, which has just two species (*Myrothamnus flabellifolius* and *Myrothamnus moschata*). Both are shrubby, xerophytic, and flowering plants (Figure 2.9). It is mostly prevalent in Southern African countries and other tropical areas (Erhabor et al., 2020). The plant grows between rocky outcrops with very shallow soils and spends nearly half of the year in a dormant condition due to dehydration (Cheikhyoussef et al., 2015). This shrub has developed the unusual capacity to survive prolonged periods of extreme dehydration (desiccation) (Nako, 2014). It is used in traditional medicine to treat respiratory problems, inflammation, wounds, heart problems, and renal problems. It is also used as a tonic and to moisturize the skin, as well as to cure chest problems, epilepsy, and mental illnesses (Marks et al., 2022).

Figure 2.9 *Myrothamnus flabellifolius* Welw. (Mukangambanzhe) (A), shrub, (B) leaves, (C) leaves with flowers.

Mimusops zeyheri, popularly known as Transvaal red milkwood, is a member of the Sapotaceae family (Figure 2.10). It is evergreen and reaches a height of 15 m, with non-aggressive lateral root systems. They are frost-hardy and drought-tolerant, and the tree is mostly pest-free due to its high amount of latex in above-ground organs, with the exception of an unnamed fruit borer that attacks mature fruits (Mngadi et al., 2019). The fruit tree is native, and it has a lot of potential to help with economic and nutritional initiatives in dry and semi-arid areas (Venter and Venter, 1996). The first leaf flush comes in late winter (July to August), while the second, accompanied by blossoms, occurs from late spring to early summer when the fruits are ready for harvest (Nkuna, 2018). *M. zeyheri* fruit has the greatest vitamin C content per unit of any locally accessible fruit, including native and alien (Dube et al., 2016). The plant is well-known in rural communities for its several applications. In traditional medicine, a decoction of *M. zeyheri's* bark and leaves is used to cure wounds and ulcers. The root is used as an infusion to treat candidiasis and other health conditions (Omotayo et al., 2020).

Figure 2.10 *Mimusops zeyheri* Sond. (Mububulu), (A) leaves and (B) fruits.

Capparis tomentosa, often known as woolly caper-bus in English, is a member of the Capparaceae family (Figure 2.11). The plant is distributed in Botswana, Eritrea, Ethiopia, Lesotho, Namibia, South Africa, Swaziland (as a native), and Kenya (as an exotic) (Akoto et al., 2008). It's a scrambling shrub that may grow up to 10 meters tall in some cases. The twigs and leaves are yellow-green and covered with silky, velvety hairs. The oblong leaves are roughly 50 mm and 20 mm in length, with a pair of sharp, hook-like thorns at the stem and leaf base junction. The stamens on the white and pink blossoms are many. The fruit is spherical, pink to orange in hue, and stalked. The seeds are encased in meaty, grey fruit pulp (Gebrehiwot and Chaithanya, 2020). The plant is widely used to cure a wide range of diseases, including mental illness, snake bites, chest pains, impotency, and barrenness (Gebrehiwot et al., 2019). It is also used to treat wounds, such as leprosy, and has been used in cooking and medicine. The flower buds are traded on the worldwide market (Bussmann et al., 2021).

Figure 2.11 *Capparis tomentosa* Lam. (Muobadali), (A) leaves and (B) fruit.

In the next chapter, extraction of selected medicinal plants, phytochemical analyses, and biological activity will be discussed in depth.

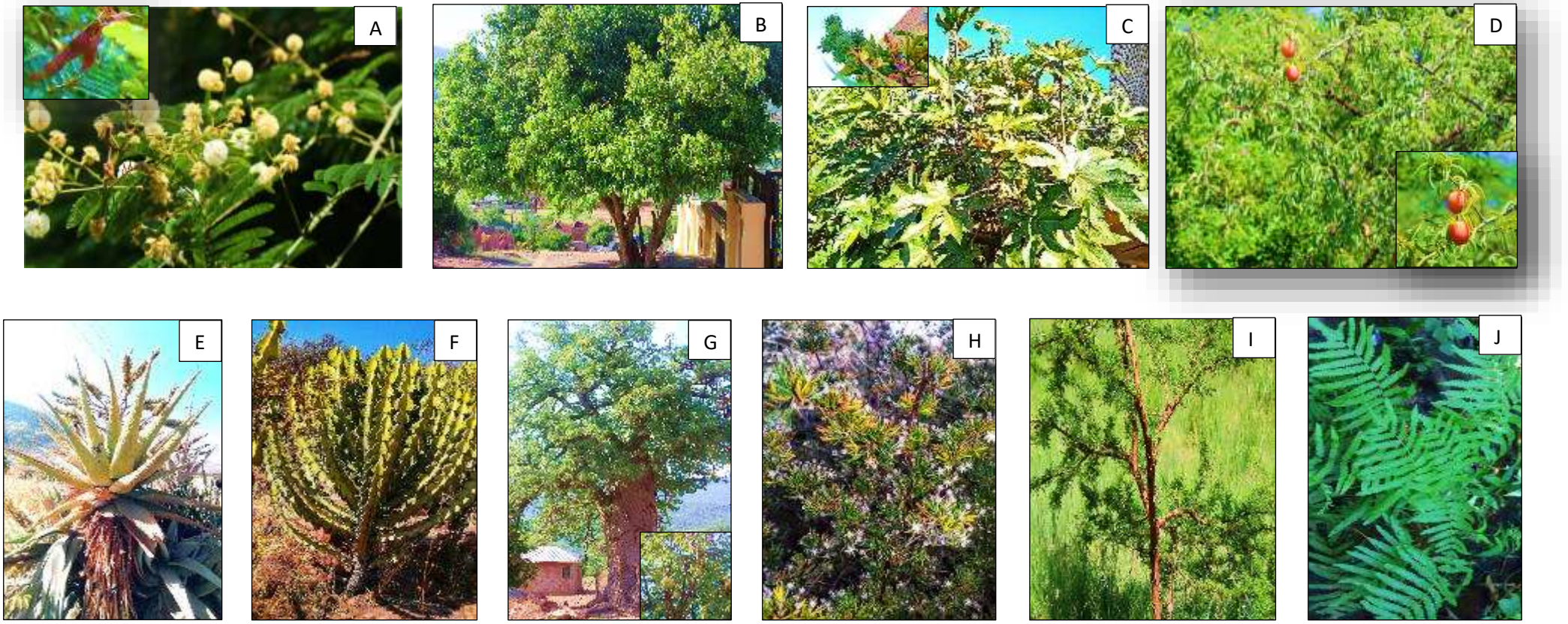


Figure 2.12 Medicinal plants used in Makhado Local Municipality, Vhembe District for the treatment of “u wela”. Left to right A: *Pterolobium stellatum* (Forssk.) Brenan, B: *Syzygium cumini* L., C: *Ricinus communis* L., D: *Ximenia caffra* Sond., E: *Aloe marlothii* A.Berger , F: *Euphorbia ingens* E.Mey. ex Boiss., G: *Adansonia digitata* A L., H: *Asparagus suaveolens* Burch., I: *Vachellia davyi* (N.E.Br) Kyal. and Boatwr. J: *Pteridium aquilinum* (L.) Kuhn subsp.

CHAPTER 3

SCREENING OF SELECTED PLANTS AGAINST BACTERIA AND FUNGAL PATHOGENS

3.1 Introduction

Medicinal plants contain a variety of bioactive substances that could be beneficial to both individuals and societies' well-being. These substances have a distinct physiological effect on the human body (Ahamed et al., 2017). They are synthesized from compounds with distinct activities that can be used to remedy a variety of health problems and persistent illnesses (Nandagoapalan et al., 2016), hence understanding the relationship between phytoconstituents and plant bioactivity is important. The demand for plant-derived medications has recently grown as a result of the conservative implementation of organic components (Niazian, 2019). The side effects of synthetic medications on host tissues as well as the multidrug resistance of organisms increase the demand for natural products (Vidya et al., 2019).

Extraction of medicinal plants is the separation of active plant compounds or secondary metabolites such as alkaloids, flavonoids, terpenes, saponins, steroids, and glycosides from dormant material with the use of a suitable solvent and a conventional extraction procedure. Phytochemicals are plant compounds that are not nutritious but may have defensive or disease-prevention qualities. Plants can produce a vast range of chemical compounds that act as a defence against predators (Sandhya et al., 2006). The vast range of secondary compounds plants produce is a result of the complicated intermediate metabolism and many of the compounds produced by plants are active against human, and animal diseases, and pests that plants are not subjected to.

Infectious microorganisms emanating from microorganisms such as fungi, and bacteria are the leading cause of severe infections especially in underdeveloped nations, resulting in more deaths and illnesses in immunocompromised people (Danish et al., 2020). It is estimated that less than ten percent of the world's plant species have been chemically and pharmacologically examined for their potential therapeutic value (Panda et al., 2017). The variety and frequency of sexual infections

resulting from harmful microorganisms have grown, making them a major cause of sickness and mortality in young people. Since immemorial times, medicinal herbs have been effective treatments for STIs (Nthulane et al., 2020). Plants have historically played an essential role in the production of therapeutic drugs since they are substantial-sources-of-distinctive-and-innovative-chemicals-for-medication-development (Pereira et al., 2019).

Phytochemical analysis is the most basic approach for identifying the presence and number of secondary metabolites in plant extracts (Al-Tohamy et al., 2018). The chemical fingerprinting generates a distinctive chemical pattern for the plant extract, which aids in its identification (Shawky et al., 2018). Examples of plant secondary metabolites-include-alkaloids,-flavonoids,-terpenoids,-steroids,-carbohydrates,-proteins, and saponins (Verma and Singh, 2020).

Earlier studies used agar diffusion to determine the antimicrobial activity of plant extracts. This has been replaced by the serial dilution microplate determination of minimum inhibitory concentrations in leading scientific journals (Eloff, 2019). Micro-dilution methods are used to determine the minimum inhibitory concentration (MIC), which is the lowest concentration of antimicrobial agent that may inhibit the growth of pathogens. The micro-dilution assay is a dependable and frequently used method for antimicrobial resistance testing, which leads to the establishment of MIC of compounds that might possess antimicrobial activity. The bioautography assay is used to determine the presence of active compounds in plant extracts. The assay is less expensive, straightforward, and available, with the added benefit of identifying antimicrobial metabolites and biologically active compounds in plant extracts against bacteria and fungi (Nuthan et al., 2020). The essence of this assay is the separation and detection of active compounds in plant extracts, which is done directly on a TLC plate (Fathoni et al., 2021). INT (p-iodonitrotetrazolium violet) is sprayed on the TLC plates as an indicator of growth, hence a clear zone is visible on bioautograms against the pinkish background (Elisha et al., 2017).

3.2 Materials and methods

3.2.1 Plant selection and collection

A database on ethnomedicinal plants of over 300 plant species used for therapeutic purposes obtained from four Districts of Limpopo Province (Capricorn, Mopani, Vhembe, and Waterberg) and Mpumalanga Province (Ehlanzeni District) has been created in the Department of Biodiversity, ethnomedicinal laboratory. In the current study,-eight-plant-species-(*Elaeodendron-transvaalense*,-*Albizia-versicolor*,-*Xantho cercis zambesiaca*, *Cassia abbreviata*, *Anthocleista grandiflora*, *Myrothamnus flabellifolius*, *Mimusops zeyheri,* and *Capparis tomentosa*) were selected based on the treatment of “u wela” from ethnobotanical data and the availability of the plant species

Plant materials were collected in February-April 2021 from Nzhelele in Vhembe District, Makhado Municipality, Limpopo Province (Figure 3.1 and Table 3.1). The plants were stored in open mesh orange bags at room temperature of 25°C to ensure efficient drying of the material. Plants were identified at the Larry Leach Herbarium at the University of Limpopo. Voucher specimens were prepared and deposited at the Larry Leach Herbarium. Plant materials such as leaves stems, and bark roots were allowed to dry for 3-5 weeks and ground to a fine powder.

3.2.2 Ethical Considerations

The research was conducted in Vhembe District, Limpopo Province. Permission for plant collection was granted by the local authority and “Indunas”. The proposal was approved by the Turfloop Research Ethics Committee for ethical clearance prior to the study.

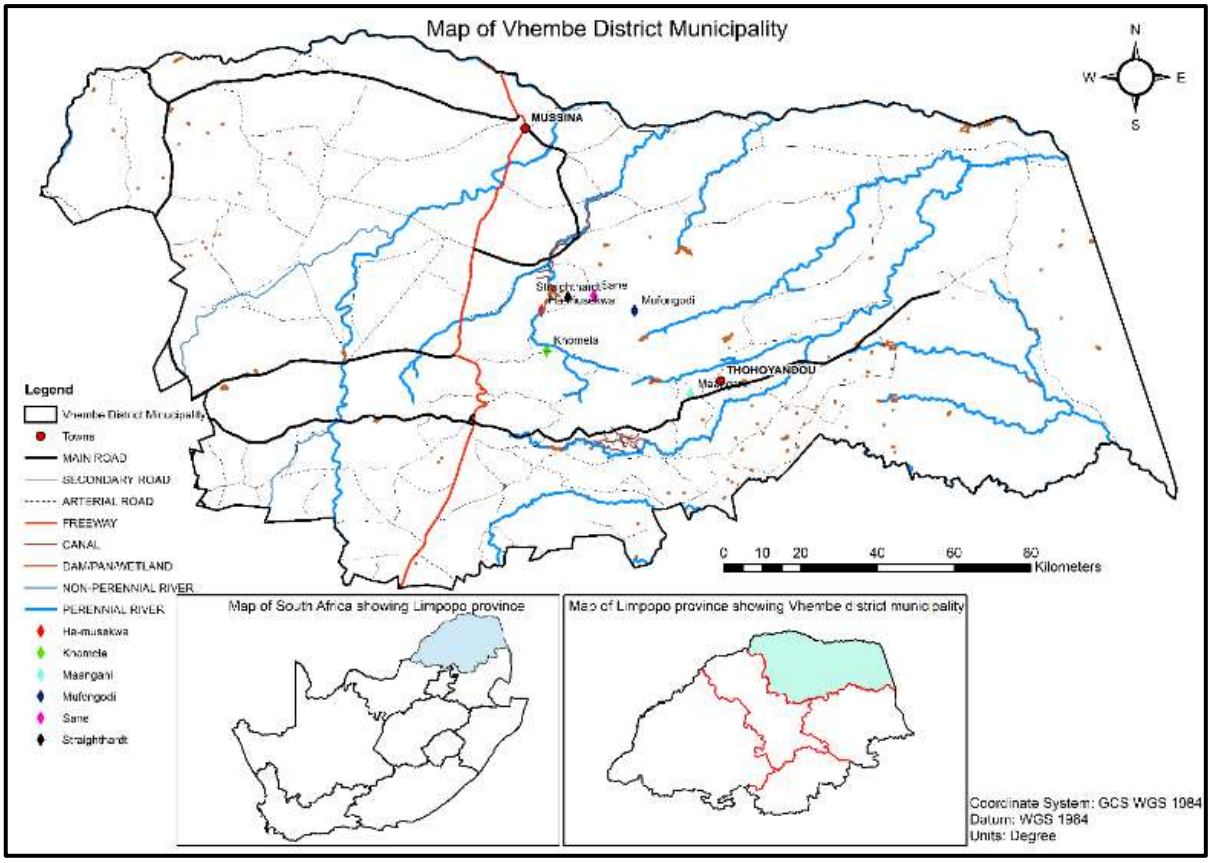


Figure 3.1 A map showing the study areas in Vhembe District, Makhado Local Municipality.

In the current study, eight plant species belonging to different families were collected from their natural habitat (Table 3.1) in the Vhembe District of Makhado Local Municipality, Limpopo Province. The plants were selected based on the treatment of “u wela” from ethnobotanical data and the availability of the plant species.

Table 3.1 Medicinal plants used for the treatment of “u wela” in Makhado Local Municipality.

Family	**Scientific Name**	**Vernacular name**	**Voucher specimen**	**Plant part**	**Location**
Celastraceae	*Elaeodendron transvaalense*	Mulamanamana	TT01	Bark	Sane
Fabaceae	*Albizia versicolor*	Muvhambangoma	TT02	Bark	Ha-Musekwa
Fabaceae	*Xanthocercis zambesiaca*	Mutshato	TT03	Bark	Maangani
Caesalpiniaceae	*Cassia abbreviata*	Muboma	TT04	Roots	Maangani
Gentianaceae	*Anthocleista grandiflora*	Muene-ene	TT05	Bark	Khomela
Myrothamnaceae	*Myrothamnus flabellifolius*	Mukangambanzhe	TT06	Whole plant	Straighthardt
Sapotaceae	*Mimusops zeyheri*	Mubululu	TT07	Bark	Khomela
Capparaceae	*Capparis tomentosa*	Muobadali	TT08	Bark	Mufongodi

3.2.3 Plant extraction

Plant parts such as roots, leaves, and bark were cut into small pieces and allowed to dry at room temperature (25°C). Dried finely ground 5g of plant material was extracted with 50 ml of various extractants such as hexane, dichloromethane, acetone, methanol, and ethyl-acetate in polyester plastic tubes. The mixtures were shaken vigorously for 3–5 min on an orbital shaking machine at a speed of 150 rpm. The plant material was centrifuged at 3500 rpm for 5 minutes and filtered using Whatman No.1

filter paper. The supernatants were decanted into weighed vials. The process was repeated in triplicates and the extracts were combined. The solvent was removed under a stream of cold air at room temperature. The aqueous extract was dried using a freeze-dryer.

3.2.4 Phytochemical analysis

Thin-Layer Chromatography (TLC) was used to investigate the chemical constituents of various plant extracts. The chemical components of each plant extract were analysed using Thin-Layer-Chromatography-(TLC)-plates-(ALIGRAM-SIL-g/UV-254-MACHEREY-NAGEL, Merck). The 10 mg/ml acetone extract of each plant sample (10 µl) was loaded on TLC plates and developed with different eluent solvent systems such as ethyl-acetate: methanol: water: [EMW], chloroform: ethyl-acetate: formic acid [CEF] and benzene: ethanol: ammonium hydroxide [BEA] (Kotze and Eloff, 2002). Development of the chromatograms was under eluent saturated conditions. TLC plates with chemical compounds that were not visible under UV light were sprayed with vanillin-sulphuric acid spray reagent for detection (Stahl, 2013).

3.2.5 Fungal strains and inoculum quantification

C. albicans (ATCC 10231) was obtained from the Department of Veterinary Tropical Diseases at the University of Pretoria. This is an opportunistic fungal pathogen that causes “makuma” (Masevhe et al., 2015). For the quantification of fungi, the Neubauer haemocytometer cell-counting method was used for counting the number of cells for each fungal culture (Aberkane et al., 2002). The inoculum of each isolate was prepared by growing the fungus on Sabouraud dextrose agar for seven days at 35ºC. The final inoculum concentration was adjusted to approximately 1.0×10^{6} cells/ml.

3.2.6 Bacterial strains and inoculum quantification

Bacterial strains of *E. coli* (ATTC 10536), and *S. aureus* (ATTC 25923) were obtained from the University of Pretoria, Department of Paraclinical Sciences. The *N. gonorrhoeae* (ATTC 49226) strain was purchased from the KwikStik company. The bacterial pathogen causes sexually transmitted diseases in humans. The isolates were maintained on Mueller Hinton agar for seven days at 35ºC. Before the bacterial cultures were used, they were diluted with sterile Muller Hinton broth to a turbidity that

matches 0.5 McFarland standard (1.0×10^6 Colony Forming Unit (CFU)/ml^{-1}) (Mulu et al., 2004).

3.3 Determination of antifungal activity

3.3.1 Micro-dilution assay

The micro-plate method was used to determine the antifungal activity of plant extracts (Eloff, 1998; Masoko and Eloff, 2005). The dried plant extracts were dissolved in acetone and serially diluted (50%) with water in 96-well microtiter plates. Acetone was used as a negative control and Amphotericin B was used as a reference anti-fungicide. A 100 µl of fungal culture was added to each well in the microplate and incubated for 24h. As an indicator of growth, 40µl of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) dissolved in water was added to the microplate. Microplates were incubated for three to five days at 35ºC at 100% relative humidity. The minimum inhibitory concentration (mg/ml) was recorded as the lowest concentration that inhibits the growth of fungi.

3.3.2 Bioautography assay

The bioautography assay was used to determine the number of active compounds in the plant extracts. The TLC plates were loaded with each plant extract and developed using different eluent solvent systems such as CEF, BEA, and EMW. TLC plates were allowed to dry under a stream of cold air to remove all the solvents. The developed TLC plates were sprayed with an overnight culture of *C. albicans* until wet. The plates were incubated overnight, sprayed with 2 mg/ml solution of p-iodonitrotetrazolium violet, and further incubated overnight at 35°C in a chamber at 100% relative humidity in the dark. The white areas in the plates indicated the inhibition of fungal growth.

3.4 Determination of antibacterial activity

3.4.1 Micro-dilution assay

The microplate method described (3.3.1) was used to determine the antibacterial activity of plant extracts (Eloff, 1998).

3.4.2 Bioautography assay

The bioautography assay was used to determine the number of antibacterial compounds present in the plant extracts. The assay is explained in detail in section 3.3.2. The developed TLC plates were sprayed with an overnight culture of *E. coli*, *S.*

aureus, and *N. gonorrhoeae* until wet. The white areas in the plates indicated the inhibition of the test organism (Begue and Kline, 1972).

3.5 Results and discussion

3.5.1 Percentage of mass extracted

Methanol extracted the highest quantity of plant material from the bark of *A. grandiflora* (15.6%), followed by acetone (6.2%) and ethyl-acetate (5%) bark extract from *E. transvaalense* (Figure 3.2*).* Methanol extracts had more polar organic compounds. Furthermore, methanol has the ability to extract both lipophilic and hydrophilic compounds from plants due to its high volatility (Selvaraj et al., 2020). Similarly, in a study conducted by Malada et al. (2022), methanol extracted the highest mass of dried leaf material from *Mystroxylon aethiopicum*, followed by water, acetone, and ethyl-acetate.

Acetone has the ability to dissolve both polar and non-polar compounds (Maulidiyah et al., 2023), hence the substantial quantity of plant material extracted by acetone as indicated in Figure 3.2. Eloff et al. (2017) also observed similar results, wherein acetone extracted a mass that was 5.4 times less than that of methanol from the leaves of *Melianthus comosus.* The optimum yield of ethyl-acetate extract was observed in *E. transvaalense.* The intermediary polarity of ethyl-acetate allows for the extraction of polar and non-polar compounds. The lowest extraction efficiency was observed with hexane, this extract contains only highly non-polar compounds, and it yielded only 3% compared to 15.6% with methanol. The bark of *A. grandiflora* therefore contains more polar compounds than non-polar compounds.

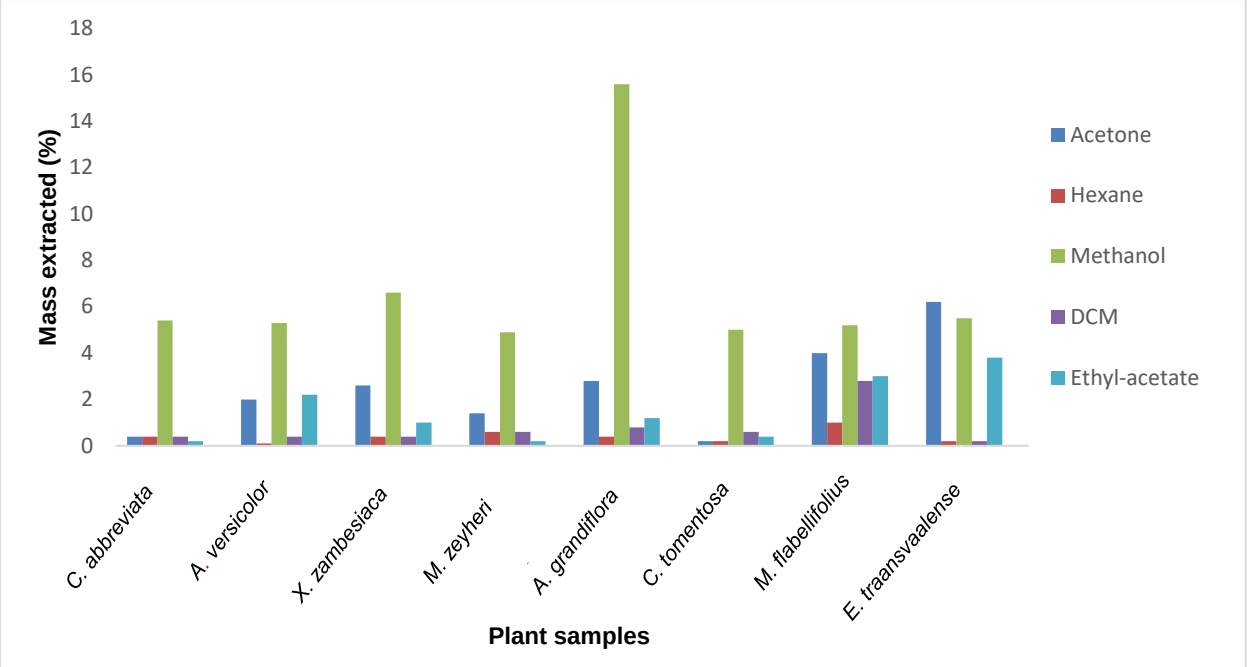


Figure 3.2 Percentage mass of material extracted from 4g of powdered plant material.

3.5.2 Phytochemical analysis

The chromatograms demonstrated different coloured bands, indicating the diversity of the compounds present within the plant extracts (Figure 3.3-3.8). When two compounds are compared under the same circumstances, the compound with the higher R_f value is less polar because it does not interact as strongly as the polar silica gel stationary phase.

The TLC chromatograms were developed using different eluent solvent systems based on different polarities, BEA (non-polar), CEF (intermediate polarity), and EMW (polar). The separated compounds were visualised under ultraviolet light at the wavelength of 365 nm and 254 nm before being sprayed with the vanillin-sulphuric acid spray reagent (Figure 3.3). Compounds in the plant extracts were separated more effectively by the BEA (54%), followed by the CEF (33%) and the least compounds were separated by the EMW (13%) solvent system, indicating that more compounds were non-polar. The *C. abbreviata* extracts had the highest number of bands, followed by *C. tomentosa* and *A. versicolor*.

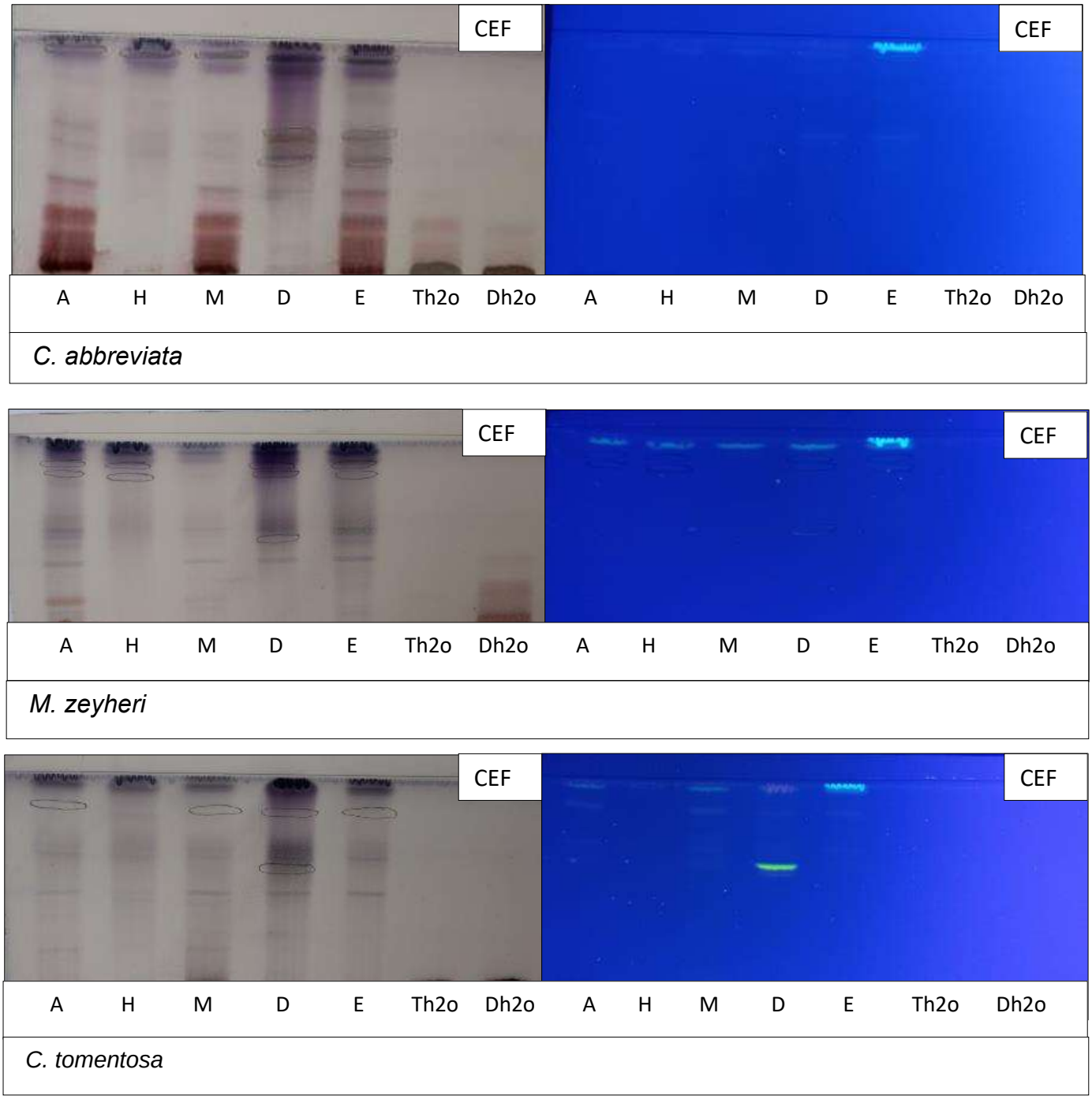


Figure 3.3 TLC chromatograms of *C. abbreviata* , *M. zeyheri* and *C. tomentosa* developed in CEF sprayed with vanillin-sulphuric acid (Lanes on the left) and viewed under UV Light (Lanes on the right): (A = acetone, H = hexane, M = methanol, D = DCM, E = ethyl-acetate, Th2o =Decoction and Dh2o = aqueous).

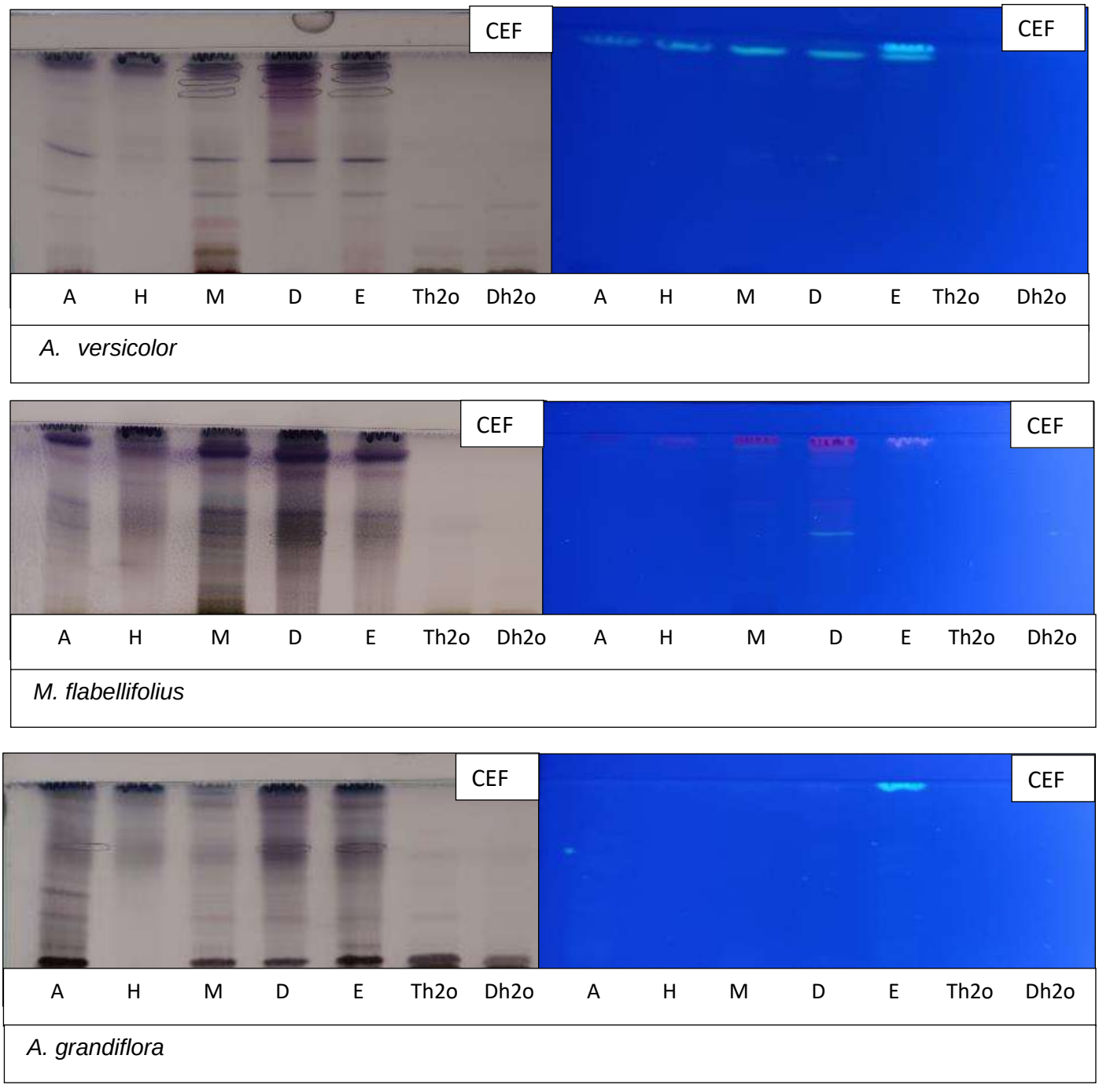


Figure 3.4 TLC chromatograms of *A .versicolor, M. flabellifolius* and *A. grandiflora* developed in CEF sprayed with vanillin-sulphuric acid (Lanes on the left) and viewed under UV Light (Lanes on the right): (A = acetone, H = hexane, M = methanol, D = DCM, E = ethyl-acetate, Th2o =Decoction and Dh2o = aqueous).

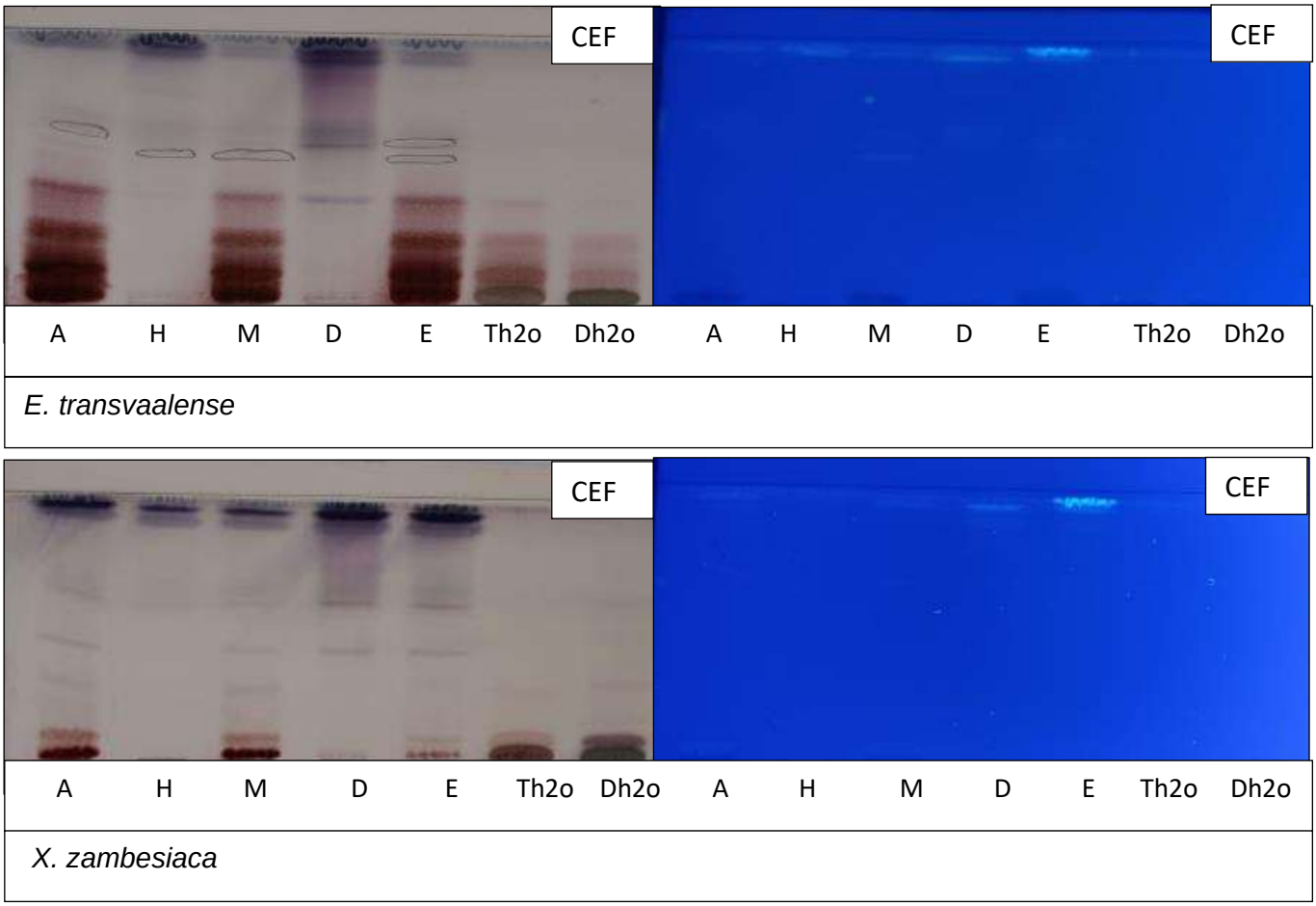
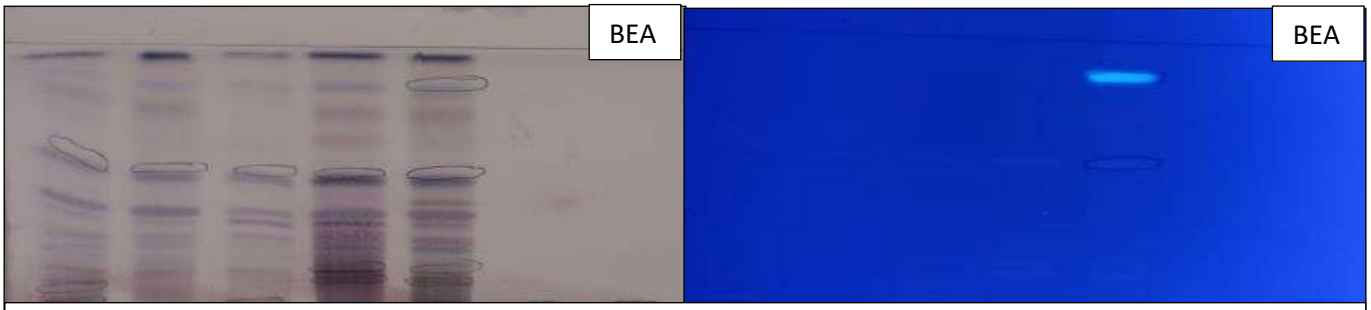
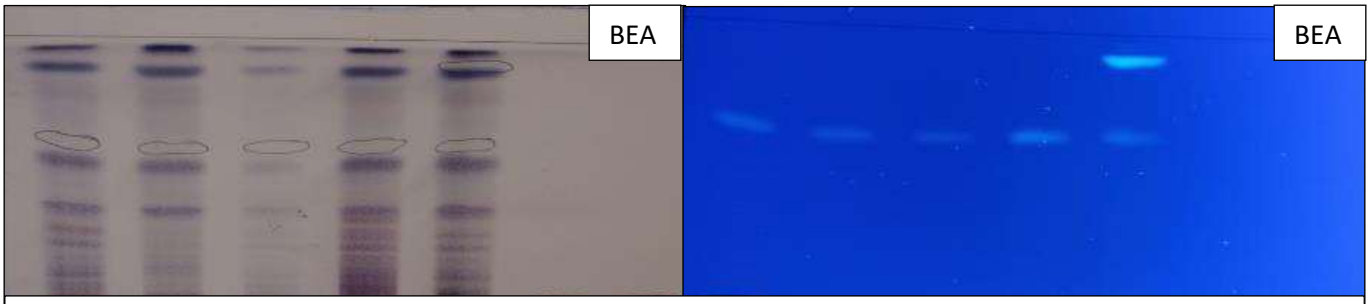


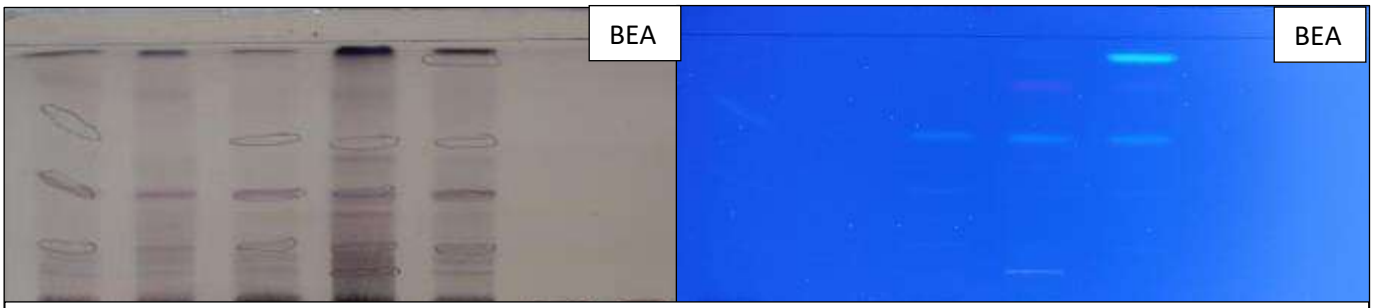
Figure 3.5 TLC chromatograms of *E. transvaalense* and *X. zambesiaca* developed in CEF sprayed with vanillin-sulphuric acid (Lanes on the left) and viewed under UV Light (Lanes on the right): (A = acetone, H = hexane, M = methanol, D = DCM, E = ethyl acetate, Th2o =Decoction and Dh2o = aqueous).



