ANTIMICROBIAL PROPERTIES AND PHYTOCHEMICAL ANALYSIS OF MEDICINAL PLANTS USED FOR THE TREATMENT OF EAR INFECTIONS

By

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science 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.

Chauke, S (Ms) **Date**

DEDICATION

I dedicate this work to my loving and supportive parents Mr. D.M and Mrs. L. Chauke.

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ABSTRACT

Ear infections are a major health concern that negatively affects the health and welfare of individuals across the globe. The infection is caused by a wide spectrum of bacterial, fungal, and viral pathogens. Treatments of ear infections involve the use of antimicrobials such as antibiotics, antifungals, and antivirals. However, most microbial pathogens have developed resistance to the available antimicrobial drugs. Hence, the study aimed to identify plant species used in traditional medicine as a remedy for ear infections and investigate their antifungal activities against the selected fungal pathogens (*Aspergillus fumigatus* and *Candida albicans*). These fungal pathogens cause ear infections in humans. Eight plant species including *Carpobrotus edulis* L., *Cotyledon orbiculata* L., *Dichrostachys cinerea* (L.) Wight & Arn., *Erythrina lysistemon* Hutch., *Flacourtia indica* (Burm. f.) Merr., *Psidium guajava* L., *Ricinus communis* L., and *Sansevieria hyacinthoides* (L.) Druce were selected from the ethnomedicinal plant's database of over 300 medicinal plants used for therapeutic purposes in humans.

Fresh and dried leaves of selected plants were extracted with solvents of various polarities such as acetone, hexane, methanol, and water. In the current study, methanol extracted a larger quantity (30.75%) of plant materials followed by acetone (6.5%) from dried leaf extract of *C. edulis* and *P. guajava*. Acetone extracted more plant material (8.05%) from fresh leaf extract of *C. orbiculata*. Acetone was the second-best solvent for extracting a larger quantity of dried leaf materials as compared to other solvents.

Thin layer chromatography (TLC) was used to analyse the phyto-constituents 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 TLC chromatograms were visualized under UV radiation at 360 nm. In TLC chromatograms separated with BEA, chemical components with a similar R_f value of 0.88 were observed in acetone, hexane, and methanol-dried leaf extracts of *R. communis* and *S. hyacinthoides*. Surprisingly, TLC chromatograms separated in BEA, dried leaf extracts contained the highest number of phyto-constituents with a total of 73 followed by 30 in CEF and EMW (29). However, in chromatograms of fresh leaf extracts a total

of 12 compounds were visible in BEA, followed by 5 compounds in EMW, and 1 in CEF. Therefore, the BEA solvent system was the best eluent for separating compounds. In addition, different bands were observed after spraying the TLC plates with vanillin reagent.

Antifungal activities of plant extracts were determined using serial microdilution assay against the selected fungal pathogens (*Aspergillus fumigatus* and *Candida albicans*). Noteworthy activities (0.02 mg/ml) against *C. albicans* were observed from *P. guajava* acetone extract and *S. hyacinthoides* acetone fresh leaf extracts. The methanol-dried leaf extract of *C. edulis* was active against *A. fumigatus* with MIC of 0.02 mg/ml while fresh leaf extract was active with MIC of 0.31–2.5 mg/ml. The dried leaf water extracts of *C. edulis* and *D. cinerea* had an excellent activity of 0.02 mg/ml against *A. fumigatus.*

The bioautography assay was used to determine the number of active components in different plant extracts. Antifungal compounds were visible in dried leaf extracts of *P. guajava*, *R. communis*, and *S. hyacinthoides*. A total of 19 antifungal compounds were observed against *A. fumigatus*. Dried leaf extracts of *P. guajava*, *R. communis*, and *S. hyacinthoides* had an active component with an R_f value of 0.88 against *A. fumigatus*. In TLC bioautograms developed in BEA, two active compounds with similar R_f values of 0.20 were visible in acetone and methanol extract of *P. guajava* against *C. albicans.*

The results of this study support the traditional use of the selected plant species to combat ear infections and related ailments in humans. The crude extracts have the potential to serve as an ototopic. The antifungal compounds also have the potential to be isolated and used in the formulation of ototopical drugs that may help lift the health burden caused by ear infections across the globe.

ABBREVIATIONS

A	Acetone
A.F	*Aspergillus fumigatus*
AIDS	Acquired Immunodeficiency Syndrome
AOE	Acute Otitis Externa
AOM	Acute Otitis Media
BEA	Benzene: Ethyl acetate: Acetone
C.A	*Candida albicans*
CEF	Chloroform: Ethyl acetate: Formic acid
COE	Chronic Otitis Externa
COM	Chronic Otitis Media
CSOM	Chronic Suppurative Otitis Media
EMW	Ethyl acetate: Methanol: Water
H	Hexane
HIV	Human Immunodeficiency Virus
INT	p-Iodonitrotetrazolium Violet
M	Methanol
MIC	Minimum Inhibitory Concentration
NOE	Necrotizing Otitis Externa
OE	Otitis Externa
OI	Otitis Interna
OM	Otitis Media
R_f	Retention Factor
SD	Sabouraud Dextrose
TLC	Thin-Layer Chromatography
URTI	Upper Respiratory Tract Infection
URVI	Upper Respiratory Viral Infection
W	Distilled Water

Table of Contents

CHAPTER 1 1

INTRODUCTION 1

1.1 Medicinal plants 1

1.2 Rationale 2

1.3 Aim 4

1.4 Objectives 4

1.5 Outline of study 5

CHAPTER 2 6

LITERATURE REVIEW 6

2.1 Introduction 6

2.2 Indigenous knowledge 6

2.3 Importance of traditional medicine 6

2.4 Conservation of medicinal plants 7

2.5 Use of medicinal plants in drug production 7

2.6 Traditional treatments for ear infections 9

2.7 Ear infections 9

2.7.1 Types of ear infections 9

2.7.2 Causes or risk factors of ear infections 12

2.7.3 Microbial pathogens that cause ear infections 13

2.8 Extracranial complications of ear infections 14

2.8.1 Labyrinthitis 14

2.8.2 Facial nerve paralysis 14

2.8.3 Mastoiditis 14

2.9 Ear infections and URTIs 14

2.10 Ear infections and HIV/AIDS 15

2.11 Antibiotic treatment of ear infections caused by bacteria 15

2.11.1 Oral antibiotics 15

2.11.2 Topical antibiotics 16
2.12 Antifungal treatment of ear infections caused by fungi 16
2.13 Antimicrobial drug resistance 17
2.14 Fungal pathogens 17
2.14.1 *Aspergillus fumigatus* 17
2.14.2 *Candida albicans* 18
2.15 Bacterial pathogens 18
2.15.1 *Escherichia coli* 18
2.15.2 *Pseudomonas aeruginosa* 19
2.15.3 *Staphylococcus aureus* 19
2.16 Botanical descriptions of the plant species used in the study 20
2.16.1 *Carpobrotus edulis* L. 20
2.16.1.1 Ethnomedicinal uses 21
2.16.2 *Cotyledon orbiculata* L. 22
2.16.2.1 Ethnomedicinal uses 22
2.16.3 *Dichrostachys cinerea* (L.) Wight & Arn. 23
2.16.3.1 Ethnomedicinal uses 23
2.16.4 *Erythrina lysistemon* Hutch. 24
2.16.4.1 Ethnomedicinal uses 24
2.16.5 *Flacourtia indica* (Burm. f.) Merr. 25
2.16.5.1 Ethnomedicinal uses 26
2.16.6 *Psidium guajava* L. 26
2.16.6.1 Ethnomedicinal uses 27
2.16.7 *Ricinus communis* L. 28
2.16.7.1 Ethnomedicinal uses 29
2.16.8 *Sansevieria hyacinthoides* (L.) Druce 30
2.16.8.1 Ethnomedicinal uses 30

2.17 Conclusion 32
CHAPTER 3 33
PLANT EXTRACTION AND PHYTOCHEMICAL ANALYSIS OF SELECTED PLANT SPECIES 33
3.1 Introduction 33
3.2 Materials and methods 34
3.2.1 Plant selection and collection 34
3.2.2 Extraction of dried plant material 35
3.2.3 Extraction of fresh plant material. 36
3.2.4 Phytochemical analysis 38
3.3 Results and discussion 38
3.3.1 Extraction of dried and fresh plant material 38
3.3.2 Phytochemical analysis 41
3.4 Conclusion 43
CHAPTER 4 44
ANTIFUNGAL ACTIVITIES OF SELECTED PLANT SPECIES AGAINST FUNGAL PATHOGENS THAT CAUSE EAR INFECTIONS 44
4.1 Introduction 44
4.2 Methods and materials 45
4.2.1 Fungal strains and inoculum quantification 45
4.2.2 Serial microdilution assay 45
4.2.3 Bioautography assay 46
4.3 Results and discussion 46
4.3.1 Serial microdilution assay 46
4.3.2 Plant extracts with excellent antifungal activity 47
4.3.3 Total activity of plant extracts 47
4.3.4 Bioautography assay 52
4.4 Conclusion 57

CHAPTER 5 58
CONCLUSION 58
REFERENCES 61

List of Figures

Figure 2. 1 The structure of the ear with indications of the main compartments of the ear, namely: Outer ear, middle ear, and inner ear (Payne and Wong, 2022). 10
Figure 2. 2 Flow chart indicating the three parts of the ear and the type of infection. Images from left to right: Otoscopic images of OE (Osguthorpe and Nielsen, 2011), OM (Bhutta *et al*., 2017), and 3D T2 TSE MRI scan of OI (Wu *et al*., 2014). 12
Figure 2. 3 Microscopic image of *Aspergillus fumigatus* (Adhavan, 2020). 17
Figure 2. 4 Microscopic image of *Candida albicans* (Tyavambiza, 2018)..... 18
Figure 2. 5 Microscopic image of *Escherichia coli* (Singh *et al*., 2015)..... 19
Figure 2. 6 Microscopic image of *Pseudomonas aeruginosa* (Tyavambiza, 2018). . 19
Figure 2. 7 Microscopic image of *Staphylococcus aureus* (Tyavambiza, 2018). 20
Figure 2. 8 Images of *Carpobrotus edulis* L. (Bilomu ra ku nava) leaves (A) and flower (B). 21
Figure 2. 9 *Cotyledon orbiculata* L. (Bilomu ra tindleve) whole plant (A), leaves (B) and flowers (C). 23
Figure 2. 10 *Dichrostachys cinerea* (L.) Wight & Arn. (Ndzhenga) whole plant (A), leaves (B), and flowers (C). .....
Figure 2. 11 *Erythrina lysistemon* Hutch. (Muvale/ Nsisimbana) whole plant (A), leaves (B), flowers (C), and pods (D)..... 25
Figure 2. 12 *Flacourtia indica* (Burm. f.) Merr. (Xivambula) whole plant (A), leaves (B), and flowers (C). 26
Figure 2. 13 *Psidium guajava* L. (Mugwava) whole plant (A), leaves (B), flowers (C), and fruit (D). 28
Figure 2. 14 *Ricinus communis* L. (Nhlampfurha) whole plant (A), leaves (B), flowers and fruit (C). 30
Figure 2. 15 *Sansevieria hyacinthoides* (L.) Druce (Mhangane) whole plant (A)..... 31
Figure 3. 1 A map showing study areas in Bushbuckridge Local Municipality. 35
Figure 3. 2 *S*chematic representation of the extraction procedure for dried and fresh leaf materials. 37
Figure 3. 3 The percentage of crude extracts from dried leaves of different plants extracted using acetone, hexane, and methanol. 40

Figure 3. 4 Percentage of crude extracts from fresh leaves of succulent plants extracted using acetone, hexane, and methanol. 40
Figure 3. 5 TLC chromatograms of dried leaf extracts of *C. edulis*, *C. orbiculata*, *D. cinerea*, *E. lysistemon*, *F. indica*, *P. guajava*, *R. communis*, and *S. hyacinthoides* in BEA, CEF, and EMW. Lanes from left to right: A= Acetone, H= Hexane, M= Methanol, and W= water. 42
Figure 4. 1 Bioautograms of *F. indica*, *P. guajava*, *R. communis,* and *S. hyacinthoides* leaf extracts developed in BEA, CEF and EMW solvent systems with their corresponding chromatograms. White bands indicating compounds with antifungal activity against *A. fumigatus*. Lanes from left to right: A= Acetone, H= Hexane, M= Methanol and W= Distilled water. 53
Figure 4. 2 Bioautograms of *R. communis* and *S. hyacinthoides* leaf extracts developed in CEF and EMW solvent systems with their corresponding chromatograms. White bands indicating compounds with antifungal activity against *A. fumigatus*. Lanes from left to right: A= Acetone, H= Hexane, M= Methanol and W= Distilled water. 54
Figure 4. 3 Bioautogram and corresponding chromatogram of *F. indica* and *P. guajava* in BEA with white bands indicating compounds with antifungal activity against *C. albicans*. Lanes from left to right: A=Acetone, H=Hexane, M=Methanol and W= Distilled water. 55

List of tables

Table 2. 1 Drugs derived from plants or plant products..... 8

Table 4. 1 Minimum inhibitory concentrations of fresh and dried leaves extracts of eight different plant species tested against two fungal pathogens……..49

Table 4. 2 Plant extracts with excellent antifungal activity (0.02 mg/ml) against the tested fungal pathogens at 24 and 48 hours. 50

Table 4. 3 Total activity of plant extracts against *A. fumigatus* and *C. albicans*. 51

Table 4. 4 R_f values of compounds inhibiting fungal growth separated with BEA and EMW using dried leaf extracts of *P. guajava*. 55

Table 4. 5 R_f values of compounds inhibiting fungal growth separated with BEA, CEF and EMW using dried leaf extracts of *R. communis.* 55

Table 4. 6 R_f values of compounds inhibiting fungal growth separated with CEF and EMW using dried leaf extracts of *S. hyacinthoides.*..... 56

CHAPTER 1

INTRODUCTION

1.1 Medicinal plants

Medicinal plants continue to play an important role in securing the health of people in African developing countries (Agidew, 2022). In South Africa, local people in rural and urban areas rely on herbal medicines for their primary healthcare since it is cheap, easily accessible, and safe (Fennel *et al*., 2004). Hence, medicinal plants are receiving global recognition due to their importance in healthcare (Aburigal *et al*., 2022). In Limpopo province, local people and traditional health practitioners use medicinal plants to combat various ailments in humans (Shikwambana and Mahlo, 2020). The local community prefers medicinal plants for medicinal purposes since they are effective, easily accessible, and low-cost. Hence, there is a growing interest in the study of medicinal plants (Larida *et al*., 2022). Herbal medicine is a major component of South African cultures and traditions. This medicine is also known as phytomedicine which uses plants for medicinal purposes. More so, herbal medicines are used to vitalise the immune system to prevent and cure diseases (Taylor *et al*., 2001).

Medicinal plants are an essential source of medicine with effectual properties to treat various ailments in humans. More importantly, these plants contain active components that are used to cure diseases and ease pain (Madhu *et al*., 2016). The chemical compounds present in the different plant parts may have physiological properties in the human body. Plant parts such as flowers, fruits, leaves, resins, rhizomes, roots, seeds, and stems are used in traditional medicine (Alqethami and Aldhebiani, 2021). Medicinal plants provide a great source of information for a wide diversity of chemical compounds that can be used to derive the drugs that will be effective against various ailments in humans (Yadav *et al*., 2014). Furthermore, medicinal plant drugs are divided into two categories. Firstly, they are incorporated in mixtures containing different compounds, like infusions, essential oils, tinctures, or extracts. Secondly, they are used as pure, chemically defined active principles (Taylor *et al*., 2001).

Plants undergo a metabolic process that results in primary and secondary metabolites. Primary metabolites such as amino acids, simple sugars (glucosides), proteins, and lipids are involved in cellular processes. Secondary metabolites are chemically active

compounds produced in response to stress with complex structures and more limited distribution than primary metabolites (Kumari *et al*., 2017). Secondary metabolites are classified into different groups based on their structure. The most important of these secondary metabolites with medicinal properties are alkaloids, glycosides, flavonoids, tannins, saponins, and resins. These bioactive compounds vary depending on the plant in which they are produced (Alqethami and Aldhebiani, 2021).

The chemical compounds that are naturally found in plants are called phytochemicals. They are responsible for the colour and organoleptic properties of the plant. Phytochemicals could be available as dietary supplements, but the potential health benefits of phytochemicals are derived from the consumption of the whole plant. Several phytochemicals have a wide range of activities that helps give immunity against long-term diseases (Yadav *et al*., 2017). It has been discovered that phytochemical compounds have antibacterial and antifungal activities (Rangasamy *et al*., 2007). In this dissertation, the study investigates medicinal plants used for the treatment of ear infections by traditional health practitioners and local people residing in Limpopo province (Capricorn District) and Mpumalanga province (Ehlanzeni District). Based on the literature, the study has never been conducted previously in the two Districts.

1.2 Rationale

An ear infection is caused by bacteria, fungi, and viruses in the ear (Wolk, 2016). These microorganisms accumulate in the ear discharge and cause inflammation, especially in the middle and external ear (Appiah-Korang *et al.*, 2014). Furthermore, the infections may lead to ear tumours, metastatic tumours, or primary tumours in the surrounding organs (Mahmoudian-Sani *et al.*, 2017). The overuse and misuse of antibiotics have caused microbial pathogens to be more resistant to methicillin and vancomycin (Aneja *et al.*, 2012). Recently, drugs such as quinolones, amoxicillin-clavulanate, levofloxacin, moxifloxacin, and ciprofloxacin are used to combat ear infections in humans (Szmuilowicz and Young, 2019). However, some of these drugs may be toxic and ineffective due to antimicrobial drug resistance developed by pathogens (Chong *et al.*, 2021). Screening of medicinal plants with potential antimicrobial properties can lead to the discovery of novel antifungal agents that could resolve fungal ear infections. Therefore, the study focused on investigating medicinal

plants that are used by traditional health practitioners for the treatment of ear infections.

