THE ANTIOXIDANT, CYTOTOXICITY AND ANTIMICROBIAL ACTIVITIES OF MEDICINAL PLANTS USED FOR MALE SEXUAL HEALTH IN VENDA, LIMPOPO PROVINCE

by

Munyadziwa Rosette Nelwamondo

DISSERTATION

Submitted in fulfilment of the requirements of the degree of

MASTER OF SCIENCE

In

MICROBIOLOGY

In the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Molecular and Life Sciences)

At the

UNIVERSITY OF LIMPOPO

Supervisor: Prof. P. Masoko

Co-supervisor: Prof. M.S. Mphosi

2022

Declaration

I, **Munyadziwa Rosette Nelwamondo**, declare that the dissertation entitled "**The antioxidant, cytotoxicity and antimicrobial activities of medicinal plants used for male sexual health in Venda, Limpopo Province**" submitted to the University of Limpopo, for the degree of Master of Science in Microbiology 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.

………………………   ..…………………..

MR Nelwamondo (Ms)   Date

Dedication

I dedicate this craft of work and scholarly effort to the loving memory of my number one cheer leader, mutahabvu Vhamusanda Vho-Nemutandani who always spoke highly of me and undoubtingly believed in me. A special dedication to my mother, Matodzi Rosinah Nelwamondo, and my father, Thovhele A.C "Ndaedzo" Nelwamondo, as a sign of honour- I love and appreciate the both of you dearly. I pray you live long enough to share many more of my milestones, accolades, and victories, thank you for your blessings for my dreams and future endeavours. "Lwamondo ḽa Matshele na Mmado- Kheyo yo ṱangana, aa"

Acknowledgements

I thank the Almighty God, for giving me the strength, boldness, and courage to complete my research project. Greater is He on the inside of me, than he that is in this world (1 John 4:4).

I thank Prof. Peter Masoko (Supervisor) and Prof. Maboko Mphosi (Co-Supervisor), for their affable scientific guidance, consistent motivation and for trusting me with this project.

I thank Mr. Herbert Ramabulana for sharing his indigenous knowledge of the selected plants, and Dr. Bronwyn Egan, for providing scientific identifications of the plant material used in this study.

My sincere gratitude is extended to Ms. Refilwe Kudumela and Ms. Mutshidzi Malada, for their assistance in the laboratory, and Mr. Mash Matotoka, for conducting cytotoxicity tests.

I thank the University of Limpopo and CSIR for providing extensive financial support.

I would also like to thank Ms. Lerato Madileng, for the psychological and emotional support, and for going beyond her line of work to ensure that I become a better and healthy person.

To my mentors, friends and everyone who helped me in one way or another during the study, but is not mentioned here, I extend my sincere gratitude to you, your positive perspectives have helped mold me into a disciplined, responsible, mature person.

Lastly, my greatest gratitude goes to the Nelwamondo and Madavha family at large, namely, my *mum*, my brothers; Lufuno, Nnekedzeni and Thivhonali, and my sisters; Mudzunga, Yolanda, Palesa, my nieces and nephews and my youth ministry group and mentees thank you for the love, prayers, support and patience.

Table of Contents

Declaration…………………………………………………………………………………i

Dedication…………………………………………………………………………………ii

Acknowledgements iii

List of abbreviations xiii

List of figures………………………………………………………………………….xvii

List of tables…………………………………………………………………………..xxii

Conference presentations xxv

Abstract……………………………………………………………………………xxvi

Chapter 1: Introduction 1

References……………………………………………………………………………...3

Chapter 2: Literature review.. 6

2.1. Traditional medicinal plants 6

2.2. Sexual and reproductive health 8

2.3. Traditional medicinal plants as a source of sexual health treatment.............. 10

2.4. Plant secondary metabolites of therapeutic relevance.................................. 11

2.4.1. Phenolic compounds 12

2.4.2. Flavonoids 13

2.4.3. Alkaloids 15

2.4.4. Saponins 16

2.4.5. Steroids 17

2.4.6. Tannins 18

2.5. Biological activities of plants 19

2.5.1. Antimicrobial activity 21

2.5.2. Antioxidant activity 22

2.6. Safety of plant extracts 23

2.7. Proximate composition 24

2.8. Extraction, separation, and purification of bioactive compounds 25

2.9. Test organisms for antimicrobial activity 26

2.9.1. Bacteria of clinical significance in public health sector 26

2.9.2. Fungus of clinical significance 27

2.10. Medicinal plants used in study 28

2.10.1. *Elephantorrhiza burkei* 29

2.10.2. *Securidaca longepedunculata* 30

2.10.3. *Wrightia natalensis* 31

2.11. Aim and objectives 32

2.11.1. Aim 32

2.11.2. Objectives 32

References........34

Chapter 3: Isolation and identification of microbial contaminants and nutrient evaluation of medicinal plants 54

3.1. Introduction 54

3.2. Methodology and analytical procedures 55

3.2.1 Sample procurement 55

3.2.2. Isolation of microbial contaminants 56

3.2.3. Proximate composition 57

3.3. Results 57

3.3.1. Gram staining of bacteria isolates 57

3.3.2. Wet mount of fungal isolates 58

3.3.3. Microbial identification by VITEK 2 system 59

3.3.4. Proximate composition 60

3.4. Discussion 61

3.5. Conclusion 63

References........ 64

Chapter 4: Extraction and phytochemical screening 68

4.1. Introduction 68

4.2. Methodology 69

4.2.1. Extraction procedure 69

4.2.2. Phytochemical analysis 69

4.3. Results 75

4.3.1. Preliminary phytochemical screening 75

4.3.2. Phytochemical analysis 76

4.4. Discussion 80

4.5. Conclusion 84

References........ 85

Chapter 5: Antioxidant activity assays 89

5.1. Introduction 89

5.2. Methods and materials 91

5.2.1. Qualitative antioxidant activity assay 91

5.2.2. Quantitative antioxidant activity assay 91

5.2.3. Ferric reducing power assay 92

5.3. Results 93

5.3.1. Qualitative DPPH assay on TLC 93

5.3.2. Quantification of Antioxidant activity 93

5.4. Discussion 97

5.5. Conclusion 99

References 101

Chapter 6: Antimicrobial activity assays 106

6.1. Introduction 106

6.2. Methods and Materials 108

6.2.1. Test microorganisms 108

6.2.2. Qualitative antibacterial assay 108

6.2.3. Quantitative antibacterial assay 109

6.3. Results 112

6.3.1. Bioautography assay 112

6.3.2. Broth Micro-dilution assay 114

6.3.3. Synergistic, additive, and antagonistic effects 117

6.4. Discussion 121

6.5. Conclusion 124

References…………………………………………………………………………..125

Chapter 7: Cytotoxicity assay.. 131

7.1. Introduction 131

7.2. Methods and materials 132

7.3. Results.. 133

7.4. Discussion 134

7.5. Conclusion 135

References…………………………………………………………………………..137

Chapter 8: Bioactivity guided isolation of antibacterial compounds........................ 140

8.1. Introduction 140

8.2. Methods and materials 141

8.2.1. Serial exhaustive extraction 141

8.2.1.1. Phytochemical analysis 142

8.2.1.2. TLC-DPPH assay 142

8.2.1.3. Bioautography assay 142

8.2.1.4. Broth micro-dilution assay 142

8.2.2. Isolation of antibacterial and antioxidant compounds 143

8.2.3. Second open column chromatography 146

8.3. Results 147

8.3.1. Serial exhaustive extraction 147

8.3.2. Isolation of antibacterial compounds from acetone extracts 153

8.3.3. Isolation of antibacterial compounds from 100% Ethyl acetate fraction 160

8.3.4. Isolation of antibacterial compounds from grouped subfractions........... 165

8.4. Discussion 171

8.5. Conclusion 174

References………………………………………………………………………..175

Chapter 9: General discussion, conclusion and recommendation 178

9.1. General discussion 178

9.2. Conclusion 179

9.3. Recommendation.. 179

References……………………………………………………………………………..181

List of abbreviations

A	Acetone
Ac	Absorbance of control
$AlCl_3$	Aluminium Chloride
AOAC	Association of Official Analytical Chemists
As	Absorbance of sample
Avg	Average
AmB	Amphotericin-B
Gen	Gentamicin
ATCC	American Type Culture Collection
BEA	Benzene: Ethanol: Ammonium hydroxide (18:2:0.2)
CEF	Chloroform: Ethyl acetate: Formic acid (10:8:2)
CO_2	Carbon dioxide
D/DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2- diphenyl-1-picrylhydrazyl radical

E	Ethyl acetate
EB	*Elephantorrizha burkei*
EMW	Ethyl acetate: Methanol: Water (40:5.4:5)
FRAP	Ferric Reducing Antioxidant Power
G	Gram
GAE	Gallic acid equivalence
H	hexane
H2SO4	Sulphuric acid
HCl	Hydrochloric acid
INT	p- iodonitrotetrazolium violet
M	Methanol
mg/mL	Milligrams per millilitre
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant Staphylococcus aureus
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NA	Nutrient agar

Na_2CO_3	Sodium Carbonate
$NaNO_3$	Sodium Nitrate
NaOH	Sodium Hydroxide
QE	Quercetin equivalence
Rf	Retardation factor
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SANBI	South African National Biodiversity Institute
SD	Standard deviation
SDA	Sabourad dextrose agar
SEE	Serial exhaustive extraction
SL	*Securidaca longepedunculata*
STD	Sexually Transmitted Disease
STI	Sexually Transmitted nfection
TLC	Thin-layer Chromatography
UNIN	University of the North

UTIs	Urinary tract infections
UV	Ultraviolet
v/v	volume per volume
W	Water
w/v	weight per volume
WHO	World Health Organization
WN	*Wrightia natalensis*

List of figures

Chapter 2

Figure 2.1: Structure and nomenclature for phenol and substitution patterns of phenolic compounds 13

Figure 2.2: The basic structure of flavonoids.. 14

Figure 2.3: Major flavonols, flavones and polymethoxylated flavones..................... 14

Figure 2.4: Structure of chelerythrine. 16

Figure 2.5: An overview of saponin 17

Figure 2.6: (**a**) The four-fused-ring steroid skeleton uses letter designations for each ring and the numbering of the carbon atoms. (**b**) The cholesterol molecule............. 18

Figure 2.7: Diagrammatic representation of hydrolysable and condensed tannins . 19

Figure 2.8: Method for obtaining active biological compounds from plants. 20

Figure 2.9: *Enterococcus faecalis* cells viewed under conventional light microscope 26

Figure 2.10: Gram's staining of *S. aureus* at 100X resolution 27

Figure 2.11: *Candida albicans* growing on sabouraud agar 28

Figure 2.12: *Elephantorrhiza burkei*; **A**- Shrub, **B**- Flower and **C**- Roots................. 29

Figure 2.13: *Securidaca longepedunculata* (Fresen) with flowers. 30

Figure 2.14: *Wrightia natalensis*; **A**- Shrub, **B**- Leaves and **C**- Pods (seeds).......... 31

Chapter 3

Figure 3.1: **A**- *E. burkei* (+ve) and **B**- Herbal treatment (+ve) 58

Figure 3.2: **A**- *W. natalensis,* **B**- *S. longepedunculata* and **C**- Herbal treatment. 58

Chapter 4

Figure 4.1: The crude extract mass of *E. burkei, S. longepedunculata, W. natalensis* and medicinal treatment extracted with five different solvents. **Key:** H-Hexane, D-Dichloromethane A- Acetone, M- Methanol, E-Ethanol, and W-Water. 75

Figure 4.2: The chromatograms *W. natalensis*, *S. longepedunculata*, *E. burkei* and herbal treatment extracted with different solvents and developed in BEA, CEF and EMW mobile systems and visualised under UV-light at 254 nm (**A**), 365nm (**B**) and (**C**) was sprayed with vanillin-sulphuric acid reagent.. 76

Figure 4.3: The gallic acid standard curve for total phenol 78

Figure 4.4: The gallic acid standard curve for total tannin analysis. 78

Figure 4.5: The quercetin calibration curve for total flavonoids analysis. 79

Chapter 5

Figure 5. 1: The chromatograms of medicinal plants and herbal treatment extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with 0.2% DPPH in methanol……………………………………………………………93

Figure 5.2: Percentage free radical DPPH inhibition of *S. longepedunculata* and *E. burkei*……………………………………………………………………………………94

Figure 5.3: Percentage free radical DPPH inhibition of *W. natalensis* and herbal treatment. 95

Figure 5.4: Ferric reducing power of *S. longepedunculata* and *W. natalensis* at varying concentrations expressed as Absorbance 700nm. Ascorbic acid was used as standard to which samples were compared. 96

Figure 5.5: Ferric reducing power of *E. burkei* and herbal treatment at varying concentrations expressed as Absorbance 700nm. Ascorbic acid was used as standard to which samples were compared. 97

Chapter 6

Figure 6.1: The chromatograms of medicinal plants and herbal treatment extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with *E. faecalis* (**A**) and *S. aureus* (**B**). 112

Figure 6.2: The chromatograms of medicinal plants and a treatment extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with *C. albicans.* 113

Chapter 7

Figure 7.1: Percentage cell viability of medicinal plants and herbal treatment with water extracts on human monocytic cell line (THP-1) 134

Figure 7.2: Percentage cell viability of medicinal plants and herbal treatment with acetone extracts on human monocytic cell line (THP-1) 134

Chapter 8

Figure 8.1: The chromatograms of *E. burkei* root extracts with different solvents and developed in BEA, CEF and EMW mobile systems and visualised under UV-light at 365 nm (**A**), 254 nm (**B**) and (**C**) was sprayed with vanillin-sulphuric acid reagent.148

Figure 8.2: The chromatograms of *E. burkei* root extracts with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with 0.2% DPPH in methanol. 149

Figure 8.3: The chromatograms of *E. burkei* extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with *E. faecalis* and *S. aureus* cultures and visualised under 2mg/mL INT. 152

Figure 8.4: The chromatograms of *E. burkei* extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with *C. albicans* cultures and visualised under 2mg/mL INT. 153

Figure 8.5: The chromatograms of *E. burkei* extracted with acetone and developed in BEA, CEF and EMW mobile systems were then visualised under UV-light at 365 nm (**A**), 254 nm (**B**) and (**C**) was sprayed with vanillin-sulphuric acid reagent. 155

Figure 8.6: The chromatograms *E. burkei* acetone fractions developed in BEA, CEF and EMW mobile systems sprayed with 0.2% DPPH in methanol. 156

Figure 8.7: The chromatograms of *E. burkei* acetone fractions developed in BEA, CEF and EMW mobile systems sprayed with *E. faecalis, S. aureus* and *C. albicans* cultures and visualised under 2mg/mL INT. 159

Figure 8. 8: The chromatograms of *E. burkei* acetone fractions developed in hexane: Ethyl acetate (100%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%) and 100% Ethyl acetate mobile systems and visualised under UV-light at 365nm (A), 254nm (B) and (C) was sprayed with vanillin-sulphuric acid reagent. 161

Figure 8 9: The chromatograms of *E. burkei* acetone fractions developed in hexane: Ethyl acetate (100%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%) and 100% mobile systems sprayed with *E. faecalis.* 162

Figure 8.10: The chromatograms of *E. burkei* acetone fractions (100% Ethyl acetate) developed in BEA mobile systems and visualised under 254 nm UV light. 163

Figure 8.11: The chromatograms of *E. burkei* acetone fractions (100% Ethyl acetate) developed in BEA mobile systems and visualised under 365 nm UV light. 164

Figure 8.12: The chromatograms of *E. burkei* acetone fractions (100% Ethyl acetate) developed in BEA mobile systems sprayed with vanillin-sulphuric acid reagent. ... 165

Figure 8.13: The phytochemical analysis of the grouped fractions visualised under a UV light at 254 nm (**A**) and 365 nm (**B**) and (**C**) was sprayed with vanillin-sulphuric acid reagent 166

Figure 8.14: The chromatograms *E. burkei* acetone fractions developed in mobile systems sprayed with 0.2% DPPH in methanol (**A**) for antioxidant activity and sprayed with *E. faecalis* (**B**) for antibacterial activity. 168

Figure 8.17: Flow diagram of the isolation process of the active fraction. 171

List of tables

Chapter 3

Table 3.1: Bacterial contaminants of the medicinal plants and herbal treatment using Vitek 2 system 59

Table 3.2: Proximate composition of medicinal plants' roots material and herbal treatment material. 60

Chapter 4

Table 4.1: Phyto-constituents present in the plants and herbal treatment. 77

Table 4.2: The total phenol, tannin and flavonoids content measured in different plants and herbal treatment. 79

Chapter 6

Table 6.1: Outcome combination based on FIC index value. 111

Table 6.2: The minimal inhibitory concentration indexes of the selected plant extracts (mg/mL) against the test organisms after 24 hours incubation. 114

Table 6.3: The minimal inhibitory concentration indexes of the selected plant extracts (mg/mL) against the test organism after 48 hours incubation. 115

Table 6.4: The total activity of the medicinal plants and herbal treatment extracts (ml/g) against bacteria. 116

Table 6.5: The total activity of the medicinal plants and herbal treatment extracts (ml/g) against fungus. 116

Table 6.6: Antibacterial activity of extract combinations against *Staphylococcus aureus.* 118

Table 6.7: Antibacterial activity of extract combinations against *Enterococcus faecalis.* 119

Table 6.8: Antifungal activity of extract combinations against *C. albicans* 120

Table 6.9: Minimal inhibitory concentration indexes of the 1:1:1 combinations of the selected plants against bacteria. 121

Table 6.10: Minimal inhibitory concentration indexes of the 1:1:1 combinations of the selected plants against fungus. 121

Chapter 8

Table 8.1: The MIC values of *E. burkei* plant extracts (mg/mL) against the test organisms after 24 hours incubation. 150

Table 8.2: The MIC values of *E. burkei plant* extracts (mg/mL) against the test organisms after 48 hours incubation 151

Table 8.3: The mass (g) of fractions collected from column chromatographic separation of *E. burkei* acetone extracts using different solvents. 154

Table 8.4: MIC values (mg/mL) of fractions from column chromatography against two tested bacteria. 157

Table 8.5: MIC values (mg/mL) of fractions from column chromatography against *C. albicans* fungus. 158

Table 8.6: The mass of combined sub-fraction 1 based on their biological activity. 160

Table 8.7: The mass of combined sub-fraction 2 based on their biological activity. 166

Table 8.8: The total phenol, tannins, and flavonoids content of differen plant parts..167

Table 8.9: The MIC values (ug/mL) of the grouped sub-fraction 2 against *E. faecalis*………………………………………………………………………………….170

Conference presentations

Munyadziwa R. Nelwamondo, Peter Masoko and Maboko S. Mphosi. *Investigating safety, antimicrobial activity, phytochemical and cytoxicity of medicinal plants used as sexual enhancers, in Vhembe district, Limpopo Province*, University of Limpopo Faculty Research Day, The Ranch Resort, September 2019.

Munyadziwa R. Nelwamondo, Peter Masoko and Maboko S. Mphosi. *Antimicrobial properties of medicinal plants used for male sexual health in Limpopo Province.* Indigenous Plant Usage Forum (IPUF), (virtual), July 2021.

Munyadziwa R. Nelwamondo, Peter Masoko and Maboko S. Mphosi. *Antimicrobial properties of medicinal plants used for male sexual health in Limpopo Province*. University of Limpopo Faculty Research Day, Bolivia Lodge, October 2021.

Munyadziwa R. Nelwamondo, Peter Masoko and Maboko S. Mphosi. *Antimicrobial properties of medicinal plants used for male sexual health in Limpopo Province*. World Antimicrobial Awareness week (WAAW), (virtual), November 2021.

Abstract

Sexually transmitted infections represent one of the main health problems related to reproductive and sexual function, constituting one of the main causes of infertility. Plants have been employed traditionally in treating diseases for centuries. *Elephantorrhiza burkei, Securidaca longepedunculata* and *Wrightia natalensis* medicinal plants were selected for this study based on their use in traditional medicine to treat sexual health problems in Limpopo Province. The aim of the study was to determine antimicrobial and antioxidant properties of the selected plants. Different extraction procedures coupled with solvents of varying polarities were used in the extraction of the plant materials. Possible microbial contaminants were identified using the VITEK 2 instrument. Qualitative phytochemical analysis was determined using standard chemical tests and Thin-Layer Chromatography. Total polyphenol content was quantified calorimetrically. Antioxidant activity was quantified using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing power assays. *In vitro* antimicrobial activities were determined using a broth micro-dilution assay and bioautography. Combinational effects of the addition of multiple plant species on antimicrobial activity were investigated for additive, synergistic and antagonistic interactions. The plant species phytochemical profile contained polyphenols that are known to have antimicrobial effects against two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212), a Gram-negative bacterium, as well as the fungus, *Candida albicans* (ATCC 10231). *Elephantorrhiza burkei* exhibited high antioxidant activity and antimicrobial activity against the test pathogens, although most of the interactions were antagonistic and indifferent. Toxicity was found in the herbal treatment and acute toxicity was found in the selected plants. Based on the good correlations which were found in *E. burkei,* it was selected for bioactivity guided by an isolation of antibacterial compounds. *Elephantorrhiza burkei* was not successfully isolated, but the fraction collected showed exceptional biological activity that validates its usage against disease-causing pathogens related to sexually transmitted infections, which compromises male sexual health. This study suggests that the evaluated plants are potential sources of novel anti-infective agents. Further *in vivo* and *in vitro* studies are recommended for all the plants, respectively.

Chapter 1: Introduction

In both developed and developing countries worldwide, bacterial agents of sexually transmitted infections (STIs) are a serious public health burden (Igietseme *et al.,* 2015). Annually, 499 million instances of STIs are reported, with most infections occurring in adults aged 15 to 49. Over a quarter of STIs occurs in sub-Saharan Africa (Maema *et al.,* 2020). The high prevalence of STIs in this region is related to high-risk sexual practices among young adults, socio-demographics, and economic factors, as well as inadequate access to healthcare facilities (Lewis, 2011).

Infection and inflammation of the genital tract are responsible for 13 – 15% of infertility disturbances in couples (Henkel *et al.,* 2007; Chen and Haidl, 2019). Infertile men's sperm and genital tracts have been found to have a variety of pathogenic microorganisms (Farsimadan and Motamedifar, 2020). These pathogens cause many inflammatory disorders within the male genital tract, with *Chlamydia trachomatis* as the most common (Schill *et al.,* 2006). Other common microorganisms that have been previously studied are *Escherichia coli, Proteus mirabilis, Streptococcus faecalis, Neisseria gonorrhea and Pseudomonas aeruginosa* (Burstein and Zenilman, 1999, Schill *et al.,* 2006, Chen and Haidl, 2019).

Modern remedies of genital tract infections include ceftriaxone and azithromycin for gonococcal urethritis; and ofloxacin, levofloxacin and amoxicillin for non-gonococcal urethritis. Some individuals are unable to tolerate these recommended drugs with patients sometimes experiencing significant nausea or vomiting while on the treatment (Workowski and Berman, 2011). Workowski and Berman (2011) stated that other patients experience resistance when treated with azithromycin (Workowski and Berman, 2011).

Infection treatment with antibiotics over a prolonged period may cause further sperm defects (Zeyad and Hammadeh, 2017). Empirical antibiotic regimens are used to treat sexually transmitted infections. This is linked to the overuse of broad-spectrum

antibiotics, which can lead to bacterial resistance, as well as insufficient or improper antimicrobial treatment (Rumyantseva et al., 2015). Although countless antibiotics have been produced by pharmaceutical companies for decades, a crisis which we are faced by is that many antibiotics are no longer effective in fighting the simplest infections. In order to aid the discovery of new drugs and possibly find improved applications of traditional medicine (Koné and Atindehou, 2008). Hence, there is a need to search for natural products for drug development because medicinal plants have medicinal value.

Traditional remedies from plants have been used to treat male reproductive related disorders for ages in South Africa (Abdillahi and van Staden, 2013). Traditional medicines, usually made from plants, have been shown to be useful in the treatment of infectious diseases, such as sexually transmitted infections (Semenya and Potgieter, 2013). This is because plants and plant parts have been noted to have disease preventing and healing effects (Komape *et al.,* 2017). Medicinal plants represent a rich source of antimicrobial agents with better activity against multi-drug resistant bacteria and their antimicrobial potential have been accepted (Anwar *et al.,* 2009).

Plants used in the study were selected based on information provided by Mr Herbert Ramabulana, an herbalist from Dzanani (personal communication) and approval ethics (REC-0310111-31) is attatched. Mr. Ramabulana highlighted that the powdered roots concoction *of Elephantorrhiza burkei, Securidaca longepedunculata* (Fresen) and *Wrightia natalensis* collectively (taken orally) is used in Dzanani, Vhembe District, Limpopo Province for sexual impotence, erectile dysfunction, and sexually transmitted diseases. This study focused on sexually transmitted infections by investigating three medicinal plants against microbes responsible for some of these infections.

References

Abdillahi, H. S. and van Staden, J. 2013. Application of medicinal plants in maternal healthcare and infertility: a South African perspective. *Planta Medica*, *79*(07), pp.591-599.

Anwar, A. Leong, K.M. Ng, M.L. Chu, J.J. and Garcia-Blanco, M.A. 2009. The polypyrimidine tract-binding protein is required for efficient dengue virus propagation and associates with the viral replication machinery. *Journal of Biological Chemistry*, *284*(25), pp.17021-17029.

Burstein, G.R. and Zenilman, J.M. 1999. Nongonococcal urethritis—a new paradigm. *Clinical Infectious Diseases*, *28*(1), pp.S66-S73.

Chen, S.J. and Haidl, G. 2019. Male genital tract infections and Leukocytospermia. In *Oxidants, antioxidants and impact of the oxidative status in male reproduction*, Academic Press, London, pp.101-104.

Farsimadan, M. and Motamedifar, M. 2020. Bacterial infection of the male reproductive system causing infertility. *Journal of Reproductive Immunology*, pp.103-183.

Henkel, R. Maab, G. Jung, A. Haidl, G. Schill, W.B. and Schuppe, H.C. 2007. Age-related changes in seminal polymorphonuclear elastase in men with asymptomatic inflammation of the genital tract. *Asian Journal of Andrology*, *9*(3), pp.299-304.

Igietseme, I.U. Omosun, Y. and Black, M.C. 2015. Bacterial sexually transmitted infections (STIs): A clinical overview. In *Molecular medical microbiology*, 2nd edition, Academic Press, pp.1403-1420.

Komape, N.P.M., Bagla, V.P., Kabongo-Kayoka, P. and Masoko, P. 2017. Anti-mycobacteria potential and synergistic effects of combined crude extracts of selected medicinal plants used by Bapedi traditional healers to treat tuberculosis related symptoms in Limpopo Province, South Africa. *BMC Complementary and Alternative Medicine*, *17*(1), pp.-13.

Koné, W.M. and Atindehou, K.K., 2008. Ethnobotanical inventory of medicinal plants used in traditional veterinary medicine in Northern Côte d'Ivoire (West Africa). South African Journal of Botany, *74*(1), pp.76-84.

Lewis, D.A. 2011. HIV/sexually transmitted infection epidemiology, management, and control in the IUSTI Africa region: Focus on sub-Saharan Africa. *Sexually Transmitted Infections*, *87*(2), pp.ii10-ii13.

Maema, L.P. Potgieter, M.J. and Samie, A. 2020. Treatment of sexually transmitted infections by Bapedi traditional health practitioners. *Indian Journal of Traditional Knowledge*, *19*(3), pp.533-541.

Rumyantseva, T., Golparian, D., Nilsson, C.S. Johansson, E., Falk, M., Fredlund, H., van Dam, A., Guschin, A. and Unemo, M. 2015. Evaluation of the new AmpliSens multiplex real-time PCR assay for simultaneous detection of *Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium,* and *Trichomonas vaginalis*. *Journal of Pathology Microbiology and Immunology*, *123*(10), pp.879-886.

Schill, W.B., Comhaire, F.H. and Hargreave, T.B. 2006. *Andrology for clinician*. Springer Science and Business Media, Springer-Verlag Berlin, Heidelberg, Germany, pp.1323-1325.

Semenya, S.S. and Potgieter, M.J. 2013. Sexually transmitted infections and their diagnoses: Bapedi experience. *African Health Sciences*, *13*(4), pp.1047-1053.

Workowski, K.A. and Berman, S.M. 2011. Centers for disease control and prevention sexually transmitted disease treatment guidelines. *Clinical Infectious Diseases*, *53*(3), pp.S59-S63.

Zeyad, A. Amor, H. and Hammadeh, M.E. 2017. The impact of bacterial infections on human spermatozoa. *International Journal of Women's Health and Reproduction Sciences*, *5*(4), pp.243-252.

Chapter 2: Literature review

2.1. Traditional medicinal plants

Traditional medicine is an imperative component of the health care system of most countries in the world (Bhalla, 2020). Medicinal usage of herbal medicines suggests extensive historical use, and this is indeed true for many products that are accessible as "traditional herbal medicines" (Hosseinzadeh *et al*., 2015). With over 60% of the world's population and about 80% in developing countries relying directly on medicinal plants for their medical need, traditional medicine is very much regarded as the preferred primary health care system in many communities, due to their affordability, accessibility, and low cost (Bhalla, 2020). World Health Organisation (WHO, 2002) reported that up to 80% of the population in Africa use traditional medicine to serve their health needs which are indigenous to different cultures, whether justifiable or not (Che *et al*., 2017).

The number of people depending on traditional herbal medication results from trust and faith in these medications since they have been used for primary health care needs for centuries (van Vuuren *et al*., 2014). Plants used for medicinal purposes are mostly accessed through traditional healers who play a great part in providing accessible health care to communities (SANBI, 2006). In South Africa, few medicinal plants have been utilised to their full capacity in terms of commercialisation (Street and Prinsloo 2013).

A noteworthy number of modern drugs have been isolated from natural sources, most of which are used in traditional medicine. In essence, traditional medicine has been a large source of medicinal agents over the past thousands of years (Sivakrishnan, 2018). Approximately 28% (about 50 000 to 80 000) of plant species worldwide reported to have ethnomedicinal uses and this high number is worthy to consider (Louhaichi *et al*., 2011). The high number of plant species that are used for medicinal purposes is noteworthy, particularly in South Africa, a country with a strong

history of traditional healing. South Africa hosts a variety of around 30,000 flowering plant species, of which 3 000 plant species are used for medicinal purpose across the country (van Wyk and Gericke, 2000 and Xego *et al.*, 2016). Semenya and Potgieter, conducted an ethnobotanical survey on indigenous plant species used by Bapedi traditional healers to treat sexually transmitted infections by in three districts of the Limpopo Province (Semenya and Potgieter, 2013). Data was collected from 34 traditional healers via a semi-structured questionnaire, supplemented by field observations. Results showed that 37 species from 33 genera belonging to 24 families, mostly Asteraceae (10.8%), Asphodelaceae, Fabaceae and Hyacinthaceae (8.1%, each) are used to treat STIs such as chlamydia, gonorrhoea, HIV/AIDS, syphilis and other STIs (Semenya and Potgieter, 2013). Most research in this province focussed on the VhaVenda (Mabogo, 1990; Madzibane and Potgieter, 1999; Samie *et al.*, 2005; Tshikalange *et al.*, 2005; Mulaudzi *et al.*, 2011) and VaTsonga (Mashabane *et al.*, 2001).

Medicinal plants are being harvested worldwide from the forest and this creates pressure on both plant biodiversity and ecosystems (Oyedeji, 2018). The use of herbal remedies is common in the Limpopo Province (South Africa) due to the increase in the cost of conventional modern medicine and the lack of accessibility of healthcare providers (Semenya, and Maroyi, 2012). As reported by Rinne (2001), local communities consult traditional healers regularly because of their proximity. Furthermore, traditional healers are familiar with the patient's culture and their treatments are affordable. In addition, upon usage of Western medicine (antibiotics include) as a treatment for sexually transmitted infections (STIs), patients are expected to undergo repeat testing three months, and this makes the overall cost of conventional treatment somewhat expensive (Rinne, 2001).

Traditional herbal medicines may be full plants, raw plant materials, plant preparations and plant finished products that are used to maintain health, diagnose, prevent, improve or treat disease (Noor *et al.*, 2013). Medicinal plants are both used as crude

extracts directly from plants or indirectly as modern medicines such as drugs (Rekha *et al.,* 2013). Various parts of plants such, bark, stem, leaf, root, etc. are used to prevent, relieve symptoms, or revert abnormalities back to normalcy (Bhalla, 2020). In the Limpopo region, 61% of the medicinal plant material traded is in the form of roots, while whole plants are 21%, bark material 15% and 2% comprise fruits and leaves (Tshisikhawe, 2002).

More than 1/3 of the plants in the book *Medicinal Plants of South Africa* by van Wyk *et al*. (2013) are harvested for their roots. From an ethnobotanical survey of two regions in Limpopo (Lwamondo) and Mpumalanga (Jongilanga), the roots were observed to be the most frequently used parts in the formulation of herbal remedies (Mahwasane *et al*., 2013; Tshikalange *et al*., 2016). Similarly, Semenya and Potgieter (2013) reported roots as the most preferred parts for the herbal formulation used by the Bapedi traditional healers in South Africa for the treatment of sexual dysfunction. This may be due to the perception by the traditional healers that potent healing power is contained in the roots (Tshisikhawe, 2021), and that they are traditionally considered to be “strong medicine” (Mahwasane *et al.,* 2013).

The root is the most metabolite rich plant part which contributes to its efficacy in the treatment of sexual dysfunction (Semenya and Potgieter, 2013; Adnan *et al.,* 2014). Herbalists always make sure that the plant from which materials are harvested should not die (Tshisikhawe, 2021). When collecting roots, they collect lateral roots, avoiding taproots in the process. The roots are also covered with soil after collection (Tshisikhawe, 2021).

2.2. Sexual and reproductive health

Reproductive health care is said to be the second most widespread health care challenge on the African continent (Chinnoch, 1996). As more past than present infections appear to impair male fertility, it is important to treat any acute infection adequately and appropriately (Low *et al*., 2006). Disease is a possible outcome of infection, rather than an equivalent clinical state (Anderson, 2019). Some diseases,

such as erectile dysfunction and sexual impotence, are worthy of mention as they are regarded as petty and yet play an important role in ensuring family stability, sexually transmitted diseases control (HIV/AIDS included) and economic productivity (Kamatenesi-Mugisha and Oryem-Origa, 2005). Infertility constitutes a definite burden to the socioeconomic development of many African nations because it affects the life of families, couples, and individuals and in most cases, always leads to decreased levels of personal well-being. Hence, infertility is now considered a public health concern (de La Rochebrochard, 2009).

Treatment of sexually transmitted infections poses many challenges in developing countries (Lichtenstein, 2003 and Mårdh, 2004). In response, the World Health Organisation (WHO, 1991) introduced syndromic management guidelines. This strategy primarily focuses on treating patients according to the symptoms they present, rather than delaying treatment until laboratory test confirmation of the infection (Erasmus, 2012). Symptoms used to diagnose sexually transmitted infections included genital ulcers, vaginal discharge, urethral discharge, and swollen testicles, to mention a few (Maema *et al.,* 2020). Genital bacterial infections are a major cause of urethritis, which is characterised as a gonococcal or non-gonococcal infection in the male genital tract. The most common symptom of urethritis is urethral discharge (Igietseme *et al.,* 2015). The inflammation of the urethra and periurethral glands in men is caused by etiological agents of sexually transmitted infections. About half of the cases are characterised by a purulent, mucopurulent, serous, or even hemorrhagic (Leos-Alvarado *et al.,* 2020).

Infections can affect various sites of the male reproductive tract, such as the epididymis, testis, and male accessory sex organs (Pellati *et al*., 2008). Local traditional healers have always played an important role in reproductive health problems for the natives in the communities due to geographical and socio-economic factors (Hegde *et al.,* 2007). Also, plants have been used for years to treat infertility and related reproduction problems (Abdillahi and van Staden, 2013). In South Africa, herbal products that are sold by informal traders are usually suggested to be

aphrodisiacs, blood cleansers, energy boosters and detoxifiers (Ndhlala *et al.,* 2009). These plants have properties that can treat sexual dysfunction in men by cleansing the reproductive system and acting as sexual enhancers (Afolayan and Yakubu, 2009).

2.3. Traditional medicinal plants as a source of sexual health treatment

In the last few decades, with more rigorous research for therapies based on natural systems, plants are used extensively to cure different diseases. Tshisikhawe (2021) reported that Dr. Nthatho Motlana believes that the 80% figure is conservative since 99% of patients do consult traditional healers and then go for Western medicine. Such communities usually use Western medicine as a complementary treatment (Tshisikhawe, 2021). Hence there is a need to search for plants of medicinal value.

Plants as important source of medicine plays a key role in world health (Bhalla, 2020). In many African countries, there is a high prevalence rate of diseases such as gonorrhoea and syphilis, thus making STIs a major public health problem (Tshikalange *et al.,* 2005). A high number of cases are never reported to the health authorities; instead, patients go to traditional healers for treatments (Green, 1992). Both patients and traditional healers appear to believe that STIs are treated ineffectively by Western medicine. Some of those who go to a hospital may even come back to the traditional healers to “cleanse” the system after treatment by Western medicine (Moss *et al.,* 1999).

Plants are used in treatment of many illnesses, including sexually transmitted infections (STIs) (Ndubani, 1997; Ndubani and Höjer, 1999). The search for biologically active extracts based on traditionally used plants is still relevant due to the appearance of microbial resistance of many antibiotics and the occurrence of fatal, opportunistic infections (Tshikalange *et al*., 2005).

According to studies, a considerable portion of the African population relies on herbal treatments for their basic health care needs, particularly in the case of STIs. The same

applies in South Africa, as evidenced by various studies such as Hutchings *et al.,* (1996), Ndubani and Höjer (1999), Kambizi and Afolayan (2001), Ssegawa and Kasenene (2007), Namukobe *et al.,* (2011), Maroyi (2011) and Muthee *et al.,* (2011), which reported that a considerable portion of the African population relies on herbal treatments for their primary health care needs related to STIs.

The resistance of gonorrhoea and other STIs to antibiotics has increased rapidly in recent years and has reduced treatment options (WHO, 2016). Medicinal plants are being used because they possess numerous bioactive compounds that can fight against various types of diseases caused by pathogens (Senthilmurugan-Viji *et al.,* 2013). Several herbal treatments have been validated for their effect on infertility and can therefore aid as a source for the identification of new chemical leads that are beneficial in sexual health (Chauhan *et al.,* 2014). Medicinal plants are used as natural resources in the development of new drugs (Shakya, 2016). The importance of bioactive compounds found in herbal products in the treatment of male infertility and related sexual disorders is indisputable (Abdillahi and van Staden, 2012).

2.4. Plant secondary metabolites of therapeutic relevance

Plants produced phytochemical compounds (also known as secondary metabolites) that play a role of defence in the plant (Kinghorn *et al.,* 2003). They can inhibit the growth of problematic pathogens (Harvey *et al.,* 2015), which may ultimately be used as a lead for drug development. These include flavonoids, tannins, terpenoids, steroids, alkaloids, and many others, which have rich medicinal value such as anti-microbial and antioxidant activities (Ganesh *et al.,* 2020).

These secondary metabolites are found in leaves, barks, stems, roots, fruits, and seeds of the plant (Yadav and Aragwala, 2011). They are usually found in plants in small quantities and their accumulation in different organs of the plant is not the same (Sasnovskaya, 2021). The therapeutic effectiveness of plants is founded in these

secondary metabolites which cure many diseases (Shakya, 2016). Medicinal plants characteristically contain mixtures of different chemical compounds that may act additively, individually, or in synergy to improve health (Gurib-Fakim, 2006). The pharmacological effect of medicinal products from plants depends on the content of biologically active substances in them and belongs to different classes of chemical compounds in varied composition (Sasnovskaya, 2021).