C. abbreviata



M. zeyheri



C. tomentosa

Figure 3.6 TLC chromatograms of *C. abbreviata, M. zeyheri* and *C. tomentosa* developed in BEA sprayed with vanillin-sulphuric acid (Lanes on the left) and viewed under UV Light (Lanes on the right): (A = acetone, H = hexane, M = methanol, D = DCM, E = ethyl acetate, Th2o =Decoction and Dh2o = aqueous).

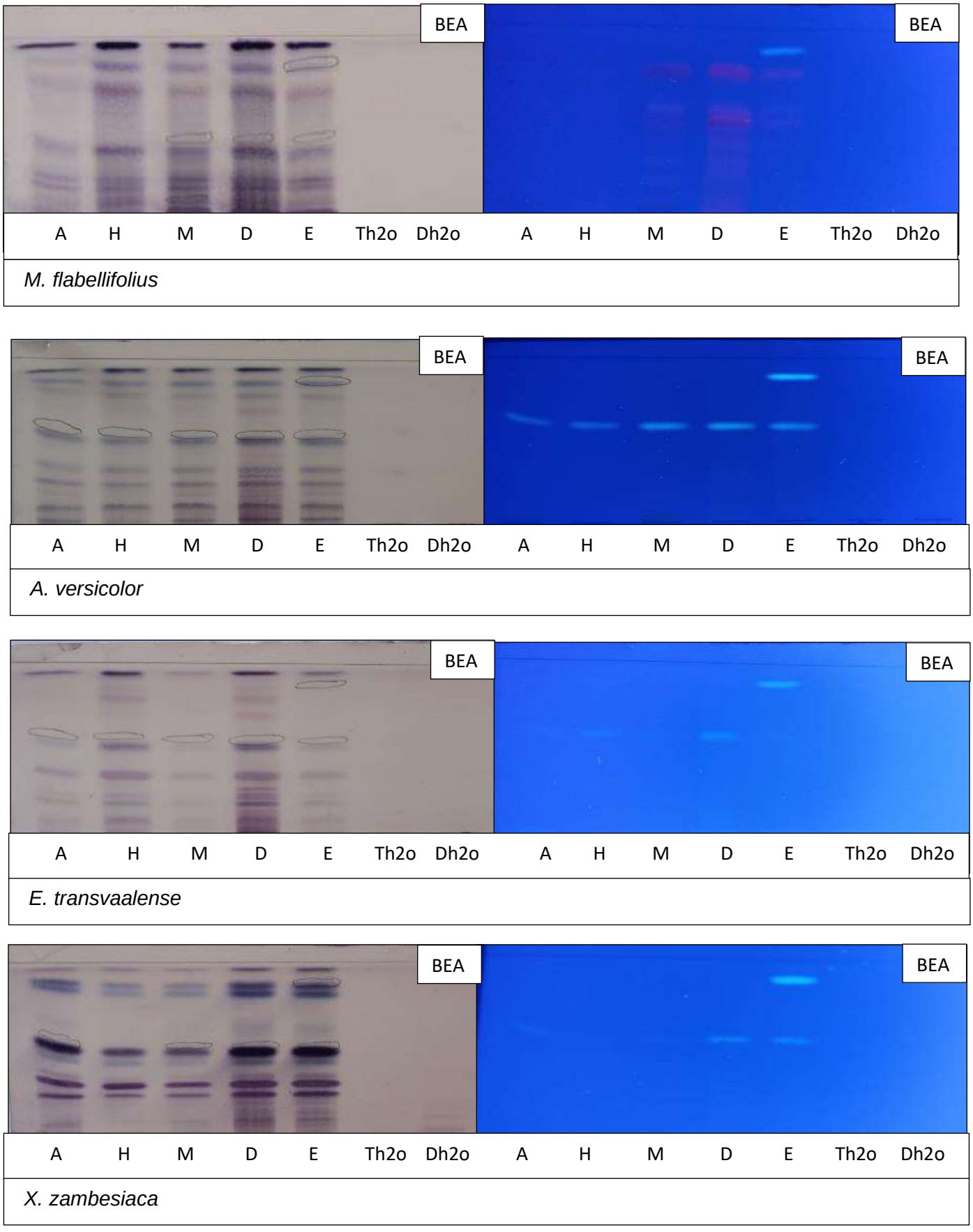


Figure 3.7 TLC chromatograms of *M. flabellifolius, A. versicolor, E. transvaalense* and *X. zambesiaca* developed in BEA sprayed with vanillin-sulphuric acid (Lanes on the left) and viewed under UV Light (Lanes on the right): (A = acetone, H = hexane, M = methanol, D = DCM, E = ethyl acetate, Th2o =Decoction and Dh2o = aqueous).

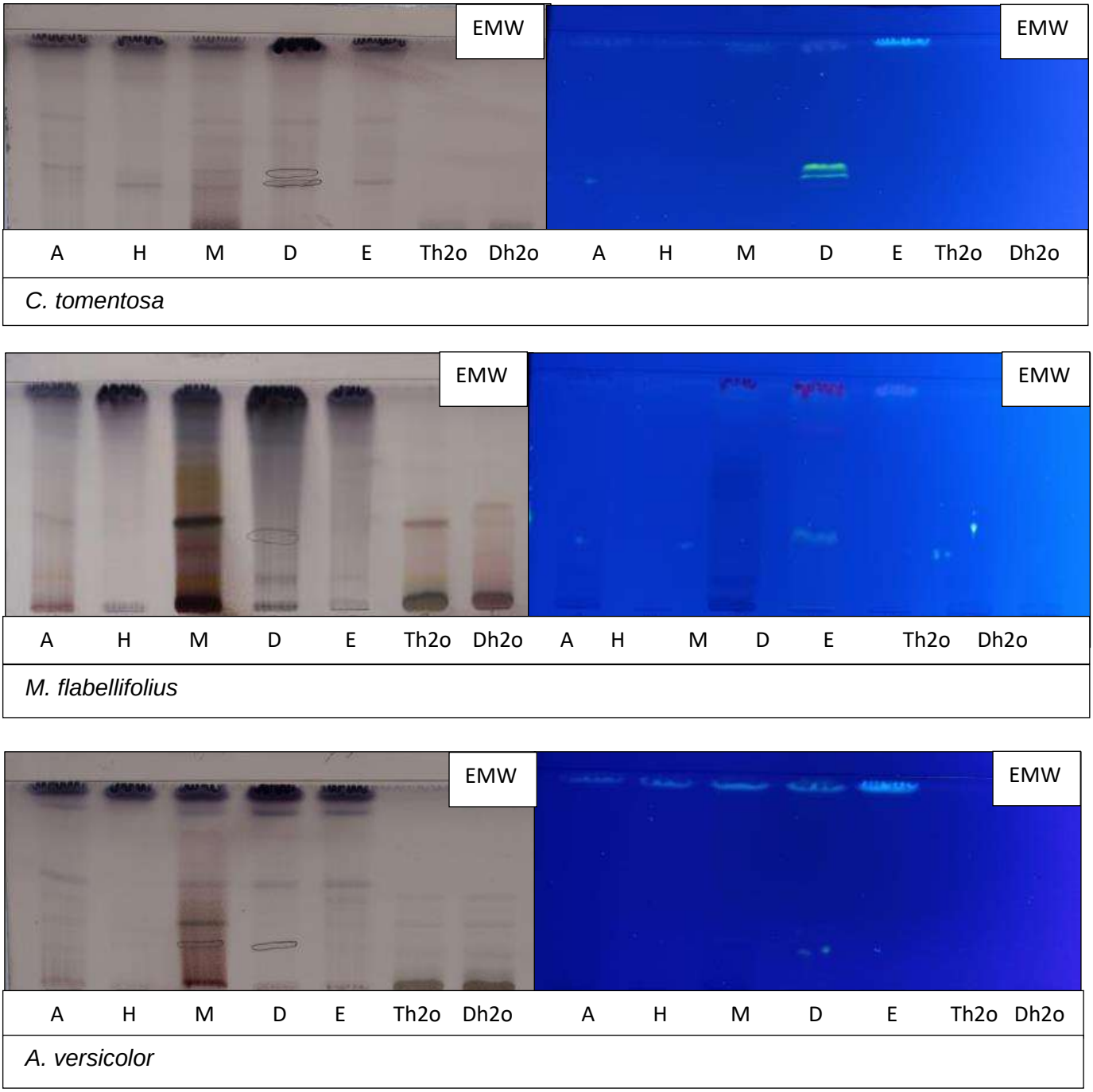


Figure 3.8 TLC chromatograms of C. *tomentosa, M. flabellifolius* and *A. versicolor* developed in EMW sprayed with vanillin-sulphuric acid (Lanes on the left) and viewed under UV Light (Lanes on the right): (A = acetone, H = hexane, M = methanol, D = DCM, E = ethyl acetate, Th2o =Decoction and Dh2o = aqueous).

3.6 Antifungal activity of plant extracts against *Candida albicans*

3.6.1 Serial dilution method

The antifungal activity of plant extracts was determined against *C. albicans* using serial dilution assay. All plant extracts were active against the tested fungal pathogen (Table 3.1).

After examining the antimicrobial activity of acetone leaf extracts of 537 South African tree species against 6 important pathogens the following classification was developed: outstanding activity < 0.02 mg/ml, excellent activity 0.021-0.04 mg/ml, very good activity 0.041 - 0.08 mg/ml, good activity 0.081 - 0.16 mg/ml, average activity 0.161 - 0.32 mg/ml and weak activity > 0.32 mg/ml (Eloff, 2021).

Plant extracts of *A. versicolor* and *C. abbreviata* had excellent antifungal activity with a low MIC value of 0.02 mg/ml-0.03 mg/ml. Noteworthy anti-candida activity was observed in aqueous extracts of *E. transvaalense, A. versicolor, X. zambesiaca, M. zeyheri,* and *C. abbreviata* with MIC values of 0.02 mg/ml. These results support the traditional use of water for the preparation of medication by traditional health practitioners. Methanol bark extracts of *X. zambesiaca* had a weak activity with MIC value of 1.25 mg/ml. Similar results were obtained with methanol stem extracts of *X. zambesiaca* showing less activity against *C. albicans* (Ngobeni, 2016).

Notably, DCM (root) and acetone (bark) extracts of *A. grandiflora* and *E. transvaalense* respectively were inactive with very high MIC values of 2.5 mg/ml. Contrastingly, it was found that the antimicrobial activities of ethanol extracts of aerial leaves and stem of *C. tomentosa* inhibited the growth of *C. albicans* (Gebrehiwot and Chaithanya, 2020). The root extracts of *C. abbreviata* had excellent activity against *C. albicans* with a low MIC value of 0.02 mg/ml.

Table 3.2 Minimum inhibitory concentrations (mg/ml) of selected plant species against *Candida albicans.*

Plant species		*Elaeodendron transvaalense*							*Albizia versicolor*							*Xanthocercis zambesiaca*							
Fungi	Time (Hrs.)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	Amp B
C. a	24	**0.02**	**0.02**	**0.02**	1.25	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	0.03	**0.02**	**0.02**	**0.02**	1.25	1.25	1.25	1.25	**0.02**	**0.02**	**0.02**	**0.03**	< 0.02
	48	**0.02**	**0.02**	**0.02**	1.25	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	0.03	**0.02**	**0.02**	**0.02**	1.25	1.25	1.25	1.25	**0.02**	**0.02**	**0.02**	**0.03**	< 0.02
	72	**0.02**	**0.02**	**0.02**	2.5	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	0.03	**0.02**	**0.02**	**0.02**	2.5	1.25	1.25	1.25	**0.02**	**0.02**	**0.02**	**0.03**	< 0.02
Plant species		*Cassia abbreviata*							*Anthocleista grandiflora*							*Capparis tomentosa*							
C. a	24	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	1.25	**0.02**	**0.02**	**0.02**	1.25	0.62	2.5	2.5	**0.02**	**0.02**	**0.02**	**0.02**	0.15	0.31	< 0.02
	48	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	1.25	**0.02**	**0.02**	**0.02**	1.25	0.62	2.5	2.5	**0.02**	**0.02**	**0.02**	**0.02**	0.15	0.31	< 0.02
	72	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	2.5	**0.02**	**0.02**	**0.02**	1.25	0.62	2.5	2.5	**0.02**	**0.02**	**0.02**	**0.02**	0.15	0.31	< 0.02
Plant species		*Mimusops zeyheri*							*Myrothamnus flabellifolius*							Amp B							
C. a	24	1.25	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	0.15	0.31	0.31	**0.03**	0.31	0.31	0.15	**0.03**	< 0.02							
	48	1.25	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	0.15	0.31	0.31	**0.03**	0.31	0.31	0.15	**0.03**	< 0.02							
	72	1.25	0.15	**0.02**	**0.02**	**0.02**	**0.02**	0.15	0.31	0.31	**0.03**	0.31	0.31	0.15	**0.03**	< 0.02							

A = acetone, H = hexane, M = methanol, D = dichloromethane, E = ethyl-acetate, Lab H_2O= aqueous

Abbreviations: *C. a*. – *Candida albicans*

3.6.2 Total activity

The total activities of plant extracts are shown in Table 3.2

The total activity of the plant crude extracts was calculated using the following formula:

$$\text{Total activity} = \frac{\text{Quantity of material in mg extracted from 1g of plant material}}{\text{Minimum inhibitory concentration (mg/ml)}}$$

The total activity denotes the extent to which the active compounds in one gram may be diluted while still inhibiting microbial growth, hence assisting in the selection of potential plants (Eloff et al., 2008). The total activity includes both the mass extracted and the antimicrobial activity and is used to assess the potential uses of extracts from different plant species (Famuyide et al., 2019). This is a significant measure for comparing the efficacy of various plant extracts. The ethyl-acetate extracts exhibited the highest total activity as compared to other extractants (Table 3.2). The highest total activity was found in the ethyl-acetate extract of *C. tomentosa* (5688 ml/g) against *C. albicans*. The lowest total activity was observed in the acetone extract of *C. tomentosa* (27.11 ml/g) against *C. albicans.*

Table 3.3 Total activities (mg/ml) of selected plant species against *Candida albicans*.

Total activity																					
Plant species		*Elaeodendron transvaalense*					*Albizia versicolor*					*Xanthocercis zambesiaca*					*Mimusops zeyheri*				
Extractants																					
Fungi	Time (Hrs.)	A	H	M	D	E	A	H	M	D	E	A	H	M	D	E	A	H	M	D	E
C. a	24	3472	3915	3587	36.46	3352	3524	3568	2261	3864	3420	53.56	54.29	53.19	5304	3300	52.11	4292	40.60	3365	4439
	48	3472	3915	3587	36.46	3352	3524	3568	2261	3864	3420	53.56	54.29	53.19	5304	3300	52.11	4292	40.60	3365	4439
	72	3472	3915	3587	36.46	3352	3524	3568	2261	3864	3420	53.56	54.29	53.19	5304	3300	52.11	4292	40.60	3365	4439
Average		3472	3915	3587	36.46	3352	3524	3568	2261	3864	3420	53.56	54.29	53.19	5304	3300	52.11	4292	40.60	3365	4439
Plant species		*Cassia abbreviata*					*Anthocleista grandiflora*					*Capparis tomentosa*					*Myrothamnus flabellifolius*				
C. a	24	3410	4135	3448	3419	3372	54.50	3432	4115	4191	65.80	27.11	3415	3472	3338	5688	296.67	221.41	997.71	283.87	265.32
	48	3410	4135	3448	3419	3372	54.50	3432	4115	4191	65.80	27.11	3415	3472	3338	5688	296.67	221.41	997.71	283.87	265.32
	72	3410	4135	3448	3419	3372	54.50	3432	4115	4191	65.80	27.11	3415	3472	3338	5688	296.67	221.41	997.71	283.87	265.32
Average		3410	4135	3448	3419	3372	54.50	3432	4115	4191	65.80	27.11	3415	3472	3338	5688	296.67	221.41	997.71	283.87	265.32

Extractants: A = acetone, H = hexane, M = methanol, D = dichloromethane, E = ethyl-acetate

Abbreviations: *C. a*. – *Candida albicans*

3.6.3 Plant extracts with excellent antifungal activity

Cassia abbreviata extracts had the best antifungal activity with a MIC value of 0.02 mg/ml (Table 3.3). The leaves, bark, and roots of *C. abbreviata* have been used for centuries by traditional health practitioners for the treatment of hernia, vomiting, syphilis, and bilharzia (Mongalo and Mafoko, 2013). Furthermore, the root of *C. abbreviata* is prescribed to individuals diagnosed with oral and vaginal candidiasis. Medication can be administered orally, anally, and nasally (Ibrahim et al., 2022). A total number of 5 plant extracts with 0.02 mg/ml was found in *E. transvaalense*, *A. versicolor,* and *C. tomentosa.*

The bark of *E*. *transvaalense* is used to treat fever, and bladder infections and to alleviate bodily discomfort (Khumalo et al., 2021). In folk remedies, the plant is used for the treatment and management of ailments including stomach-related complaints, herpes simplex, stroke, and haemorrhoids (More, 2021). *A. versicolor* has many therapeutic properties. Its roots, bark, and leaves are applied in traditional medicine as therapy for allergic reactions, leukoderma, malaria, and syphilis (Balkrishna et al., 2022). The roots and leaves of *C. tomentosa* are used by traditional health practitioners to treat malaria either alone or in conjunction with other plant species (Tekulu et al., 2020). Furthermore, it is traditionally used to treat headaches, erectile dysfunction, and infertility in women (Gebrehiwot et al., 2019). *A. grandiflora* and *X. zambesiaca* had the lowest number of excellent antifungal activity. The bark of *A. grandiflora* is used for the treatment of diarrhoea and high blood pressure (Mudau et al., 2022).

Table 3.4 Plant extracts with excellent antifungal activity (0.02 mg/ml) against *Candida albicans*.

Extractants	*E. transvaalense*	*A. versicolor*	*X. zambesiaca*	*M. zeyheri*	*C. abbreviata*	*A. grandiflora*	*C. tomentosa*	*M. flabellifolius*	Average
A	1	1	0	0	1	0	0	0	3
H	1	1	0	0	1	1	1	0	5
M	1	0	0	1	1	1	1	0	5
D	0	1	1	1	1	1	1	0	6
E	1	1	1	1	1	0	1	0	6
D H_2O	1	1	1	1	1	0	1	0	6
T H_2O	1	0	0	0	1	0	0	0	2
Total	5	5	3	4	6	3	5	0	33

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol, E= ethyl-acetate, Dh2o = aqueous and Th2o =Decoction mixture

3.6.4 Bioautography assay

Bioautography assay was used to determine the number of active compounds in the various plant extracts (Table 3.4-3.6). The BEA solvent system is widely recognized for separating non-polar compounds, the CEF solvent system for separating compounds of intermediate polarity, and the EMW solvent system for separating polar compounds (Kotze and Eloff, 2002). The R_f values were determined by dividing the distance travelled by the target compound by the distance travelled by the solvent front. The presence of white bands against the pink background on the chromatograms confirmed that the plant extracts inhibited the growth of *C. albicans* (Figure 3.9-3.11). A total of 111 active compounds were separated by EMW, followed by 47 active compounds separated by CEF and 41 active compounds separated by BEA. Anti-candidal compounds were observed in all plant extracts, except for the *M. zeyheri* bark extract and C. *abbreviata.*

In bioautograms developed in CEF, anti-candidal compounds were visible in plant extracts of *C. tomentosa, A. grandiflora, A. versicolor, M. flabellifolius,* and *X. zambesiaca*. The findings indicate that the eluent solvent system separated more polar compounds. Similar results were confirmed with EMW separating more phytochemicals from plant extracts (Ajmal, 2019). Antifungal compounds were observed in decoction and aqueous extracts of *C. abbreviata, A. versicolor,* and *E. transvaalense* in TLC bioautograms developed in EMW against *C. albicans* with R_f values ranging from 0.55-0.90*.* More active compounds (68) were found in the extracts of *M. flabellifolius.* Anti-candidal compounds were also visible in methanol, DCM, and ethyl-acetate extracts of *X. zambesiaca* developed in EMW with R_f values ranging between 0.90-0.93. No active compounds were observed in the ethyl-acetate and decoction extracts of *E. transvaalense* separated with BEA and CEF against the tested pathogen. According to Khumalo et al. (2019), the dichloromethane crude extract of *E. transvaalense* had better activity against the tested pathogen. More importantly, the incubation period and humidity must be regulated. The roots and bark of *E. transvaalense* are combined with the roots of *Peltophorum africanum* and used as a herbal remedy for female infertility and for anti-inflammatory reasons (Maroyi and Semenya, 2019).

The findings support traditional health practitioners' use of water in the preparation of remedial medications from this plant. This is in contrast to many publications focusing on the antimicrobial activity of different extractants (Kotze et al., 2002).

Table 3.5 R_f values of roots extracts of *C. abbreviata* and *C. tomentosa* against *C. albicans*.

Solvent system	Extractants							
	R_f values	A	H	M	D	E	Dc	Aq
	Plant species							
	C. abbreviata							
EMW	0.89		√					
	0.86		√		√	√		
	0.84				√	√		√
	0.80							
	0.75				√			
	0.22					√		
	0.21	√		√				
	C. tomentosa							
EMW	0.95				√			
	0.80	√	√	√	√			
	0.79				√			
	0.68	√	√	√	√			
	0.60	√	√	√				
	0.14			√	√			
CEF	0.94	√	√	√	√	√		
	0.35				√			

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol, Dc = Decoction extract and Aq= Aqueous.

Table 3.6 R_f values of roots extracts of *M. flabellifolius* and *A. versicolor* against *C. albicans.*

Solvent system	Extractants							
	R_f values	A	H	M	D	E	Dc	Aq
Plant species								
M. flabellifolius								
CEF	0.93	√	√	√	√	√		
BEA	0.87	√	√	√	√	√		
	0.56	√	√	√	√	√	√	√
	0.45	√	√	√	√	√	√	√
	0.44			√	√			
	0.43	√	√	√	√			
EMW	0.93	√	√	√	√			
	0.90	√	√	√	√	√	√	√
	0.80	√	√	√	√			
	0.75	√	√	√	√		√	√
	0.71		√	√	√	√		
	0.48		√	√	√			
	0.39		√	√	√	√		
	0.35		√	√	√			
	0.11							
	0.02					√	√	√
A. versicolor								
EMW	0.95	√	√	√	√	√		
	0.90			√	√	√	√	√
	0.89		√	√	√	√	√	
	0.39				√			
CEF	0.95	√	√	√	√	√		
	0.93	√	√	√	√	√		
	0.83							
	0.56				√			
	0.49	√			√	√		

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol, Dc = Decoction extract and Aq= Aqueous.

Table 3.7 R_f values of roots extracts of *A. grandiflora, E. transvaalense* and *X. zambesiaca* against *C. albicans.*

Solvent system	Extractants							
	R_f values	A	H	M	D	E	Dc	Aq
	Plant species							
	A. grandiflora							
CEF	0.93	√	√	√	√	√		
	0.87	√	√	√	√	√		
BEA	0.92		√			√		
	0.71					√		
	0.39	√			√		√	
	0.13	√			√	√	√	
	0.12			√			√	
EMW	0.87	√	√	√	√	√		
	E. transvaalense							
EMW	0.85		√		√	√	√	
	0.84							
	0.83	√	√		√	√	√	
	0.66		√	√	√			√
	0.60			√				
	0.55					√	√	
	X. zambesiaca							
EMW	0.93	√	√	√	√	√		
	0.90	√	√	√	√	√		
CEF	0.93	√	√	√	√	√		
	0.86	√	√		√	√		
	0.75	√	√					
	0.69	√						
BEA	0.55	√	√	√	√	√		

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol, Dc = Decoction extract and Aq= Aqueous.

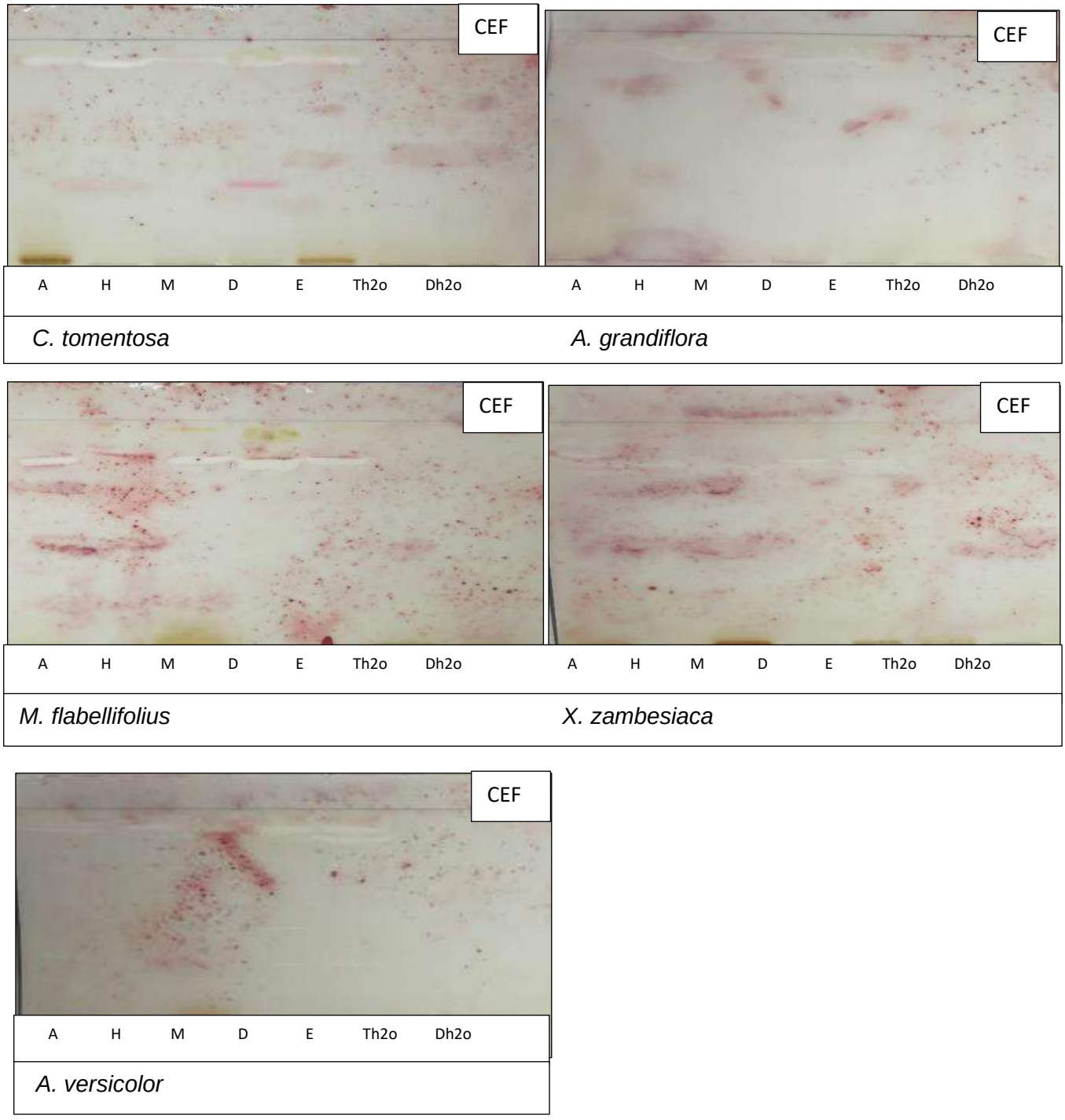


Figure 3.9 Bioautograms of extracts of *C. tomentosa, A. grandiflora*, *M. flabellifolius*, *X. zambesiaca*, and *A. versicolor* developed in CEF sprayed with *Candida albicans*. White areas indicate inhibition of fungal growth. Lanes from left to right: (A = acetone, H = hexane, M = methanol, D = DCM, E = ethyl acetate, Th2o =Decoction extract and Dh2o = aqueous).

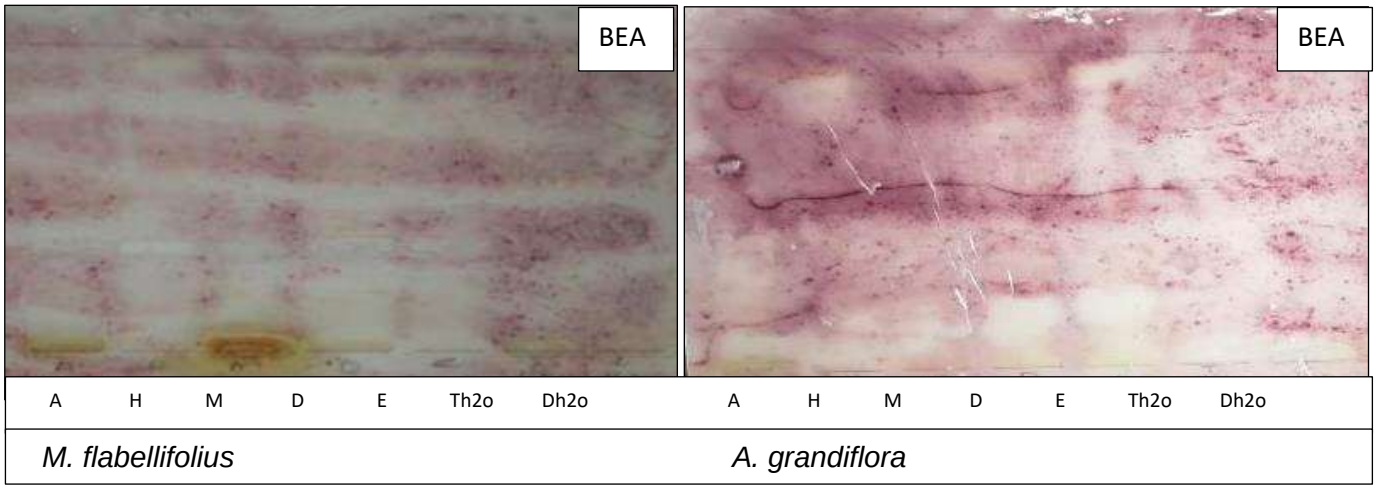


Figure 3.10 Bioautograms of extracts of *M. flabellifolius, A. grandiflora* and *X. zambesiaca* developed in BEA, sprayed with *Candida albicans*. White areas indicate inhibition of fungal growth. Lanes from left to right: (A = acetone, H = hexane, M = methanol, D = DCM, E = ethyl acetate, Th2o =Decoction extract and Dh2o = aqueous).

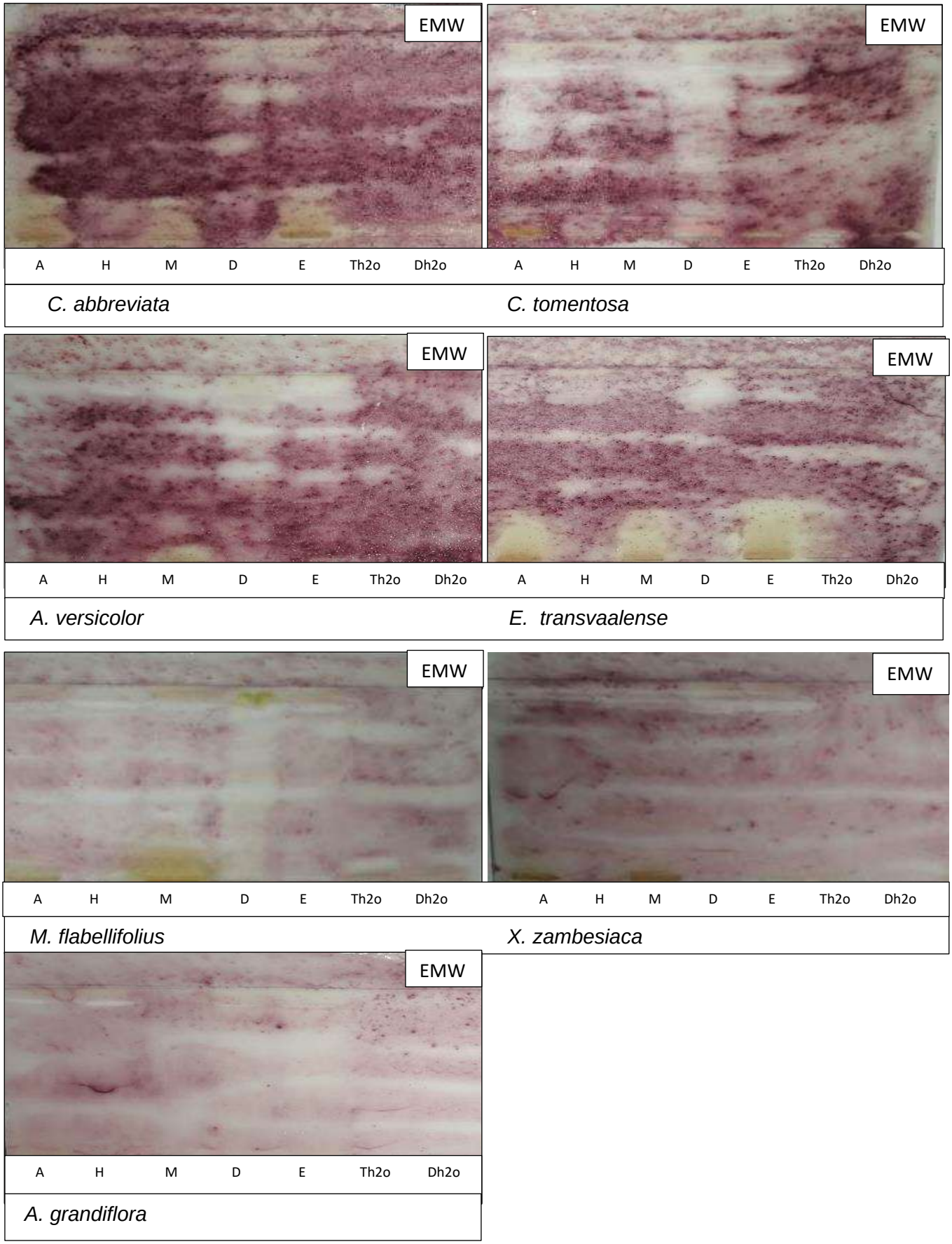


Figure 3.11 Bioautograms of extracts of *C. abbreviata, C. tomentosa, A. versicolor*, *E. transvaalense, M. flabellifolius*, *X. zambesiaca, and A. grandiflora*, developed in EMW, sprayed with *Candida albicans*. White areas indicate inhibition of fungal growth. Lanes from left to right: (A = acetone, H = hexane, M = methanol, D = DCM, E = ethyl acetate, Th2o =Decoction extract and Dh2o = aqueous).