South Africa is rich in the knowledge of plants used in traditional medicines which need to be justified by scientific evidence (Dyubeni and Buwa, 2012). Plants are a good source of bioactive compounds with antimicrobial activity (Gunatilaka, 2006). Furthermore, these plants contain phytochemicals that have therapeutic properties that are affordable and less toxic (Otimanam *et al.*, 2022). The World Health Organization (WHO) also regards medicinal plants as the best source of a variety of drugs (Yadav and Agarwala, 2011). About 78% of new chemical compounds derived from medicinal plants are used as a potential alternative to treat various infections in humans (Mustafa *et al.*, 2017). *Aspergillus fumigatus* and *Candida albicans* are the most common fungal pathogens responsible for ear infections (Prasad *et al.*, 2014).

The use of plant-based natural products in drug development may lead to the discovery of new bioactive compounds that can provide effective curative agents for microbial infections. According to Dewatisari *et al.* (2022), medicinal plants are the solution to the rising concern of antimicrobial drug resistance. In previous studies, notable antimicrobial activities of some plant extracts have been observed (Kebede *et al.*, 2021). Ear infections in children are a major health concern and may be associated with hearing impairment, and delayed speech, and language development (Karunanayake *et al.*, 2016). These infections affect over 90% of children with 20% of the infections being chronic (Ryan *et al.*, 2020). It is estimated that 60% of the cases of ear infections in humans may lead to disabling hearing loss or death in extreme conditions (Hailu *et al.*, 2016). Hence, these infections account for a large proportion of the health concerns in developing countries (Sahu *et al.*, 2014). Without the discovery of new compounds through various methods such as the screening of medicinal plants, this major health burden may continue to rise.

The invention of a new antimicrobial drug or eardrop solution from plant-based natural products could be a solution. Most treatments for ear infections are western, however, some of these drugs are less effective because of resistance to antimicrobial drugs (Yang *et al.*, 2022) and may have adverse side effects on humans (Magdy *et al.*, 2022) hence, there is a need to investigate a different approach that is used in traditional

medicine. Local people and traditional health practitioners use different medicinal plants and approaches to treat ear infections and other diseases. However, there is a lack of scientific evidence to support the ethnomedicinal use of these plants to combat ear infections and related ailments. Therefore, there is a need to focus research on traditional medicine and phytotherapy since they can provide novel drugs that are effective, cheap, and non-toxic (Mahmoudian-Sani *et al.*, 2017).

1.3 Aim

The study aimed to select medicinal plants used to treat ear infections from a database of ethnomedicinal plants and to determine the antimicrobial activity of the selected plants against fungal pathogens.

1.4 Objectives

The objectives were to:

i. select eight plant species used to treat ear infections from a database of ethnomedicinal plant species for further phytochemical analysis and biological assays.
ii. investigate the chemical components of various plant extracts.
iii. determine the antifungal activity of acetone, hexane, methanol, and water extracts of selected plants against *Aspergillus fumigatus* and *Candida albicans*.
iv. investigate the effectiveness of the fresh and dried leaf materials against the tested fungal pathogens.
v. determine the number of antifungal compounds in different plant extracts.

1.5 Outline of study

Chapter 1 entails the general background of the importance of medicinal plants, the rationale, aim, and objectives of the study.

Chapter 2 entails a more detailed literature review of the use of medicinal plants in traditional medicine and drug production as well as a literature review of the different types of ear infections, causes, complications, and treatments. The botanical descriptions and literature review of ethnomedicinal uses of selected plants are discussed, and the conclusion is also given.

Chapter 3 deals with the methods employed during plant extraction and phytochemical analysis and their respective results, discussions, and conclusions.

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

Chapter 5 focuses on the summary and overall conclusion of the study. Recommendations for future work have been given and the references that were used for the study are listed.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter deals with a literature review on medicinal plants, traditional medicine, treatment of ear infections, antimicrobials used for ear infections, indigenous knowledge, and conservation of medicinal plants. Thorough literature reviews were conducted and compared with the previous and current findings on the uses of different plant species.

2.2 Indigenous knowledge

Indigenous knowledge is the set of knowledge, rules, standards, skills, and mentalities generated by native people of an area (Kebebew, 2016). The native people are responsible for protecting their indigenous knowledge (Emmanuel and Didier, 2012). This indigenous knowledge is transferred from one generation to the next through oral communication (Shikwambana and Mahlo, 2020). However, it is quite difficult to obtain traditional knowledge from indigenous people due to the lack of trust (Zerabruk and Yirga, 2012). There is a lack of documentation on the use of medicinal plants to combat various diseases in humans, in Limpopo and Mpumalanga provinces. Hence, it necessitated the research to focus on the medicinal usage of plant species for the treatment of ear infections and related ailments in humans.

2.3 Importance of traditional medicine

Traditional medicine refers to the knowledge, beliefs, and practices that are applied in the diagnosis, prevention, and elimination of diseases (Kebebew, 2016). Traditional health practitioners provide healthcare needs to the community based on cultural, religious, spiritual, and social beliefs (Zuma *et al.*, 2016). Additionally, South African traditional health practitioners are divided into diviners, faith healers, and herbalists (De Andrade and Ross, 2005). Many people in urban and rural areas across Africa prefer traditional medicine than conventional medicine (Rainatou *et al.*, 2021). South Africans believe that traditional medicine is a form of healthcare practice that different communities use to treat various ailments. It was found that nearly 80% of the South African population utilises traditional medicine for primary healthcare (Mahwasane *et*

al., 2013). The use of plants in traditional medicine is regarded as a form of cultural practice (Zizka *et al.*, 2015). Hence, traditional knowledge of medicinal plants is best sourced from traditional health practitioners. Moreover, traditional health practitioners also believe that medicinal plants must be respected so that they can be effective in treating ailments (Shinwari, 2010). Furthermore, many significant modern drugs have been discovered based on traditional knowledge of medicinal plants (Fabricant and Farnsworth, 2001).

2.4 Conservation of medicinal plants

The increasing population growth is a factor that may lead to the loss of medicinal plants in the wild due to agricultural practices and deforestation (Zerabruk and Yirga, 2012). Overharvesting of medicinal plants is on the rise since communities depend on medicinal plants for their healthcare needs (Chen *et al.*, 2016; Bukuluki *et al.*, 2014). According to Heywood (2017), the loss of medicinal plants decreases the chances of novel drug discovery. It is, therefore, important to preserve or document the ethnomedicinal knowledge of plants since it is at risk of diminishing (Khan *et al.*, 2018). Hence, local people and traditional health practitioners are encouraged to grow medicinal plants in their homes. More importantly, they should be taught the conservation measures that need to be taken when collecting endangered plant species.

Traditional health practitioners and local people have methods of conserving medicinal plants. They preserve medicinal plants through selective harvesting, domesticating medicinal plants, and growing medicinal plants at burial sites and sacred forests. In addition, traditional health practitioners may hide the names, uses, and locations of some medicinal plants (Kibonde, 2020). Medicinal plants can also be conserved *in situ* and *ex situ*. *In situ* conservation includes bio reserves, natural parks, and wild nurseries. *Ex situ* methods of conservation include botanic gardens, field gene banks, and seed banks (Kadam and Pawar, 2020).

2.5 Use of medicinal plants in drug production

Medicinal plants are used to complement conventional medicine due to their manifold uses (De Oliveira Melo *et al.*, 2022). They have affordable and less toxic phytochemicals with potential therapeutic properties (Otimanam *et al.*, 2022). These

phytochemicals have good molecular properties such as greater rigidity, lower mass, fewer heavy metals, and structural diversity (Mathur and Hoskins, 2017). They are in demand since they can also produce physiological and pharmacological effects in living cells (Mathur and Hoskins, 2017). Medicinal plants increase the chances of new drug discovery since they are rich in chemically diverse components. The new drugs can be in the form of pure compounds or homogenous extracts (Mustafa *et al.*, 2017). Hence, plants are used as raw materials for the extraction of active compounds that can be used in drug development (Singh, 2015). These active compounds can be found in different plant parts such as the bark, flowers, leaves, roots, seeds, and stems. Medicinal plants form the basis of many modern medicines (Khumalo *et al.*, 2022). Hence, medicinal plants are regarded as the main source of novel drugs (Srinivas *et al.*, 2013). Several drugs have been developed or isolated from medicinal plants as recorded in table 2.1 (Anand *et al.*, 2019).

Table 2. 1 Drugs derived from plants or plant products

Plant species	**Plant-derived drugs/molecules**
Allium sativum L.	Allicin (diallylthiosulfnate)
Artemisia annua L.	Artemisinin
Atropa belladonna L.	Tiotropium bromide (Spiriva®)
Camptotheca acuminate Decne.	Camptothecin
Cannabis sativa L.	Cannabidiol and tetrahydrocannabinol
Catharanthus roseus (L.) G. Don	Vinblastine and vincristine
Colchicum autumnale L.	Colchicine
Digitalis purpurea L.	Digoxin and digitoxin
Galanthus woronowii Losinsk.	Galantamine (Reminyl®)
Filipendula ulmaria (L.) Maxim	Aspirin
Papaver somniferum L.	Codeine, Papaverine and Apomorphine hydrochloride (Apokyn®)
Taxus brevifolia Nutt.	Paclitaxel (Taxol®)
Taxus brevifolia Nutt. *Taxus chinensis* (Pilg.) Rehder	Paclitaxel

2.6 Traditional treatments for ear infections

Traditional health practitioners are more knowledgeable about various traditional methods used for the treatment of different ailments in humans, including ear infections. There are different ways used by traditional healers to treat ear infections. Predominantly, the majority of traditional health practitioners use a variety of plants to treat ear infections. However, some traditional health practitioners use chicken fat, snake fat, millipede, lizard fat, water buffalo fat, sardine fat, powdered tortoise, powdered owl, and powdered monkey brain to treat ear infections (De Andrade and Ross, 2005).

2.7 Ear infections

2.7.1 Types of ear infections

Infections of the ear are clinically called otitis (Neves *et al.*, 2018). Otitis is an inflammation of the ear (Aldhaher *et al.*, 2018). Hence, an ear infection is a term denoted to the inflammation of the ear caused by infectious organisms such as bacteria, yeasts, and viruses (Hegde *et al.*, 2021). Otitis is divided into otitis externa (OE), otitis media (OM), and otitis interna (OI) for the outer, middle, and inner ear respectively (Figure 2.1) (Szmuilowicz and Young, 2019). Symptoms of ear infections include clumsiness, fever, fluid draining from the ear, fussiness, loss of appetite, ringing sounds in the ear, and vomiting (Ayub *et al.*, 2015).

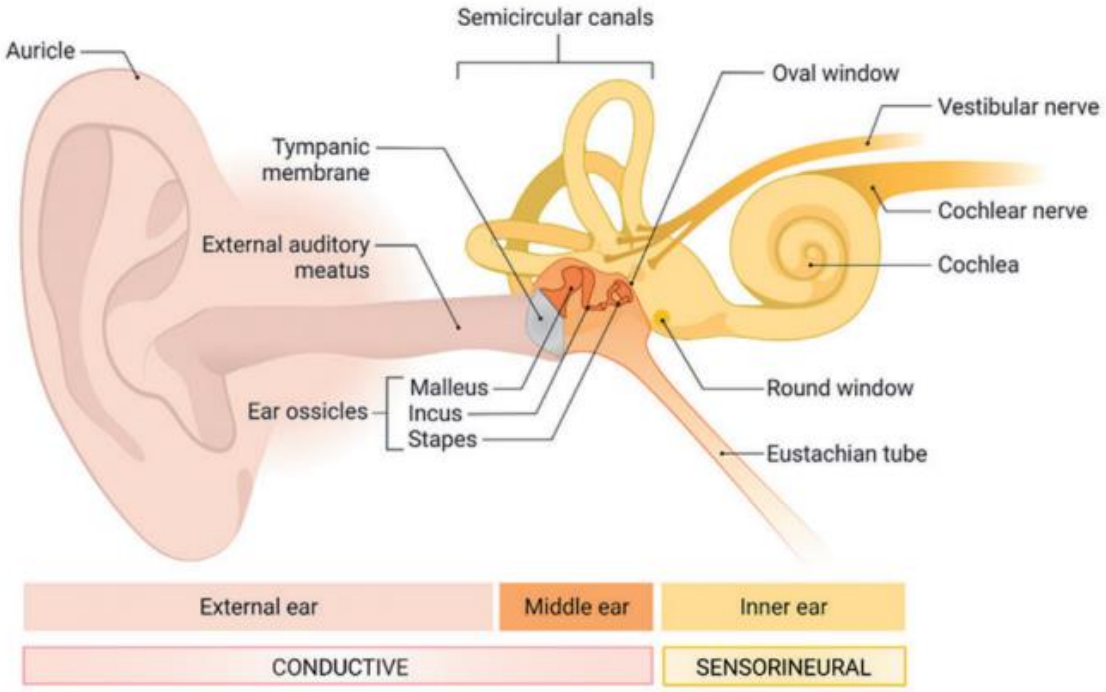

Figure 2. 1 The structure of the ear with indications of the main compartments of the ear, namely: Outer ear, middle ear, and inner ear (Payne and Wong, 2022).

OE is an inflammation of the outer ear canal that may extend to the soft tissues surrounding the outer ear (Harris and Viljoen, 2021). OE can be acute or chronic (Bulut *et al.*, 2021). Acute otitis externa (AOE) is usually caused by bacteria while chronic otitis externa (COE) is caused by fungi, allergies, or dermatitides (Osguthorpe and Nielsen, 2021). Factors that contribute to OE include excessive sweating, stress, wearing a hearing aid, and removal of ear wax (Bhat *et al.*, 2015). Symptoms of OE include severe ear pain, blood-stained otorrhea, swelling (Bhat *et al.*, 2015), severe headache, a feeling of fullness in the ear, and hearing loss (Alnawaiseh *et al.*, 2011).

OM refers to infections of the middle ear which are usually related to upper respiratory tract infections (URTIs) (Aldhaher *et al.*, 2018). The main causes of OM are bacteria and viruses (Cho *et al.*, 2015). However, in very rare cases fungal pathogens are also responsible for OM (Bennett *et al.*, 2016). These microorganisms can enter the middle ear from the external ear through a perforated tympanic membrane (Gaur and Khan, 2019). OM can be acute or chronic (Olive-Busom *et al.*, 2021). Chronic Suppurative Otitis Media (CSOM) is the continual discharge through a chronic perforation of the tympanic membrane. Acute otitis media (AOM) results from a viral infection in the

respiratory tract leading to damage to the eustachian tube (Siyad and Venkataramanan, 2021). The presence of dense or watery fluid that can cause temporary hearing loss is considered a symptom of both acute and chronic OM (Sabir *et al.*, 2021; Won *et al.*, 2021).

OI is the inflammation of the inner ear which involves the sensory organs (Ayub *et al.*, 2015). OI is mostly regarded as one of the complications following middle ear infections (Madsen *et al.*, 2001). It is also believed that OI can extend from the brain in the presence of meningitis (Madsen *et al.*, 2001). The types of microbial pathogens responsible for OI are of bacterial, fungal, and viral origin (Gheorghe *et al.*, 2021). Symptoms of OI include labyrinthitis also known as vertigo (Ayub *et al.*, 2015).

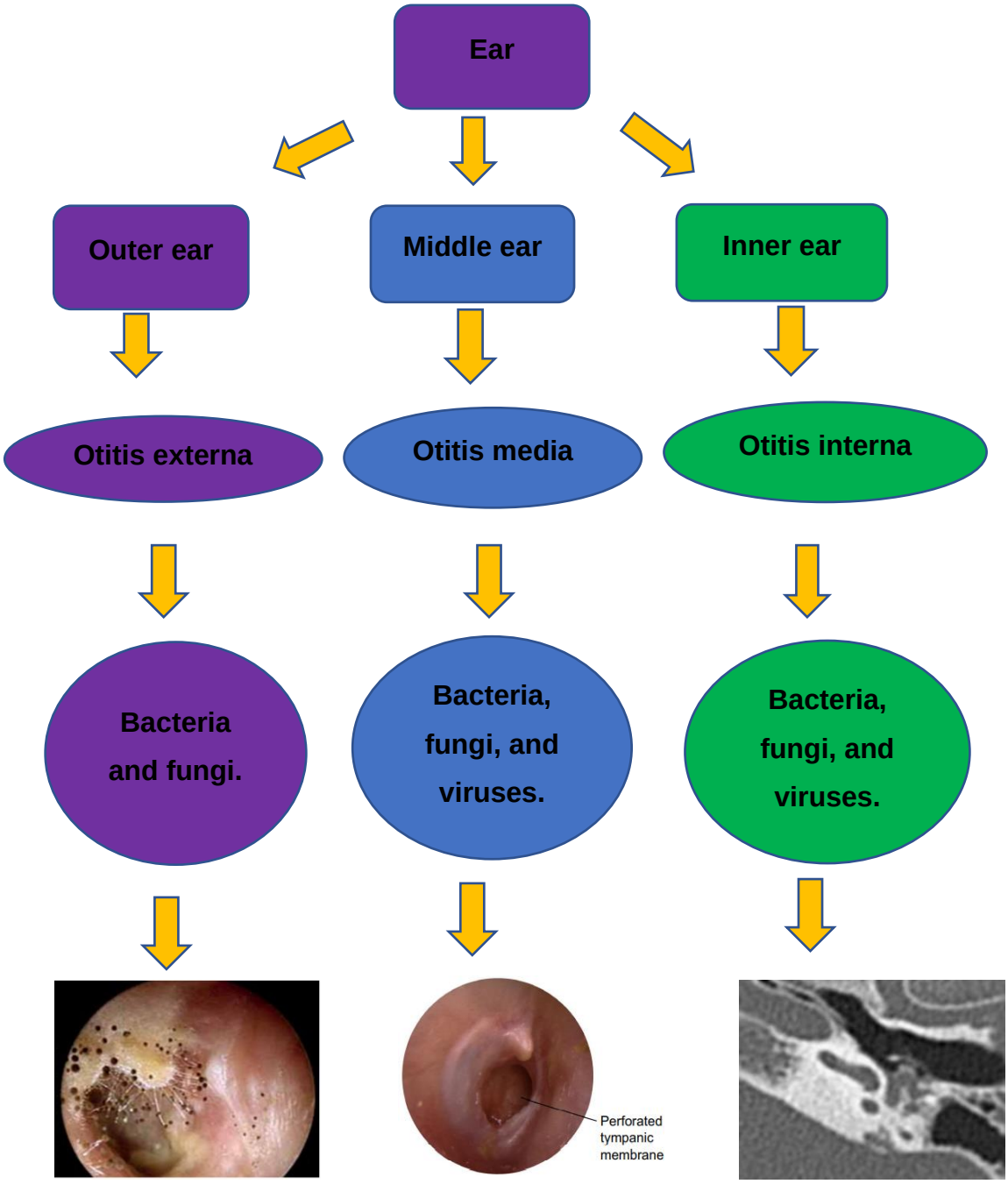

Figure 2. 2 Flow chart indicating the three parts of the ear and the type of infection. Images from left to right: Otoscopic images of OE (Osguthorpe and Nielsen, 2011), OM (Bhutta *et al.*, 2017), and 3D T2 TSE MRI scan of OI (Wu *et al.*, 2014).