Medicinal plants contain antioxidant, aphrodisiac and adaptogenic properties. Biologically active substances include alkaloids, glycosides, tannins, coumarins, and terpenoids (Sasnovskaya, 2021). Phytoconstituents with known structures have been classified in appropriate chemical groups and the active crude extracts. About 317 phytochemicals are listed for antioxidant potential; 340 plants as aphrodisiac and antioxidants and 40 plants are listed for adaptogenic nature (Shoba *et al.,* 2016). The study of phenolics has led to the discovery of the biological and physiological properties, which include anti-microbial, antioxidant, anti-inflammatory (Manach et al., 2005). The amplified interest and market in natural products are rapidly becoming more important to rural African communities as income generating activity that can be further investigated for the improvement of new, novel herbal drugs (Shoba *et al.,* 2016). Below some medicinally relevant phytochemical compounds are discussed.

2.4.1. Phenolic compounds

Phenolic compounds are a main class of secondary metabolites in plants and are divided into phenolic acids and polyphenols. Phenols are a member of a group of aromatic chemical compounds with weakly acidic properties and are characterised by a hydroxyl (OH) group attached directly to an aromatic ring (Okuwu, 2004). As will be seen below in **Figure 2.1**, the compounds are found combined with mono- and polysaccharides, linked to one or more phenolic group, or can occur as derivatives, such as ester or methyl esters (Minatel, 2017). The pentose phosphate, shikimate and phenylpropanoid pathways in plants are responsible for the production of phenolics (Randhir *et al.,* 2004).

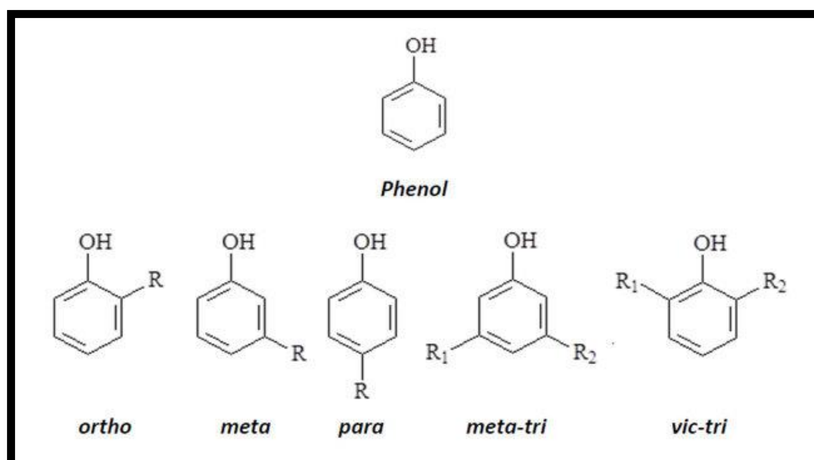

Figure 2.1: Structure and nomenclature for phenol and substitution patterns of phenolic compounds (Mu'Azu *et al*., 2017).

Phenolic compounds, abundant in plants are an essential part of the human diet and are of considerable interest due to their antioxidant properties. Phenolic compounds have antioxidant activity due to their ability to scavenge free radical (Zhao *et al.,* 2014). The antioxidant activity of phenolic compounds depends on the structure, the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings (Balasundram *et al.,* 2006).

2.4.2. Flavonoids

Most of them are known to be pigments in higher plants and derivatives of phenylalanine and tyrosine amino acids. (Khoddami *et al.,* 2013). Their chemical structure is based upon the C6-C3-C6 carbon skeleton. These are yellow polyphenolic compounds like flavones and contain the ketone group. Quercetin and kaempferol represent the common flavonoids found in plants and are represented in **Figure 2.2** Flavonoids are polyphenolic compounds that are ubiquitously present in foods, ingredients, and medicines derived from plants. A common group of flavonoids is the diphenylpropanes, which have one or more phenolic groups (**Figure 2.3**). This

phytochemical has attracted much attention because of its possible roles in the prevention of a wide range of degenerative diseases and exhibits properties like hepatoprotection, antioxidant, antimicrobial and antistress, e.g., silymarin and carotenoids (Kaladhar *et al.*, 2014). It is known that these compounds are syndissertationed by plants in response to microbial infections. Flavonoids have been shown to have anti-microbial activities with greater potency against Gram-negative bacteria (Martini *et al.,* 2004).

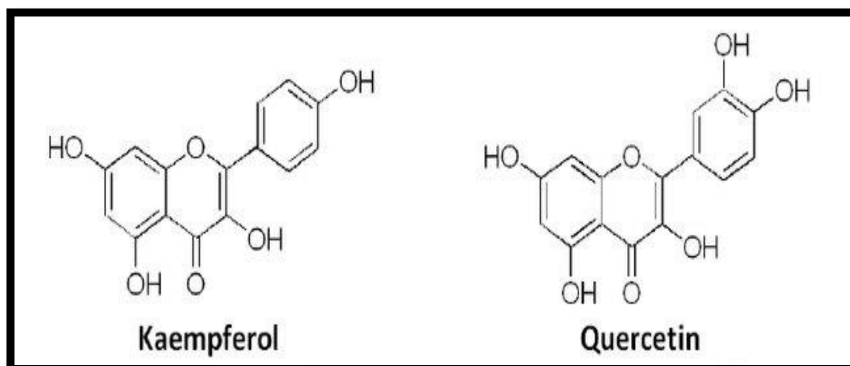

Figure 2.2: The basic structure of flavonoids (Nishiumi *et al*., 2011).

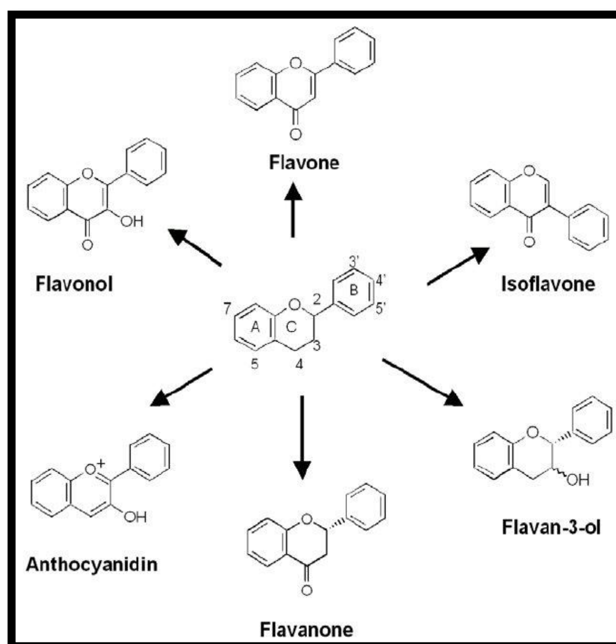

Figure 2.3: Major flavonols, flavones and polymethoxylated flavones (Nishiumi *et al.*, 2011).

2.4.3. Alkaloids

Alkaloids are basic and nitrogen containing compounds that are synthesised by many living organisms' alkaloids, are low-molecular-weight structures and form approximately 20% of plant-based secondary metabolites (Frolova, 2016). Alkaloids are known both in traditional and modern medicine to have several pharmacological activities, including antimicrobial activity (Othman *et al.,* 2019); they can be found in 300 plant families (Cushnie *et al.,* 2014). Alkaloids rank among the most efficient and therapeutically significant plant substances (Okwu, 2005).

They include compounds such as isoquinoline, imidazole, quinoline, atropine and piperdine alkaloids, among others (Irchhaiya *et al.,* 2015). Morphine, which was isolated from opium poppy *Papaver somniferum* in 1805, was the first medically useful example of an alkaloid (Fessenden and Fessenden, 1982; Mokgoatsane, 2011). One specific alkaloid that is known to possess antibacterial properties is Chelerythrine (illustrated in **Figure 2.4**). It is found in the plants, *Zanthoxylum clava-herculis* and *Zanthoxylum rhoifolium* (Gibbons *et al.,* 2003; Tavares *et al., 2*014). It is an effective selective and mobile-permeable protein kinase C capable of exhibiting antibacterial activity against *Staphylococcus aureus* and other human pathogen activity (Gibbons *et al.,* 2013; Tavares *et al.,* 2014).

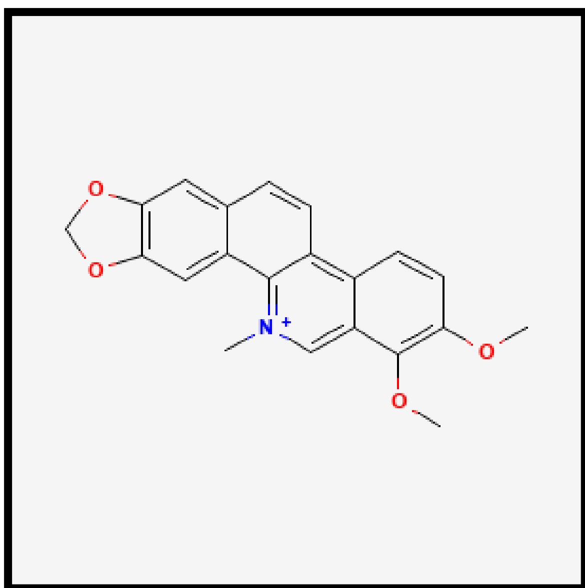

Figure 2.4: Structure of chelerythrine PubChem (www.pubchem.com Accessed 26 September 2021).

2.4.4. Saponins

Saponins are glycosides with triterpenoid or spirostane aglycones that demonstrate various pharmacological effects against mammalian diseases (Xu *et al.,* 2016). Saponins have therapeutic validation on the basis that anti-microbial, anti-inflammatory, and cytotoxic activities have been attributed to their presence in plant extracts (Abbas *et al.,* 2015). When dissolved in water to form aqueous solutions, these compounds have the characteristic of forming a persistent mass of bubbles, commonly referred to as a froth upon vigorous shaking (Sparg *et al.,* 2004). **Figure 2.5** is an overview of saponin (www.sciencedirect.com Accessed 9 August 2021).

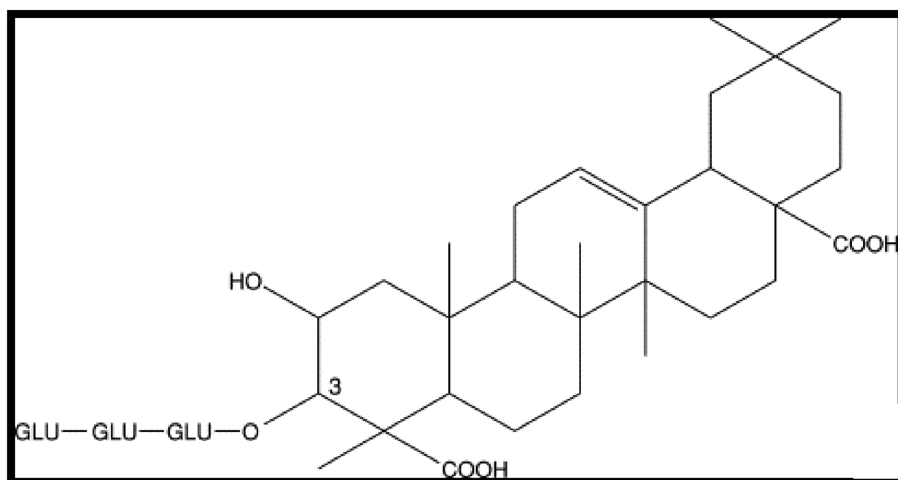

Figure 2.5: An overview of saponin (www.sciencedirect.com Accessed 9 August 2021).

2.4.5. Steroids

Plant steroids constitute a diverse group of natural products (Gunaherath *et al.,* 2006). Plant steroids possess many interesting medicinal, pharmaceutical and agrochemical activities like anti-tumour, immunosuppressive, hepatoprotective, antibacterial, plant growth hormone regulator, sex hormone, anthelminthic, cytotoxic and cardiotonic activity (Yokota, 1997). Epimedium is classified as a 'kidney-yang tonifying' herb that is frequently used to treat disorders such as impotence, premature ejaculation, and infertility, in combination with other herbs (Indran *et al.,* 2014). In **Figure 2.6**, a simple representation of steroid skeleton and cholesterol is provided.

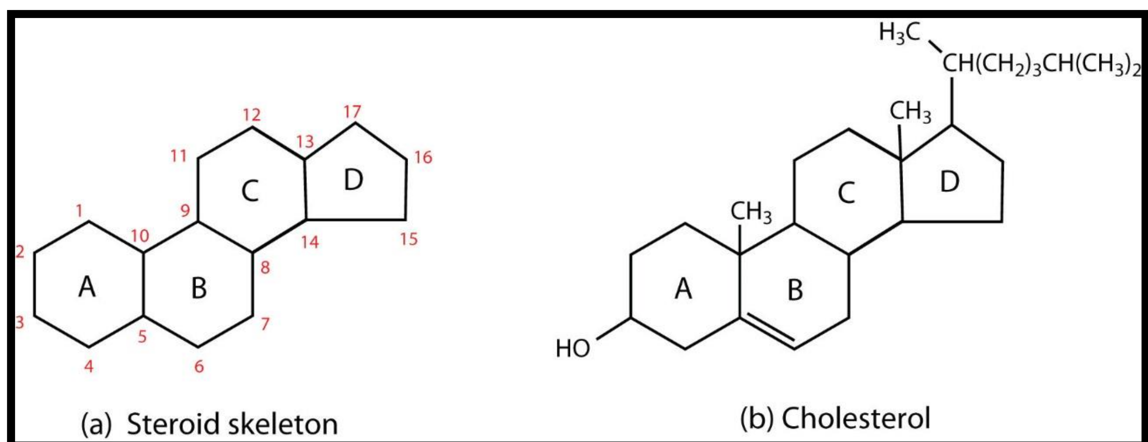

Figure 2.6: (**a**) The four-fused-ring steroid skeleton uses letter designations for each ring and the numbering of the carbon atoms. (**b**) The cholesterol molecule (https://saylordotorg.github.io/ Accessed 10 July 2019).

2.4.6. Tannins

These are amorphous complex phenolic substances classified based on the hydrolysis product. It is an astringent, bitter plant polyphenolic compound, e.g., tannic acid, gallic acid (Kaladhar *et al*., 2014). Tannins can inactivate microbial enzymes, adhesins and cell envelope transport proteins, which could be their mode of antimicrobial action (Mokgoatsane, 2011). They act as a barrier for microorganisms and defend plants because of their potential to shape complexes with protein, starches, and different macromolecules (**Figure 2.7**). They bind to the cell walls of the microorganism's inhibiting growth and protease activity. Tannins have been reported to act as antioxidants in vertebrates and pro-oxidants in the presence of oxygen (Barberhenn and Constable, 2011). Tannins are not toxic and many of them have P-vitamin activity (Sasnovskaya, 2021).

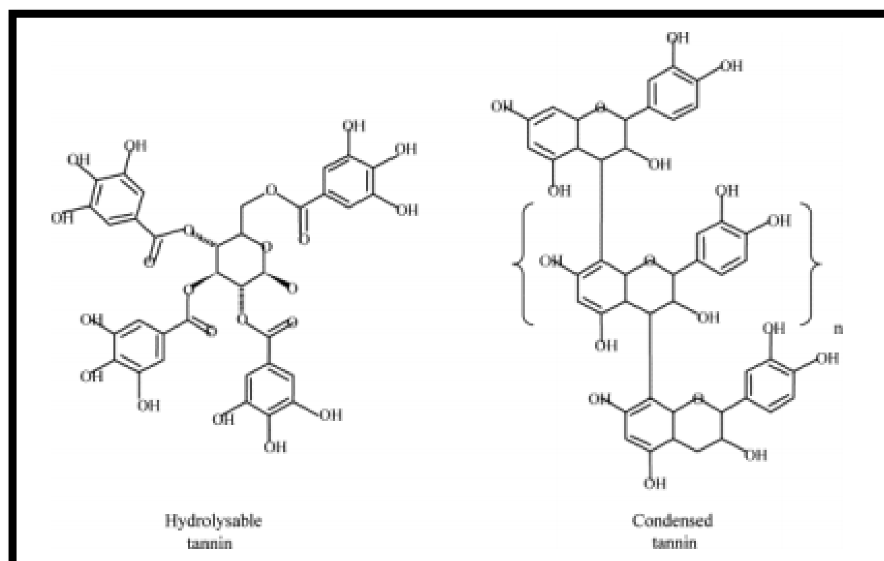

Figure 2.7: Diagrammatic representation of hydrolysable and condensed tannins **(**Krause *et al.,* 2005).

2.5. Biological activities of plants

A single plant species can produce numerous bioactive compounds that are neither stringently required for metabolic processes nor to form part of nutrition. The production of the compounds is subject to the interaction of the plant with the environment in which it is supposed to thrive (Okem *et al.,* 2015). Special attention is directed towards those plants which have grown in the wild. Their success relies on particularly high chemical diversity and a wide spectrum of biological activities due to the valuable content of nutrients, alkaloids, polyphenols, and phytoestrogens, that definitely establish their potential as novel pharmacological entities for medicinal treatment (Santini *et al.,* 2017). Medicinal plants contain a broad range of secondary metabolites with antioxidant and antimicrobial properties (Alhage *et al.,* 2018). On the other hand, the prevalence of infectious diseases poses a major problem, since pathogens have acquired resistance to the available antibiotics (Eloff, 1998).

Therefore, there is a great need to develop new drugs with a different mechanism of action and novel structures (Kudumela and Masoko, 2018). Drug discovery from medicinal plants is an extensive process that follows several steps (**Figure 2.8**) guided by the biological activity of interest. The successful isolation of compounds with desired activity from plant extracts is determined through several bioassays. It must be noted, however, that some compounds tend to lose activity with each step of purification because they may act synergistically to exhibit one biological activity. Masoko and Nemudzhivhadi (2015) reported that once compounds are separated, they may lose activity.

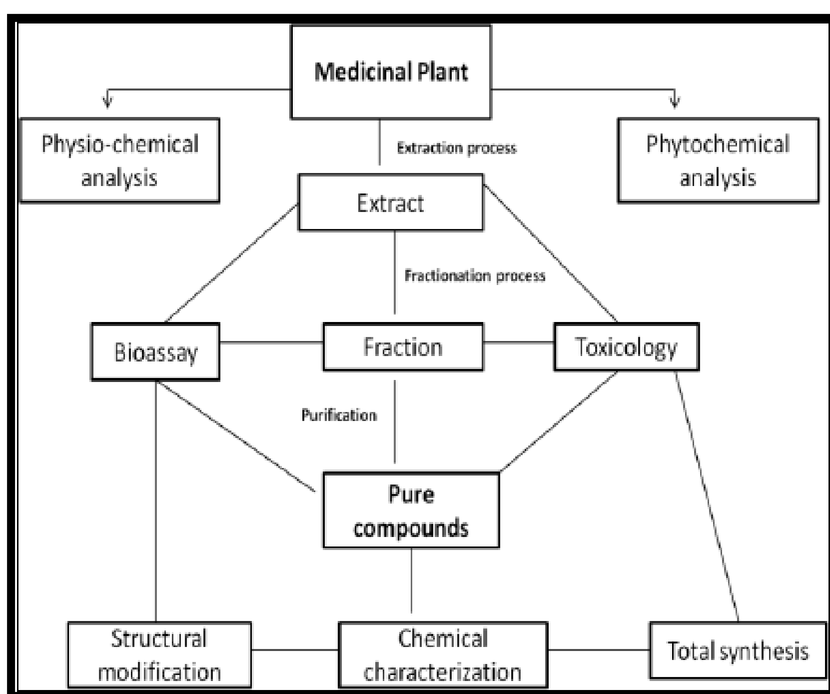

Figure 2.8: Method for obtaining active biological compounds from plants (Shakya, 2016).

Despite several advancements in the field of synthetic drug chemistry and antibiotics, plants continue to be one of the major raw materials for drugs treating various human ailments. Clinical and pharmaceutical investigations have elevated the status of medicinal plants by identifying the role of active principles present in them and elaborating on their mode of action in human and animal systems (Jain *et al.,* 2019).

2.5.1. Antimicrobial activity

For centuries, humankind has effectively used various components of plants or their extracts for the treatment of many diseases, including bacterial infections (Murugan *et al.,* 2013). Traditional medicinal plants are a natural source of new drugs of high value to humankind. Plants have been used throughout the whole world as raw drugs or traditional medicines. Many plant extracts have been shown to inhibit the growth of microorganisms, thus making plants a rich source of antibacterial compounds (Vashist and Jindal, 2012). Secondary metabolites are usually considered to play a role in defence reactions of plants against infections by pathogenic microorganisms. The antibacterial activity of medicinal plants is due to the presence of secondary metabolites, including tannins, alkaloids, flavonoids, and other phenolic compounds (Chitemerere and Mukanganyama, 2011).

In recent years, antibacterial compounds have been screened from several medicinal plants based on their traditional use in rural areas for similar purposes. The presence of antibacterial compounds from medicinal plants is a well-known fact and provided information that led to the development of new antimicrobial drugs. Many of the modern medicines have been derived from natural sources, including plants and many of these drugs were isolated based on the use of such plants traditionally (Katiyar *et al.,* 2012).

Traditional medicine has indeed been an effective complement to the scientific form of health care. It is an alternative health care system. The search for unexplored plants or plant group with substantial antibacterial action has attained massive importance these days, due to a growing concern about the attainment of antibiotic- resistance by the pathogenic microorganism (Bishnoi *et al.,* 2016). There are several methods used in medical research laboratories to test for bioactive compounds with antimicrobial activity, which includes bioautography and microbroth dilution methods.

Medicinal plants represent a rich source of antimicrobial agents with better activity against multi-drug resistant bacteria and their antimicrobial potential was accepted long before the discovery of microorganisms (Anwar *et al.,* 2009). Medicinal plants have a diverse mixture of biologically active compounds that may act in killing or inhibiting the growth of disease-causing microorganisms (Alvin *et al.,* 2014). Therefore, the screening of natural products for the discovery and development of novel antimicrobial agents is imperative (Yala *et al.,* 2001; Edward-Jones, 2013; Aumeeruddy-Elalfi *et al.,* 2016).

2.5.2. Antioxidant activity

Medicinal plants are a rich source of many bioactive compounds with various biological activities, including antioxidant activity. Antioxidants are defined as compounds that prevent free radicals from oxidizing by reacting with them (Özyazici, 2021). In other words, they are reactions that enable the stopping of free radical-producing reactions and repairing the damage to lipid, protein, and DNA molecules (Karaaslan *et al.*, 2014). Oxygen plays an important role in the biological systems of aerobic forms of life, although its derivatives are highly toxic. Free radicals are produced either by-products or end products of some biochemical reactions that contribute to the development and maintenance of cellular life. High levels of free radicals in normal systems results in an imbalance between antioxidants and free radicals (Kumar *et al.,* 2012).

Antioxidants can be examined in two classes, natural and synthetic. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and propyl gallate (PG) are examples of synthetic antioxidants that are currently commercialised (Taghvaei and Jafari, 2015). Vitamins (A, C, E), carotenoids and phenolic compounds are the most important natural antioxidants that occur naturally in plant and animal tissues or that are released by the processing of food (Lourenço *et al.*, 2019). The most important factor in the antioxidant effect of herbal products is due to flavonoids, cinnamic acid derivatives and phenolic compounds such as coumarins. Medicinal plants containing phenolic compounds, such as phenols,

tannins and flavonoids, are rich sources of natural antioxidants that are non-toxic and easily accessible (Shakya, 2016).

There are several methods that are currently used in medicinal plant research laboratory to test for the presence of antioxidant activity of traditional medicinal plant extracts. The common procedures involve the determination of the ability of plants extracts to scavenge free radicals using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals (Molan *et al.,* 2012). The free radical scavenging assays are based on the rate of discolouration of coloured radical compounds while the metal ions reduction assays are based on the reduction of metal complexes (Marques *et al.,* 2014).

One of the most basic ways to determine the antioxidant potential of medicinal plants is by determining their free radical scavenging activity (Chigayo *et al.,* 2016). Other numerous assays have been frequently used to estimate antioxidant capacities of plant extracts. These include ferric reducing antioxidant power (FRAP), chelating effect and phosphomolybdenum assays (Sarikurkcu and Tepe, 2015).

2.6. Safety of plant extracts

The mere fact that a product is "natural" may not signify that the product is safe. Some natural products may be contaminated with potentially hazardous substances, such as toxic metals and pathogenic microorganisms (WHO, 2004). Evidence of human contamination of herbs has also been reported (Kineman *et al*., 2002). Microbial contamination is assumed to occur through handling by personnel who are infected with pathogenic bacteria during harvesting/collection, post-harvest processing and the drug manufacturing process. Some herbal products may be containing toxic constituents, such as liver toxins or carcinogens, which are chemicals with the potential to cause cancer (WHO, 2004). Therefore, it is of great importance that the cytotoxicity of the plant extracts be investigated for scientific validation.

Prolonged use of more popular herbal remedies may potentially lead to genotoxic effects (Fennel *et al.,* 2004). Populations who stay in poor areas and are dependent on medicinal plants are at a great risk of encountering toxicity effects of plants as well as long term effects emanating from continued use of medicinal plants. There are also concerns regarding the dosage of traditional medicine taken by people due to inadequate knowledge (Masoko and Nxumalo, 2013), because plants may be therapeutic at one dose and potentially toxic at another (McGaw *et al.,* 2007). It is important to analyse medicinal plants' toxicity for traditional healers and other consumers to have knowledge on their safety and dosage (Bagla, 2011). Moreover, it is important to evaluate these effects to know as to whether people can or cannot continue using medicinal plants for their needs without being at the risk of the long-term toxic effects of these plants (Bagla, 2011).

2.7. Proximate composition

The proximate composition of foods includes moisture, ash, lipid, protein, and carbohydrate contents. These food components may be of interest in the food industry for product development, quality control (QC) or regulatory purposes. Analyses used may be rapid methods for QC or more accurate, but time-consuming official methods. Sample collection and preparation must be considered carefully to ensure analysis of a homogeneous and representative sample, and to obtain accurate results. Estimation methods of moisture content, ash value, crude lipid, total carbohydrates, starch, total free amino acids, and total proteins are put together in a lucid manner.

Medicinal plants used for various purposes, especially health and food, must comply with certain quality standards. Research has demonstrated that nutrition plays a crucial role in the prevention of chronic disease and now with the recognition that typical foods may provide prophylactic benefits, efforts are being directed towards promoting the "functional diet" (Ramalingum and Mahomoodally, 2014). The new concept of functional foods has been identified as a promising field to boost nutritional sciences to the forefront of preventive medicines for both existing and emerging diseases of humankind (Ramalingum and Mahomoodally, 2014). Health professionals

now recognise that a synergy of drug therapy and nutrition might confer optimum outcomes in the fight against diseases (Ramalingum and Mahomoodally, 2014).

2.8. Extraction, separation, and purification of bioactive compounds

Separation and purification of compounds from medicinal plant materials usually begins with their extraction. But, since plant extracts usually occur as mixtures of various bioactive compounds with different polarities, their separation remains a big challenge (Sasidharan *et al.,* 2011). The most important factor that must be considered before designing an isolation protocol is the nature of the target compound present in the crude extracts or fractions. However, it is more difficult to design an isolation protocol for a crude extract where the types of compounds present are totally unknown. In this situation, it is advisable to carry out qualitative tests for the presence of various types of compounds. The yield of compounds at the end of the isolation and purification process is important in natural product research where compounds can be quantified and become structurally elucidated (Sarker *et al.,* 2006).

Chromatography is the general name for the separation, recognition, and purification of substances in a mixture in a two-phase system, one of which is stationary, and the other is a mobile phase (Özyazici, 2021). There are several separation techniques that are used to separate and purify compounds of interest from crude extracts, and these include thin-layer chromatography, column chromatography and preparative thin-layer chromatography plates. The second step of separation and purification of compounds involves the use of column chromatography and preparative thin-layer chromatography, to separate components from crude extracts of plants based on the polarities using one or two solvents of varying polarities into fractions for further purification. The final step involves the use of thin-layer chromatography (TLC) plates to confirm the purity of separated compounds (Visht and Chaturvedi, 2012). Pure compounds are further analysed for structure elucidation and characterisation using nuclear magnetic resonance (NMR) and Mass spectrometry (MS).

2.9. Test organisms for antimicrobial activity

2.9.1. Bacteria of clinical significance in public health sector

The disease-causing bacteria such as two Gram-positive bacteria *Staphylococcus aureus* and *Enterococcus faecalis* which are found in most healthy individuals can cause urinary tract infections (Zeyad and Hammadeh, 2017). Also, a fungus *Candida albicans* which causes semen candidiasis was used as a test microorganism. It is the most important sexually transmitted fungal infection; this microorganism affects male fertility potential (Castrillón-Duque *et al.*, 2018).

2.9.1.1. *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive lactic acid bacterium commonly found in mammalian intestine and plants. *Enterococcus faecalis* causes life threating diseases such as endocarditis and bacteraemia, urinary tract infections, and meningitis (Murray, 1998). Although *E. faecalis* is often susceptible to ampicillin, treatment failure of 60% and lack of bactericidal activity of cell wall–active agents (i.e., penicillin G, ampicillin, vancomycin), it prompted efforts to identify combination therapies that would yield a bactericidal effect in severe infections (Baddour *et al.,* 2005; Miro *et al.,* 2013; Baddour *et al.,* 2015).

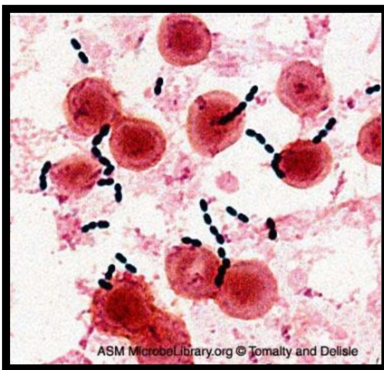

Figure 2.9: *Enterococcus faecalis* cells viewed under conventional light microscope (Todar, 2009).

2.9.1.2. *Staphylococcus aureus*

The genus *Streptococcus* comprises over 50 different species of Gram-positive, non-sporulating cocci-shaped bacteria. It is found in a diverse range of environments, including soils, or as part of the natural human microflora (Wright *et al.,* 2016). *Staphylococcus aureus* is a Gram-positive facultative bacterium. This bacterium is known to be a common animal and human pathogen, which causes a wide range of clinical infections. The infections caused by this pathogen include bacteremia, pneumonia endovascular, infective endocarditis, skin, and soft tissue infections (Tong *et al.,* 2015; Giersing *et al.,* 2016). In **Figure 2.10** the *S. aureus* is illustrated and the black arrow shows the grapes-like morphology.

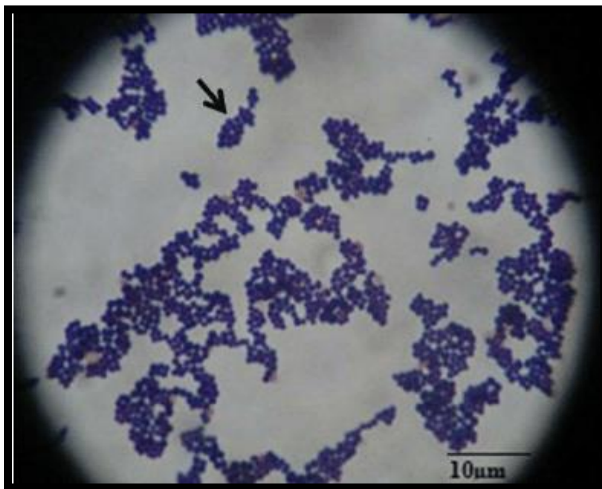

Figure 2.10: Gram's staining of *S. aureus* at 100X resolution (Mahan, 2015).

2.9.2. Fungus of clinical significance

2.9.2.1. *Candida albicans*

Candida albicans is an opportunistic pathogenic yeast (Gow, 2017); it is a common member of the human gut flora. It can also survive outside the human body (Bensasson *et al.,* 2019). It is detected in the gastrointestinal tract and mouth in 40–60% of healthy adults. It is usually a commensal organism, but it can

become pathogenic in immunocompromised individuals under a variety of conditions. It is one of the few species of the genus *Candida* that causes the human infection candidiasis, which results from an overgrowth of the fungus. *Candida albicans* commonly colonises the urethra. It can be found in the semen and may affect in vitro fertilisation. *Candida albicans* has an inhibitory effect on human sperm motility and impairs the ultrastructure of human spermatozoa, which could be associated with male infertility (Tian *et al*., 2007).

Figure 2.11: *Candida albicans* growing on sabouraud agar sponsored by Science History Images / Alamy Stock Photo https://commons.wikimedia.org/wiki Accesed 12 January 2021).

2.10. Medicinal plants used in study

A medicinal plant is defined as any plant containing biologically active substances that can be used for therapeutic purposes or which contain precursors of chemo-pharmaceutical syndissertation (Jain *et al*., 2019; Sasnovskaya, 2021). Most indigenous plants have vernacular names which were derived in several ways, similarly the Vhavenḓa have Tshivenḓa names. These (Tshisikhawe, 2021). For instance, there are names based on functional significance; such names are usually related to the utilisation of the plants by people inhabiting some or all regions of Venḓa. The name may indicate the purpose for which the plant is used, e.g., *gumululo (Elephantorrhiza burkei*), which means cleansing or washing away in Tshivenḓa (Mabogo, 1990).

2.10.1. *Elephantorrhiza burkei*

Elephantorrhiza burkei (also known as Gumululo in Tshivenḓa) is a genus of flowering plants in legume family, Fabaceae, and the subfamily Caesalpinioideae. This plant thrives on rocky ridges and is well adapted on slopes in woodland, grassland and scrubveld of Gauteng, Limpopo, North-West, Mpumalanga in South Africa and places like Zambia and Botswana in Africa (Foden and Potter, 2019). The two pod valves of *E. burkei* roll back and persist with their margins for many months, while the pods of *E. elephantina* generally disintegrate and disappear more rapidly. *Elephantorrhiza burkei* is known in the system of traditional medicine (Irwin and Barneby, 1982). Various parts of the tree are used in traditional medicine for treating everything from blackwater fever, headache, toothache, and stomach-ache to using it as a natural abortion agent. An infusion is made from the root and drunk as an aphrodisiac (Mabogo, 1990). It is understood to gather all undesirable and harmful substances in the body and make them available for excretion (Magwede *et al.,* 2019). So, it works like a blood cleanser by flushing out any harmful substances from the blood streams.

Figure 2.12: *Elephantorrhiza burkei*; **A**- Shrub, **B**- Flower and **C**- Roots (adapted from plantzafrica.com Accessed 9 October 2020) and (van Wyk *et al*., 2013).

2.10.2. ***Securidaca longepedunculata***

Securidaca longepedunculata (also known as Violet tree in English) or Mpesu (in Tshivenḓa) is a slender tree, from the polygalacea family with beautiful flowers. It is a highly regarded medicinal and magical tree, especially by the Vhavenḓa people of the Limpopo Province where it can be found. It also occurs in the North-West and Limpopo Provinces of South Africa, in Mozambique and is widely distributed in tropical Africa. Mpesu is known to be a natural aphrodisiac (Mabogo, 1990). The roots and bark are taken orally either as powdered or as infusions for treating chest complaints, headache, constipation abortion, tuberculosis, infertility problems, venereal diseases, and for vaginal itching (Ndou, 2013; Mustapha, 2013). The root bark is nibbled for the sweet and pleasant taste, which is probably due to the presence of methyl salicylate, but it is also liked for its aphrodisiac effects (Mabogo, 1990; Tshisikhawe, 2021). Mpesu has compounds that are extracted from roots and these natural chemicals relax the muscles of the penis and relax the brain of the user, it is also used as a sexual booster (Namadina *et al.,* 2020). It treats diabetes and microbial infections; it also possesses anti-inflammatory properties that help to reduce arthritic pains (Ojewole, 2008).

Figure 2.13: *Securidaca longepedunculata* (Fresen) with flowers (adapted from plantzafrica.com Accessed 9 October 2020).

2.10.3. *Wrightia natalensis*

Wrightia natalensis (also known as saddle-pod in English) and Musunzi (in Tshivenḓa) grows as a small to medium-sized deciduous tree. *Wrightia natalensis* is native to Zimbabwe, Mozambique, Swaziland and South Africa-namely in Limpopo and Kwazulu Natal . Its fragrant flowers feature a creamy yellow corolla. The roots are chewed fresh, and the juice is swallowed by herd boys and young men. *Wrightia natalensis* succulent roots are a rich source of water and have aphrodisiac properties and is, as such, used medicinally in this regard (Mabogo, 1990; Obi *et al.*, 2002; Tshisikhawe *et al.,* 2021). *Wrightia natalensis* is also used for skin infections, blood poisoning, respiratory infections, and gonorrhoea (Obi *et al.*, 2002).

Figure 2.14: *Wrightia natalensis*; **A**- Shrub, **B**- Leaves and **C**- Pods (seeds) (adapted from www.malawiflora.com).

Medicinal plants often provide three major kinds of benefits: financial benefits to individuals who harvest, process, and sell or distribute them; health benefits to the individuals who consume them as medicines; and society-wide benefits, such as

taxation income, job opportunities, and a healthier labour force (Smith-Hall *et al.,* 2012). Nevertheless, development of plants or plant extracts with potential medicinal uses and applications is dulled by weak scientific evidence, insufficient financing, and poor practices in the process of drug development (Ahn, 2017). Plants used in Tshivenḓa traditional medicine are generally compounded for effective or multiple treatment, the proportions differing from practitioner to practitioner and from treatment to treatment. In general, the combinations include the main remedies that are known to be effective for the treatment (Tshisikhawe, 2021).

2.11. Aim and objectives

2.11.1. Aim

The study aimed to isolate and characterise antimicrobial compounds from *Elephantorrhiza burkei, Securidaca longipedunculata* and *Wrightia natalensis* used in male sexual health and evaluate toxicological effects.

2.11.2. Objectives

The specific objectives of the study were to:

i. Isolate and identify microbial contaminants and nutrient evaluation.

ii. Phytochemically analyse (extraction and screening) and determine qualitative phytochemical constituents.

iii. Evaluate the antimicrobial activity of crude extracts of selected plants using minimum inhibitory concentration assay and bioautography.

iv. Determine the presence of antioxidant compounds in the selected plants using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and quantitative total antioxidant activity assay using ferric reducing power (FRAP) assay.

v. Perform synergistic/antagonistic study of the plant extracts.

vi. Isolate bioactive compounds with antimicrobial activity using column chromatography and preparative TLC.

vii. Investigate the cytotoxicity effects of the plant extracts and isolated compounds on Kmst-6 cell line (PC-201-012) using cell viability assay.

References

Abbas, G. Rauf, K. and Mahmood, W. 2015. Saponins: The phytochemical with an emerging potential for curing clinical depression. *Natural Product Research*, 29, pp.302–307.

Abdillahi, H. S. and van Staden, J. 2013. Application of medicinal plants in maternal healthcare and infertility: A South African perspective. *Planta Medica*, *79*(07), pp. 591-599.

Abdillahi, H.S. and Van Staden, J. 2012. South African plants and male reproductive healthcare: conception and contraception. *Journal of Ethnopharmacology*, *143*(2), pp.475-480.

Adnan, M. Ullah, I. Tariq, A. Murad, W. Azizullah, A. Khan, A.L. and Ali, N. 2014. Ethnomedicine use in the war affected region of northwest Pakistan. *Journal of Ethnobiology and Ethnomedicine*, *10*(1), pp.1-16.

Ahn, K. 2017. The worldwide trend of using botanical drugs and strategies for developing global drugs. *BMB Reports*, *50*(3), pp.111.

Alhage, J. Elbitar, H. Taha, S. and Benvegnu T. 2018. *In vitro* assessment of antioxidant, antimicrobial, cytotoxic, anti-inflammatory, and antidiabetic activities of *Campanula retrorsa* crude extracts. *Pharmacognosy Research*, *10*, pp.397-403.

Alvin, A., Miller, K.I. and Neilan, B.A. 2014. Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. *Microbiological Research*, *169*(7-8), pp.483-495.

Anderson, J.B. 2019. STD (sexually transmitted disease) or STI (sexually transmitted infection): Should we choose. *Am Sex Health Assoc*.

Anwar, A. Leong, K.M. Ng, M.L. Chu, J.J. and Garcia-Blanco, M.A. 2009. The polypyrimidine tract-binding protein is required for efficient dengue virus propagation and associates with the viral replication machinery. *Journal of Biological Chemistry*, *284*(25), pp.17021-17029.