3.7 Antibacterial activity of plant extracts against *E. coli* and *S. aureus*

3.7.1 Serial dilution method

The antibacterial activity of plant extracts was determined using the serial dilution assay (Table 3.7). Acetone was used as a negative control and gentamicin as a positive control. In the current study, all plant extracts were active against the tested pathogens. The acetone and methanol extracts of *E. transvaalense, X. zambesiaca, C. abbreviata, A. grandiflora, M. zeyheri, M. flabellifolius* had excellent antibacterial activity against *E. coli* and *S. aureus* with low MIC values ranging from 0.02-0.03 mg/ml (Table 3.7). Previously, it was found that the chloroform leaf extracts of *A. grandiflora* had the ability to inhibit the growth of *S. aureus* (Rotich, 2022). Plant extracts with low MIC values could be a good source of bioactive components with antimicrobial potency. Poor activity was observed in the DCM and ethyl-acetate bark and root extracts of *A. versicolor* and *C. tomentosa* against *S. aureus* with MIC value of 2.5 mg/ml. The hexane extracts of *E. transvaalense, C. abbreviata, M. flabellifolius, A. grandiflora,* and *C. tomentosa* indicated low antibacterial activity with MIC values ranging from 0.31-0.62 mg/ml against *S. aureus* (Table 3.7). Gentamicin with the MIC value of 0.002 mg/ml has the ability to inhibit the growth of the tested microorganisms.

Table 3.8 Minimum inhibitory concentrations (mg/ml) of selected plant species against the bacterial pathogens.

MIC values (mg/ml)																
Plant species		*Elaeodendron transvaalense*							*Albizia versicolor*							
Extractants																
Bacteria	Time (Hrs.)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	Gent
E. c	24	0.02	0.03	0.02	1.25	0.15	0.31	0.15	2.5	0.03	0.03	0.02	0.02	0.03	0.02	< 0.02
	48	0.02	0.03	0.02	1.25	0.15	0.31	0.15	2.5	0.03	0.03	0.02	0.02	0.03	0.02	< 0.02
	72	0.02	0.03	0.02	1.25	0.15	0.31	0.15	2.5	0.03	0.03	0.02	0.02	0.03	0.02	< 0.02
S. a	24	0.03	0.62	0.02	0.15	0.15	0.02	0.02	0.03	0.02	0.02	2.5	2.5	0.07	0.03	< 0.02
	48	0.03	0.62	0.02	0.15	0.15	0.02	0.02	0.03	0.02	0.02	2.5	2.5	0.07	0.03	< 0.02
	72	0.03	0.62	0.02	0.15	0.15	0.02	0.02	0.03	0.02	0.02	2.5	2.5	0.07	0.03	< 0.02
Plant species		*Anthocleista grandiflora*							*Capparis tomentosa*							
E. c	24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.15	0.02	0.02	0.02	0.15	0.02	0.02	< 0.02
	48	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.15	0.02	0.02	0.02	0.15	0.02	0.02	< 0.02
	72	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.15	0.02	0.02	0.02	0.15	0.03	0.03	< 0.02
S. a	24	0.31	0.31	0.03	0.15	0.62	0.03	0.03	0.31	0.62	0.03	2.5	2.5	0.62	0.31	< 0.02
	48	0.31	0.31	0.03	0.15	0.62	0.03	0.03	0.31	0.62	0.03	2.5	2.5	0.62	0.31	< 0.02
	72	0.31	0.31	0.03	0.15	0.62	0.03	0.03	0.31	0.62	0.03	2.5	2.5	0.62	0.31	< 0.02

Gentamicin was used as a positive control.

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol, E= ethy-acetate, Dh2o =aqueous and Th2o =Decoction mixture

Abbreviations: *E.c* – *Escherichia coli*, *S.a* – *Staphylococcus aureus* and Gent – Gentamicin

Table 3.8 Continued. Minimum inhibitory concentrations (mg/ml) of of selected plant species against the bacterial pathogens.

MIC values (mg/ml)																
Plant species		*Xanthocercis zambesiaca*							*Cassia abbreviata*							
Extractants																
Bacteria	Time (Hrs.)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	Gent
E. c	24	0.02	0.02	0.02	0.62	0.13	0.02	0.02	0.02	0.03	0.02	0.02	0.02	0.03	0.03	< 0.02
	48	0.02	0.02	0.02	0.62	0.13	0.02	0.02	0.02	0.03	0.02	0.02	0.02	0.03	0.03	< 0.02
	72	0.02	0.15	0.02	0.15	0.02	0.02	0.02	0.02	0.15	0.02	0.07	0.03	0.07	0.15	< 0.02
S. a	24	0.02	0.15	0.02	0.15	0.02	0.02	0.02	0.02	0.15	0.02	0.07	0.03	0.07	0.15	< 0.02
	48	0.02	0.15	0.02	0.15	0.02	0.02	0.02	0.02	0.15	0.02	0.07	0.03	0.07	0.15	< 0.02
	72	0.02	0.15	0.02	0.15	0.02	0.02	0.02	0.02	0.15	0.02	0.07	0.03	0.07	0.15	< 0.02
Plant species		*Mimusops zeyheri*							*Myrothamnus flabellifolius*							
E. c	24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	2.5	2.5	< 0.02
	48	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	2.5	2.5	< 0.02
	72	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	2.5	2.5	< 0.02
S. a	24	0.02	0.02	0.02	0.31	0.31	0.02	0.02	0.02	0.31	0.02	0.03	0.07	0.02	0.02	< 0.02
	48	0.02	0.02	0.02	0.31	0.31	0.02	0.02	0.02	0.31	0.02	0.03	0.07	0.02	0.02	< 0.02
	72	0.02	0.02	0.02	0.31	0.31	0.02	0.02	0.02	0.31	0.02	0.03	0.07	0.02	0.02	< 0.02

Gentamicin was used as a positive control.

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol, E= ethy-acetate, Dh2o =aqueous and Th2o =Decoction mixture

Abbreviations: *E.c* – *Escherichia coli*, *S.a* – *Staphylococcus aureus* and Gent – Gentamicin

3.7.2 Total activity

The total activities of plant extracts are shown in Table 3.8.

The total activity of the plant crude extracts was calculated using the formula described in chapter 3, section 3.6.2.

The highest total activity (4599 ml/g) was observed in the acetone plant extracts of *M. flabellifolius* against both *E. coli* and *S. aureus*, followed by the total activity of hexane extracts of *M. zeyheri* (4292 ml/g) and DCM extracts of *A. grandiflora* (4191 ml/g). The lowest activity (27 ml/g) was observed in DCM and ethyl-acetate extracts of *C. tomentosa* and *A. versicolor* against the tested bacterial pathogens.

Table 3.9 Total activity of selected plant species against the bacterial pathogens.

Plant species		*Elaedendron transvaalense*					*Albizia versicolor*					*Xanthocercis zambesiaca*				
Fungi	Time	A	H	M	D	E	A	H	M	D	E	A	H	M	D	E
E. c	24	3472	2609	3587	73.	447	29	2379	2261	3864	3416	3348	3393	3325	113	508
	48	3472	2609	3587	73.	447	29	2379	2261	3864	3416	3348	3393	3325	113	508
Average		3472	2609	3587	73	447	29	2379	2261	3864	3416	3348	3393	3325	113	508
S. a	24	2315	126.3	3586	609	447	2349	3569	3392	31	27	3348	452	3325	467	3300
	48	2315	126	3586	609	447	2349	3569	3392	31	27	3348	452	3325	467	3300
Average		2315	126	3586	609	447	2349	3569	3392	31	27	3348	452	3325	467	3300
Plant species		*Cassia abbreviata*					*Anthocleista grandiflora*					*Capparis tomentosa*				
E. c	24	3410	2757	3448	3419	3372	3406	3432	4115	4191	4113	452	3415	3472	3338	592
	48	3410	2757	3448	3419	3372	3406	3432	4115	4191	4113	452	3415	3472	3338	592
Average		3410	2757	3448	3419	3372	3406	3432	4115	4191	4113	452	3415	3472	3338	592
S. a	24	3410	551	3448	977	2247	220	221	2743	559	133	219	110	2314	27	36
	48	3410	551	3448	977	2247	220	221	2743	559	133	219	110	2314	27	36
Average		3410	551	3448	977	2247	220	221	2743	559	133	219	110	2314	27	36
Plant species		*Mimusops zeyheri*					*Myrothamnus flabellifolius*									
E. c	24	3257	4292	4060	3365	4439	4599	3435	2328	4400	4053					
	48	3257	4292	4060	3365	4439	4599	3435	2328	4400	4053					
Average		3257	4292	4060	3365	4439	4599	3435	2328	4400	4053					
S. a	24	3257	4292	4060	218	286	4599	222	3492	2933	1158					
	48	3257	4292	4060	218	286	4599	222	3492	2933	1158					
Average		3257	4292	4060	218	286	4599	222	3492	2933	1158					

Extractants: A = acetone, H = hexane, M = methanol, D = dichloromethane and, E= ethy-acetate

Abbreviations: *E.c – Escherichia coli* and *S.a – Staphylococcus aureus.*

3.7.3 Plant extracts with excellent antibacterial activity

M. zeyheri had the highest number of plant extracts (8) with excellent antbacterial activity with MIC value of 0.02 mg/ml (Table 3.9). The bark and roots of *M. zeyheri* are used by traditional health practitioners for the treatment of gonorrhea (Monyela, 2021). The root infusion of *M. zeyheri* is used for the treatment of candidiasis. In our findings, *X. zambesiaca* and *C. abbreviata* had a total number of 6 compounds, followed by *A. grandiflora* and *M. flabellifolius* with a total of 5 compounds with a low MIC value of 0.02 mg/ml. *E. transvaalense* and *C. tomentosa* had the lowest total (3) number of plant extracts with good antibacterial activity. The root maceration of *C. tomentosa* is used to treat headaches and is administered nasally or orally (Tekulu et al., 2020).

Table 3.10 Plant extracts with excellent antibacterial activity (0.02 mg/ml) against the tested bacterial pathogens.

	Plant species								
Extractants	*E. transvaalense*	*A. versicolor*	*X. zambesiaca*	*C. abbreviata*	*A. grandiflora*	*C. tomentosa*	*M. zeyheri*	*M. flabellifolius*	Average
A	1	0	2	2	1	0	2	2	10
H	0	1	1	0	1	1	2	1	7
M	2	1	2	2	1	1	2	0	11
D	0	1	0	1	1	1	1	1	6
E	0	1	1	1	1	0	1	1	6
Total	3	4	6	6	5	3	8	5	40

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol and E= ethyl- acetate.

3.7.4 Bioautography assay

The bioautography assay was used to determine the number of active compounds present in different plant extracts (Figure 3.12-3.14). A total of 9 compounds were observed in bioautograms separated with BEA, followed by CEF (3). No antifungal compounds were observed in EMW bioautograms. This indicates that the bioactive compound was relatively non-polar and was not separated by the polar eluent. This is supported by the BEA bioautograms, which separated more active compounds as compared to other solvent systems (Meela et al., 2019). These findings suggest that the most separated compounds were non-polar compounds (Table 3.10). In bioautograms developed in BEA, active compounds were visible in the acetone, DCM, and ethyl-acetate extracts of *A. versicolor* with R_f values of 0.24. A similar compound was observed in the DCM extract of *E. transvaalense.* with the same R_f value of 0.24 against *E. coli.* Leaf extract of *Adenia gummifera* developed in BEA displayed strong bands of inhibition against *E. coli* and *S. aureus* (Jambwa et al., 2023). Active compounds with R_f values of 0.85 were visible in the acetone, DCM, and ethyl-acetate bark extracts of *A. versicolor* developed in CEF against *S. aureus* (Table 3.11). There were no antibacterial compounds found in several plant extracts with high antibacterial activity, indicating probable synergism.

Table 3.11 R_f values of compounds inhibiting bacterial growth separated with BEA and CEF using extracts of *A. Versicolor* and *E. transvaalense.*

A. Versicolor									
		A		H		M		D	E
Solvent system	R_f values	*E. c*	*S. a*	*E. c*	*S. a*	*E. c*	*S. a*	*E. c* *S.a*	*E.c* *S.a*
BEA	0.24	√						√	√
	0.27		√		√		√		
CEF	0.85		√					√	√
E. transvaalense									
BEA	0.24							√	
	0.36		√		√				

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol and E= ethyl- acetate

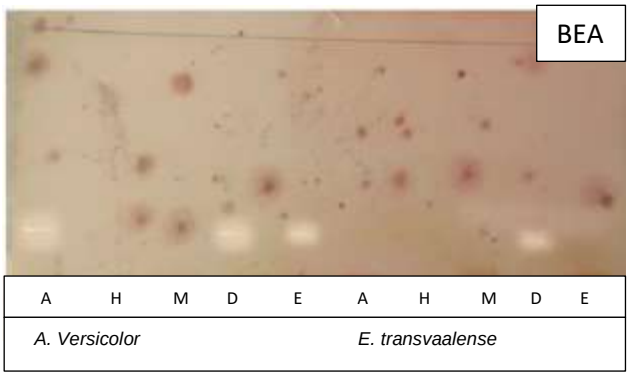


Figure 3.12 Bioautogram of extracts of *A. versicolor* and *E. transvaalense,* developed in BEA, and sprayed with *E. coli*. White areas indicate inhibition of bacterial growth. Lanes from left to right: (A = acetone, H = hexane, D = DCM, M = methanol and E= ethyl-acetate).

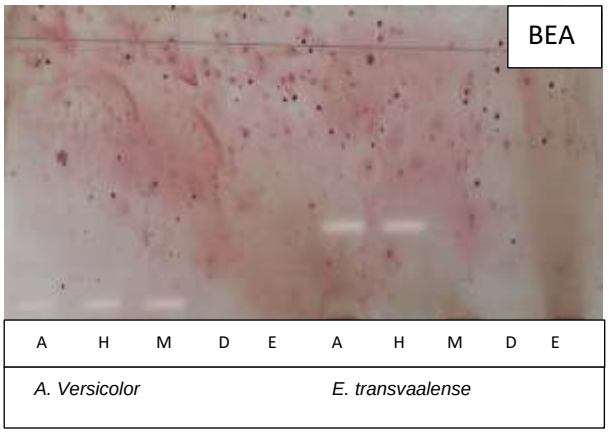


Figure 3.13 Bioautogram of extracts of *A. versicolor* and *E. transvaalense* developed in BEA, sprayed with *S. aureus*. White areas indicate inhibition of bacterial growth. Lanes from left to right: (A = acetone, H = hexane, D = DCM, M = methanol and E= ethyl-acetate).

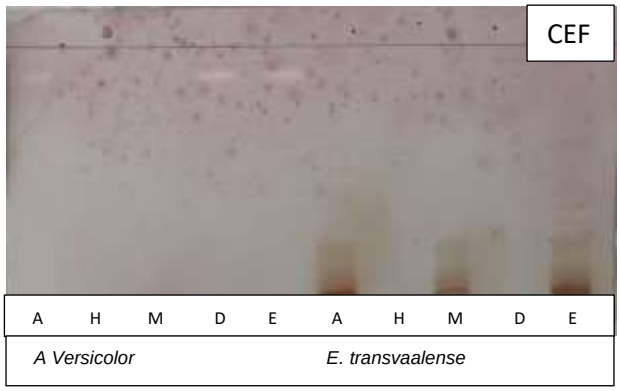


Figure 3.14 Bioautogram of extracts of *A. versicolor* and *E. tranvaalense,* developed in CEF, sprayed with *S. aureus.* White areas indicate inhibition of bacterial growth. Lanes from left to right: (A = acetone, H = hexane, D = DCM, M = methanol and E= ethyl-acetate).

3.8. Antibacterial activity of plant species against *N. gonorrhoeae*

3.8.1 Micro-dilution assay

The antibacterial activity of plant extracts was determined using a micro-dilution assay (Table 3.11). In the current study, all plant extracts were active against the tested pathogen. The hexane, aqueous, and decoction extracts of *C. abbreviata, M. zeyheri, A. grandiflora*, *A. versicolor, X. zambesiaca, M. flabellifolius,* and *C. tomentosa* had excellent antibacterial activity against *N. gonorrhoeae* with low MIC values ranging from 0.02-0.03 mg/ml (Table 3.11). These findings support the use of water by traditional health practitioners. Similar results were found with water extracts of *C. abbreviata* to be active against *N. gonorrhoeae* (Mulubwa and Prakash, 2015).

Ethanol root extracts of *C. abbreviata* were reported to have good activity against gonorrhea and syphilis (Prinsloo et al., 2018). Poor activity was observed in the methanol, DCM, extracts of *M. zeyheri, M. flabellifolius* against *N. gonorrhoeae* with MIC values ranging from 1.25-2.5 mg/ml.

Table 3.12 Minimum inhibitory concentrations (mg/ml) of selected plant species against *Neisseria gonorrhoeae.*

Plant species		*Elaeodendron transvaalense*							*Albizia versicolor*							*Xanthocercis zambesiaca*							
Bacteria	Time (Hrs.)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	Gent
G. n	24	0.02	0.02	0.02	0.02	0.03	0.62	0.31	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	< 0.02
	48	0.02	0.02	0.02	0.02	0.03	0.62	0.31	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.15	0.03	0.02	0.02	< 0.02
	72	0.03	0.03	0.02	0.02	0.03	2.5	2.5	0.02	0.02	0.03	0.07	0.03	0.02	0.02	0.02	0.03	0.02	0.15	0.03	0.02	0.03	< 0.02
Plant species		*Cassia abbreviata*							*Anthocleista grandiflora*							*Capparis tomentosa*							
G. n	24	0.07	0.02	0.62	0.07	0.02	0.02	0.02	1.25	0.02	0.02	0.62	1.25	0.02	0.02	0.02	0.02	0.02	0.31	0.02	0.03	0.03	< 0.02
	48	0.07	0.02	0.62	0.07	0.02	0.02	0.02	1.25	0.02	0.02	0.62	1.25	0.03	0.02	0.02	0.02	0.02	0.31	0.02	0.03	0.03	< 0.02
	72	0.07	0.02	2.5	1.25	0.02	0.02	0.02	1.25	0.02	0.02	1.25	1.25	0.03	0.02	0.07	0.15	0.03	0.31	0.02	0.03	0.03	< 0.02
Plant species		*Mimusops zeyheri*							*Myrothamnus flabellifolius*							Gent							
G. n	24	0.02	0.02	2.5	1.25	0.02	0.02	0.02	1.25	0.02	1.25	0.62	0.02	0.02	0.03	< 0.02							
	48	0.07	0.02	2.5	1.25	0.02	0.02	0.02	1.25	0.02	1.25	0.62	0.02	0.03	0.03	< 0.02							
	72	0.07	0.02	2.5	2.5	0.07	0.02	0.02	1.25	0.03	2.5	2.5	0.02	0.03	0.03	< 0.02							

Gentamicin was used as a positive control.

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol, E= ethyl-acetate, Dh2o = Distilled aqueous and Th2o =Decoction mixture

Abbreviations: *G.n= Neisseria gonorrhoeae,* Gent= Gentamicin.

3.8.2 Total activity

The total activity of the plant crude extracts has been explained and calculated using the formula expressed in section 3.6.2. The highest total activity (1611ml/g) was observed in plant extracts of *C. tomentosa* against *N. gonorrhoeae* (Table 3.12). The lowest activity (13 ml/g) was observed in the methanol extract of *M. zeyheri* against the tested bacteria.

Table 3.13 Total activities of plant extracts in ml/g tested against *N. gonorrhoea.*

Plant species		*Elaeodendron transvaalense*					*Albizia versicolor*					*Xanthocercis zambesiaca*				
Bacteria	Time (Hrs.)	A	H	M	D	E	A	H	M	D	E	A	H	M	D	E
N. g	24	1591	1752	1585	1556	1038	1563	1560	1033	1764	1593	1528	1621	1577	1444	1435
	48	1591	1752	1585	1556	1038	1563	1560	1033	1764	1593	1528	1080	1577	193	956
	72	1060	1168	1585	1556	1038	1563	1560	1033	504	1062	1528	1080	1577	193	956
Average		1414	1557	1585	1556	1038	1563	1560	1033	1344	1416	1528	1261	1577	610	1116
Plant species		*Cassia abbreviata*					*Anthocleista grandiflora*					*Capparis tomentosa*				
N. g	24	459	1567	50	452	1561	25	1481	1567	50	24	1592	1582	1772	1595	1611
	48	459	1567	50	452	1561	25	1481	1567	50	24	1592	1582	1772	213	1611
	72	459	1567	12	25	1561	25	1481	1567	25	24	212	211	1181	213	1611
Average		459	1567	37	310	1561	25	1481	1567	42	24	1132	1125	1575	674	1611
Plant species		*Mimusops zeyheri*					*Myrothamnus flabellifolius*									
N. g	24	1785	1579	13	29	1508	25	1558	32	52	1552					
	48	510	1579	13	29	1508	25	1558	32	52	1552					
	72	510	1579	13	14	431	25	1038	16	13	1552					
Average		935	1579	13	24	1149	25	1384	27	39	1552					

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol, E= ethyl-acetate

Abbreviations: *N.g= Neisseria gonorrhoeae*

3.8.3 Plant extracts with excellent antibacterial activity

Albizia versicolor and *Cassia abbreviata* had more plant extracts with good antibacterial activity containing MIC values of 0.02 mg/ml (Table 3.13). Traditionally, *A. versicolor* has been reported to have several therapeutic applications. The roots and bark are used to cure anaemia, enlarged glands, disorders caused by sexually transmitted diseases, backache, and are used as an anthelmintic. The bark is used to treat flu, headaches, and sinus infections, and pulverized bark may be sniffed for the same effect.

The *Albizia* species have been reported to contain a variety of phytochemicals, most notably triterpenoids, saponins, and lignanoids (He et al., 2020). However, cattle and sheep have died as a result of consuming *A. versicolor* pods (Bastianello et al., 1992). In a study conducted by Chisamile et al. (2023), it was revealed that *C. abbreviata* root and stem decoction are used in Tanzania and Mozambique to cure diarrhea, stomach pains, and syphilis. The leaves, and barks have been used to cure earache, and fruits can be used to treat eye infections (Osunga et al., 2023). A total number of 3 plant extracts with 0.02 mg/ml was found in *X. zambesiaca, M. zeyheri,* and *A. grandiflora,* followed by *E. transvaalense* with a total number of 2 plant extracts with excellent antibacterial activity. *C. tomentosa* and *M. flabellifolius* had the lowest number of extracts with good activity.

Table 3.14 Plant extracts with excellent antibacterial activity (0.02 mg/ml) against *Neisseria gonorrhoeae*.

	Plant species								
Extractants	*E. transvaalense*	*A. versicolor*	*X. zambesiaca*	*M. zeyheri*	*C. abbreviata*	*A. grandiflora*	*C. tomentosa*	*M. Flabellifolius*	Average
A	0	1	1	0	0	0	0	0	2
H	0	1	0	1	1	1	0	0	4
M	1	0	1	0	0	1	0	0	3
D	1	0	0	0	0	0	0	0	1
E	0	0	0	0	1	0	1	1	3
D H_2O	0	1	1	1	1	0	0	0	4
T H_2O	0	1	0	1	1	1	0	0	4
Total	2	4	3	3	4	3	1	1	21

Extractants: A = acetone, H = hexane, D = dichloromethane, M = methanol, E= ethyl- acetate, Dh2o = Distilled water and Th2o =Decoction mixture.

3.8.4 Bioautography assay

The active compounds were observed in TLC bioautograms separated with CEF. Similar active compounds with an R_f value of 0.85 were observed in bioautograms developed in CEF against *N. gonorrhoeae*. No active compounds were visible in TLC bioautograms developed in EMW and BEA, a possible reason may be the synergy between the compounds found in the plant extract. Another reason may be that some of the active compounds evaporated during the drying period of the TLC plates. Based on the literature, there is limited information on the antibacterial activity of *A. versicolor.* Therefore, there is a need to explore the biological activity of these plant species against the fungal and bacterial pathogens.

3.9 Conclusion

The plant extracts were active against the tested fungal pathogens with MIC values ranging between 0.02 and 2.5 mg/ml. The aqueous and decoction extracts possess strong antibacterial activity against the bacteria pathogens. The plant extracts were active against the tested pathogens and showed a degree of excellent activity with MIC values ranging from 0.02-0.03 mg/ml. In bioautography assay, more active compounds were observed in TLC bioautograms separated with BEA when compared to CEF and EMW eluent solvent systems. No active compounds were observed in TLC bioautograms developed in CEF. This study revealed that some of the plants used for the treatment of “u wela” have the ability to be utilised for medicinal purposes against *N. gonorrhoeae*. The findings confirm the effectiveness of the use of water by traditional health practitioners and the local people when preparing their medications. *A. versicolor* had good antimicrobial activity against the tested microorganism. This plant species might be a potential primary source of gonorrhea treatments.

In the next chapter, the isolation of active compounds from the barks *of A. versicolor* will be investigated.

CHAPTER 4

INVESTIGATION OF PLANT SPECIES SELECTED FOR ISOLATION OF ACTIVE COMPOUNDS

4.1 Introduction

In this chapter, *Albizia versicolor* was chosen for further phytochemical analysis and isolation of antimicrobial compounds. The plant species was selected based on excellent biological activity and active compounds were observed in the plant extracts against the tested microorganisms.

A. versicolor is an evergreen tree that grows up to 20 meters tall and has a broad, rounded to flat crown. Young branches have thick brownish to yellowish hairs, whereas older branches and stems have corky, greyish-brown, and rough bark (Orwa et-al.,-2009).-The-seed-pods-contain-a-toxic-substance-known-as-tetramethoxypyri doxine. It is the cause of the illness of albiziosis. Poisoning often happens mid-winter when pods are blown from trees and consumed by animals. It causes a range of neurological symptoms that eventually lead to death (Hyde et al., 2022). The perennial plant has been broadly described in chapter 2, section 2.11. Previously, it was reported that the plant contains the presence of carbohydrates, flavonoids, peltogynoid, and triterpenes. This tree has traditionally been used to treat hypertension (Mudau et al., 2020), and other medicinal applications are discussed in chapter 3, section 3.6.3. The bark of this multi-branched tree is smooth, and the leaves are clustered on the tips of inflexible branches. A decoction of the bark is used to purify the stomach and as a laxative, relieving stomach pains. The leaves are chewed, and the juices are consumed to cure sore throats and diabetes (Deutschländer et al., 2009). To the best of our knowledge, there is a lack of information on the isolation of *A. versicolor* plant species.

Plants manufacture a diverse range of secondary metabolites, and it is these metabolites that serve as the foundation for many commercial pharmaceutical medications as well as herbal therapies derived from medicinal plants. Secondary metabolites in plants are derivatives of primary metabolites generated by plants because of various physiological changes. The plants contain chemical components

that possess biological activity that can benefit human health through the pharmaceutical and food sectors (Ashraf et al., 2018).

Bioassay-guided fractionation can be used to successfully separate plant extracts by exposing them to chemical fractionations and bioassays to reduce the complexity and identify specific chemicals with important biological effects. Bioassay-guided fractionation is performed, using the following main procedures: (i) extraction of metabolites from biomass using solvents, (ii) chromatographic fractionation of the resultant extract, (iii) bioassay screening of each fraction, (iv) isolation of the molecule(s) from bioactive fractions, and (v) identification and assessment of the isolated molecules' bioactivity (Nothias et al., 2018).

Effective chromatographic separation of analytes in a mixture is dependent on the identification of an appropriate chromatographic technique, followed by the refinement of the separation's test conditions. Column-chromatography,-and-(TLC) thin-layer-chromatography techniques are used to identify unknown compounds in plant extracts (Mroczek et al., 2020). Furthermore, column chromatography is a technique preferred for separating, identifying, and purifying a mixture of chemical components based on the interaction of a stationary (solid) and mobile (liquid) phase. The components of the mixture-disperse-themselves-between-the-phases-by-adsorption,-partition,-ion exchange,-or-size-exclusion-(Ebere-et-al.,-2019).-Separation-by-thin-layer-chroma-tography is dependent on polarity, with certain compounds adhering closely to their adsorbent (Kagan and Flythe, 2014).

In this chapter, solvent-solvent fractionation will be carried out using solvents of various polarities. The biological activity of the fractions will be determined using serial dilution and bioautography assays against the selected bacterial and fungal pathogens.

4.2 Methods and materials

4.2.1 Plant collection and storage

The barks of *A. versicolor* were collected in the Vhembe District. The extraction procedure is described in chapter 3, section 3.2.

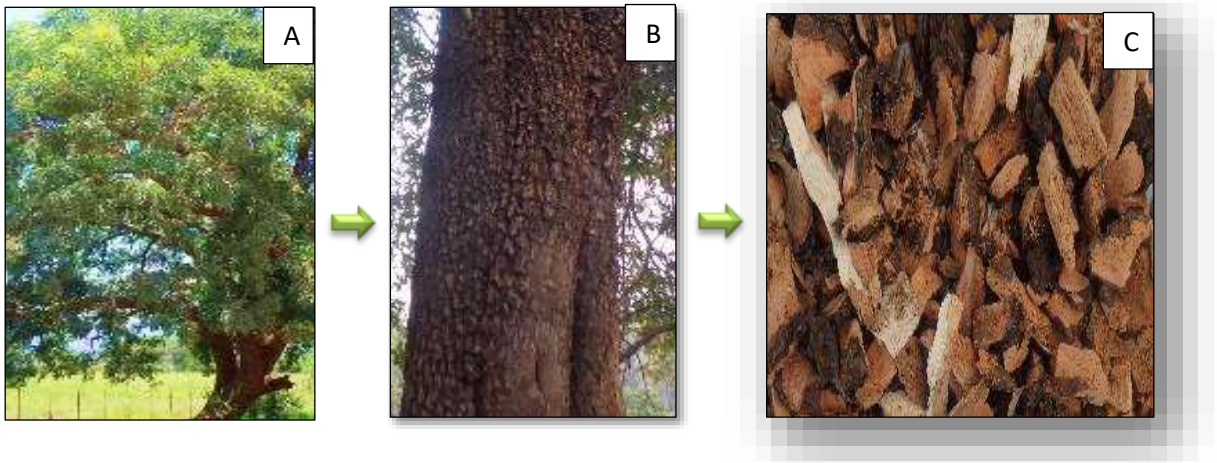


Figure 4.1 A picture of (A) whole plant, (B), fresh bark, and (C), dried bark of *Albizia versicolor* taken from the Vhembe District.

4.2.2 Serial exhaustive extraction

Finely ground bark material (500 g) was serially extracted using hexane, chloroform, acetone, and methanol (1500 ml), respectively. The plant material was washed three times. The extracts were filtered into pre-weighed beakers using the Whatman No.1 filter paper, concentrated at 60 °C with a rotary evaporator (Buchi Rotavapor-R), and transferred into pre-weighed labelled beakers (150 ml). The plant extracts were evaporated under a stream of cold air at room temperature.

4.2.3 Phytochemical analysis

Phytochemical analysis was used to analyse the chemical components of plant extract. The procedure is described in chapter 3, section 3.2.4.

4.2.4 Antifungal activity

4.2.4.1 Micro-dilution assay

The antifungal activity of the plant extracts was determined against the fungal pathogens using a micro-dilution assay described in chapter 3, section 3.3.1.

4.2.4.2 Bioautography assay

The number of active compounds present in the plant extracts was determined using the bioautography assay described in chapter 3, section 3.3.2.

4.2.5 Antibacterial activity

4.2.5.1 Micro-dilution assay

The antibacterial activity of the plant extracts was determined against the bacteria using a micro-dilution assay described in chapter 3, section 3.3.1.

4.2.5.2 Bioautography assay

The number of active compounds present in the plant extracts was determined against the bacteria using the bioautography assay described in chapter 3, section 3.3.2.

4.3 Results and discussion

4.3.1 Serial exhaustive extraction

Figure 4.2 represents the mass extracted from 500g of *A. versicolor.* Methanol (57%) extracted a large quantity of plant material, followed by chloroform (32%), acetone (7%), and hexane (4%), (Figure 4.2). Other researchers found that methanol extracted a greater amount of plant material compared to other solvents (Dhawan and Gupta, 2017). More importantly, methanol and acetone are used to extract polar compounds (Santos-Buelga et al., 2012). As a result, *A. versicolor* bark extracts contain more polar compounds than non-polar compounds.

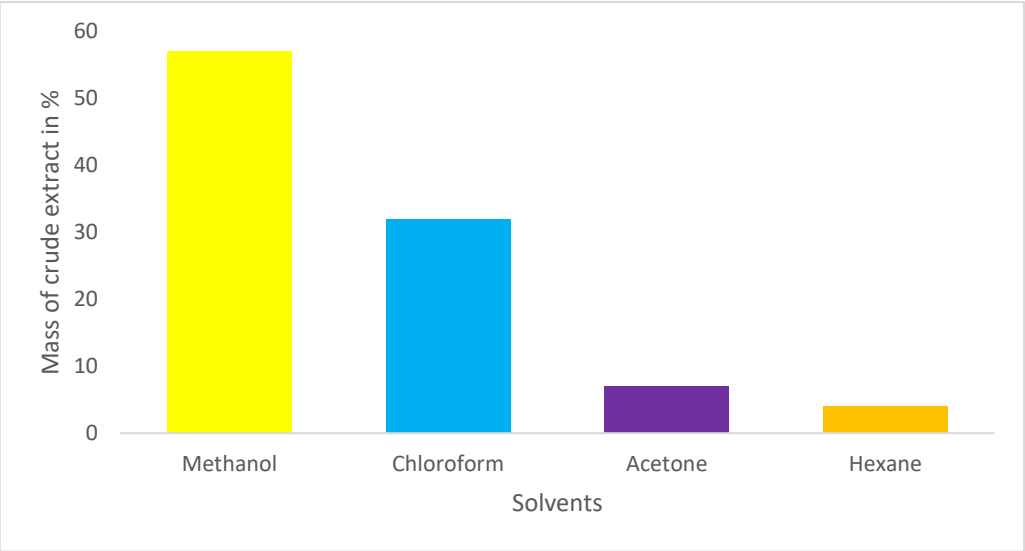


Figure 4.2 Quantity of plant material sequentially extracted from 500 g of *A. versicolor*, bark with different extractants. Lanes from left to right: methanol, chloroform, acetone, and hexane.

4.3.2 Phytochemical analysis of crude extracts

Thin-layer chromatography was used to investigate the chemical compounds present in the bark extracts of *A. versicolor* (Figure 4.3). The TLC fingerprints revealed a wide range of chemical compounds in the crude plant extracts separated by BEA, CEF and EMW. The majority of the extracts were separated by the BEA (26) as compared to CEF (20) and EMW (17). The findings may suggest that *A. versicolor* primarily contains a significant proportion of non-polar compounds. Similarly, a study investigated by Mokoka et al. (2010) reported that BEA was the eluent solvent system since it separates non-polar compounds.

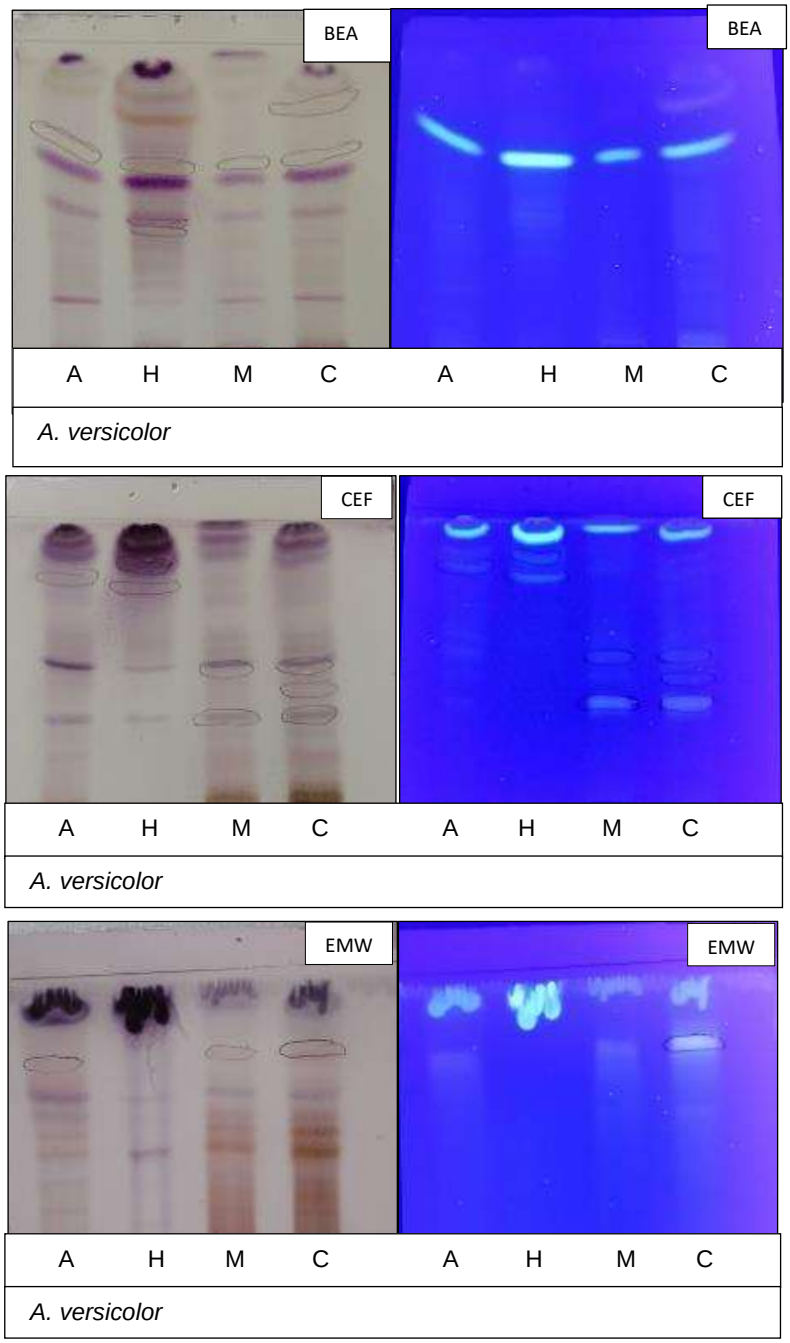


Figure 4.3 TLC chromatograms of *A. versicolor* developed in BEA, CEF and EMW sprayed with vanillin-sulphuric acid. Lanes from left to right: A = acetone, H = hexane, M = methanol and C = chloroform).