2.7.2 Causes or risk factors of ear infections

Ear infections are caused by genetic and environmental factors with the most significant environmental factor being air pollution (Deng *et al.*, 2017). Some immune

responses are associated with the genetic makeup of an individual. Therefore, genetic makeup can increase the chances of ear infections in an individual (Hammaren-Malmi *et al.*, 2005). High levels of ear infections can be caused by a lack of breastfeeding, poor hygiene and nutrition, passive smoking, and inadequate healthcare (Karkos *et al.*, 2004). Breastfeeding protects children from ear infections, while formula feeding may increase the chances of developing ear infections (Curry *et al.*, 2002). Pacifier usage also increases the chances of middle ear infections (Curry *et al.*, 2002). However, the use of ear cleaners (89.1%), ear manipulation (95.6%), and swimming in the sea (9.1%), and in pools (5%) are the most important factors causing ear infections (Curry *et al.*, 2002). Rates of OM are high in winter and low during the summer season, corresponding to rates of URTIs. Kiakojuri *et al.* (2019) found that ear infections were more frequent in autumn (57.3%) than in other seasons and lowest during spring.

2.7.3 Microbial pathogens that cause ear infections

Viruses are one of the causative pathogens of ear infections, especially middle ear infections. Viruses cause inflammation in the middle ear, extending to the outer ear. They include adenovirus, coronavirus, enterovirus, types A and B influenza virus, rhinovirus, and parainfluenza virus (Ayub *et al.*, 2015). In other types of infection, such as AOM, these viruses interact with bacteria. The bacteria include *Haemophilus influenzae*, *Moraxella catarrhalis,* and *Streptococcus pneumoniae* (Pettigrew *et al.*, 2011). Martin *et al.* (2005) found that fungal pathogens associated with ear infections include *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Candida albicans,* and *Candida tropicalis*. It was found that some species of bacteria such as *Klebsiella*, *Pseudomonas*, *Staphylococcus,* and *Streptococcus* were responsible for outer ear infections (Enoz *et al.*, 2009). Additionally, inner ear infections can be caused by bacteria such as (*Neisseria meningitidis*), fungi (*Aspergillus niger*), and viruses (cytomegalovirus and zika virus) (Gheorghe *et al.*, 2021).

2.8 Extracranial complications of ear infections

2.8.1 Labyrinthitis

Labyrinthitis is one of the most common complications of OM (Trinidad *et al.*, 2005). It is caused by the spread of infection through the oval or round window of the middle ear into the inner ear, which often leads to vertigo and sensorineural hearing loss (Bennett *et al.*, 2016).

2.8.2 Facial nerve paralysis

Facial nerve paralysis can be caused by acute or chronic OM (Ciorba *et al.*, 2015). The facial nerve is responsible for the movement and sensation of the face (Gao *et al.*, 2022). It is prone to a tumour, infection, trauma, surgery, and other injuries. The recovery of the facial nerve takes time thereby affecting the psychological and social activities of people (Gao *et al.*, 2022). Facial nerve paralysis has adverse mental and physical effects such as dysarthria, impaired mastication, poor peripheral vision, keratopathy, nasal obstruction, and oral incompetence (Wang *et al.*, 2022).

2.8.3 Mastoiditis

Mastoiditis is regarded as an inflammation of the mastoid air cells, a portion of the temporal bone. The majority of the time it occurs as a consequence of AOM. Mastoiditis forms a cascade of events leading to fever, headache, lethargy, and pain behind the ear (Patel and Olympia, 2022).

2.9 Ear infections and URTIs

URTIs are infections involving the ear, nose, and throat (Njoroge and Bussmann, 2006). The ear is connected to the nose and throat through the eustachian tube which extends to the nasopharynx (Alberti, 2001). URTIs include ear infections, epiglottitis, laryngitis, pharyngitis, rhinitis, sinusitis, and tonsillitis (Bhuvaneshwari *et al.*, 2020). Ear infections, mainly OM are regarded as a complication of Covid-19 (Raad *et al.*, 2021). AOM is also a complication of upper respiratory viral infections (URVIs). It occurs in about 50% of children with URVIs caused by an adenovirus, respiratory syncytial virus, or coronavirus. In addition, about one-third occurs in those associated with the influenza virus, parainfluenza virus, enterovirus, or rhinovirus. Co-colonization of bacteria in the nasopharynx also increases the risk of AOM in children (Sawada *et al.*, 2019). Human rhinovirus is also involved in AOM in children, and it has been

reported to be associated with antibiotic failure in mixed bacterial-viral OM (Pitkaranta *et al.*, 1998).

2.10 Ear infections and HIV/AIDS

Human immunodeficiency virus (HIV) is a retrovirus that infects cells of the immune system impairing their function and resulting in the deterioration of the immune system. The virus infects and damages helper T-cells, weakening both cell-mediated and humoral immunity. Destruction of both cell-mediated and humoral immunity predisposes an individual to develop acquired immunodeficiency syndrome (AIDS) with ear, nose, and throat manifestations (Shija *et al.*, 2020). It exposes the patients to opportunistic infections such as AOM or CSOM. Furthermore, a low CD4 cell count increases the chances of middle ear fluid build-up paralleling the chances of developing OM (Obasikene *et al.*, 2014). Children that are HIV-positive are more susceptible to middle ear infections. The greater immunosuppression in HIV-positive children is related to both high rates and severity of OM (Torre III *et al.*, 2016). A study revealed that HIV-positive adults may have auditory and otologic disorders such as tinnitus (26%), vertigo (17%), hearing loss (27.5%), and middle ear abnormalities (41%) (Dawood *et al.*, 2020).

2.11 Antibiotic treatment of ear infections caused by bacteria

2.11.1 Oral antibiotics

There are broad differences in the antibiotic prescriptions given to patients suffering from ear infections. Antibiotic prescription for ear infections depends on the aetiology of each specific ear infection. For instance, neomycin is effective only against *S. aureus* and *Proteus* sp. while polymyxin B is effective against the *aeruginosa* genus and anaerobes (Ayub *et al.*, 2015). In addition, oral antibiotic treatments are recommended for immunosuppressed individuals (Viswanatha and Naseeruddin, 2011). Oral antibiotics used for the treatment of ear infections include azithromycin, amoxicillin, co-amoxiclav, benzylpenicillin, cephalosporins, clindamycin, gentamycin, and vancomycin (Pantagia *et al.*, 2021). However, amongst those that are used amoxicillin is a preferred oral antibiotic against gram-positive bacteria (Ayub *et al.*, 2015). Oral antibiotics have adverse side effects such as diarrhoea, rash, and vomiting (Hullegie *et al.*, 2021). In a study conducted by Zhang and Chen (2019), it was found that the use of oral antibiotics may increase the risk of colon cancer. Amongst the

adverse side effects, oral antibiotics also increase the chances of microorganisms developing resistance to the antibiotics (Hoskison *et al.*, 2013).

2.11.2 Topical antibiotics

Topical antibiotics are considered the most effective mode of treatment for ear infections with clinical cure rates of 80% within ten days of treatment (Mughal *et al.*, 2021). Topical antibiotics reach the pathogens on infected tissue at high concentrations than systemic antibiotics (Bhat *et al.*, 2015). Topical fluoroquinolones such as ciprofloxacin and ofloxacin are reported to be more effective as compared to any other kind of antibiotic (Vivero-Lopez *et al.*, 2021). It is also reported that ear drops containing quinolones are safer to use in the middle ear (Bhat *et al.*, 2015). Quinolones are structural derivatives of quinoline from quinine which was obtained from the bark of the *Chinchona* plant (Heeb *et al.*, 2011). However, about 58% of cultures have developed resistance to fluoroquinolones (Noonan *et al.*, 2018).

In addition, aminoglycosides such as amikacin and gentamicin are preferred to treat gram-negative bacteria through a topical application (Ayub *et al.*, 2015). Aminoglycoside ear drops are applied with care because they are toxic to the ear (ototoxic) (Bhat *et al.*, 2015). For instance, gentamicin causes adverse side effects including ataxia, imbalance, oscillating vision, hearing loss, and vertigo (Wooltorton, 2002). Examples of aminoglycoside ear drops such as kanamycin, tobramycin, streptomycin, and dihydrostreptomycin are also toxic (Matz *et al.*, 2004).

2.12 Antifungal treatment of ear infections caused by fungi

Ear infections caused by *Aspergillus* and *Candida* species are treated using topical antifungals such as clotrimazole cream (Ayub *et al.*, 2015), ketoconazole cream, and cresylate otic drops (Ho *et al.*, 2006). However, ketoconazole and other topicals such as salicylic acid and griseofulvin are less effective (Debta *et al.*, 2020). Examples of effective topicals include amphotericin B, econazole cream, and thiomersal (Debta *et al.*, 2020). Drugs such as antihistamines and decongestants (e.g., acetaminophen and phenylpropanolamine) are used. However, these have side effects including dizziness, dry mouth, headache, high blood pressure, and sedation (Ayub *et al.*, 2015). Topical formulations such as acetic acid, aluminium acetate, boric acid, silver nitrate, and topical steroids are used for AOE (Bhat *et al.*, 2015).

2.13 Antimicrobial drug resistance

Resistance to antibiotics exhibited by microbial pathogens continues to escalate due to the overuse and misuse of antibiotics (Aneja *et al.*, 2012). The use of systemic antimicrobials increases the development and recurrence of resistant organisms (Schaefer and Baugh, 2012). The failure of patients to complete their course of antibiotics also contributes to the development of resistance. A large number of antibiotic eardrops are sold in the market without a doctor's prescription which condones the development of resistant microbes (Gaur and Khan, 2019). For example, *Staphylococcus aureus* has developed a methicillin-resistant strain that is difficult to treat (Thapaliya *et al.*, 2017). Additionally, the bacterial pathogen *Escherichia coli* is resistant to ceftriaxone and amoxiclav antibiotics (Hussein, 2022). *C. albicans* is resistant to the antifungal drug fluconazole while *A. fumigatus* is resistant to triazoles (Gow *et al.*, 2022).

2.14 Fungal pathogens

2.14.1 *Aspergillus fumigatus*

A. fumigatus is an airborne fungus (Figure 2.3) resulting in high mortality rates in patients suffering from invasive fungal infections (Heinekamp *et al.*, 2015). The fungus commonly targets immunocompromised individuals thereby causing life-threatening invasive diseases (Schrettl *et al.*, 2008). *A. fumigatus* is the second most common cause of fungal infections causing various diseases such as aspergilloma, invasive aspergillosis, and allergic bronchopulmonary aspergillosis (Kaur and Singh, 2013). *A. fumigatus* is also a common pathogen isolated from OE (Prasad *et al.*, 2014).

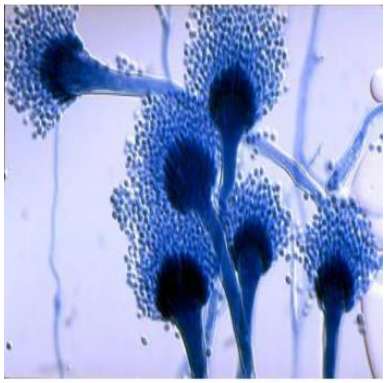

Figure 2. 3 Microscopic image of *Aspergillus fumigatus* (Adhavan, 2020).

2.14.2 *Candida albicans*

C. albicans is a dimorphic fungus (Figure 2.4) that can grow as yeast and in other cases as hyphae in response to external stimuli. However, there are other morphological forms of *C. albicans* including the opaque, the pseudohyphal cell, and the chlamydospore (Whiteway and Bachewich, 2007). It is part of the human microbiome inhabiting the gastrointestinal tract, reproductive tract, oral cavity, and skin of most humans. In healthy individuals, it is often harmless but alterations in the host microbiota can enable *C. albicans* to overgrow and cause an infection (Nobile and Johnson, 2015). *C. albicans* causes two major types of infections in humans known as superficial infections (oral or vaginal candidiasis), and life-threatening systemic infections (Mayer *et al.*, 2013). In OE, *C. albicans* is the most common fungal pathogen (Prasad *et al.*, 2014).

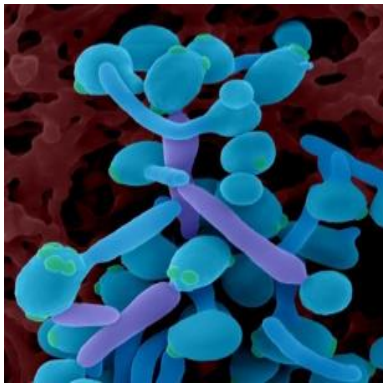

Figure 2. 4 Microscopic image of *Candida albicans* (Tyavambiza, 2018)

2.15 Bacterial pathogens

2.15.1 *Escherichia coli*

E. coli is a normal inhabitant of the animal and human gut (Figure 2.5). This bacterium can be found in soil, vegetation, and water. It is one of the common causes of bloodstream infections, OM, urinary tract infections, and wounds. The prevalence and vulnerability of *E. coli* show geographic variations in various environments and populations (Kibret and Aber, 2011).

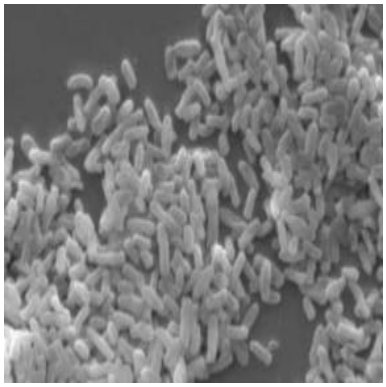

Figure 2. 5 Microscopic image of *Escherichia coli* (Singh *et al.*, 2015).

2.15.2 *Pseudomonas aeruginosa*

P. aeruginosa is a gram-negative rod-shaped bacterium (Figure 2.6) with a wide distribution in diverse environments, including water, soil, plants, animals, and a tendency to be present in locations associated with human activity (Spernovasilis *et al.*, 2021). *P. aeruginosa* causes opportunistic infections in both animals and humans. According to the World Health Organization (WHO), *P. aeruginosa* is resistant to the currently available drugs such as fluoroquinolones and penicillins (Langendonk *et al.*, 2021). Infections of the ear skin caused by *P. aeruginosa* range from mild to life-threatening which include OE, necrotizing otitis externa (NOE), and perichondritis (Spernovasilis *et al.*, 2021).

Figure 2. 6 Microscopic image of *Pseudomonas aeruginosa* (Tyavambiza, 2018).

2.15.3 *Staphylococcus aureus*

S. aureus (Figure 2.7) is both a commensal bacterium and a human pathogen (Tong *et al.*, 2015). *S*. *aureus*, a gram-positive bacterium, asymptomatically colonizes approximately 30% of the population and can infect nearly every tissue in the body. *S*.

aureus readily adapts its metabolic and virulence responses in different tissues, causing superficial (e.g., folliculitis) and invasive infections (e.g., osteomyelitis) (Ford *et al.*, 2021). *S. aureus* is the most invasive species and aetiological agent of human and animal maladies, including skin infections, abscesses, food poisoning, toxic shock syndrome, septicemia, endocarditis, and pneumonia. *S. aureus* is one of the most prominent causes of nosocomial- and community-acquired bacterial infections worldwide (Malachowa and DeLeo, 2010). Methicillin-resistant *S*. *aureus* (MRSA) constitutes the major threat among antibiotic-resistant agents that cause deaths (Sharaf *et al.*, 2021).

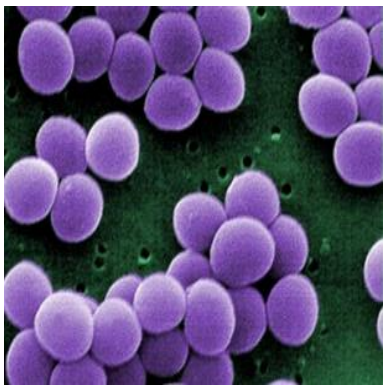

Figure 2. 7 Microscopic image of *Staphylococcus aureus* (Tyavambiza, 2018).

2.16 Botanical descriptions of the plant species used in the study

Eight plant species used for the treatment of ear infections in Capricorn District, Limpopo province and Ehlanzeni District, Mpumalanga province 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) and Mpumalanga province (Ehlanzeni District). The pictures of the selected plant species were taken from five villages: Gottenburg, Hlalakahle, Hlavekisa, Hluvukani, and Thorndale (Figure 2.8–Figure 2.14).

2.16.1 *Carpobrotus edulis* L.