Baddour, L.M. Wilson, W.R. Bayer, A.S. Fowler Jr, V.G. Bolger, A.F. Levison, M.E. Ferrieri, P. Gerber, M.A. Tani, L.Y. Gewitz, M.H. and Tong, D.C. 2005. Infective endocarditis: diagnosis, antimicrobial therapy, and management of complications: a statement for healthcare professionals from the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease, Council on Cardiovascular Disease in the Young, and the Councils on Clinical Cardiology, Stroke, and Cardiovascular Surgery and Anesthesia, American Heart Association: endorsed by the Infectious Diseases Society of America. *Circulation*, *111*(23), pp.394-e434.

Baddour, L.M. Wilson, W.R. Bayer, A.S. Fowler Jr, V.G. Tleyjeh, I.M. Rybak, M.J. Barsic, B. Lockhart, P.B. Gewitz, M.H. Levison, M.E. and Bolger, A.F. 2015. Infective endocarditis in adults: diagnosis, antimicrobial therapy, and management of complications: A scientific statement for healthcare professionals from the American Heart Association. *Circulation*, *132*(15), pp.1435-1486.

Bagla, P.P. 2011. Piercing the veil of ayurveda. *Science*, *334*(6062). pp.149.

Balasundram, N. Sundram, K. and Samman, S. 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, *99*(1), pp.191-203

Bensasson, D. Dicks, J. Ludwig, J.M. Bond, C.J. Elliston, A. Roberts, I.N. and James, S.A. 2019. Diverse lineages of *Candida albicans* live on old oaks. *Genetics*, *211*(1), pp.277-288.

Bhalla, E. 2020. Traditional uses of medicinal plants for treatment of widespread diseases: A review. *Epidemiology and Transmission of Infectious Diseases*, pp.150.

Bishnoi, A, Singh, V, Sharma, V. and Alam, A. 2016. Antibacterial Activity of *Anoectangium clarum* mitt. (Bryophyta: Pottiaceae) against some pathogenic bacteria. *Sikkim Medical University Journal*, *3*(1) pp. 650-654.

Castrillón-Duque, E.X. Suárez, J.P. and Maya, W.D.C. 2018. Yeast and fertility: Effects of *in vitro* activity of *Candida* spp. on sperm quality. *Journal of Reproduction and Infertility*, *19*(1), pp 49-55.

Chauhan, N.S. Sharma, V. Dixit, V.K. and Thakur, M. 2014. A review on plants used for improvement of sexual performance and virility. *BioMed Research International.*

Che, C.T. George, V. Ijinu, T.P. Pushpangadan, P. and Andrae-Marobela, K. 2017. Traditional Medicine in Pharmacognosy, Academic Press, pp.15-30.

Chigayo, K. Mojapelo, P.E.L. and Moleele, S.M. 2016. Phytochemical and antioxidant properties of different solvent extracts of *Kirkia wilmsii* tubers. *Asian Pacific Journal of Tropical Biomedicine*, *6* (12), pp.1037–1043.

Chinnoch, P.P. 1996. Reproductive health. *Africa Health*, *18*(3), pp.19.

Cushnie, T.T. Cushnie, B. and Lamb, A.J. 2014. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. *International Journal of Antimicrobial Agents*, *44*(5), pp.377-386.

De La Rochebrochard, E. Quelen, C. Peikrishvili, R. Guibert, J. and Bouyer, J., 2009. Long-term outcome of parenthood project during in vitro fertilization and after discontinuation of unsuccessful *in vitro* fertilization. *Fertility and Sterility*, *92*(1), pp.149-156.

Eloff, J.N. 1998. Which extracting solvent should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*, *60*(1), pp.1-8.

Erasmus, L.J.C. Potgieter, M.J. Semenya, S.S. and Lennox, S.J. 2012. Phytomedicine versus gonorrhoea: The Bapedi experience. *African Journal of Traditional, Complementary and Alternative Medicines*, *9*(4), pp.591-598.

Fessenden, R.J. and Fessenden, J.S. 1982. Organic chemistry, 2nd edition, Willard Grant Press, Boston, Mass, United States of America. Pp.729-791.

Foden, W. and Potter, L. 2005. Plumbago auriculata Lam. National assessment: Red List of South African plants version 2017.1. *Published at http://redlist. sanbi. or g/species. php*, pp.3567-1.

Frolova, O. O. 2016. Biologically active substances of plants from *Salix L*. genus. *Pharmacy and Pharmacology 4(*2), pp.41-59

Ganesh, M.S. John, D.N.A.A. and Radhika, D.J. 2020. A comparative study of antibacterial potential of various extracts of different parts of *Jatropha Curcas* Linn against bacterial strains. *International Journal of Life Science and Pharma Research*, *10*(2), pp.83-87.

Gibbons, S. Leimkugel, J. Oluwatuyi, M. and Heinrich, M. 2003. Activity of *Zanthoxylum clava-herculis* extracts against multi-drug resistant methicillin-resistant *Staphylococcus aureus* (mdr-MRSA). *Phytotherapy Research*, *17*(3), pp.274-275.

Giersing, B.K. Dastgheyb, S.S. Modjarrad, K. and Moorthy, V. 2016. Status of vaccine research and development of vaccines for *Staphylococcus aureus*. *Vaccine*, *34*(26), pp.2962-2966.

Gow, N.A.R. 2017. Microbe Profile: *Candida albicans*: a shape-changing, opportunistic pathogenic fungus of humans. *Microbiology. 163* (8), pp.1145–1147.

Green, E.C. 1992. Sexually transmitted diseases, ethnomedicine and health policy in Africa. *Social Science and Medicine* 35, pp.121–130.

Gunaherath, B. Kamel, G. and Gunatilaka, A.L. 2006. Plant steroids: occurrence, biological significance, and their analysis. *Encyclopedia of Analytical Chemistry: Applications, Theory and Instrumentation*, pp.1-26.

Gurib-Fakim, A. 2006. Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*, *27*(1), pp.1-93.

Harvey, A.L., Edrada-Ebel, R. and Quinn, R.J. 2015. The re-emergence of natural products for drug discovery in the genomics era. *Nature Reviews Drug Discovery*, *14*(2), pp.111-129.

Hegde, H.V. Hegde, G.R. and Kholkute, S.D. 2007. Herbal care for reproductive health: ethno medicobotany from Uttara Kannada district in Karnataka, India. *Complementary Therapies in Clinical Practice*, *13*(1), pp.38-45.

Hosseinzadeh, S. Jafarikukhdan, A. Hosseini, A. and Armand, R. 2015. The application of medicinal plants in traditional and modern medicine: a review of *Thymus vulgaris*. *International Journal of Clinical Medicine*, *6*(09), pp.635.

Hutchings, A. 1996. *Zulu medicinal plants: An inventory*, University of Natal press, Durban, South Africa.

Igietseme, I.U. Omosun, Y. and Black, M.C. 2015. Bacterial sexually transmitted infections (STIs): A clinical overview. *Molecular Medical Microbiology,* 2nd edition, Academic Press, pp.1403-1420.

Ilodibia, C.V. Ewere, F.U. Akachukwu, E.E. Adimonyemma, R.N. Igboabuchi, N.A. and Okeke, N.F., 2016. Proximate composition, vitamin, and anatomical studies on *Gomphrena celosioides*. *Annual Research and Review in Biology*, *10*(3) pp.1-6.

Indran, I.R. Zhang, S.J. Zhang, Z.W. Sun, F. Gong, Y. Wang, X. Li, J. Erdelmeier, C.A. Koch, E. and Yong, E.L. 2014. Selective estrogen receptor modulator effects of epimedium extracts on breast cancer and uterine growth in nude mice. *Planta medica*, *80*(01), pp.22-28.

Irchhaiya, R. Kumar, A. Yadav, A. Gupta, N. Kumar, S. Gupta, N. Kumar, S. Yadav, V. Prakash, A. and Gurjar, H. 2015. Metabolites in plants and its classification. *World Journal of Pharmacy and Pharmaceutical Sciences*. *4*(1), pp.287-305.

Irwin, H.S. and Barneby, R.C. 1982. *The American cassiinaea synoptical revision of leguminosae tribe cassieae subtribe cassiinae in the New World* (No. 580.744747 M4/v. 35/2).

Jain, C. Khatana, S. and Vijayvergia, R. 2019. Bioactivity of secondary metabolites of various plants: a review. *International Journal of Pharmaceutical Sciences and Research*, *10*(2), pp.494-498.

Kaladhar, D.S.V.G.K, Saranya, K.S., Vadlapudi, V. and Yarla, N.S. 2014. Evaluation of anti-inflammatory and anti-proliferative activity of *Abutilon indicum* L. plant ethanolic leaf extract on lung cancer cell line A549 for system network studies. *Cancer Science and Therapy*, *6*, pp.195-200.

Kamatenesi-Mugisha, M. and Oryem-Origa, H. 2005. Traditional herbal remedies used in the management of sexual impotence and erectile dysfunction in western Uganda. *African Health Sciences*, *5*(1), pp.40-49.

Kambizi, L. and Afolayan, A.J., 2001. An ethnobotanical study of plants used for the treatment of sexually transmitted diseases (njovhera) in Guruve District, Zimbabwe. *Journal of Ethnopharmacology*, *77*(1), pp.5-9.

Katiyar, C. Gupta, A. Kanjilal, S. and Katiyar, S. 2012. Drug discovery from plant sources: An integrated approach. *Ayu*, *33*(1), pp.10.

Kennedy, D.O. and Wightman, E.L. 2011. Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function. *Advances in Nutrition, 2*, pp.32–50.

Khoddami, A. Wilkes, M.A. and Roberts, T.H. 2013. Techniques for analysis of plant phenolic compounds. *Molecules*, *18*(2), pp.2328-2375.

Kineman, B. Nahikian-Nelms, M.L. Frazier, C. Somarajan, N. and Galati, V. 2001. A pilot investigation of the microbial contamination of herbal supplements: A potential

risk for immunocompromised populations. *Journal of the American Dietetic Association*, *101*(9), pp.A-59.

Krause, D.O. Smith, W.J. Brooker, J.D. and McSweeney, C.S. 2005. Tolerance mechanisms of streptococci to hydrolysable and condensed tannins. *Animal Feed Science and Technology*, *121*(1-2), pp.59-75.

Kudumela, R.G. and Masoko, P. 2018. *In vitro* assessment of selected medicinal plants used by the Bapedi Community in South Africa for treatment of bacterial infections. *Journal of Evidence-Based Integrative Medicine*, *23,* pp.1-11.

Leos-Alvarado, C. Llaca-Díaz, J. Flores-Aréchiga, A. Pérez-Chávez, F. and Casillas-Vega, N. 2020. Male urethritis: A review of the ideal diagnostic method. *Actas Urológicas Españolas (English Edition)*.

Lichtenstein, B. 2003. Stigma as a barrier to treatment of sexually transmitted infection in the American deep south: issues of race, gender and poverty, *Social Science and Medicine*, *57*(12), pp.2435-2445.

Louhaichi, M. Salkini, A.K. Estita, H.E. and Belkhir, S. 2011. Initial assessment of medicinal plants across the Libyan Mediterranean coast. *Advances in Environmental Biology*, *5*(2), pp.359-370.

Lourenço, S.C. Moldão-Martins, M. and Alves, V.D., 2019. Antioxidants of natural plant origins: From sources to food industry applications. *Molecules*, *24*(22), pp.4132.

Low, N., Broutet, N., Adu-Sarkodie, Y., Barton, P., Hossain, M. and Hawkes, S., 2006. Global control of sexually transmitted infections. *The Lancet*, *368*(9551), pp.2001-2016.

Mabogo, D.E.N. 1990. *The Ethnobotany of Vhavenda*. M.Sc. Dissertation. University of Pretoria, Pretoria, South Africa.

Maema, L.P.P. Potgieter, M.J. and Samie, A. 2020. Treatment of sexually transmitted infections by Bapedi traditional health practitioners. *Indian Journal of Traditional Knowledge*, *19*(3), pp.533-541.

Magwede, K. van Wyk, B.E. and van Wyk, A.E. 2019. An inventory of Vhaveṋḓa useful plants. *South African Journal of Botany*, *122*, pp.57-89.

Mahwasane, S.T. Middleton, L. and Boaduo, N. 2013. An ethnobotanical survey of indigenous knowledge on medicinal plants used by the traditional healers of the Lwamondo area, Limpopo Province, South Africa. *South African Journal of Botany*, *88*, pp.69-75.

Manach, C., Williamson, G., Morand, C., Scalbert, A. and Remesy, C. 2005. Bioavailability and bioefficacy of polyphenols in humans: Review of 97 bioavailability studies. *American Journal of Clinical Nutrition*, *8*, pp. 230-242.

Mårdh, P.A. 2004. How widespread are STDs? Need for improvement in surveillance systems, interpretation of test results and screening programs, as exemplified by genital chlamydial infection. *Reviews in Gynaecological Practice, 4*(3), pp.141-147.

Maroyi, A. 2011. An ethnobotanical survey of medicinal plants used by the people in Nhema communal area, Zimbabwe. *Journal of Ethnopharmacology*, *136*(2), pp.347-354.

Marques, S.S. Magalhães, L.M. Tóth, I.V. and Segundo, M.A. 2014. Insights on antioxidant assays for biological samples based on the reduction of copper

complexes—the importance of analytical conditions. *International Journal of Molecular Sciences*, *15*(7), pp.11387-11402.

Martini, N.D. Katerere, D.R.P. and Eloff, J.N. 2004. Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). *Journal of Ethnopharmacology*, *93*(2-3), pp.207-212.

Mashabane, L.G., Wessels, D.C.J., Potgieter, M.J., 2001. The utilization of *Colophospermum mopane* by the VaTsonga in the Gazankulu region (eastern Northern Province, South Africa). South African Journal of Botany, *67*, pp.199-205.

Masoko, P. and Nemudzivhadi, V. 2015. Isolation and chemical structural characterization of the mixture of two related phytosterols from *Ricinus communis* L. (Euphorbiaceae) Leaves. *European Journal of Medicinal Plants*, *10*(2), pp.1-11.

Masoko, P. and Nxumalo, K.M. 2013. Validation of antimycobacterial plants used by traditional healers in three Districts of the Limpopo Province. *Evidence-Based Complementary and Alternative Medicine*, 586247: 7.

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

McGaw, L.J., van der Merwe, D. and Eloff, J.N. 2007. *In vitro* anthelmintic, antibacterial and cytotoxic effects of extracts from plants used in South African ethnoveterinary medicine. *The Veterinary Journal*, *173*(2), pp.366-372.

Minatel, I.O. Borges, C.V. Ferreira, M.I. Gomez, H.A.G. Chen, C.Y.O. and Lima, G.P.P. 2017. Phenolic compounds: Functional properties, impact of processing and

bioavailability. Phenolic Compounds Biological Activity. InTech, Rijeka, Croatia, pp.1-24.

Miro, J.M. Pericas, J.M. and del Rio, A. 2013. A new era for treating *Enterococcus faecalis* endocarditis: Ampicillin plus short-course gentamicin or ampicillin plus ceftriaxone: that is the question. *Circulation*, *127*(17), pp.1763-1766.

Mokgoatsane, S.I. 2011. *The isolation and characterization of an antibacterial compound from Terminalia sambesiaca (Combretaceae)*, MSc dissertation, North-West University. Available at: http://dspace.nwu.ac.za/handle/10394/1. (Accessed 3 June 2020).

Molan, A. L, Faraj, A. M. and Mahdy, A. S. 2012. Antioxidant activity and phenolic content of some medicinal plants traditionally used in Northern Iraq. *Phytopharmacology*, *2*(2), pp.224-233.

Moss, W. Bentley, M. Maman, S. Ayuko, D. Egessah, O. Sweat, M. Nyarang'o, P. Zenilman, J. Chemtai, A. Halsey, N. 1999. Foundations for effective strategies to control sexually transmitted infections: voices from rural Kenya. *AIDS Care, 11*, pp.95–113.

Mu'Azu, N.D. Jarrah, N. Zubair, M. and Alagha, O. 2017. Removal of phenolic compounds from water using sewage sludge-based activated carbon adsorption: a review. *International Journal of Environmental Research and Public Health*, *14*(10), pp.1094.

Murrary, B. E. 1998. Diversity among the multidrug-resistant enterococci. *Emerging Infectious Diseases, 4*(1), pp.46-65.

Murugan, T. Wins, J.A. and Murugan, M. 2013. Antimicrobial activity and phytochemical constituents of leaf extracts of *Cassia auriculata*. *Indian Journal of Pharmaceutical Sciences*, *75*(1), pp.122.

Mustapha, A.A. 2013, Ethno-medico-botanical uses of *Securidaca longepedunculata* Fresen (family-polygalaceae) from Keffi local government, Nasarawa state, Nigeria, *Journal of Natural Remedies*, *13*(2), pp.133-137.

Muthee, J.K. Gakuya, D.W. Mbaria, J.M. Kareru, P.G. Mulei, C.M. and Njonge, F.K., 2011. Ethnobotanical study of anthelmintic and other medicinal plants traditionally used in Loitoktok district of Kenya. *Journal of Ethnopharmacology*, *135*(1), pp.15-21.

Namadina, M.M. Shawai, R.S. Musa, F.M. Sunusi, U. Aminu, M.A. Nuhu, Y. and Umar, A.M. 2020. Phytochemical and antimicrobial activity of *Securidaca longipedunculata* root against urinary tract infection pathogens. *ChemSearch Journal*, *11*(2), pp.90-98.

Namukobe, J. Kasenene, J.M. Kiremire, B.T. Byamukama, R. Kamatenesi-Mugisha, M. Krief, S. Dumontet, V. and Kabasa, J.D. 2011. Traditional plants used for medicinal purposes by local communities around the Northern sector of Kibale National Park, Uganda. *Journal of Ethnopharmacology*, *136*(1), pp.236-245.

Ndhlala, A.R. Stafford, G.I. Finnie, J.F. and van Staden, J. 2009. In vitro pharmacological effects of manufactured herbal concoctions used in KwaZulu-Natal South Africa. *Journal of Ethnopharmacology, 122*, pp.117-122.

Ndou, A.P. 2006. *Securidaca longepedunculata* Fresen, Walter Sisulu National Botanical Garden. http://www.plantzafrica.com/plantqrs/securidlong.htm (Accessed 3 June 2019).

Ndubani, P. 1997. Knowledge about and herbal treatment of sexually transmitted diseases among the Goba of Chiawa, Zambia. *Central African Journal of Medicine, 43*, pp.283–287.

Ndubani, P. and Höjer, B. 1999. Traditional healers and the treatment of sexually transmitted illnesses in rural Zambia. *Journal of Ethnopharmacology*, *67*(1), pp.15-25.

Nishiumi, S. Miyamoto, S. Kawabata, K. Ohnishi, K. Mukai, R. Murakami, A. Ashida, H. and Terao, J. 2011. Dietary flavonoids as cancer-preventive and therapeutic biofactors. *Front in Bioscience*, *3*(3), pp.1332-1362.

Noor, R. Huda, N. Rahman, F. Bashar, T. and Munshi, S.K. 2013. Microbial contamination in herbal medicines available in Bangladesh. *Bangladesh Medical Research Council Bulletin*, *39*(3), pp.124-129.

Obi, C.L. Potgieter, N. Randima, L.P. Mavhungu, N.J. Musie, E. Bessong, P.O. Mabogo, D.E.N. and Mashimbye, J. 2002. Antibacterial activities of five plants against some medically significant human bacteria: Research in action. *South African Journal of Science*, *98*(1), pp.25-28.

Ojewole, J. A. O. 2008. Analgesic, anti-inflammatory and hypoglycaemic Effects of *Securidaca longepedunculata* (fresen) [polygalaceae] root-bark aqueous extract. *Inflammopharmacology, 16*(4), pp.174-181.

Okem, A. Stirk, W.A. Street, R.A. Southway, C. Finnie, J.F. and van Staden, J. 2015. Effects of Cd and Al stress on secondary metabolites, antioxidant and

antibacterial activity of *Hypoxis hemerocallidea* Fisch. and CA Mey. *Plant Physiology and Biochemistry*, *97*, pp.147-155.

Okwu, D.E. 2005. Phytochemicals, vitamins and mineral contents of two Nigeria medicinal plants. *International Journal of Molecular Medicine and Advance Sciences*, *1*(4), pp.375-381.

Othman, L. Sleiman, A. and Abdel-Massih, R.M. 2019. Antimicrobial activity of polyphenols and alkaloids in middle eastern plants. *Frontiers in Microbiology*, *10*, pp.911.

Oyedeji, O.O. 2018. Cultivation of medicinal plants in South Africa: A solution to quality assurance and consistent availability of medicinal plant materials for commercialization. *Academia Journal of Medicinal Plants, 6*(7), pp.168-177.

Özyazici, A.P.D.G. 2021. New development on medicinal and aromatic plants. Iksad Publications, Turkey, pp.119.

Pellati, D. Mylonakis, I. Bertoloni, G. Fiore, C. Andrisani, A. Ambrosini, G. and Armanini, D. 2008. Genital tract infections and infertility. *European Journal of Obstetrics and Gynecology and Reproductive Biology, 140*(1), pp.3-11.

PlantsZAfrica: http://pza.sanbi.org/ (Accessed 5 February 2021.

PubChem: https://pubchem.ncbi.nlm.nih.gov (Accessed 6 June 2020).

Ramalingum, N. and Mahomoodally, M.F. 2014. The therapeutic potential of medicinal foods. *Advances in Pharmacological Sciences*.

Randhir, R. Lin, Y.T. Shetty, K. and Lin, Y.T. 2004. Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia Pacific Journal of Clinical Nutrition*, *13*(3), pp. 295-307.

Rekha, D. Tamil-Selvi, S. Bharathidasan, R. Panneerselvam, A. Ilakkiya, R. and Jayapal, R. 2013. Study of medicinal plants used from koothanoallur and marakkadai, Thiruvarur district of Tamil nadu, India. *Hygeia Journal for Drugs and Medicines*, 5(1), pp.164-170.

Rinne, E. 2001. Water and healing-experiences from the traditional healers in Ile-Ife. Nigeria. *Nordic Journal of African Studies*, *10*(1), pp.41-65.

Santini, A. Tenore, G.C. and Novellino, E. 2017. Nutraceuticals: A paradigm of proactive medicine. *European Journal of Pharmaceutical Sciences*, *96*, pp.53-61.

Sarikurkcu, C. and Tepe, B. 2015. Biological activity and phytochemistry of firethorn (*Pyracantha coccinea* M.J. Roemer). *Journal of Functional Foods*, *19*, pp.669-675.

Sarker, S.D. Latif, Z. and Gray, A.I. 2006. Natural product isolation. In *Natural Products Isolation*, Humana Press, Totowa, New Jersey, America. Pp.1-25.

Sasidharan, S. Chen, Y. Saravanan, D. Sundram, K.M. and Latha, L.Y. 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines, 8*(1). Pp.1-10.

Sasnovskaya, K.A. 2021. The main biologically active substances of plants, their characteristics and application.

Semenya, S.S. and Maroyi, A. 2012. Medicinal plants used by the Bapedi traditional healers to treat diarrhoea in the Limpopo Province. South Africa. *Journal of Ethnopharmacology*, *144*(2), pp.395-401.

Semenya, S.S. and Potgieter, M.J. 2013. Sexually transmitted infections and their diagnoses: Bapedi experience. *African Health Sciences*, *13*(4), pp.1047-1053

Senthilmurugan-Viji, G. Vasanthe, B. and Suresh, K. 2013. Screening and antibacterial activity analysis of some important medicinal plants. *International Journal of Innovation and Applied Studies,* 2(2), pp.146-152.

Shakya, A.K. 2016. Medicinal plants: Future source of new drugs. *International Journal of Herbal Medicine, 4*(4), pp.59-64.

Sivakrishnan, S. 2018. Traditional herbal medicine: A review. *International Journal of Respiratory Analyst, 5*(4), pp.610-614.

Smith-Hall, C. Larsen, H.O. and Pouliot, M. 2012. People, plants, and health: a conceptual framework for assessing changes in medicinal plant consumption. *Journal of Ethnobiology and Ethnomedicine*, *8*(1), pp.1-11.

South African National Biodiversity Institute (SANBI). 2006. A South African response to the Global Strategy for Plant Conservation. SANBI Biodiversity Series 1 Annual Report 2005–2006, SANBI, Claremont, pp.28.

Sparg, S. Light, M. and van Staden, J. 2004. Biological activities and distribution of plant saponins. *Journal of Ethnopharmacology*, *94*, pp.219–243.

Ssegawa, P. and Kasenene, J.M, 2007. Medicinal plant diversity and uses in the Sango bay area, Southern Uganda. *Journal of Ethnopharmacology*, *113*(3), pp.521-540.

Street, R.A. and Prinsloo, G. 2013. Commercially important medicinal plants of South Africa: a review. *Journal of Chemistry*.

Taghvaei, M. and Jafari, S. M. 2015. Application and stability of natural antioxidants in edible oils in order to substitute synthetic additives*. Journal of Food Science and Technology, 52*, pp.1272-1282.

Tavares, L.D.C. Zanon, G. Weber, A.D. Neto, A.T. Mostardeiro, C.P. Da Cruz, I.B. Oliveira, R.M. Ilha, V. Dalcol, I.I. and Morel, A.F., 2014. Structure-activity relationship of benzophenanthridine alkaloids from *Zanthoxylum rhoifolium* having antimicrobial activity. *Public Library of Science*, *9*(5), pp.e97000.

Thite, S. V. Chavan, Y. R. Aparadh, V. T. and Kore, B. A. 2013. Preliminary phytochemical screening of some medicinal plants. *International Journal of Pharmaceutical Chemical and Biological Sciences, 3*(1), pp.87-90.

Tian, Y.H. Xiong, J.W. Hu, L. Huang, D.H. and Xiong, C.L. 2007. *Candida albicans* and filtrates interfere with human spermatozoal motility and alter the ultrastructure of spermatozoa: an *in vitro* study. *International Journal of Andrology*, *30*(5), pp.421-429.

Todar, K. 2009. Textbook of bacteriology, Department of Bacteriology. University of Wisconsin-Madison. http://www.textbookofbacteriology.net/kt_toc.html Accessed 2019/10/29.

Tong, S.Y. Davis, J.S. Eichenberger, E. Holland, T.L. and Fowler, V.G. 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clinical Microbiology Reviews*, *28*(3), pp.603-661.

Tshikalange, T.E. Meyer, J.J.M. and Hussein, A.A. 2005. Antimicrobial activity, toxicity and the isolation of a bioactive compound from plants used to treat sexually transmitted diseases. *Journal of Ethnopharmacology*, *96*(3), pp.515-519.

Tshikalange, T.E. Mophuting, B.C. Mahore, J. Winterboer, S. and Lall, N. 2016. An ethnobotanical study of medicinal plants used in villages under Jongilanga tribal council, Mpumalanga, South Africa. *African Journal of Traditional, Complementary and Alternative Medicines*, *13*(6), pp.83-89.

Tshisikhawe M. P. 2021, *BOT 2649 Ethnobotany I Study guide,* University of Venda, Thoyandou, South Africa.

Tshisikhawe, M.P. 2002. Trade of indigenous medicinal plants in the Northern Province, Venda region: their ethnobotanical importance and sustainable use. 31 M.Sc. Dissertation, University of Venda for Science and Technology, Thohoyandou, South Africa.

Van Vuuren, S. Williams, V.L. Sooka, A. Burger, A. and van der Haar, L. 2014. Microbial contamination of traditional medicinal plants sold at the Faraday muthi market, Johannesburg, South Africa. *South African Journal of Botany*, *94*, pp.95-100.

Van wyk, B. E. and Gericke, N. 2000. People’s plants: A guide to useful plants of Southern Africa, Briza Publications.

Visht, S. and Chaturvedi, S. 2012. Isolation of natural products. *Journal of Current Pharma Research*, *2*(3), pp.584.

World Health Organization, 1991. Management of patients with sexually transmitted diseases: report of a WHO Study Group. Geneva, Switzerland: World Health Organization.

World Health Organization, 2002. The WHO strategy for traditional medicine: Review of the global situation and strategy implementation in the Eastern Mediterranean Region.

World Health Organization, 2004. WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems. Geneva, Switzerland: World Health Organization.

World Health Organization, 2016. Progress report of the implementation of the global strategy for prevention and control of sexually transmitted infections: 2006–2015, at http://www.who.int/reproductivehealth/publications/rtis/STI-progress.pdf?ua=1 (accessed 22 October 2016).

Wright, M.H. Jean Arnold, M.S. Lee, C.J. Courtney, R. Greene, A.C. and Cock, I.E., 2016. Qualitative phytochemical analysis and antibacterial activity evaluation of *Indian Terminalia* spp. against the Pharyngitis causing pathogen *Streptococcus pyogenes*, *Pharmacognosy Communications*, *6*(2).

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

Xu, X.H. Li, T. Fong, C.M.V. Chen, X. Chen, X.J. Wang, Y.T. Huang, M.Q. and Lu, J.J. 2016. Saponins from Chinese medicines as anticancer agents. *Molecules*, *21*(10), pp.1326.

Yokota, T. 1997. The structure, biosyndissertaion and function of brassinosteriods. *Trends in Plant Science*, *2*(4), pp.137-143.

Zeyad, A. Amor, H. and Hammadeh, M.E. 2017. The impact of bacterial infections on human spermatozoa. *International Journal of Women's Health and Reproduction Sciences*, *5*(4), pp.243-252.

Zhao, H.X. Zhang, H.S. and Yang, S.F. 2014. Phenolic compounds and its antioxidant activities in ethanolic extracts from seven cultivars of Chinese jujube, *Food Science and Human Wellness*, *3*(3), pp.183-190.

Chapter 3: Isolation and identification of microbial contaminants and nutrient evaluation of medicinal plants

3.1. Introduction

Herbal medicines are not given "regulatory categories", which means there is no national regulation of herbal medicines; however, under draft regulations, herbal medicines will, at least in part, be regulated in the same way as conventional pharmaceuticals (WHO, 2019). The safety of most herbal products is essentially compromised by a lack of suitable quality controls. It has become vital, therefore, to furnish the general public, including healthcare professionals, with adequate information to facilitate a better understanding of the risks associated with the use of these products and to ensure that all medicines are safe and are of suitable quality (Ekor, 2014).

Microbial contamination is an unwanted introduction or the presence of various forms of microorganisms in traditional herbal medicines (Walther *et al.,* 2016). These traditional herbal treatments are used in different formulations and may contain different kinds of microorganisms, which mostly adhere to leaves, stems, seeds, roots and flowers (Sabitha *et al.,* 2012). Most of these microorganisms may originate from the soil in which these plants grow. Microbial contaminants may accumulate from starting, intermediate or fully processed traditional medicine (Walther *et al.,* 2016). Hygiene during the manufacturing processes and storage is integral. This is because under unsterile conditions, pathogenic microorganisms can easily contaminate the products. Microbial contaminations in consumables are a concern as they potentially jeopardise the health of consumers. Microbial contamination from bacteria, fungi and moulds from the environment may pose a threat to the health of a patient and an even more significant threat to the health of an immunocompromised patient (van Vuuren *et al.,* 2014; Walther *et al.,* 2016).

Nowadays, there is a tendency to keep medicines in bottles of various sizes as they become readily and freely available (Tshisikhawe, 2021). Some medicines such as

bark, roots or leaves are left unprocessed until needed, while others are processed immediately and stored in powder form. The fact that most practices in traditional medicine look unhygienic should be considered in light of the comparison between traditional and modem medical standards. This can, however, be improved through retraining programmes. Stored traditional medicine must be prevented from interacting with its container, just as with atmospheric gases, moisture, or any other substances (Tshisikhawe, 2021).

Quality is an important issue in the production and utilisation of medicinal and aromatic plants (Özyazici, 2021). Analyses of plants are of great importance in the quality control of herbal materials. Physical analyses are performed in medicinal plants. Physical analyses include thousand grain weight, foreign matter, moisture/dry matter, moisture, coarseness, grinding size, Brix, pH, refractive index, sieve analysis, specific gravity, and colour. Physical analyses are also important as herbal materials are analysed after the plant is dried (European Directorate for the Quality of Medicines, 2007; Yetim and Kesmen, 2012). Substances defined as secondary metabolites are generally less than 5% dry weight in medicinal plants (Özyazici, 2021).

3.2. Methodology and analytical procedures

3.2.1 Sample procurement

The selected plants were *Elephantorrhiza burkei* UNIN 1220024, *Securidaca longepedunculata* (Fresen) UNIN 1220025 and *Wrightia natalensis* UNIN 1220023. The plants were collected at Ha-Maelula Dzanani on the Witvlag road (22.9831 °S, 30.1402 E) in the Vhembe District of Limpopo Province. The collection was conducted during the late summer (October 2019) and material was stored in airtight containers. Voucher specimen and tree labels were used to verify the identity of the plants. The plant samples were deposited at Larry Leach Herbarium (UNIN) for voucher specimen identification at the University of Limpopo. The plant material was identified by the curator of Larry Leach Herbarium, Dr B Egan. The plant materials were washed and

dried at an ambient temperature in the dark. The roots of the plant were separated and ground to a fine powder using a warring blender and stored in an airtight container in the dark until use. A new herbal treatment encompassing a combination (ratio: 1:1:1) of these selected plants was also provided by the entrepreneur as a manufactured capsule in zip-lock plastics. The study was conducted at the University of Limpopo, Department of Biochemistry and Microbiology.

3.2.2. Isolation of microbial contaminants

The plant samples and new herbal treatment samples were extracted by mixing 0, 25 g of each sample with 1ml sterile distilled water and incubating in shaker at room temperature. Samples were cultured under aseptic conditions on nutrient agar (NA) and sabouraud dextrose agar (SDA) plates immediately after extraction, using the spread-plate technique. A 100x serial dilution of each concoction was made, and 100 μL of dilutions were spread-plated on the above-mentioned media using a sterile bend glass rod. For bacterial growth, the NA plates were incubated at 37 ℃ for 24 hrs. For fungal species isolation, the SDA plates were incubated at 30 ℃ for 48 hrs.

3.2.2.1. Purification of isolates using Gram stain procedure and Wet mount procedures

To enable purification, the isolates were sub-cultured on fresh agar plates and subjected to multiple Gram staining procedures (Smith and Hussey, 2005) for bacteria and Wet mount procedure for fungi. A conventional light microscope was used to visualise the isolates for a rapid purification. Different magnification factors were used for proper visualisation under the microscope The isolates were visualised under light microscopy and under the microscopic factor of 40X-100X.

3.2.2.2. Identification of pure isolates using the VITEK 2 system

The VITEK 2 instrument was used for the identification of the pure cultures of the bacterial isolates. The instrument is housed at the Limpopo Agro-food Technology

Station (LATS) at the University of Limpopo. The manufacturer's protocol was followed for analysis. The VITEK 2 system is a fully automated microbiology system utilising growth-based technology system and operates *in vitro*. A suspension of a pure culture was prepared by suspending isolated colonies in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in 12 x 75 mm clear plastic (polystyrene) test tubes using a sterile swab. The test kit card with transferred suspensions was placed in the VITEK incubator (Pincus, 2006). The VITEK system analyses the card as the growth of the organism, which occurs and gives an identity of the organism (Pincus, 2006).

3.2.3. Proximate composition

The proximate analysis (moisture content, ash analysis, protein content, ash) of the medicinal plants and herbal treatment were determined by the official methods of AOAC (2012), Hedge and Hofreiter (1962), and Sadasivam (1992). Ash was determined using (Muffle furnace, Gravimetric Method at 550 ℃) and protein by dumas methods using (Leco Truspec N). Moisture content was determined using (Max 50) and Energy was determined using (Leco AC 600) (Horwitz, 2003; Hussain *et al.*, 2011).

3.3. Results

3.3.1. Gram staining of bacteria isolates

Gram-positive bacteria were observed to have a purplish-violet colour and Gram-negative bacteria were observed to have a pinkish colour. In **Figure 3.1.A** and **3.1.B**, images were observed under microscopic evaluation and Gram-positive bacteria was visualised.

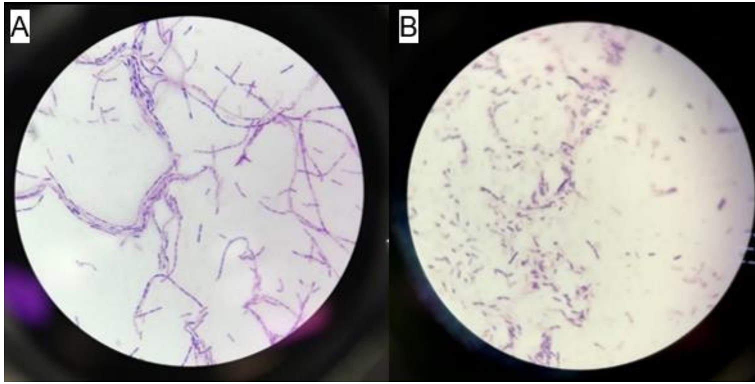

Figure 3.1: **A**- *E. burkei* (+ve) and **B**- Herbal treatment (+ve)

Key: Gram-positive (+ve)

3.3.2. Wet mount of fungal isolates

The isolates were visualised under light microscopy. Images were observed under microscopy. In **Figure 3.2.A**, the fungal isolates were observed to be small, and circular based on morphology and because they were too many to count in number. In **Figure 3.2.B**, the fungal isolates were observed to be very small, and filamentous based on morphology and were too many to count in number. In **Figure 3.2.C**, the fungal isolates were observed to be irregular and budding based on morphology and being too many to count in number.

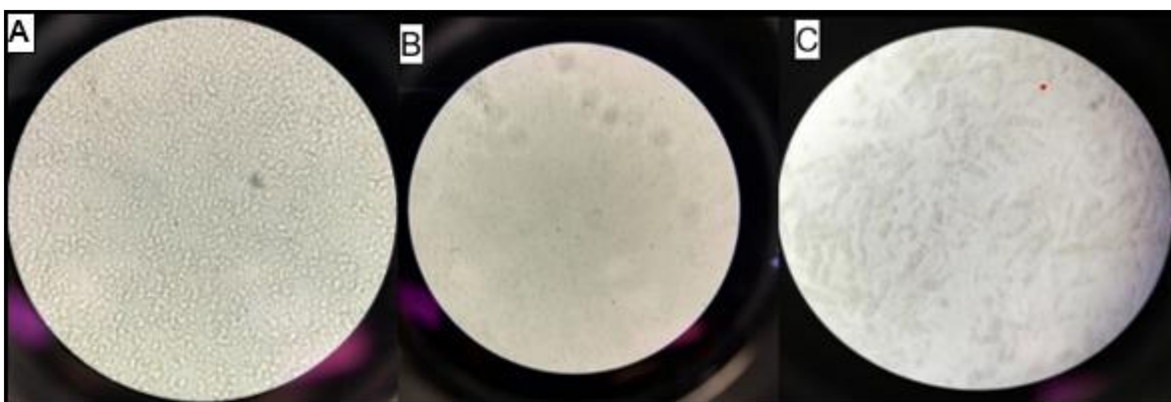

Figure 3.2: **A**- *W. natalensis,* **B**- *S. longepedunculata* and **C**- Herbal treatment.

3.3.3. Microbial identification by VITEK 2 system

Bacterial colonies were identified using the VITEK 2 system. The genus and species name were all identified with a high identification score (**Table 3.1**), which was considered either excellent, very good, good, or acceptable. Gram-positive and Gram-negative bacteria were isolated. All the identified bacteria were found to be pathogenic based on the infections they cause.