4.3.3 Antifungal activity

4.3.3.1 Serial dilution method

The antifungal activity of *A. versicolor* bark extracts was determined against *C. albicans, C. neoformans*, and *A. fumigatus* using serial dilution assay (Table 4.1). All the extracts were active against the tested fungal pathogens. Excellent activity was observed in the acetone, hexane, and chloroform extracts of *A. versicolor* against *C. albicans*, and *A. fumigatus* with low MIC values ranging between 0.02-0.03 mg/ml. Contrastingly, very poor activity was observed in the acetone extracts tested against *C. neoformans.* Notably, the methanol bark extract showed moderate activity against *C. neoformans* with a MIC value of 0.13 mg/ml. However, it was reported that methanol seed extract of *Extracta japonica* had excellent activity against *C. neoformans* (Bisso et al., 2022).

Table 4.1 Minimum inhibitory concentrations (mg/ml) of *A. versicolor* bark extracts tested against the fungal pathogens.

Plant species		*A. versicolor*				
Fungi	Time (Hrs.)	A	H	M	C	Amp B
Candida albicans	24	0.02	0.02	0.02	0.03	< 0.02
	72	0.03	0.02	0.03	0.03	< 0.02
Cryptococcus neoformans	24	2.5	0.02	0.13	0.02	< 0.02
	48	2.5	0.03	0.13	0.03	< 0.02
Aspergillus fumigatus	24	0.02	0.02	0.02	0.02	< 0.02
	48	0.02	0.02	0.02	0.02	< 0.02

Extractants: A = acetone, H = hexane, M = methanol, C = chloroform

4.3.3.2 Bioautography assay

Bioautography was used to determine the number of active compounds in the plant extracts. More active compounds were visible in BEA as compared to CEF and EMW, The chloroform extracts displayed four compounds, followed by hexane (3) extracts against *A. fumigatus* (Figure 4.4). Similar antifungal compounds were observed in the acetone and hexane extracts tested against *C. albicans*, with the same R_f value of 0.23. Interestingly there was no activity in the bioautograms developed in CEF and EMW, this may be due to synergistic effects. Shekwa et al. (2023), observed active compounds in the stem extract of *Carissa bispinosa* stem in bioautograms developed in BEA.

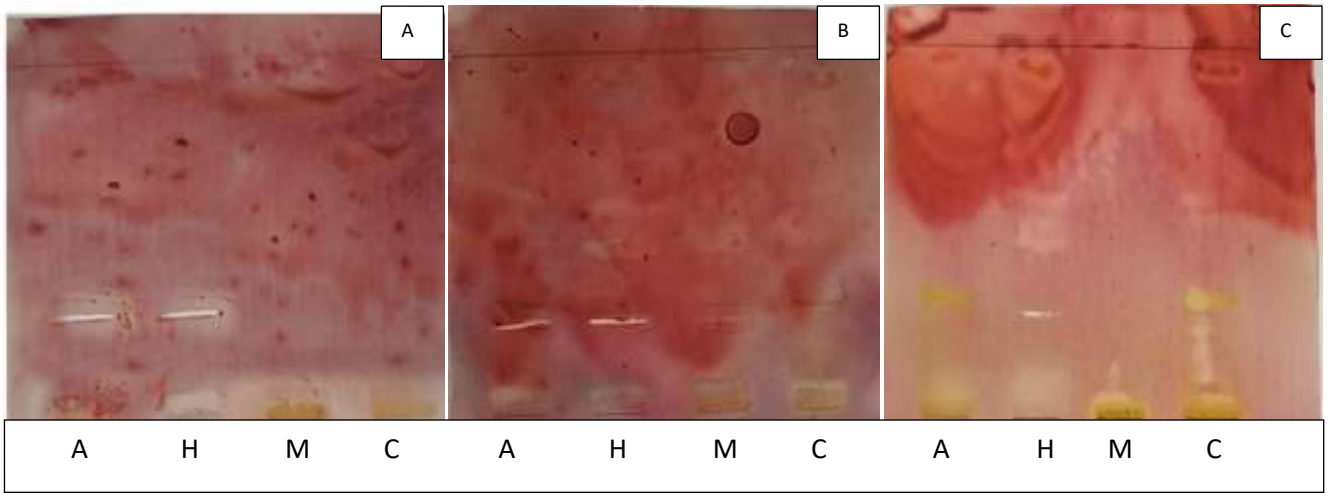


Figure 4.4 Bioautograms of bark extracts of *A. versicolor* developed in BEA, sprayed with A= *C. albicans,* B= *C. neoformans* and C= *A. fumigatus.* White areas indicate inhibition of fungal growth. Lanes from left to right: (A = acetone, H = hexane, M = methanol and C = chloroform).

CHAPTER 5

ISOLATION OF ANTIMICROBIAL COMPOUNDS FROM THE BARK OF *A. VERSICOLOR*

5.1 Introduction

A. versicolor (chapter 4) was selected for further phytochemical analysis and isolation of active compounds based on good antimicrobial activity against the tested microorganism.

Plant extracts contain a significant range of phytochemicals and have shown high-level efficacy against infections (Manandhar et al., 2019). However, there have been few extensive analyses of these plants for their possible involvement as phytochemical entities and antibacterial treatment (Dubale et al., 2023). Plants contain secondary metabolites that have been capitalized as potential drugs and are used in preliminary pharmacological designs, as well as running microbial tests (Elshafie et al., 2023). The extraction of bioactive compounds from plant materials has received a lot of interest as a result of their prospective medicinal benefits (Xiang et al., 2022).

Albizia is a genus from the Fabaceae family with over 150 species (El Khodary et al., 2021). A study conducted by Mohamed et al. (2013) discovered that *Albizia* species are high in phenolic and saponin compounds. The vast array of compounds produced by plants provides a wide range of chemical structures that could lead to innovative forms of antimicrobial activities (Gorlenko et al., 2020). The stem bark extracts of *A. versicolor* are used for the treatment of venereal diseases and as a laxative (Rukunga and Waterman, 1996). In Africa, *Albizia* species are used in traditional medicine to cure rheumatism, cough, diarrhea, and bruises (Mustafa et al., 2023; Yusuf et al., 2022).

Isolation of antifungal compounds aims at targeting pure compounds from plant material that inhibit the fungi of interest. Column chromatography is a technique that is used to isolate the bioactive compounds from the plant extracts. Furthermore, column chromatography and TLC techniques are the most affordable procedures and are suitable for sample purification (Heftmann, 2011). In this chapter, column

chromatography was used to isolate compounds from the acetone extracts of *A. versicolor*.

5.2 Materials and methods

The summary of the methodology is presented in Figure 5.1. Ground-powdered bark (500 g) materials of *A. versicolor* were serially extracted with a solvent of increasing polarities such as acetone, hexane, methanol, and chloroform. The extracts were concentrated using a rotary evaporator. The plant extracts were concentrated using a rotary evaporator at 40°C. The concentrated extracts were transferred into pre-weighed beakers and placed under a stream of cold air in a fume hood for complete dryness. The plant extracts (10mg/ml) were tested for antifungal activity using both micro-dilution and bioautography assays. The method is described in chapter 3, sections 3.1.1 and 3.3.2)

The procedure for the isolation of antifungal compounds from the bark of *A. versicolor* is explained in a schematic representation in Figure 5.1.

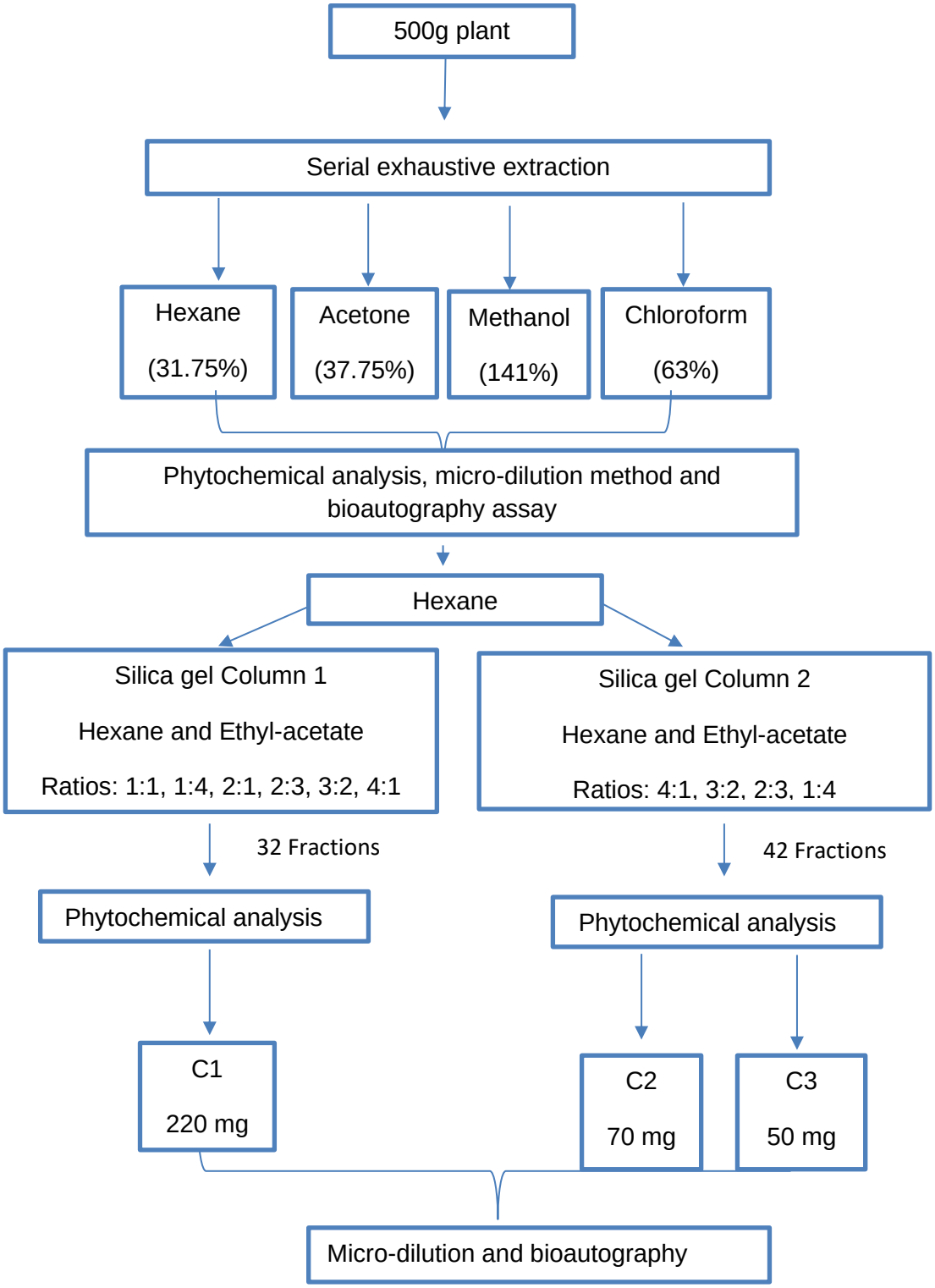


Figure 5.1 Schematic diagram of serial exhaustive extraction of *A. versicolor* bark, and isolation of bioactive compounds using column chromatography.

5.3 Isolation of antifungal compounds

5.3.1 Column chromatography

Silica gel (170 g) was mixed with 400 ml hexane to form a slurry and packed into a glass column to a height of 47 cm and a diameter of 5 cm (denoted as column i). The acetone crude extract (2 g) was mixed with silica gel (1 g) and allowed to dry at room temperature. Hexane (100%) was initially used to elute the column, followed by the same volume of each of the following solvent mixtures: hexane and ethyl-acetate (1:1, 1:4, 2:1, 2:3, 3:2, and 4:1). Thirty-two fractions were collected in 50 ml tubes. TLC chromatograms of the fractions were prepared in duplicate and developed in BEA, CEF, and EMW. One set was sprayed with vanillin-sulphuric reagent spray as the reference chromatograms for visualizing compounds (Figures 5.2-5.5) and the other was sprayed with *C. albicans* (Figure 5.9) to locate the antifungal compounds present in the fractions. The antifungal compounds visible on TLC plates with similar R_f values were combined. Fractions with similar pure spots on TLC chromatograms were grouped together and evaporated to dryness under a reduced pressure of 45 °C. The combined fractions were further analysed using TLC fingerprinting.

5.4 Results and discussion

5.4.1 Compound 1 (column i)

Fractions 11-16 (Figure 5.4) from the first column (Ci) contained a pure compound C1. The pooled fraction was concentrated under vacuum at 45°C and transferred to a pre-weighed glass vial to dry completely.

5.4.2 Compound 2 (column ii)

Silica gel (13 g) was dissolved in hexane and used to pack a column ii (20 × 1.0 cm). Hexane: EtOAC (1:4) fractions (0.13 g) were mixed with a small portion of silica gel and allowed to dry. Ethyl-acetate (4:1), (3:2), (2:3), and (1:4). The mixture was spread on top of the column, and $CHCl_3$: EtOAC (2:3) was used as an eluent solvent system. Fractions of 10 ml volume were collected. Fractions 8-10 (Figure 5.4) from the second column (Cii) contained a pure compound C2 and was concentrated in a rotavapor under vacuum at 45 ºC and transferred into a pre-weighed vial and put under a stream of cold air to complete dryness.

5.4.3 Compound 3 (column iii)

Silica gel (20 g) was dissolved in hexane and used to pack the column iii (20 × 1.0 cm). Hexane: EtOAC (1:4) fractions (0.20 g) were mixed with a small portion of silica gel and allowed to dry. Ethyl-acetate (1:4), (2:3), (3:2), (4:1), (96:4), (95:3), (92:1), and (80:20) was used as an eluent solvent system. Fractions of 10 ml volume were collected. Fractions 6-7 (Figure 5.4) contained a pure compound C3.

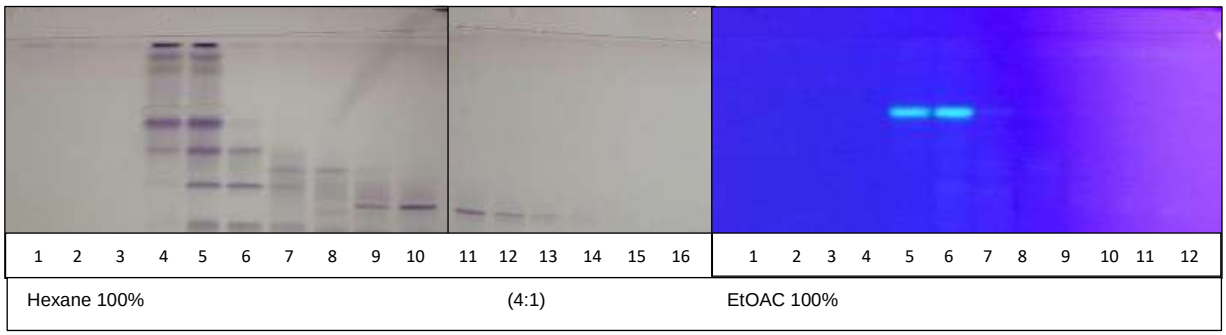


Figure 5.2 Fractions collected from column i developed in BEA, sprayed with vanillin-sulphuric acid spray reagent (Right) and viewed under UV Light (Left).

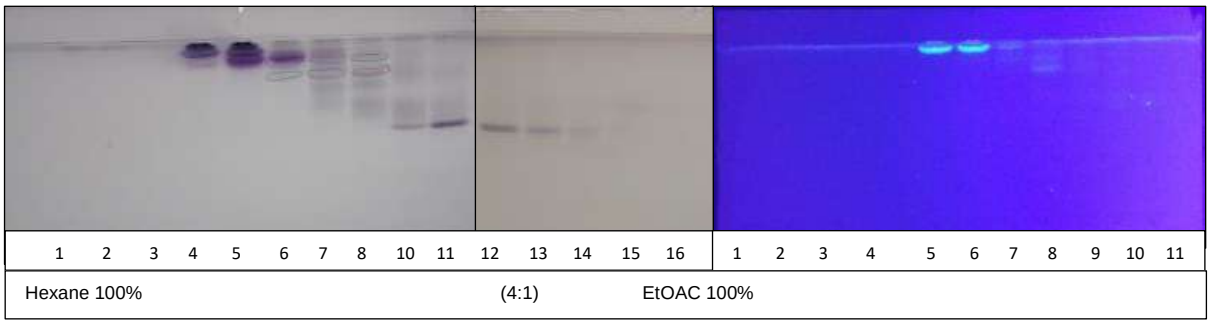


Figure 5.3 Fractions collected from column i developed in CEF, sprayed with vanillin-sulphuric acid spray reagent, (Right) and viewed under UV Light (Left).

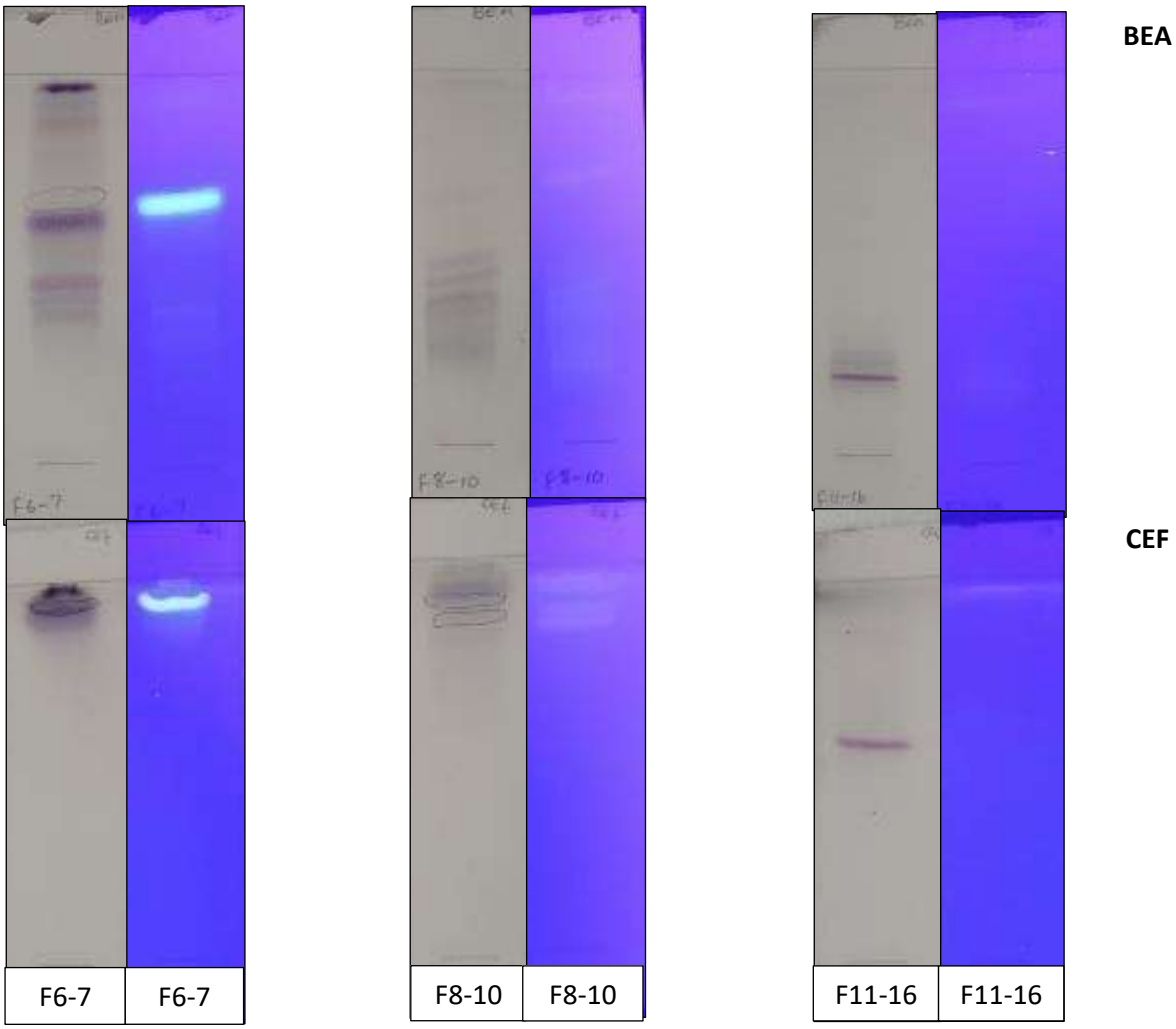


Figure 5.4. Fractions resulting from the combination of fractions 6-7, fractions 8-10 and fractions 11-16 developed in BEA (top) and CEF (below), sprayed with vanillin-sulphuric reagent spray (Left) and viewed under UV light (Right).

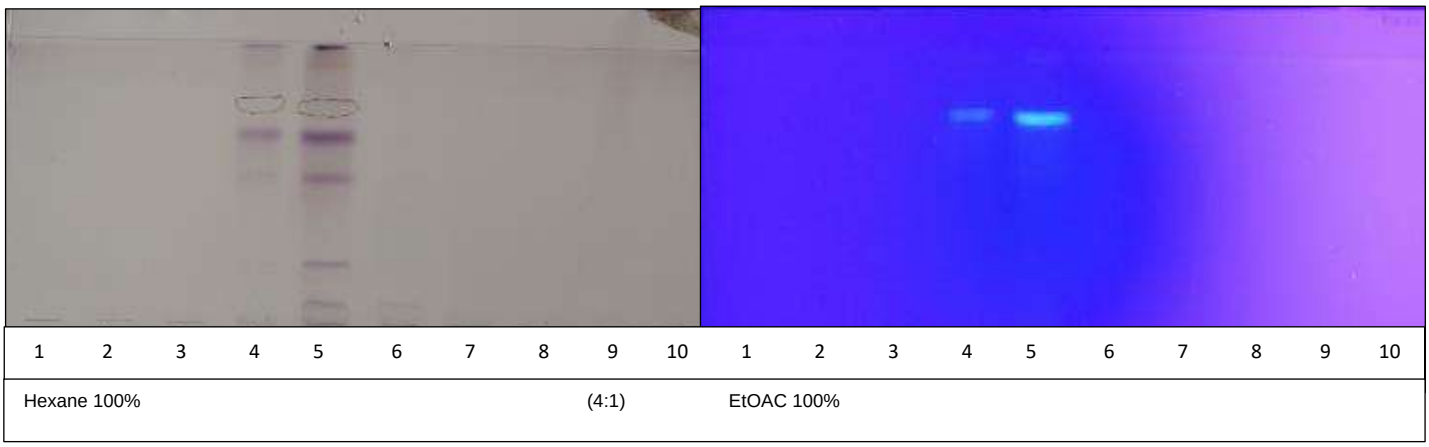


Figure 5.5. Fractions collected from column ii developed in BEA, sprayed with vanillin-sulphuric acid spray reagent, and viewed under UV Light.

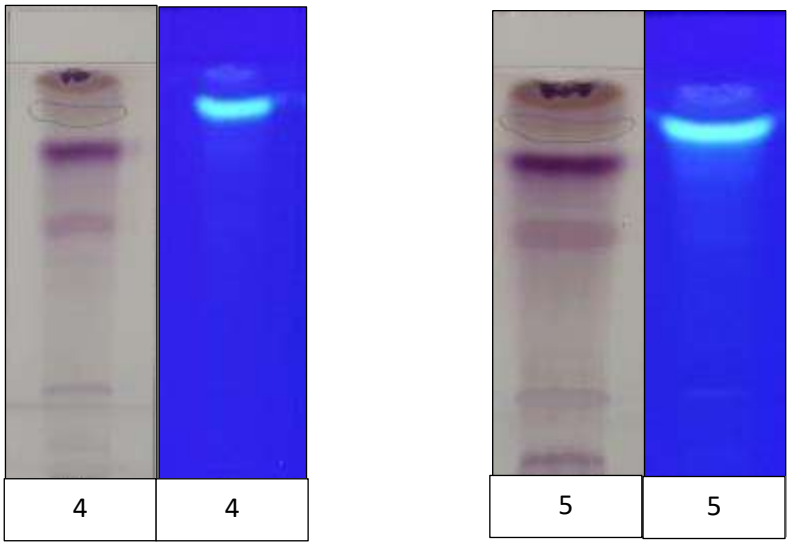


Figure 5.6 Fractions 4 and 5 developed in BEA resulted in similar bands. TLC plates viewed under UV light, and subjected to phytochemical analysis.

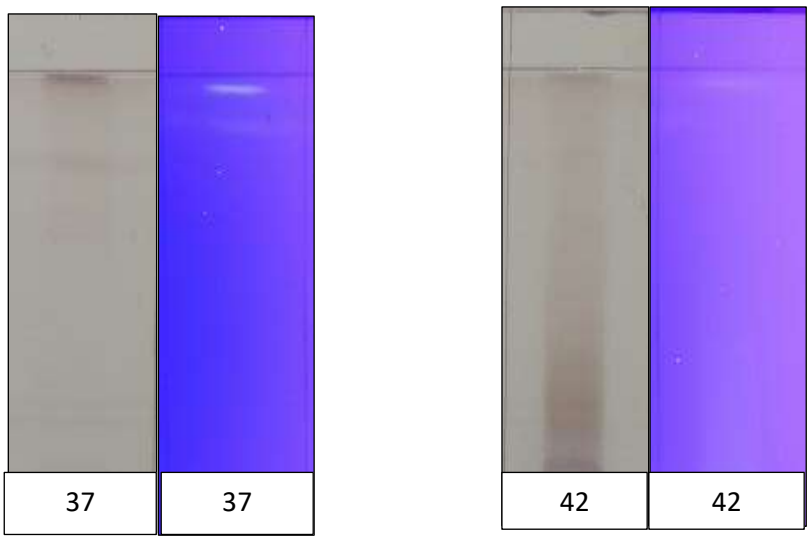


Figure 5.7 Fractions 37 and 42 developed in BEA.TLC plates viewed under UV light, and subjected to phytochemical analysis, resulting in each one having a single diluted compound.

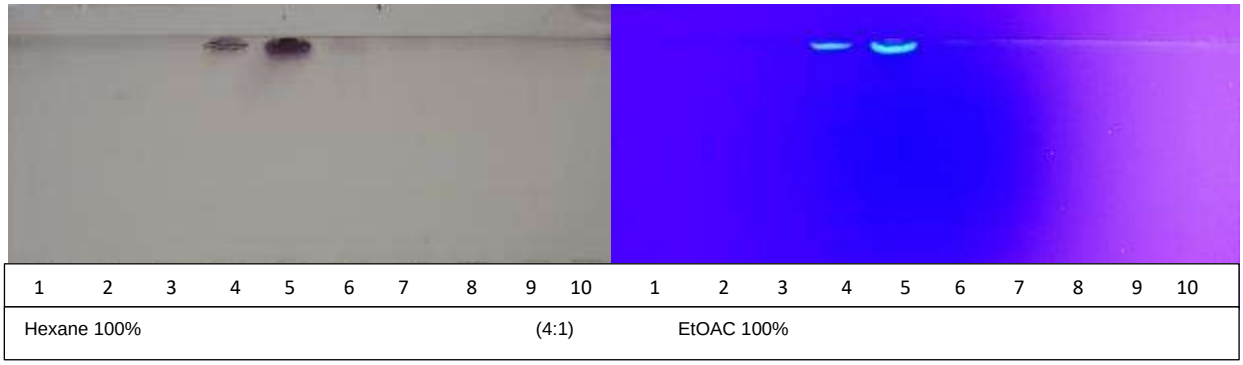


Figure 5.8 Fractions collected from column ii developed in CEF, sprayed with vanillin-sulphuric acid spray reagent (left), and viewed under UV Light (right).

5.4.4 Fractions obtained from column i

The bioautography assay was used to determine the number of active compounds from fractions obtained from column I, using three eluent solvent systems. Fractions were tested for antifungal activity against *C. albicans* and antifungal compounds were observed in bioautograms developed in BEA (Figure 5.9). Interestingly fractions 7 and 8 showed excellent activity against *C. albicans*, with the same R_f of value of 0.31.

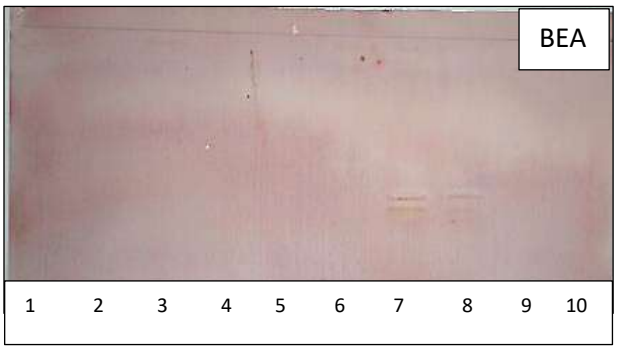


Figure 5.9 Fractions from column (i) developed in BEA, tested against *C. albicans*.

CHAPTER 6

STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS FROM THE BARK OF *ALBIZIA VERSICOLOR*

6.1 Introduction

In this chapter, NMR and mass spectroscopy will be used to identify the active compounds isolated from the bark of *A. versicolor* in chapter 5.

Spectroscopic is a technique used to identify the chemical structure of a pure molecule. Nuclear magnetic resonance (NMR) and mass spectroscopy (MS) are research techniques used to determine the molecular structure and weight of isolated compounds (Marcone-et-al.,,-2013). NMR spectroscopy is the most preferred technology due to its long-term and ability to measure metabolite levels, high degree of experimental repeatability (Emwas et al., 2019). Furthermore, 1-dimensional proton 1H-NMR is the initial stage in structural characterisation. When allocating protons, the chemical shift, multiplicity, coupling constants, and integration are all elements to consider. This technique is used to investigate the effects of ionizing radiation on molecules. It is dependent on chemical events in the gas phase that consume sample molecules during the production of ionic and neutral species (Khalizov et al., 2020). Furthermore, a mass spectrometer creates numerous ions from a sample, separates them based on their unique mass-to-charge ratio (m/z), and then measures the relative abundance of each ion type (Terziev et al., 2019).

The initial stage in the mass spectrometric examination of chemicals is the generation of gas phase ions of the molecule, which is accomplished mostly by electron ionization. This molecular ion becomes fragmented. In this chapter, NMR and MS were used to identify the isolated compounds extracted from the bark of *A. versicolor.*

6.2 Materials and methods

6.2.1 Structure elucidation

Nuclear Magnetic Resonance (NMR) was used for the identification of isolated antifungal compounds. All samples were sent to the Department of Chemistry at the University of Limpopo for NMR analysis and structure elucidation.

6.2.2 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) (1D and 2D) spectroscopy and mass spectrometry were used to identify the isolated compounds. 1H NMR and 2D NMR experiment data were acquired on a 400 MHz NMR spectrometer (Bruker 400 MHz). HPLC-HR-ESI-MS was performed on Sciex Exion LC-MS X500R QTOF. Each sample was dissolved in 0.6 ml chloroform ($CDCl_3$) and transferred into NMR tubes (5mm).

6.2.3 Mass Spectrometry Preparation

The analysis was carried out on an AB Sciex Exion LC system connected to a Sciex X500R QTOF (quadruple time of flight) mass spectrometer equipped with electrospray ionisation (ESI) probe. A 10 µL aliquot was injected through an autosampler. Separation was achieved using a Phenomenex Luna C 18 column (100 ×2 mm, 2 µm particle size). Mobile phases A and B consisted of water and methanol with 0.1% formic acid respectively. Gradient elution was done as follows: 0 min, 90% A and 10% B: 1 min, 90% A and 10% B: 10 min, 1% A and 99% B: 27 min, 1% A and 99% B: 28 min 95% A and 5% B: 30 min 95% A and 5% B. The flow rate used was 0.4 ml/min at 40 °C and the injection volume of 10 µL. The mass spectrometry was operating in a positive mode by the IDA experiment with a scan time of 0.588 seconds. Nitrogen (N_2) was used as the desolvation gas. Ion source gas 1 and 2 at 50 psi and 70 psi respectively, curtain gas at 30 psi, CAD gas at 7, Ion source temperature 400 °C, spray voltage 5500 V, declustering potential at 40 V, and collusion energy 10 V. The full scan data were acquired from TOF MS m/z 100 to 1000 Da with an accumulation time of 0.25 s. Explorer software was used for data acquisition and processing.

6.3 Results and discussion

6.3.1 Compound 1

Compound 1 was isolated as a cream-white powder (25 mg). Based on 1H and 13C NMR spectra compound 1 was identified as 10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol and it is similar to lupeol but not identical. The spectral data (Figure 6.1) is in agreement with the literature (Kartika et al., 2020; and Rukunga and Waterman, 2001). Lupeol is a unique bioactive compound derived from a variety of plants (Das et al., 2022) with potent anti-inflammatory, and carcinoma properties in both *in-vivo* and *in-vitro* systems (Sharma and Gupta, 2022). Lupeol has shown positive outcomes in the treatment of a variety of human ailments including skin

infections, and neurological conditions (Sohag et al., 2022). The compound lupeol has recently been isolated from the stem barks of *Justicia secunda* (Bako et al., 2023), the whole plant of *Dischidia alboflava* (Linh et al., 2023), and roots of *Euclea natalensis* (Rashed, 2022).

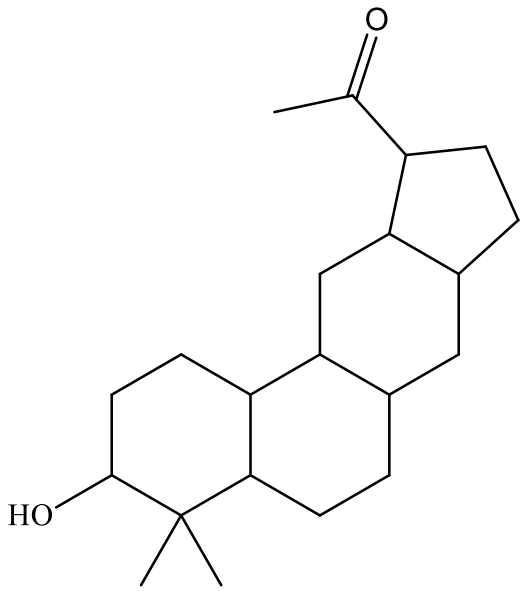
The 1H-NMR of the isolated fraction revealed peaks in the aliphatic region. The proton spectrum of fraction 1 showed signals ranging from 0.81 to 5.29 ppm assignable to methyl (–CH_3), methylene (–CH_2), and methane (–CH) protons. The quartet at 5.07 ppm with a coupling constant of 6.0 Hz corresponds to the hydroxyl group (-OH). The 13C-NMR on, the other hand, revealed tertiary carbon atoms. These tertiary carbon signals resonate at 40.2, 41.0, 43.4, 49.4, 51.4, 51.3, 55.2 and 56.0 ppm. The primary and secondary carbons resonate at the region 13.1-37.2 ppm. Another signal in the carbon spectrum at 174.9 ppm is assigned to the quaternary carbon of the carbonyl group. The Heteronuclear Single-Quantum Correlation (HSQC) experiment showed coupling between the proton at 5.09 ppm and the carbon signal at 70.1 ppm. This was assigned the tertiary carbon with the hydroxyl group. The multiplicity of the proton signals resonating at the region 3.30-4.47 ppm on the proton NMR spectrum showed direct attachment to carbon signals around 29.2-51.3 ppm. This correlation corresponds to the assigned protons and carbons in the aliphatic region on the NMR spectra. This multiplicity of the protons in the aliphatic region is a result of coupling due to the proximity of the proton atoms. This characterization conforms to the proposed structure below.

Table 6.1 H-NMR data in deuterated chloroform.

ppm	Multiplicity	Coupling constant	(Kartika et al., 2020)
0.8	m		0.85
1.18	m		1.15
2.11	s		2.14
3.31	dd	8.4 and 18	
3.56	s		
3.86	d	6.8	3.87
4.10	dd	4.8 and 12.4	
4.20	d	10.4	4.22
4.33	d	7.6	
4.4	d	4.8	
5.10	-		5.06
5.28	-		5.29

Table 6.2 H-NMR data in deuterated chloroform

ppm	Multiplicity
12.3	CH_3
13.9	CH_3
14.2	CH_3
19.1	CH_3
21.2	CH_3
22.8	CH_2
29.3	CH_2
31.9	CH_2
32.0	CH_2
34.4	CH_2
37.2	CH_2
40.3	CH_2
40.9	CH_2
49.4	CH
51.3	CH
55.2	CH
63.23	CH
101.2	C
129.5	C
139.6	C
174.9	C



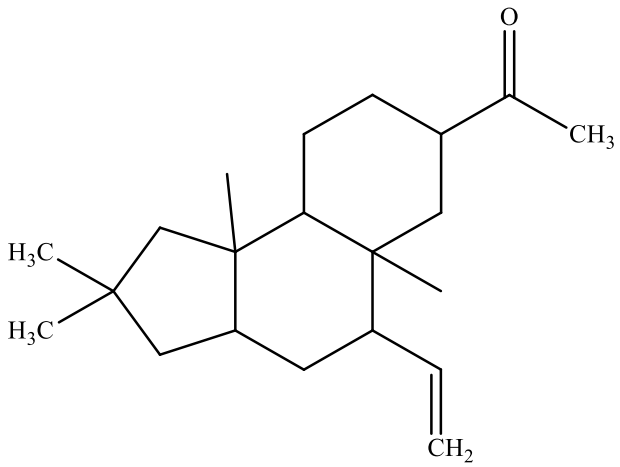
$C_{21}H_{34}O_2$

Figure 6.1 10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol.

6.3.2 Compound 2

Compound 2 was isolated as a cream-white solid (15mg). The compound was identified-as-1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1cyclopenta[a]naphthalen-7-yl)ethenone.-Compound-2 showed a resemblance to a triterpenoid lupeol, however with a few variations. A triterpenoid lupeol is pentacyclic, with a wide range of biological activities (Toledo et al., 2021). It may be found in fruits and vegetables, as well as medicinal plants (Park et al., 2023). Triterpenoid lupeol is present in vegetables such as white cabbage, and tomato, as well as fruits such as olives, figs, and mango (Dharsan et al., 2019). The fruits of the medicinal plant *Rosa beggeriana* produce triterpenoid lupeol and have been documented to treat kidney stones (Aituarova et al., 2023), however in a study conducted by (Raj, 2023), it was concluded that lupeol's therapeutic effectiveness in the prevention and management of urolithiasis needed more investigations.

The ^{1}H-NMR and ^{13}C-NMR of the isolated compound 2 (1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone)-revealed-a-similar-trend as compared to those of compound 1 (10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol) with few exceptions. The proton and carbon spectra of compound 2 showed signals resonating at the aliphatic region corresponding to the methyl (–CH_3), methylene (–CH_2), and methane (–CH) protons. The ^{13}C-NMR on the other hand, revealed signals at 139.6 ppm and 129.5 ppm corresponding to the secondary carbons of the alkene. In the Heteronuclear Single-Quantum Correlation (HSQC) spectrum, these secondary carbons align with the hydrogen atoms at 1.95 ppm.