Carpobrotus edulis L. with the synonym *Mesembryanthemum edule* L. belongs to the Aizoaceae family (Figure 2.8). It has several common names including sour fig (English), suurvy (Afrikaans), umgongozi (Zulu), and igcukuma (Xhosa) (Rocha *et al.*, 2017). The herb is native to South Africa and commonly found in the Eastern Cape,

Northern Cape, KwaZulu-Natal, Free State, and Western Cape provinces (Omoruyi *et al.*, 2020). The plant thrives on sandy soils and dunes but can be found in all soil types in all provinces of South Africa (van der Watt and Pretorius, 2001). It is a ground-cover succulent plant that occupies coastal ecosystems with Mediterranean climatic conditions (Hafsa *et al.*, 2016). The plant has green-coloured leaves that grow up to 10.8cm in length (Omoruyi et al., 2012) that are thin, blade-like, and succulent (Chokoe *et al.*, 2008). The leaves can also have red, orange, or purple-coloured margins (Akinyede *et al.*, 2020). This perennial herb is resistant to drought and wind conditions (Omoruyi *et al.*, 2020).

2.16.1.1 Ethnomedicinal uses

C. edulis has been used as a form of traditional medicine in South Africa. The leaves, flowers, or fruits of the plant are used raw, or boiled in water and administered orally to treat various bacterial and fungal infections (Mudimba and Nguta, 2019). The Khoi-Khoi and San use the leaf juice to treat diarrhoea and tuberculosis, as a mouthwash for gum infections and sore throat, or applied topically to burn wounds. Traditional health practitioners in the Eastern Cape province also use the plant to treat constipation, diabetes mellitus, high blood pressure, and intestinal worms (Rocha *et al.*, 2017). The leaf juice of *C. edulis* has been recorded for its use to treat sinusitis, infantile eczema, and internal chest conditions, as well as in soothing bites caused by spiders, ticks, and blue bottle stings (Van der Watt and Pretorius, 2001). Other authors have documented that *C. edulis* is used in traditional medicine to treat blood pressure, chilblains, earache, toothache, and vaginal and oral thrush (Akinyede *et al.*, 2020).

Figure 2. 8 Images of *Carpobrotus edulis* L. (Bilomu ra ku nava) leaves (A) and flower (B).

2.16.2 *Cotyledon orbiculata* L.

Cotyledon orbiculata L. belongs to the family of Crassulaceae (Amabeoku *et al.*, 2007). It is a succulent shrub with woody branches and thick and fleshy leaves that may vary from green to grey, often with a red line around the leaf margins and covered with a waxy layer on the surface (Figure 2.9). The leaves are obovate to narrowly ovoid-shaped. The flowers are yellow to orange-red coloured, usually hanging, tubular and bell-shaped, carried in clusters on the ends of an elongated flower stalk. It is found in Angola, Lesotho, Mozambique, Namibia, South Africa, and Swaziland in sandy or rocky soils, riverbanks, grassland, scrub, fynbos, and karoo biomes at an altitude ranging from 50 m to 3000 m above sea level (Maroyi, 2019). It has been listed in the Red Data List of South Africa due to its over-use (Kumari *et al.*, 2016).

2.16.2.1 Ethnomedicinal uses

The leaves are used to treat corn and warts while the juice from the leaves is used to treat ear and toothaches, and as a hot poultice to treat boils and inflammation (Amabeoku *et al.*, 2007). The traditional use of *C. orbiculata* includes the treatment of epilepsy using juice from the leaves (Stafford *et al.*, 2005). In Lesotho, the plant is listed as one of the medicinal plants used to treat skin infections as well as boils and mouth ulcers (Moteetee and Kose, 2017). The leaves, leaf sap, and roots are used to treat sexually transmitted diseases such as gonorrhoea, syphilis, and venereal diseases (Maroyi, 2019). The plant is sun-dried and ground into fine powder by the Bapedi people of South Africa to wake fainted individuals whereas fresh leaves are crushed, and the juice is used to relieve aching feet or sniffed to induce sneezing as a way to treat various ailments (Mogale *et al.*, 2019). The leaves and roots are also used to treat chronic diarrhoea (Masafu *et al.*, 2016). It is used to treat haemorrhoids and skin rashes (Maroyi, 2019).

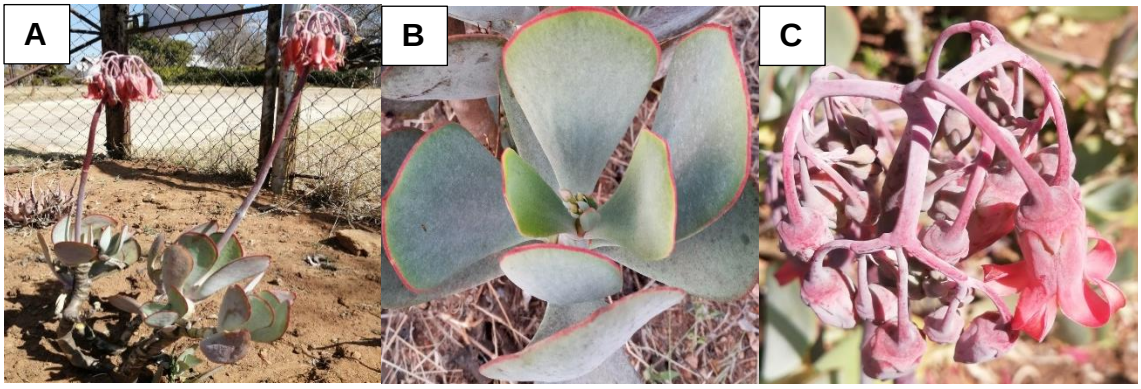

Figure 2. 9 *Cotyledon orbiculata* L. (Bilomu ra tindleve) whole plant (A), leaves (B) and flowers (C).

2.16.3 *Dichrostachys cinerea* (L.) Wight & Arn.

Dichrostachys cinerea (L.) Wight & Arn. belongs to the Fabaceae plant family (Figure 2.10). It is commonly referred to as a sickle bush, Christmas tree, or Chinese lantern tree (Abdullahi and Yusha'u, 2021). The plant is indigenous to South Africa (Abou Zeid *et al.*, 2009) and found in tropical and subtropical areas (Mishra *et al.*, 2009). *D. cinerea* is a thorny shrub that can grow up to 8 metres with thorns that can also bear leaves. The inflorescence consists of a large amount of pink, white, or mauve filaments, which derive from the leaf axes, on approximately 4 cm stems. The flowers may produce a light fragrance; however, the sterile flowers are located under the fertile ones and each bearing a yellow stamen (Saez and Alfayate, 2020).

2.16.3.1 Ethnomedicinal uses

Fresh leaves of the plant are chewed to relieve diarrhoea, earache, as well as toothache. The leaves and bark are used to heal wounds while an infusion of the roots is used to treat abdominal pains, cough, and pneumonia. Powdered roots are also sniffed to stop nose bleeds, while leaves and roots are smoked to relieve head colds, epilepsy, and tuberculosis (Abdullahi and Yusha'u, 2021). The bark of the plant is used to treat dysentery, elephantiasis, and toothache while the leaves are used to treat boils, gonorrhoea, and as a laxative. The plant has also been used as an aphrodisiac and as an astringent for scorpion bites (Lavanya and Ambikapathy, 2016). It is also reported that the tender shoots of the plants are applied to the eye to treat eye infections while the roots are used to treat calculi, joint pains, renal troubles, and the uterus (Jayakumari *et al.*, 2012). Mishra *et al.* (2009) reported that the plant is used

as a vermifuge and to treat headaches, leprosy, and syphilis. A decoction of the plant roots is used to treat snakebite victims (Mishal *et al.*, 2008).

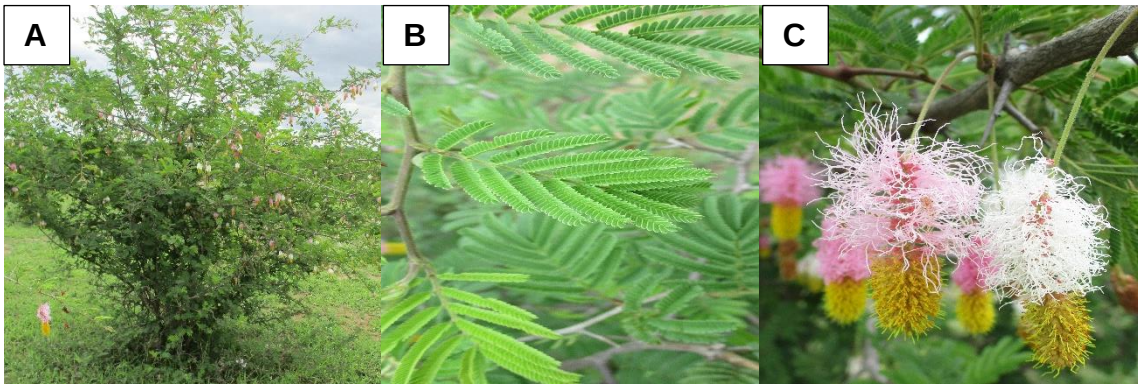

Figure 2. 10 *Dichrostachys cinerea* (L.) Wight & Arn. (Ndzhenga) whole plant (A), leaves (B), and flowers (C).

2.16.4 *Erythrina lysistemon* Hutch.

Erythrina is a genus within the family Fabaceae and comprises over 110 species. The species are found in tropical and subtropical regions across the globe (Figure 2.11). They can be trees, herbs, or shrubs characterized by orange or red-coloured flowers. *E. lysistemon* is a deciduous tree that reaches up to 12 metres in height (Juma and Majinda, 2004). *E. lysistemon* has a spreading crown and red flowers. It is widely distributed from the North of Tanzania to the Eastern Cape of South Africa (Dao *et al.*, 2009). *E. lysistemon* is one of the species of *Erythrina* indigenous to South Africa (Pillay *et al.*, 2001).

2.16.4.1 Ethnomedicinal uses

Species of *Erythrina* are largely used in folk medicine in many parts of the world (Nde *et al.*, 2012) to treat various ailments such as female infertility, stomach pain, and gonorrhoea (El-Masry *et al.*, 2002). In South Africa, the plant is used in traditional medicine to treat abscesses, arthritis, earache, and wounds (Dao *et al.*, 2009), whereas in India the stem bark paste is used to treat asthma, rheumatism, stomach-ache, dysentery, eczema, dermatitis, and other skin infections (Akter *et al.*, 2016). The Xhosa and Zulu people of South Africa use the leaves and bark to treat abscesses, bronchitis, earache, purulent sores, respiratory infections, sprains, strained ligaments, toothache, tuberculosis and to disinfect wounds (Pillay *et al.*, 2001). The plant is also known for the treatment of eye infections using its leaves (Akter *et al.*, 2016). The plant

is used in folk medicine by Asian, African, and South Americans as a tranquilizer and anti-anxiety as well as for the treatment of amenorrhea, dizziness, eye trouble, headache, sterility, asthma, malaria, epilepsy, liver disorder, and inflammation (Son and Elshamy, 2021).

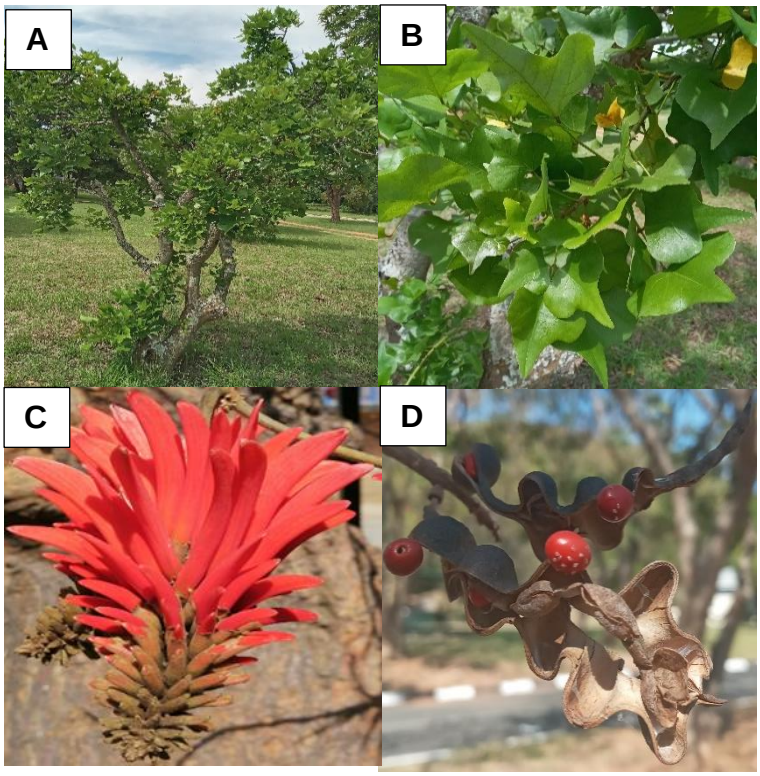

Figure 2. 11 *Erythrina lysistemon* Hutch. (Muvale/ Nsisimbana) whole plant (A), leaves (B), flowers (C), and pods (D).

2.16.5 *Flacourtia indica* (Burm. f.) Merr.

Flacourtia indica (Burm. f.) Merr. is a branched, deciduous, and dioecious shrub (Figure 2.12) or small tree usually up to 5 m high belonging to the Salicaceae family, formerly known as Flacourtiaceae (Nguyen *et al.*, 2021). The plant is native to African and Asian countries and is commonly known as the Indian plum, Governor's plum, and Madagascar plum (Nguyen *et al.*, 2021). It is indigenous to the Indian Peninsula (Nandhini *et al.*, 2019). The plant is characterized by thorny branches and crenate, deciduous, and obovate or ovately shaped leaves (Tiwari, 2017). It also comprises small, yellow-coloured flowers either in a simple or compound raceme. The fruits of the plant are edible dark red or black-coloured indehiscent drupes (Tiwari, 2017).

2.16.5.1 Ethnomedicinal uses

All parts of the plant are used globally in traditional medicine to treat a wide variety of ailments. The leaves are used in Ayurvedic medicine to treat asthma, bronchitis, cough, phthisis, and pneumonia whereas, in Bengal, the leaves are known to be effective against snakebites as well as the treatment of schistosomiasis, malaria, and diarrhoea. The juice of the fresh leaves is used to treat fevers, dysentery, diarrhoea, and intestinal worms. Leaf decoctions are used for gynaecological disorders and hydroceles (Nguyen *et al.*, 2021). The fruits of the plant are used to treat jaundice and enlarged spleen, while the seeds combined with turmeric are used to prevent rheumatic pain, the bark is applied to the body to treat intermittent fever and the root is used to treat nephritic colic (Kundu *et al.*, 2013). The leaves of *F. indica* are used to treat measles whereas the bark is used to treat chicken pox (Kigen *et al.*, 2016).

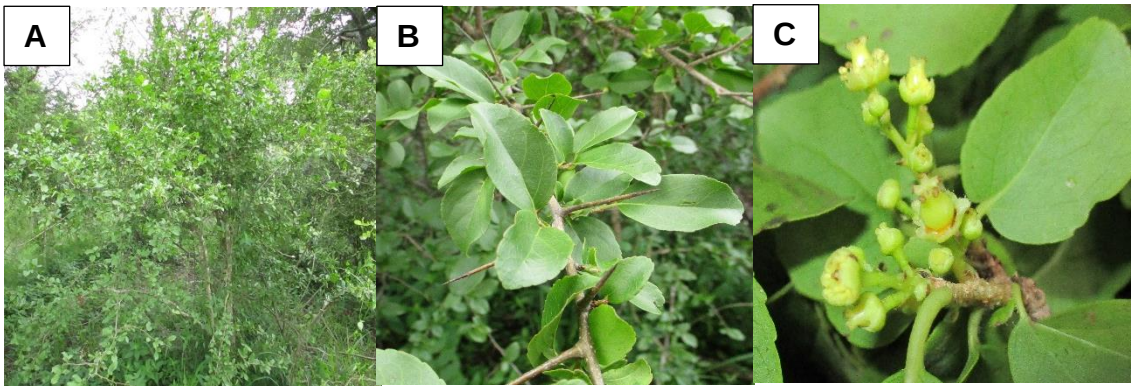

Figure 2. 12 *Flacourtia indica* (Burm. f.) Merr. (Xivambula) whole plant (A), leaves (B), and flowers (C).

2.16.6 *Psidium guajava* L.

Psidium guajava L. belongs to the plant family of Myrtaceae and subfamily Myrtoideae (Morais-Braga *et al.*, 2016) (Figure 2.13). It is commonly referred to as guava (Fernandes *et al.*, 2014). It is native to tropical America (Sanches *et al.*, 2005) and Mexico (Soliman *et al.*, 2016). However, in South Africa *P. guajava* is regarded as an invasive species (Ruwanza and Thondhlana, 2022). It flourishes in all tropical and subtropical (Khadhri *et al.*, 2014) areas but it can adapt to varying climatic conditions although it prefers dry climates (Soliman *et al.*, 2016). It grows as an evergreen shrub or a small tree that can reach up to 15 m in height (Jaiarj *et al.*, 1999). However, the plant is capable of growing up to 1500 m in height (Ekeleme *et al.*, 2017).

It is characterized by a thin, smooth, copper-coloured bark that flakes off, showing a greenish layer beneath (Kamath *et al.*, 2008). The guava fruit is exotic and can either be round, ovoid, or pear-shaped with an average diameter that ranges from 4-10cm (Ngbolua *et al.*, 2018) and a weight that can be from as little as one ounce to as much as one pound (Rishika and Sharma, 2012). It is a berry-type of fruit (Morais-Braga *et al.*, 2016) that is yellow with pink flesh and tan-coloured seeds (Joseph and Priya, 2011). It produces white flowers that are about 1 inch in diameter, borne singly or in small clusters in axils of leaves of recent growth (Rishika and Sharma, 2012). It has dark green leaves (Kumar *et al.*, 2021) that can grow up to 6 inches in length and 2 inches wide (Biswas *et al.*, 2013).