Table 3.1: Bacterial contaminants of the medicinal plants and herbal treatment using Vitek 2 system

Identified species	Gram	Identification Score (%)	Isolated	Infection	Reference
Staphylococcus vitulinus	+ve	97	Isolated from food	Urinary tract infections (UTIs) and pelvic inflammatory disease	Webster *et al., 1994*
Micrococcus luteus	+ve	94	Soil, dust, water and air, and normal microbiota of the mammalian skin	Urinary catheter infections	Madigan *et al.,* 1997, Kundrat, 2015
Enterobacter cloacae	+ve	87	Farm animals, pets, wild animals, and various food products of animal origin.	Urinary tract infections (UTIs)	Sanders and Sanders, 1997
Staphylococcus lentus	-ve	93	Pets, farm animals, wild animals, and food of animal origin	Infection of peritoneum, blood, and urine	Hay and Sherris, 2020
Raoultella ornithinolytica	-ve	86	Soil	Biliary infections	Hajjar, 2020, Hadano, 2012

Key: Gram-positive (+ve) and Gram-negative (-ve)

3.3.4. Proximate composition

The proximate analysis (moisture content, ash analysis and protein content) of the medicinal plants and herbal treatment were determined (Table 3.2). The medicinal plants and herbal treatment had relatively low protein content and *W. natalensis* had no protein content at all. High energy yield was observed in all samples.

Table 3.2: Proximate composition of medicinal plants' roots material and herbal treatment material.

Medicinal plants	**Protein**	**Moisture**	**Ash**	**Energy**
	%	g/100g		(cal/g)
S. longepedunculata	4.7	11.28 ± 0,94	0.44	3914,36 ± 0,47
W. natalensis	0	9.70 ± 0,78	0.37	4243,32 ± 0,37
E. burkei	3.3	10.80 ± 1.0	0.43	4112,93 ± 0,5
Herbal treatment	3.1	10.70 ± 0,58	1.04	4175,90 ± 0,52

Individual readings (n = 3) were averaged and presented with standard deviations.

3.4. Discussion

Normally, herbs and herbal materials may carry many bacteria and moulds, often originating from the soil in which they grow or derived from manure. Even though a large range of bacteria and fungi form the naturally occurring microflora of herbal medicines, aerobic spore-forming bacteria usually predominate and are implicated in human health. Due to the 'professional secrecy' of herbalists, it is difficult to establish comprehensive quality criteria for herbal drugs. However, to improve the purity and safety of the products, basic hygiene during preparation and standardisation of some physical characteristics such as moisture content, pH, and microbiological contamination levels are desirable (Bala *et al.,* 2017).

The VITEK 2 is an automated microbial identification system that provides highly accurate and reproducible results, as shown in multiple independent studies (Pincus, 2006). An identification score of 96-99 % is considered excellent; it was the range score for S*taphylococcus vitulinus*, 93-95% very good, which was the score for *Micrococcus luteus* and *Staphylococcus lentus*, 89-92 % good, 85-88% acceptable, which was the range score of *Enterobacter cloacae* and *Raoultella ornithinolytica* (Pincus, 2006). Overall, these are good and acceptable results based on identification scores.

Pathogens may cause harm or worsen the existing condition of the patient (de Freitas Araújo and Bauab, 2016). Thus, it is important to establish which possible infections these pathogens can cause. In the instance of bacteria identified in Table 3.1, infections associated with the reproductive system were identified, i.e. urinary tract infections (UTIs) and pelvic inflammatory disease (caused by *Staphylococcus vitulinus* and *Enterobacter cloacae),* urinary catheter infections (caused by *Micrococcus luteus)*, infection of peritoneum, blood, and urine (caused by *Staphylococcus lentus)* and biliary infections (caused by *Raoultella ornithinolytica)*. Such infections extensively contradict the purpose of the usage of selected medicinal plants for the

male reproductive system, as these pathogens can exert adverse effects and worsen already existing infections of the male reproductive system. However, this will be based on the concentration of the bacterial load which can in future be investigated.

As indicated in Table 3.1, both Gram-negative and Gram-positive bacteria were identified. The presence of pathogenic microorganisms such as *Salmonella, Escherichia coli, Staphylococcus aureus, Shigella* spp and other Gram-positive and Gram-negative strains of bacteria in herbal medicines pose a hazard to consumers (Walther *et al.,* 2016). Thus, in principle, medicine has no clinical relevance if it is active against the indicated ailment, but its ingestion or application is unsafe and vice versa (Moreira *et al.,* 2014). In section 3.3.2, the isolated fungal contaminants were visualised using a microscope, and the morphology and number of colonies observed were noted. The place of isolation of the microorganisms includeds soil and some are air-borne which can possibly be the point of contamination of the samples. However, none of these fungal contaminants were identified using Vitek 2. This could be due to the machine not being sensitive enough to identify these contaminants or the fungal load being too small to be detected, more sensitive methods can be employed in future.

The nutritional significance of plant species is measured by their content of carbohydrates, proteins, fats, oils, vitamins, minerals, and water, which are responsible for growth in flora and fauna species. Fats, protein, and carbohydrates are vital nutrients for life expectancy. The quantity and quality of proteins in the plants are important for selecting plant species for nutritive significance (Day, 2013; Prajna *et al.*, 2015). In the samples analysed, the protein content was found to be significantly low and *W. natalensis* had no protein, thus, these medicinal plants have no nutritive significance.

In this study, it was observed that the energy content of *S. longepedunculata* was 3914.36 cal/g, energy content for *E. burkei* was 4112.93 cal/g, energy content for *W. natalensis* was 4243.32 cal/g, and energy content for the herbal treatment was

4254.42 cal/g (Table 3.2). The highest energy content was *W. natalensis* followed by the herbal treatment, but overall, all the samples have high energy levels, which is good for the consumer.

Moisture content is important as an agent in chemical reactions and is a factor in the perishability and preservation of foods (Mosele *et al.*, 2011). The higher degree of food spoilage is mainly explained by a higher moisture content which is found mostly in the leaves (75%) (Ilodibia *et al.*, 2016). In the samples analysed, low moisture content was determined, which means that these plants will not spoil quickly. The ash content in a food material determines the consistency of the material, identifying it as carbon-free and showing the organic, inorganic, and impurity content found in the sample. In Table 3.2, the total ash content of *S. longepedunculata* had the value of 0.44 g/100g, which was closely relative to *E. burkei* that had the value of 0.43 g/100g. The highest total ash content was the herbal treatment with the value of 1.04 g/100g and the lowest total ash content was *W. natalensis* with the value of 0.37 g/100g. The soluble and insoluble minerals in the sample are also predicted by the total ash content (Ilodibia *et al.*, 2016).

3.5. Conclusion

The microbial pathogens identified could possibly be pathogenic depending on bacterial load, which can be harmful to the consumers. Moreover, this contamination poses a threat to the health of patients that are dependent on the medicines. This highlights a lack of quality control over the harvesting, preparation, and packaging of the products. Mineral analysis can further help in validating and ensuring the safety of the medicinal plants and herbal treatment. This finding highlights the importance of research to support the development of traditional herbal medicine practices that provide appropriate, safe, and effective treatments. Based on proximate composition, the samples analysed are high in energy that is beneficial to the consumer, but low in protein content, which is not recommended for nutritive value.

References

AOAC, 2012. Official methods of analysis of association of official analytical chemists, Association of Official Analytical Chemists, Arlington, Virginia, USA.

Bala, J.D. Kuta, F.A. Abioye, O.P. Adabara, N.U. Abdulsalam, R. Adelere, I.A. Adel, A.S. AL-Gheethi, K.H. and Ademujimi, M. 2017. Isolation and characterisation of microorganisms contaminating herbal infusion sold in Minna, Nigeria. *Journal of Science, Technology, Mathematics and Education*, *13*(2), pp.10-15.

Day, L. 2013. Proteins from land plants–potential resources for human nutrition and food security. *Trends in Food Science and Technology*, *32*(1), pp.25-42.

de Freitas Araújo, M.G. and Bauab, T.M. 2012. Microbial quality of medicinal plant materials. *Latest Research into Quality Control*, *4*, pp.67-81.

de Sousa Lima, C.M. Fujishima, M.A.T. de Paula Lima, B. Mastroianni, P.C. de Sousa, F.F.O. and **da Silva, J.O**., 2020. Microbial contamination in herbal medicines: a serious health hazard to elderly consumers. *BMC complementary medicine and therapies*, *20*(1), pp.1-9.

Ekor, M. 2014. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology*, *4*, pp.177.

European Directorate for the Quality of Medicines. 2007. *European pharmacopoeia*. Council of Europe.

Hadano, Y. Tsukahara, M. Ito, K. Suzuki, J. Kawamura, I. and Kurai, H. 2012. *Raoultella ornithinolytica* bacteremia in cancer patients: report of three cases. *Internal Medicine*, *51*(22), pp.3193-3195.

Hajjar, R. Ambaraghassi, G. Sebajang, H. Schwenter, F. and Su, S.H. 2020. *Raoultella ornithinolytica*: emergence and resistance. *Infection and Drug Resistance*, *13*, pp.1091.

Hay, C.Y. and Sherris, D.A. 2020*. Staphylococcus lentus sinusitis*: a new sinonasal pathogen. *Ear Nose and Throat Journal*, *99*(6), pp.NP62-NP63.

Hedge, J.E. Hofreiter, B.T. and Whistler, R.L. 1962. Carbohydrate chemistry, Academic Press, New York, pp.17.

Horwitz, B. 2003. The elusive concept of brain connectivity. *Neuroimage*, *19*(2), pp.466-470.

Hussain, J. ur Rehman, N. Al-Harrasi, A. Ali, L. Ullah, R. Mabood, F. Hussain, H. and Ismail, M. 2011. Nutritional prospects and mineral compositions of selected vegetables from Dhoda sharif Kohat. *Journal of Medicinal Plants Research*, *5*(29), pp.6509-6514.

Kundrat, L. 2015. Environmental Isolate Case Files: *Micrococcus luteus*. https://blog.microbiologics.com/environmental-isolate-case-files-*micrococcus-luteus* (Accessed 5 August 2020).

Madigan, M.T. Martinko, J.M. and Parker, J. 1997. Brock biology of microorganisms (Vol. 11), Upper Saddle River: Prentice Hall, New Jersey, pp.145-165.

Moreira, D.L. Teixeira, S.S. Monteiro, M.H.D. De-Oliveira, A.C.A.X. and Paumgartten, F.J.R. 2014. Traditional use and safety of herbal medicines. *Brazilian Journal of Pharmacognosy*, 24, pp.248–257.

Mosele, M.M., Hansen, Å.S., Hansen, M., Schulz, A. and Martens, H.J. 2011. Proximate composition, histochemical analysis and microstructural localisation of nutrients in immature and mature seeds of marama bean (*Tylosema esculentum*)–An underutilised food legume. *Food Chemistry, 127*, pp.1555-1561.

Özyazici, A.P.D.G. 2021. New development on medicinal and aromatic plants. Iksad Publications, Turkey, pp.119.

Pincus, D.H. 2006. Microbial identification using the bioMérieux Vitek® 2 system. *Encyclopedia of Rapid Microbiological Methods. Bethesda, MD: Parenteral Drug Association*, pp.1-32.

Prajna, P.S. and Rama Bhat, P. 2015. Phytochemical and mineral analysis of root of *Loeseneriella arnottiana* (Wight). *International Journal of Current Research in Biosciences and Plant Biology*, *2*(3), pp.67-72.

Sabitha, S. Kabshawi, M. and Mehairbi, M. 2012. Medicinal plants diversity and their conservation status in the United Arab Emirates (UAE). *Journal of Medicinal Plants Research*, *6*(7), pp.1304–1322.

Sadasivam, S. and Manickam, A. 1992. Biochemical methods for agricultural sciences, Wiley Eastern Limited, New Dellhi, pp. 221-222.

Sanders Jr, W.E. and Sanders, C.C. 1997. *Enterobacter* spp.: Pathogens poised to flourish at the turn of the century. *Clinical Microbiology Reviews*, *10*(2), pp.220-241.

Smith, A.C. and Hussey, M.A., 2005. Gram stain protocols. *American Society for Microbiology*, *1*, p.14.

Tshisikhawe M. P. 2021, *BOT 2649 Ethnobotany I Study guide,* University of Venda, Thoyandou, South Africa.

Van Vuuren, S. Williams, V.L. Sooka, A. Burger, A. and van der Haar, L. 2014. Microbial contamination of traditional medicinal plants sold at the Faraday muthi market, Johannesburg, South Africa. *South African Journal of Botany*, *94*, pp.95-100.

Walther, C. Marwa, Seni, Hamis. and Silago. 2016., Microbial contamination of traditional liquid herbal medicinal products marketed in Mwanza city: Magnitude and risk factors. *Pan African Medical Journal*, 23, pp.1–8.

Webster, J.A. Bannerman, T.L. Hubner, R.J. Ballard, D.N. Cole, E.M. Bruce, J.L. Fiedler, F. Schubert, K. and Kloos, W.E. 1994. Identification of the *Staphylococcus sciuri* species group with EcoRI fragments containing rRNA sequences and description of *Staphylococcus vitulus* sp. *International Journal of Systematic and Evolutionary Microbiology*, *44*(3), pp.454-460.

Yetim, H. and Kesmen, Z. 2012. Gıda Analizleri, Erciyes Üniversitesi Yayınları, Kayseri, pp.163.

Chapter 4: Extraction and phytochemical screening

4.1. Introduction

People who reside in rural settlements largely depend on traditional medicine (Aleebrahim-Dehkordy *et al.,* 2017). Boiling fresh herbal portions with water as a means of extraction, thus a decoction, was traditionally a more favoured method used to prepare herbal concoctions. Various plant parts such as leaves, flowers, stems, and/or roots from different or the same plant were used as herbal medicine (Tshisikhawe, 2021). The characteristic(s) of the recipes and the manner of preparation is based on traditional theories and beliefs. Phytochemical studies involve careful search for the biological activities of the compounds isolated. Findings should be interpreted considering traditional use and ways of preparation should be considered when interpreting traditional use (Tshisikhawe, 2021).

To analyse the phytochemical profile of a complex mixture, techniques such a thin-layer chromatography (TLC) become useful and powerful. It is time efficient and a quick resolution towards challenges involved in discriminating and developing fingerprints for major chemical classes that are present in mixtures (Zeng *et al.,* 2008). Several standard tests are performed to check for the presence of different bioactive constituents in crude extracts. These include the Wagner's and Hager's test for alkaloids, Borntrager's test for anthraquinones, Ferric chloride test for tannins, Keller killiani test for cardiac glycosides, Frothing and Haemolysis test for saponins, Salkowski's test for steroids and the lead acetate and sodium hydroxide test for flavonoids (Patil and Khan, 2016).

The chemical constituents present in herbal medicine or plants are a part of the physiological functions of living flora. Hence, they are believed to have better compatibility with the human body (Sen *et al.,* 2010). The chapter aimed to determine the phytochemical constituents, TLC phytochemical fingerprint and quantification of phytoconstituents of the selected plants and herbal treatment.

4.2. Methodology

4.2.1. Extraction procedure

The ground roots (1 g) were separately extracted with 10 ml of different solvents of varying polarity (hexane, dichloromethane (DCM), acetone, methanol, and distilled water), using 50 ml centrifuge bottles. The samples were placed in a shaking incubator at 2000 rpm for 30 min to mix the plant material with solvents and extract more compounds from the sample. Extraction on each plant material was carried out two times per solvent. Next, the extracts were filtered into universal bottles. The filtrates were placed under a fan to evaporate the solvents. The extracts were left to dry on a fan and later weighed to obtain the crude extract weight, then by reconstitutions to a final concentration of 10 mg/mL acetone.

4.2.2. Phytochemical analysis

The chemical properties of the phytochemicals present in medicinal plants of the plant species and herbal treatments were evaluated using qualitative and quantitative analytical methods.

4.2.2.1. Qualitative phytochemical fingerprint profiles

The chemical profile of extracts was determined by thin-layer chromatography (TLC) to confirm the presence of bioactive compounds extracted in various extracts mentioned in Section 4.2.2.2. The plates used were aluminium-backed TLC plates (Merck, silica gel 60 F254) at a size of 10 × 10 cm. All the extracts were reconstituted with acetone to a concentration of 10 mg/mL. Using a micropipette, 10 µL of each of the reconstituted extracts were loaded onto the TLC plates 1 cm from the bottom of the plates. The TLC plates were developed in solvent systems of varying polarity, i.e., ethyl acetate/ methanol/ water (40:5.4:5): [EMW] (polar/ neutral); chloroform/ ethyl

acetate/ formic acid (5:4:1): [CEF] (intermediate polarity/acidic); benzene/ ethanol/ ammonia hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). At the end of the chromatographic development, the plates were removed from the chromatographic tank, air dried under a fume-hood cabinet and observed under ultraviolet (UV) light (365 nm) for fluorescing compounds. To detect the presence of the secondary metabolites in the roots extracts which were not visible under UV light, vanillin-sulphuric acid reagent [0.1 g vanillin (Sigma ®): 28 ml methanol: 1 ml concentrated sulphuric acid] was sprayed on the TLC plates. The plates were subsequently heated at 110 ℃ for 1 to 2 minutes for optimal colour development. The plates were scanned and analysed.

4.2.2.2. Qualitative phytochemical screening

The plant material and herbal treatment were examined for the presence of phytoconstituents' classes. That is, alkaloids, tannins, saponins, steroids, terpenes, flavonoids, phlabotannins and cardiac glycosides were screened using standard chemical tests.

4.2.2.2.1. Alkaloids

The Drangendoff's reagent method described by Harborne (1973) was used. Ground and powdered leaves (0.2 g) were extracted with 95% ethanol in a Soxhlet extractor for six hours and the ethanolic extract was evaporated to dryness using a vacuum evaporator at 45℃. The residue was redissolved in 5 ml of 1% HCl and 5 drops of Drangendoff's reagent was added. Colour change was observed to draw the inference.

4.2.2.2.2. Flavonoids

Diluted ammonia (5 ml) solution was added to a portion of each plant extract's aqueous filtrate, followed by an addition of concentrated sulphuric acid. The sample was observed for colour changes to draw inference (Borokini and Omotayo, 2012).

4.2.2.2.3. Saponin

The persistent frothing test for saponin described by Odebiyi and Sofowora (1978) was used. One gram of the powdered roots sample was suspended in 30 ml tap water. The mixture was vigorously shaken and heated. The sample was observed for the formation of froth to draw an inference.

4.2.2.2.4. Phlabobatannin

The sample (0.2 g) of powdered roots was dissolved in 10 ml of distilled water and filtered. The filtrate was boiled with 2% HCl solution. The sample was used to form a coloured precipitate to draw inference (Borokini and Omotayo, 2012).

4.2.2.2.5. Tannins

Trease's and Evans's (1989) method was adopted. Powdered roots samples (0.5 g) were dissolved in 5 ml of distilled water, then boiled gently and cooled. The solution (1 ml) was put in a test tube and three drops of ferric chloride solution were added. The sample was observed for a blue-black, green or blue-green colour to draw an inference.

4.2.2.2.6. Terpenes/ terpenoids

The Salkowski test was used to test for the presence of terpenes. The powdered roots sample was mixed in 2 ml of chloroform, and 3 ml concentrated sulphuric acid (H2SO4) was carefully added to form a layer. The sample was observed for a colour change to draw inference (Sofowora, 1982).

4.2.2.2.7. Steroids

Acetic anhydride (2 ml) was added to 0.5 g powdered roots of each plant sample and was followed by adding 2 ml of sulphuric acid. The sample was observed for a colour change to draw inference (Borokini and Omotayo, 2012).

4.2.2.2.8. Cardiac glycosides

The Keller-Killani test was used to determine the presence of cardiac glycosides in the plant material. The powdered root sample (5 mg) studied was treated with 2 ml of glacial acetic acid, containing one drop of ferric chloride solution. This was underplayed with 1 ml of concentrated sulphuric acid. The sample was observed for colour changes to draw inference (Sofowora, 1982).

4.2.2.3. Quantitative phytochemical analysis

The total phenolic, flavonoid and tannin contents of the plants and the concoctions were determined. The choice of the selected phytoconstituents was because the above-mentioned phytoconstituents are associated with several key biological activities such as antimicrobial, antioxidant and anti-inflammatory activities (Akindele and Adeyemi, 2007).

4.2.2.3.1. Total phenolic content determination

A solvent mixture of 70% aqueous acetone was used to extract the powdered leaves. This was followed by the determination of the total phenolic content using spectrophotometric method described by Singleton *et al.* (1999) with minor modifications. The Folin-Ciocalteau method was used, where 0.1 mL of extract and 0.9 mL of distilled water were mixed in a 25 mL volumetric flask. To this mixture, 0.1 mL of Folin-Ciocalteau phenol reagent (Sigma ®) was added and the mixture was shaken well. One milliliter of 7% Sodium carbonate (Na2CO3) solution (Sigma ®) was added to the mixture after 5 minutes. The volume was made up to 2.5 mL with distilled

water. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared in the same manner. The mixtures were incubated for 90 minutes at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV)/visible spectrophotometer. The formula obtained from the standard curve of gallic acid was used for the determination of total phenolic content. Total phenolic content was expressed as mg of GAE/g of extract (Tambe and Bhambar, 2014).

4.2.2.3.2. Total tannin content determination

The tannin content was determined using the Folin-Ciocalteau reagent method. About 0.1 mL of the 70% aqueous acetone extract was added to a 10 mL volumetric flask containing 5 mL of distilled water. To this mixture, 0.2 mL of 2 M Folin- Ciocalteau phenol reagent and 1 mL of 35% Na2CO3 solution was added, and this was made up to 10 mL with distilled water. The mixture was shaken well and kept at room temperature for 30 minutes. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared in the same manner. Absorbance for test samples and standard solutions were measured against the blank at 725 nm with a UV/Visible spectrophotometer. The formula obtained from the standard curve of gallic acid was used for the determination of total tannin content. The tannin content was expressed as mg of GAE/g of extract (Tambe and Bhambar, 2014).

4.2.2.3.3. Total flavonoid content determination

Total flavonoid content was determined by the aluminum chloride colorimetric assay. One milliliter of 70% aqueous acetone extract was mixed with 4 mL of distilled water in a 10 mL volumetric flask; this was followed by an addition of 0.30 mL of 5% sodium nitrite. About 0.3 mL of 10 % aluminum chloride (Sigma ®) was added to the mixture after 5 minutes. After 5 minutes, 2 mL of 1 M Sodium hydroxide (Sigma ®) was added and this was made up to 10 mL with distilled water. A set of reference standard

solutions of quercetin (0.0313, 0.0625, 0.125, 0.25, 0.5 mg/mL) were prepared in the same manner. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with a UV/Visible spectrophotometer. The formula obtained from the standard curve of quercetin was used for the determination of total flavonoid content. The total flavonoid content was expressed as mg of QE/g of extract (Tambe and Bhambar, 2014).

4.2.2.3.4. Statistical analysis

All the experiments were carried out in triplicates, and the data were presented as mean ± standard deviation. Calculations were carried out using MS Office Excel 2010. All data were calculated using a linear regression formula (y=mx+c) obtained from each standard curve.

4.3. Results

4.3.1. Preliminary phytochemical screening

The grounded root material of *Elephantorrizha burkei, Securidaca longepedunculata, Wrightia natalensis* and herbal treatment were extracted using five different solvents of different polarity, namely: hexane, dichloromethane (DCM), acetone, methanol, and distilled water. **Figure 4.1** represents the quantity in milligrams of the plant material extracted from 1 g of the ground root powder using the different solvents. Methanol was the best extracting solvent in the plant materials. It extracted a crude mass of 199 mg (*E. burkei),* 143 mg (*S. longepedunculata)* and 74 mg (*W. natalensis).* Water was the best extracting solvent with a crude mass of 73 mg for the herbal treatment.

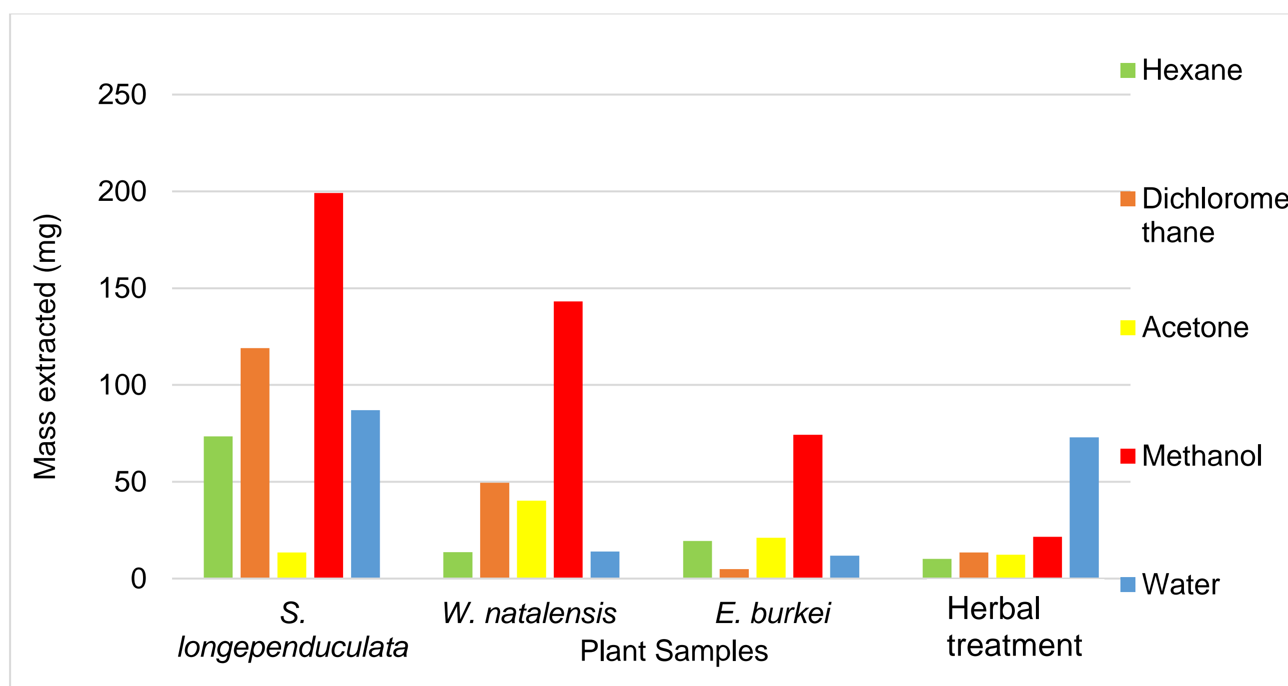

Figure 4.1: The crude extract mass of *E. burkei, S. longepedunculata, W. natalensis* and medicinal treatment extracted with five different solvents. **Key:** H-Hexane, D-Dichloromethane A- Acetone, M- Methanol, E-Ethanol, and W-Water.

4.3.2. Phytochemical analysis

4.3.2.1. Qualitative phytochemical fingerprint profiles

Thin-layer chromatography was employed for an analysis of phytochemicals contained in the different extracts of the selected plants and herbal treatment. All the extracts were developed in different solvent systems, namely; BEA (non-polar), CEF (intermediate polar) and EMW (polar). Phytochemicals were visualised by the fluorescence of bands detected by UV light (254 nm) in **Figure 4.2.A** and UV light (365 nm) in **Figure 4.2.B**. More fluorescent compounds were observed on chromatograms developed in EMW although they were not separated. Vanillin-sulphuric acid was used as a spraying reagent for the visualisation of the different separated compounds. Phytochemicals were visualised as purple and red in colour, as illustrated in **Figure 4.2.C**. Separated bands were more resolved in BEA than CEF in all the plants. Overall, *E. burkei* showed the most phytoconstituents.

Figure 4.2: The chromatograms *W. natalensis*, *S. longepedunculata*, *E. burkei* and herbal treatment extracted with different solvents and developed in BEA, CEF and EMW mobile systems and visualised under UV-light at 254 nm (**A**), 365nm (**B**) and (**C**) was sprayed with vanillin-sulphuric acid reagent.

4.3.2.2. Qualitative phytoconstituent screening

Different phytoconstituents present in *W. natalensis, S. longepedunculata, E. burkei* and herbal treatment, which are responsible for the therapeutic activity, are presented in **Table 4.1.** All the phytoconstituents tested for using standard chemical test were found to be present, namely; flavonoids, alkaloids, tannins, phlabotannins, steroids, terpenes, saponins, cardiac glycosides.

Table 4.1: Phyto-constituents present in the plants and herbal treatment.

Phytoconstituents	*W. natalensis*	*S. longepedunculata*	*E. burkei*	Herbal treatment
Flavonoids	+	+	+	+
Alkaloids	+	+	+	+
Tannins	+	+	+	+
Phlabotannins	+	+	+	+
Steroids	+	+	+	+
Terpenes	+	+	+	+
Saponins	+	+	+	+
Cardiac glycosides	+	+	+	+

Key: + present; - absent

4.3.2.3. Quantitative phytochemical analysis

The total phenol, tannin, and flavonoid content *W. natalensis, S. longepedunculata, E. burkei* and herbal treatment were measured and quantified from standard curves (**Figures 4.3 to 4.5**), with each value being an average of 3 analyses ± standard deviation. The linear curves indicate a positive linear relationship between the concentration of the phytochemicals and the absorbance. The total flavonoids were

the most abundant in *E. burkei* extracts, the phenols and tannins were most abundant in *S. longepedunculata*.

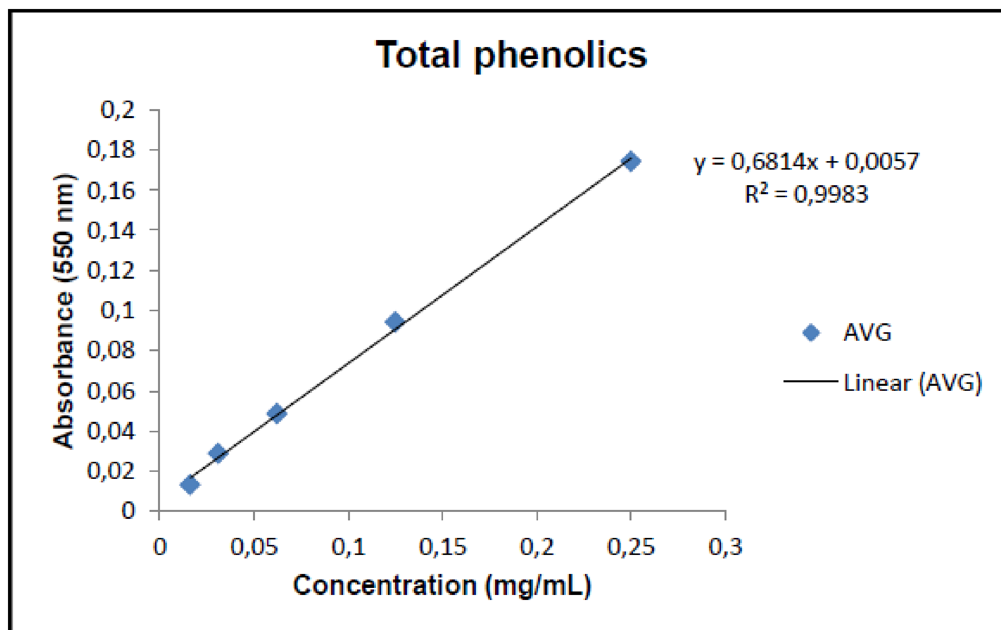

Figure 4.3: The gallic acid standard curve for total phenol

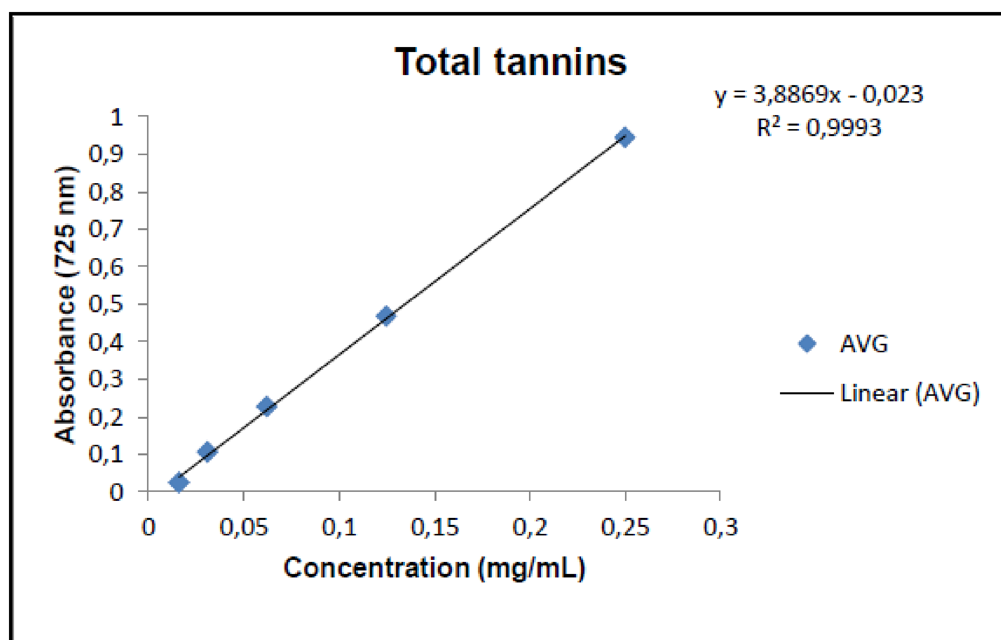

Figure 4.4: The gallic acid standard curve for total tannin analysis.

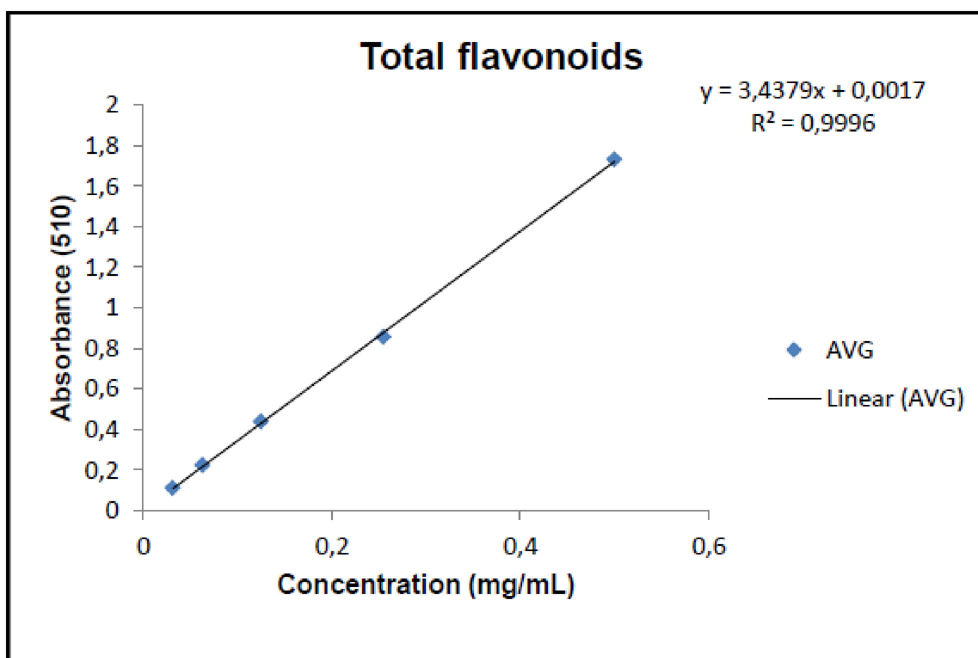

Figure 4.5: The quercetin calibration curve for total flavonoids analysis.

Table 4.2: The total phenol, tannin and flavonoids content measured in different plants and herbal treatment.

Solvents	**Phenolic content (mg TAE/g extract)**	**Tannin content (mg TAE/g extract)**	**Flavonoid content (mg QAE/g extract)**
	W. natalensis		
Hexane	30,00 ± 0,91	67,75 ± 13,48	9,42 ± 0,77
DCM	1,1 ± 18,38	16,81 ± 12,76	12,90 ± 6,06
Acetone	28,64± 1,31	12,28 ± 7,40	10,58 ± 0,87
Methanol	20,80± 23,35	0,47 ± 4,59	3,43 ± 0,44
Water	15,63, ± 677,00	30,82 ± 7,52	8,26 ± 2,58
	S. longepedunculata		
Hexane	3,18 ± 159,03	157,12 ± 10,75	52,24 ± 1,17
DCM	2,24 ± 33,12	66,96 ± 26,22	11,36 ± 4,93
Acetone	117,90± 69,00	97,18 ± 28,59	11,07 ± 7,21
Methanol	7,90± 1,67	16,34 ± 27,09	4,98 ± 2,41
Water	29,63± 84,34	95,42 ± 23,27	10,10 ± 0,60

		E. burkei	
Hexane	27,36 ± 139,03	17,86 ± 1,15	214,33 ± 5,46
DCM	3,43 ± 18,96	31,30 ± 2,16	18,03 ± 4,44
Acetone	7,72 ± 50,47	50,44 ± 1,32	130,82 ± 7,76
Methanol	15,20 ± 55,57	27,27 ± 3,30	9,037 ± 1,02
Water	4,39 ± 22,06	13,83 ± 5,29	6,62 ± 0,93
		Herbal treatment	
Hexane	5,80 ± 0,16	54,37 ± 3,05	38,90 ± 6,04
DCM	3,85 ± 14,36	19,37± 4,36	0,34 ± 1,10
Acetone	12,38 ± 12,57	46,31 ± 11,73	9,52 ± 4,35
Methanol	22,06 ± 18,65	70,23 ± 1,15	3,53 ± 0,73
Water	3,81 ± 15,09	13,78 ± 0,68	1,21 ± 0,67

Key: TAE: tannic acid equivalence per gram of plant extract and QE: quercetin equivalence. Values are mean of triplicates ± standard deviation (SD) (n=3).

4.4. Discussion

Plants produce a diverse assortment of phytochemicals that are essential for their development and endurance in the environment. Associated biological activities of these compounds have enabled plants to be used as therapeutic agents against various ailments (Sasnovskaya, 2021). The plants analysed in this study were selected because of their ethnopharmacological effects, i.e. erectile dysfunction, sexual impotence, and microbial infections as prescribed by traditional herbalists in Dzanani, Vhembe District, Limpopo Province.

The dried and ground root of *Elephantorrizha burkei, Securidaca longepedunculata, Wrightia natalensis* and herbal treatment were extracted with solvents of varying polarity from non-polar to polar, namely; hexane, ethyl acetate, acetone, methanol, and water. Extraction and separation of the bioactive components within the plant material is done to remove the non-active components; hence, organic solvents are used to allow maximum extraction of entities with various biological activities (Eloff, 1998). The mass extracted from each plant material using the different solvents are

represented in **Figure 4.1**. The best extracting solvent was methanol for the plant materials, namely, *S. longepedunculata* (199 mg), followed by *W. natalensis* (143 mg) and lastly, *E. burkei* (74 mg). This is supported by a study done by Boeng and Melo (2014), which shows that methanol has the capability to dissolve polar and non-polar compounds. This suggests that the compounds present in *E. burkei, S. longepedunculata, W. natalensis* were soluble in non-polar solvents.

The traditional methods of preparation of herbal concoctions mostly involve the use of water as a solvent, which is advantageous to them because it is readily available and has no side effects. However, studies on medicinal plants' bioactive constituents have shown that water is not an efficient extracting solvent since some phytoconstituents extracted by this solvent may not have desired biological activities (Parekh and Chanda, 2007; Das *et al*., 2010). In this study, water (73 mg) was the best extracting solvent for the herbal treatment. This validates the notion that when patients consume herbal treatment with water, they are most likely to have the utmost beneficial results based on all phytoconstituents extracted. Eloff (2004) reported on the importance of knowing the concentration of the extracts when analysing medicinal plants. The extracts were dried and reconstituted in acetone to a final concentration of 10 mg/ml. This solvent was reported to be harmless against bacteria (Eloff, 1998b). Therefore, it will not inhibit bacterial growth in the subsequent bioassays.