$C_{21}H_{34}O$

Figure 6.2 1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone.

6.4 Conclusion

The structure elucidation of three isolated compounds from the bark of *A. versicolor* was determined using Nuclear Magnetic Resonance spectroscopy and Mass Spectrometry. Amongst the three compounds, only two compounds were successfully identified as 10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol-and-1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1cyclopenta[a]naphthalen-7-yl)ethenone.

Although comparable to lupeol, compounds 10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol-and-1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1cyclopenta[a]naphthalen-7-yl)ethenone are novel compounds and there is no literature to confirm the two isolated compounds. Therefore, to the best of our knowledge, the two compounds are being reported for the first time in this study. Compound 3 was not identified due to the presence of a long chain of fatty acids.

In the next chapter, the antioxidant activity of selected plant species will be determined using the DPPH assay.

CHAPTER 7

ANTIOXIDANT ACTIVITY OF SELECTED PLANT SPECIES USING DPPH ASSAY

7.1 Introduction

In this chapter, the presence of antioxidant activities will be determined using quantitative and qualitative free radical scavenging. The three plant species namely, *A. versicolor* (Bark), *M. flabellifolius* (whole plant), and *E. transvaalense* (bark) were selected based on good antifungal activity against the tested microorganisms.

Oxidative stress is caused by an imbalance in the cellular equilibrium of pro-oxidant and antioxidant species (Ghazizadeh et al., 2020). Oxidative stress is defined by an extreme rise in reactive oxygenated species (ROS), which is accompanied by insufficient antioxidant defense or failure of the cells' buffering mechanism to maintain redox equilibrium. It is also defined as the disruption of redox signaling and regulation (Pisoschi et al., 2021). Furthermore, ROS causes numerous changes in biomolecules that eventually define clinical symptoms (Bjørklund et al., 2022).

Antioxidants are compounds that prevent oxidation. They are also known as "free radical scavengers" as they employ radicals to produce minor reactive species (Csicsor and Tombácz, 2022). Antioxidants are classified into two groups based on their source: exogenous antioxidants and endogenous antioxidants (Zawada et al., 2022), they help to prevent diseases such as aging, cancer, diabetes, inflammation, liver disease, cardiovascular disease, cataracts, nephrotoxicity, and neurological disorders (Neha-et-al.,-2019). Due to their preventive activities in food and pharmaceutical goods against oxidative degradation in the body, as well as against oxidative stress-mediated disease processes, antioxidants have gained popularity. Screening for antioxidant capabilities in plants and plant-derived substances necessitates the use of suitable methodologies that address the mechanism of antioxidant activity and concentrate on the kinetics of the processes involving antioxidants (Gulcin, 2020).

Current approaches for assessing antioxidant activity rely on spectrophotometric methods including hydrogen atom transfer (HAT) and single electron transfer (SET). These assays include the 2,2′-casino-bis (3-ethylbenzothiazoline-6-sulfonic-acid-(ABTS)-assay,-the-2,2-diphenyl-1-(2,4,6-tri-nitrophenyl)-hydrazyl-(DPPH)-radical scavenging activity assay, the oxygen radical absorbance capacity (ORAC) assay, the ferric reducing antioxidant potential (FRAP) assay, and the cupric reducing antioxidant capacity (CUPRAC assay) (Sirivibulkovit et al., 2018). The DPPH test is one of the simplest and most extensively used procedures among them (Celiz et al., 2020). It is based on the antioxidant reducing the violet DPPH radical through a hydrogen atom transfer process, resulting in the color shift to stable pale-yellow DPPH molecules (Majeed et al., 2021).

The antioxidant activity is determined by measuring the residual violet DPPH radical at 515 - 520 nm using a UV-Vis spectrophotometer. This method gives important information on the antioxidant's ability to donate hydrogen atoms, the reaction's reducing capacity, and the mechanism of the free radical-antioxidant interaction. The test is also easy because it simply requires the DPPH radical reagent and the antioxidant (Munteanu and Apetrei, 2021).

In this chapter, the antioxidant activity of plant extracts of *A. versicolor* (bark), *M. flabellifolius* (whole plant), and *E. transvaalense* (bark), will be determined using qualitative and quantitative DPPH free radical-scavenging assay.

7.2 Materials and methods

7.2.1 Plant extraction

The plant materials were extracted as described in chapter 3, section 3.2.3.

7.2.2 Qualitative DPPH radical-scavenging assay

The qualitative screening for antioxidant activity was determined using DPPH (1,1-diphenyl-2-picrylhydrazine) assays with some minor modifications (Braca et al., 2002). TLC plates were loaded with 10 µl of plant extract and dried before being developed in three eluent solvent systems (BEA, CEF, and EMW). The plates were sprayed with the solution of DPPH (0.2%) in methanol. Yellow bands with radical scavenger

capacity on a purple backdrop suggested the presence of antioxidant compounds. The yellow band indicates the radical scavenger present in the plant extract.

7.2.3 Quantitative DPPH free radical-scavenging assay

The DPPH (2,2-diphenyl-2-picrylhydrazine) free radical-scavenging assay was performed with various modifications, as previously described by Ammar et al. (2009). The crude extracts isolated from *A. versicolor*, *M. flabellifolius*, and *E. transvaalense* at various concentrations (15, 30, 60, 120, and 250 µg /ml) were blended with a 0.2 mM of DPPH solution in methanol and the centrifuge tubes were vigorously shaken. The centrifuge tubes were incubated in the dark for 30 minutes at room temperature, and the absorbance at 517 nm was measured spectrophotometrically. Methanol hydroxide and ascorbic acid solution were employed as positive and negative controls, respectively. The test solutions were generated in triplicates. The degree of discoloration (from purple to yellow) showed the compounds' free-radical scavenging effectiveness. The antioxidant activity of the extracts was measured using the formula below, Where Ab= absorbance of the blank and Ac= absorbance of the samples.

$$\% \text{ inhibition} = \frac{Ab-Ac}{Ab} X\ 100$$

7.3. Results and Discussion

7.3.1 Qualitative DPPH free radical scavenging activity assay on TLC plates

The DPPH free radical scavenging technique is frequently used to assess the antioxidant properties of an extract. This technique is a quick, easy, and frequently used approach for testing antioxidant activity (Le et al., 2019). The plant extracts antioxidant activity was measured using a spectrophotometer, adopting the 2, 2, diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. In the presence of antioxidants, DPPH changes color from purple to light yellow, indicating that it has decreased (Zamani et al., 2018).

The results below depict the antioxidant activity of *A. versicolor* bark extracts using the DPPH solution (Figure 7.1). The TLC chromatograms separated with EMW displayed

excellent separation when compared to CEF and BEA. The R_f values are presented in Table 7.1. The antioxidant compounds are represented by the yellow bands against the purple background.

In TLC chromatograms separated with BEA, similar antioxidant compounds were visible in the acetone extract with the same R_f values of 0.36 each. Antioxidant compounds were visible in the acetone, methanol, and chloroform extracts with R_f values ranging from 0.07-0.87 in TLC chromatograms developed in CEF. Noticeably, more compounds were observed in the methanol and chloroform extracts with the same R_f values ranging from 0.40-0.75 in the TLC chromatograms developed in EMW. Antioxidant activity was observed in the chloroform extracts, hence they possessed strong antioxidant activity compared to other solvents. However, no antioxidant compound was present in the hexane extract. Previously, it was reported that the hexane was unable to separate antioxidant compounds from *Coffea arabica* leave extracts Marcheafave et al. (2019), Based on our findings, it may suggest that methanol and chloroform extracts have therapeutic qualities and require further investigation.

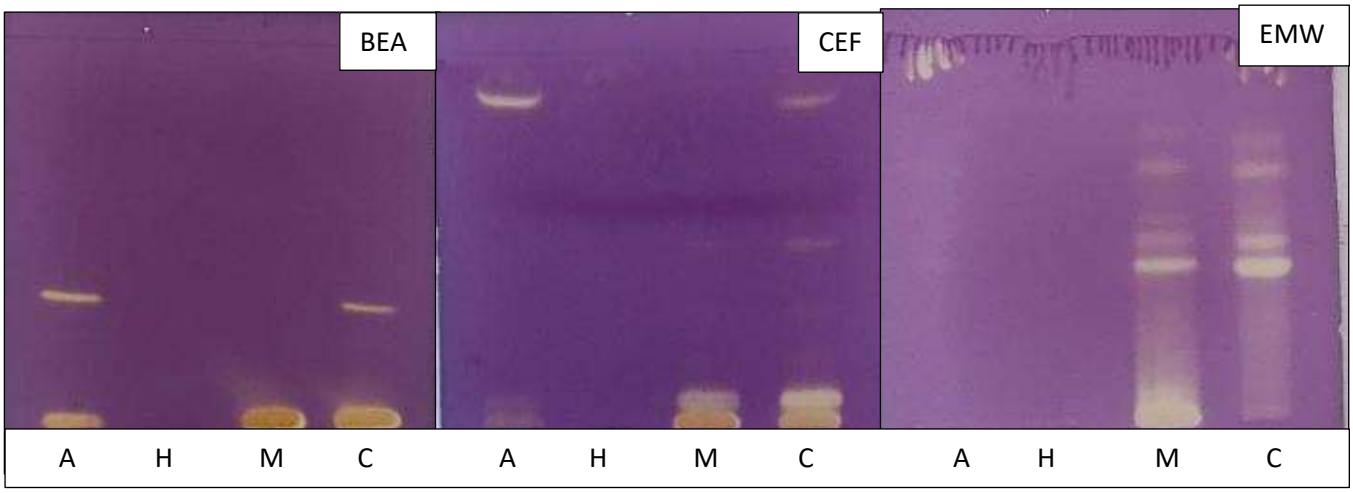


Figure 7.1 TLC chromatograms of *A. versicolor extracted* with acetone, hexane, MeOH and chloroform (left to right), developed in BEA, CEF and EMW, and sprayed with 0.2% DPPH solution.

Table 7.1 R_f values of antioxidant compounds separated with BEA, CEF and EMW bark extracts of *A versicolor* and sprayed with DPPH solution.

Solvent systems	R_f values	Acetone	Hexane	Methanol	Chloroform
BEA	0.36	√			√
CEF	0.07			√	√
	0.18				√
	0.36			√	√
	0.48			√	√
	0.87	√			√
EMW	0.40			√	√
	0.43			√	√
	0.48			√	√
	0.63			√	√
	0.75			√	√

7.3.2 Quantitative antioxidant activity assay

The antioxidant activity of the plant extracts was determined using 2, 2, diphenyl-1-picrylhydrazyl (DPPH) reduction with comparison to the ascorbic acid, as shown in figures 7.2-7.4 The antioxidant activity of the plant extracts was represented as a percentage inhibition and the values used were mean of triplicates ± standard deviation (Figure 7.2, 7.3, and 7.4).

The methanol extracts of *A. versicolor* (Figure 7.2) demonstrated the overall highest antioxidant activity, whereas the hexane extract had the lowest. In a study conducted by Johari and Khong (2019), the methanol extract of *Pereskia bleo* displayed greater antioxidant activity when contrasted to the hexane extracts, hence reporting similar results as the current study. This further emphasizes that the methanol extracts had greater potential to scavenge free radicals than other extracts. The plant extracts showed significant antioxidant activity below 30 µg/ml concentration. The roots and bark of *A. versicolor* are used for several ailments such as anaemia, enlarged glands, and disorders caused by sexual activity and backaches (Fern, 2022).

The methanol extract of *M. flabellifolius* (Figure 7.3) demonstrated the highest activity under the 15, 120, and 250 µg/ml concentrations, whereas the ethyl-acetate extract had the lowest activity under the 30, 120, and 250 µg/ml concentrations. These findings indicate that *M. flabellifolius* is efficiently extracted by polar solvents when compared to solvents that are moderately polar or non-polar. Similar findings were reported by Ezez and Tefera (2021), who discovered that ethyl-acetate was less effective than methanol in separating ginger extracts. *M. flabellifolius* leaf decoction and infusion have been used to treat individuals with immunological deficiencies. Asthma, infectious illnesses, respiratory, inflammation, and epilepsy are among the medical applications (Nantapo and Marume, 2022). The leaves and stem of *M. flabellifolius* curl in the winter seasons, before hydration following rainfall, the leaves curl upwards to reduce exposure to the searing sunlight (Erhabor et al., 2020).

The South African Red Data list classifies *E. transvaalense* as near threatened (Rasethe et al., 2019). The stem bark of *E. transvaalense* is widely used in traditional medicine in Southern Africa, mostly for gastrointestinal tract diseases and skin illnesses (Khumalo et al., 2019). The acetone extract of *E. transvaalense* (Figure 7.4) demonstrated the highest activity under the 120 µg/ml concentration, whereas the chloroform extract had the lowest activity under the 15 and 60 µg/ml concentrations. Based on the literature, there is limited information on the antioxidant activity of *A. versicolor, M. flabellifolius* and *E. transvaalense.*

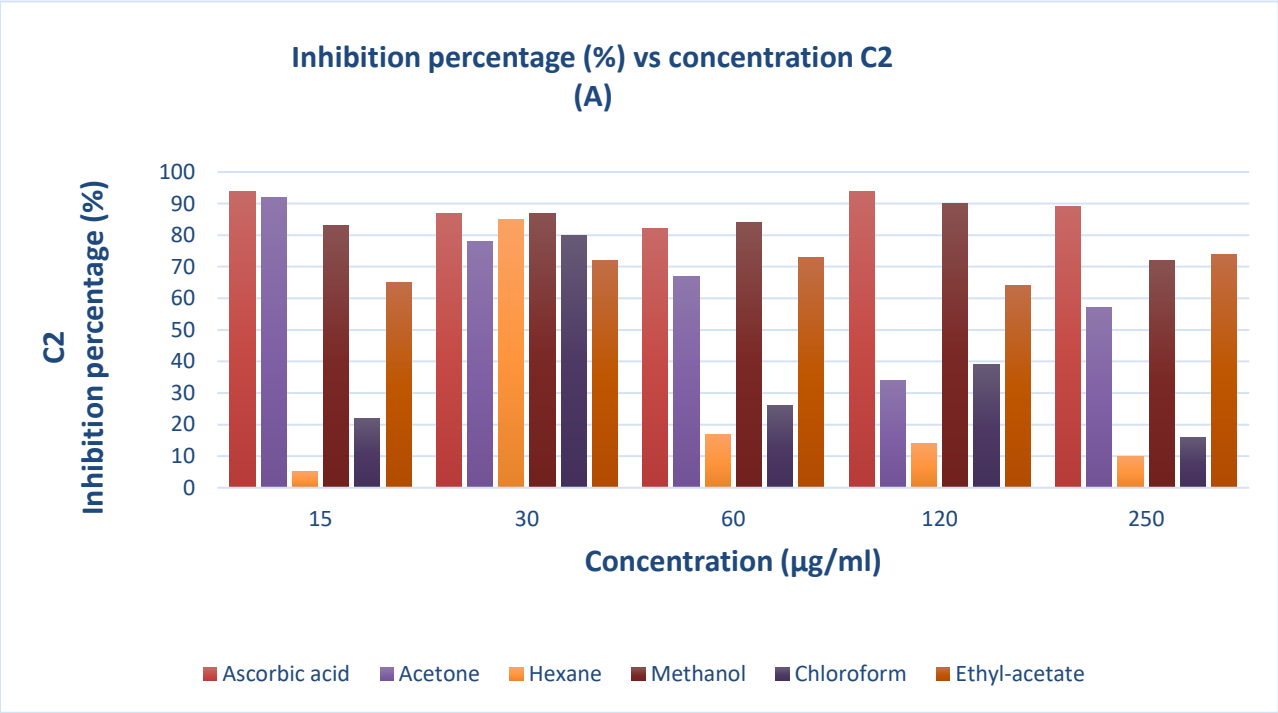


Figure 7.2 The percentage free radical (DPPH) inhibition of *A. versicolor.*

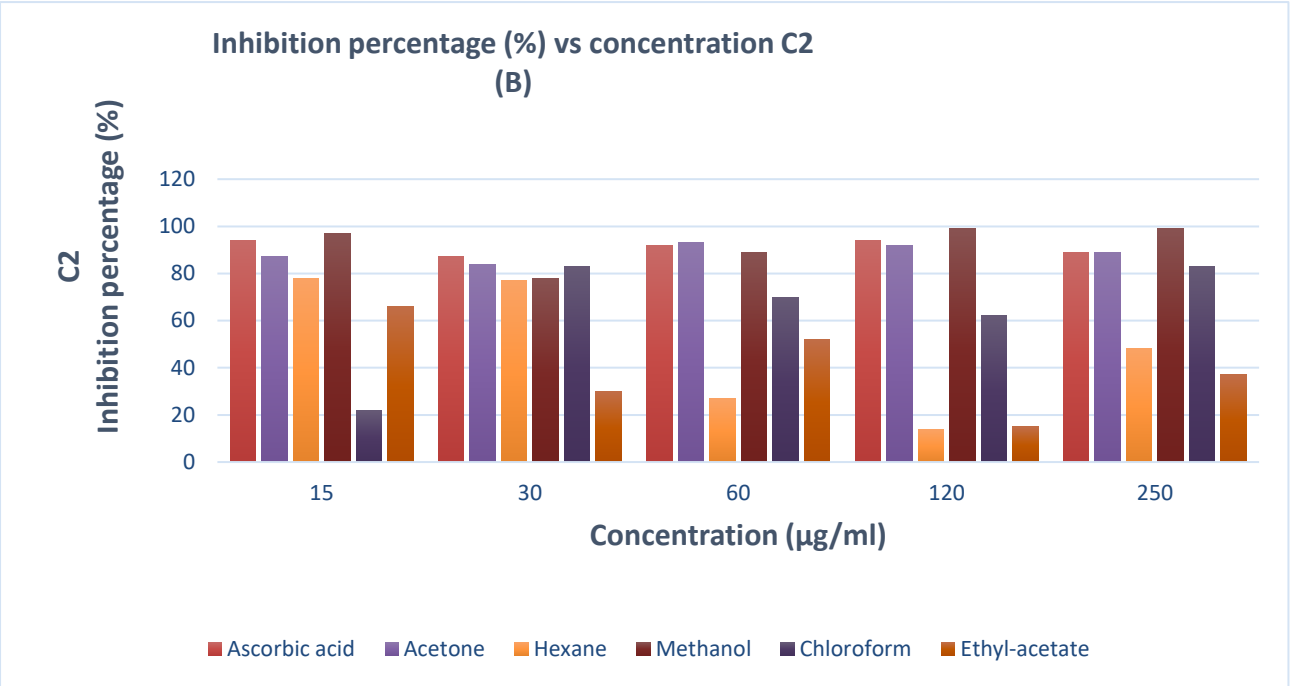


Figure 7.3 The percentage free radical (DPPH) inhibition of *M. flabellifolius.*

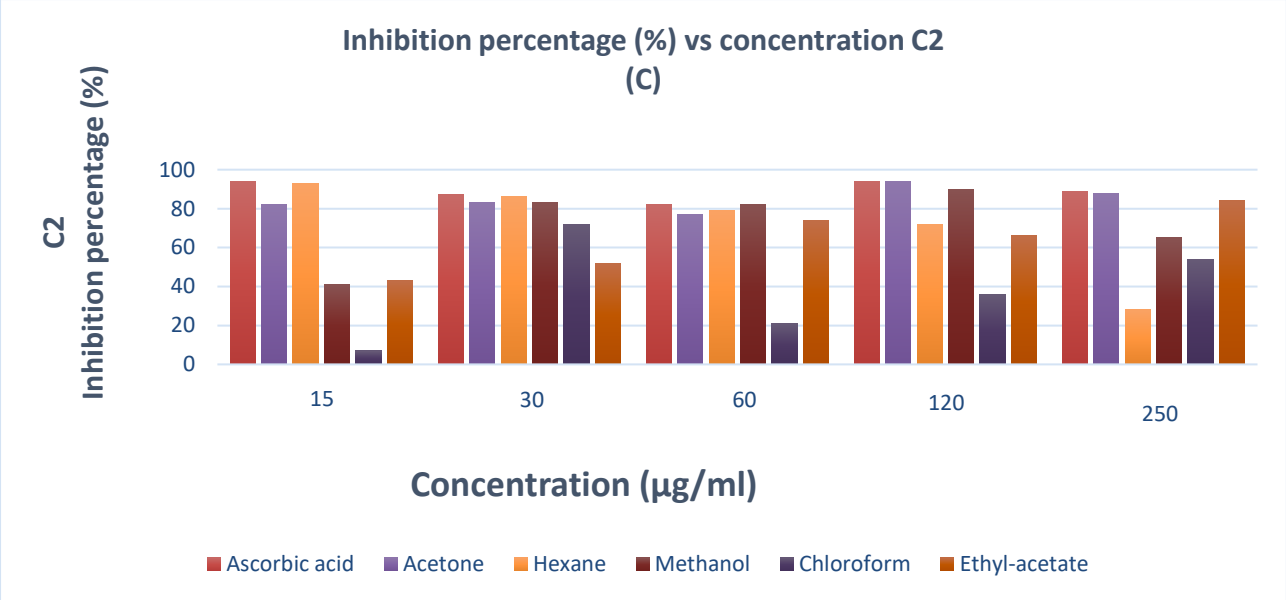


Figure 7.4 The percentage free radical (DPPH) inhibition of *E. transvaalense.*

7.4 Conclusion

The bark extracts of *A. versicolor* showed strong antioxidant activity by inhibiting DPPH. The chloroform extracts possess strong antioxidants in the qualitative assay as compared to other solvents. In the quantitative assay, methanol had the highest antioxidant-activity-followed-by-hexane. As-such,-further-biological-investigations and isolation of these antioxidant molecules are necessary before they may be utilised as natural antioxidant supplements.

The local community should be taught conservation to avoid extinction and over-exploitation of endangered plant species.

In the next chapter, the antifungal and cytotoxicity activities of isolated compounds will be discussed.

CHAPTER 8

ANTIFUNGAL AND CYTOTOXICITY ACTIVITIES OF ISOLATED COMPOUNDS

8.1 Introduction

The toxicity of the bark extracts of *A. versicolor* will be determined against the Vero kidney monkey cells. The cytotoxicity will be determined using the MTT test (3-(4, 5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide).

Medicinal plants contain antiviral, antibacterial, antifungal, and anthelminthic properties (Joshi et al., 2020). Therefore, the development of modern treatment systems relies heavily on drug discovery from natural sources based on ethnopharmacological investigations. The extraction from medicinal plants and characterisation of the active compounds they contain aid in the development of novel medications to treat a variety of ailments (Hassan and Ullah, 2019). Therefore, experiments to evaluate the safety and efficacy of different plant species in treating various pathological disorders are required in the future (Andrade et al., 2018).

Medicinal plants consist of secondary metabolites, these are mostly employed for biologically beneficial purposes, but some are very toxic. Toxicity refers to the ability to create adverse consequences or harm in a single cell, or whole body (Rocha et al., 2022). Crude extracts and pure compounds of medicinal plants play an important role in drug discovery; nevertheless, it is crucial to determine the toxic effects of the plant extracts due to the different side effects they might possess on both humans and animals. The level of toxicity in plants can be determined using cell cultures and isolated organs. The most preferred method for cytotoxicity determination is with cell cultures since it is cheap and effective (Indrayanto et al., 2021). For example, Vero cells are regularly used to evaluate the effects of chemicals, toxins, and other substances on mammalian cells at the molecular level (Ammerman et al., 2008).

Toxic effects caused by heavy consumption of secondary metabolites, for example, excessive intake of glycosides, alkaloids, and terpenoids result in lesions in the nervous system (Sharma et al., 2019). Condensed tannins can result in mucosal

toxicity, hence causing a reduction in the ability to absorb nutrients. Moreover, saponins result in the impairing of growth due to reduced food intake (Kaur et al., 2021; Milgate and Roberts, 1995).

Toxicity in medicinal natural products may arise due to the ingestion of cardiotonic plants and the usage of potentially toxic plants (Thomson et al., 2000; Mbi-Nkwain, 2014). Toxicological studies are significant in identifying hazardous stages of drugs and in evaluating the safety of drugs. Medical practitioners who provide natural products should be trained and informed on customer safety (Curry et al., 2011).

In this chapter, the toxic effects and antimicrobial activity of plant extracts and isolated compounds were investigated.

8.2 Materials and methods

8.2.1 TLC fingerprinting

The chemical components of the isolated compounds were analysed using TLC plates. The method is described in chapter 3, section 3.2.4.

8.2.2 Antifungal activity

8.2.2.1 Micro-dilution assay

The microplate method described in chapter 3 section 3.3.1 was used to determine the minimum inhibitory concentration (MIC) of the isolated compounds.

8.2.2 2 Bioautography assay

The number of active isolated compounds was determined using the bioautography method described in chapter 3 section 3.3.2.

8.2.3 Cytotoxicity activity

The cytotoxicity of plant crude extracts and isolated compounds were determined using-the-(3-(4,5-dimethylthiazol)-2,5-diphenyltetrazoliumbromide)-(MTT)-assay-described by Mosmann (1983) and McGaw et al. (2007) against African green monkey Vero kidney cells. The cells were obtained from the Department of Veterinary Tropical Diseases (University of Pretoria). Minimal Essential Medium (MEM, Whitehead Scientific) was used to maintain the cells, supplemented with 0.1% gentamycin

(Virbac) and 5% fetal calf serum (Highveld Biological). Two hundred µl of the cell suspension was pipetted into each sterile 96-well microtitre plate.

The microtitre plates were incubated at 37 °C in a 5% CO_2 incubator for 48 h with a test compound or extract. After incubation of 4h in 30 µl of 5 mg/ml MTT solution, the MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Biotek Synergy) at a wavelength of 570 nm. Doxorubicin chloride was used as a positive control. The LC_{50} values were calculated as the concentration of the test compound resulting in a 50% reduction of absorbance compared to untreated cells.

8.3 Results and discussion

8.3.1 Phytochemical analysis of isolated compounds

The phytochemical analysis of compounds visualized under UV light and sprayed with vanillin-sulphuric-acid-spray-reagent.-Compound-1-(10-isopropyl-4,4-dimethyl Hexad ecahydro-1H-cyclopenta[b]phenanthren-3-ol) was visible on the TLC chromatograms with an R_f value of 0.58 (Figure 8.1). Compound 2 (1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethanone)-was observed in CEF chromatograms with R_f values of 0.70.

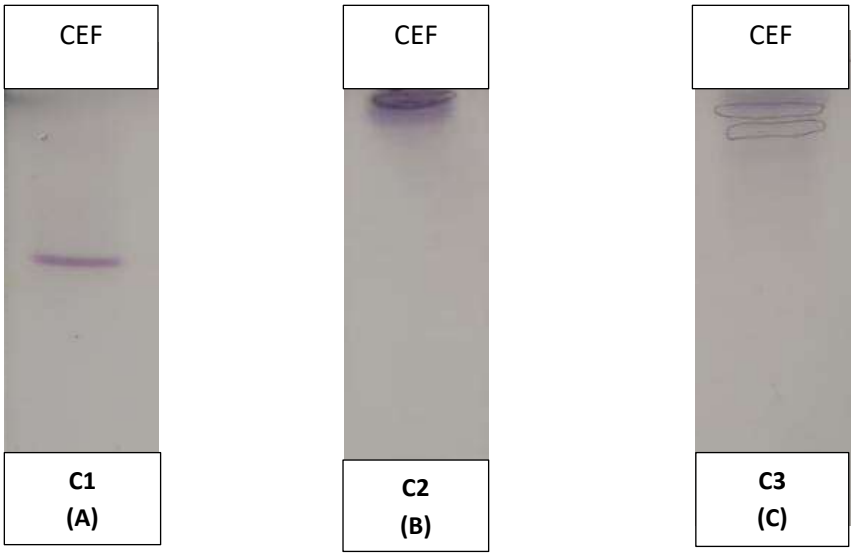


Figure 8.1 TLC fingerprints of compound 1 (a), compound 2 (b) and compound 3 (c), isolated from the hexane and ethyl-acetate fraction of *A. versicolor* developed in CEF and sprayed with vanillin-sulphuric acid reagent spray.

8.3.2 Antifungal activity of isolated compounds

8.3.2.1 Micro-dilution method

Compounds obtained from column i and column ii were tested for antifungal activity against *C. albicans* and *C. neoformans*. Table 8.1 shows the minimum inhibitory concentrations-of-the-compounds.-10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol-and-2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone had excellent antifungal activity against the tested pathogens with the lowest MIC value ranging from 0.02-0.07 mg/ml.

Table 8.1. The minimum inhibitory concentrations of compounds obtained from columns i and ii against *C. albicans* and *C. neoformans.*

C. albicans			
Time	Compound	MIC (mg/ml)	Amp B
24	C1	0.02	<0.02
48		0.02	<0.02
24	C2	0.07	<0.02
48		0.07	<0.02
C. neoformans			
24	C1	0.02	<0.02
48		0.02	<0.02
24	C2	0.02	<0.02
48		0.02	<0.02

C1=10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol.

C2=1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone.

8.3.2.2 Bioautography assay against the fungal pathogen

The bioautography assay was used to determine the activeness of the isolated compounds against *C. albicans* and *C. neoformans* (Figure 8.2-8.4). Antifungal compounds were observed in compound 1 (10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol)-against the fungal pathogen with R_f values of 0.31. In TLC bioautograms separated with CEF, an active compound was observed in compound-2-(1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone)-and-compound-1-(10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol) against *C. albicans* with R_f values ranging from 0.58-0.90. The most separated compounds were non-polar components. The results are comparable with the study conducted by Sakong (2012), which indicated that lupeol extracted from *Calodendrum capense* demonstrated good efficacy against *A. fumigatus, C. albicans, and C. neoformans* with R_f values varying from 0.39 to 0.95.

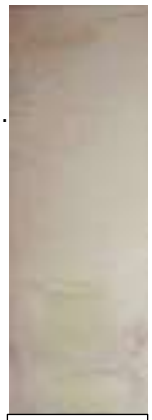


Figure-8.2-Bioautograms-of-10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol developed in BEA, sprayed with *C. albicans.* White areas indicate inhibition of fungal growth.

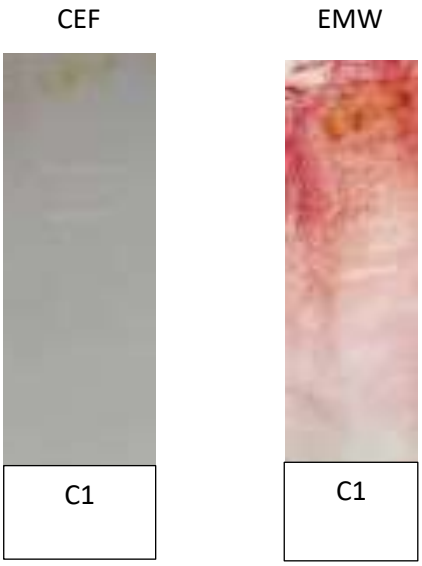


Figure-8.3-Bioautograms-of-10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta [b]phenanthren-3-ol developed in CEF (left) and EMW (right), sprayed with *C. albicans*. White areas indicate inhibition of fungal growth.

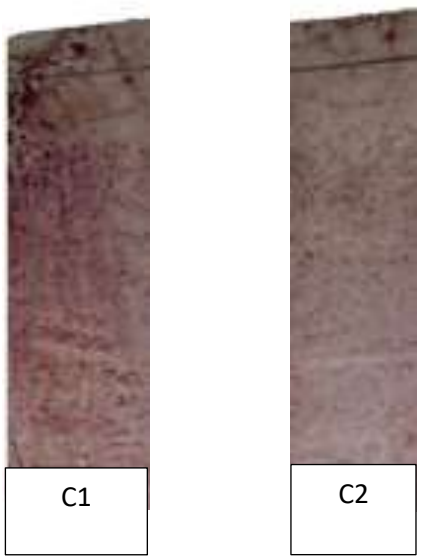


Figure-8.4-Bioautograms-of-10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclpenta [b]phenanthren-3-ol,-and-1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopen-ta[a]naphthalen-7-yl)ethenone were developed in BEA and sprayed with *C. neoformans*. White areas indicate inhibition of fungal growth.

8.3.3 Antibacterial activity

8.3.3.1 Micro-dilution method

Micro-dilution assay was used to determine the biological activity of compounds 10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol,-and-1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)-ethenone against *N. gonorrhoeae.* Table 8.2 shows the minimum inhibitory concentrations-of-the-compounds-10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol-and-1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone.-Both-the-compounds-had-excellent antibacterial activity against the tested pathogen with the lowest MIC value ranging from 0.02-0.07 mg/ml. These compounds are similar to the lupeol but not identical. Based on the literature, there is a lack of information on the biological activity of the two isolated compounds.

Lupeol has an extensive spectrum of medicinal effects against a vast range of disorders, such as cardiovascular issues, HIV, and microbiological infections (Amoussa et al., 2016). A study investigated by Rosandy et al. (2021), revealed that the antibacterial activity of triterpenoid lupeol extracted from the bark of *Dehaasia cuneata* showed poor inhibition activity against *E. coli*, and methicillin-resistant *S. aureus*. However in a study conducted by Harizon et al. (2015), contrasting results were observed, the lupeol extracted from the bark of *Salix alba* had good MIC activity against *S. aureus* and *Streptococcus mutans.* To the best of our knowledge, this compound was reported for the first time tested against *N. gonorrhoeae,* showcasing good activity against the tested pathogen using the micro-dilution assay and the bioautography assay.

Table 8.2. The minimum inhibitory concentrations of isolated compounds against *N. gonorrhoeae*.

N. gonorrhoeae.			
Time	Compound	MIC (mg/ml)	Gentamicin
24	C1	0.02	<0.02
48		0.02	<0.02
24	C2	0.02	<0.02
48		0.02	<0.02

C1=10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol.

C2=1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1cyclopenta[a]naphthalen-7-yl)ethenone.

8.3.3.2 Bioautography assay against the bacterial pathogen

The bioautography assay was used to determine the activity of the isolated compounds against *N. gonorrhoeae* (Figure 8.5). For bioautography assay, 10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol-and-1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta-[a]-naphthalen-7-yl)ethen-one-showed single compounds active against *N. gonorrhoeae* with R_f value of 0.31. Active compounds were observed in TLC bioautograms separated in CEF with an R_f value of 0.70. A study conducted by Malewska (2022) revealed that lupeol extracted from the leaves of *Diospyros lanceifolia* showed good activity against *Pseudomonas aeruginosa* and *E. coli.* Bioautography, according to Mongalo et al. (2022), is an effective method for assessing the antibacterial activity of substances derived from various plant-based sources. However, such substances may be hazardous to both animals and people and determining such cytotoxic effects is difficult.

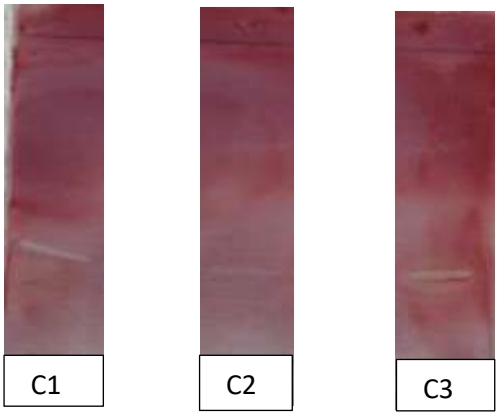


Figure 8.5 Bioautograms of 10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta [b]phenanthren-3-ol (compound 1), 1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone (compound 2) and compound 3 developed in BEA, sprayed with *N. gonorrhoeae.* White areas indicate inhibition of bacterial growth.

Figure-8.6-Bioautogram-of-10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta [b]phenanthren-3-ol (compound 1), developed in CEF and sprayed with *N. gonorrhoeae*. White areas indicate inhibition of bacterial growth.

8.3.4 Cytotoxicity assay

The cytotoxicity of three compounds was determined against African green monkey Vero kidney cells using the MTT assay as described in chapter 8, section 8.2.3. Vero monkey cells are among the most widely used continuous mammalian cell lines for a variety of purposes, including the detection of toxins and the evaluation of the toxicity of drugs or plant extracts (Oon et al., 2021). Doxorubicin was used as a positive control.-Compound-2-(1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a] naphthalen-7-yl)ethenone) was not toxic at the highest concentration tested with LC_{50} > 200 against the Vero monkey cells. The crude extract was less cytotoxic with LC_{50}-=-1.06-mg/ml.-Compound-1-(10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopent a[b] phenanthren-3-ol) was relatively non-toxic with LC_{50} of 0.179 mg/ml (Figure-8.7) Compound 2 was less toxic at a concentration greater than 200 mg/ml against the Vero kidney monkey cells. Previously, it was reported that plant extracts with LC_{50} values greater than 0.1 mg/ml are not considered toxic (Kabongo-Kayoka et al., 2016). The stem bark of *Albizia julibrissin* consists of triterpenoid saponins which have exhibited cytotoxic properties against various cancer cell types (Han et al., 2021).

Triterpenoid lupeols have the ability to prevent liver damage, work as anti-genotoxics, and contain antioxidant characteristics, therefore can be employed as supplements to lessen toxic effects in human cells (Khan et al., 2023). A recent study by Khunoana et al. (2022) investigated the cytotoxicity of the lupeol compound extracted from the *Ptaeroxylon obliquum* leaf extracts, and the results revealed that the chloroform fractions were relatively non-toxic to (African green monkey kidney cells) Vero cells with IC50 values as high as 284 µg/mL. Furthermore, according to Shai et al. (2008), lupeol extracted from the leaves of *Curtisia dentata* was discovered to be non-toxic but rather cytostatic, since it does not kill Vero cells but rather inhibits their development and proliferation. Different human and animal cell lines must be used to confirm and validate toxicity (Mongalo et al., 2022).

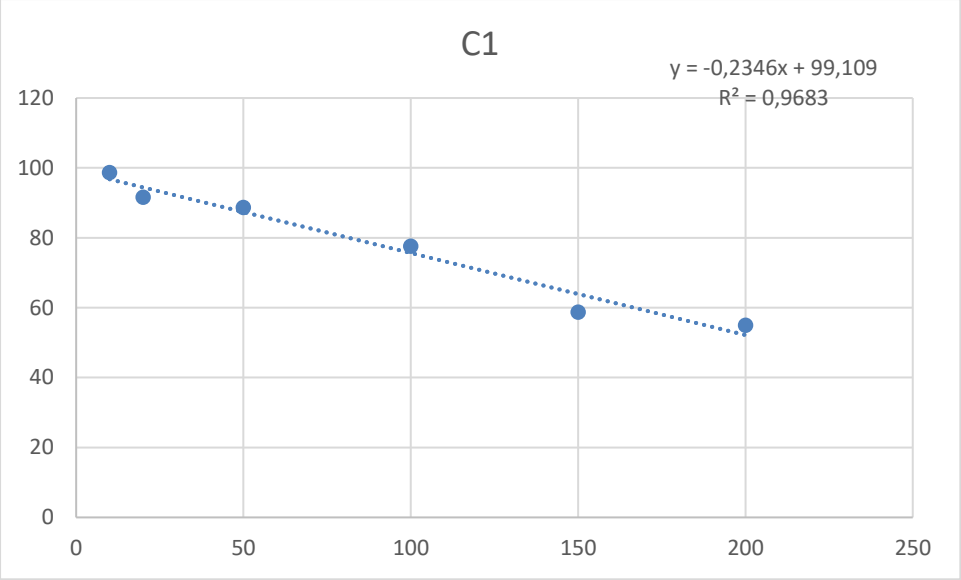


Figure 8.7 Cytotoxicity of 10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol with LC_{50}= 0.179 mg/ml against Vero cells.