2.16.6.1 Ethnomedicinal uses

The bark, fruits, leaves, and roots of the plant all possess medicinal value in traditional medicine (Rishika and Sharma, 2012). The plant has been long used in traditional medicine to treat various ailments in many parts of the world. In South Africa, *P. guajava* leaves are used to treat flu (Ruwanza and Thodhlan, 2022) and diabetes (Mbara *et al.*, 2022). The decoction of the bark and leaves is used to treat diarrhoea, dysentery, vomiting, and sore throats, and to regulate menstrual cycles in India (Kamath *et al.*, 2008). The bark is used to treat diarrhoea in children whereas the leaves are used to relieve coughs, pulmonary disorders, wounds, and ulcers, while the fruit is used as a tonic, laxative, and anthelmintic (Khadri *et al.*, 2014). The leaves are chewed to relieve toothache while an aqueous extract of the leaves is used to lower blood glucose levels in individuals with diabetes (Soliman *et al.*, 2016). The whole plant is used to treat female-related disorders such as dysmenorrhoea, miscarriages, uterine bleeding, and premature labour (Rishika and Sharma, 2012). In America, plant leaf extracts are used in herbal formulas for bowel health, and to induce weight loss, while in Brazil the fruit and leaves are used to treat anorexia, cholera, diarrhoea, digestive problems, dysentery, gastric insufficiency, skin problems, sore throat, ulcers, and vaginal discharge (Soliman *et al.*, 2016).

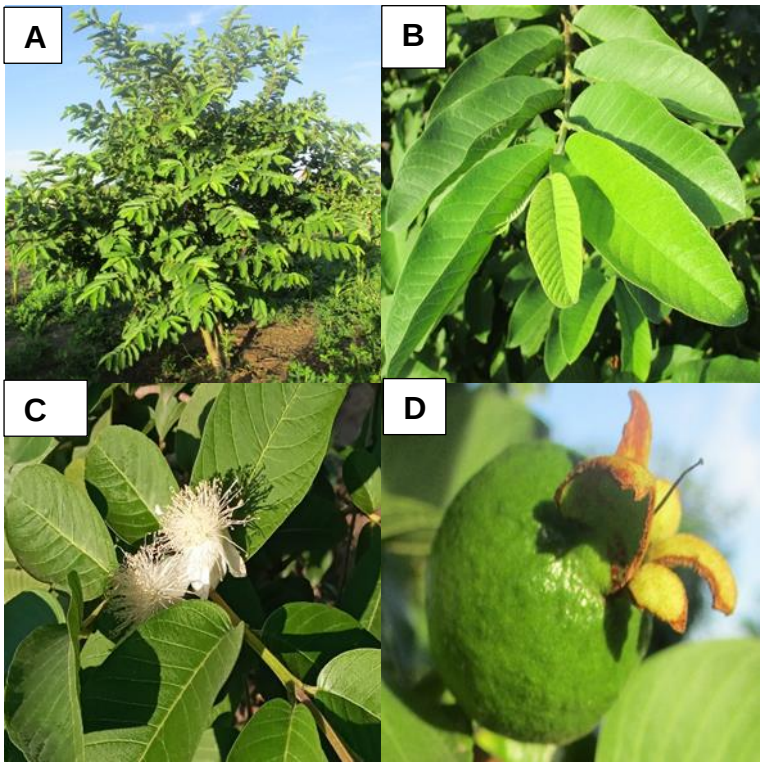

Figure 2. 13 *Psidium guajava* L. (Mugwava) whole plant (A), leaves (B), flowers (C), and fruit (D).

2.16.7 *Ricinus communis* L.

Ricinus communis L. is a member of the Euphorbiaceae family (Inayor and Ibraheem, 2014) (Figure 2.14). The plant is commonly known as the castor plant or palm of Christ (Jena and Gupta, 2012). According to Manoj (2017), the plant is an evergreen herbaceous or semi-woody, large shrub or small tree that reaches 5 m in height. It is a fast-growing, suckering perennial herb oilseed crop (Abdul *et al.*, 2018). The plant is indigenous to Africa (Shobha *et al.*, 2019) but is an occupant of tropical and temperate regions of the world (Sandhu *et al.*, 2014). It is resistant to droughts and frost (Nemudzivhadi and Masoko, 2015). It thrives in clay and sandy soils (Vasco-Leal *et al.*, 2021). According to Jain and Nafis (2011), the plant is known to be highly poisonous all over the world. Its phenotypic diversity is wide, with variations in growth habits, the colour of the leaves, stem, and seeds, and oil content (Riberio *et al.*, 2016).

Flowers are monoecious, large, and arranged on the thick rachis of an oblong panicle. The fruit is greenish, deeply grooved, tricoccus capsules, dehiscing longitudinally and septically into six valves. It has smooth seeds that are flattened and oval (Jeyaseelan and Jashothan, 2012). When ripe, the capsule, which contains three seeds, becomes

hard and cracked (Sbihi *et al.*, 2018). Leaves are alternate, split like a palm, ovate lobes, sharp tips, green or brown on the surface, and sharp saw teeth on the edge (Park, 2018). The colour of leaves changes from light green to dark red in relation to the amount of anthocyanin pigmentation present (Sbihi *et al.*, 2018). It is a xerophyte and heliophile plant with a deep tap root system (Riberio *et al.*, 2016).

2.16.7.1 Ethnomedicinal uses

All parts of the plant including the flowers, fruits, leaves, stems, and roots are known for their medicinal use in traditional medicine (Inayor and Ibraheem, 2014). Although all parts of the plant have medicinal uses, the most used part of the plant in traditional medicine is the leaf (Santos *et al.*, 2018). The leaves are used by nursing mothers to increase the flow of milk (Kota and Manthri, 2011). They are used in the form of a poultice on sores, boils, and swellings (Kumar, 2017). In addition, the leaves are used against viral infections, biliousness, burns, earache, malaria, and night blindness while the stem is used for the treatment of cancer and hypoglycemia (Ramanjaneyulu *et al.*, 2017). The leaves and roots extracts as well as the seed oil are used in traditional medicine as a laxative and to cure abscesses, backache, dropsy (edema), headache, hypoglycemia, inflammation, liver disorder, rheumatism, warts, and ringworms (Inayor and Ibraheem, 2014). In South Africa, people from Ga-Mashashane inhale steamy vapour from leaves to treat flu and sexually transmitted infections (Papo *et al.*, 2022).

The hot water extract of dried aerial parts is used as an anthelmintic, a bronchodilator, and a purgative (Sbihi *et al.*, 2018). The flowers are used to ease glandular and vaginal pain whereas the fruits are used for treating tumours, piles, liver, and spleen diseases. The bark is used as purgative, abortion, ascites, asthma, bronchitis, carination (expulsion of gas from stomach and intestines), hypoglycemia, leprosy, pains, rectum, and rheumatism diseases. Castor oil is also used for combating fungal infections, relieving menstrual pains when applied to the lower abdomen, and reducing stretch marks (Masoko and Nemudzivhadi, 2015).

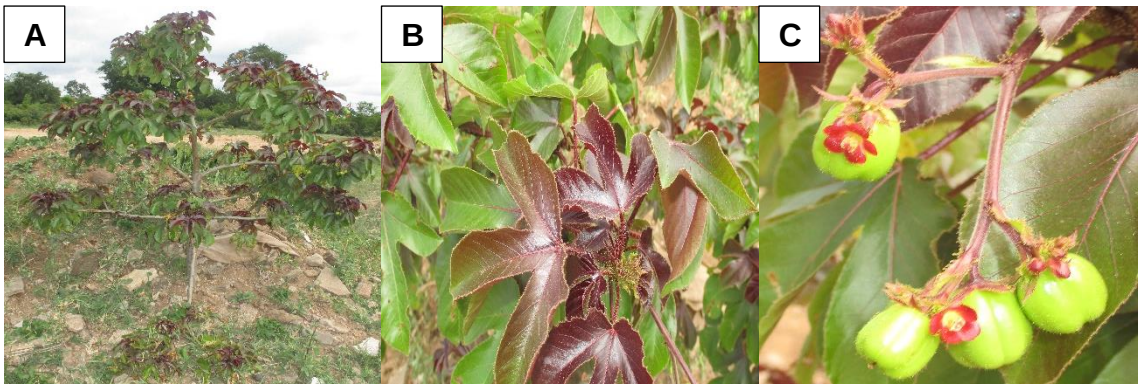

Figure 2. 14 *Ricinus communis* L. (Nhlampfurha) whole plant (A), leaves (B), flowers and fruit (C).

2.16.8 *Sansevieria hyacinthoides* (L.) Druce

Sansevieria hyacinthoides (L.) Druce belongs to the Asparagaceae family of plants (Takawira-Nyenya *et al.*, 2018) (Figure 2.15). *S. hyacinthoides* is a South African indigenous plant (Van der Burg *et al.*, 2012). *Sansevieria* is a genus of xerophytic perennial herbs that occur mostly in dry tropical and subtropical habitats (Takawira-Nyenya and Stedje, 2011). The plant is a stemless, evergreen succulent herb that can reach up to 60 cm in height (Mwachala and Mbugua, 2007). The plant is commonly called mother-in-law's tongue, devil's tongue, jinn's tongue, bow string hemp, snake plant, or snake tongue (Islam *et al.*, 2020).

2.16.8.1 Ethnomedicinal uses

The leaves of the plant are used in traditional medicine to treat intestinal worms (McGaw *et al.*, 2000). Leaves and rhizomes are used to treat earache, stomach-ache, toothache, haemorrhoids, ulcers, diarrhoea, and intestinal parasites whereas in general *Sansevieria* species are known for the treatment of chest diseases and as a cough remedy in traditional medicine (Nielsen *et al.*, 2012). In the traditional medical treatment of earaches and haemorrhoids, leaves are heated, and the warm juice is squeezed into the affected area. The leaf sap is also applied directly to infected sores, cuts, and grazes (Philip *et al.*, 2011). Roots and rhizomes of the plant are used in the treatment of blood disorders, heart diseases, fever, gonorrhoea, itch, leprosy, rheumatism, and glandular enlargements (Sultana *et al.*, 2011).

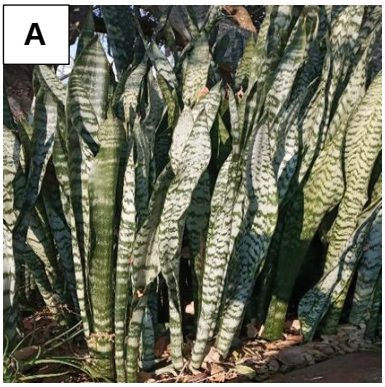

Figure 2. 15 *Sansevieria hyacinthoides* (L.) Druce (Mhangane) whole plant (A).

2.17 Conclusion

There are diverse types of ear infections that pose a health threat to the lives of humans. To date, there are a lot of antimicrobial drugs employed against ear infections. However, this threat may continue to be on the rise due to antimicrobial drug resistance developed by many microbial pathogens. The traditional knowledge of medicinal plants used by locals and traditional health practitioners in treating ear infections may partly assist in dealing with antimicrobial drug resistance. The medicinal plants selected in this study prove to be of significant value in traditional medicine due to their manifold uses in treating various ailments and diseases in South Africa and the world collectively. Additionally, previous studies conducted on these plants indicate their potential as a source of safe medicine.

CHAPTER 3

PLANT EXTRACTION AND PHYTOCHEMICAL ANALYSIS OF SELECTED PLANT SPECIES

3.1 Introduction

The fresh and dried leaves of eight selected plant species as shown in chapter 2 were extracted using solvents of varying polarities. Thin-layer chromatography (TLC) was used to investigate the chemical components present in different plant extracts.

Plants produce a wide array of chemical compounds that have antimicrobial properties. These chemical compounds are divided into primary or secondary metabolites with respect to their function (Erb and Kliebenstein, 2020). Primary metabolites are vital for the growth and development of plants (Fernie and Pichersky, 2015). However, plants synthesize secondary metabolites in response to abiotic and biotic factors and for defence against these factors (Neilson *et al.*, 2013). They are synthesized in all plant organs such as the bark, flowers, leaves, roots, seeds, and stems (Chiocchio *et al.*, 2021). Secondary metabolites include alkaloids, curcumins, flavonoids, glucosides, lignans, phenolics, plant steroids, saponins, and terpenes (Alamgir, 2018). Furthermore, these secondary metabolites are potential candidates for antimicrobial or therapeutic drugs (Anand *et al.*, 2019).

Extracting medicinally active compounds from plant tissues requires the use of selective solvents following a standard procedure (Handa, 2008). The yield of plant extraction highly depends on the method of extraction and the solvent used for extraction. A suitable solvent should have the ability to quickly absorb and preserve the extract, evaporate at low heat, not dissociate the compounds, and have low toxicity (Muhamad *et al.*, 2017). A solvent with similar polarity to the solute is most likely to dissolve the solute (Altemimi *et al.*, 2017). Hence, the extraction of polar compounds is made using polar solvents such as methanol and water, while non-polar solvents such as dichloromethane and hexane are used to extract non-polar compounds (Abubakar and Haque, 2020).

There are various conventional methods for plant extraction such as maceration, infusion, decoction, percolation, Soxhlet, or hot continuous extraction (Abubakar and Haque, 2020). Conventional methods were used from ancient times by indigenous people, and they form a basis for new advanced extraction techniques (Belwal *et al.*, 2018). Among the most advanced extraction methods are microwave-assisted extraction, ultrasound-assisted extraction, accelerated solvent extraction, and supercritical fluid extraction (Azwanida, 2015). However, in this study, the infusion method of extraction was used. The infusion method involves soaking the powdered plant material in a cold or hot solvent for a short period (Abubakar and Haque, 2020).

The phytochemical analysis involves the use of different tests to determine the presence and absence of certain chemical substances. TLC is a rapid and sensitive method for determining the composition of a mixture. More importantly, it is used to verify the identity and purity of a compound, and to determine the compositions of different extraction solvents (Cai, 2014). Following TLC, various coloured chemical constituents should be visible after visualizing under ultraviolet light or spraying with a reagent spray (Velavan, 2015). Advantages of TLC include faster runs, better separation of compounds, and choice between different adsorbents. Hence, it is one of the most useful tools for assaying phytochemicals (Kumar *et al.*, 2013).

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. The database has sixty different plants used as medicine for ear-related ailments. In the proposed study, the eight most frequently used plant species were selected based on the availability and accessibility of the plant parts used. The plant species were collected from five different villages (Gottenburg, Hlalakahle, Hlavekisa, Hluvukani, and Thorndale) in Bushbuckridge Local Municipality, Ehlanzeni District, Mpumalanga province, South Africa. For each selected plant, only the plant part used to treat ear infections was collected. Collected plants were identified using literature and the Larry

Leach herbarium at the University of Limpopo. Voucher specimens were deposited at the herbarium.

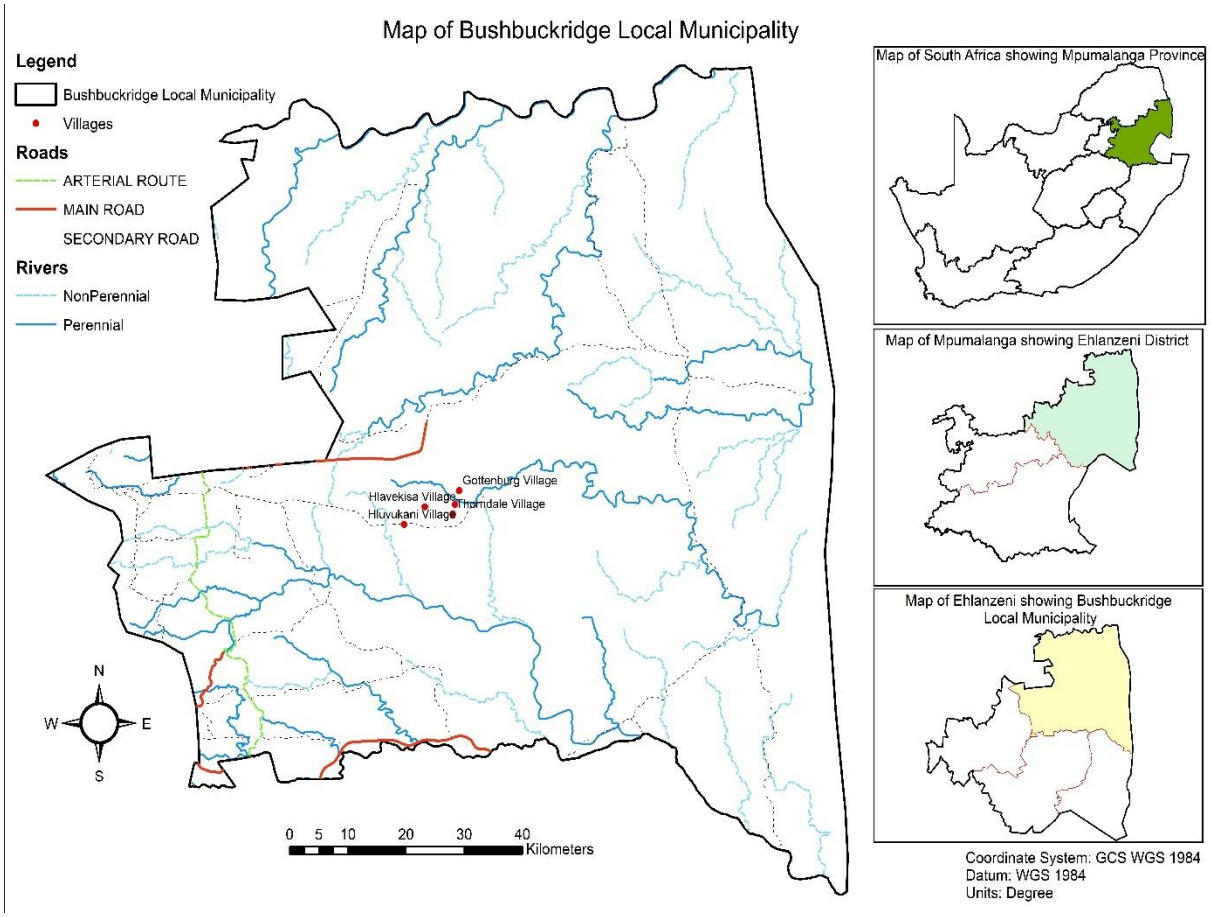

Figure 3. 1 A map showing study areas in Bushbuckridge Local Municipality.