Plants use phytochemicals as a defence mechanism, but they are also helpful to human beings when consumed (Ivey *et al*., 2015). Therefore, it was important to evaluate and screen the different secondary metabolites. Preliminary phytochemical screening (Table 4.1) revealed the presence of terpenoids, flavonoids, phlabotannins, tannins, steroids, alkaloids, saponins and cardiac glycosides in all the plants and the herbal treatment. The results are very similar to the study of Abubakar *et al*., 2016 for phytochemicals present in *S. longepedunculata*. These secondary metabolites (alkaloids, saponins, tannins and flavonoids) are known to have activity against several pathogens and aid the antimicrobial activities of *S. longepedunculata* and

support their traditional use for the treatment of various illnesses (Hassan *et al.*, 2004; Sharma *et al.,* 2012). In addition, the tested phytoconstituents are generally known to have various biological roles. Therefore, knowing the plant's phytochemical composition is vital in identifying compounds with desired effects (Das *et al.*, 2010; Mehmood *et al.*, 2015). For example, terpenoids have been reported to have been applied in the treatment of microbial infections (Krzyzanowska *et al.*, 2010).

To evaluate and determine the number and type of compounds in each extract of the selected plants, thin-layer chromatography was used. The separated fluorescing compounds were visualised using ultraviolet light at the wavelength of 365 nm and 245 nm. Under the UV light at the wavelength of 365 nm, fluorescence bands were observed in *E. burkei* (in all mobile phases) and *S. longepedunculata* (in EMW and BEA mobile system), showing a diversity of compounds. Some compounds were not fluorescing; therefore, there was a need to detect them by spraying the plates with vanillin-sulphuric acid reagent. The compounds observed using vanillin reagent and UV light of 365 nm were similar. However, the compounds separated on the polar (EMW) mobile phase had a high number of bands with distinct purple and red colours, more than any other mobile phase for *E. burkei.* Thin-layer chromatography helps to reveal compounds that can be targeted for further analysis, such as antimicrobial assays and isolation of the respective compounds (Móricz *et al.*, 2017).

The Folin Ciocalteau and aluminium chloride methods determined the total phenolic, tannin, and flavonoid contents, respectively. The quantitative measure of the tannin content, phenolic content and flavonoid content was performed to estimate the concentration of the major phytochemicals in the plants, crude extracts.

As seen in Table 4.2, the *E. burkei* total phenolic content ranged from 4.4 to 27.36 mgGAE/g of dichloromethane and hexane extract, respectively, while the total tannin content ranged from 13.83 to 50.44 mg GAE/g of water and acetone extract, respectively. The total flavonoid content ranged from 6.62 to 15.367 mg QE/g of water and hexane extract, respectively. This shows that hexane is a good extracting solvent.

On the other hand, water poorly extracted solvent for polyphenols contained in *E. burkei.* For *S. longepedunculata*. Total phenolic content ranged from 2.24 to 117.90 mg GAE/g of dichloromethane and acetone extracts, respectively, while the total tannin content ranged from 16.34 to 157.12 mg GAE/g of methanol and hexane extracts, respectively. The total flavonoid content ranged from 4.98 to 52.24 mgQE/g of methanol and hexane extracts, respectively. This shows that dichloromethane and hexane are good extracting solvents whereas methanol and acetone are poor extracting solvents for polyphenols in *S. longepedunculata*. For *W. natalensis*, total phenolic content ranged from 1.10 to 30.00 mg GAE/g of dichloromethane and hexane extracts, respectively, while the total tannin content ranged from 0.47 to 67.75 mg GAE/g of methanol and hexane extracts, respectively. The total flavonoid content ranged from 3.43 to 12.90 mg QE/g of methanol and dichloromethane extracts, respectively. This shows that hexane is a good extracting solvent and methanol is a poor extracting solvent for polyphenols contained in *W. natalensis*. For the herbal treatment, total phenolic content ranged from 3.81 to 22.06 mg GAE/g of water and methanol extracts, respectively, while the total tannin content ranged from 13.78 to 70.23 mg GAE/g of water and hexane extracts, respectively. The total flavonoid content ranged from 0.34 to 38.80 mgQE/g of dichloromethane and hexane extracts, respectively. This shows that hexane is a good extracting solvent and water is a poor extracting solvent for the herbal treatment's polyphenols.

Overall, the total flavonoids were the most abundant in the analysed plant and herbal treatment, followed by phenols, the least were the tannins (Table 3.2), and followed by hexane extracting solvent, which is no polar. The study demonstrated that using different solvents of varying polarities aids the extraction of different phytochemicals that were best resolved in non-polar BEA mobile system.

4.5. Conclusion

The different phytoconstituents present in the plant extract and the herbal treatment are used for various health conditions. Therefore, the claimed pharmacological effects of these medicines need also to be investigated to validate their use in treating the indicated ailments. Furthermore, the screened major phytoconstituents are known to possess both antibacterial and antioxidant activities. Therefore, the plants will be analysed further for their potential as natural antioxidant and antimicrobial agents.

References

Abubakar, U.S., Abdullahi, M.S., Hadiza, R.J., Joseph, M., Binta, I.K. and Habiba, G.U. 2018. Antibacterial activity of Securidaca longipedunculata stem bark against some clinical bacterial isolates. *Journal of Pharmacognosy and Phytochemistry*, *7*(6), pp.1967-1970.

Akindele, A.J. and Adeyemi, O.O. 2007. Anti-inflammatory activity of the aqueous leaf extracts of *Byrsocarpus coccineus*, *Fitoterapia*, *78*(1), pp.25-28.

Aleebrahim-Dehkordy, E. Tamadon, M.R. Nasri, H. Baradaran, A. Nasri, P. and Beigrezaei, S. 2017. Review of possible mechanisms of analgesic effect of herbs and herbal active ingredient. *Journal of Young Pharmacists*, *9*(3), pp.303-306.

Boeng, J. and Melo, C. 2014. Mapping the energy consumption of household refrigerators by varying the refrigerant charge and the expansion restriction. *International Journal of Refrigeration*, *41*, pp.37-44.

Borokini, T.I. and Omotayo, F.O. 2012. Phytochemical and ethnobotanical study of some selected medicinal plants from Nigeria. *Journal of Medicinal Plants Research*, *6*(7), pp.1106-1118.

Das, K. Tiwari, R.K.S. and Shrivastava, D.K. 2010, Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research*, *4*(2), pp.104-111.

Eloff, J.N. 1998. Which extracting solvent should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*, *60*, pp.1-8.

Eloff, J.N. 1998b. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica*, *64*(8), pp.711-713.

Eloff, J.N. 2004. Quantification the bioactivity of plant extracts during screening and bioassay guided fractionation. *Phytomedicine*, *11*(4), pp.370-371.

Harborne, J.B. 1973. Phytochemical methods, 3rd edition, Chapman and Hall Limited, London, pp.35–203.

Hassan, M.A. Oyewale, A.O. Amupitan, J.O. Abdullahi, M.S. and Okonkwo, E.M. 2004. Preliminary phytochemical and antimicrobial investigation of crude extract of root bark of *Deteriummi crocarpum*. *Journal of Chemical Science Nigeria*, *29*, pp.36-49.

Ivey, K.L. Hodgson, J.M. Croft, K.D. Lewis, J.R. and Prince, R.L. 2015. Flavonoid intake and all-cause mortality. *The American Journal of Clinical Nutrition*, *101*(5), pp.1012-1020.

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

Krzyzanowska, J. Czubacka, A. and Oleszek, W. 2010. Dietary phytochemicals and human health. *Bio-Farms for Nutraceuticals: Functional Food and Safety Control by Biosensors*, *698,* pp.74–99.

Mehmood, B. Dar, K.K. Ali, S. Awan, U.A. Nayyer, A.Q. Ghous, T. and Andleeb, S. 2015, *In vitro* assessment of antioxidant, antibacterial and phytochemical analysis

of peel of *Citrus sinensis*. *Pakistan Journal of Pharmaceutical Sciences, 28*(1), pp.231-239.

Móricz, Á.M. Krüzselyi, D. and Ott, P.G. 2017. Separation and detection of bioactive essential oil components by over pressured layer chromatography coupled with bioactivity tests. *JPC-Journal of Planar Chromatography-Modern TLC*, *30*(2), pp.121-125.

Odebiyi, O.O. and Sofowara, E.A. 1978. Phytochemical screening of Nigerian medicinal plants II, *Llodydia*, *41*, pp.234–246.

Parekh, J. and Chanda, S. 2007. Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. *African Journal of Biomedical Research*, *10*(2), pp.175-181.

Patil, M.B. and Khan, P.A. 2016. Review: Techniques towards the plant phytochemical study. *International Journal of Science Information*, *1*(3), pp.157-172.

Sasnovskaya, K.A. 2021. The main biologically active substances of plants, their characteristics and application.

Sen, S. Chakraborty, R. Sridhar, C. Reddy, Y.S.R. and De, B. 2010. Free radicals, antioxidants, diseases and phytomedicines: Current status and future prospect. *International Journal of Pharmaceutical Sciences Review and Research*, *3*(1), pp.91-100.

Sharma, A.K. Mayank, G. Ragini, T. Gopal, N. Akhoury, S. Kumar, S. Yamini, B. T. Dharmendra, K. 2012. Comparative *in vitro* antimicrobial and phytochemical

evaluation of methanolic extract of root, stem and leaf of *Jatropha curcas* Linn. *Pharmacognosy Journal*, *4*(30) pp.34-40.

Singleton, V.L., Orthofer, R. and Lamuela-Raventós, R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*, *299*, pp.152-178.

Sofowora, A., 1982. Medicinal plants and traditional medicine in Africa, John Wiley and Sons Limited, Chichester, England, pp.142-145.

Tambe, V.D. and Bhambar, R.S. 2014. Estimation of total phenol, tannin, alkaloid and flavonoid in *Hibiscus Tiliaceus* Linn. wood extracts. Research and reviews. *Journal of Pharmacognosy and Phytotherapy*, *2*(4), pp.2321-6182.

Trease, G.E. and Evans, W.C. 1989. Textbook of pharmacognosy. 14th edition. W.B. Sanders, London.

Tshisikhawe M. P. 2021, *BOT 2649 Ethnobotany I Study guide,* University of Venda, Thoyandou, South Africa.

Zeng, Z.D. Liang, Y.Z. Jiang, Z.H. Chaub, F.T. and **Wang, J.R.** 2008. Quantification of target components in complex mixtures using alternative moving window factor analysis and two-step iterative constraint method. *Talanta 74*, pp.1568–1578.

Chapter 5: Antioxidant activity assays

5.1. Introduction

Free radicals are generated in our body by several systems. A balance among free radicals and antioxidants is an important matter for appropriate physiological function. If free radicals become greater than the ability of the body to control them, a case known as oxidative stress appears, and because of that, a number of human diseases arise in the body (Alkadi, 2020). Free radical scavengers act as hydrogen donors, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents (Thakur, 2020).

South African plants with known antioxidant activities may be investigated as infertility remedies since antioxidants improve various processes of male reproductive function such as spermato-genesis and steroidogenesis (Sheweita *et al*., 2005; Murugesan *et al.,* 2007; Elumalai *et al*., 2009). In male urogenital tract infection, reactive oxygen species (ROS), highly reactive molecules with half-life time in the nano-second range, are thought to be particularly responsible for the damage of spermatozoa and thus for compromised fertility (overviews in Drach *et al.,* 1978; Henkel *et al*.,1998; Comhaire *et al.,* 1999; Ochsendorf, 1999). This is because the sperm plasma membrane is composed of a particularly high amount of polyunsaturated fatty acids like docosahexanoic acid (Darin-Bennett *et al*., 1978), and is therefore extraordinarily susceptible to oxidation. Once spermatozoa are exposed to unphysiologically high levels of oxidative stress as it is in the case of urogenital tract infections, the function of the sperm membrane is impaired even in DNA damage (Aitken, 1995, Henkel *et al.,* 2005).

Antioxidants have the capability to remove radicals with the help of free radical chain breaking trades, for that reason decreasing oxidative strain (Pourmorad *et al.,* 2006). Components attributing antioxidant interest can act independently or via synergy with different components (Dai and Mamper, 2010). To exhibit antioxidant activity, a

compound should possess chelating potential which is dependent on the arrangement of functional groups within the compound (Thompson *et al*., 1976).

Oxidative stress is a key factor in the pathophysiology of a number of diseases. It results from prooxidative and antioxidative cellular imbalance between reactive oxygen species (ROS) production and the ability of biological systems' defence mechanisms to eliminate the cellular stress disturbances (Nita and Grzybowski, 2016). One common pathological denominator in both CVD (cardiovascular diseases) and ED is oxidative stress, that is, the overproduction of reactive oxygen species (ROS), in particular, superoxide (O_{2-}) and hydrogen peroxide (H_2O_2) (Mahajan and Gajare, 2012). Thus, there is direct relationship between oxidative stress, sexual impotency and psychoactive mechanisms that significantly alters nitrogen oxide inhibition mechanisms, as stated above. Therefore, it is necessary to evaluate the potential of natural herbs and extracts to correct disorders and disabilities which evolve in the manifestation of erectile dysfunction (Mahajan and Gajare, 2012). The antioxidant activity of natural substances can be evaluated by examining their scavenging abilities against stable or unstable radicals using spectrophotometry (Dlamini *et al.,* 2019).

Various methods are used to determine the antioxidant capacity. One of these methods is DPPH radical scavenging activity. It is one of the frequently used methods to determine the radical scavenging activity of extracts obtained from plants (Koçak *et al*., 2020; Maduraiveeran *et al*., 2021; Onbasli and Yuvali, 2021). The compound is purple and when sprayed on TLC plates, colour change occurs to show the presence of activity. The 2, 2-diphenyl-1-picrylhydrazyl DPPH acts as a stable radical, aiding in the detection of compounds with the ability to scavenge free radicals or to prevent the procedure of oxidation from taking place. Molecules that are capable of donating electrons react with DPPH resulting in yellow colour change on TLC plate (Naik, *et al.,* 2003). Since this analysis method is safe and economical, it has been used by many researchers to determine antioxidant activity (Arslan Burnaz *et al*., 2017; Hara *et al*., 2017, 2018; Koçak *et al*., 2020).

5.2. Methods and materials

5.2.1. Qualitative antioxidant activity assay

The antioxidant activity of the plant extracts was evaluated using a method described by Braca *et al.* (2002). All extracts from section 4.2.1 were tested for their antioxidant activity by reconstituting acetone to 10 mg/mL. A volume of 10 µL of each extract was loaded by spotting in 1 cm wide line TLC plates and developed in BEA, CEF and EMW solvent systems, and then dried at room temperature. The developed TLC plates were then sprayed with 0.2% (w/v) of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma®) in methanol as an indicator. The presence of antioxidant activity was observed as yellow spots on the plate against a purple background.

5.2.2. Quantitative antioxidant activity assay

Free radical scavenging activity of the concoctions and the plant decoctions was quantified using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method reported by Chigayo *et al*. (2016) with modifications. Briefly, different concentrations of the extracts (250-15.63 µg/mL) were prepared to a volume of 1 mL of the solution. L-ascorbic acid was used as a standard by preparing the same concentration range as the extracts. To this 1 mL solutions, 2 mL of 0.2 mmol/L DPPH solution (dissolved in methanol) was added and vortexed thoroughly. All the prepared mixtures were left to stand in the dark for 30 minutes. The control solution was prepared by adding 2 mL of 0.2 mmol/L DPPH to 1mL of distilled water. After the elapsed time, the solutions were analysed with a UV/VIS spectrophotometer. The experiment was conducted in triplicates and repeated three times. The absorbance of the solutions was read at 517 nm and the percentage antioxidant potential was calculated using the equation below.

$$\%\ inhibition = \frac{Ac - As}{Ac} \times 100$$

Ac was an absorbance of the control solution, while As was the absorbance of the extracts.

5.2.3. Ferric reducing power assay

A method described by Oyaizu (1986) was used to determine antioxidant capacity using the reducing power assay. Five different concentrations of the samples (625 – 39 µg/mL) were prepared by serially diluting a stock solution of 1 250 µg/mL. The different concentrations (2.5 mL) were mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of (1% w/v in distilled water) potassium ferricyanide (Rochelle) in test tubes, respectively. The mixtures were vortexed after an addition of solutions. Methanol was used as blank and ascorbic acid was used as positive control. In a test tube, a volume of 2.5 mL plant extract, 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1% w/v in distilled water) were added and mixed well. The mixture was incubated at 50 ℃ for a period of 20 minutes. Thereafter, 2.5 mL of trichloroacetic acid (10% w/v in distilled water) was added to the mixture and centrifuged at 650 rpm for 10 minutes. Five millilitres of the supernatant were transferred to another test tube with 5 mL distilled water and 1 mL ferric chloride (0.1% w/v in distilled water) solution and mixed (all samples were done in triplicates). The absorbance was then measured after 60 minutes of incubation at 700 nm using a spectrophotometer (Beckman Coulter-DU730) and recorded for all samples.

5.3. Results

5.3.1. Qualitative DPPH assay on TLC

The screening of antioxidant compounds in the plant extracts and herbal treatment was conducted using DPPH. Yellow zones on the chromatograms against a purple background (**Figure 5.1**) indicated antioxidant compounds. The *E. burkei* plant extracts had prominent antioxidant activity whilst the *S. longepedunculata* showed very minimal antioxidant activity. *Wrightia natalensis* and herbal treatment showed no antioxidant activity. The results indicated that the compounds that exhibit antioxidant activity are polar, as observed EMW mobile system and intermediate polar as observed on the BEA mobile system.

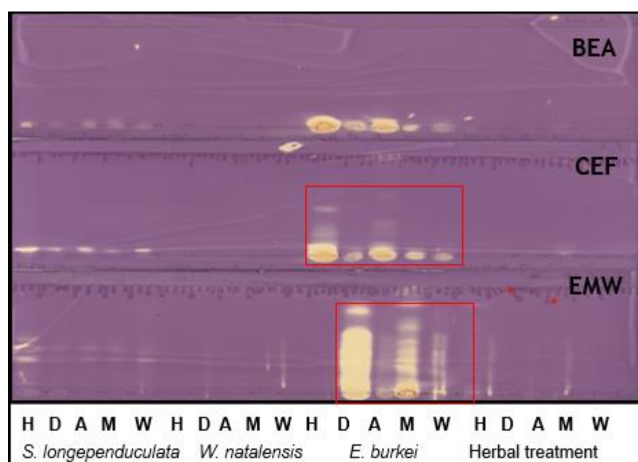

Figure 5. 1: The chromatograms of medicinal plants and herbal treatment extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with 0.2% DPPH in methanol. **Key:** H- Hexane, D- Dichloromethane A- Acetone, M- Methanol, E- Ethanol, and W- Water.

5.3.2. Quantification of Antioxidant activity

The antioxidant activity of the plant extracts and the herbal treatment was quantified using two assays. The free radical scavenging assay determined the proton (H_+) donating ability of the extracts to the radical DPPH. The ferric reducing power assay

(FRAP) was used to determine the electron (e-) donating ability of the extracts to reduce Fe^{3+} to Fe^{2+}.

5.3.2.1 Qualitative DPPH antioxidant assay

The antioxidant activity of the plant extracts was quantified using 2, 2, diphenyl-1-picrylhydrazyl (DPPH) reduction and compared with ascorbic acid, as illustrated in **Figure 5.2** and **Figure 5.3**. The antioxidant activity of the plant extracts was expressed as the percentage inhibition. The antioxidant activity increases with an increase in concentration of the plant extract. The *E. burkei* hexane and acetone plant extracts had the highest antioxidant activity while the herbal treatment had the lowest activity for all extractions, namely; hexane, dichloromethane, acetone, and water extract.

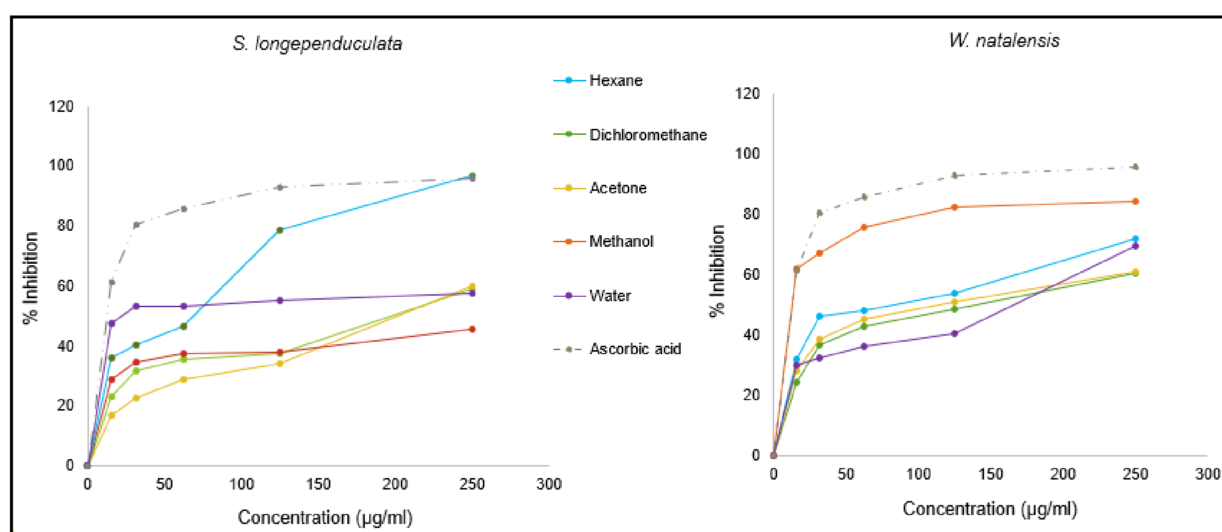

Figure 5.2: Percentage free radical DPPH inhibition of *S. longepedunculata* and *E. burkei*. Ascorbic acid was used as standard to which samples were compared.
Key: H- Hexane, D- Dichloromethane A- Acetone, M- Methanol, E-Ethanol, and W-Water.

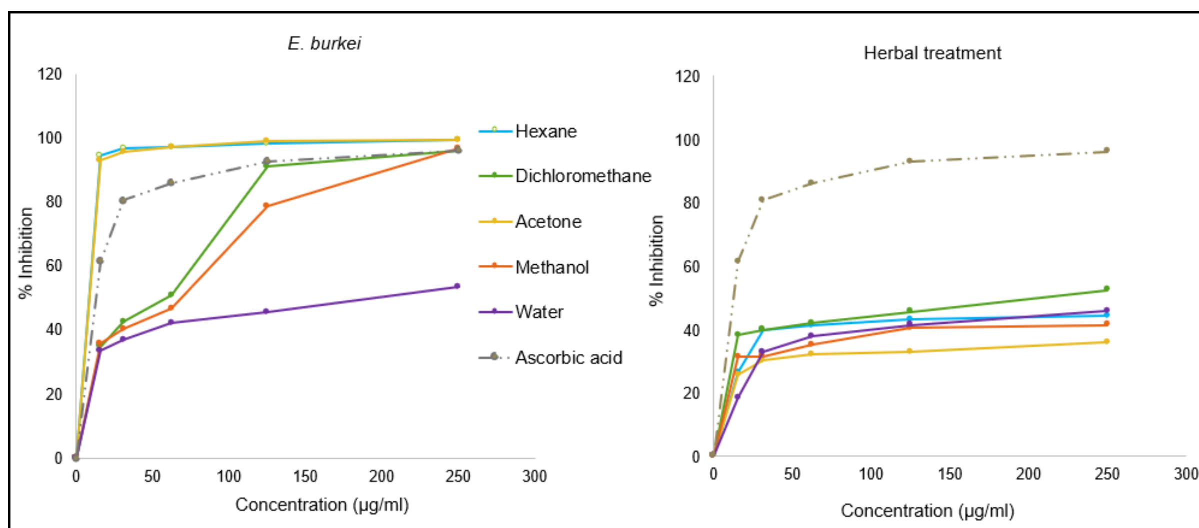

Figure 5.1: Percentage free radical DPPH inhibition of *W. natalensis* and herbal treatment. Ascorbic acid was used as standard to which samples were compared.

Key: H- Hexane, D- Dichloromethane A- Acetone, M- Methanol, E- Ethanol, and W- Water.

5.3.2.2. Free radical scavenging activity assay

The reducing power of the plant materials were determined using ferric reducing antioxidant potential (FRAP) assay. Ascorbic acid was used as standard and generally all the plant parts tested had very low ferric reducing power/ antioxidant capacity when compared to the standard reference (**Figure 5.4** and **Figure 5.5**). The *E. burkei* hexane and acetone extracts had a high ferric reducing capacity as compared to other plant parts, while the herbal treatment hexane extracts had a very low ferric reducing power.

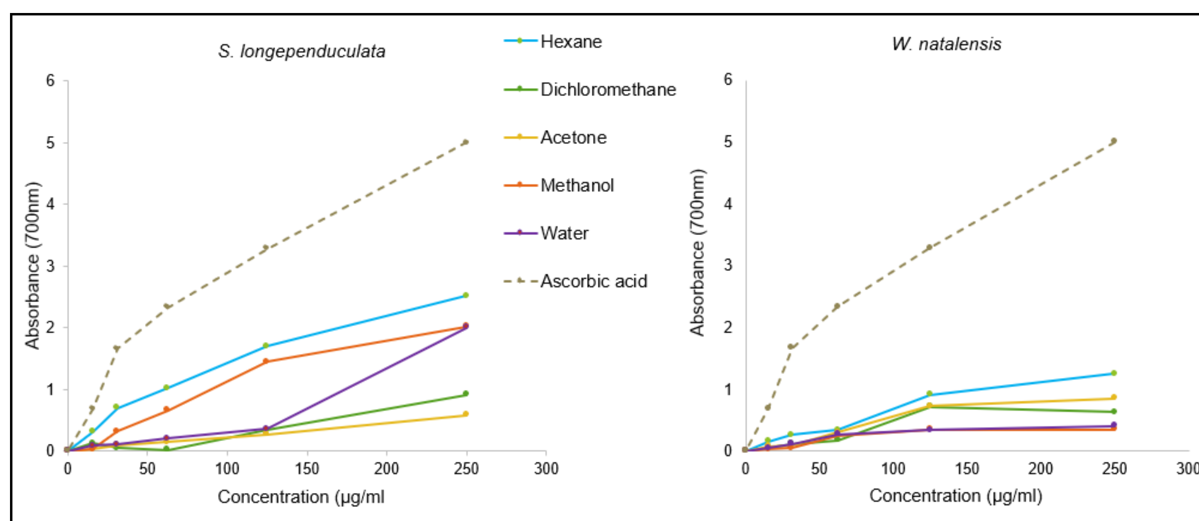

Figure 5.2: Ferric reducing power of *S. longepedunculata* and *W. natalensis* at varying concentrations expressed as Absorbance 700nm. Ascorbic acid was used as standard to which samples were compared.

Key: H- Hexane, D- Dichloromethane A- Acetone, M- Methanol, E- Ethanol, and W- Water.

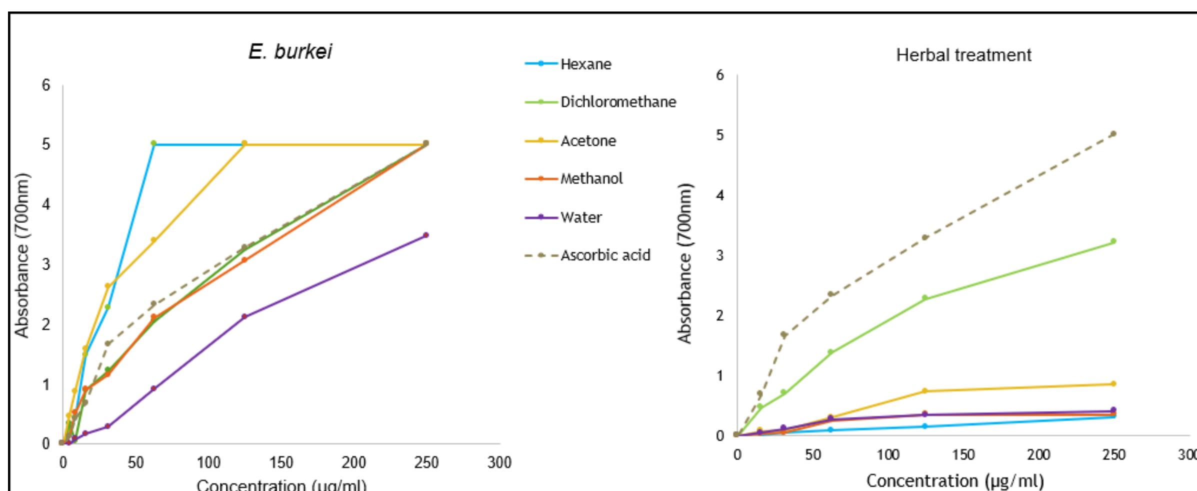

Figure 5.3: Ferric reducing power of *E. burkei* and herbal treatment at varying concentrations expressed as Absorbance 700nm. Ascorbic acid was used as standard to which samples were compared.

Key: H- Hexane, D- Dichloromethane A- Acetone, M- Methanol, E- Ethanol, and W- Water.

5.4. Discussion

Overproduction of oxidants (reactive oxygen species and reactive nitrogen species) in the human body is responsible for the pathogenesis of some diseases (Zhang *et al.,* 2015). Increased presence of ROS in male and female genitals from endogenous and exogenous sources results in cellular damage and as such, compromises the vitality of sperm cells and other organelles in males and interferes with oocyte maturation in females. In some cases, oxidative stress is the result of bacterial infection, which in turn results in infertility. In other instances, certain disease conditions result in an endogenous increase of free radicals which in turn results in cellular damage and infertility. Agarwal *et al.* (2014); Hazra and Parmar (2014) reported that a favourable trend towards natural products has developed due to reports from medicinal centres

regarding the potential teratogenic, carcinogenic and mutagenic effects of synthetic antioxidants in experimental animals, including primates.

The antioxidant activity of the extracts was evaluated using thin-layer chromatography (TLC), by separating the extracts in different solvent systems (BEA, CEF and EMW). The chromatograms were air-dried and sprayed with 0.2% free radical (DPPH) solution. The reagent DPPH is a dark-purple coloured crystalline powder that is composed of free radicals that, upon encounter with antioxidant, get reduced to a yellow-coloured substance (Gonçalves *et al.*, 2013). The presence of antioxidant activity was observed by the development of a yellow colour against a purple background formed by the DPPH solution upon spraying. High antioxidant activity was visualised in EMW mobile system for *E. bukei* and *S. longepedunculata.* For the same plant extracts, antioxidant activity was visualised in BEA mobile system although they were fainter. This meant that the antioxidant activity was lesser than that of the EMW mobile system. No antioxidant activity was visualised for the herbal treatment and *S. longepedunculata.* These phytochemicals were reported to have several activities, including antioxidant activity. Regarding the interactions, they may be synergistic, additive, or antagonistic towards a biological activity (Wang *et al.*, 2011).

Free radical scavenging activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a widely used technique to assess antioxidant activity due to its time efficiency and accuracy in quantification. The antioxidant activity of the plant extracts was compared to the standard (ascorbic acid) in a concentration dependent manner. *E. burkei* had the highest scavenging activity with hexane extract. This same trend was also noted for *S. longepedunculata* and the herbal treatment. The antioxidant activity increases with the increase in concentration of the plant extract. *E. burkei* hexane and acetone demonstrated high scavenging powers which were greater than the control, until they remarkably reached the maximum threshold of reducing power at about 10 µg/ml, which shows that this plant has the potential to be a natural antioxidant.

The reducing power assay measures the antioxidant capacity of the plant extracts. This method is based on the ability of antioxidants within the extracts to reduce the Fe^{3+}/ferricyanide complex to its ferrous (fe^{2+}) form by donating electrons. The concentration of Fe^{2+} ions were taken as a measure of the Perl's Prussian Complex. Therefore, an increase in absorbance signalled an increase in reducing the power capacity of the plant extracts (Lee *et al.*, 2012). The antioxidant activity of the plant extracts was compared to the standard (ascorbic acid) in a concentration dependent manner. Hexane extracts in *S. longependucalta, W. natalensis* and herbal treatment showed the highest reducing powers. The antioxidant activity increases with an increase in concentration of the plant extract, *E. burkei* hexane and acetone demonstrated high reducing powers that where greater than the control, until they reached the maximum threshold of reducing power. Both the assays show that the hexane extracts had the most activity. This suggests that the antioxidant compounds are non-polar and have the potential to be used as a natural antioxidant with more powers than the natural antioxidant – the ascorbic acid.

This study supports the idea that the balance between ROS generation and antioxidant capacity in the semen plays a critical role in the pathophysiology of genital tract inflammations and their impact on sperm functions (Sikka, 2001). The identification of these powerful antioxidants in medicinal plants used to treat male infertility will be a useful tool in primary healthcare and in bioprospecting these plants for the development of fertility agents (Abdillahi and van Staden, 2013).

5.5. Conclusion

The presence of potent antioxidant compounds with free radical scavenging and reducing power abilities were demonstrated in *E. burkei* hexane and acetone extracts. This observed antioxidant efficacy could be due to the presence of polyphenols that were present in this plant in high amounts. This chemical diversity of the herbal

treatment and the individual plant extracts, therefore, prompts an investigation to understand the effect these chemicals have on the biological activity.

References

Abdillahi, H. S. and van Staden, J. 2013. Application of medicinal plants in maternal healthcare and infertility: A South African perspective. *Planta Medica*, *79*(07), pp.591-599.

Agarwal, A. Virk, G. and Ong, C, du Plessis. SS. 2014. Effect of oxidative stress on male reproduction. *World Journal Mens Health*, *32*, pp.1.

Aitken RJ, 1995. Mechanisms and prevention of lipid peroxidation in human spermatozoa. In: Fenichel P, Parinaud J (eds.), *Human Sperm Acrosome Reaction*, 236. John Libbey and Company Limited, London, pp.339–353

Alkadi, H. 2020. A review on free radicals and antioxidants. *Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders)*, *20*(1), pp.16-26.

Arslan Burnaz, N. Küçük, M. and Akar, Z. 2017. An on-line HPLC system for detection of antioxidant compounds in some plant extracts by comparing three different methods. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *1052*, pp.66-72.

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

Chigayo, K. Mojapelo, P.P.E.L. and Moleele, S.M. 2016. Phytochemical and antioxidant properties of different solvent extracts of *Kirkia wilmsii* tubers. *Asian Pacific Journal of Tropical Biomedicine, 6*, pp.1037–1043.

Comhaire, F.H. Mahmoud, A.M.A. Depuydt, C.E. Zalata, A.A. and Christophe, A.B. 1999. Mechanisms and effects of male genital tract infection on sperm quality and fertilizing potential: The andrologist's viewpoint. *Human Reproduction Update*, *5*(5), pp.393-398.

Dai, J. and Mumper, R.J. 2010. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules*, *15*(10), pp.7313-7352.

Darin-Bennett, A. Poulos, A. and White, I.G., 1976. The fatty acid composition of the major phosphoglycerides of ram and human spermatozoa. *Andrologia*, *8*(1), pp.37-45.

Dlamini, L.M. Tata, C.M. Djuidje, M.C.F. Ikhile, M.I. Nikolova, G.D. Karamalakova, Y.D. Gadjeva, V.G. Zheleva, A.M. Njobeh, P.B. and Ndinteh, D.T. 2019, Antioxidant and prooxidant effects of *Piptadeniastrum africanum* as the possible rationale behind its broad scale application in African ethnomedicine. *Journal of Ethnopharmacology*, 231, pp.429-437.

Drach, G.W. Fair, W.R. Meares, E.M. and Stamey, T.A. 1978. Classification of benign diseases associated with prostatic pain: prostatitis or prostatodynia. *The Journal of Urology*, *120*(2), pp.266-266.

Elumalai, P., Krishnamoorthy, G., Selvakumar, K., Arunkumar, R., Venkataraman, P. and Arunakaran, J. 2009. Studies on the protective role of lycopene against polychlorinated biphenyls (Aroclor 1254)-induced changes in StAR protein and cytochrome P450 scc enzyme expression on Leydig cells of adult rats. *Reproductive Toxicology*, *27*(1), pp.41-45.

Faydaoğlu, E. and Sürücüoğlu, M. 2014. Medical and aromatic plants antimicrobial, antioxidant activities and use opportunities. *Erzincan University Journal of Science and Technology, 6*(2), pp.233-265.

Gonçalves, S. Gomes, D. Costa, P. and Romano, A. 2013. The phenolic content and antioxidant activity of infusions from Mediterranean medicinal plants. *Industrial Crops and Products*, *43*, pp.465-471.

Hara, K. Someya, T. Sano, K. Sagane, Y. Watanabe, T. and Wijesekara, R. G. S, 2018. Antioxidant activities of traditional plants in Sri Lanka by DPPH free radical-scavenging assay, *Data in Brief, 17*, pp.870-875.

Hazra, T. and Parmar, P. 2014. Natural antioxidant use in ghee-A mini review. *Journal of Food Research and Technology*, *2*(3), pp.101-105.

Henkel, R., Franken, D.R. and Habenicht, U.F. 1998. Zona pellucida as physiological trigger for the induction of acrosome reaction. *Andrologica*, *30*(4-5), pp.275-280.

Koçak, Y. Oto, G. Meydan, İ. and Şeçkin, H. 2020. Investigation of total flavonoid, DPPH radical scavenging, lipid peroxidation and antimicrobial activity of *Allium schoenoprasum L.* plant growing in Van Region. *YYU Journal of Agricultural Science, 30*(1), pp.147-155.

Lee, W.C. Mahmud, R. Pillai, S. Perumal, S. and Ismail, S. 2012. Antioxidant activities of essential oil of *Psidium guajava* L. leaves. *APCBEE Procedia*, *2*, pp.86-91.

Maduraiveeran, H. Raja, K. and Chinnasamy, A, 2021. Antiproliferative and antioxidant properties of nematocysts crude venom from jellyfish *Acromitus flagellatus* against human cancer cell lines. *Saudi Journal of Biological Sciences*, *28*(3), pp.1954-1961.

Mahajan, R.T. and Gajare, S.M. 2012, Manifestation of erectile dysfunction with adaptogenic antioxidant aphrodisiac plants. *International Journal of Pharmaceutical and Biomedical Research*, *3*(1), pp.52-68.

Murugesan, P., Muthusamy, T., Balasubramanian, K. and Arunakaran, J. 2007. Effects of vitamins C and E on steroidogenic enzymes mRNA expression in polychlorinated biphenyl (Aroclor 1254) exposed adult rat Leydig cells. *Toxicology*, *232*(3), pp.170-182.

Naik, A.L.J. Reddy, S. and Rayalu, D.J. 2013. Phytochemical analysis, TLC profiling and antimicrobial activity of *Tephrosia purpurea*. *International Journal of Pharmaceutical and Life Sciences*, *4*(2), pp.2375-2379.

Nita, M. and Grzybowski, A. 2016. The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. *Oxidative Medicine and Cellular Longevity.*

Ochsendorf, F.R. 1999. Infections in the male genital tract and reactive oxygen species. *Human Reproduction Update*, *5*(5), pp.399-420.

Onbasli, D. and Yuvali, G, 2021*. In vitro* medicinal potentials of *Bryum capillare*, a moss sample, from Turkey. *Saudi Journal of Biological Sciences, 28*(1), pp.478- 483.

Oyaizu, M. 1986. Studies on product of browning reaction prepared from glucose amine. *Japanese Journal of Nutrition, 44*, pp.307–315.

Pourmorad, F. Hosseinimehr, S.J. and Shahabimajd, N. 2006. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *African Journal of Biotechnology*, *5*(11).

Prior, R.L. Wu, L. and Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food chemistry*, *53,* pp.4290-4302.

Sheweita, S.A., Tilmisany, A.M. and Al-Sawaf, H. 2005. Mechanisms of male infertility: role of antioxidants. *Current Drug Metabolism*, *6*(5), pp.495-501.

Sikka, S.C. 2001. Relative impact of oxidative stress on male reproductive function, *Current Medicinal Chemistry, 8(*7), pp.851-862.

Thakur, M. Singh, K. and Khedkar, R., 2020. Phytochemicals: Extraction process, safety assessment, toxicological evaluations, and regulatory issues: In Functional and Preservative Properties of Phytochemicals, Academic Press, pp.341-361.

Thompson, M. Williams, C.R. and Elliot, G.E.P. 1976. Stability of flavonoid complexes of copper (II) and flavonoid antioxidant activity. *Analytica chimica acta*, *85*(2), pp.375-381.