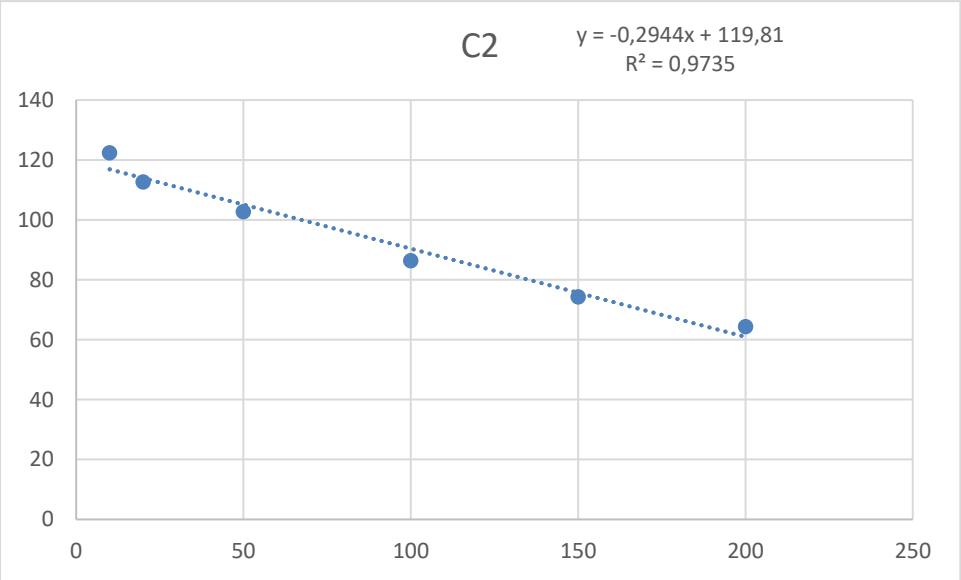


Figure 8.8 Cytotoxicity of 1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone with LC_{50}= 0.237 mg/ml against Vero cells.

8.3.5 Therapeutic index of the crude extract and isolated compounds

The therapeutic index for the two antifungal compounds was calculated using the cytotoxic concentrations of the compounds.

The therapeutic index for each fungus was calculated as follows:
Therapeutic index (TI) = LC_{50} against Vero cells in mg/ml divided by the MIC in mg/ml

Table 8.3 shows the therapeutic index of two isolated compounds against different pathogens. The highest therapeutic index was observed in compound 2 with TI = 11.85 against *C. neoformans* and *N. gonorrhoeae.* The lowest was found in compound 1 with 3.38 against *C. albicans.* More importantly, the therapeutic index of the crude extract was 53 against *C. albicans*. The higher the therapeutic index the better the compounds can be considered for use in drug discovery.

Table 8.3 The Therapeutic Index (TI) of isolated compounds against the pathogenic fungi.

Compound 1			
Fungal pathogen	**LC_{50} (mg/ml)**	**Compound MIC value (mg/ml)**	**Therapeutic Index (TI)**
Candida albicans	0.179	0.02	8.95
Cryptococcus neoformans	0.179	0.02	8.95
Compound 2			
Candida albicans	0.237	0.07	3.38
Cryptococcus neoformans	0.237	0.02	11.85

Compound 1=10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol.

Compound 2=1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone.

Table 8.4 The Therapeutic Index (TI) of isolated compounds against bacterial pathogens.

Compound 1			
Bacterial pathogen	**LC_{50} (mg/ml)**	**Compound MIC value (mg/ml)**	**Therapeutic Index (TI)**
Neisseria gonorrhoeae	0.179	0.02	8.95
Compound 2			
Neisseria gonorrhoeae	0.237	0.02	11.85

Compound 1=10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol.

Compound 2=1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone.

8.4 Conclusion

The isolated compounds were active against the tested microorganisms. In bioautography assay, similar compounds with an R_f value of 0.31 were-observed-in-10-isopropyl-4,4-dimethylhexadecahydro-1H-cyclopenta[b]phenanthren-3-ol-(compound-1)-and-1-(2,2,5a,9b-tetramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethenone-(compound-2).-The plant extract and isolated compounds were not highly toxic against the Vero kidney monkey cells. The highest therapeutic index was observed in compound 2. The findings suggest that antimicrobial drugs that are not toxic could be developed from *A. versicolor*. The study indicated that isolated compounds from *A. versicolor* can be used as a source of therapeutic agents against “u wela”. In the next chapter, the summary and conclusions of the study are given.

CHAPTER 9

SUMMARY, CONCLUSION AND RECOMMENDATION

9.1 Summary and Conclusion

The study aimed to investigate plant species used for the treatment of “u wela”, evaluate the activity of plant extracts, isolate and characterize antimicrobial compounds, which could be used to develop new antifungal agents to combat “u wela”.

The following objectives were achieved in the recent study:

(i) Select plant species used for the treatment of “u wela” from the database of Ethno-medicinal plant species for further phytochemical analysis.

The selected eight plant species were extracted with solvents of various polarities such as acetone, hexane, methanol, DCM, and ethyl-acetate. Methanol extracted the highest amount of plant extract (15.6%), followed by acetone (6.2%) and DCM (0.2%). The chemical components of plant extracts were determined using TLC. All the plant extracts revealed the presence of different chemical constituents. Chemical compounds present in plant extracts were separated more effectively by the BEA (54%), followed by the CEF (33%) and the least compounds were EMW (13%), indicating that a smaller number of compounds were found to be polar.

(ii)--Determine-the-antifungal-activity-of-the-selected-plant-species-against *Candida albicans*.

The antifungal activity of plant extracts was determined against *C. albicans* using serial dilution assay. All plant extracts were active against the tested fungal pathogen with MIC values ranging from 0.02-0.03 mg/ml. The plant extracts of *A. versicolor* and *C. abbreviata* showed significant antifungal activity with a low MIC value of 0.02 mg/ml-0.03 mg/ml against the tested fungal pathogen. *C. tomentosa* exhibited the highest total activity (5688 ml/g) in the ethyl-acetate extract and the lowest total activity was found in the acetone extract (27.11 ml/g). The aqueous and decoction extracts had excellent activity against the tested microorganisms. The findings confirm the effectiveness of the use of water by traditional health practitioners and the local people to prepare their medications.

The bioautography assay was used to determine the number of active compounds in plant extracts, using three eluent solvent systems BEA, CEF, and EMW. More active compounds were visible in the extracts of *M. flabellifolius*. No active compounds were observed in ethyl-acetate and decoction extracts of *E. transvaalense* developed in BEA and CEF. The lack of active compounds, especially in extracts with strong antifungal activity in the micro-dilution assay, suggests the probability of synergistic effects.

(iii)-Determine-the-antibacterial-activity-against-*Escherichia-colii*-and-*Staphylococcus aureus* for comparative purposes.

The plant extracts were screened for antibacterial activity against *E. coli* and *S. aureus* using the micro-dilution assay and bioautography assay. All plant extracts were active against-the-bacterial-pathogens..-The-acetone-and-methanoll-extracts-of-*E.-transvaalense,-X. zambesiaca, C. abbreviata, A. grandiflora, C. tomentosa, M. zeyheri, M. flabellifolius* showed excellent antibacterial activity against *E. coli* and *S. aureus* with low MIC values ranging from 0.02-0.03 mg/ml.

The highest total activity was found in the acetone plant extracts (4599 ml/g) of *M. flabellifolius* and the lowest was observed in the DCM and ethyl-acetate extracts of *C. tomentosa* (27 ml/g). Active compounds were visible in the extracts of *A. versicolor and E. transvaalense* separated in BEA and CEF with R_f values ranging from 0.24-0.27 against *E. coli*. The absence of active compounds in the acetone, hexane, methanol, DCM, and ethyl-acetate extracts of *E. transvaalense* developed in CEF could be attributed to the TLC plates being placed under a stream of cold air for too long, causing intermediate polar active compounds to evaporate.

(iv) Determine the antibacterial activity of the plant extracts against *Neisseria gonorrhoeae.*

The biological activity of the plant extracts was determined against *N. gonorrhoeae* using a micro-dilution assay. This bacterium causes sexually transmitted diseases in humans. Some of the symptoms are associated with “u wela”. The plant extracts had excellent activity against the tested bacteria. In the bioautography assay, CEF revealed a similar compound in the methanol extract of *A. versicolor*, with an R_f value of 0.85. This plant species might be a potential primary source used for the treatment of gonorrhea particularly, in the Vhembe District of Limpopo Province.

(v) Isolate antimicrobial compounds from the selected plant species with good activity and determine their chemical structure.

A. versicolor was selected as the potential plant species from the preliminary screening against the tested bacterial and fungal pathogens. The selected species was serially extracted with hexane, acetone, methanol, and chloroform. The acetone and hexane bark extracts of *A. versicolor* inhibited the growth of *C. albicans* and *A. fumigatus* with a MIC value of 0.02 mg/ml. More compounds were visible in acetone extracts of *A. versicolor*. Therefore, acetone fractions of *A. versicolor* showed promising results in both assays, then it was selected for isolation using column chromatography.

Nuclear Magnetic Resonance and Mass Spectroscopy were used for the identification of the isolated compounds. Amongst the three compounds, only two compounds were successfully-identified.-Compound-1-was-identified-as-10-isopropyl-4,4-dimethylhex adecahydro-1H-cyclopenta[b]phenanthren-3-ol-and-compound-2-as-1-(2,2,5a,9btet ramethyl-5-vinyldodecahydro-1H-cyclopenta[a]naphthalen-7-yl)ethanone. The two isolated compounds are similar to lupeol, however with a few variations. Based on the literature, there is no information on isolated compounds. Compound 3 was not identified due to the presence of a long chain of fatty acids. The findings indicate that *A. versicolor* is a potential plant that can be used for the traditional therapy of gonorrhea.

(vi) To determine the antioxidant activity of selected plant extracts using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay.

The qualitative DPPH free radical scavenging technique was used to assess the antioxidant properties of the *A. versicolor* bark extract. EMW separated the majority of the antioxidant compounds, followed by CEF, and BEA. In TLC separated with CEF, antioxidant compounds were visible with R_f values ranging from 0.07-0.87. Hexane was unable to separate antioxidant compounds, suggesting that the majority of the antioxidant compounds revealed were either polar or intermediately polar. In the quantitative assay, the methanol extracts of *A. versicolor* possessed strong antioxidant activity. The hexane extract had the lowest antioxidant activity compared to other extractants. More medicinal plants need to be investigated to discover prospective antioxidant sources and the adoption of appropriate approaches that address the

mechanism of antioxidant activity is required when screening for antioxidant properties in plants and plant-derived compounds.

(vii) Determine the cytotoxicity of the crude extracts and isolated compounds against Vero monkey kidney cells.

The cytotoxicity of bark extracts of *A. versicolor* was tested against African green monkey Vero kidney cells. The bark extract was relatively not toxic against the cells with LC_{50} ranging between 0.17 mg/ml and 1.06 mg/ml. *A. versicolor* is a potential plant species that could lead to the discovery of novel drugs to combat “u wela”.

The results of the study will be made available to the local community in Vhembe district, particularly on medicinal plants used for the treatment of "u wela”. There is a significant demand for scientific knowledge concerning the safety, efficacy, and toxicity of active components within traditional medicine. The screening of identified medicinal plants could provide lead to the discovery of novel drugs, which could be used to combat fungal and bacterial infections in humans.

9.2 Recommendations

In-vivo studies of the crude extract and isolated compounds

- *In-vivo,* studies using animal studies should be investigated to evaluate the safety of the crude extract and isolated compounds.
- The antibacterial activity of the plant extracts needs to be investigated against the bacteria pathogens that cause sexually transmitted diseases in humans.

REFERENCES

Aberkane, A., Cuenca-Estrella, M., Gomez-Lopez, A., Petrikkou, E., Mellado, E., Monzon, A., Rodriguez-Tudela, J.L., 2002. Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi. Journal of Antimicrobial Chemotherapy 50(5): 719-722.

Adamson, P.C., Loeffelholz, M.J., Klausner, J.D., 2020. Point-of-care testing for Sexually Transmitted Infections: a review of recent developments. Archives of pathology and laboratory medicine 144(11): 1344-1351.

Adikwu, E., Odeghe, B.O., John, F.E., 2020. Assessment of antiplasmodial activity of *Anthocleista-grandiflora*-on-*Plasmodium-berghei*-infected-mice. Discovery-Phytomedicine. Journal of Natural Products Research and Ethnopharmacology 7(3).

Ahamed,-T., Rahman, S.M., Shohael, A.M., 2017. Thin-layer chromatographic profiling and phytochemical screening of six medicinal plants in Bangladesh. International Journal of Biosciences 11(1): 131-140.

Aituarova, A., Zhusupova, G.E., Zhussupova, A., Ross, S.A., 2023. Study of the Chemical Composition of *Rosa beggeriana* Schrenk's Fruits and Leaves. Plants12(18): 3297.

Ajmal, A.A., 2019. Antibacterial activity of the crude extract and fractions of *Spirostachys africana* against multi-drug resistant bacteria. Doctoral dissertation.

Akoto, O., Oppong, I.V., Addae-Mensah, I., Waibel, R., Achenbach, H., 2008. Isolation and characterization of dipeptide derivative and phytosterol from *Capparis tomentosa Lam.* Scientific Research and Essays 3(8): 355-358.

Al-Snafi, A.E., 2015. Cardiovascular effects of *Carthamus tinctorius*: A mini-review. Asian Journal of Pharmaceutical Research 5(3): 199-209.

Al-Tohamy, R., Ali, S.S., Saad-Allah, K., Fareed, M., Ali, A., El-Badry, A., El-Zawawy, N.A., Wu, J., Sun, J., Mao, G.H., Rupani, P.F., 2018. Phytochemical analysis and assessment of antioxidant and antimicrobial activities of some medicinal plant species from Egyptian flora. Journal of Applied Biomedicine 16(4): 289-300.

Ammar, R.B., Bhouri, W., Sghaier, M.B., Boubaker, J., Skandrani, I., Neffati, A., Bouhlel, I., Kilani, S., Mariotte, A.M., Chekir-Ghedira, L., Dijoux-Franca, M.G., 2009. Antioxidant and free radical-scavenging properties of three flavonoids

isolated from the leaves of *Rhamnus alaternus* L.(Rhamnaceae): A structure-activity relationship study. Food Chemistry 116(1): 258-264.

Ammerman, N.C., Beier-Sexton, M., Azad, A.F., 2008. Growth and maintenance of Vero cell lines. Current Protocols in Microbiology 11(1): A-4E.

Amoussa, A.M.O., Lagnika, L., Bourjot, M., Vonthron-Senecheau, C., Sanni, A., 2016. Triterpenoids-from-*Acacia-ataxacantha*-DC:-Antimicrobial-and-antioxidant-activities. BMC Complementary and Alternative Medicine 16(1): 1-8.

Andrade, J.M., Faustino, C., Garcia, C., Ladeiras, D., Reis, C.P., Rijo, P., 2018. *Rosmarinus officinalis* L.: An update review of its phytochemistry and biological activity. Future Science OA 4(4): FSO283.

Anyanwu, GO, Onyeneke, CE, Rauf, K., 2015. Medicinal plants of the genus *Anthocleista*—A-review-of-their-ethnobotany,-phytochemistry-and-pharmaco-logy. Journal of Ethnopharmacology 175: 648-667.

Ashraf, M.A., Iqbal, M., Rasheed, R., Hussain, I., Riaz, M., Arif, M.S., 2018. Environmental stress and secondary metabolites in plants: An overview. Plant metabolites and regulation under environmental stress 153-167.

Astutik, S., Pretzsch, J., Ndzifon Kimengsi, J., 2019. Asian medicinal plants' production and utilization potentials: A review. Sustainability 11(19): 5483.

Bako, B., Ushie, O.A., Malu, S.P., 2023. Lupeol and lauric acid isolated from ethyl-acetate stem extract of *Justicia Secunda* and their antimicrobial activity. Journal of Chemical Society of Nigeria (48): 1.

Balkrishna, A., Chauhan, M., Dabas, A., Arya, V., 2022. A Comprehensive Insight into the phytochemical, pharmacological potential, and traditional medicinal uses of *Albizia lebbeck* (L.) Benth. Evidence-Based Complementary and Alternative Medicine 2022.

Bamigboye, S.O., Tshisikhawe, P.M., Taylor, P.J., 2017. Detecting threat to *Encephalartos transvenosus* (Limpopo cycad) through indigenous knowledge in Limpopo Province, South Africa. Indian Journal of Traditional Knowledge 16(2): 251-255.

Bansal, A., Priyadarsini, C., 2021. Medicinal Properties of Phytochemicals and their Production.

Barberá,-M.J.,-Serra-Pladevall,-J.,-2019.-Gonococcal-infection:-An-unresolved-problem. Enfermedades infecciosasy microbiologia clinica (English ed.) 37(7): 458-466.

Barrientos-Durán, A., de Salazar, A., Alvarez-Estévez, M., Fuentes-López, A., Espadafor, B., Garcia, F., 2020. Detection of Sexually Transmitted Disease–causing pathogens from direct clinical specimens with the multiplex PCR-based STD direct flow chip Kit. European Journal of Clinical Microbiology and Infectious Diseases 39: 235-241.

Bastianello, S.S., Labuschagne, L., Erasmus, G.L., 1992. Experimental *Albizia versicolor* poisoning in sheep and its successful treatment with pyridoxine hydrochloride.

Begue, W.J., Kline, R.M., 1972. The use of tetrazolium salts in bioautographic procedures. Journal of Chromatography A 64(1): 182-184.

Bensandy, O.O., Augustine, U.A., Comfort, M.C., 2012. Phytochemical screening of *Anthocleista grandiflora gilg.* Stem bark. Global Journal of Research on Medicinal Plants and Indigenous Medicine 1(4): 114.

Bhuda, M., Khazamula, E., 2022. Towards finding a cure for COVID-19: the role of African traditional medicine as an indicator of development. African Journal of Development Studies (formerly Afrika Journal of Politics, Economics and Society) 2022(1): 137-153.

Bisso,-B.N.,-Kayoka-Kabongo,-P.N.,-Tchuenguem,-R.T.,-Dzoyem,-J.P.,-2022.-Phyto-chemical analysis and antifungal potentiating activity of extracts from loquat-(*Eriobotrya-japonica*)-against-*Cryptococcus-neoformans*-clinical-isolates. Advances-in-Pharmaco- logical and Pharmaceutical Sciences 2022.

Bjørklund, G., Shanaida, M., Lysiuk, R., Antonyak, H., Klishch, I., Shanaida, V., Peana, M., 2022. Selenium: An Antioxidant with a critical role in anti-aging. Molecules 27(19): 6613.

Botha, C.J., Venter, E., 2002. *Albizia* species.

Braca, A., Sortino, C., Politi, M., Morelli, I., Mendez, J., 2002. Antioxidant activity of flavonoids from *Licania licaniaeflora*. Journal of Ethnopharmacology 79(3): 379-381.

Brummitt, R.K., Harder, D.K., Lewis, G.P., Lock, J.M., Polhill, R.M., Verdcourt, B., 2007. Leguminosae. Flora Zambesica 3, 3. http://epic.kew.org.

Bussmann,-R.W.,-Paniagua-Zambrana,-N.Y.,-Njoroge,-G.N.,-2021.-*Capparis-tomentosa*-Lam.-*C.-apparidaceae-Gynandropsis-gynandra*-(L.)-Briq.-*C-leomaceae.* Ethnobotany of the Mountain Regions of Africa 251-260.

Cameron, C.E., 2018. Syphilis vaccine development: requirements, challenges and opportunities. Sexually Transmitted Diseases 45(9): 17.

Cavanaugh, C.E., Floyd, L.J., Penniman, T.V., Hulbert, A., Gaydos, C., Latimer, W.W., 2011. Examining racial/ethnic disparities in Sexually Transmitted Diseases among recent heroin-using and cocaine-using women. Journal of Women's Health 20(2): 197-205.

Celiz, G., Renfige, M., Finetti, M., 2020. Spectral analysis allows using the DPPH* UV–Vis assay to estimate antioxidant activity of colored compounds. Chemical Papers 74(9): 3101-3109.

Charles, N.N.O., Bonareri, N.L., 2020. Ethnomedicinal survey of plants used by Abagusii traditional healers of Southwest Kenya in the treatment of Sexually Transmitted Diseases. International Journal of Advanced Research 6(9): 260-265.

Chauke, M.A., Shai, L.J., Mogale, M.A., Tshisikhawe, M.P., Mokgotho, M.P., 2015. Medicinal plant use of villagers in the Mopani District, Limpopo Province, South Africa. African Journal of Traditional, Complementary and Alternative Medicines 12(3): 9-26.

Cheikhyoussef, A., Summers, R.W., Kahaka, G.K., 2015. Qualitative and quantitative analysis-of-phytochemical-compounds-in-Namibian-*Myrothamnus-flabellifolius* International Science and Technology Journal of Namibia 071-083.

Chen, S.L., Yu, H., Luo, H.M., Wu, Q., Li, C.F., Steinmetz, A., 2016. Conservation and sustainable-use-of-medicinal-plants:-Problems,-progress,-and-prospects. Chinese Medicine 11(1): 1-10.

Chiarelli, T.J., Grieshaber, N.A., Omsland, A., Remien, C.H., Grieshaber, S.S., 2020. Single-inclusion-kinetics-of-*Chlamydia-trachomatis*-development.MSystems-5(5): 10-1128.

Chisamile, W.A., Sonibare, M.A., Kamanula, J.F., 2023. Ethnobotanical Study of Traditional Medicinal Plants Used for the Treatment of Infectious Diseases by Local Communities in Traditional Authority (T/A) Mbelwa, Mzimba District, Northern Region, Malawi. J 6(1): 115-139.

Chiu, S.F., Huang, P.J., Cheng, W.H., Huang, C.Y., Chu, L.J., Lee, C.C., Lin, H.C., Chen, L.C., Lin, W.N., Tsao, C.H., Tang, P., 2021. Vaginal microbiota of the Sexually Transmitted Infections caused by *Chlamydia trachomatis* and

Trichomonas vaginalis in women with vaginitis in Taiwan. Microorganisms 9(9): 1864.

Cole, M.J., Tan, W., Fifer, H., Brittain, C., Duley, L., Hepburn, T., Lawrence, T., Montgomery, A.A., Sprange, K., Thandi, S., Churchward, C., 2020. Gentamicin, azithromycin and ceftriaxone in the treatment of *gonorrhoea*: The relationship between antibiotic MIC and clinical outcome. Journal of Antimicrobial Chemotherapy 75(2): 449-457.

Crane, M.A., Popovic, A., Stolbach, A.I., Ghanem, K.G., 2021. Reporting of Sexually Transmitted Infections during the COVID-19 pandemic. Sexually Transmitted Infections 97(2): 101-102.

Csicsor, A., Tombácz, E., 2022. Screening of humic substances extracted from leonardite for free radical scavenging activity using DPPH method. Molecules 27(19):6334.

Curry, S.E., Cortland, C.I., Graham, M.J., 2011. Role-modeling in the operating room: medical student observations of exemplary behaviour. Medical Education 45(9): 946-957.

Dangarembizi, R., Chivandi, E., Dawood, S., Erlwanger, K., Mazuru, G., Michael, M. , Samie, A., 2015. The fatty acid composition and physicochemical properties of the underutilised *Cassia abbreviata* seed oil. Pakistan Journal of Pharmaceutical Sciences 28(3): 1006-1007.

Danish, P., Ali, Q., Hafeez, M.M., Malik, A., 2020. Antifungal and antibacterial activity of *Aloe vera* plant extract. Biological and Clinical Sciences Research Journal (1): 2020.

Das, A.K., Hossain, U., Ghosh, S., Biswas, S., Mandal, M., Mandal, B., Brahmachari, G., Bagchi, A., Sil, P.C., 2022. Amelioration of oxidative stress-mediated inflammation and apoptosis in pancreatic islets by Lupeol in STZ-induced hyperglycaemic mice. Life Sciences 305: 120769.

Defossez, E., Pitteloud, C., Descombes, P., Glauser, G., Allard, P.M., Walker, T.W., Fernandez-Conradi, P., Wolfender, J.L., Pellissier, L., Rasmann, S., 2021. Spatial and evolutionary predictability of phytochemical diversity. Proceedings of the National Academy of Sciences 118(3): e2013344118.

Deutschländer, M.S., Lall, N., Van De Venter, M., 2009. Plant species used in the treatment-of-diabetes-by-South-African-traditional-healers:-An-inventory. Pharmaceutical Biology 47(4): 348-365.

Dharsan,-R.,-Vishnpriya,-V.,-Ponnulakshmi,-R.,-Gayathri,-B.,-Madhan,-K.,-Shyama-ladevi, B., Selvaraj, J., 2019. Attenuation of diabetic nephropathy by a plant sterol lupeol: A biochemical analysis. Drug Invention 12: 1402-1405.

Dhawan, D., Gupta, J., 2017. Research article comparison of different solvents for phytochemical extraction potential from *Datura metel* plant leaves International Journal of Biological and Chemical Sciences11(1): 17-22.

Dong, J., Ma, X., Wei, Q., Peng, S., Zhang, S., 2011. Effects of growing location on the contents of secondary metabolites in the leaves of four selected superior clones of *Eucommia ulmoides*. Industrial Crops and Products 34(3): 1607-1614.

Dubale, S., Kebebe, D., Zeynudin, A., Abdissa, N., Suleman, S., 2023. Phytochemical screening and antimicrobial activity evaluation of selected medicinal plants in Ethiopia. Journal of Experimental Pharmacology 51-62.

Dube, Z.P., Mashela, P.W., Mathabatha, R.V., 2016. Mediterranean fruit fly on *Mimusops zeyheri* indigenous to South Africa: A threat to the horticulture industry. Pest Management Science 72(8): 1517-1520.

Dzerefos, C.M., Witkowski, E.T., Kremer-Köhne, S., 2017. Aiming for the biodiversity target with the social welfare arrow: Medicinal and other useful plants from a critically endangered grassland ecosystem in Limpopo Province, South Africa. International Journal of Sustainable Development and World Ecology 24(1): 52-64.

Ebere, E.C., Obinna, I.B., Wirnkor, V.A., 2019. Applications of column, paper, thin-layer and ion exchange chromatography in purifying samples: Mini-review. SF Journal of Pharmaceutical and Analytical Chemistry.

El Khodary, Y.A., Ayoub, I.M., El-Ahmady, S.H., Ibrahim, N., 2021. Molecular and phytochemical variability among genus *Albizia*: A phylogenetic prospect for future breeding. Molecular Biology Reports 48: 2619-2628.

Elisha, I.L., Botha, F.S., McGaw, L.J., Eloff, J.N., 2017. The antibacterial activity of extracts of nine plant species with good activity against *Escherichia coli* against five other bacteria and cytotoxicity of extracts. BMC complementary and Alternative Medicine, 17(1): 1-10.

Eloff, J.N., 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta medica 64(08): 711-713.

Eloff, J.N., Katerere, D.R., McGaw, L.J., 2008. The biological activity and chemistry of the Southern African *Combretaceae*. Journal of Ethnopharmacology 119(3): 686-699.

Eloff, J.N., Angeh, I.E., McGaw, L.J., 2017. Solvent-solvent fractionation can increase the antifungal activity of a *Melianthus comosus* (Melianthaceae) acetone leaf extract to yield a potentially useful commercial antifungal product. Industrial Crops and Products 110: 103-112.

Eloff, J.N., 2019. Avoiding pitfalls in determining antimicrobial activity of plant extracts and publishing the results. BMC Complementary and Alternative Medicine 19(1): 1-8.

Eloff, J.N., 2021. A proposal towards a rational classification of the antimicrobial activity of acetone tree leaf extracts in a search for new antimicrobials. Planta Medica 87(10/11): 836-840.

Elshafie, H.S., Camele, I., Mohamed, A.A., 2023. A Comprehensive review on the biological, agricultural and pharmaceutical properties of secondary metabolites based-plant origin. International Journal of Molecular Sciences 24(4): 3266.

Emwas, A.H., Roy, R., McKay, R.T., Tenori, L., Saccenti, E., Gowda, G.N., Raftery, D., Alahmari, F., Jaremko, L., Jaremko, M., Wishart, D.S., 2019. NMR spectroscopy for metabolomics research. Metabolites 9(7): 123.

Erhabor,-J.O.,-Komakech,-R.,-Kang,-Y.,-Tang,-M.,-Matsabisa,-M.G.,-2020.-Ethnopharmacological importance and medical applications of *Myrothamnus flabellious* Welw. (Myrothamnaceae)-A review: Journal of Ethnopharmacology 252: 112576.

Ezez, D., Tefera, M., 2021. Effects of solvents on total phenolic content and antioxidant activity of ginger extracts. Journal of Chemistry 2021: 1-5.

Famuyide, I.M., Aro, A.O., Fasina, F.O., Eloff, J.N., McGaw, L.J., 2019. Antibacterial and antibiofilm activity of acetone leaf extracts of nine under-investigated South African *Eugenia* and *Syzygium* (*Myrtaceae*) species and their selectivity indices. BMC Complementary and Alternative Medicine 19(1): 1-13.

Fathoni, A., Hudiyono, S., Budianto, E., Cahyana, A.H., Agusta, A., 2021. Metabolite detection and antibacterial activity of fungal endophytic extracts isolated from *Brotowali* (*Tinospora crispa*) plants using TLC-bioautography assay. In IOP Conference Series: Materials Science and Engineering 1011(1): 012041.

Fern, K. 2022. *Albizia versicolor Welw. ex Oliv.* Useful Tropical Plants. Online. http://tropical.theferns.info/viewtropical.php?id=Albizia+versicolor.

Frickmann, H., 2019. Diversification of the prevention of Sexually Transmitted Infections. Future Microbiology 14(17): 1465-1468.

Friedman, D.S., O'Byrne, P., 2020. Extragenital testing increases case detection of *gonorrhea* and *chlamydia*: The impact of implementing nucleic acid amplification testing. Canada Communicable Disease Report= Releve des Maladies transmissibles au Canada 46(9): 285-291.

Gangan, S.S., Nirmale, V.H., Metar, S.Y., Chogale, N.D., Pai, R., Patil, S.D., Patil, K.D., Balange, A.K., 2014. Validation of indigenous knowledge used in the management of Bivalve fishery of South Konkan coast of Maharashtra. Journal of Marine Biology Association 42.

Gebrehiwot,-S.,-Chaithanya,-K.K.,-2020.-Traditional-uses,-phytochemistry,-and-pharma-cological properties of *Capparis tomentosa Lam*.: a review. Drug Invent Today 13(7): 1006-1011.

Gebrehiwot, S., Giday, M., Erko, B., Mekonnen, Y., 2019. *In-vivo* antimalarial activity of *Capparis tomentosa Lam.* in mice infected with *Plasmodium berghei*. Journal of Ayurvedic and Herbal Medicine 5(2): 44-48.

Geremew, R.A., Agizie, B.M., Bashaw, A.A., Seid, M.E., Yeshanew, A.G., 2017. Prevalence of selected Sexually Transmitted Infection (STI) and associated factors among symptomatic patients attending Gondar town hospitals and health centers. Ethiopian Journal of Health Sciences 27(6): 589-600.

Ghasemi, P.A., Momeni, M., Bahmani, M., 2013. Ethnobotanical study of medicinal plants used by Kurd tribe in Dehloran and Abdanan Districts, Ilam Province, Iran. African Journal of Traditional, Complementary and Alternative Medicines 10(2): 368-385.

Ghazizadeh, H., Mirinezhad, M.R., Seyedi, S.M.R., Sadabadi, F., Ahmadnezhad, M., Jaberi, N., Pasdar, A., Ferns, G.A., Esmaily, H., Ghayour-Mobarhan, M., 2020. Prognostic factors associating with pro-oxidant-antioxidant balance; neutrophils to lymphocytes ratio, vitamin D, heat shock protein 27, and red cell distribution width. Archives of Medical Research 51(3): 261-267.

Gorlenko, C.L., Kiselev, H.Y., Budanova, E.V., Zamyatnin Jr, A.A., Ikryannikova, L.N., 2020. Plant secondary metabolites in the battle of drugs and drug-resistant bacteria: New heroes or worse clones of antibiotics. Antibiotics 9(4): 170.

Gowda, B.C., Sundharam, B.S., Mahadesh, J., 2014. Oral-toxicology. Journal of Forensic Dental Sciences 6(1): 3.

Grygiel-Górniak, B., Folga, B.A., 2023. *Chlamydia trachomatis*—An Emerging Old Entity. Microorganisms 11(5): 1283.

Gulcin, İ., 2020. Antioxidants and antioxidant methods: An updated overview. Archives of Toxicology 94(3): 651-715.

Gunatilaka, A.L., 2006. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. Journal of Natural Products 69(3): 509-526.

Gyles, C., 2011. The growing problem of antimicrobial resistance. The Canadian Veterinary Journal 52(8): 817.

Hamidi, M.R., Jovanova, B., Panovska, T.K., 2014. Toxicological evaluation of the plant products using Brine Shrimp (*Artemia salina L*.) model. Macedonian Pharmaceutical Bulletin 60(1): 9-18.

Han, Q., Qian, Y., Wang, X., Zhang, Q., Cui, J., Tu, P., Liang, H., 2021. Oleanane-type saponins and prosapogenins from *Albizia julibrissin* and their cytotoxic activities. Phytochemistry 185: 112674.

Harizon, Pujiastuti, B., Kurnia, D., Sumiarsa, D., Shiono, Y., Supratman, U., 2015. Antibacterial triterpenoids from the bark of *Sonneratia alba* (Lythraceae). Natural Product Communications 10(2): 1934578X1501000215.

Hassan, A., Ullah, H., 2019. Antibacterial and antifungal activities of the medicinal plant *Veronica biloba*. Journal of Chemistry 2019: 1-7.

He,-Y.,-Wang,-Q.,-Ye,-Y.,-Liu,-Z.,-Sun,-H.,-2020.-The-ethnopharmacology,-phytochemistry,-pharmacology-and-toxicology-of-genus-*Albizia*:-A-review. Journal-of-Ethnopharmacology 257: 112677.

Heftmann,-E.,-2011.-Chromatography:-Fundamentals-and-applications-of-chromatographic and electrophoretic methods. Part A: Fundamentals and Techniques. Elsevier

Homa, M., Manikandan, P., Saravanan, V., Revathi, R., Anita, R., Narendran, V., Panneerselvam, K., Shobana, C.S., Aidarous, M.A., Galgóczy, L., Vágvölgyi, C.,-2018.-Exophiala-dermatitidis-endophthalmitis:-case-report-and-literature-review.-Mycopathologia 183(3): 603-609.

Hyde, M.A., Wursten, B.T., Ballings, P., Coates Palgrave, M. 2022. *Albizia versicolor*. FlorauofuZimbabwe.uOnline.https://www.zimbabweflora.co.zw/speciesdata/species.php?species_id=125730.

Ibrahim, N.B.B.R., Puchooa, D., Govinden-Soulange, J., Facknath, S., 2022. Chemical profiling and biological activity of *Cassia abbreviata Oliv*. South African Journal of Botany 146: 325-339.

Indrayanto, G., Putra, G.S., Suhud, F., 2021. Validation of *in-vitro* bioassay methods: Application in herbal drug research. Profiles of Drug Substances, Excipients and Related Methodology 46: 273-307.

Jambwa, P., Nkadimeng, S.M., Mudimba, T.N., Matope, G., McGaw, L.J., 2023. Antibacterial and anti-inflammatory activity of plant species used in traditional poultry ethnomedicine in Zimbabwe: A first step to developing alternatives to antibiotic poultry feed additives. Journal of Ethnopharmacology 300: 115687.

Jamshidi-Kia, F., Lorigooini, Z., Amini-Khoei, H., 2018. Medicinal plants: History and future perspective. Journal of Herb Medicine Pharmacology (7): 1.

Jennings, L.K. and Krywko, D.M., 2018. Pelvic inflammatory disease.

Jobe, M.C., Ncobela, C.N., Kunene, N.W., Opoku, A.R., 2019. Effects of *Cassia abbreviata* extract and stocking density on growth performance, oxidative stress and liver function of indigenous chickens. Tropical Animal Health and Production 51(8): 2567-2574.

Johari, M.A., Khong, H.Y., 2019. Total phenolic content and antioxidant and antibacterial activities of *Pereskia bleo*. Advances in Pharmacological and Pharmaceutical Sciences, 2019.

Johnson, A., Jackson, J.B., 2021. Sexually Transmitted Infections among college students. Microbiological Infectious Diseases 5(1): 1-4.

Jose, S., 2012. Agroforestry for conserving and enhancing biodiversity. Agroforestry Systems 85(1): 1-8.

Joshi, B., Panda, S.K., Jouneghani, R.S., Liu, M., Parajuli, N., Leyssen, P., Neyts, J. , Luyten, W., 2020. Antibacterial, antifungal, antiviral, and anthelmintic activities of medicinal plants of Nepal selected based on ethnobotanical evidence. Evidence-Based Complementary and Alternative Medicine.

Kabongo-Kayoka, P.N., Eloff, J.N., Obi, C.L., McGaw, L.J., 2016. Antimycobacterial activity and low cytotoxicity of leaf extracts of some African *Anacardiaceae* tree species. Phytotherapy Research 30(12): 2001-2011.

Kagan, I.A., Flythe, M.D., 2014. Thin-layer chromatographic (TLC) separations and bioassays of plant extracts to identify antimicrobial compounds. Journal of Visualized Experiments (85): 51411.

Kartika, L., Wanda, D., Susanti, H., 2020. A phenomenological study of Indonesian mothers' in caring for children in the acute phase of kawasaki disease.