3.2.2 Extraction of dried plant material

Drying of plant materials (bark, leaves, or roots) for each plant was done at room temperature depending on the plant part that is recorded on the database. The dried plant material was ground into fine particles using a grinding machine (Trapp TRF 400 animal ration shredder hammer mill foliage, Jaraqua do sul-sc, Brazil). Several solvents of varying polarities including acetone, hexane, methanol, and water were used to extract 4 g of plant material at a ratio of 1 g: 10 ml. The plant extracts were centrifuged at 3500 rpm for 5 minutes. The supernatants were filtered using Whatman no. 1 filter paper, into labelled weighed glass beakers. Extraction was repeated three times and the extracts were combined. After centrifuging three times the pellet was discarded. A fume hood at room temperature was used to evaporate the solvents. A freeze dryer was used to dry aqueous extracts. Prior to phytochemical analysis and

bioassay testing, crude extracts were re-dissolved in acetone since they can dissolve polar and non-polar compounds, and it has been proven to be non-toxic to microorganisms (Eloff, 1998).

3.2.3 Extraction of fresh plant material.

The leaves of the plants were rinsed, wiped till dry, and chopped into small pieces. Each 40 g of plant material was ground using a pestle and mortar and extracted with 400 ml solvents of varying polarities such as acetone, hexane, methanol, and water. The extracts were filtered into labelled pre-weighed glass vials using a Whatman no 1 filter paper. The process was repeated three times and the extracts were combined. The ground material was discarded. The solvents were removed under a stream of cold air in a fume hood at room temperature. Aqueous extracts were stored in a freezer until use. Prior to phytochemical analysis and bioassay testing, crude extracts were re-dissolved in acetone. Aqueous extracts were thawed at room temperature prior to phytochemical analysis and biological assays.

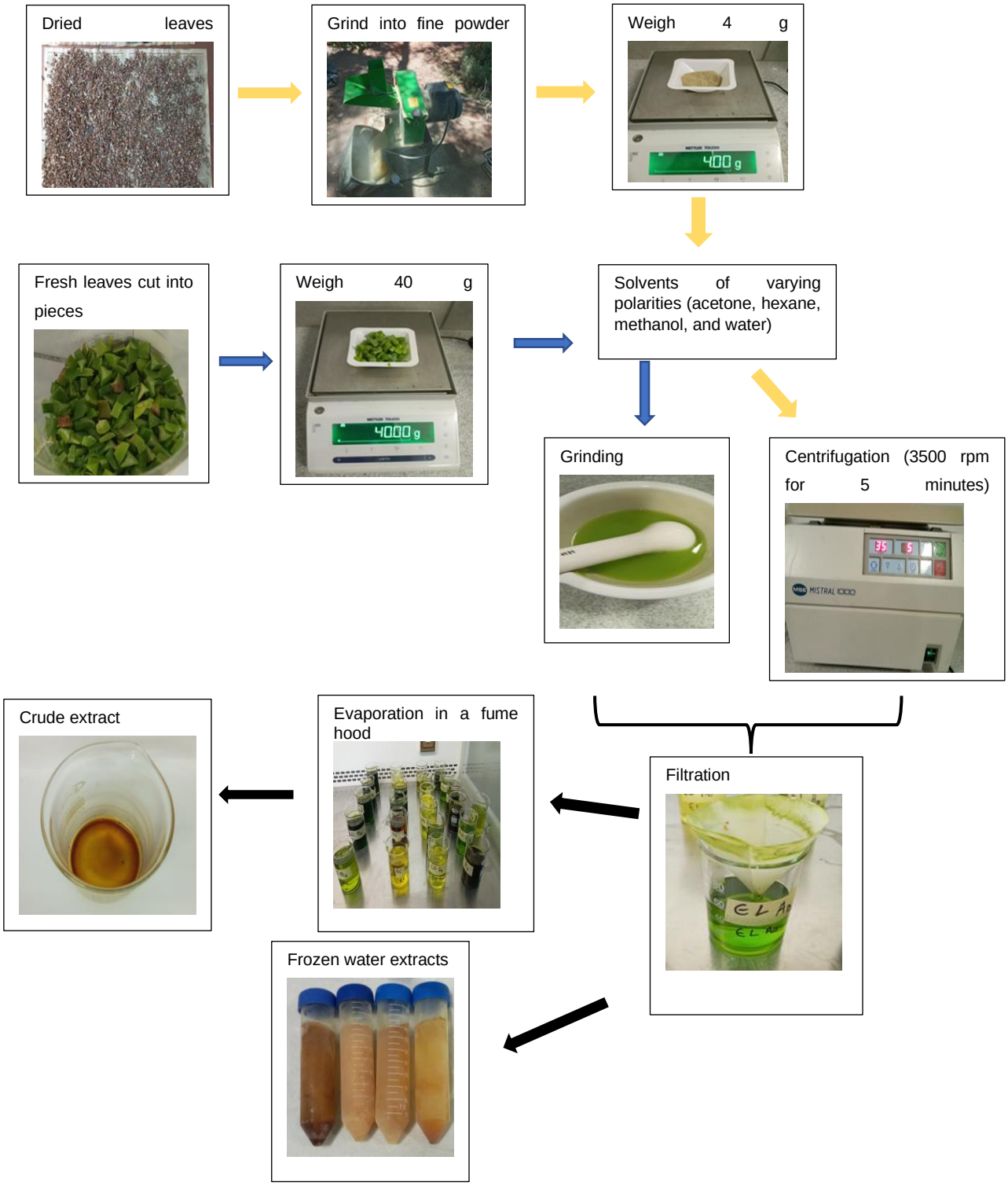

Figure 3. 2 *Schematic representation of the extraction procedure for dried and fresh leaf materials.*

3.2.4 Phytochemical analysis

The chemical components of crude extracts were determined using TLC plates. The TLC plates were developed using three different eluents: Ethyl acetate: Methanol: Water [EMW; 40:5.4:4], Chloroform: Ethyl acetate: Formic acid [CEF; 5:4:1], and Benzene: Ethanol: Ammonium hydroxide [BEA; 90:10:1] (Kotze and Eloff, 2002). BEA, CEF, and EMW are regarded as non-polar or basic, intermediate polar or acidic, and polar respectively. The TLC plates were loaded with 10 µl of every plant extract and developed in eluent solvents. Separated chemical compounds were visualized under Ultraviolet light at 360 nm using a Camac universal UV lamp TL-600. Vanillin-sulphuric acid reagent was used for detecting chemical components that cannot be detected under UV light (Stahl, 1969).

3.3 Results and discussion

3.3.1 Extraction of dried and fresh plant material

Methanol extracted a large quantity of plant material (30.75%) from the leaf of *C. edulis* (Figure 3.3). However, the highest quantity of acetone extract was observed from *P. guajava* with a percentage mass of 6.5% compared to the other plants (Figure 3.3). Polar solvents such as methanol are good extractants of more hydrophilic compounds (Cosa *et al.*, 2006). Furthermore, methanol is efficient in extracting low molecular-weight polyphenols (Muhamad *et al.*, 2017). This could indicate that the plants contain polar compounds that are soluble in methanol. This high quantity observed in acetone extract could be due to the ability of acetone to dissolve polar and non-polar compounds (Velavan, 2015).

Amongst all the plants (Figure 3.3), the yield of hexane extract was highest in *R. communis*. Hexane is a non-polar solvent used to extract non-polar compounds, but, in some cases, it is used to remove chlorophyll (Cosa *et al.*, 2006). Similar to the results of this study, Lekganyane *et al.* (2012) found that methanol extracted the highest quantity of crude from *R. communis* followed by acetone and hexane. The results of this study also revealed that methanol extracted the highest quantity of crude from *D. cinerea* dried leaves followed by hexane and acetone (Figure 3.3). However, Shandukani *et al.* (2018) found that methanol extracted the highest quantity of crude from dried leaves of *D. cinerea* followed by acetone and hexane. The results of this study and Shandukani *et al.* (2018) differ regarding the levels of acetone and hexane

extracts. The differences could be resulting from physiological variations, genetic factors, evolution, environmental conditions, geographical location, and the season in which the plant material was collected (Figueiredo *et al.*, 2008).

The fresh and dried succulent plants were extracted with various solvents. Results of dried leaves extracts and fresh leaves extracts are shown in figures 3.3 and 3.4. Methanol extracted the highest percentage of crude (7.93%) from fresh leaves of *C*. *edulis* followed by acetone (5.38%) and lastly hexane (0.15%) (Figure 3.4). Similarly, methanol extracted the highest percentage of crude (30.75%) from dried leaves of *C*. *edulis* followed by acetone (2.5%) and hexane (1.75%) (Figure 3.3). However, for *C. orbiculata* acetone extracted the highest percentage of crude (8.05%) from fresh leaves (Figure 3.4) while methanol extracted the highest mass of crude (4.5%) from dried leaves (Figure 3.3).

Hexane was more efficient in dried leaves (1.25%) of *C*. *orbiculata* than in fresh leaves (0.075%). Methanol extracted the highest quantity of plant material from both fresh (2.15%) and dried leaves (9%) of *S. hyacinthoides*. However, the yield of acetone extract is higher in dried leaves (1%) than in fresh leaves (0.83%) of *S. hyacinthoides*. Furthermore, hexane extract yield is higher in dried leaves (1.25%) than in fresh leaves (0.1%). Overall, plant extraction is more efficient using dried powdered plant material than fresh plant material. The low crude yield from fresh leaf extracts may result from the high quantity of water present in fresh leaves which may affect the solubility of the phytochemicals (Eloff, 1998). Furthermore, dried powdered plant material has increased surface area which allows more contact between samples and the solvent of extraction. While grinding using a pestle and mortar yields small coarse particles that are less effective in plant extraction (Azwanida, 2015). According to Azwanida (2015), drying plant material at room temperature is an efficient way to preserve compounds that are heat liable. Hence, dried plant samples not only yield a higher quantity of extract but also have a longer shelf life as they are less prone to microbial contamination than fresh samples (Belwal *et al.*, 2018).

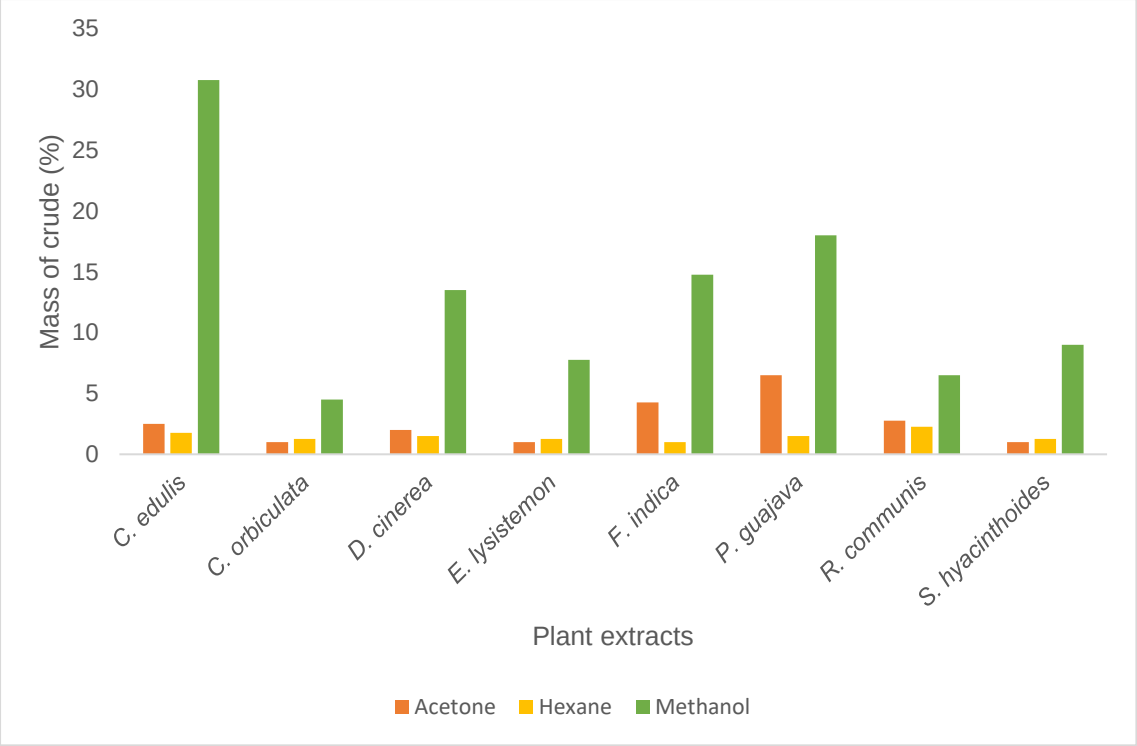

Figure 3. 3 The percentage of crude extracts from dried leaves of different plants extracted using acetone, hexane, and methanol.

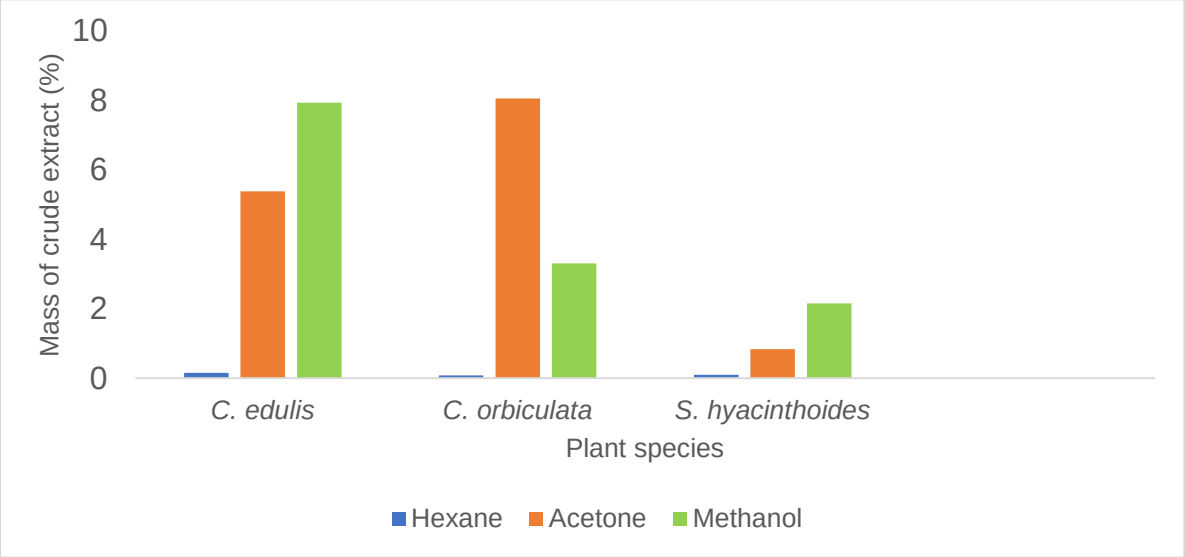

Figure 3. 4 Percentage of crude extracts from fresh leaves of succulent plants extracted using acetone, hexane, and methanol.

3.3.2 Phytochemical analysis

TLC was used to investigate the chemical components of different plant extracts. The TLC plates were separated using BEA, CEF, and EMW solvent systems. The resulting chromatograms were visualized under UV radiation. Different coloured bands were observed, and their retention factor (R_f) was calculated by dividing the distance travelled by the compound by the distance travelled by the solvent front from the original spot (Kumar *et al.*, 2013). More coloured bands of compounds were observed from dried leaf extracts than from fresh leaf extracts. For instance, a total of 12 compounds were observed from dried leaf extracts of *C. orbiculata* while only 1 compound was observed from fresh leaves of *C. orbiculata*. In addition, a total of 73 chemical components were observed from chromatograms of dried leaf extracts developed in BEA, 30 in CEF, and 29 in EMW. However, in chromatograms of fresh leaf extracts, BEA contained a total of 12 compounds followed by EMW with 5 and CEF with 1 compound.

In TLC chromatograms separated with CEF, a chemical component of R_f value of 0.36 was observed in acetone, hexane, and water extracts of *F. indica*. This suggests that the plant extracts may contain the same compound due to similar R_f values (Kumar *et al.*, 2013). Chemical components with an R_f value of 0.88 were visible in acetone, hexane, and methanol extracts of both *R. communis* and *S. hyacinthoides* developed using BEA. Therefore, *R. communis* and *S. hyacinthoides* may contain the same chemical component. A chemical compound with an R_f value of 0.77 was observed in the acetone extract of *D. cinerea* developed in CEF, however, a compound with a similar R_f value was also discovered and identified to be 3-Hydroxy-4-methoxy-benzoic-acid (Abou Zeid *et al.*, 2009). Evidently, different coloured bands of chemical constituents were visible on the chromatograms after spraying with vanillin sulphuric acid reagent (Figure 3.5). Which further indicates the presence of various compounds.