Wang, S., Meckling, K.A., Marcone, M.F., Kakuda, Y. and Tsao, R. 2011. Synergistic, additive, and antagonistic effects of food mixtures on total antioxidant capacities. *Journal of Agricultural and Food Chemistry*, *59*, pp.960–968.

Zhang, Y.J. Gan, R.Y. Li, S. Zhou, Y. Li, A.N. Xu, D.P. and Li, H.B. 2015., Antioxidant phytochemicals for the prevention and treatment of chronic diseases, *Molecules, 20*(12), pp.21138-21156.

Chapter 6: Antimicrobial activity assays

6.1. Introduction

Over 30 pathogens are responsible for sexually transmitted infections (Maema *et al.,* 2020). Different types of bacteria such as *Chlamydia trachomatis, Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Enterococcus faecalis, Neisseria gonorrhoeae Helicobacter pylori,* and *Pseudomonas aeruginosa* are among the most common isolated bacteria. They are isolated from bacteria found in the semen and genital tracts of infertile men relatively affecting semen quality and interfering with male fertility (Farsimadan and Motamedifar, 2020). These bacteria have a severe deleterious impact on sperm parameters and DNA integrity when infected (Zeyad and Hammadeh, 2017).

Medicinal plants have been hailed for their biological activities, such as antioxidant, anti-inflammatory, antidiabetic, antibacterial and antifungal activities, to mention but a few (Ahmed *et al.,* 2012; Masoko and Makgapeetja, 2015). The presence of antibacterial and antifungal compounds from medicinal plants is a well-known fact and provides information that leads to the development of new antimicrobial drugs (Abraham and Thomas, 2012). The available drugs and treatments have limited efficacy, unpleasant side effects, and contraindications in certain disease conditions, hence, the need for natural products which are cheaper (Kotta *et al.,* 2012). This has led to the exploration of plants for alternative therapeutic strategies, especially in developing countries where access to quality healthcare is lacking and herbal remedies being their primary source of healthcare.

Medicinal plants represent a rich source of antimicrobial agents with better activity against multi-drug resistant bacteria and their antimicrobial potential was accepted long before the discovery of microorganisms by humankind (Anwar *et al.,* 2009). Medicinal plants have a diverse mixture of biologically active compounds that may act in killing or inhibiting the growth of disease-causing microorganisms (Alvin *et al.,*

2014). Therefore, the screening of natural products for the discovery and development of novel antimicrobial agents is imperative (Aumeeruddy-Elalfi *et al.,* 2016).

Some parasites and micro-organisms are known to develop resistance to synthetic chemotherapeutic agents, e.g., strains of malaria parasites *(Plasmodium falciparum* or *P. vivax)* develop resistance to chloroquine, whereas some micro-organisms develop resistance to antibiotics. No resistance is known to develop because of treatment by traditional medicines, but this may occur since traditional practitioners do not keep medical files or records. It is also possible that traditional medicines may provide a solution to this problem because of their multicomponent nature.

Considering the development of resistance in those infectious diseases with existing drugs, one strategy employed in traditional herbal medicine to overcome this phenomenon is the combination of herbal remedies. To this effect, some authors have explored the combination of antibiotics with plant extract (Dawoud, *et al.,* 2013), while others have focused on plant extract combinations to achieve a more potent antimicrobial activity (Ncube *et al.,* 2012).

Herbal remedies are usually prepared by mixing different plant portions or extracts with the intention to increase the medicinal value of the remedy as opposed to using one plant species (Cano and Volpato, 2004). Some interesting outcomes have been found with the use of a mixture of natural products to treat diseases, most notably the synergistic effects and poly-pharmacological application of plant extracts (Jain *et al.*, 2019). This expected increase in the therapeutic efficacy of the remedies is attributed to synergistic interactions between diverse phytochemicals. These synergistic interactions may either increase the effectiveness towards a single or a combination of ailments (Cano and Volpato, 2004). This chapter also aimed at determining the antagonistic and synergistic effects of the selected medicinal plants and herbal treatment.

6.2. Methods and Materials

6.2.1. Test microorganisms

This study used the following test organisms: two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212) as well as the fungus, *Candida albicans (*ATCC 10231). These bacterial and fungal species were maintained on nutrient agar and sabouraud dextrose agar (SDA), respectively, at 4 ℃. The cultures were, at a later stage, inoculated in nutrient broth and incubated at 37 ℃ (bacteria) for 24 hours and 30 ℃ (fungus) for 48 hours before screening tests.

6.2.2. Qualitative antibacterial assay

6.2.2.1. Bioautography assay

The Bioautography procedure was done according to the method described by Begue and Kline (1972). The plates were loaded with 20 µL of the plant extracts and separated, as described in section 4.2.2.1, and placed under a stream of air for a period of five days to allow the solvents used for development to evaporate. The actively growing cultures of the test microorganisms mentioned above were sprayed on the plates until they were completely wet and then were incubated at 37 ℃ for 24 hours under 100% relative humidity. Following incubation, the plates were sprayed with an aqueous solution of 2 mg/mL ρ-iodonitrotetrazolium chloride (INT) (Sigma) and further incubated for 2-3 hours. The bioautograms were observed for bacterial growth, clear zones against the red-pink background and indicated growth inhibition by the compounds with antibacterial activity.

6.2.3. Quantitative antibacterial assay

6.2.3.1. Broth micro-dilution assay

The minimum inhibitory concentration (MIC) values were determined using the serial microplate broth dilution methods developed by Eloff (1998b). The plant extracts were dissolved in acetone to give a final concentration of 10 mg/mL. The bacterial species were inoculated into 150 mL nutrient broth and incubated at 37 ℃ for 24 hours, this served as the stock culture. From the stock culture, 10 mL was removed and inoculated in 150 mL nutrient broth and incubated at 37 ℃ for 24 hours. Hundred microlitres of the plant extract was serially diluted (50%) with sterile distilled water in 96-well microtitre plates, and 100 µL of the bacterial culture was added into each well. Acetone was used as a negative control; the microtitre plates were covered and incubated at 37 ℃ for 24 hours. Following incubation, 40 µL of 0.2% ρ-iodonitrotetrazolium chloride (INT) (sigma) dissolved in water was added to each well as an indicator. The covered plates were further incubated for 30 minutes at 37 ℃ at relative humidity. The plates were observed for clear wells (activity) which resulted from the reduction of the purple colour and the MIC values were recorded as the lowest concentration that inhibited bacterial growth. The tests were done in triplicates. Total activity of the extracts was calculated by dividing the MIC values with the mass extracted from 1 g of the plant material. The resultant values indicated the volume to which the amount obtained from 1 g of the plant material could be diluted to and still inhibit the growth of the test microorganisms (Eloff, 2001).

6.2.3.2. Synergistic/antagonistic study

The effects of the combinations of selected different plants were studied. The micro-dilution assay described was used to determine the combinational effects on the antimicrobial activity of various mixtures.

Stock solutions (10 mg/mL) of acetone extracts of each plant were prepared by re-dissolving the extracts in acetone. For 1:1 test combination, 50 µL of each of the two extracts were mixed to make up a volume of 100 µL in the first wells of a 96-well microtiter plate. Each extract contributed 33.3 µL for the 1:1:1 combination, respectively, to make up 100 µL in the first wells of a 96-well microtiter plate (Ncube *et al.,* 2012). MIC values were determined for each of these combinations to establish any interaction effect following the antibacterial assays described in section 6.2.3.1. Following investigations of the independent MIC of the selected plants, the synergistic or antagonistic interactions between the plants were investigated. This was achieved by determining the MIC of the combinations exhibiting antibacterial activity to establish any interaction effect. The fractional inhibitory concentration (FIC) was calculated for the 1:1 combinations of the plants. This was determined with the equation below, where (i) and (ii) represented the different 1:1 plant combination (Mabona *et al.,* 2013). To determine these effects, the fractional inhibitory concentration (FIC) of the extracts was used. The method by van Vuuren and Viljoen (2011) for the determination of FIC values was adopted as seen below:

$$\mathrm{FIC(i)}\ \frac{\text{MIC of (a)in combination with (b)}}{\text{MIC of (a)independently}}$$

$$\mathrm{FIC(ii)}\ \frac{\text{MIC of (b)in combination with (a)}}{\text{MIC of (b)independently}}$$

The FIC value for each extract in a combination was calculated by dividing the MIC value of the combination by the MIC value of each plant decoction placed in the combination (Equation). The fraction inhibitory index (ΣFIC) was then calculated by adding the two FIC values of the plant extracts in a combination.

The value of the fractional index provides information regarding the outcome of a combination. Briefly, ΣFIC values ≤ 0.5 demonstrate synergistic interactions. For additive effects of the combinations, ΣFIC are > 0.5 - 1.00.

The summary of the interpretations is tabulated

$$FIC = \frac{MIC\ (combination\ a,b)}{MIC\ (a)}$$

$FIC\ index = \Sigma FIC = FICA + FICB$

Where MIC is the minimum concentration of the extract that was able to inhibit microbial growth. FICA is the FIC for the first extract in the combination and FICB is of the second extract. The definitions on the interpretation of the combinational effects of plant extracts at a 1:1 ratio is shown in table 6.1.

Table 6.1: Outcome combination based on FIC index value.

FIC index value	**Outcome of combination**
$\Sigma FIC \leq 0.5$	Synergistic
$\Sigma FIC > 0.5 - 1.00$	Additive
$\Sigma FIC > 1.00 - \leq 4.00$.	Indifferent
$\Sigma FIC > 4.00$	Antagonistic

6.3. Results

6.3.1. Bioautography assay

Figure 6.1 and **Figure 6.2** represent potent antibacterial activities of the extracts of the selected plants against *E. faecalis, S. aureus* and *C, albicans. E. burkei. C, albicans. Elephantorizzha burkei* was the most active plant with all the extracts excluding water, showing antimicrobial activity (circled in green) against the two bacteria and one fungus on the chromatograms developed in BEA, CEF and EMW.

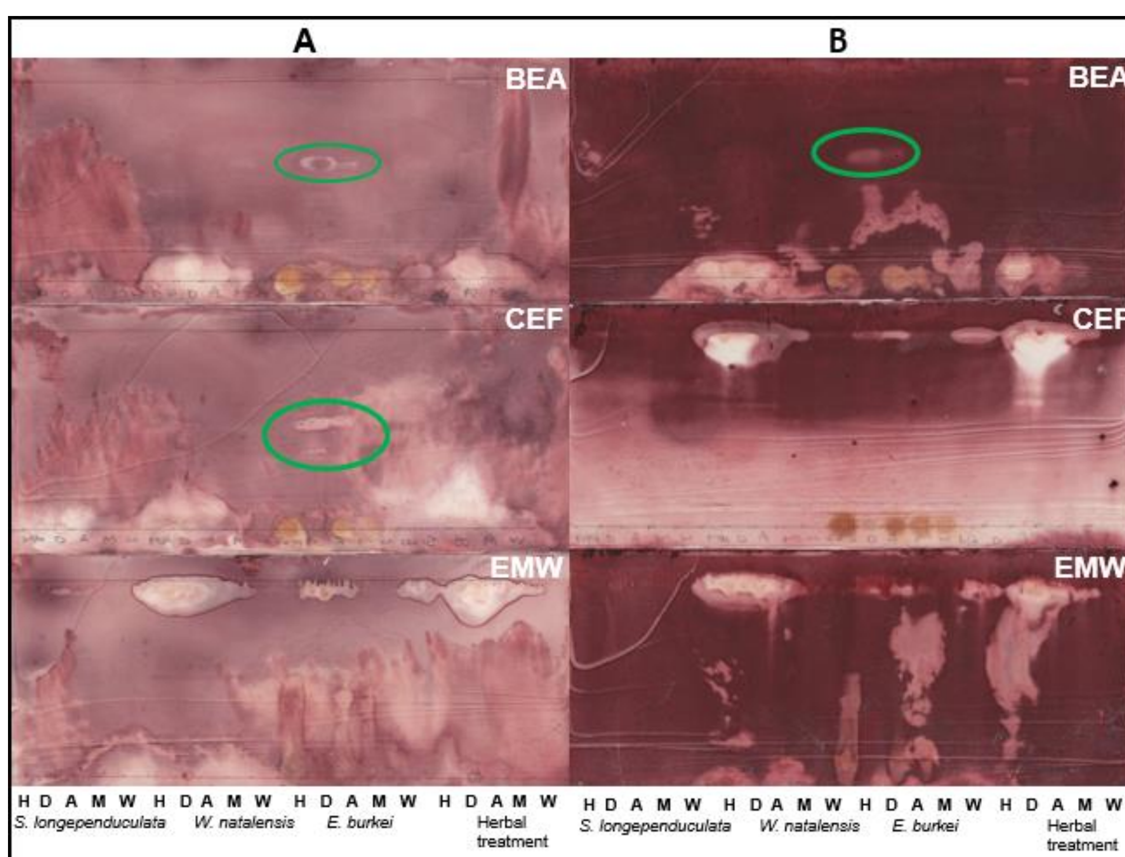

Figure 6.1: The chromatograms of medicinal plants and herbal treatment extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with *E. faecalis* (**A**) and *S. aureus* (**B**).

Key: H- Hexane, D- Dichloromethane A- Acetone, M- Methanol, E- Ethanol and W- Water.

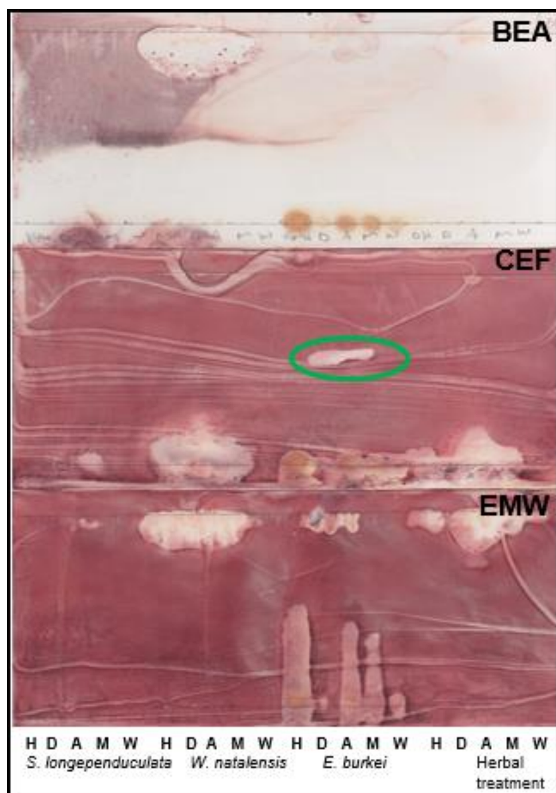

Figure 6.2: The chromatograms of medicinal plants and a treatment extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with *C. albicans.*

Key: H- Hexane, D- Dichloromethane, A- Acetone, M- Methanol, E- Ethanol and W- Water.

6.3.2. Broth Micro-dilution assay

The quantitative microdilution method in **Table 6.2** and **Table 6.3** confirms the antibacterial activity of the plant extracts where the MIC values ranged from 0.6-2.5 mg/mL for bacteria and 0.04-2.5 mg/mL. The lowest average MIC was for *E. burkei* plant extracts against *C. albicans* (0.04 mg/mL) followed by *S. aureus* (0.16 mg/mL) and *E. faecalis* (0.16 mg/mL). Total activity is represented in **Tables 6.4** and **6.5**. *Elephantorizzha burkei* showed the highest antibacterial activity against *C. albicans.*

Table 6.2: The minimal inhibitory concentration indexes of the selected plant extracts (mg/mL) against the test organisms after 24 hours incubation.

Microorganisms	*W. natalensis*					*S. longepedunculata*					*E. burkei*					Herbal treatment					**Average**	Gen (mg/mL)
	H	D	A	M	W	H	D	A	M	W	H	D	A	M	W	H	D	A	M	W		
S. aureus	0.32	0.16	0.16	2.5	2.5	1.25	0.64	0.64	0.64	2.5	0.16	0.32	0.16	0.64	0.64	1.25	2.5	0.15	1.25	1.25	**0.98**	>2.5
E. faecalis	0.64	0.32	0.64	2.5	2.5	2.5	0.64	0.64	0.64	2.5	0.16	0.32	0.16	0.64	0.64	1.25	2.5	0.32	0.64	2.5	**1.13**	0.08
Average	**0.4**	**0.2**	**0.4**	**2.5**	**2.5**	**1.8**	**0.64**	**0.64**	**0.64**	**2.5**	**0.16**	**0.32**	**0.16**	**0.64**	**0.64**	**1.25**	**2.5**	**0.4**	**0.9**	**1.8**		

Key: H- Hexane, D- Dichloromethane, A- Acetone, M- Methanol, W- Water and Gen-Gentamicin

Table 6.3: The minimal inhibitory concentration indexes of the selected plant extracts (mg/mL) against the test organism after 48 hours incubation.

Microorganisms	*W. natalensis*					*S. longepedunculata*					*E. burkei*					Herbal treatment					**Average**	AmB (mg/mL)
	H	D	A	M	W	H	D	A	M	W	H	D	A	M	W	H	D	A	M	W		
C. albicans	0.2	0.2	0.32	0.16	0.2	0.18	0.16	0.16	0.12	0.12	0.2	0.2	0.2	0.04	0.04	0.2	0.2	0.2	0.18	0.18	**0.17**	0.16
Average	**0.2**	**0.2**	**0.32**	**0.16**	**0.2**	**0.18**	**0.16**	**0.16**	**0.12**	**0.12**	**0.2**	**0.2**	**0.2**	**0.04**	**0.04**	**0.2**	**0.2**	**0.2**	**0.18**	**0.18**		

Key: H- hexane, D- dichloromethane, A- acetone, M- methanol, W- water and AmB- Amphotericin B

Table 6.4: The total activity of the medicinal plants and herbal treatment extracts (ml/g) against bacteria.

Microorganisms	*W. natalensis*					*S. longepedunculata*					*E. burkei*					Herbal treatment					**Average**
	H	D	A	M	W	H	D	A	M	W	H	D	A	M	W	H	D	A	M	W	
S. aureus	42	309	251	57	6	59	186	21	311	35	61	15	131	116	184	8	5	78	17	29	96
E. faecalis	22	155	63	57	6	29	186	21	311	35	61	15	131	116	184	8	5	19	34	29	74
Average	25	180	132	57	6	49	140	21	260	35	43	15	131	87	139	8	5	39	23	29	85

Key: H- Hexane, D- Dichloromethane, A- Acetone, M- Methanol and W- Water

Table 6.5: The total activity of the medicinal plants and herbal treatment extracts (ml/g) against fungus.

Microorganisms	*W. natalensis*					*S. longepedunculata*					*E. burkei*					Herbal treatment					**Average**
	H	D	A	M	W	H	D	A	M	W	H	D	A	M	W	H	D	A	M	W	
C. albicans	68	248	201	716	70	386	744	84	1659	725	97	25	105	1855	295	50	67	62	120	406	68

Key: H- Hexane, D- Dichloromethane, A- Acetone, M- Methanol and W- Water

6.3.3. Synergistic, additive, and antagonistic effects

Combinational effects of the addition of multiple plant species on antimicrobial activity were investigated. The combinations were of a 1:1 ratio and 1:1:1 ratio and their minimum inhibitory concentrations were used to assess the fractional inhibitory index values (ΣFIC) as described in **Table 6.1** to evaluate the synergistic or antagonistic effects of the combinations of plant extracts on the antibacterial activity (**Table 6.6 – 6.10**). Most interactions were antagonistic and indifferent against test microorganism.

Table 6.6: Antibacterial activity of extract combinations against *Staphylococcus aureus.*

Plant combination 1:1	MIC (mg/mL)	FIC 1. 2.		FIC Index	Outcomes
WN_A+WN_W	0.5	0.78	2	2.78	Indifferent
WN_A+SL_A	0.6	0.94	3.75	4.7	Antagonistic
WN_A+SL_W	0.5	0.78	2	2.78	Indifferent
WN_A+EB_A	0.6	0.94	3.75	4.7	Antagonistic
WN_A+EB_W	0.5	0.78	0.78	1.56	Indifferent
WN_W+SL_A	1	0.4	6.25	6.65	Antagonistic
WN_W+SL_W	2.5	1	1	2	Additive
WN_W+EB_A	1.7	0.68	0.68	1.36	Additive
WN_W+EB_W	2.5	1	15.63	16.63	Antagonistic
EB_A+SLA	0.8	5	5	10	Antagonistic
EB_A+SL_W	2.5	15.63	1	16.63	Antagonistic
EB_A+EB_W	2.5	15.63	3.91	19.7	Antagonistic
SLA+SL_W	1	6.25	0.4	6.67	Antagonistic

Key: WN= *Wrightia natalensis,* SL=*Securidaca longepedunculata* EB= *Elephantorrizha burkei*, $_A$= Acetone $_W$=Water.

Table 6.7: Antibacterial activity of extract combinations against *Enterococcus faecalis.*

Plant combination 1:1	MIC (mg/mL)	FIC		FIC Index	Outcomes
		1.	2.		
WN_A+WN_W	2.5	3.9	1	4.9	Antagonistic
WN_A+SL_A	2.5	3.9	1	4.9	Antagonistic
WN_A+SL_W	2.5	3.9	1	4.9	Antagonistic
WN_A+EB_A	1.3	2.03	2.03	4.06	Antagonistic
WN_A+EB_W	1.5	2.03	2.03	4.06	Antagonistic
WN_W+SL_A	2.5	1	3.91	4.91	Antagonistic
WN_W+SL_W	2.5	1	1	2	Indifference
WN_W+EB_A	0.4	0.16	0.625	0.78	Additive
WN_W+EB_W	1.3	0.52	2.03	2.55	Indifference
EB_A+SLA	0.6	0.94	0.24	4.7	Antagonistic
EB_A+SL_W	0.8	1.25	0.32	1.55	Indifferent
EB_A+EB_W	2.1	3.28	3.28	6.56	Antagonistic
SLA+SL_W	0.8	1.25	0.32	155	Indifferent

Key: WN= *Wrightia natalensis,* SL=*Securidaca longepedunculata* EB= *Elephantorrizha burkei*, $_A$= Acetone $_W$=Water

Table 6.8: Antifungal activity of extract combinations against *C. albicans*

Plant combination 1:1	MIC (mg/mL)	FIC		FIC Index	Outcomes
		1.	2.		
WN_A+WN_W	2.5	7.8	12.5	20.3	Antagonistic
WN_A+SL_A	0.5	1.56	0.08	1.64	Indifferent
WN_A+SL_W	1.9	5.9	15.8	21.8	Antagonistic
WN_A+EB_A	1.9	5.9	9.5	15.4	Antagonistic
WN_A+EB_W	2.5	7.8	62.5	70.3	Antagonistic
WN_W+SL_A	0.8	1.5	1.8	3.3	Indifferent
WN_W+SL_W	2.5	12.5	20.83	33.3	Antagonistic
WN_W+EB_A	0.4	2	2	4	Indifferent
WN_W+EB_W	2.5	12.5	62.5	75	Antagonistic
EB_A+SLA	0.1	0.5	0.83	1.33	Indifferent
EB_A+SL_W	0.1	0.5	0.63	1.13	Indifferent
EB_A+EB_W	0.1	0.5	2.5	3	Indifferent
SLA+SL_W	2.5	15.63	20.83	36.46	Antagonistic

Key: WN= *Wrightia natalensis,* SL=*Securidaca longepedunculata,* EB= *Elephantorrizha burkei*, A= Acetone W=Water.

Table 6.9: Minimal inhibitory concentration indexes of the 1:1:1 combinations of the selected plants against bacteria.

	Plant combination 1:1:1			
	SL_A + WN_A+EB_A	SL_w + WN_w+EB_w	Average	Gen (mg/mL)
S. aureus	1	2.5	1.8	2.5
E. faecalis	1.6	2.5	2.0	0.02
Average	1.9	2.4		

Key: WN= *Wrightia natalensis,* SL=*Securidaca longepedunculata,* EB= *Elephantorrizha burkei*, $_A$= Acetone $_W$=Water. Gen=Gentamicin

Table 6.10: Minimal inhibitory concentration indexes of the 1:1:1 combinations of the selected plants against fungus.

	Plant combination 1:1:1		
	SL_A + WN_A+EB_A	SL_w + WN_w+EB_w	AmB (mg/mL)
C. albicans	2.5	2.5	0.16

Key: WN= *Wrightia natalensis,* SL=*Securidaca longepedunculata,* EB= *Elephantorrizha burkei*, $_A$= Acetone $_W$=Water. AmB= Amphotercin-B

6.4. Discussion

Indigenous people of Africa largely depend on local plants to restore their health (Cordier and Steenkamp, 2015). This is mainly because of their traditional values. Therefore, it is worthwhile to investigate plants used traditionally as medicines to ascertain whether their medicinal value can be validated by showing antimicrobial activity (Taylor *et al.*, 2001). Most STIs are curable by appropriate antimicrobial treatment. However, the constant emergence of multi-drug resistant pathogens is

threatening the efficacy of the currently used antibiotics (Bandow *et al.*, 2003; Parekh and Chanda, 2007a). Hence, the present study sought to examine these valuable indigenous medicinal plants based on their local uses as treatments for male sexual health. The researcher hoped to determine the antimicrobial activity of the plants and herbal treatment based on the phytochemical composition. Tests were carried out to see whether the growth of organisms, known to be the causative agents, would be inhibited. The investigation of plant extracts for antibacterial activity is the first step towards the discovery of novel therapeutic agents against the resistant pathogenic organisms (Eloff, 2019).

Screening of antibacterial potential of the extracts was employed using both qualitative and quantitative methods. Gram-positivebacteria *S. aureus* and *E. faecalis* are found in most healthy individuals although they can cause urinary tract infections (Zeyad and Hammadeh, 2017). Fungus *Candida albicans* causes semen candidiasis. It is the most important sexually transmitted fungal infection; this microorganism affects male fertility potential (Castrillón-Duque *et al.*, 2018). It was also used as a test microorganism, as recommended by Orchard and van Vuuren (2017). For bioautographic assay, a compound showing antimicrobial activity was visualised on the TLC plates in the *E. burkei* plant extracts. The antimicrobial compound was seen in **Figure 6.1** (against *S, aureus*) on BEA mobile system, in **Figure 6.2** (*E. faecalis*) on BEA and CEF mobile systems and **Figure 6.3** (*C. albicans*) on CEF mobile system. This suggests that the antimicrobial compounds range from non-polar to intermediate polar.

The microdilution assay was performed to quantify the concentration at which the plant inhibits a spectrum bacterium (Szewczyk and Wisniewski, 2007). MIC values were reported in **Table 6.2**. MIC values equal or less than 1.0 mg/mL were considered as noteworthy/significant antimicrobial activity because it indicates good antimicrobial properties. The plant material and herbal treatment showed noteworthy microbial activity. The *E. burkei* hexane and acetone extracts against *E. facaelis* had an MIC value of 0.16 mg/mL. Similarly, *E. burkei* hexane and acetone extracts against *E. faecalis* had an MIC value of 0.16 mg/mL. The lowest activity was noted on all water extracts from plant material and herbal plant against the two bacterial strains. The

results indicated that although the herbal medicine was prepared using plants with antimicrobial activities, the concentrations of the components may have been diluted in the final preparation to inhibit bacterial growth. Dilute concentrations also have negative implications on the efficacy of the concentrations of the concoctions prepared by the traders to treat specified infections. Noteworthy microbial activity against the fungus *C. albicans* strain was observed with the *E. burkei* methanol and water extract with an impressive MIC value if 0.04 mg/mL for both extracts, respectively. Overall noteworthy activity of the plants and herbal treatment was observed against fungus *C. albicans*, with excellent values ranging from 0.04-0.19 mg/mL, which is roughly as good as the positive control amphotericin-B (0.17mg/mL).

Some researchers focus on combinations of plants with conventional antibiotics, while others focus on plant-plant combinations to improve efficacy (Chung *et al.,* 2011, Ncube *et al*., 2012; Moussaoui and Alaoui, 2016; Komape *et al*., 2017). Thus, the herbal antimicrobial efficacies of these plant species and their combinations against pathogens that are prevalent in sexually transmitted infections were analysed. For 1:1: 1, only a combination of *E. burkei*, *W. natalensis* and *S. longepedunculata* acetone extracts showed noteworthy MIC values of 1 mg/mL. This combination equates solely to the herbal treatment, as mentioned in section 3.1. The 1:1: ratio of combinational effects were mostly observed in the plants and herbal treatment against the bacteria test microorganisms. The Fractional Inhibitory Concentration index (FIC) was used to evaluate the synergistic or antagonistic effects of the combinations of plant extracts on the antibacterial activity. Based on FIC indexes, additive effects were noted with the combination of *W. natalensis* (water extracts) *and S. longepedunculata* (water extracts) against *S. aureus*, and *W. natalensis* (water extracts) and *E. burkei* (acetone extracts) against *S. aureus*. Additive effects were also noted with *W. natalensis* (water extracts) and *E. burkei* (acetone extracts) against *E. faecalis*. Overall, there were no synergistic effects, which suggested that the plants have more effective antimicrobial activity as entities.

Although hundreds of plants species have been tested for antimicrobial properties, the majority of these have not been adequately evaluated (Vashist and Jindal, 2012). Previous studies on Tshivenḓa medicinal plants have concentrated on the antibacterial activity and quite a few studies have targeted the antifungal activities of Tshivenḓa medicinal plants (Obi *et al.,* 2003; Tshikalange *et al.,* 2005; McGaw *et al.,* 2007). Although good MIC and high activity for *E. burkei* against fungus *C. albicans* were noted, only antagonistic and indifferent effects were noted during combination effects, which suggested that the plants show best microbial activity independently. These findings validate the continued use of the plant species in treating STIs, especially those caused by fungal and bacterial pathogens (Chauke *et al.*, 2015). In the future is a more in-depth approach to various strains i.e. clinical and resistant strains as these may respond differently (Van Vuuren and Holl, 2017).

6.5. Conclusion

The results support the use of the tested plants in traditional medicine. This chapter shows that the plants exhibit antibacterial activity of natural origin and various mechanisms of antibacterial activity. Bioautography demonstrated that antibacterial compounds in plant extracts and herbal treatment extracts range from non-polar to intermediate polar. It was also indicated in this chapter that the extracts of the selected plants have antibacterial and antifungal compounds.

References

Abraham, J. and Thomas, T.D. 2012. Antibacterial activity of medicinal plant *Cyclea peltata* (Lam) Hooks and Thoms. *Asian Pacific Journal of Tropical Disease*, *2*, pp.280-284.

Ahmed, A.S. Esameldin, E. Elgorashi, E.E. Moodley, N. McGaw, L.J. Naidoo, V. and Eloff, J.N. 2012. The antimicrobial, antioxidative, anti-inflammatory activity and cytotoxicity of different fractions of four South African *Bauhinia* species used traditionally to treat diarrhoea. *Journal of Ethnopharmacology*, 143, pp.826-839.

Alvin, A., Miller, K.I. and Neilan, B.A. 2014. Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. *Microbiological Research*, *169*(7-8), pp.483-495.

Anwar, A. Leong, K.M. Ng, M.L. Chu, J.J. and Garcia-Blanco, M.A. 2009. The polypyrimidine tract-binding protein is required for efficient dengue virus propagation and associates with the viral replication machinery. *Journal of Biological Chemistry*, *284*(25), pp.17021-17029.

Aumeeruddy-Elalfi, Z., Gurib-Fakim, A. and Mahomoodally, M.F. 2016. Chemical composition, antimicrobial and antibiotic potentiating activity of essential oils from 10 tropical medicinal plants from Mauritius. *Journal of Herbal Medicine*, *6*(2), pp.88-95.

Bandow, J.E., Brötz, H., Leichert, L.I.O., Labischinski, H. and Hecker, M. 2003. Proteomic approach to understanding antibiotic action. *Antimicrobial Agents and Chemotherapy*, *47*(3), pp.948-955.

Begue, W.J. and Kline, R.M. 1972. The use of tetrazolium salts in bioautographic procedure. *Journal of Chromatography*, *88*, pp 182-184.

Cano, J.H. and Volpato, G. 2004. Herbal mixtures in the traditional medicine of Eastern Cuba. *Journal of Ethnopharmacology*, *90*(2-3), pp.293-316.

Castrillón-Duque, E.X. Suárez, J.P. and Maya, W.D.C. 2018. Yeast and fertility: Effects of *in vitro* activity of *Candida* spp. on sperm quality. *Journal of Reproduction and Infertility*, *19*(1), pp 49-55.

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

Chung, P.Y. Navaratnam, P. and Chung, L.Y. 2011. Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against *Staphylococcus aureus* strains, *Annals of Clinical Microbiology and Antimicrobials*, *10*(1), pp.25- 30.

Cordier, W. and Steenkamp, V. 2015. Evaluation of four assays to determine cytotoxicity of selected crude medicinal plant extracts *In vitro. British Journal of Pharmaceutical Research, 7*, pp.16-21.

Dawoud, M.E.A., Mawgoud, Y.A. and Dawoud, T.G. 2013. Synergistic interactions between plant extracts, some antibiotics and/or their impact upon antibiotic-resistant bacterial isolates. *African Journal of Biotechnology*, *12*(24).

Eloff, J.N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica*, *64*(8), pp 711-713.

Eloff, J.N. 2001. Antibacterial activity of Marula (*Sclerocarya birrea* (A. rich.) *Hochst. subsp*. caffra (Sond.) Kokwaro) (Anacardiaceae) bark and leaves. *Journal of Ethnopharmacology*, *76*(3), pp.305-308.

Eloff, J.N. 2019, Avoid pitfalls in determining antimicrobial activity of plant extracts and publishing the results. *BMC Complementary and Alternative Medicine*, *19,* pp.106.

Farsimadan, M. and Motamedifar, M. 2020. Bacterial infection of the male reproductive system causing infertility. *Journal of Reproductive Immunology*, pp.103-183.

Jain, C. Khatana, S. and Vijayvergia, R. 2019. Bioactivity of secondary metabolites of various plants: a review. *International Journal of Pharmaceutical Sciences and Research*, *10*(2), pp.494-498.

Komape, N.P.M., Bagla, V.P., Kabongo-Kayoka, P. and Masoko, P. 2017. Anti-mycobacteria potential and synergistic effects of combined crude extracts of selected medicinal plants used by Bapedi traditional healers to treat tuberculosis related symptoms in Limpopo Province, South Africa. *BMC Complementary and Alternative Medicine*, *17*(1), pp.1-13.

Kotta, S. Khan, A.W. Pramod, K. Ansari, S.H. Sharma, R.K. and Ali, J. 2012. Exploring oral nanoemulsions for bioavailability enhancement of poorly water-soluble drugs. *Expert Opinion on Drug Delivery*, *9*(5), pp.585-598.

Maema, L.P.P. Potgieter, M.J. and Samie, A. 2020. Treatment of sexually transmitted infections by Bapedi traditional health practitioners. *Indian Journal of Traditional Knowledge, 19*(3), pp.533-541.

Masoko, P. and Makgapeetja, D.M. 2015. Anti-bacterial, antifungal and antioxidant activity of *Olea africana* against pathogenic yeast and nosocomial pathogens. *BMC Complementary and Alternative Medicine*, *15*, pp.409.

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

McGaw, L.J., van der Merwe, D. and Eloff, J.N. 2007. *In vitro* anthelmintic, antibacterial and cytotoxic effects of extracts from plants used in South African ethnoveterinary medicine. *The Veterinary Journal*, *173*(2), pp.366-372

Moussaoui, F. and Alaoui, T. 2016. Evaluation of antibacterial activity and synergistic effect between antibiotic and the essential oils of some medicinal plants. *Asian Pacific Journal of Tropical Biomedicine*, *6*(1), pp.32-37.

Ncube, B. Finnie, J.F. and van Staden, J. 2012. In vitro antimicrobial synergism within plant extract combinations from three South African medicinal bulbs. *Journal of Ethnopharmacology*, *139*(1), pp.81-89.

Obi, C.L. Potgieter, N. Bessong, P.O. Masebe, T. Mathebula, H. Molobela, P. and Jäger, A.K. 2003, In vitro antibacterial activity of Venda medicinal plants. *South African Journal of Botany*, *69*(2), pp.199-203.

Orchard, A. and van Vuuren, S.F. 2017. Commercial essential oils as potential antimicrobials to treat skin diseases. *Evidence Based Complementary Alternative Medicine*, 2017: ID 4517971: Doi.org/10.1155/2017/4517971.

Parekh, J. and Chanda, S. 2007. Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. *African Journal of Biomedical Research*, *10*(2), pp.175-181

Szewczyk, G. and Wisniewski, K. 2007. Dish and household cleaning, In *Handbook for Cleaning/Decontamination of Surfaces*, pp.125-195.

Taylor, J.L.S. Rabe, T. McGaw, L.J. Jäger, A.K. and van Staden, J., 2001. Towards the scientific validation of traditional medicinal plants, *Plant Growth Regulation*, *34*(1), pp.23-37.

Tshikalange, T.E. Meyer, J.J.M. and Hussein, A.A. 2005. Antimicrobial activity, toxicity, and the isolation of a bioactive compound from plants used to treat sexually transmitted diseases. *Journal of Ethnopharmacology*, *96*(3), pp.515-519.

Van Vuuren, S. and Holl, D. 2017. Antimicrobial natural product research: A review from a South African perspective for the years 2009–2016. *Journal of Ethnopharmacology*, *208*, pp.236-252.

Van Vuuren, S. and Viljoen, A. 2011. Plant-based antimicrobial studies–methods and approaches to study the interaction between natural products. *Planta Medica*, *77* (11), pp. 1168–1182.

Vashist, H. and Jindal, A. 2012. Antimicrobial activities of medicinal plants–Review. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, *3*(1), pp.222-230.

Zeyad, A. Amor, H. and Hammadeh, M.E. 2017. The impact of bacterial infections on human spermatozoa. *International Journal of Women's Health and Reproduction Sciences*, *5*(4), pp.243-252.

Chapter 7: Cytotoxicity assay

7.1. Introduction

Plants commonly used in traditional medicine are assumed to be safe. However, this is not usually the case, since several medicinal plants have been found to be toxic and cause damage on genetic material and even kill cells (Abdillahi and van Staden, 2013). Among the few plants whose biological activities have been evaluated scientifically, some have been reported to be poisonous or toxic and yet are still being used by pregnant women (Abdillahi and van Staden, 2013).

Toxicological evaluation of medicinal plants is often neglected since prolonged use with no effects is considered proof of their safety. However, historical, traditional usage is not always a reliable guarantee of safety since it is difficult for traditional healers to detect or monitor delayed consequences such as mutagenicity and long-term adverse effects (Ernst, 1998). Toxicological evaluation, such as carcinogenesis of medicinal plants, is very important, especially if the plant contains compounds with known mutagenic activities (Chhaabra *et al*., 2003).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that is converted to an insoluble purple formazan by metabolically active cells. The tetrazolium ring is cleaved by succinate dehydrogenase present in the mitochondria. Due to the impermeability of the resulting formazan, this purple product becomes collected inside healthy cells. Upon resuspension, the absorbance of the purple colour gives an estimation of the number of viable cells (Mosmann, 1983).

Kokwaro (1974), Sofowora (1982) and Arnold and Gulumian (1984), in their studies on African medicinal plants, described methods of preparation that display great uniformity all over the continent. Each method of preparation has, through experience, been found suited to the extraction of the active principles in desired proportions and chemical forms in relation to the treatments for which they are needed. This is

particularly important with drugs that may become toxic or ineffective when improper methods are used. Most root tubers are eaten raw although some may be eaten after following proper instruction of cooking. Drying and boiling removes the toxicity of tubers (Tshisikhawe, 2021).

Insufficient information regarding dosage taken by adults and those given to children poses a serious challenge as medicinal plants may contain other compounds that are toxic and can cause adverse effects to the patients (Masoko and Nxumalo, 2013). Scientific investigations of medicinal plants not only aim to validate their biological activities, but also include cytotoxic studies to ensure that medicinal plant(s) are also safe to use (McGaw *et al.,* 2008). The aim of this chapter was to test for cytotoxicity in the medicinal plants and herbal treatment samples.