Kaur, N., Agarwal, A., Sabharwal, M., Jaiswal, N., 2021. Natural Food Toxins as Anti-nutritional factors in plants and their reduction strategies. Food Chemistry: The Role of Additives, Preservatives and Adulteration 217-248.

Khalizov, A.F., Guzman, F.J., Cooper, M., Mao, N., Antley, J., Bozzelli, J., 2020. Direct detection of gas-phase mercuric chloride by ion drift-chemical ionization mass spectrometry. Atmospheric Environment 238: 117687.

Khan, I.H., Javaid, A., 2019. Antifungal, antibacterial and antioxidant components of ethyl-acetate extract of *quinoa stem.* Plant Protection 3(03): 130.

Khan, M.A., Singh, D., Fatma, H., Akhtar, K., Arjmand, F., Maurya, S., Siddique, H.R., 2023.-Antiandrogen-enzalutamide-induced-genetic,-cellular,-and-hepatic-damages:-Amelioration-by-triterpene-lupeol.-Drug-and-Chemical-Toxicology 46(2): 380-391.

Khumalo, G.P., Sadgrove, N.J., Van Vuuren, S.F., Van Wyk, B.E., 2021. South Africa's best bark medicines prescribed at the Johannesburg Muthi markets for skin, gut, and lung infections: MIC's and Brine Shrimp Lethality. Antibiotics 10(6): 681.

Khumalo, G.P., Sadgrove, N.J., Van Vuuren, S.F., Van Wyk, B.E., 2019. Antimicrobial lupenol triterpenes and a polyphenol from *Elaeodendron transvaalense,* a popular Southern African medicinal bark. South African Journal of Botany122: 518-521.

Khunoana, E. T., Eloff, J. N., Ramadwa, T. E., Nkadimeng, S. M., Selepe, M. A., McGaw, L. J., 2022. *In-vitro* antiproliferative activity of *Ptaeroxylon obliquum* leaf extracts, fractions and isolated compounds on several cancer cell lines. Applied Sciences 12(21): 11004.

Kojima, N., Klausner, J.D., 2019. Disparities in the testing, treating, suppressing, and preventing HIV infection among underserved populations. AIDS Research and Human Retroviruses 35(9): 786.

Kotze, M., Eloff, J.N., Houghton, P.J., 2002. Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). South African Journal of Botany 68(1) :62-67.

Lazzarin, S., Giacomelli, A., Moschese, D., Antinori, S., Gervasoni, C., 2023. Drug shortages highlight the need for expanded treatment options for *Syphilis*. Clinical Infectious Diseases 396.

Le, D.D., Nguyen, D.H., Zhao, B.T., Min, B.S., Song, S.W., Woo, M.H., 2019. Quantitation and radical scavenging activity evaluation of iridoids and phenylethanoids from the roots of *Phlomis umbrosa* (*Turcz.*) using DPPH free radical and DPPH-HPLC methods, and their cytotoxicity. Natural Product Sciences 25(2): 122-129.

Leitgeb, N., 2010. Safety of electromedical devices: Law-risks-opportunities. Springer Science and Business Media.

Leteane, M.M., Ngwenya, B.N., Muzila, M., Namushe, A., Mwinga, J., Musonda, R., Moyo, S., Mengestu, Y.B., Abegaz, B.M., Andrae-Marobela, K., 2012. Old plants newly discovered: *Cassia sieberiana* DC and *Cassia abbreviata* Oliv. root extracts inhibit *in-vitro* HIV-1c replication in peripheral blood mononuclear cells (PBMCs) by different modes of action. Journal of Ethnopharmacology 141(1): 48-56.

Li, B., Webster, T.J., 2018. Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections. Journal of Orthopaedic Research 36(1): 22-32.

Lin, S.R., Chang, C.H., Hsu, C.F., Tsai, M.J., Cheng, H., Leong, M.K., Sung, P.J., Chen, J.C., Weng, C.F., 2020. Natural compounds as potential adjuvants to cancer therapy: Preclinical evidence. British Journal of Pharmacology 177(6): 1409-1423.

Linh, N.T.T., Cham, B.T., Hung, D.V., Lien, G.T.K., Quan, T.D., Thuy, T.T., Anh, N.T.H., 2023. Triterpenoids from *Dischidia alboflava* whole plant. Tạp chí Khoa học và Công nghệ-Đại học Đà Nẵng 67-70.

Lu, H., Zhang, X., Khan, S.A., Li, W., Wan, L., 2021. Biogenic synthesis of MnO_2 Nanoparticles with leaf extract of *Viola betonicifolia* for enhanced antioxidant, antimicrobial, cytotoxic, and biocompatible applications. Frontiers in Microbiology 12: 761084.

Maema, L.P., Potgieter, M.J., Samie, A., 2019. Ethnobotanical survey of invasive alien plant species used in the treatment of Sexually Transmitted Infections in Waterberg District, South Africa. South African Journal of Botany 122: 391-400.

Mahomoodally, M.F., 2013. Traditional medicines in Africa: An appraisal of ten potent African medicinal plants. Evidence-Based Complementary and Alternative Medicine 2013.

Majeed, S., Danish, M., Mohamad Ibrahim, M.N., Sekeri, S.H., Ansari, M.T., Nanda, A., Ahmad, G., 2021. Bacteria-mediated synthesis of iron oxide nanoparticles and their antibacterial, antioxidant, cytocompatibility properties. Journal of Cluster Science 32(4): 1083-1094.

Makgahlela, M.W., Sodi, T., 2016. Cultural conceptions of a bereavement-related illness in a South African indigenous community. Journal of Psychology in Africa 26(6): 541-545.

Malada, P.M., Mogashoa, M.M., Masoko, P., 2022. The evaluation of cytotoxic effects, antimicrobial activity, antioxidant activity and combination effect of *Viscum rotundifolium* and *Mystroxylon aethiopicum*. South African Journal of Botany 147: 790-798.

Malewska,-T.,-2022. Biological and phytochemical analysis of three medicinally important-plants:-*Prunus-persica,-Diospyros-lanceifolia*-and-*Holboellia-latifolia* (Doctoral-dissertation, Macquarie University).

Malik,-M.,-Hussain,-S.,-Sajjad,-N.,-Nazir,-S.,-Gondal,-M.U.,-Arshad,-J.,-2021.-Physicoche-medical analysis, qualitative and quantitative investigation of atropine in medicinal plant *Atropa belladonna*. European Journal of Medical Research 8(5): 149-157.

Manandhar, S., Luitel, S., Dahal, R.K., 2019. *In-vitro* antimicrobial activity of some medicinal plants against human pathogenic bacteria. Journal of Tropical Medicine 2019.

Marcheafave, G.G., Tormena, C.D., Pauli, E.D., Rakocevic, M., Bruns, R.E., Scarminio, I.S., 2019. Experimental mixture design solvent effects on pigment extraction and antioxidant activity from *Coffea arabica L*. leaves. Microchemical Journal 146: 713-721.

Marcone, M.F., Wang, S., Albabish, W., Nie, S., Somnarain, D., Hill, A., 2013. Diverse food-based-applications-of-nuclear-magnetic-resonance-(NMR)-technology. Food Research International 51(2): 729-747.

Marks, R.A., Mbobe, M., Greyling, M., Pretorius, J., McLetchie, D.N., VanBuren, R., Farrant, J.M., 2022. Variability in functional traits along an environmental gradient-in-the-South-African-resurrection-plant-*Myrothamnus-flabellafolia.* Plants 11(10): 1332.

Maroyi,-A.,-Semenya,-S.S.,-2019.-Medicinal-uses,-phytochemistry-and-pharma-cological properties of *Elaeodendron transvaalense*. Nutrients 11(3): 545.

Masevhe, N.A., McGaw, L.J., Eloff, J.N., 2015. The traditional use of plants to manage *candidiasis*-and-related-infections-in-Venda,-South-Africa.-Journal-of-Ethno-pharmacology 168: 364-372.

Masoko, P., Eloff, J.N., 2005. The diversity of antifungal compounds of six South African-*Terminalia*-species-(Combretaceae)-determined-by-bioautography. African Journal of Biotechnology 4(12).

Maulidiyah, M., Susilowati, P.E., Saprin, S., Diraa, L., Natsir, M., Usman, U., Nurlansi, N., Nurdin, M., 2023. Inhibitory activity of *Candida albicans* fungi by acetone extract of the *Lichen Usnea* sp. In AIP Conference Proceedings 27191.

Mbi-Nkwain, A.C., 2014. Catholic University of Cameroon, (catuc) bamenda.

McGaw, L.J., Steenkamp, V., Eloff, J.N., 2007. Evaluation of *Athrixia* bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. Journal of Ethnopharmacology 110(1): 16-22.

McLaughlin, S.E., Golden, M.R., Soge, O.O., Berzkalns, A., Thibault, C.S., Barbee, L.A., 2023. *Pharyngeal gonorrhea* in heterosexual male and female sex partners of persons with *gonorrhea*. Sexually Transmitted Diseases 50(4): 203-208.

Meela, M.M., Mdee, L.K., Masoko, P., Eloff, J.N., 2019. Acetone leaf extracts of seven invasive weeds have promising activity against eight important plant fungal pathogens. South African Journal of Botany 121: 442-446.

Milgate,-J.,-Roberts,-D.C.K.,-1995.-The-nutritional-and-biological-significance-of saponins. Nutrition Research 15(8): 1223-1249.

Mngadi, S., Moodley, R., Jonnalagadda, S.B., 2019. Elemental composition and nutritional value of the edible fruits of Transvaal red milkwood (*Mimusops zeyheri*)-and-impact-of-soil-quality.-Environmental-Monitoring-and-Assessment 191(3): 1-12.

Mohamed, T.K., Nassar, M.I., Gaara, A.H., El-Kashak, W.A., Brouard, I., El-Toumy, S.A.,-2013.-Secondary-metabolites-and-bioactivities-of-*Albizia-anthelmintica.* Pharmacognosy Research 5(2): 80.

Mokoka, T.A., McGaw, L.J., Eloff, J.N., 2010. Antifungal efficacy of ten selected South African plant species against *Cryptococcus neoformans*. Pharmaceutical Biology 48(4): 397-404.

Mongalo,-N.I.,-Mafoko,-B.J.,-2013.-*Cassia-abbreviata-Oliv*.-A-review-of-its-ethno-medicinal-uses,-toxicology,-phytochemistry,-possible-propagation-techniques-and-Pharmcology.-African-Journal-of-Pharmacy-and-Pharmacology-7(45): 2901-2906.

Mongalo, N.I., Raletsena, M.V., 2022. An Inventory of South African medicinal plants used in the management of sexually transmitted and related opportunistic infections: An appraisal and some scientific evidence (1990–2020). Plants 11(23): 3241.

Monyela, S., 2021. Characterisation of mmupudu (*Mimusops zeyheri)* leaf rust in Limpopo Province (Doctoral dissertation).

Monzón, J., Moyer-Horner, L., Palamar, M.B., 2011. Climate change and species range dynamics in protected areas. Bioscience 61(10): 752-761.

Moore, S., Rosenthal, D., Mitchell, A., 2020. Youth, AIDS and Sexually Transmitted Diseases. Psychology Press.

More, G.K., 2021. Metabolomics and cell-based assay analysis of selected South African plants to determine anti-rift valley fever virus activity (Doctoral dissertation).

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65(1-2): 55-63.

Mroczek, T., Dymek, A., Widelski, J., Wojtanowski, K.K., 2020. The bioassay-guided fractionation and identification of potent acetylcholinesterase inhibitors from *Narcissus* CV ‘hawera’ using optimized vacuum liquid chromatography, high-resolution mass spectrometry and bioautography. Metabolites 10(10): 395.

Mudau, G., Odeyemi, S., Dewar, J., 2020. Vhavenda herbal remedies as sources of antihypertensive-drugs:-ethnobotanical-and-ethnopharmacological-studies. Oxidative Medicine and Cellular Longevity 2020: 1-18.

Mudau, T.E., Olowoyo, J.O., Amoo, S.O., 2022. Ethnobotanical assessment of medicinal plants used traditionally for treating diabetes in Vhembe District, Limpopo Province, South Africa. South African Journal of Botany 146: 304-324.

Mulaudzi, F.M., 2001. Synergy between indigenous knowledge systems, modern health care system and scientific research-A vision for the 21st century. Health SA Gesondheid 6(4): 14-20.

Mulaudzi, F.M., 2007. The cultural beliefs of the Vhavenda on the causes and transmission of Sexually Transmitted Infections. Health SA Gesondheid 12(3):46-54.

Mulaudzi, F.M., Makhubela-Nkondo, O.N., 2006. Indigenous healers' beliefs and practices concerning Sexually Transmitted Diseases. Curationis 29(1): 46-53.

Mulaudzi, R.B., Ndhlala, A.R., Kulkarni, M.G., Finnie, J.F., Van Staden, J., 2011. Antimicrobial properties and phenolic contents of medicinal plants used by the Venda-people-for-conditions-related-to-venereal-diseases. Journal-of-Ethno-pharmacology (2): 330-337.

Mulaudzi, R.B., Ndhlala, A.R., Van Staden, J., 2015. Ethnopharmacological evaluation of a traditional herbal remedy used to treat gonorrhoea in Limpopo Province, South Africa. South African Journal of Botany 97: 117-122.

Mulu, A., Tessema, B., Derbie, F., 2004. *In-vitro* assessment of the antimicrobial potential of honey on common human pathogens. Ethiopian Journal Health Development 18: 107-112.

Mulubwa, M.W.I.L.A., Prakash, S.H.I.V., 2015. Antimicrobial activity and potency of *Cassia-abbreviata-Oliv*-stem-bark-extracts. International-Journal-of-Pharma-cology.-Pharmacology 7: 426-428.

Munteanu, I.G., Apetrei, C., 2021. Analytical methods used in determining antioxidant activity: A review. International Journal of Molecular Sciences 22(7): 3380.

Mustafa, A., Hanif, U., Sardar, A.A., Jan, H.A., 2023. Ethnomedicinal study of medicinal plants used by the population of Taunsa Sharif, Dera Ghazi Khan, Punjab, Pakistan. Ethnobotany Research and Applications 26: 1-27.

Muswede,-N.J.,-Tshivhase,-L.,-Mavhandu-Mudzusi,-A.H.,-2021.-Condom-use-edu-cation, promotion and reasons for condom use: Perspectives of healthcare providers and young adults in Vhembe District, Limpopo Province. South African Family Practice (63): 4.

Nako, N., 2014. Chemical studies on some natural products from *Myrothamnus flabellifolius.*

Nandagoapalan, V., Doss, A., Marimuthu, C., 2016. Phytochemical analysis of some traditional medicinal plants. Bioscience Discovery 7(1): 17-20.

Nantapo, C.W.T., Marume, U., 2022. Exploring the potential of *Myrothamnus flabellifolius* Welw. (Resurrection tree) as a phytogenic feed additive in animal nutrition. Animals 12(15): 1973.

Nazer, M., Abbaszadeh, S., Darvishi, M., Kheirollahi, A., Shahsavari, S., Moghadasi, M., 2019. The most important herbs used in the treatment of Sexually Transmitted Infections in traditional medicine. Sudan Journal of Medical Sciences 14(2): 41-64.

Ndhlala, A.R., Stafford, G.I., Finnie, J.F., Van Staden, J., 2011. Commercial herbal preparations in KwaZulu-Natal, South Africa: The urban face of traditional medicine. South African Journal of Botany 77(4): 830-843.

Neha, K., Haider, M.R., Pathak, A., Yar, M.S., 2019. Medicinal prospects of antioxidants: A review. European Journal of Medicinal Chemistry 178: 687-704.

Ngobeni, B., 2016. Pharmacological evaluation of extracts from *Buxus macowanii*, *Polygala-myrtifolia,-Scilla-sp.*-and-*Xanthocercis-zambesiaca* (Doctoral-dissertation, Bloemfontein: Central University of Technology, Free State).

Ngobeni, B., Manduna, I.T., Malebo, N.J., Mashele, S.S., 2023. Phytotherapy for Sexually Transmitted-Infections-in-Thaba-Nchu,-Free-State-Province,-South-Africa. Pharmacognosy Journal 15: 1.

Niazian, M., 2019. Application of genetics and biotechnology for improving medicinal plants. Planta 249: 953-973.

Niehaus, I., 2013. Averting danger: Taboos and bodily substances in the South African lowveld. Critical African Studies 5(3): 127-139.

Nizami, G., Sayyed, R.Z., 2018. Phytochemicals with anticancer potential: Methods of extraction, basic structure, and chemotherapeutic action in anticancer plants: Properties and Application. Springer 431-453.

Nkuna, T.S., 2018. Effects of soil type, salinity and *Vesicula arbuscular mycorrhiza* on growth and foliar elements in *Mimusops zeyheri* (sond.) indigenous fruit trees (Doctoral dissertation).

Nothias, L.F., Nothias-Esposito, M., Da Silva, R., Wang, M., Protsyuk, I., Zhang, Z., Sarvepalli, A., Leyssen, P., Touboul, D., Costa, J., Paolini, J., 2018. Bioactivity-

based molecular networking for the discovery of drug leads in natural product bioassay-guided fractionation. Journal of Natural Products 81(4): 758-767.

Nthulane, N.P., Mosebi, S., Tshikalange, T.E., Nyila, M.A., Mankga, L.T., 2020. Antimicrobial and anti-inflammatory activities of selected medicinal plants against pathogens causing Sexually Transmitted Infections. Journal of Herbal medicine Pharmacology 9(2): 130-137.

Nuthan, B.R., Rakshith, D., Marulasiddaswamy, K.M., Rao, H.Y., Ramesha, K.P., Mohana, N.C., Siddappa, S., Darshan, D., Kumara, K.K.S., Satish, S., 2020. Application of optimized and validated agar overlay TLC–bioautography assay for detecting the antimicrobial metabolites of pharmaceutical interest. Journal of Chromatographic Science 58(8): 737-746.

Nyatsanza, F., Tipple, C., 2016. *Syphilis*: presentations in general medicine. Clinical Medicine 16(2): 184.

Nycz, B.T., Pretty, K., Gomez-Trujillo, A., Sanchez, B., Dominguez, S.R., 2020. Description of enteropathic *Escherichia coli* species in pediatric patients at a quaternary children's hospital. Journal of the Pediatric Infectious Diseases Society 9(5): 573-579.

Oladeji, O.M., Kopaopa, B.G., Mugivhisa, L.L., Olowoyo, J.O., 2023. Investigation of heavy metal analysis on medicinal plants used for the treatment of skin cancer by traditional practitioners in Pretoria. Biological Trace Element Research 1-9.

Omotayo, A.O., Ijatuyi, E.J., Ogunniyi, A.I., Aremu, A.O., 2020. Exploring the resource value of transvaal red milk wood (*Mimusops zeyheri*) for food security and sustainability: An appraisal of existing evidence. Plants 9(11): 1486.

Oon, Y.N., Chen, R.J., Kuan, J.M., Sit, N.W., 2021. Research article Bioactivity of medicinal plant extracts against human fungal pathogens and evaluation of toxicity using Vero cells. Tropical Biomedicine 38(3): 469-475.

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., Simons, A., 2009. Agroforestree Database: A tree reference and selection guide. Version 4.

Osunga, S., Amuka, O., Machocho, K.A., Getabu, A., Onani, O.M., 2023. Some members of genus *Cassia (Senna*): Their Ethnobotany, Potency and Prospects of Drug discovery.

Panda, R.M., Behera, M.D., Roy, P.S., 2017. Investigating the influence of environmental heterogeneity on plant species richness pattern of the Eastern Himalaya. Dim 1: 54-58.

Park, J.S., Rehman, I.U., Choe, K., Ahmad, R., Lee, H.J., Kim, M.O., 2023. A triterpenoid lupeol as an antioxidant and anti-neuroinflammatory agent: Impacts on oxidative stress in Alzheimer's disease. Nutrients 15(13): 3059.

Patwardhan, B., Partwardhan, A., 2005. Traditional medicine: Modern approach for affordable global health. World Health Organization 1-172.

Peerzada, S., Khan, M.T., Akhtar, M.F., Saleem, A., Hamid, I., Akhtar, B., Ali, S., Ahmed, S., Raza, M., 2020. Phytochemical, anti-inflammatory, anti-nociceptive and cytotoxic basis for the use of *Haloxylon stocksii*. Pakistan Journal of Pharmaceutical Sciences 33(2): c887-894.

Pereira, A.S., Den Haan, H., Peña-García, J., Moreno, M.M., Pérez-Sánchez, H., Apostolides, Z., 2019. Exploring African medicinal plants for potential anti-diabetic compounds with the DIA-DB inverse virtual screening web server. Molecules 24(10): 2002.

Peuchant, O., Lhomme, E., Martinet, P., Grob, A., Baïta, D., Bernier, C., Gibaud, S.A., Le Hen, I., Le Naour, E., Trignol-Viguier, N., Lanotte, P., 2022. Doxycycline versus azithromycin for the treatment of anorectal *Chlamydia trachomatis* infection in women concurrent with vaginal infection (chlazidoxy study): A-multicentre,-open-label, randomised, controlled, superiority trial. The Lancet Infectious Diseases 22(8): 1221-1230.

Phillipson, J.D., 2001. Phytochemistry and medicinal plants. Phytochemistry 56(3): 237-243.

Phumthum, M., Srithi, K., Inta, A., Junsongduang, A., Tangjitman, K., Pongamornkul, W., Trisonthi, C., Balslev, H., 2018. Ethnomedicinal plant diversity in Thailand. Journal of Ethnopharmacology 214: 90-98.

Pisoschi, A.M., Pop, A., Iordache, F., Stanca, L., Predoi, G., Serban, A.I., 2021. Oxidative stress mitigation by antioxidants-an overview on their chemistry and influences on health status. European Journal of Medicinal Chemistry 209: 112891.

Poddar, S., Sarkar, T., Choudhury, S., Chatterjee, S., Ghosh, P., 2020. Indian traditional medicinal plants: A concise review. International Journal of Botany Studies 5(5): 174-190.

Prinsloo, G., Marokane, C.K., Street, R.A., 2018. Anti-HIV activity of Southern African plants: Current developments, phytochemistry and future research. Journal of Ethnopharmacology 210: 133-155.

Raj, R., 2023. Virtual exploration of lupeol from *Bhadra, Aerva lanata* linn for antiurolithiatic activity. Frontline Medical Sciences and Pharmaceutical Journal 3(07): 06-11.

Ramavhale, T.T., Mahlo, S.M., 2019. A survey of South African plant species used for the treatment of “u wela” in Venda culture, Limpopo Province. International Journal of Traditional and Complementary Medicine 15: 1–9.

Rasethe, M., Potgieter, M., Pfab, M., 2022. The utilization of selected threatened or protected-plant-species:-A-case-of-Limpopo-Province,-South-Africa.-Sustain-ability 14(3): 1073.

Rasethe, M.T., Semenya, S.S., 2019. The population, utilization and local management of *Elaeodendron transvaalense* in the Blouberg Municipality, Limpopo-Province,-South-Africa. Biodiversitas-Journal-of-Biological-Diversity 20: 10.

Rasethe, M.T., Semenya, S.S., Maroyi, A., 2019. Medicinal plants traded in informal herbal medicine-markets-of-the-Limpopo-Province,-South-Africa. Evidence-Based-Complementary and Alternative Medicine 2019.

Rashed,-K.N.Z., 2022. Phytochemicals, bioactivities of *Euclea natalensis*: A review. International Journal of Biomedical and Advance Research 13(9): 5705.

Rizzo, A., Fiorentino, M., Buommino, E., Donnarumma, G., Losacco, A., Bevilacqua, N., 2015. *Lactobacillus crispatus* mediates anti-inflammatory cytokine interleukin-10 induction in response to *Chlamydia trachomatis* infection *in-vitro*. International Journal of Medical Microbiology 305(8): 815-827.

Rocha, J.D., Carneiro, F.M., Fernandes, A.S., Morais, J.M., Borges, L.L., Chen-Chen, L., de Almeida, L.M., Bailão, E.F.L.C., 2022. Toxic potential of Cerrado plants on different organisms. International Journal of Molecular Sciences 23(7): 3413.

Rosandy, A.R., Ishak, S.S.O., Sabri, N.A., Ahmad, W.Y.W., Al Muqarrabun, L.M.R., 2021. Antibacterial activity of lupeol from the bark of *Dehaasia cuneate* (Lauraceae). Current Research on Bioscience and Biotechnology *2*(2): 145-148.

Rotich, W., 2022. Botanical aspects, chemical overview, and pharmacological activities of 14 plants used to formulate a Kenyan Multi-Herbal Composition (CareVid™). Scientific African: 01287.

Ruddock, P.S., Charland, M., Ramirez, S., López, A., Towers, G.N., Arnason, J.T., Liao, M., Dillon, J.A.R., 2011. Antimicrobial activity of flavonoids from *Piper lanceaefolium* and other Colombian medicinal plants against antibiotic susceptible and resistant strains of *Neisseria gonorrhoeae*. Sexually Transmitted Diseases 82-88.

Rukunga,-G.M.,-Waterman,-P.G.,-1996.-Kaempferol-glycosides-from-*Albizia*-*versicolor.* Bulletin of the Chemical Society of Ethiopia (1): 10.

Rukunga, G.M., Waterman, P.G., 2001. Triterpenes of *Albizia versicolor* and *Albizia schimperana* stem barks. Fitoterapia 72(2): 188-190.

Sabbag,-C.,-2022.-The-present-and-future-of-gastronomy-against-global-threats.-gastronomy, hospitality, and the future of the restaurant industry: Post-COVID-19 Perspectives 139-164.

Saidi, T.A., Tshipala-Ramatshimbila, T.V., 2006. Ecology and management of a remnant *Brachystegia spiciformis* (miombo) woodland in North-Eastern Soutpansberg, Limpopo Province. South African Geographical Journal 88(2): 205-212.

Sakong, B.M., 2012. Isolation and characterization of compounds from *Calodendrum capense* (Rutaceae) and *Lydenburgia cassinoides* (Celastraceae) for treatment of fungal and bacterial infections in immunocompromised patients (Doctoral dissertation, University of Pretoria).

Sandhya, B., Thomas, S., Isabel, W., Shenbagarathai, R., 2006. Ethnomedicinal plants used by the Valaiyan community of Piranmalai hills (reserved forest), Tamilnadu, India pilot study. African Journal of Traditional, Complementary and Alternative Medicines 3(1): 101-114.

Sanguinetti, M., Posteraro, B., Lass-Flörl, C., 2015. Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. Mycoses 58: 2-13.

Santana, N., Teixeira, J., 2018. Prevalence and predictors associated with Sexually Transmitted Diseases in women, south of Brazil. International Journal of Infectious Diseases 73: 335.

Santos-Buelga, C., Gonzalez-Manzano, S., Dueñas, M., Gonzalez-Paramas, A.M., 2012. Extraction and isolation of phenolic compounds. Natural Products Isolation 427-464.

Schippmann,-U.,-Leaman,-D.J.,-Cunningham, A.B., 2002. Impact of cultivation and gathering-of-medicinal-plants-on-biodiversity:-Global-trends-and-issues-Biodiversity and the Ecosystem Approach in Agriculture, Forestry and Fisheries.

Schranz, A.J., Barrett, J., Hurt, C.B., Malvestutto, C., Miller, W.C., 2018. Challenges facing a rural opioid epidemic: Treatment and prevention of HIV and *hepatitis C*. Current HIV/AIDS Reports15(3): 245-254.

Selvaraj, P., Neethu, E., Rathika, P., Jayaseeli, J.P.R., Jermy, B.R., AbdulAzeez, S., Borgio, J.F., Dhas, T.S., 2020. Antibacterial potentials of methanolic extract and silver-nanoparticles-from-marine-algae. Biocatalysis-and-Agricultural-Biotechnology 28: 101719.

Shai, L.J., McGaw, L.J., Aderogba, M.A., Mdee, L.K., Eloff, J.N., 2008. Four pentacyclic triterpenoids with antifungal and antibacterial activity from *Curtisia dentata* (Burm. f) CA Sm. leaves. Journal of Ethnopharmacology 119(2): 238-244.

Sharma, M., Dwivedi, J., Kumar, B., Singh, B., Rawat, A.K.S., 2019. Plant-based secondary metabolites for health benefits: Classification and processing. In phytochemicals from medicinal plants: Scope, Applications, and Potential Health Claims. CRC Press 33-64.

Sharma, D., Gupta, N., 2022. Lupeol: An Alternative Approach towards Cancer Treatment.

Shawky, E., Abou El Kheir, R.M., 2018. Rapid discrimination of different *Apiaceae* species based on HPTLC fingerprints and targeted flavonoids determination using multivariate image analysis. Phytochemical Analysis 29(5): 452-462.

Shekwa, W., Maliehe, T.S., Masoko, P., 2023. Antimicrobial, antioxidant and cytotoxic activities of the leaf and stem extracts of *Carissa bispinosa* used for dental health care. BMC Complementary Medicine and Therapies 23(1): 1-14.

Shirindi, M.L., Makofane, M.D.M., 2015. Ritual impurities: Perspectives of women living with HIV and AIDS. African Journal for Physical Health Education, Recreation and Dance 21(3.2): 941-952.

Silva, N.C.C., Fernandes Júnior, A.J.J.O.V.A., 2010. Biological properties of medicinal plants: a review of their antimicrobial activity. Journal of Venomous Animals and Toxins Including Tropical Diseases 16(3): 402-413.

Sinha, S., Samad, A., 2021. Major viral diseases of *Salvia spp*., diagnosis, and their management strategies. In virus diseases of ornamental plants. Springer 211-230.

Sirivibulkovit, K., Nouanthavong, S., Sameenoi, Y., 2018. based DPPH assay for antioxidant activity analysis. Analytical Sciences 34(7): 795-800.

Sobeh, M., Mahmoud, M.F., Abdelfattah, M.A., Cheng, H., El-Shazly, A.M., Wink, M., 2018.-A-proanthocyanidin-rich-extract-from-*Cassia-abbreviata*-exhibits-anti-oxidant-and-hepatoprotective-activities-*in-vivo*.-Journal-of-Ethnopharma-cology 213: 38-47.

Sohag, A.A.M., Hossain, M.T., Rahaman, M.A., Rahman, P., Hasan, M.S., Das, R.C., Khan,-M.K.,-Sikder,-M.H.,-Alam,-M.,-Uddin,-M.J.,-Rahman,-M.H.,-2022.-Mole-cular Pharmacology and therapeutic advances of the pentacyclic triterpene lupeol. Phytomedicine 99: 154012.

Srivastava, J., Chandra, H., Nautiyal, A.R., Kalra, S.J., 2014. Antimicrobial resistance (AMR) and plant-derived antimicrobials (PDAms) as an alternative drug line to control infections. 3 Biotechnology 4(5): 451-460.

Stahl, E. ed., 2013. Thin-layer chromatography: a laboratory handbook. Springer Science and Business Media.

Street, R.A., 2016. Unpacking the new proposed regulations for South African traditional health practitioners. SAMJ: South African Medical Journal 106(4): 325-326.

Street, R.A., Stirk, W.A., Van Staden, J., 2008. South African traditional medicinal plant-trade-challenges-in-regulating-quality,-safety-and-efficacy. Journal-of-Ethnopharmacology 119(3): 705-710.

Tekulu, G.H., Hiluf, T., Brhanu, H., Araya, E.M., Bitew, H., Haile, T., 2020. Anti-inflammatory and anti-nociceptive property of *Capparis tomentosa Lam*. root extracts. Journal of Ethnopharmacology 253: 112654.

Terziev, V., Petkova-Georgieva, S., 2019. Toxic chemical compounds detection for the society social health care prevention. IJASOS-International E-journal of Advances in Social Sciences (15): 5.

Thomson, L.J., Glenn, D.C., Hoffmann, A.A., 2000. Effects of sulfur on trichogramma egg-parasitoid-in-vineyards: Measuring-toxic-effects-and-establishing-release windows. Australian Journal of Experimental Agriculture 40(8): 1165-1171.

Toledo, C.R., Paiva, M.R.B., Castro, B.F.M., Pereira, V.V., de Freitas Cenachi, S.P., Vasconcelos-Santos, D.V., Fialho, S.L., Silva-Cunha, A., 2021. Intravitreal lupeol:-A-new-potential-therapeutic-strategy-for-non-infectious-uveitis.-Biomedicine and Pharmacotherapy 143: 112145.

Torrone, E.A., Morrison, C.S., Chen, P.L., Kwok, C., Francis, S.C., Hayes, R.J., Looker, K.J., McCormack, S., McGrath, N., van de Wijgert, J.H., Watson-Jones, D., 2018. Prevalence of Sexually Transmitted Infections and bacterial vaginosis among women in sub-Saharan Africa: An individual participant data meta-analysis of 18 HIV prevention studies. PLoS medicine 15(2): e1002511.

Tóth, Z., Forgács, L., Locke, J.B., Kardos, G., Nagy, F., Kovács, R., Szekely, A., Borman, A.M., Majoros, L., 2019. *In-vitro* activity of rezafungin against common and-rare-*Candida*-species-and-*Saccharomyces-cerevisiae*. Journal-of-Antimicrobial-Chemotherapy, 74 (12)1 3505-3510.

Tree, N., Atiner's Conference Paper Series Biology 2015-1916.

Tshikalange,-T.E.,-Lawal,-F.,-2020.-*Elaeodendron-transvaalense*.-In Underexplored-medicinal plants from Sub-Saharan Africa. Academic Press 87-91.

Turner, N.J., Ignace, M.B., Ignace, R., 2000. Traditional ecological knowledge and wisdom of aboriginal peoples in British Columbia. Ecological Applications 10(5): 1275-1287.

Van Wyk, A.E., Smith, G.F., 2001. Regions of floristic endemism in Southern Africa: A review with emphasis on succulents. Umdaus press.

Van Wyk, A.S., Prinsloo, G., 2018. Medicinal plant harvesting, sustainability and cultivation in South Africa. Biological Conservation 227: 335-342.

Venter, F., Venter, J.A., 1996. Making the most of indigenous trees. Briza Publications.

Verma, A.K., Singh, S., 2020. Phytochemical analysis and *in-vitro* cytostatic potential of ethnopharmacological important medicinal plants. Toxicology Reports 7: 443-452.

Vermeulen, W.J., 2009. The sustainable harvesting of non-timber forest products from natural forests in the southern Cape, South Africa: Development of harvest systems and management prescriptions (Doctoral dissertation, Stellenbosch: University of Stellenbosch).

Vidya,-A.G.,-Vijayan,-A.,-Jyothis,-L.J.,-Nair,-R.,-Suja,-K.P.,-2019.-Evaluation-of-anti-fungal-efficacy-of-some-medicinal-plants-on-*Candida*-spp.-causing-vulvovaginitis.

Vyas, S., Kothari, S.L., Kachhwaha, S., 2019. Nootropic medicinal plants: Therapeutic alternatives for Alzheimer's disease. Journal of Herbal Medicine 17: 100291.

Whiting, C., Schwartzman, G., Khachemoune, A., 2023. *S*yphilis in dermatology: recognition and management. American Journal of Clinical Dermatology 24(2): 287-297.

Williams, V.L., Balkwill, K., Witkowski, E.T., 2000. Unraveling the commercial market for-medicinal-plants-and-plant-parts-on-the-Witwatersrand,-South-Africa. Economic Botany 54(3): 310-327.

Williams, V.L., Victor, J.E., Crouch, N.R., 2013. Red listed medicinal plants of South Africa: status, trends, and assessment challenges. South African Journal of Botany 86: 23-35.

World Health Organization (WHO)., 2002. WHO Traditional Medicine Strategy 2002-2005. Geneva:-World-Health-Organization,-(WHO/EDM/TRM/2002.1).

World Health Organization, 2018. Report on global Sexually Transmitted Infection surveillance 2018.

Xego, S., Kambizi, L., Nchu, F., 2016. Threatened medicinal plants of South Africa: Case-of-the-family-Hyacinthaceae.-African-Journal-of-Traditional,-Comple-mentary and Alternative Medicines 13(3): 169-180.

Xiang, B., Zhou, X., Qin, D., Li, C., Xi, J., 2022. Infrared assisted extraction of bioactive compounds from plant materials: Current research and future prospect. Food Chemistry 371: 131192.

Yusuf, S., Sanyinna, Y.M., Audu, B.S., Wakawa, A.I., Aforo, B., 2022. Comparative acute toxicity of aqueous extracts of *Tephrosia vogelii* and *Albizia gummifera* on *Clarias gariepinus* (African-Catfish) Fingerlings. International Journal of Science and Technology Research 2: 37-46.

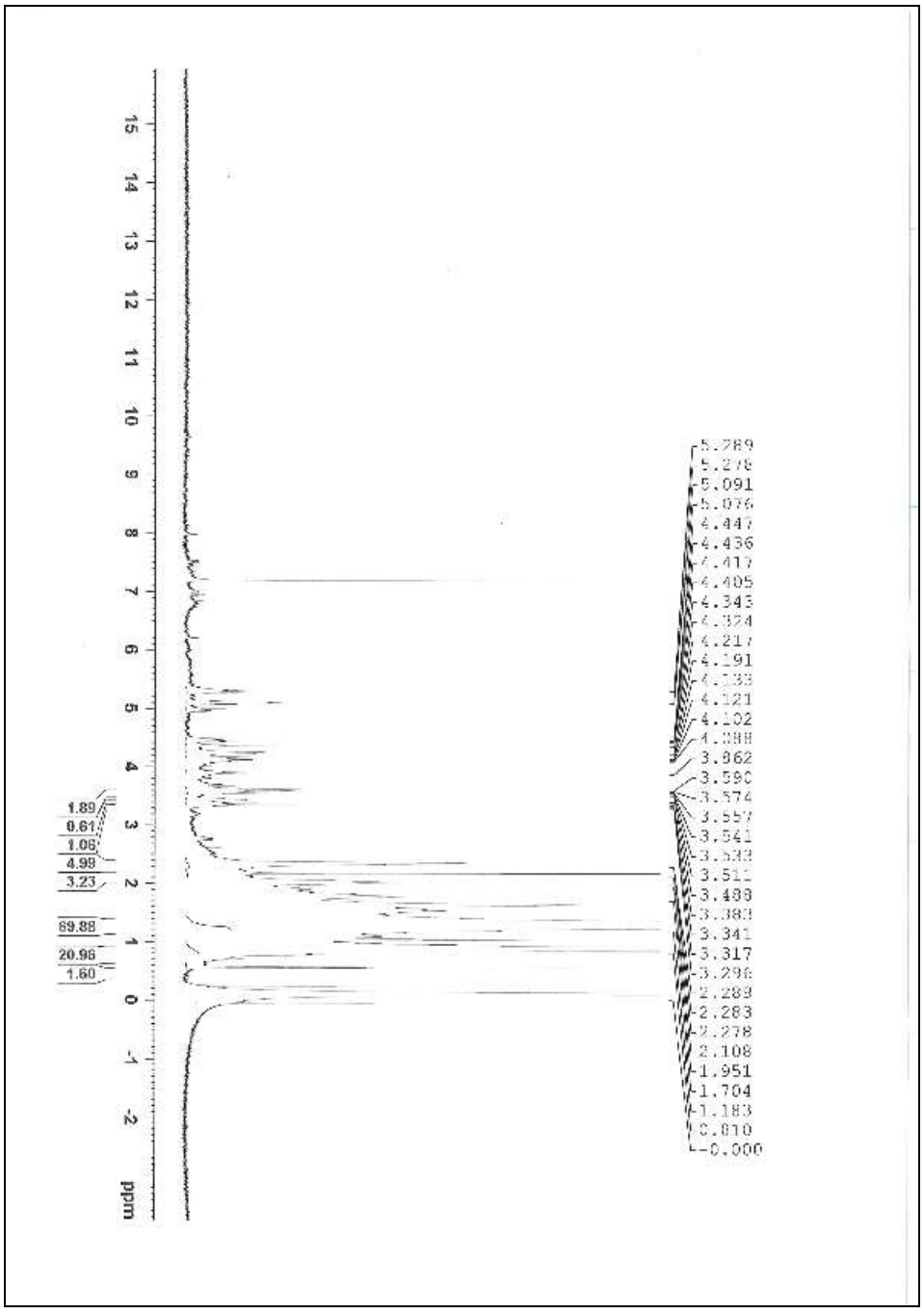
Zamani, M., Delfani, A.M., Jabbari, M., 2018. Scavenging performance and antioxidant activity of γ-alumina nanoparticles towards DPPH free radical: Spectroscopic and DFT-D studies. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 201: 288-299.