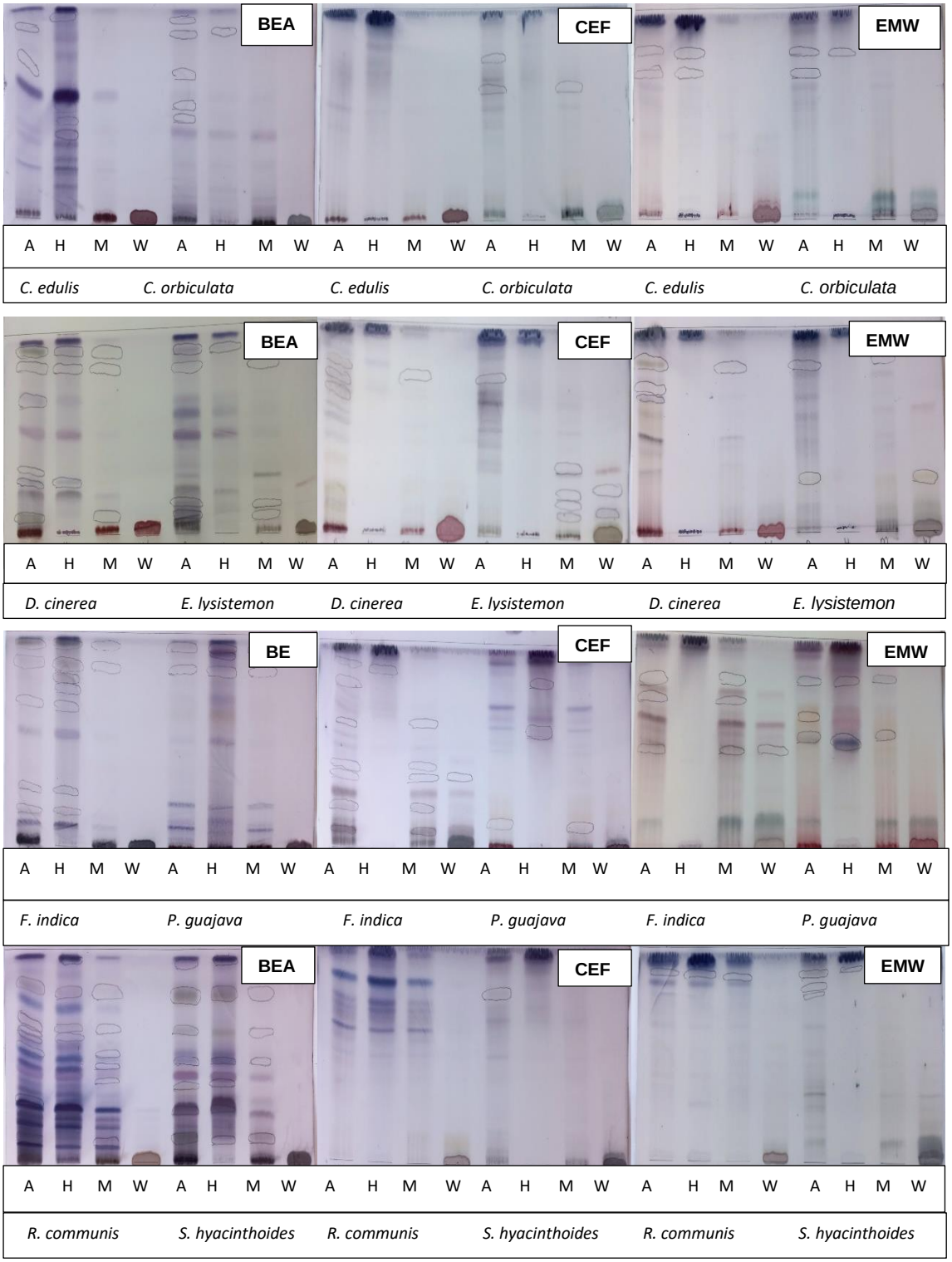

Figure 3. 5 TLC chromatograms of dried leaf extracts of *C. edulis*, *C. orbiculata*, *D. cinerea*, *E. lysistemon*, *F. indica*, *P. guajava*, *R. communis*, and *S. hyacinthoides* in BEA, CEF, and EMW. Lanes from left to right: A= Acetone, H= Hexane, M= Methanol, and W= water.

3.4 Conclusion

Drying of plant material before plant extraction and phytochemical analysis yields higher quantities of plant material. Hence, the most efficient form of extraction is with using dried plant samples. In this study, methanol extracted a large quantity of plant material in both fresh and dried leaf materials. Methanol was the best solvent for extraction in terms of high yields followed by acetone. Compounds from dried plant samples separated better than extracts from fresh samples when subjected to thin-layer chromatography and visualized using both UV light and vanillin sulphuric acid reagent. However, the BEA solvent system was the most effective in separating more compounds. Furthermore, no chemical components were visible in aqueous extracts. This may indicate that the solvent systems used in this study were not able to separate the chemical constituents of aqueous extracts.

CHAPTER 4

ANTIFUNGAL ACTIVITIES OF SELECTED PLANT SPECIES AGAINST FUNGAL PATHOGENS THAT CAUSE EAR INFECTIONS

4.1 Introduction

Eight plant species selected from Chapter 2 were screened for antifungal activity against the selected fungal pathogens using the serial microdilution assay. The bioautography assay was also used to determine the presence of active compounds in the plant extracts. The selected plant species are used by the local people and traditional health practitioners for the treatment of ear infections and related ailments.

Antimicrobial activity tests are used for drug discovery and for determining the therapeutic effects of these drugs (Balouiri *et al.*, 2016). *In vitro* laboratory, methods for determining antimicrobial activities of plant extracts include diffusion methods, dilution methods, and bioautography assay (Armengol *et al.*, 2021). Diffusion methods are divided into agar-disk diffusion, agar-well diffusion, agar plug, cross streak, and poisoned food. Alternatively, dilution methods are divided into agar dilution and broth microdilution (Armengol *et al.*, 2021).

Micro-dilution methods are the most appropriate for determining the minimum inhibitory concentrations (MIC) of a drug (Eloff, 1998). The MIC is the lowest concentration of drug that suppresses the growth of a microorganism after a certain period of incubation (Eloff, 1998). When the concentration drops below the MIC, the microorganism can regrow (Mouton and Vinks, 2005). Micro-dilution methods are used extensively because they use very low quantities of reagents and samples. The method is also highly sensitive, producing reliable results (Silva *et al*., 2011). In addition, this method is known to be highly effective and less laborious (Armengol *et al.*, 2021). In this study, the broth microdilution assay is used to determine the minimum inhibitory concentrations of plant extracts against fungal pathogens that cause ear infections.

Another method used in this study to test for antimicrobial activities of plant extracts is the bioautography method. In the direct bioautography method, the microorganism

grows directly on the TLC plate (Sasidharan *et al.*, 2011). The bioautography assay is a quick and sensitive screening method for the detection of antimicrobial compounds (Horvath *et al.*, 2010). This method combines chromatographic separation and *in situ* activity determination (Shahverdi *et al.*, 2007). Which allows the localization of antimicrobial activity directly onto the microorganism upon application to a chromatographic plate (Navarro *et al.*, 1998). The method is highly effective in the detection of antimicrobial compounds since it allows the localization of activity thereby facilitating the target-directed isolation of active compounds (Rahalison *et al.*, 1991). The position of the inhibition zones can be compared with the TLC fingerprint with reference to R_f values (Sasidharan *et al.*, 2011). This further prevents wasting time through unnecessary isolation of inactive compounds (Shahverdi *et al.*, 2007). In this specific study, this method is used to determine the number of antifungal compounds from different plant extracts against the test microorganisms.

4.2 Methods and materials

4.2.1 Fungal strains and inoculum quantification

Aspergillus fumigatus (ATCC 204305), and *Candida albicans* (ATCC 10231), were obtained from the University of Pretoria. The Sabouraud dextrose (SD) agar and broth were autoclaved at 121°C for 15 minutes and cooled in the biosafety cabinet before subculture. For each isolate, fungi were grown on SD agar for a week at 35°C and subcultured into SD broth under aseptic conditions in a biosafety cabinet using a delta lab sterile swab. Viable cells of each fungal culture were counted using a haemocytometer (Aberkane *et al.*, 2002). For microdilution and bioautography assay, the final concentration of inoculum was adjusted to 1.0×10^6 cells/ml and 2.0×10^6 cells/ml respectively.

4.2.2 Serial microdilution assay

The microplate method was used to assess the MIC of each plant extract (Eloff, 1998). In 96-well microtiter plates, 100 µl of a fungal culture was loaded into each well of 10 mg/ml plant extracts serially diluted two-fold with water (Eloff, 1998). Plant extracts were carried out in triplicates and repeated three times to confirm results. Residues of different extracts were redissolved in acetone to make a final concentration of 10 mg/ml. The plant extracts (100 µl) were serially diluted (50%) with water in 96 well microtitre plates (Masoko and Eloff, 2005). Acetone was used as a negative control

and Amphotericin B was used as a reference antibiotic. Hundred microliters (100 µl) of fungal culture were added to the microplate and incubated for 24 and 48 hrs. As an indicator of growth, 40 µl p-iodonitrotetrazolium violet (INT) of 0.2 mg/ml dissolved in water was added to the micro plate wells. Covered microtiter plates were incubated for 24 and 48 hrs at 35°C and 100% relative humidity. The lowest extract concentration at which fungal growth was suppressed was regarded as the MIC.

4.2.3 Bioautography assay

Each plant extract (10 µl) was loaded onto the TLC plates in a volume of 10 µl, and eluted with BEA, CEF, and EMW. The plates were kept in a fume hood overnight to allow the solvents to evaporate, then sprayed with an overnight culture of *A. fumigatus* and *C. albicans* until fully drenched. To detect antifungal compounds, the plates were incubated for a night at 35°C in a dark chamber with 100% relative humidity, then sprayed with 2 mg/ml INT solution and incubated for up to 6 hours. White areas indicated where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the fungi

4.3 Results and discussion

4.3.1 Serial microdilution assay

The antifungal activities of plant extracts were determined against the tested microorganisms (Table 4.1). The Amphotericin-B was active against *C. albicans* with MIC of 0.08–0.16 mg/ml which corresponds to the low MIC of 0.25 µg/ml observed by Haslene-Hox *et al.* (2022). The MIC of amphotericin-B against *A. fumigatus* was 0.02 mg/ml which is supported by the MIC of 83 µg/ml obtained by Francisco *et al.* (2022).

Acetone fresh leaf extract of *C. edulis* was active against *A. fumigatus* with MIC of 0.02 mg/ml (24 hours) and 0.63 mg/ml (48 hours). Lehtinene *et al.* (2006) obtained similar results showing an increase in MIC value with an increase in the incubation period. The increase in MIC values may result from the proliferation of subpopulations that are less susceptible to the plant extract within the inoculum (Tam *et al.*, 2005). Therefore, the failure of a plant extract to kill the entire microbial population permits the growth of resistant populations which increases the MIC (Lehtinene *et al.*, 2006). However, some plant extracts such as dried leaf acetone extract of *C. edulis* had a constant MIC of 0.04 mg/ml against *A. fumigatus* from 24–48 hours.

The aqueous extracts of both fresh and dried leaves of *C. edulis* had an excellent MIC of 0.02 mg/ml against *A. fumigatus*. The results justify the use of freshly squeezed leaf juice or water extracts by traditional health practitioners when treating ear infections. In *D. cinerea* dried leaves, the methanol extract (0.63 mg/ml) was more active than the water extract (1.25 mg/ml) against *C. albicans* which corresponds to the findings of Neondo *et al.* (2012).

Moreover, dried leaf extracts had more excellent activity than fresh leaf extracts. For instance, strong antifungal activity was observed against *A. fumigatus* from *S. hyacinthoides* dried leaf hexane extract (0.04 mg/ml) while acetone fresh leaf extract had MIC of 0.31–2.5 mg/ml. Dried leaf methanol extract of *C. edulis* was also active with MIC of 0.02 mg/ml against *A. fumigatus* while methanol fresh leaves extract was active from 0.31–2.5 mg/ml. Dried leaves have better activity due to their secondary metabolites which are more stable than fresh leaves. Furthermore, the metabolites in fresh leaves could be diluted by the higher percentage of water quantity (Manilal *et al*., 2009). Earlier studies have reported that fresh plant material is less active than dried plant material (Manilal *et al.,* 2009; Takaki-Campos, 1988). However, hexane and methanol fresh leaf extracts of *C. orbiculata* were more active against *C. albicans* (0.02 mg/ml) than dried leaf extracts that had a MIC of 0.31–1.25 mg/ml. In agreement with the results of this study, Alabri *et al.* (2013) found that fresh leaves were more active than dried leaves.

4.3.2 Plant extracts with excellent antifungal activity

Excellent antifungal activities were observed from different plant extracts (Table 4.2). However, the highest number of excellent activities (0.02 mg/ml) was observed from plant extracts of *D. cinerea*.

4.3.3 Total activity of plant extracts

The total activity of plant extracts is used to measure the dilution quantity of active compound extracts from one gram of plant material that can inhibit the growth of microorganisms (Eloff *et al.*, 2008). The highest total activity (15375 ml/g) was observed against *A. fumigatus* from methanol extract of *C. edulis* dried leaves (Table 4.3). Furthermore, it implies that the quantity of plant material to kill or inhibit the test

organisms is lower. Extremely low total activities were observed in some of the plant extracts such as 110 ml/g observed from *E. lysistemon* hexane extracts against *C. albicans*. The lowest total activities indicate that the plant extracts have very low activity and high quantities of plant material are required to inhibit or kill the microorganism.

Table 4. 1 Minimum inhibitory concentrations of fresh and dried leaves extracts of eight different plant species tested against two fungal pathogens.

Fungi	Time (hours)	MIC (mg/ml)															
		C. edulis (F)				*C. edulis* (D)				*C. orbiculata* (F)				*C. orbiculata* (D			
Extractants		A	H	M	W	A	H	M	W	A	H	M	W	A	H	M	W
A. f	24	**0.02**	0.16	0.31	**0.02**	0.04	0.04	**0.02**	**0.02**	0.04	0.16	0.31	2.5	0.16	0.16	2.5	0.63
	48	0.63	0.63	2.5	0.16	0.04	0.04	**0.02**	**0.02**	0.04	0.16	0.63	2.5	0.31	0.31	2.5	0.63
C. a	24	0.04	0.04	2.5	2.5	0.31	2.5	0.31	2.5	0.31	**0.02**	**0.02**	2.5	1.25	1.25	0.31	2.5
	48	2.5	0.16	2.5	2.5	1.25	2.5	1.25	2.5	2.5	**0.02**	2.5	2.5	1.25	1.25	0.31	2.5
Average		0.80	0.25	1.95	1.30	1.64	1.27	0.40	1.26	0.72	0.09	0.87	2.5	0.74	0.74	1.41	1.57
Fungi	Time (hours)	MIC (mg/ml)															
		S. hyacinthoides (F)				*S. hyacinthoides* (D)				*D. cinerea* (D)				*E. lysistemon* (D)			
Extractants		A	H	M	W	A	H	M	W	A	H	M	W	A	H	M	W
A. f	24	**0.02**	0.31	0.31	0.16	0.04	0.04	2.5	0.31	**0.02**	2.5	**0.02**	**0.02**	**0.02**	2.5	**0.02**	0.63
	48	0.63	0.31	1.25	0.16	0.04	0.04	2.5	0.31	**0.02**	2.5	**0.02**	**0.02**	**0.02**	2.5	**0.02**	0.63
C. a	24	**0.02**	**0.02**	0.63	2.5	0.63	0.31	1.25	2.5	0.31	2.5	0.63	1.25	1.25	1.25	2.5	2.5
	48	0.31	0.16	1.25	2.5	0.63	1.25	2.5	2.5	0.31	2.5	0.63	1.25	1.25	1.25	2.5	2.5
Average		0.25	0.20	0.86	1.33	0.34	0.41	2.19	1.41	0.17	2.5	0.33	0.64	0.64	1.88	1.26	1.57
Fungi	Time (hours)	MIC (mg/ml)															
		F. indica (D)				*P. guajava* (D)				*R. communis* (D)				Amph-B			
Extractants		A	H	M	W	A	H	M	W	A	H	M	W				
A. f	24	**0.02**	**0.02**	0.16	0.31	0.63	0.63	0.63	2.5	0.63	0.63	0.31	0.31	**0.02**			
	48	**0.02**	**0.02**	0.16	0.31	0.63	0.63	0.63	2.5	1.25	1.25	0.31	0.31	**0.02**			
C. a	24	2.5	2.5	2.5	1.25	**0.02**	0.31	0.31	1.25	0.63	0.63	1.25	2.5	0.08			
	48	2.5	2.5	2.5	1.25	0.63	0.63	0.31	1.25	2.5	2.5	2.5	2.5	0.16			
Average		1.26	1.26	1.33	0.78	0.48	0.55	0.47	1.88	1.25	1.25	1.09	1.41	0.07			

Amphotericin B was used as a positive control. Extractants: A = acetone, H = hexane, M = methanol, W =distilled water. Abbreviations: *C. a*= *Candida albicans*, and *A. f* = *Aspergillus fumigatus*.

Condition of plant material: D= dried and F= fresh.

Table 4. 2 Plant extracts with excellent antifungal activity (0.02 mg/ml) against the tested fungal pathogens at 24 and 48 hours.

Extractants	Plant species											Average
	C. edulis		*C. orbiculata*		*S. hyacinthoides*		*D. cinerea*	*E. lysistemon*	*F. indica*	*P. guajava*	*R. communis*	
	F	D	F	D	F	D	D	D	D	D	D	
A	1	0	0	0	2	0	2	2	2	1	0	0.91
H	0	0	2	0	1	0	0	0	2	0	0	0.45
M	0	2	1	0	0	0	2	2	0	0	0	0.64
W	1	2	0	0	0	0	2	0	0	0	0	0.45
Total	2	4	3	0	3	0	6	4	4	1	0	2.45

Extractants: A= acetone, H= hexane, M= methanol, and W= distilled water. Condition of plant material: D= dried and F= fresh

Table 4. 3 Total activity of plant extracts against *A. fumigatus* and *C. albicans*.