7.2. Methods and materials

To determine the toxicological outcomes of the consumption of the medicinal plants and herbal treatment, their effect on cell viability of THP-1 human monocytic cell line derived from an acute monocytic leukaemia patient, was assessed. The MTT calorimetric assay described by Mosmann, (1983) was performed with modifications. The cell culture was maintained in a flask with Dulbeco minimal essential medium (DMEM, Whitehead scientific) supplemented by 10% foetal bovine serum (FBS) (Adcock-Ingram). Trypan blue was used to dye the cells and an automatic cell counter (model) was used to quantify viable cells. The cells were diluted with Roswell Park Memorial Institute (RPMI) complete media to obtain 5×10^4 cells/mL cell suspension. Two hundred microliters of the cell suspension were added into each of the wells of the 96 well microtitre plate. The plates were incubated at 37 ºC in a 5% carbon dioxide (CO_2) incubator for 24 hours.

The stock solutions of the extracts were prepared with acetone and water solvents to a concentration of 250 mg/mL dissolved in dimethyl sulfoxide (DMSO). The extracts were diluted to 1 mg/mL with complete media and a 0.25% of DMSO was maintained. One hundred microliters of the extracts (1000, 500, 250 µg/mL) prepared in a separate

95 well plate was transferred to the plate containing the cell cultures. The microtiter plates were incubated at 37 ºC in a 5% carbon dioxide incubator for 24 hours. Following incubation, 20 µL of 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dissolved in 1X PBS was added to each well and the plates were further incubated for 4 hours. After incubation, the media was removed from the plates and 100 µL of DMSO was added to each well. The plates were carefully swirled to dissolve the purple formazan crystals. Purple formazan crystals are formed when MTT is reduced by metabolically active cells. Thus, the amount of formed formazan products produced provides an indication of the number of viable cells. A microtiter plate reader (Promega) was used to measure the absorbance of the purple colour at 540 nm. Cells treated (positive control) with the extracts were compared with untreated cells (negative control).

7.3. Results

The effect of the water and acetone extracts of the selected plants on cell viability of THP-1 human monocytic cell line was determined using MTT assay. The cytotoxicity was represented as the percentage cell viability compared to the untreated cells and the treated cells with water and acetone extracts. The percentage viability of water extracts had a value ranging from <33.0 to 105.0 µg/mL (**Figure 7.1**). The percentage viability of acetone extracts had a value ranging from <34.5 to 108.0 µg/mL (**Figure 7.2**).

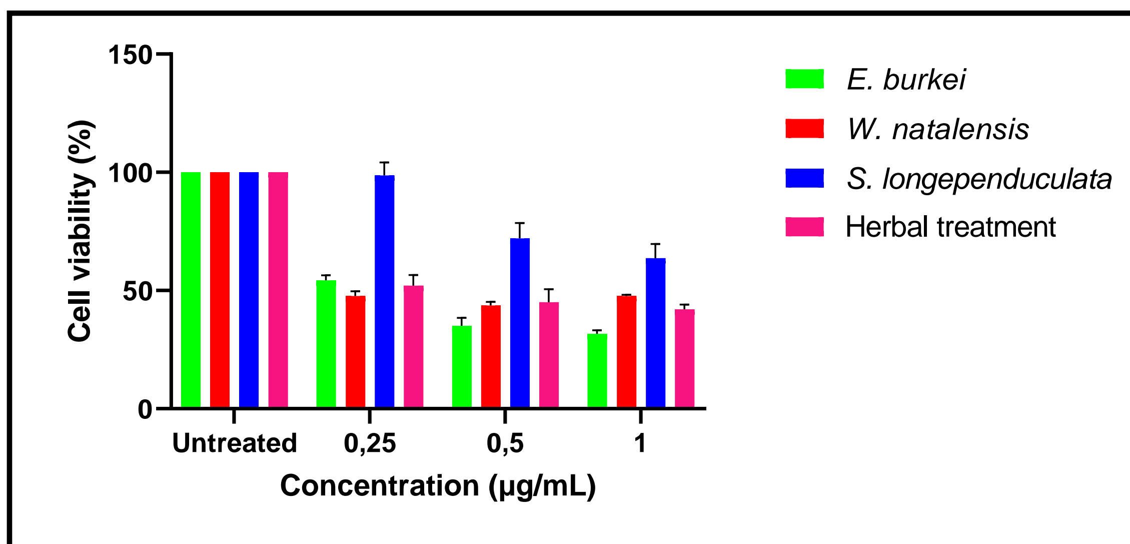

Figure 7.1: Percentage cell viability of medicinal plants and herbal treatment water extracts on human monocytic cell line (THP-1). Data represented as means duplicates ± standard deviations.

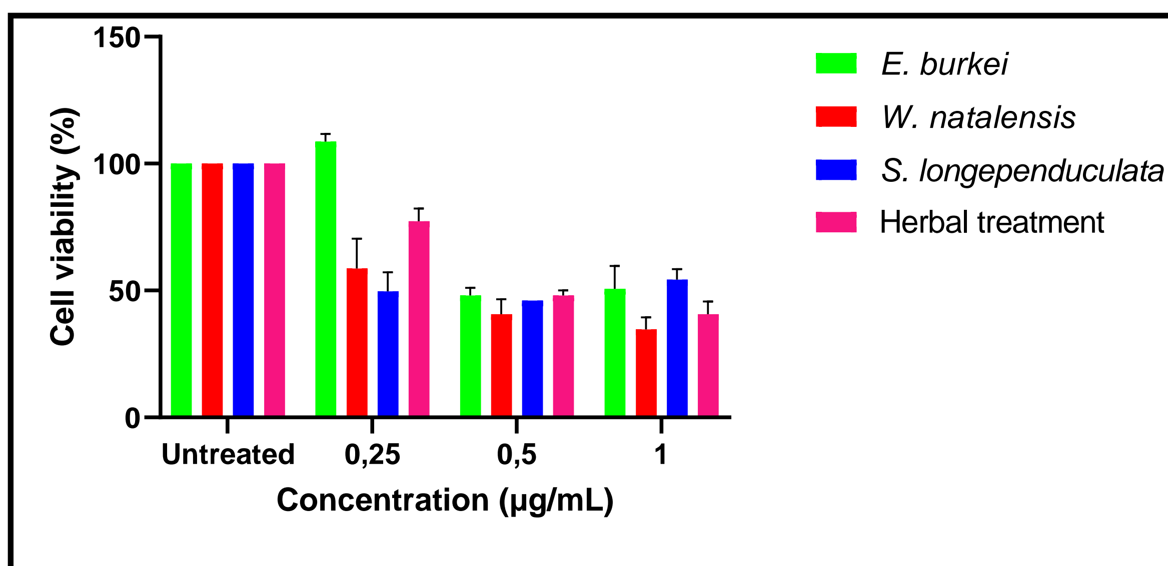

Figure 7.2: Percentage cell viability of medicinal plants and herbal treatment acetone extracts on human monocytic cell line (THP-1). Data represented as means duplicates ± standard deviations.

7.4. Discussion

The safety of many medicinal plants is questionable as traditional healers use different plants at different quantities and mostly in combinations (McGaw *et al.*, 2007).

Although there is a common misconception that medicinal plants are natural and therefore safe for consumption, such plants may be therapeutic at one dose and potentially toxic at another (McGaw *et al*., 2007). There is also a lack of safety of the plants on the human cells to support their medicinal use (Kilonzo *et al*., 2016). The MTT test as described in section 7.3 was performed at different concentrations of the acetone extract since medicinal plants are regarded to be safe in small doses and water extracts are also safe; it is the employed method of intake by the herbalist.

Cell viability assay was conducted to determine the cytotoxic effect of the samples against THP-1 human monocytic cell line derived from an acute monocytic leukemia patient. For treated cells with water extracts, *E. burkei* was found to be toxic at high concentrations with a percentage inhibition value of 33 µg/mL. In a previous study, as reported by Ajao *et al.* (2018), there were no records of reported cytotoxicity of *E. burkei* root water extracts. Implicit in this is that this study is the first to report the cytotoxicity of *E. burkei*. A decrease in concentration was also observed to decrease the toxicity. *S. longepedunculata* root water extracts were found to be toxic, which is supported by Ajao *et al. (*2018), who reported that plant extracts have acute cytotoxicity (Mongalo *et al*., 2015), and that with an increase in concentration, the extracts become more toxic. For acetone extracts, *W. natalensis* was found to be toxic with the percentage inhibition of 34.5 µg/mL. *Securidaca longepedunculata* showed no toxicity with acetone root extracts. The herbal treatment was found to be nontoxic for acetone root extracts, since it is a combination of plants and at low doses of each plant no toxicity is observed.

7.5. Conclusion

The preliminary toxicological results from this study suggest that herbal treatment may be toxic, and some plants show acute toxicity human cells. The high cytotoxicity of *E. burkei* and *W. natalensis* highlights the importance of using it with caution and at low doses. Although there is validity in the traditional use of the herbal concoctions, animal

models are required to investigate the pharma-kinetics of the concoctions. The plants listed in the study need to be evaluated *in vitro* such as genotoxicity before any further usage, *in vivo* for biological activities and further for efficacy and safety, including clinical studies where possible.

References

Abdillahi, H. S. and van Staden, J. 2013. Application of medicinal plants in maternal healthcare and infertility: A South African perspective. *Planta Medica*, *79*(07), pp.591-599.

Ajao, A.A. Sibiya, N.P. and Moteetee, A.N. 2019. Sexual prowess from nature: A systematic review of medicinal plants used as aphrodisiacs and sexual dysfunction in sub-Saharan Africa. *South African Journal of Botany*, *122*, pp.342-359.

Arnold, H.J. and Gulumian, M. 1984. Pharmacopoeia of traditional medicine in Venda, *Journal of Ethnopharmacology*, *12*(1), pp.35-74.

Chhabra, R.S., Bucher, J.R., Wolfe, M. and Portier, C. 2003. Toxicity characterization of environmental chemicals by the US National Toxicology Program: An overview. *International Journal of Hygiene and Environmental Health*, *206*(4-5), pp.437-445.

Diaz, G. Melis, M. Musinu, A. Piludu, M. Piras, M. and Falchi, A.M. 2007. Localization of MTT formazan in lipid droplets. An alternative hypodissertation about the nature of formazan granules and aggregates. *European Journal of Histochemistry*, *51*(3), pp.213-218.

Ernst, E. 1998. Harmless herbs? A review of the recent literature. *The American Journal of Medicine*, *104*(2), pp.170-178.

Kilonzo, M. Ndakidemi, P.A. and Chacha, M. 2016. *In vitro* antifungal and cytotoxicity activities of selected Tanzanian medicinal plants. *Tropical Journal of Pharmaceutical Research*, *15*(10): 2121-2130.

Kokwaro, J.O. 1974. Advantages & disadvantages of charcoal burning in Kenya. University of Nairobi.

Masoko, P. and Nxumalo, K.M. 2013. Validation of antimycobacterial plants used by traditional healers in three Districts of the Limpopo Province. *Evidence-Based Complementary and Alternative Medicine*, 586247: 7.

McGaw, L.J. Lall, N. Meyer, J.J.M. and Eloff, J.N. 2008. The potential of South African plants against Mycobacterium infections. *Journal of Ethnopharmacology*. *119*(3), pp.482-500.

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

McGaw, L.J., van der Merwe, D. and Eloff, J.N. 2007. *In vitro* anthelmintic, antibacterial and cytotoxic effects of extracts from plants used in South African ethnoveterinary medicine. *The Veterinary Journal*, *173*(2), pp.366-372

Mongalo, N.I. McGaw, L.J. Finnie, J.F. and van Staden, J. 2015. *Securidaca longipedunculata* fresen (Polygalaceae): A review of its ethnomedicinal uses, phytochemistry, pharmacological properties and toxicology. *Journal of Ethnopharmacology*, *165*, pp.215-226.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*, pp.55–63.

Sofowora, A., 1982. Medicinal plants and traditional medicine in Africa, John Wiley and Sons Limited, Chichester, England, pp.142-145.

Storket, J.C. Blázquez-Castro, A. Cañete, M. Horobin, R.W. and Villanueva, A. 2012. MTT assay for cell viability: intracellular localization of the formazan product is in lipid droplets, *Acta Histochem*, *114*(8):785-796.

Tshisikhawe M. P. 2021, *BOT 2649 Ethnobotany I study guide,* University of Venda, Thoyandou, South Africa.

Watt, J.M. and Breyer-Brandwijk, M.G. 1962. The medicinal and poisonous plants of Southern and Eastern Africa, (No. 581.96 W38).

Chapter 8: Bioactivity guided isolation of antibacterial compounds

8.1. Introduction

Medicinal plants are the "backbone" of traditional medicine and are considered to have some important ingredients that can be used in drug development and synthesis. They significantly play a critical role in the development of human cultures around the world and have a promising future (Khan, *et al*., 2019). The degree of chemical purity of phyto-medicines can subsequently be assessed using an array of analytical methods (Matotoka and Masoko, 2018), because there are millions of plants that are yet to be discovered for their medicinal activities (Singh, 2015).

Medicinal plants serve as reservoirs of potentially useful chemical compounds, which could serve as newer leads and clues for modern drug design (Majekodunmi, 2015). The percentage of natural products in modern drugs is between 35% and 50%. Most of the modern drugs are plant derived. Examples are pilocarpine, vincristine, emetine, physostigmine, digitoxin, quinine, atropine and reserpine. Screening of plants for biological activities must therefore be guided by the knowledge of medicinal folklore and poisonous plants (Tshisikhawe, 2021).

Medicines are used in solving physical health problems in the indigenous communities. These are direct and empirical application of medicines to treat a particular disease with clearly observable or detectable symptoms. The success of the treatment is totally attributed to the effects of the medicine used. Chemical compositions of such medicines play an important role. A patient treated by these medicines must show normal or natural diseases with familiar symptoms that can be treated with remedies known to be active against such complications. These include injuries and diseases such as colds, fevers, diarrhea, sexually transmitted infections STIs, etc. (Tshisikhawe, 2021).

Scientific research may take various forms. One of these forms is the screening of plants for bioactive agents. This involves biological screening (i.e. searching for the

physiological effect which a plant or extract may produce) and phytochemical screening (i.e. searching for the active compound). These approaches have one problem in common, namely, selection of material to be screened, otherwise known as sampling. Kokwaro (1976) gives guidelines on the chemical constituents of plants as well their possible medicinal effects. A very brief summary of these is given below.

Preparation of medicinal plants for experimental purposes is an initial step and key in achieving quality research outcome. It involves extraction and determination of quality and quantity of bioactive constituents before proceeding with the intended biological testing (Abubakar and Haque, 2020). Isolation of bioactive compounds employs a process that integrates separation of compounds in a mixture with their *in vitro* biological activity; this is known as bioassay guided fractionation. This process begins with the screening of different crude extracts for any biological activity; followed by the separation of the active compounds within the crude extracts and testing the fractions for biological activity. The fractions with activity are further separated and tested for biological activity until a pure compound (s) is obtained (Jamil *et al*., 2012). The separation usually involves the use of column chromatography, where the components from crude extracts are separated into fractions based on the polarities, using one or two solvents (Bucar *et al*., 2013). Thin-layer chromatography is often used to confirm the purity of the isolated compounds (Sasidharan *et al*., 2011). The aim of this chapter was to isolate bioactive compounds using bioassay guided fractionations.

8.2. Methods and materials

8.2.1. Serial exhaustive extraction

Serial exhaustive extraction (SEE) was used to extract bioactive compounds from the *Elephantorrhiza burkei* root plant material since it had promising antibacterial activity. The mass of 1 kg of the plant material(powder) was weighed and extracted into 10 litres of each solvent (hexane, dichloromethane, acetone, and methanol) in increasing polarity in a bottle. The mixture was vigorously shaken using a shaker (Thermo

Scientific MaxQ 3000) at 200 rpm. Shaking was done three times (overnight, followed by 2-hour intervals followed by 1-hour intervals). The supernatant was filtered, concentrated using rotary evaporator (Buchi R-114) at 50 ℃, and transferred into pre-weighed labelled beakers (250 ml). The remaining solvents were evaporated from the extracts under a stream of cold air at room temperature and the masses of the crude extracts were determined.

8.2.1.1. Phytochemical analysis

The chemical profiles of the *E. burkei* extracts were analysed on aluminium backed TLC plates (Merck, silica gel 60 F254) using a method developed by Kotze and Eloff, (2002) as described in **section 4.2.2.**

8.2.1.2. TLC-DPPH assay

Qualitative DPPH assay, using thin-layer chromatography was done according to the method described by (Braca *et al.,* 2002), as explained in **section 5.2.1**.

8.2.1.3. Bioautography assay

Bioautography was done according to the method described by Begue and Kline (1972), as described in **section 6.2.2.1** and the bacterial species were maintained as described in **section 6.2.1.**

8.2.1.4. Broth micro-dilution assay

The broth micro-dilution method described by Eloff (1998) was used to determine the minimum inhibitory concentrations (MIC) values of crude extracts against four tested bacterial species, as explained in **section 6.2.1.**

8.2.2. Isolation of antibacterial and antioxidant compounds

8.2.2.1. Open column chromatography

Bioassay guided fractionation was used to guide the isolation, separation, and purification of bioactive compounds with antioxidant and antibacterial activities using column chromatography from the acetone crude extracts. An open column (35 cm height × 4 cm radius) was packed with silica gel 60 (particles size 0.063 - 0.200 mm) (Fluka) using 100% hexane. The samples were mixed with small amounts of silica gel and subjected to column chromatography. The constituents of the extracts were eluted through an open column using 10 L solvents, as shown in **Table 8.1**, with increasing polarity from non-polar (hexane) to polar (methanol). The fractions were collected, and the solvents were removed under a stream of cold air at room temperature and the masses of the crude extracts were determined. Furthermore, the fractions were tested for antioxidant and antibacterial activity using TLC-DPPH (**Section 8.2.3**) and bioautography (**Section 8.2.4**) and broth micro-dilution (**Section 8.2.5**).

Table 8.1: Different percentages of solvents used for first open column chromatography.

Elution solvent	Percentages (%)
hexane	100%
hexane: Ethyl acetate	90:10
	80:20
	70:30
	50:50
	30:70
	10:90
Ethyl acetate	100
Ethyl acetate: Methanol	90:10
	80:20
	70:30
	60:40
	50:50
	40:60
Methanol	100

8.2.2.2. Determination of solvent system for second open chromatograghy

The roots extracts of *E. burkei* were extracted using serial exhaustive extraction with solvents, namely; hexane, dichloromethane (DCM), acetone and methanol, methanol extracted the highest yield followed by acetone (**Table 8.2**).

Table 8.2: Different percentages of solvents used for second open column chromatography.

	Elution solvent	Percentages (%)
1.	hexane	100%
2.	hexane: Ethyl acetate	90:10
3.		80:20
4.		70:30
5.		50:50
6.		30:70
7.		10:90
8.	Ethyl acetate	100
9.	Ethyl acetate: Methanol	90:10
10.		80:20
11.		70:30
12.		60:40
13.		50:50
14.		40:60
15.	Methanol	100

8.2.3. Second open column chromatography

The results of the biological activities of the fractions obtained from the first column (**Table 8.2**) revealed that the sub-fraction 8 [ethyl acetate 100%] exhibit high antibacterial activities and showed similar profiles. Therefore, that fraction of 0.99 g crude mass was further separated and purified for bioactive compounds.

8.2.3.1. Preparative TLC

The sub-fractions were further separated on TLC silica gel glass plates (Merck Silica gel 60 F_{254}) using 100% ethyl acetate for antimicrobial compounds. To detect UV reactive bands, the plates were visualised under UV light at 360 nm to locate the bands on the TLC plates and a small portion on the side of the glass plates was sprayed with vanillin-sulphuric acid reagent while the rest of the plates were covered with an aluminium foil. Visualised bands with vanillin sulphuric acid reagent were used as referenced line for scraping off active bands from developed TLC plates. The active compounds were immersed in the ethyl acetate, filtered using cotton wool and separated from silica gel. Purity of isolated compounds was confirmed by developing TLC plates in 100 % ethyl acetate and spraying TLC with vanillin-sulphuric acid and heated at 110 ^{o}C until colour developed. The compounds were also analysed for antimicrobial activity using bioautography assay, with *E. faecalis* as the test organism.

8.3. Results

8.3.1. Serial exhaustive extraction

8.3.1.1. Quantity of plant material extracted from *E. burkei*

The roots extracts of *Elephantorrizha burkei* (1 kg) were subjected to serial exhaustive extraction using different solvents, namely; hexane, dichloromethane (DCM), acetone and methanol. As shown in **Table 8.3**, Methanol was the best extracting solvent, extracting a total mass of 163.08 g, followed by acetone (38.32 g) and hexane (17.34 g). Meanwhile dichloromethane extracted the least (6.75 g).

Table 8.3: The mass residue extracted (g) from *E. burkei.*

Extracting solvents		Mass residue extracted (g)	
		Mass	Total
hexane	I	12.5.	17.34
	II	3.76	
	III	1.04	
Dichloromethane	I	1.39	6.75
	II	4.68	
	III	0.68	
Acetone	I	26.68	38.32
	II	8.04	
	III	3.6	
Methanol	I	148.55	163.08
	II	2.49	
	III	12.04	
Total			255.49

8.3.1.2. Phytochemical analysis

The roots extracts were analysed for their phytochemical components on TLC plates, which after developing, were viewed under UV light (**Figure 8.1 A and B**) and visualised by using vanillin-sulphuric acid reagent (**Figure 8.1. C**). In all used solvents, fluorescing compounds were observed in acetone and dichloromethane extracts with less fluorescence on hexane extract in CEF solvent system. After spraying the TLC plates with vanillin-sulphuric acid agents, more compounds were observed in EMW and CEF eluent system.

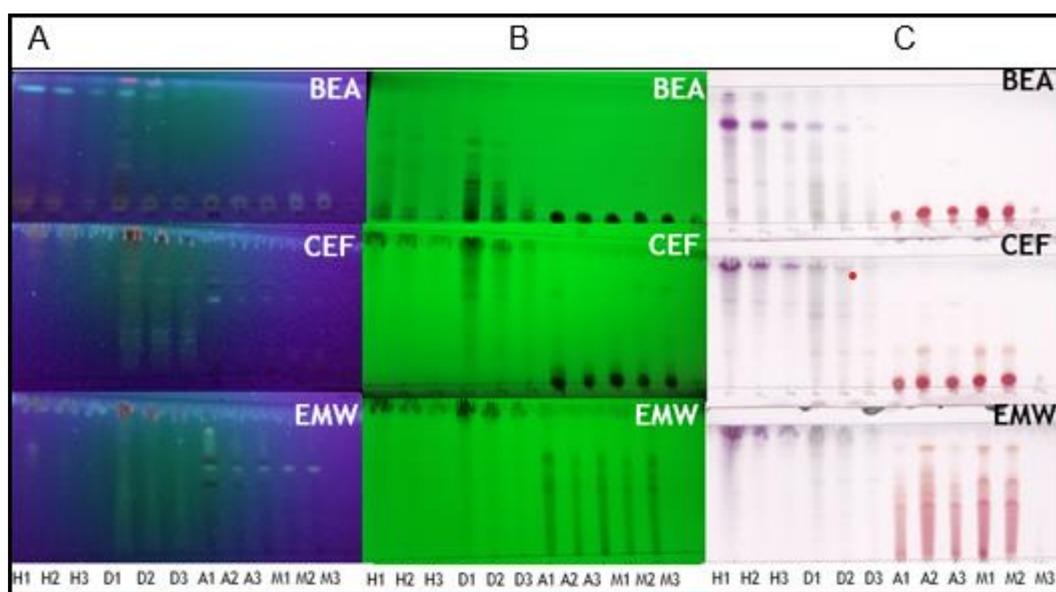

Figure 8.1: The chromatograms of *E. burkei* root extracts with different solvents and developed in BEA, CEF and EMW mobile systems and visualised under UV-light at 365 nm (**A**), 254 nm (**B**) and (**C**) was sprayed with vanillin-sulphuric acid reagent. **Key**: H- Hexane, D- Dichloromethane A- Acetone, M- Methanol and E- Ethanol.

8.3.1.3. Qualitative antioxidant activity

The roots extracts obtained by SEE method were also analysed for antioxidant activity, which was observed to be more visible in acetone extracts than all the solvent systems used (**Figure 8.1**), followed by dichloromethane extracts, while hexane and dichloromethane had no antioxidant activities.

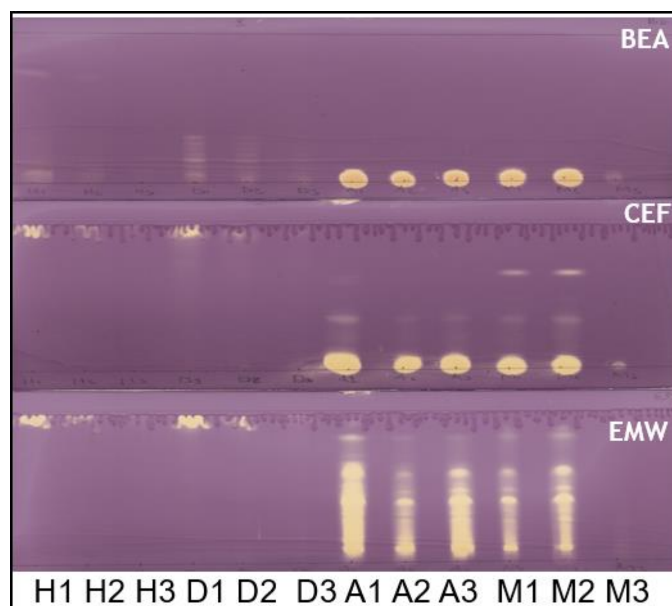

Figure 8.2: The chromatograms of *E. burkei* root extracts with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with 0.2% DPPH in methanol.

Key: H -Hexane, D- Dichloromethane A- Acetone, M- Methanol and E- Ethanol.

8.3.1.4. Broth micro-dilution assay

The quantitative micro-dilution method was used to determine the Minimum Inhibitory Concentration of the crude extracts against *S. aureus*, *E. faecalis* and *C. albicans*. As indicated on **Table 8.5**, the MIC values ranged from 0.02-2.5 mg/mL. The hexane 1 extract had the lowest average MIC of 0.03 mg/mL, followed by hexane 2 (0.09 mg/mL) and methanol 1 (0.11 mg/mL). The methanol, hexane, and acetone extracts had high activity against all tested microorganisms whereas the dichloromethane extracts had the least activity.

Table 8.1: The MIC values of *E. burkei* plant extracts (mg/mL) against the test organisms after 24 hours incubation.

Microorganisms	Hexane			Dichloromethane			Acetone			Methanol			Avg	Gen (mg/mL)
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
S. aureus	0.04	0.16	1.87	1.15	0.16	0.13	0.31	0.31	0.23	0.23	0.63	2	**0.6**	0.02
E. facaelis	0.02	0.02	0.2	0.13	0.65	2.5	0.45	1.25	0.45	0.02	0.4	0.2	**0.6**	0.02
Average	**0.03**	**0.09**	**2**	**0.64**	**0.4**	**1.3**	**0.38**	**1.4**	**0.34**	**0.11**	**0.5**	**1.1**		

Key: H- hexane, D- dichloromethane, A- acetone, M- methanol, Avg- average and Gen- Gentamicin.

Table 8.2: The MIC values of *E. burkei plant* extracts (mg/mL) against the test organisms after 48 hours incubation

Microorganism	**Hexane**			**Dichloromethane**			**Acetone**			**Methanol**			**Avg**	**AmB (mg/mL)**
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**		
C. albicans	0.6	1	2	2.5	1.25	1.67	1.25	1	1	0.5	0.63	0.02	**1.1**	0.02

Key: H- hexane, D- dichloromethane, A- acetone, M- methanol, Avg- average and AmB= Amphotericin-B

8.3.3.5. Bioautography

Bioautography was used to evaluate the antibacterial activities of the *E. burkei* extracts. The crude extracts were separated in BEA, CEF, and EMW and sprayed with *E. faecalis, S. aureus* and *C. albicans*. The white zones against pink background on the chromatograms below indicate antibacterial activity (**Figure 8.3** and **8.4**). The hexane, dichloromethane and ethyl acetate extracts had potent activity against all the tested bacteria on bioautograms developed in BEA and CEF.

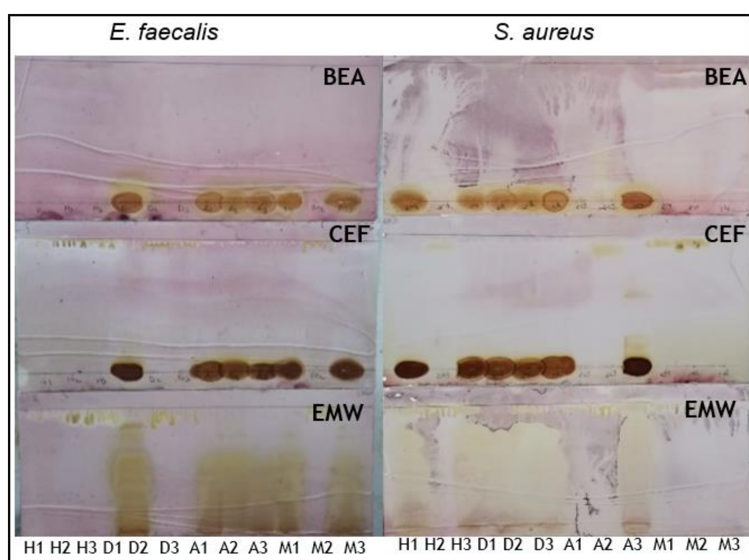

Figure 8.3: The chromatograms of *E. burkei* extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with *E. faecalis* and *S. aureus* cultures and visualised under 2mg/mL INT.

Key: H-Hexane, D- Dichloromethane A- Acetone, M- Methanol and E-Ethanol.

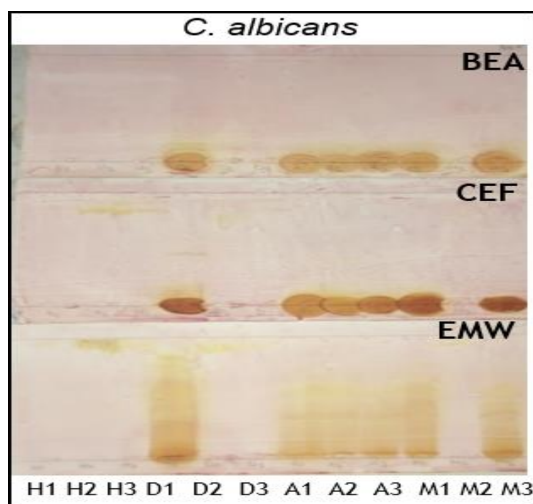

Figure 8.4: The chromatograms of *E. burkei* extracted with different solvents and developed in BEA, CEF and EMW mobile systems sprayed with *C. albicans* cultures and visualised under 2mg/mL INT.

Key: H-Hexane, D- Dichloromethane A- Acetone, M- Methanol and E-Ethanol.

8.3.2. Isolation of antibacterial compounds from acetone extracts

8.3.2.1. First open column chromatography

Acetone extracts of *E. burkei* exhibited overall potent antibacterial activity against all the tested bacteria, as such, it was chosen for the isolation of antibacterial compounds. The three extracts were combined to give a total mass of 33.14 g, which was subjected to column chromatography. Column chromatographic separation of the acetone extracts used the different percentages of the solvents listed in **Table 6.4** as eluents and the masses of the fractions collected are also listed. A total of 45.03 g was collected. The highest mass was eluted with 80% Ethyl acetate: Methanol (13.82 g), while the least was eluted with 90% hexane in ethyl acetate (0.27g).

Table 8.3: The mass (g) of fractions collected from column chromatographic separation of *E. burkei* acetone extracts using different solvents.

Elution solvent	Percentages (%)	Mass (g)
hexane	100%	0.31
hexane: Ethyl acetate	90:10	0.27
	80:20	0.36
	70:30	0.30
	50:50	0.29
	30:70	0.27
	10:90	0.28
Ethyl acetate	100	0.99
Ethyl acetate: Methanol	90:10	12.28
	80:20	13.82
	70:30	6.22
	60:40	1.81
	50:50	5.64
	40:60	1.1
Methanol	100	1.14
Total		45.08

8.3.2.2 Phytochemical analysis of 1st column chromatography fractions

Following the column chromatographic separation of the acetone extracts, the fractions collected were analysed on TLC for the phytochemicals. This involved separation and development of the plates in BEA, CEF and EMW and visualisation of the compounds under ultraviolet light and spraying with vanillin-sulphuric acid (**Figure 8.5**). The number of compounds observed decreased with increasing polarities of the eluent system.

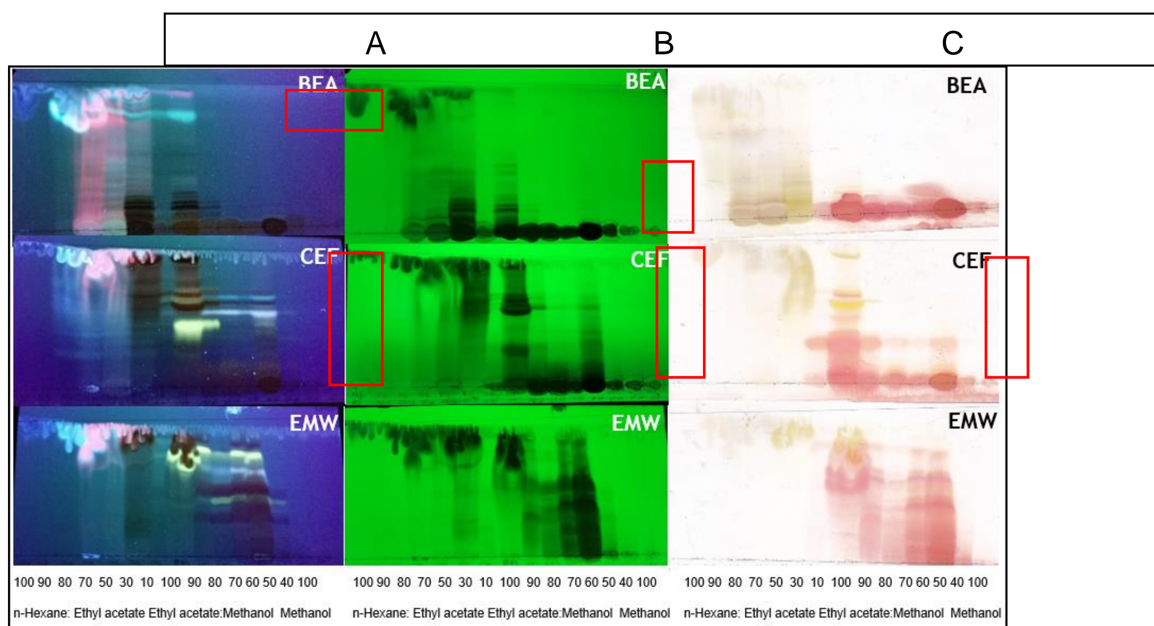

Figure 8.5: The chromatograms of *E. burkei* extracted with acetone and developed in BEA, CEF and EMW mobile systems were then visualised under UV-light at 365 nm (**A**), 254 nm (**B**) and (**C**) was sprayed with vanillin-sulphuric acid reagent.

8.3.2.3. Qualitative antioxidant activity

The root extracts obtained by the SEE method were also analysed for antioxidant activity, which was observed to be more visible in Ethyl acetate: Methanol extracts within all the solvent systems used (**Figure 8.6**), while Hexane: Ethyl acetate and Methanol had no antioxidant activities.

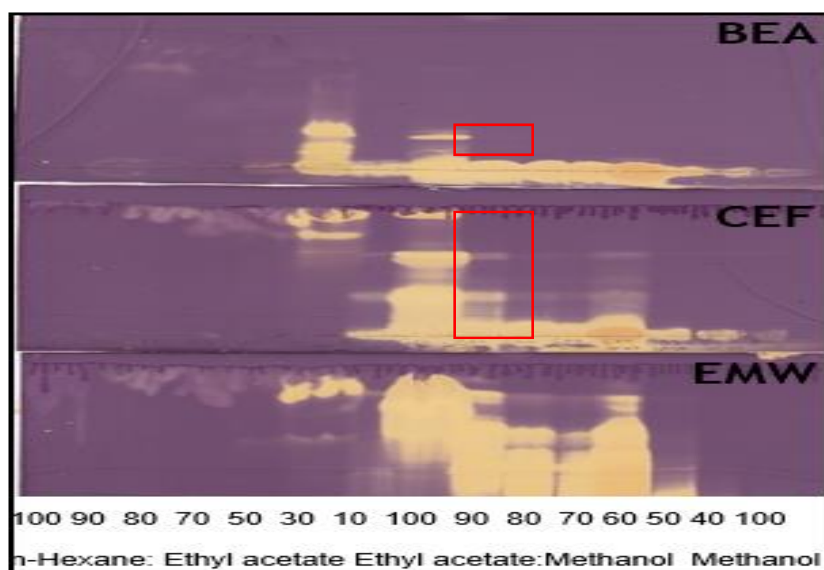

Figure 8.6: The chromatograms *E. burkei* acetone fractions developed in BEA, CEF and EMW mobile systems sprayed with 0.2% DPPH in methanol.

8.3.2.4. Broth micro-dilution assay for 1st column fractions

The broth micro-dilution results for determination of minimum inhibitory concentrations of the fractions are shown in **Table 8.8**. Fractions 7 and 8 had the highest antibacterial activity against all the tested microorganisms with the lowest MIC average (0.63 mg/mL), while fractions 1, 2, 13 and 14 had the lowest antibacterial activity with the highest average MIC (2.5 mg/mL).

Table 8.4: MIC values (mg/mL) of fractions from column chromatography against two tested bacteria.

Microorganisms	MIC values (mg/mL)																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	**Avg**	**Gen**
E. faecalis	2.5		2.5	0.63	1.25	2.5	1.25	0.63	0.32	1.25	0. 63	1,25		1.25	2.5	1,15	1.67
S. aureus	2.5		2.5	1.25	2.5	2.5	2.5	0.63	0.63	2.5	1.25	2.5		2.5	2.5	1,6	0.92
Average	2.5		2.5	0.94	1.87	2.5	1.87	0.63	**0.48**	1.87	1.87	1.87		1.87	2.5	1.4	

Key: recoloured values= noteworthy activity; Avg= average;1= 100% hexane; 2= 90% hexane: ethyl acetate; 3= 80% hexane: ethyl acetate; 4= 70% hexane: ethyl acetate; 5= 50**%** hexane: ethyl acetate; 6= 30% hexane: ethyl acetate; 7= 10% hexane: ethyl acetate; 8= 100% ethyl acetate; 9= 90% ethyl acetate: methanol; 10= 80% ethyl acetate: methanol; 11= 70% ethyl acetate: methanol; 12= 60% ethyl acetate: methanol; 13= 50% ethyl acetate: methanol; 14= 40% ethyl acetate: methanol; 15= 100% methanol.

Table 8.5: MIC values (mg/mL) of fractions from column chromatography against *C. albicans* fungus.

Microorganism	MIC values (mg/mL)															Avg
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	
C. albicans	2.5	2.5	1.25	1.25	2.5	1.25	0.63	0.2	0.03	0.03	0.05	0.2	2.5	2.5	2.5	

Key: recoloured values= noteworthy activity; Avg= average;1= 100% hexane; 2= 90% hexane: ethyl acetate; 3= 80% hexane: ethyl acetate; 4= 70% hexane: ethyl acetate; 5= 50% hexane: ethyl acetate; 6= 30% hexane: ethyl acetate; 7= 10% hexane: ethyl acetate; 8= 100% ethyl acetate; 9= 90% ethyl acetate: methanol; 10= 80% ethyl acetate: methanol; 11= 70% ethyl acetate: methanol; 12= 60% ethyl acetate: methanol; 13= 50% ethyl acetate: methanol; 14= 40% ethyl acetate: methanol; 15= 100% methanol.