Zawada, A., Machowiak, A., Rychter, A.M., Ratajczak, A.E., Szymczak-Tomczak, A., Dobrowolska, A., Krela-Kaźmierczak, I., 2022. Accumulation of advanced glycation end-products in the body and dietary habits. Nutrients 14(19): 3982.

.

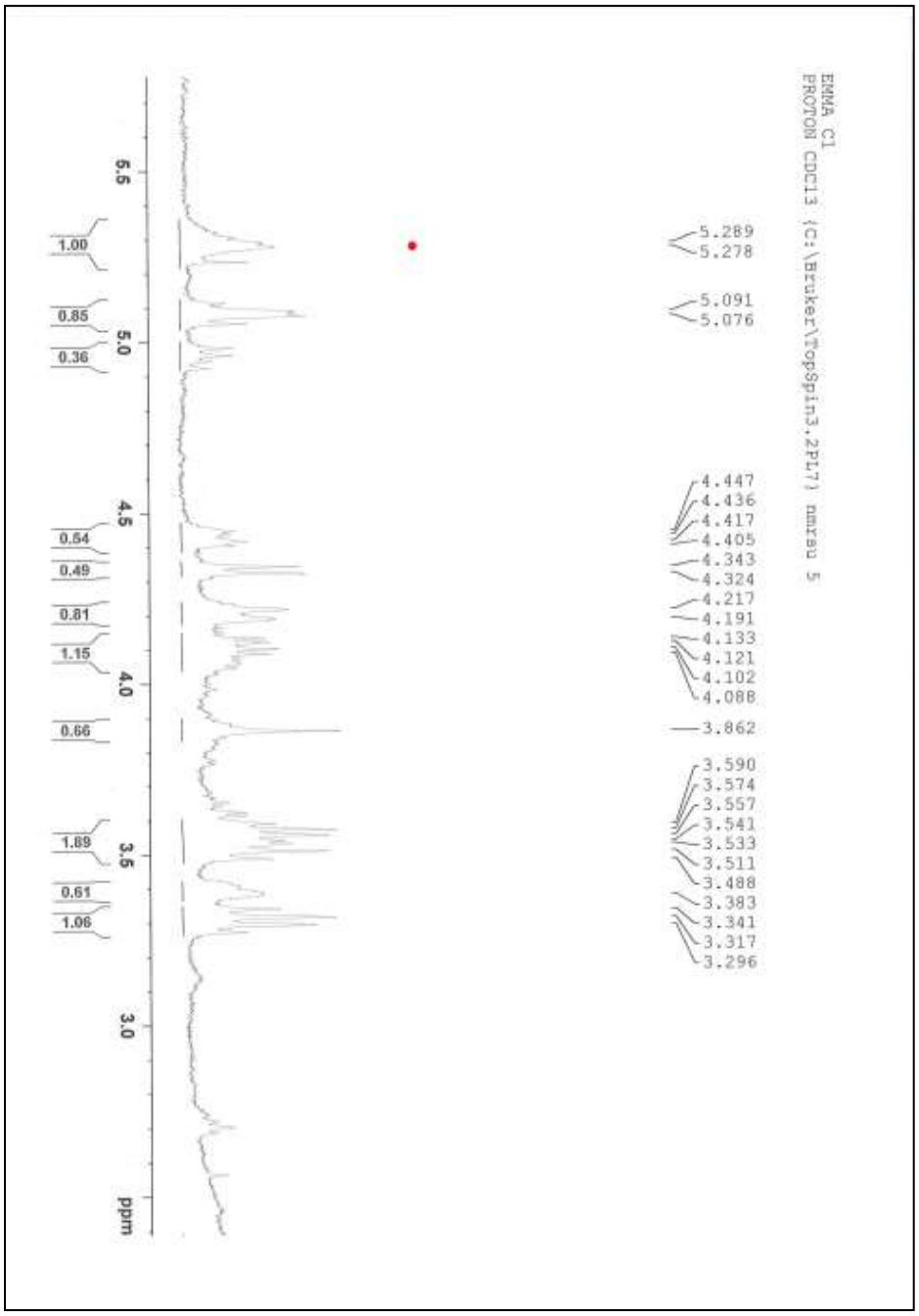
Zubaidah, S., Azis, S., Mahanal, S.U.S.R.I.Y.A.T.I., Batoro, J.A.T.I., Sumitro, S.B., 2020. Local knowledge of traditional medicinal plants use and education system on-the-young-of-Ammatoa-Kajang-tribe-in-South-Sulawesi,-Indonesia.-Bio-diversitas Journal of Biological Diversity (9): 21.

Appendix A



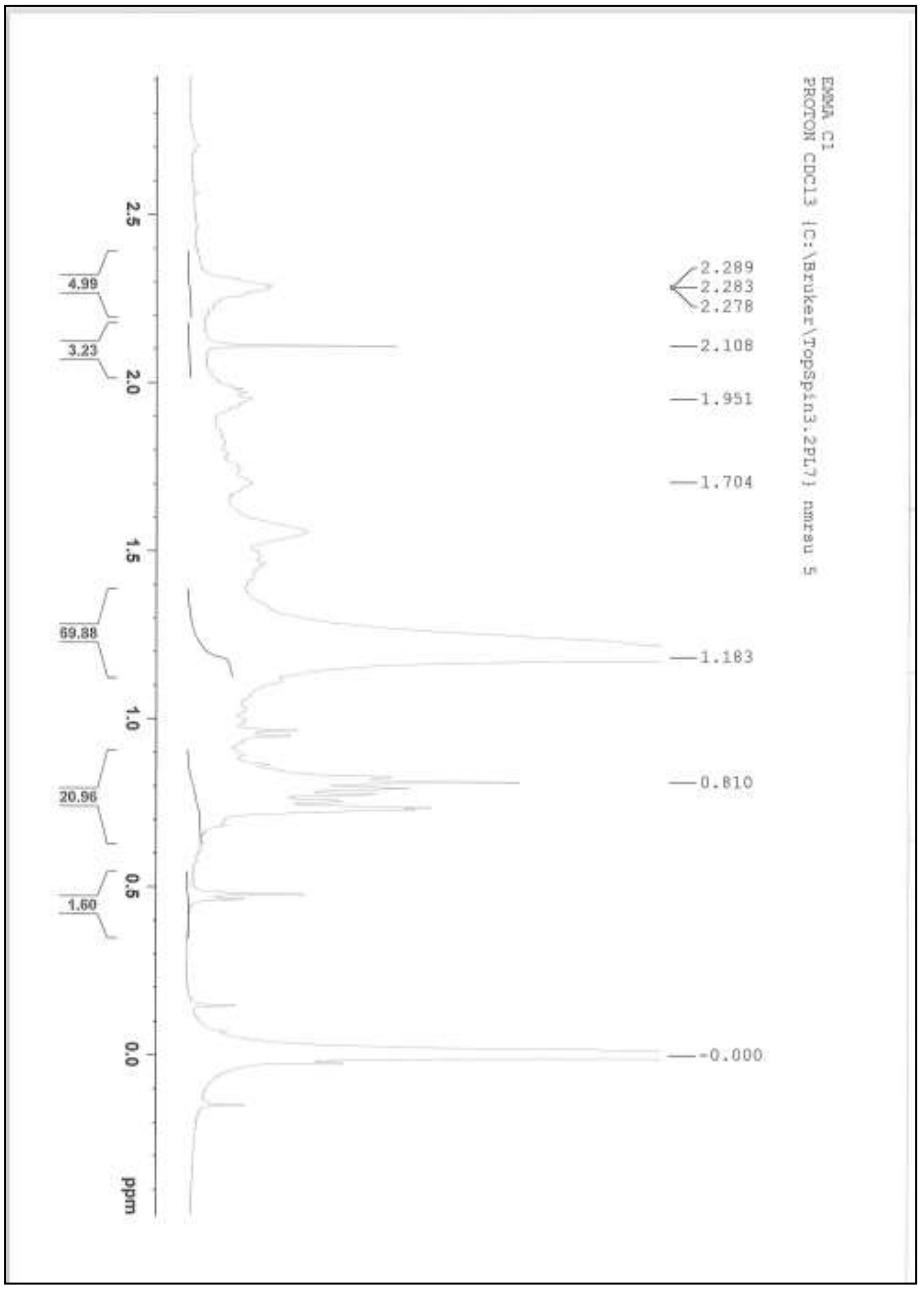
Proton (^{1}H) NMR of compound 1.

Appendix B



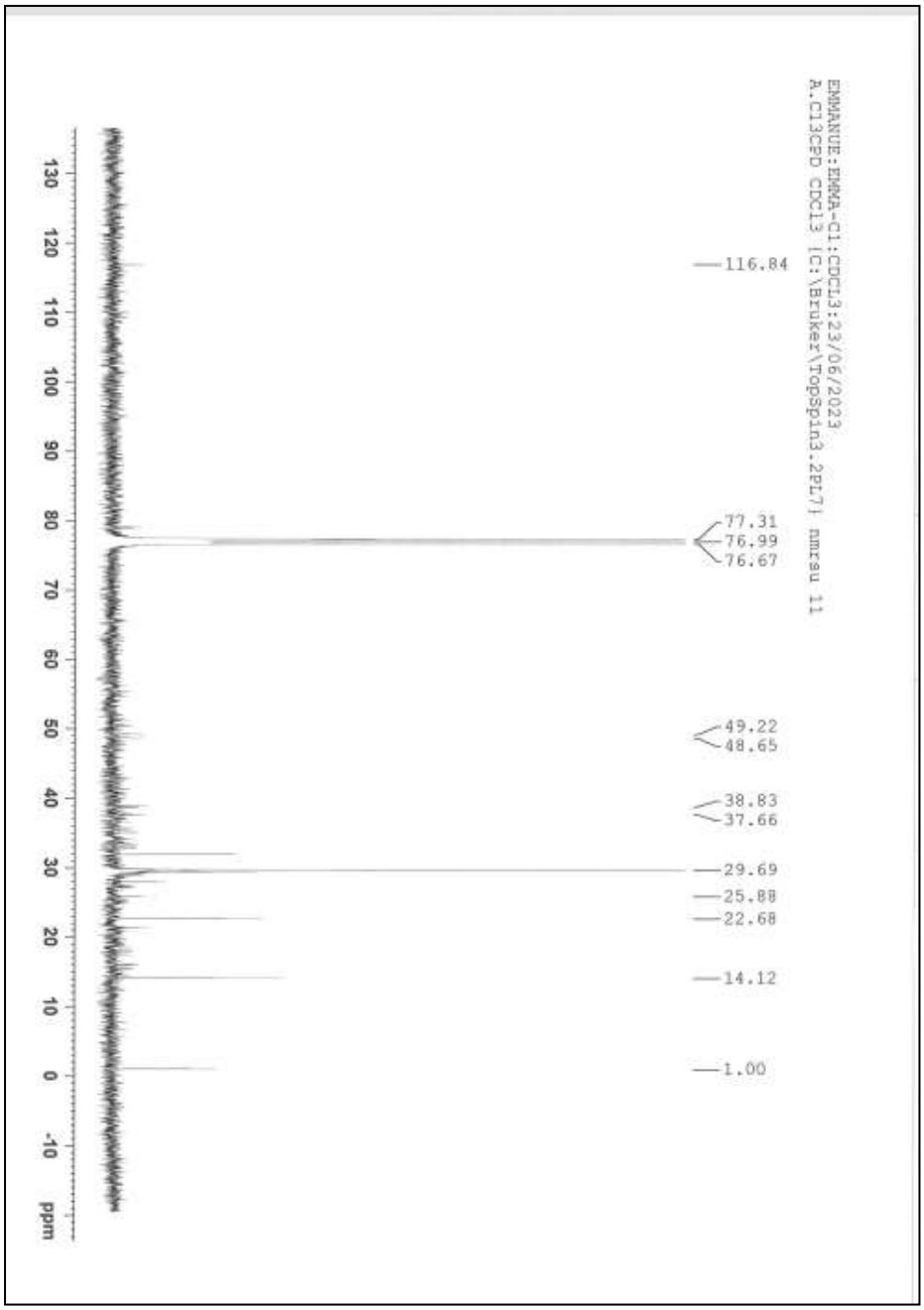
Proton NMR CDC13 spectrum of compound 1.

Appendix C



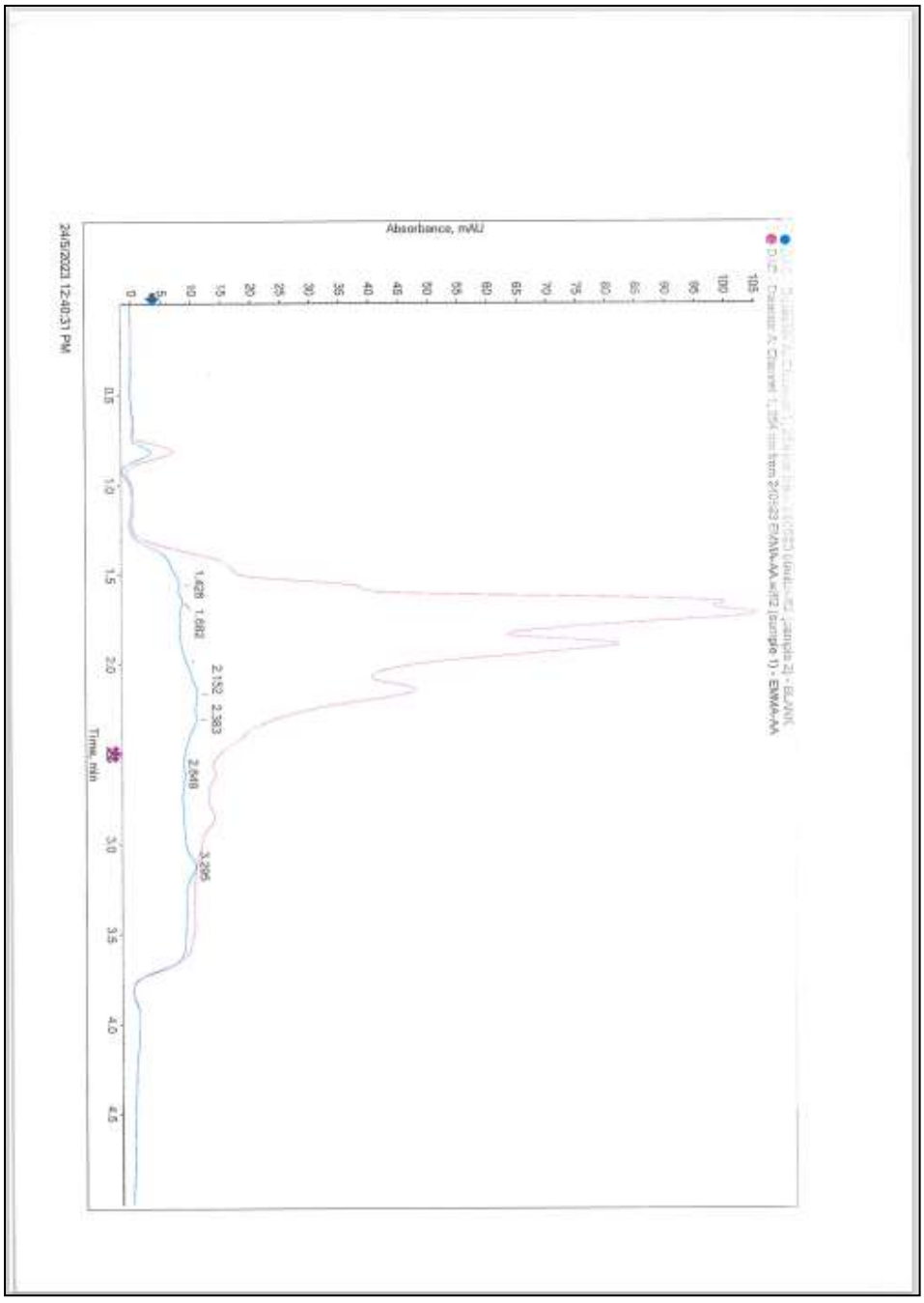
Proton NMR spectrum.

Appendix D



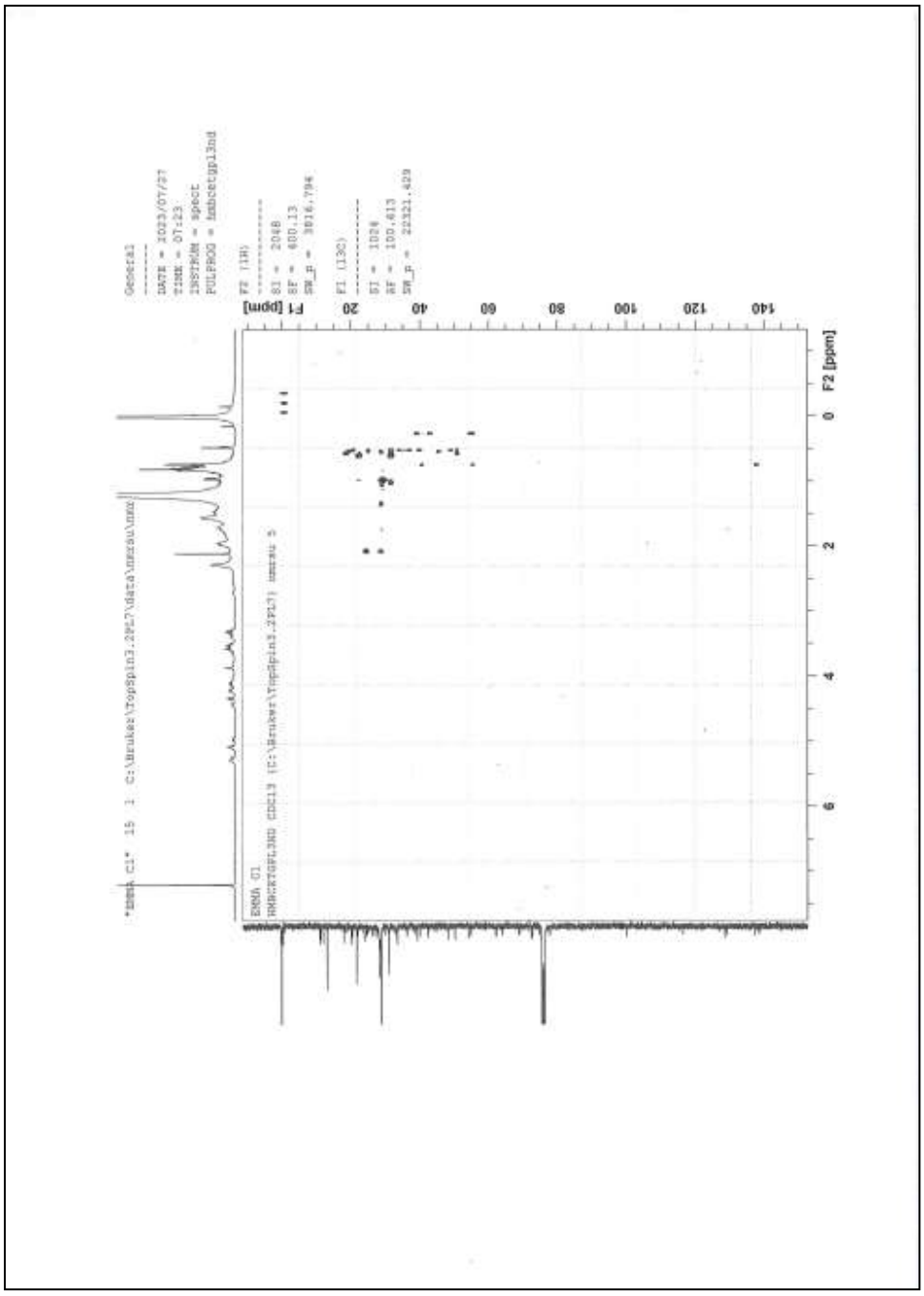
Carbon (^{13} C) NMR spectrum.

Appendix E



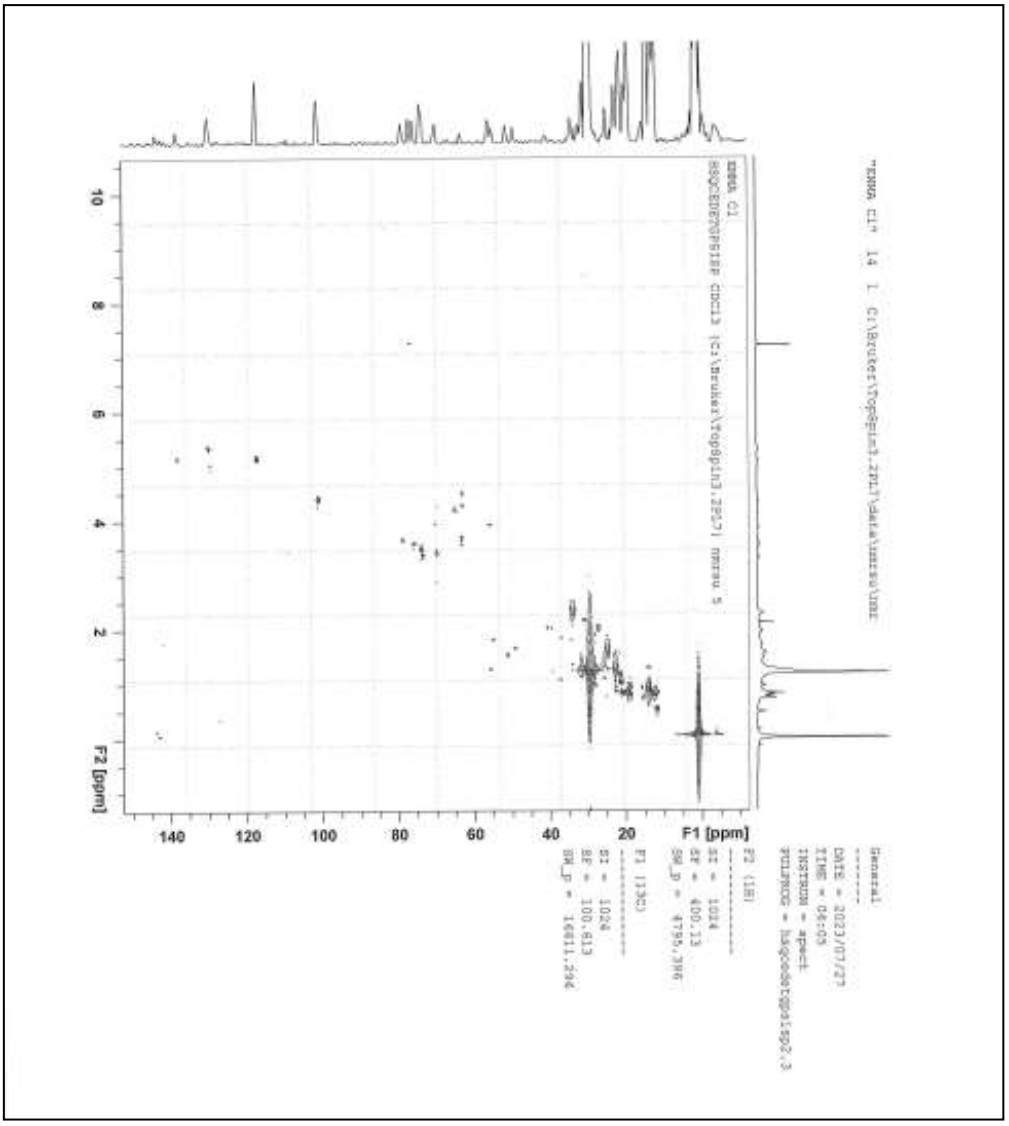
UV Chromatogram.

Appendix F



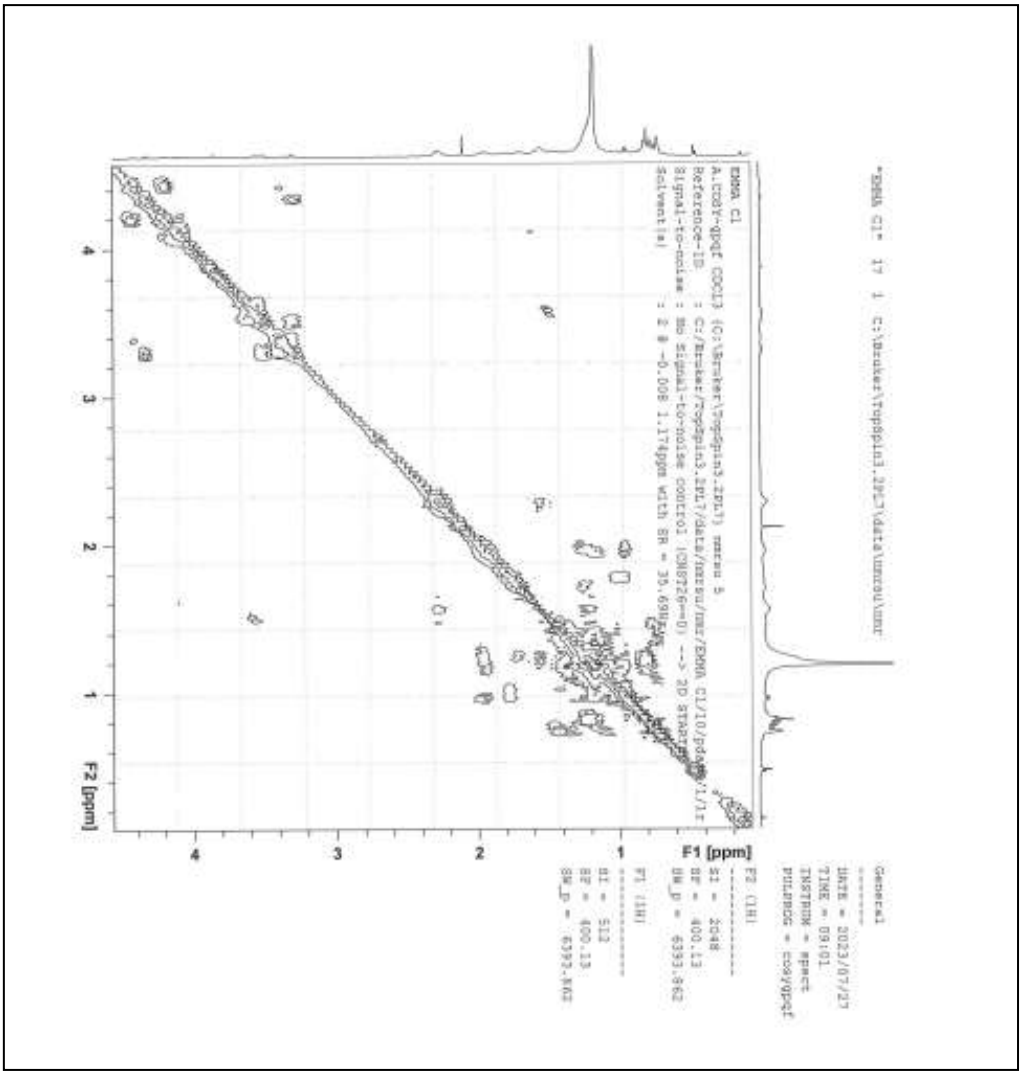
$H_{mbc\rightarrow}$Spectra.

Appendix G



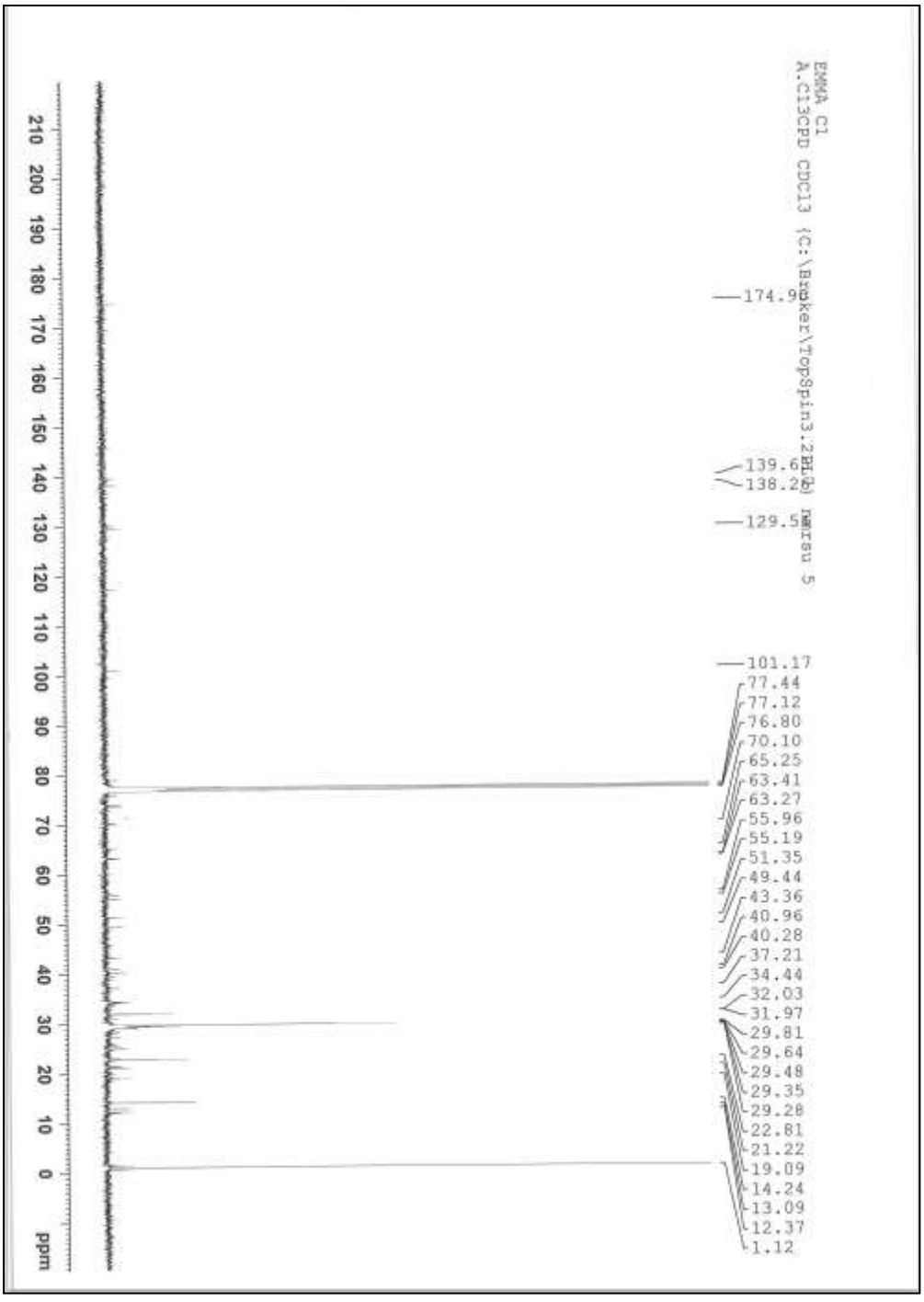
Hsqc Spectrum.

Appendix H



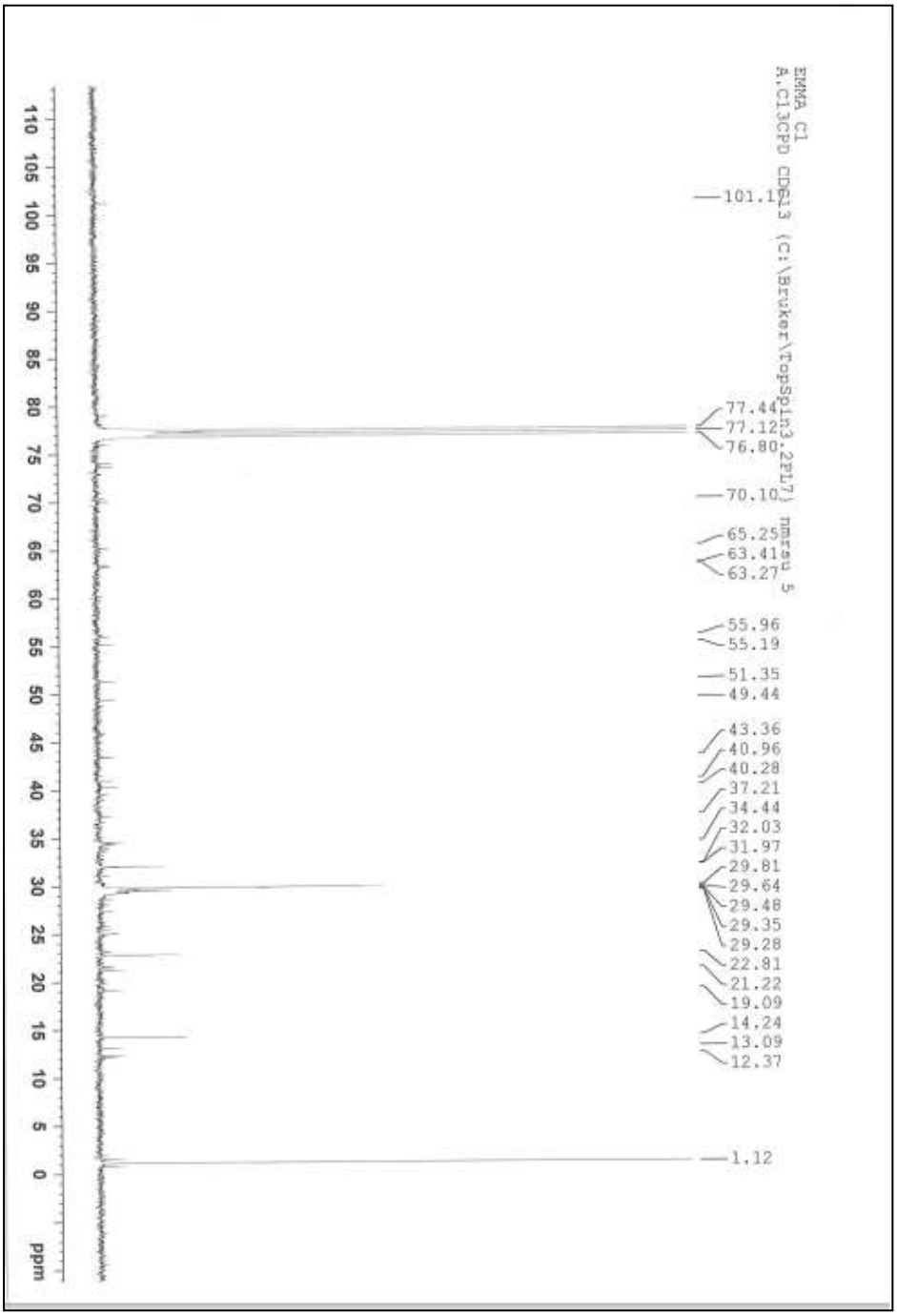
Cosy Spectrum.

Appendix I



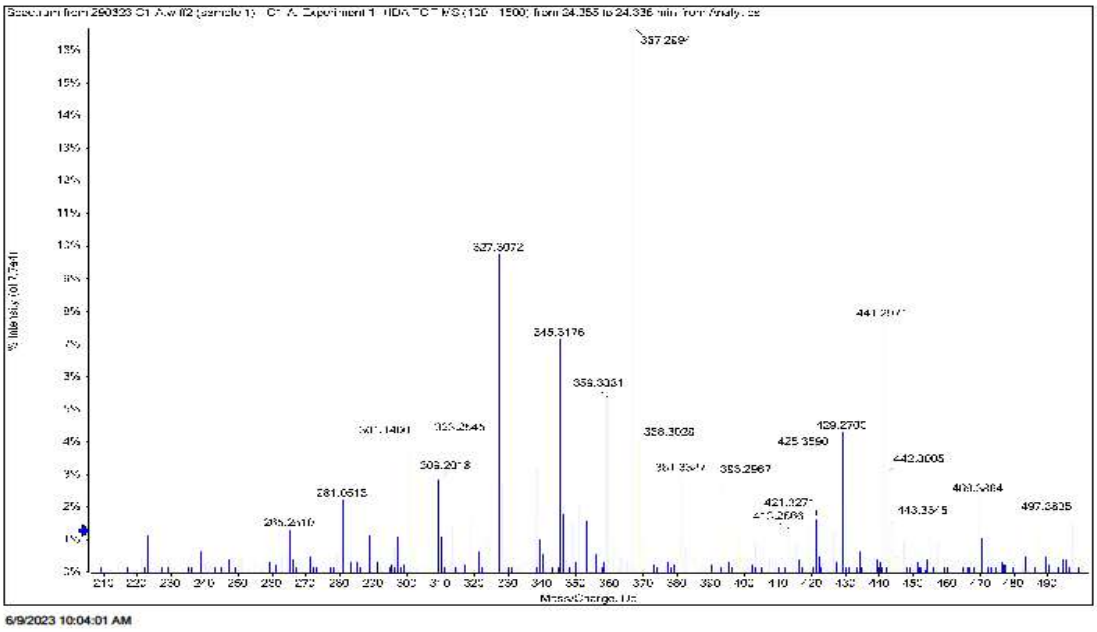
Carbon (^{13}C) spectrum.

Appendix J



Carbon (^{13}C) spectrum.

Appendix K



Mass spectrometry.