Fungi	Time (hours)	Total activity (ml/g)											
		C. edulis (F)			*C. edulis* (D)			*C. orbiculata* (F)			*C. orbiculata* (D)		
		A	H	M	A	H	M	A	H	M	A	H	M
A. f	24	2688	938	255	625	437	**15375**	2012	150	106	162	194	132
	48	185	238	131	625	437	**15375**	2012	150	521	132	148	132
C. a	24	1344	137	131	180	117	991	259	140	1650	140	132	145
	48	1222	938	131	120	117	246	132	140	1650	140	132	145
Average		1035	14.66	87.69	337.66	222	7996.99	1079	130	864	27	41.54	181
Fungi	Time (hours)	Total activity (ml/g)											
		S. hyacinthoides (F)			*S. hyacinthoides* (D)			*D. cinerea* (D)			*E. lysistemon* (D)		
		A	H	M	A	H	M	A	H	M	A	H	M
A. f	24	412	116	693	250	312	136	1000	111	**6750**	500	115	**3875**
	48	344	118	170	250	312	136	1000	111	**6750**	500	115	**3875**
C. a	24	412	150	122	158	140	172	164	111	214	118	110	131
	48	86	117	123	158	117	136	164	111	214	118	110	131
Average		216	60	34.47	132.94	168	45	532		3482	254	7.5	1953
Fungi	Time (hours)	Total activity (ml/g)											
		F. indica (D)			*P. guajava* (D)			*R. communis* (D)					
		A	H	M	A	H	M	A	H	M			
A. f	24	2125	500	921	103	123	285	143	135	209			
	48	2125	500	921	103	124	285	122	118	209			
C. a	24	120	100	159	3250	123	580	143	115	152			
	48	120	100	159	103	123	580	110	180	126			
Average		1071	252	490	889		433	30	24	124			

Plant material: D= dried, and F= fresh. Solvents: A= acetone, H= hexane, and M= methanol. Abbreviations: *A. f*= *Aspergillus fumigatus*, and *C. a*= *Candida albicans*.

4.3.4 Bioautography assay

The bioautography assay was used to determine the number of antifungal compounds from different plant extracts. Active components were visible in dried leaf extracts (Figures 4.1 and 4.2) and their R_f values were calculated (Tables 4.4–4.6). In TLC chromatograms separated with BEA, similar active compounds with R_f values of 0.20 against *C. albicans* were visible in acetone and methanol extracts of *P. guajava*. In addition, another active compound with an R_f value of 0.88 was observed from *P. guajava* acetone extract and *S. hyacinthoides* hexane and methanol extract against *A. fumigatus*. Remarkably, active components of the same R_f value of 0.88 were observed in acetone, hexane, and methanol extracts of *R. communis* against *A. fumigatus*. In essence, *P. guajava*, *R. communis,* and *S. hyacinthoides* could potentially possess the same active component.

The fresh leaf extracts of selected plants have not shown any visible active compounds on the bioautograms. However, fresh leaf acetone and water extracts of *C. edulis* have shown excellent MIC of 0.02 mg/ml against *A. fumigatus*. This indicates that the active components in *C. edulis* plant extracts act synergistically to elicit antifungal activity. However, some plant extracts such as *R. communis* have displayed low activity in MIC (0.31 to 2.5 mg/ml) but have shown a total of 12 individual active compounds in the bioautograms. This indicates that the active components act independently. In addition, the types of solvent systems used could have been a factor since they might have not separated the antifungal compounds. The lack of active compounds in some plant extracts could be due to the presence of antifungal compounds in very small quantities, denaturation, evaporation, and photo-oxidation of active compounds (Akinsanya *et al.*, 2017).

Acetone had the highest total number of active components (10) followed by hexane (8) and methanol (3). Furthermore, the EMW solvent system separated the highest number of active constituents (9), then CEF (8), and lastly BEA (4). Antifungal compounds were mostly visible against *A. fumigatus* with a total of 19 and only a total of 2 against *C. albicans*. The lack of activity from some plant extracts may be due to the presence of formic acid in the solvent system (CEF) which is toxic to yeasts including *Candida* species (Lastauskiene *et al.*, 2014). Some of the antifungal compounds were present in non-polar (hexane) extracts which agrees with previous

findings which indicate that the compounds responsible for antimicrobial activity were non-polar (Masoko and Eloff, 2005).

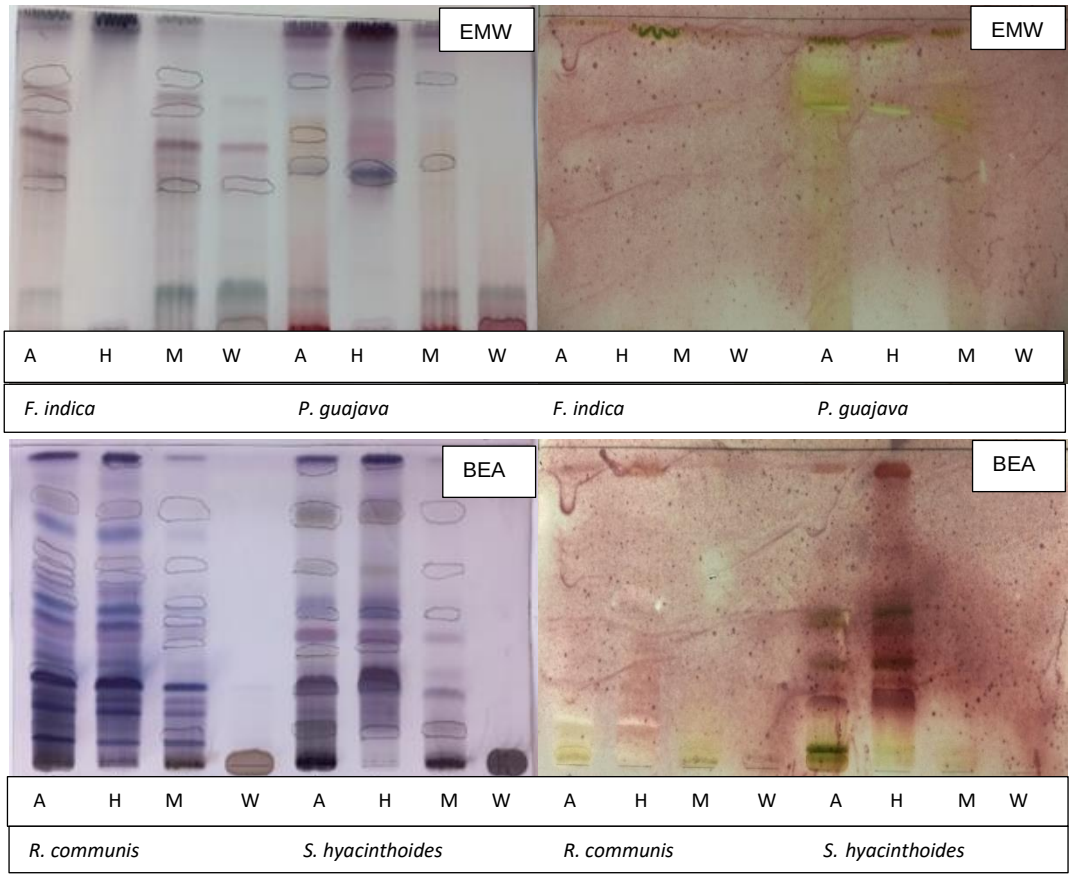

Figure 4. 1 Bioautograms of *F. indica*, *P. guajava*, *R. communis,* and *S. hyacinthoides* leaf extracts developed in BEA, CEF and EMW solvent systems with their corresponding chromatograms. White bands indicate compounds with antifungal activity against *A. fumigatus*. Lanes from left to right: A= Acetone, H= Hexane, M= Methanol and W= Distilled water.

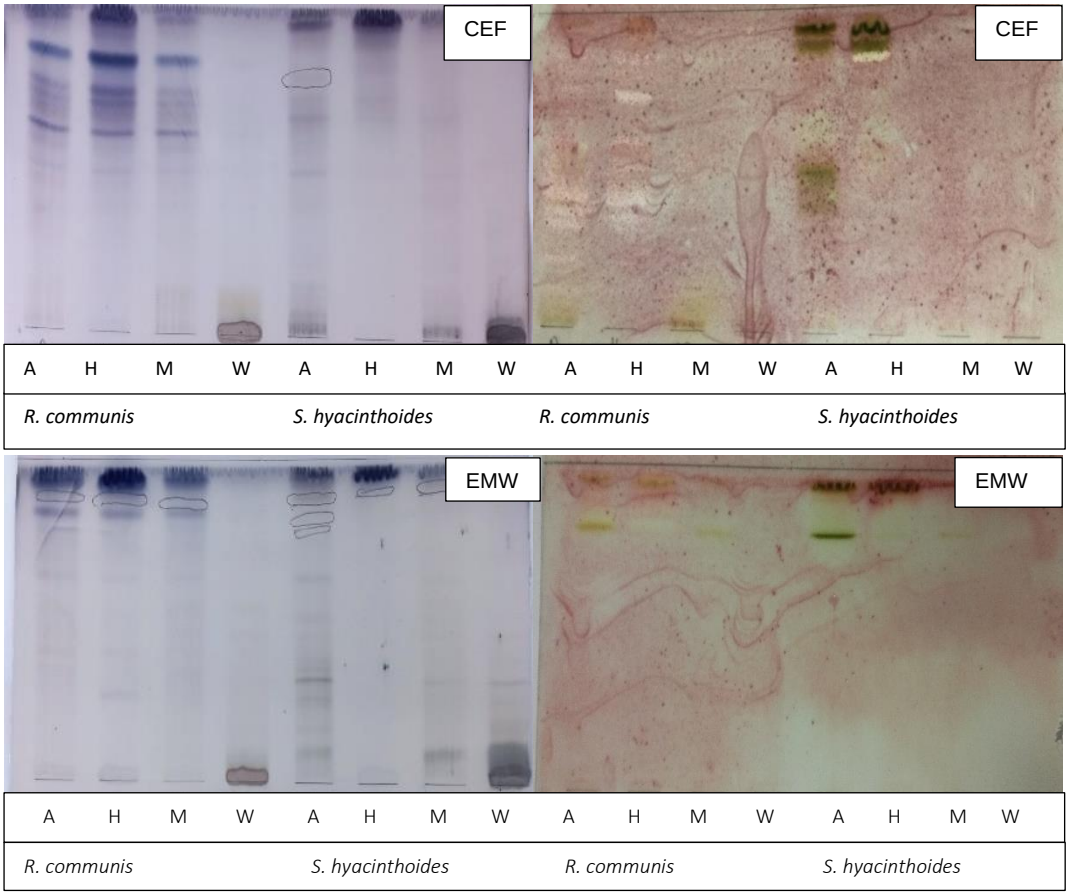

Figure 4.2 Bioautograms of *R. communis* and *S. hyacinthoides* leaf extracts developed in CEF and EMW solvent systems with their corresponding chromatograms. White bands indicate compounds with antifungal activity against *A. fumigatus*. Lanes from left to right: A= Acetone, H= Hexane, M= Methanol and W= Distilled water.

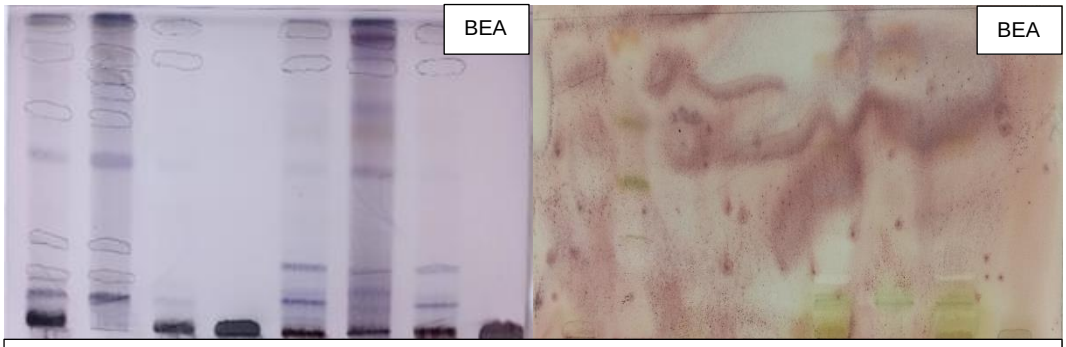

Figure 4. 3 Bioautogram and corresponding chromatogram of *F. indica* and *P. guajava* in BEA with white bands indicate compounds with antifungal activity against *C. albicans*. Lanes from left to right: A=Acetone, H=Hexane, M=Methanol and W= Distilled water.

Table 4. 4 R_f values of compounds inhibiting fungal growth separated with BEA and EMW using dried leaf extracts of *P. guajava*.

P. guajava (dried leaves)							
Solvent systems	R_f values	Acetone		Hexane		Methanol	
		A. fumigatus	*C. albicans*	*A. fumigatus*	*C. albicans*	*A. fumigatus*	*C. albicans*
BEA	0.20		√				
	0.20						√
EMW	0.79	√		√			
	0.88	√					

Table 4. 5 R_f values of compounds inhibiting fungal growth separated with BEA, CEF and EMW using dried leaf extracts of *R. communis.*

R. communis (dried leaves)							
Solvent systems	R_f values	Acetone		Hexane		Methanol	
		A. fumigatus	*C. albicans*	*A. fumigatus*	*C. albicans*	*A. fumigatus*	*C. albicans*
BEA	0.15	√		√			
CEF	0.24	√					
	0.33	√					
	0.41	√		√			
	0.44	√		√			
	0.77			√			
EMW	0.88	√		√		√	

Table 4. 6 R_f values of compounds inhibiting fungal growth separated with CEF and EMW using dried leaf extracts of *S. hyacinthoides.*

S. hyacinthoides (dried leaves)							
Solvent systems	R_f values	Acetone		Hexane		Methanol	
		A. fumigatus	*C. albicans*	*A. fumigatus*	*C. albicans*	*A. fumigatus*	*C. albicans*
CEF	0.93			√			
EMW	0.85	√					
	0.88			√		√	

4.4 Conclusion

The results of this study indicate that dried leaves have more antifungal activity as compared to fresh leaves. *C. edulis* dried leaves had excellent antifungal activity against *A. fumigatus* than fresh leaves with MIC of 0.02 mg/ml to 0.04 mg/ml. *S. hyacinthoides* acetone and hexane extracts of dried leaves had excellent antifungal activity against *A. fumigatus* than fresh leaves with MIC of 0.04 mg/ml. *D. cinerea* dried leaves acetone, methanol, and water extracts also had excellent antifungal activity against *A. fumigatus* with MIC of 0.02 mg/ml. The majority of the plants could elicit more antifungal activity against *A. fumigatus* than *C. albicans*. However, a few notable excellent antifungal activities against *C. albicans* were observed in fresh leaf extracts of *C. edulis*, *C. orbiculata,* and *S. hyacinthoides*.

P. guajava, *R. communis* and *S. hyacinthoides* dried leaves extracts contain antifungal compounds against *A. fumigatus*. In addition, *P. guajava* acetone and methanol extracts have antifungal compounds against *C. albicans*. This proves the use of these plants in traditional medicine to combat ear infections. These compounds have the potential to be isolated and can serve as novel antifungal drugs

CHAPTER 5

CONCLUSION

Fresh and dried leaves of eight plant species were extracted using a solvent of various polarities such as acetone, hexane, methanol, and water. Methanol extracted a larger quantity of plant material from the fresh and dried leaf material of *C. edulis* (30.75%) as compared to other solvents. Based on our findings, plant extraction is more efficient using dried leaves due to higher yields of crude as compared to fresh leaves. Furthermore, methanol serves as a good solvent for extracting higher quantities of crude from both fresh and dried leaves.

The chemical components of the selected eight plant species were investigated using thin-layer chromatography. Chemical components were observed from *S. hyacinthoides* dried leaf extracts which indicates the presence of different chemical components. In TLC chromatograms developed in BEA, similar chemical components with an R_f value of 0.10 were visible in plant extracts of *D. cinerea* and *E. lysistemon*, respectively. The most separated compounds were non-polar components, separated with BEA, followed by intermediate polarity, CEF and relatively polar compounds separated by EMW.

The minimum inhibitory concentration (MIC) of plant extracts was determined using the serial microdilution assay method against *A. fumigatus* and *C. albicans*. The dried leaves of *C. edulis* had an excellent antifungal activity with MIC values ranging between 0.02 to 0.04 mg/ml against *A. fumigatus* than fresh leaves. *S. hyacinthoides* acetone and hexane extracts of dried leaves were more active against *A. fumigatus* than fresh leaves with MIC of 0.04 mg/ml. In addition, fresh leaf extract *C. edulis* and *orbiculata* and *S. hyacinthoides* were active against *C. albicans with* the lowest MIC value of 0.04 mg/ml. The dried leaves possess strong antifungal activity as compared to the extract from fresh leaf materials. These results support the use of dried than fresh plant material by traditional health practitioners. Aqueous extracts of *C. edulis* and *D. cinerea* were active against *A. fumigatus*. The results validate the extensive and effective use of water extracts in traditional medicine. Therefore, the medicinal

plants used in this study have the potential to possess antifungal compounds. The crude extracts can be used to treat fungal ear infections.

The bioautography assay was used to determine the number of antifungal compounds present in different plant extracts. A total of 21 antifungal compounds were visible on TLC chromatograms developed in BEA (4), CEF (8), and EMW (9). Medicinal plant extracts contain numerous antifungal compounds that can be used to combat fungal ear infections. The presence of more than one active compound in a single plant species proves that medicinal plants are an abundant source of diverse active compounds.

The selected plant species are of significant medicinal value to the people of Bushbuckridge Local Municipality in treating ear infections. However, the leaves and other parts of the selected plants prove to have significant and diverse medicinal uses in South Africa and the world at large. The use of medicinal plants by traditional health practitioners to treat ear infections is strongly supported by the findings of this study. This shows that the traditional knowledge of medicinal plants is still relevant and serves as one of the best routes to novel drug discovery. Hence, it is essential to preserve the traditional knowledge of medicinal plants through publications and to verify these traditional uses through laboratory experiments.

Recommendations

For future studies, it is recommended that testing for the antibacterial activity of the different plant extracts against bacterial pathogens that cause ear infections such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* be conducted. Testing for the antiviral activities of different plant extracts against viruses that are responsible for ear infections is also recommended. In addition, it is recommended that the free radical scavenging properties of potential plants be investigated since ear infections are associated with inflammation that can result from excess free radicals. Cytotoxicity studies to determine whether the plant extracts are toxic or safe for use by humans through oral or topical application is further recommended. Since the current study was focused on the leaves of the plants, it is recommended that the biological activities of the different plant parts such as the bark,

roots, and fruit of potential plant species be explored. Lastly, the isolation and characterization of the antimicrobial compounds is recommended.

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