8.3.2.5. Bioautography

Bioautography was used to evaluate the antibacterial activities of the *E. burkei* extracts. The crude extracts were separated in BEA, CEF, and EMW and sprayed with *E. faecalis, S. aureus* and *C. albicans*. The white zones against pink background on the chromatograms below indicate antibacterial activity (**Figure 8.7**). The Ethyl acetate: Methanol extracts had potent activity against all the tested bacteria on bioautograms developed in BEA and CEF.

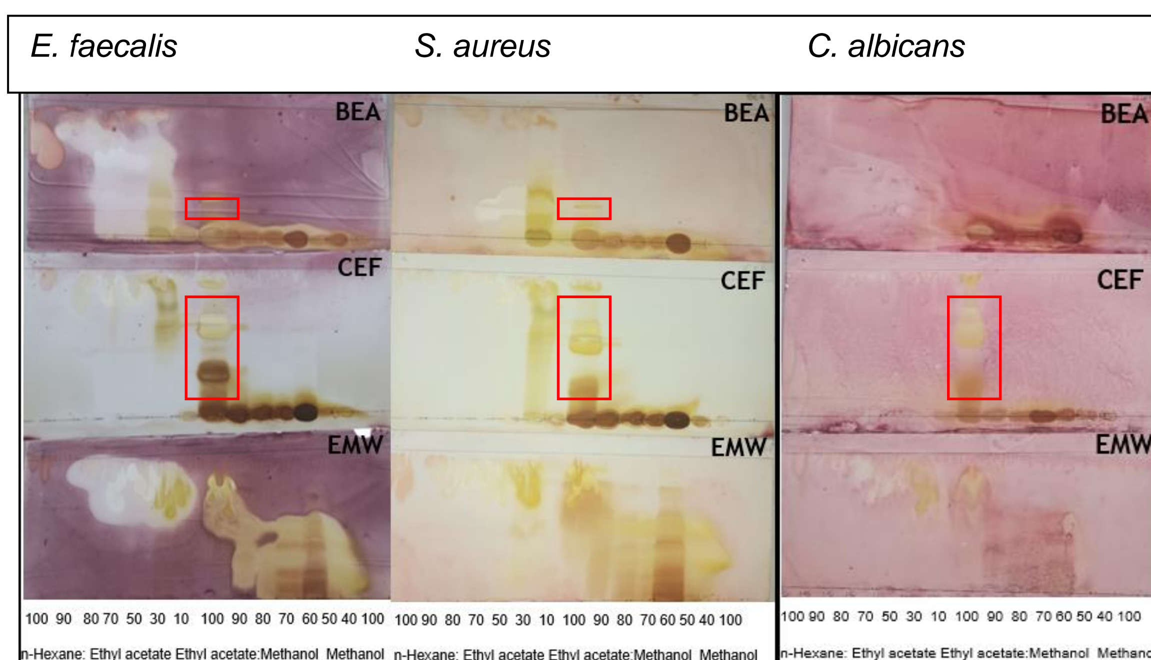

Figure 8.7: The chromatograms of *E. burkei* acetone fractions developed in BEA, CEF and EMW mobile systems sprayed with *E. faecalis, S. aureus* and *C. albicans* cultures and visualised under 2mg/mL INT.

8.3.3. Isolation of antibacterial compounds from 100% Ethyl acetate fraction

Following the column chromatographic separation of the acetone extracts; the 100% Ethyl acetate fractions exhibited overall potent antibacterial activity against all the tested microorganisms, as such, it was chosen for isolation of antibacterial compounds.

Table 8.6: The mass of combined sub-fraction 1 based on their biological activity.

	Mass (g)	**Total mass (g)**
Sub-fraction 1 (Ethyl acetate)	0.99	0.99

8.3.3.1. Phytochemical analysis of 1st column 100% Ethyl acetate fraction

The 100% Ethyl acetate fractions were collected with a mass of 0.99 g, which was redissolved with 100% Ethyl acetate and analysed on TLC mobile system for the phytochemicals using BEA (non-polar) mobile system. This involved separation and development of the plates in hexane: Ethyl acetate (100%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%) and 100% Ethyl acetate, respectively. The visualisation of the compounds was done under ultraviolet light and spraying with vanillin-sulphuric acid (**Figure 8.8**). Similar biological activities were observed on 100% ethyl acetate visualised as smears in **Figure 8.8**.

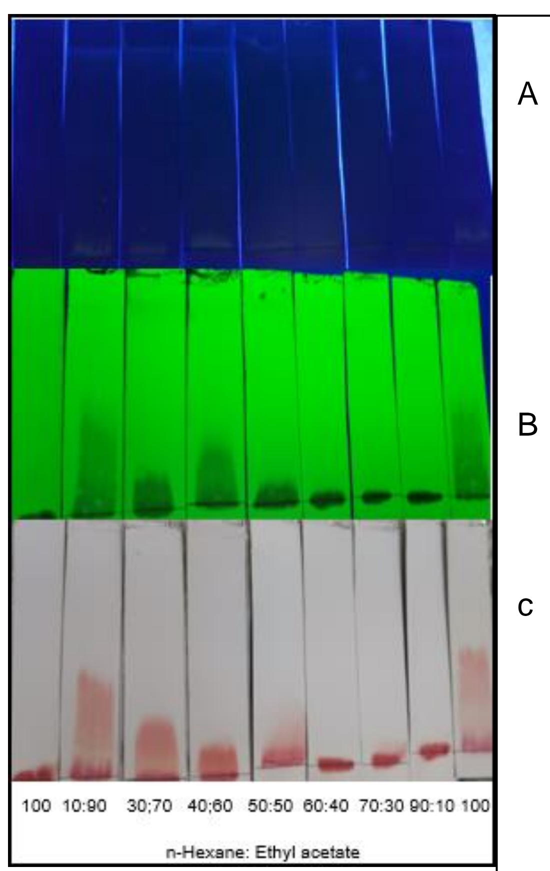

Figure 8. 8: The chromatograms of *E. burkei* acetone fractions developed in hexane: Ethyl acetate (100%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%) and 100% Ethyl acetate mobile systems and visualised under UV-light at 365nm (A), 254nm (B) and (C) was sprayed with vanillin-sulphuric acid reagent.

8.3.3.2. Bioautography assay

The qualitative antimicrobial assay was conducted for the fractions collected from first open column chromatography. The antimicrobial activity was observed on the BEA eluent systems in the fractions from 100% hexane in ethyl acetate up to 100% ethyl acetate.

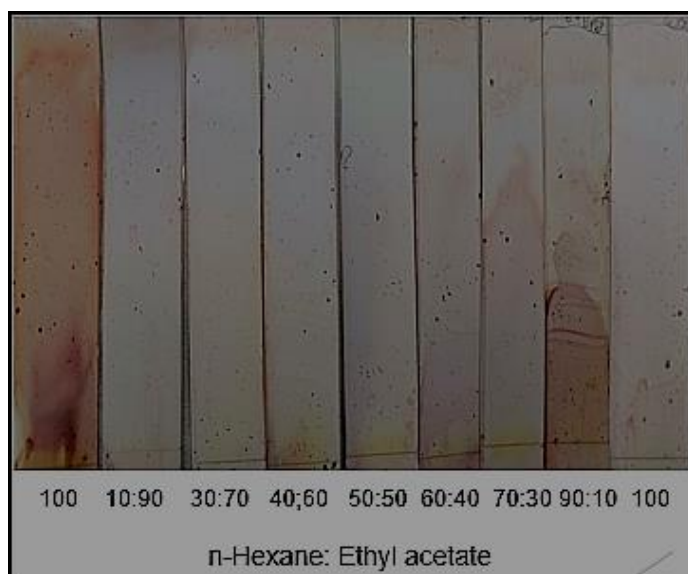

Figure 8 9: The chromatograms of *E. burkei* acetone fractions developed in hexane: Ethyl acetate (100%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%) and 100% mobile systems sprayed with *E. faecalis.*

8.3.3.3: Phytochemical analysis of 2nd column fraction

The second open column chromatography of Sub-fraction 2 (100% ethyl acetate) was carried out using BEA as the eluent system. A total of 256 fractions were collected in test tubes and spotted on TLC plates to determine their TLC profile. The TLC profile was visualised under a UV light at 365 nm (**Figure 8.10**) and 254 nm (**Figure 8.11**) and sprayed with vanillin-sulphuric acid (**Figure 8.12**). Sub-fractions were collected in test tubes and selected tubes were analysed on TLC plates to trace the target red compounds and fluorescence compounds showing biological activity.

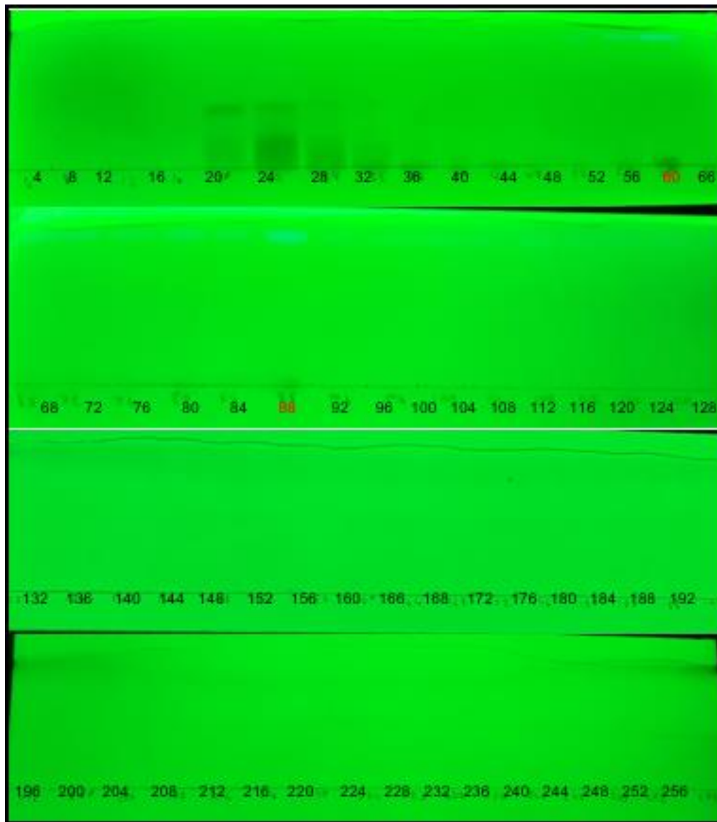

Figure 8.10: The chromatograms of *E. burkei* acetone fractions (100% Ethyl acetate) developed in BEA mobile systems and visualised under 254 nm UV light.

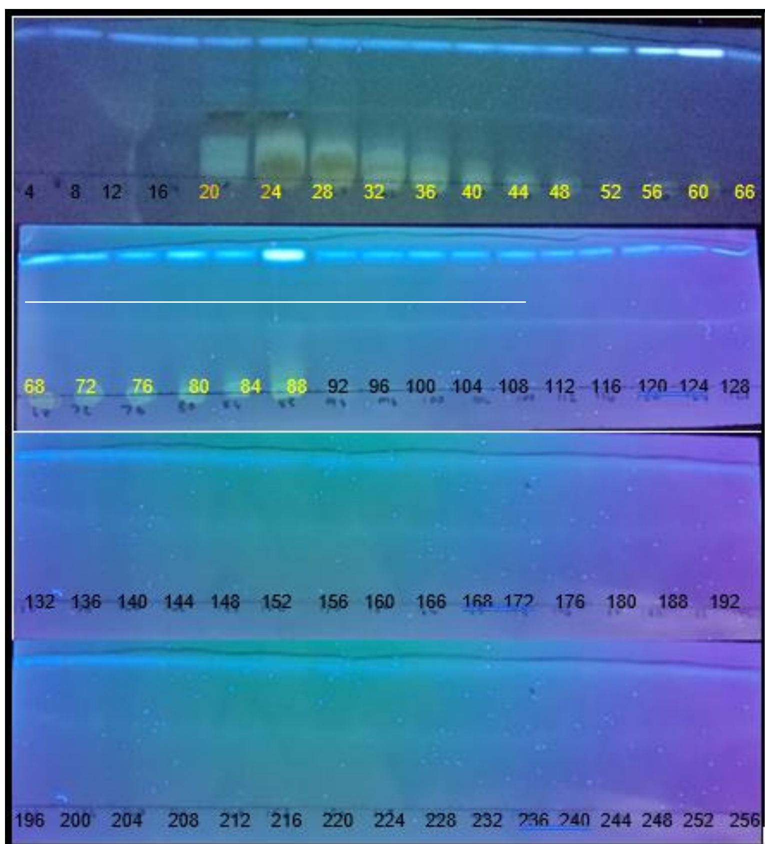

Figure 8.11: The chromatograms of *E. burkei* acetone fractions (100% Ethyl acetate) developed in BEA mobile systems and visualised under 365 nm UV light.

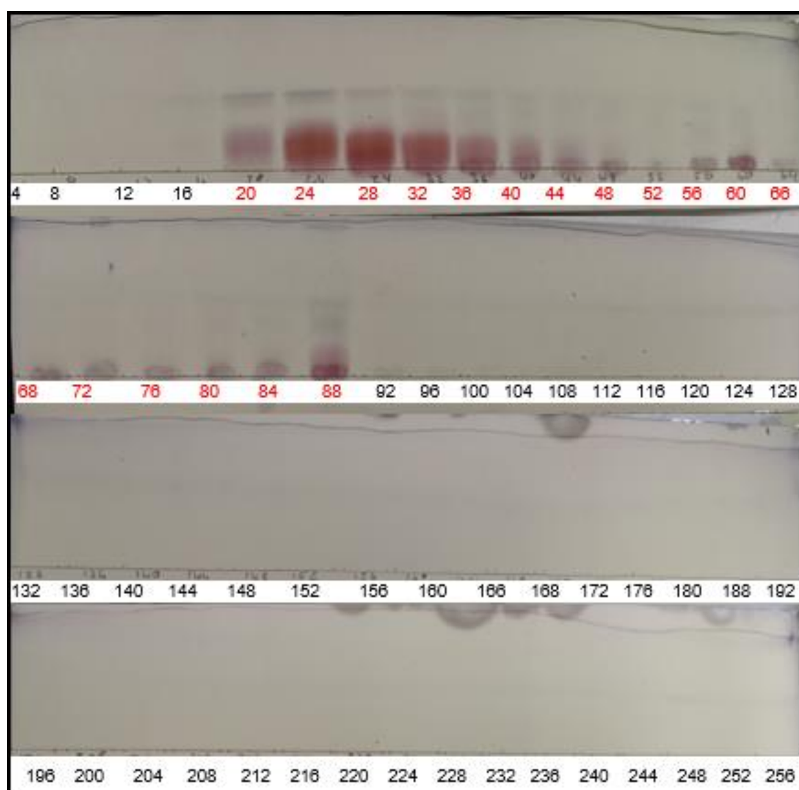

Figure 8.12: The chromatograms of *E. burkei* acetone fractions (100% Ethyl acetate) developed in BEA mobile systems sprayed with vanillin-sulphuric acid reagent.

8.3.4. Isolation of antibacterial compounds from grouped subfractions.

Following the column chromatographic separation of the acetone extracts (1st column), the 100% Ethyl acetate extracts (2nd extraction), the collected subfractions in 3rd extraction (test tubes 20-88 of 100% ethyl acetate), that is, subfraction 3, exhibited overall potent antibacterial activity against the *E. faecalis*. *E. faecalis* was the most sensitive test microorganism of the antibacterial compounds, as such, sub-fraction 3 was chosen for further biological activity analysis fraction.

Table 8.7: The mass of combined sub-fraction 2 based on their biological activity.

	Mass (g)	Total mass (g)
Sub-fraction 3 (test tube 20-88)	0.58	0.58

8.3.4.1: Phytochemical analysis of 2nd column grouped subfractions.

The Subfraction 3 obtained in **section 8.3.4** (subfraction 20-88 of 100% ethyl acetate) was the target fraction that was eluted with 100% ethyl acetate with a mass of 0,58 g. The sub-fractions were collected in test tubes and selected tubes were analysed on TLC plates using BEA mobile system to trace the target purple compounds plates using BEA mobile system to trace the target purple compounds and fluorescence compounds. The antimicrobial and antioxidant activities were observed on the same location on all TLC plates as indicated in red. This shows that the purified subfraction has biological activities.

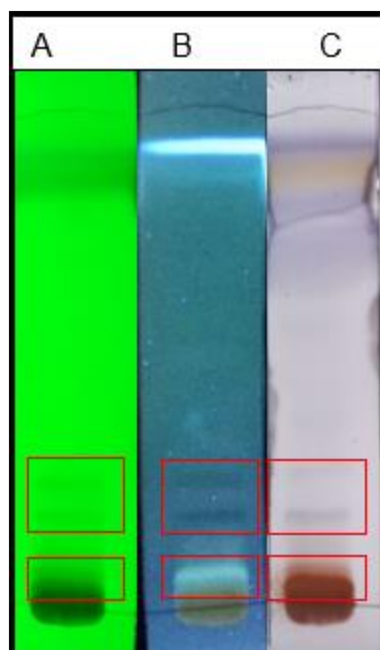

Figure 8.13: The phytochemical analysis of the grouped fractions visualised under a UV light at 254 nm (**A**) and 365 nm (**B**) and (**C**) was sprayed with vanillin-sulphuric acid reagent

8.3.4.2 Quantification of phytoconstituents

The total phenols, tannins, and flavonoids of *E. burkei* were measured, as described in **section 4.3.2.3**, and quantified from the standard curves. Each value was an average of 3 analyses ± standard deviation. The total tannins were the most abundant in the analysed subfraction of the plant, followed by the flavonoids whereas the least were the phenols (**Table 8.8**).

Table 8.8: The total phenol, tannins, and flavonoids content of differen plant parts.

Sample	**Total phenols (mg of GAE/g of sample)**	**Tannins (mg of GAE/g of sample**	**Flavonoids (mg of QE/g of sample)**
Subfraction 20-88	256.18 ± 0.16	1880.213 ± 0	327.90 ± 6.04

Key: GAE- Gallic acid equivalence, QE- Quercetin equivalence. Values are mean of triplicates ± standard deviation (SD) (n=3).

8.3.4.3. Antioxidant and antimicrobial activity of grouped subfraction

The grouped of subfractions were tested for qualitative (**Figure 8.14A)** and quantitative TLC DDPPH assay (**Figure 8.15**) and Ferric reducing assay (**Figure 8.16**) antioxidant activity. This was followed by qualitative bioautography assay (**Figure 8.14B**) and quantitative microdilution assay (**Table 8.9**) antibacterial activity against *E. faecalis*. The TLC plates were developed in BEA system and sprayed with *E. faecalis*. The results in **Figure 8.14B** below indicate that the fractions have activity against *E. faecalis,* as indicated by the yellow colour on DPPH chromatogram (**Figure 8.14A**) and white colour on the bioautography TLC chromatogram (**Figure 8.14B)**. The fraction showing the activities are indicated in red. They also have similar biological activities in both chromatographs.

Figure 8.14: The chromatograms *E. burkei* acetone fractions developed in mobile systems sprayed with 0.2% DPPH in methanol (**A**) for antioxidant activity and sprayed with *E. faecalis* (**B**) for antibacterial activity.

8.3.4.3.1 Quantitative antioxidant activity

The sub-fractions were observed to have similar TLC profile and biological activities. The purified sub-fraction 2 was quantified using DPPH assay, as described in section **5.3.2.1**, and FRAP assay, as described in **section 5.3.2.2.**

8.3.4.3.2 Quantitative DPPH antioxidant assay

The radical scavenging activity of the plant extracts was quantified from a standard curve, as described in **section 5.3.2.1**. The sub-fraction 2 that was analysed was found to have high percentage inhibition, as seen in **Figure 8.15** at about 75 µg/mL-250 µg/mL. It had the highest radical scavenging potential by reaching the maximum threshold of percentage inhibition. The positive control had the lower activity when compared to the sub-fraction.

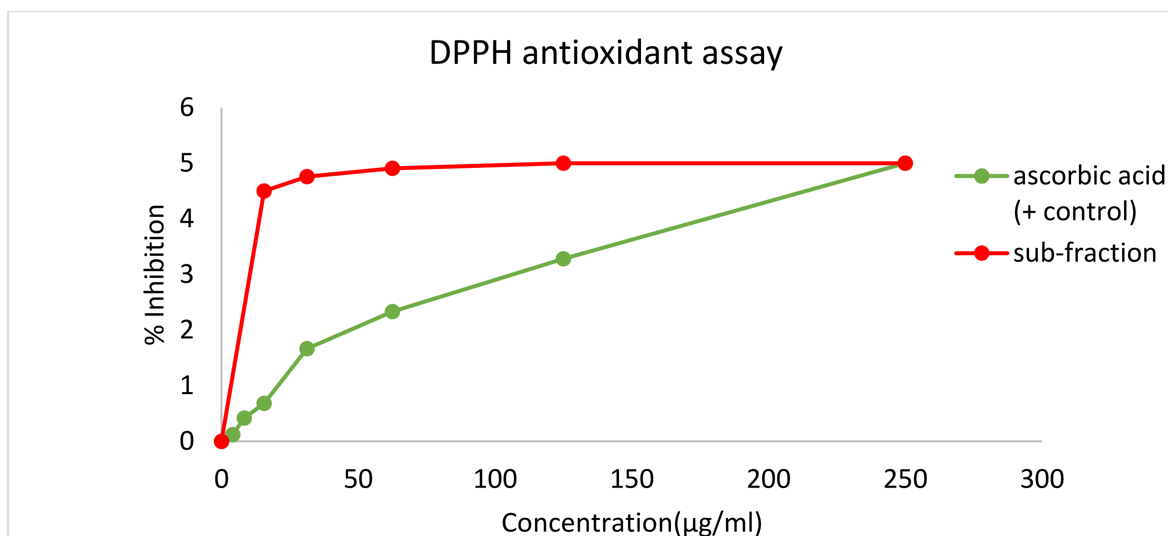

Figure 8.15: The percentage free radical (DPPH) inhibition of sub-fraction was expressed as percentage inhibition at varying concentrations. Ascorbic acid was used as standard to which samples were compared.

Key: Positive control (+ve control) Values are mean of triplicates ± standard deviation SD) (n=3).

8.3.4.3.3 Quantitative FRAP assay

The reducing power of sub-fraction 2 was determined using ferric reducing antioxidant potential (FRAP) assay, as described in **section 5.3.2.2**. The sub-fraction material was found to have high percentage inhibition as seen in **Figure 8.16** and at 250 µg/mL, 50 % percentage inhibition was reached whereas at low concentration below 60 µg/mL, there was no inhibition.

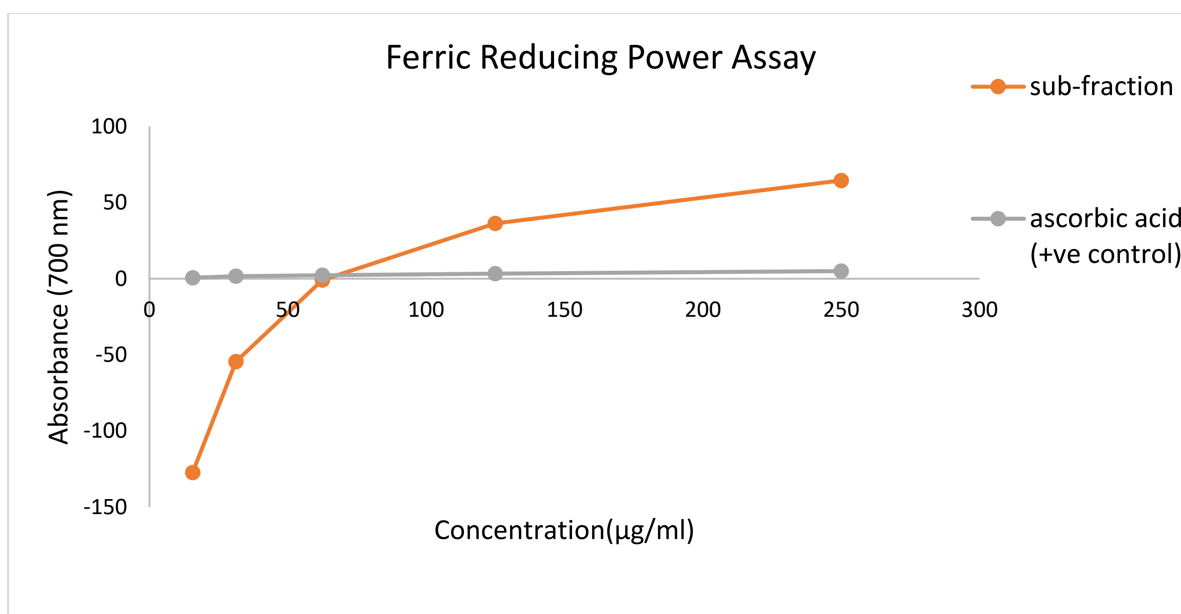

Figure 8.16: Ferric reducing power of *E. burkei* at varying concentrations expressed as absorbance 700 nm. Ascorbic acid was used as standard to which samples were compared.

Key: Positive control (+ve control). Values are mean of triplicates ± standard deviation (SD) (n=3).

8.3.3.3.4 Quantitative antimicrobial activity

The sub-fractions were observed to have similar TLC profile and biological activities. The purified sub-fraction was quantified using broth micro-dilution assay (**Table 8.9**) using *E. faecalis* test organism. The positive control (Gentamicin) was found to be 125 (µg/mL)

Table 8.9: The MIC values (ug/mL) of the grouped sub-fraction 2 against *E. faecalis.*

Samples	MIC values (µg/mL)
Sub-fraction	7.8
Positive control	125

The flow diagram below (**Figure 8. 17**) represents the summary of the whole process of isolation of the antibacterial fraction from *E. burkei* roots. The diagram shows

collected fractions, masses and the pure compound obtained. The pure fraction was, however, not further characterised because the material collected was very minimal in mass and the biological activity would have been lost during the isolation process. From large scale extraction, over 90% of plant material was lost and in 1st column chromatography, a further 75% of plant material was lost. In the 2nd column, 75% of plant material was lost and lastly, in the 3rd column, a further 50% of plant material was lost.

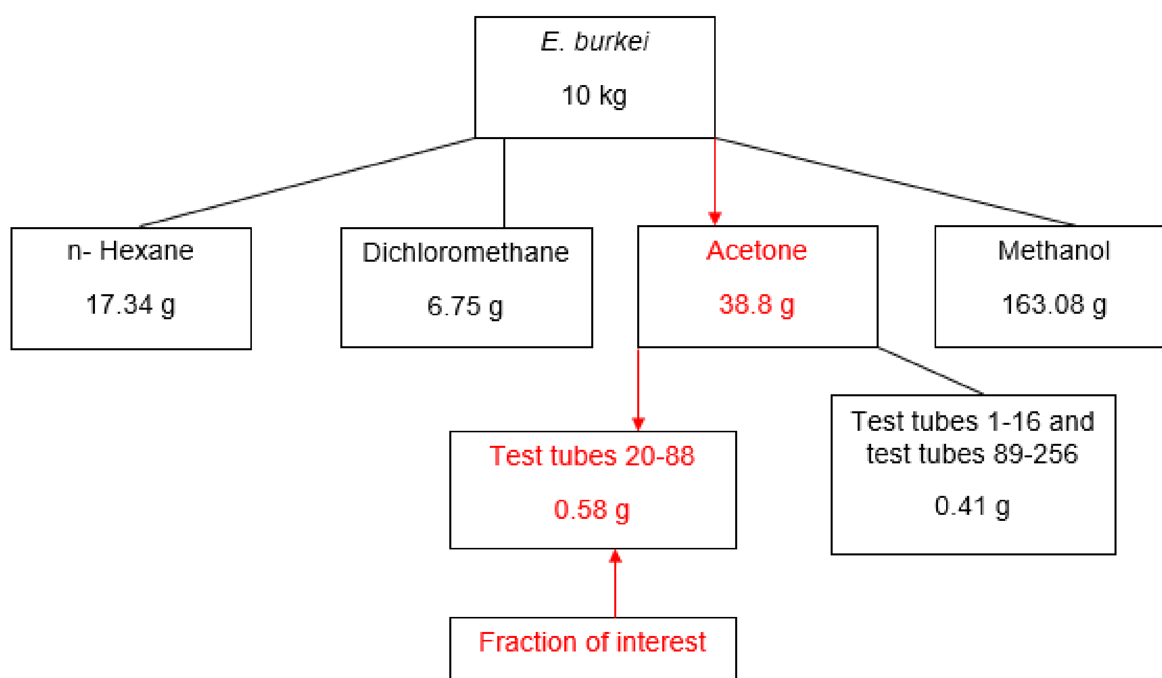

Figure 8.15: Flow diagram of the isolation process of the active fraction.

8.4. Discussion

Medicinal plants have different active compounds based on their soil diversity, geographical location, and climate conditions (Rekha *et al.*, 2013; Thite *et al.*, 2013). In addition, water content in the fresh plant material is likely to affect the solubility and separation during liquid-liquid extraction (Abdullahi and Mainul, 2021); hence, dried

plant material is always preferred. Preparation of medicinal plants for experimental purposes is an initial step and a key factor in achieving quality research outcome. It involves extraction and determination of quality and quantity of bioactive constituents before proceeding with the intended biological testing (Abdullahi and Mainul, 2021). This is always the target for the fraction of interest against the unwanted material.

The isolation of phytochemical compounds allows in-depth investigations of the bioactivity by identifying the responsible compounds and possible mechanisms of action. In traditional healthcare systems, medicinal plants are usually used in combination to heighten the biological activity of the extracts. However, compounds within a plant may have either antagonistic effects or synergistic effects when applied together (Dzoyem *et al.*, 2016). Therefore, isolated compounds may or may not show the biological activity that was exhibited by the extracts. In this study, the isolation process was guided by the antimicrobial activity of the extract, and it began with serial exhaustive extraction to allow maximum extraction of the compounds within the plant material. To extract the bioactive compounds from the roots of *E. burkei*, a large-scale extraction using a serial exhaustive method was carried out with solvents of varying polarity (hexane, dichloromethane, acetone, and methanol).

A total mass of 255.49 g was extracted from 1 kg of *E. burkei* roots (**Table 8.3**). Methanol extracted highest mass of 163.08 g, followed by hexane (17.34 g) and acetone (38.82 g) while dichloromethane extracted the least (6.75 g). Overall, only 26% of plant material was retained, the rest was considered to not have any biological activity of interest. The results indicated that *E. burkei* roots leaves contain non-polar to polar compounds since most of the plant materials were extracted from hexane and methanol. The extracts from serial exhaustive extraction were spotted on the TLC plates and developed in BEA, CEF and EMW mobile phases for phytochemical analysis (**Figure 8.1**). Most compounds were observed in the BEA mobile phase than the CEF and EMW. This suggested that *E. burkei* roots mostly contain non-polar compounds. Similar results were obtained in the preliminary screening (Chapter 4). Furthermore, similar phytochemicals were observed in acetone extracts on the

chromatogram sprayed with vanillin-sulphuric acid reagent in the BEA mobile phase visualised as red bands.

Qualitative antioxidant activity was observed in acetone extracts in EMW and CEF mobile systems. Bioautographic assay was used to evaluate the antibacterial activities using the same test organisms as in **section 5**. *E. faecalis* was the most sensitive and clear zones were visualised on the TLC plates with acetone extracts. Therefore, the three acetone extracts from SEE were collected to a further mass of 33.14 g (Subfraction 1) and subjected to first column chromatography. Phytochemical analysis of first column chromatography of Subfraction 1 was performed. Biological activity was observed on the TLC plates using 365 nm UV light and at 100%, Ethyl acetate had a distinct fluorescence in all mobile systems. The pattern was observed on TLC plates visualised with vanillin reaction, but the most resolved singular bands of compounds were seen on CEF mobile system. Qualitative antioxidant activity was visualised by yellow bands in all mobile systems at 100% Ethyl acetate, which was seen as a yellow smear against the purple TLC background. The 100% Ethyl acetate once more showed exceptional antimicrobial activity on MIC with a value of 0.02 mg/mL for *C. albicans*, 0.32 mg/mL for *E. faecalis* and 0.63 mg/mL for *S. aureus*. During bioautographic TLC assay, 100% Ethyl acetate showed distinct antimicrobial activity on all test organisms. However, *E. faecalis* was found to be the most sensitive; hence, as the study continued, it was used as the most prominent microorganism to test for microbial susceptibility.

Column chromatography was used to further fractionate the fraction to remove other non-active components. Ethyl acetate eluent (Subfraction 2) with a mass of 0.99 g was subjected to second column chromatography. Phytochemical analysis was employed and test tubes 20-88 showed biological activities and were thus grouped together (Subfraction 3). The eluent mass of the active compounds was 0.58 g and was collected and tested for all biological activities as indicated in **sections 4-6**. The

fraction of interest showed even higher polyphenol activity as compared to *E. burkei* crude extracts.

The use of plants and their extract for the preparation of herbal drugs provides the foundation to modern therapeutic sciences and thus enabled humankind to establish the empirical system of medicine. Hence, several plant species are being screened for these herbal compounds and are also isolated from the plants (Jain *et al.,* 2019). The isolation of pure compound was not successful, but the fraction collected showed acceptable and promising biological results of antimicrobial and antioxidant activities. However, the mass of material collected was a problem, therefore, better and more efficient methods should be employed.

Isolation of compounds from medicinal plants provides single active entities that may further be developed into new drugs. However, due to synergism between compounds within the extracts, the activity may be lost. Antagonistic effects also play a role in the bioactivity of new compounds, which also influence the isolation of compounds and bring new knowledge about the medicinal plants in use (Newton *et al*., 2002).

8.5. Conclusion

The isolation of the *E. burkei* acetone root extract was unsuccessful. However, a purified fraction was collected instead of isolation of pure compound. The purified fraction showed distinct antioxidant on DPPH TLC assay and antimicrobial properties against *E. faecalis*. This suggests that the purified fraction can be validated as anti-infective agent against disease causing pathogens.

References

Abdullahi R. A. and Mainul, H. 2021. Review article: Preparation of medicinal plants: basic extraction and fractionation procedures for experimental purposes. *Journal of Pharmacy and Bioallied Sciences*,*12*(1), pp.1-10.

Abubakar, A.R. and Haque, M. 2020. Preparation of medicinal plants: Basic extraction and fractionation procedures for experimental purposes. *Journal of Pharmacy and Bioallied Sciences*, *12*(1), pp.1.

Begue, W.J. and Kline, R.M. 1972. The use of tetrazolium salts in bioautographic procedure. *Journal of Chromatography*, *88*, pp.182-184.

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

Bucar, F. Wube, A. and Schmid, M., 2013. Natural product isolation–how to get from biological material to pure compounds. *Natural Product Reports*, *30*(4), pp.525-545.

Dzoyem, J.P. Aro, A.O. McGaw, L.J. and Eloff, J.N. 2016, Antimycobacterial activity against different pathogens and selectivity index of fourteen medicinal plants used in southern Africa to treat tuberculosis and respiratory ailments. *South African Journal of Botany*, *102*, pp.70-74.

Eloff, J.N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica, 64*(8), pp.711-713.

Jain, C. Khatana, S. and Vijayvergia, R. 2019. Bioactivity of secondary metabolites of various plants: a review. *International Journal of Pharmaceutical Sciences and Research*, *10*(2), pp.494-498.

Jamil, M. ul Haq, I. Mirza, B. and Qayyum, M., 2012. Isolation of antibacterial compounds from *Quercus dilatata* L. through bioassay guided fractionation. *Annals of Clinical Microbiology and Antimicrobials*, *11*(1), pp.1-11.

Khan, M. Ullah, N. Azhar, M. Komal, W. and Muhammad, W. 2019. A mini-review on the therapeutic potential of *Zingiber officinale* (ginger). *Natural Products: An Indian Journal*, *15*(1), pp.125.

Kokwaro, J.O. 1976. Medicinal plants of East Africa. East African Literature Bureau, University of Nairobi Press, pp.384.

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

Majekodunmi, S.O. 2015. Review of extraction of medicinal plants for pharmaceutical research. *Merit Research Journal of Medicine and Medical Sciences*, 3, pp.521-527.

Matotoka, M.M. and Masoko, P. 2018. Phytochemical screening and pharmacological evaluation of herbal concoctions sold at Ga-Maja, Limpopo Province. *South African Journal of Botany*, *117*, pp.1-10.

Newton, S.M. Lau, C. Gurcha, S.S. Besra, G.S. and Wright, C.W. 2002. The evaluation of forty-three plant species for *in vitro* antimycobacterial activities; isolation of active constituents from *Psoralea corylifolia* and *Sanguinaria canadensis*. *Journal of Ethnopharmacology, 79*(1), pp.57-67.

Rekha, D. Tamil-Selvi, S. Bharathidasan, R. Panneerselvam, A. Ilakkiya, R. and Jayapal, R. 2013. Study of medicinal plants used from koothanoallur and marakkadai, Thiruvarur district of Tamil nadu, India. *Hygeia Journal for Drugs and Medicines*, 5(1), pp.164-170.

Sasidharan, S. Chen, Y. Saravanan, D. Sundram, K.M. and Latha, L.Y. 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines, 8*(1). pp.1-10.

Singh, R., 2015. Medicinal plants: A review. *Journal of Plant Sciences*, *3*(1), pp.50-55.

Sofowora, A., 1982. Medicinal plants and traditional medicine in Africa, John Wiley and Sons Limited, Chichester, England, pp.142-145.

Thite, S. V. Chavan, Y. R. Aparadh, V. T. and Kore, B. A. 2013. Preliminary phytochemical screening of some medicinal plants. *International Journal of Pharmaceutical Chemical and Biological Sciences, 3*(1), pp.87-90.

Tshisikhawe M. P. 2021, *BOT 2649 Ethnobotany I study guide,* University of Venda, Thohoyandou, South Africa.

Chapter 9: General discussion, conclusion and recommendation

9.1. General discussion

Medicinal treatment refers to the direct and empirical application of medicine to treat a particular disease with clearly observable or detectable symptoms. This is when the success of the treatment is being attributed totally to the effects of the medicine used. In the far North of South Africa, the Venḓa region has remained mostly rural and has a resilient tradition of medicinal plant usage (Samie *et al.,* 2005). Many of these traditional African remedies have been shown to have a solid scientific basis and this can be seen from the number of drugs in use today from plant origins (Newman and Cragg, 2007).

Phytochemicals are used by plants as a defense mechanism, but they are also helpful to human beings. When consumed, different compounds are responsible for different activities within the plants whereas some operate synergistically for certain activities (Barbehenn and Constabe, 2011). Low protein content of medicinal plants was found in this study, and it is not favourable in relation to nutritive value. It is known that flavonoid and phenolic components, vitamins (A, E, C) in the content of plants have antioxidant activities and health benefits (Faydaoğlu and Sürücüoğlu, 2014; Koçak *et al*., 2020). It is also known that natural sources of these polyphenol compounds exhibited stronger antioxidant activity than synthetic ones (Özyazici, 2021). These polyphenols were found in abundance when quantified, which shows promising biological effects that help eradicate or alleviate diseases caused by pathogens.

The medicinal plants in this study showed promising antimicrobial activities, some even better than the antibiotics currently being used in Western medicine. All plant extracts and herbal treatment were confirmed as an exceptional antifungal due to the antimicrobial activity against *C. albicans* compound. This validated the extent to which plant species are being used in the treatment of STIs. However, there is still a need to explore the biological activity of various extracts from the species against microorganisms such as *Neisseria gonorrhoeae, Klebsiella granulomatis,*

Mycoplasma hominis and *Mycoplasma genitalium*. These microorganisms are the most common causative agents of gonorrhoea, bacterial vaginitis, donovanosis and other urogenital infections (Mongalo *et al*., 2015).

During SEE, the activity of *E. bukei* biological activity got lost. However, the fraction of interest that was purified showed remarkable biological activities, including high quantitative antioxidant activity and quantitative antimicrobial activities. The antimicrobial fraction was purified, and it had distinct antimicrobial activity as compared to crude extracts of *E. burkei*. The *in vitro* bioassays of this study indicated that *Securidaca longepedunculata* (Polygalaeae), *Wrightia natalensis* (Apocynaceae)) are also promising “future candidates” for the treatment of erectile dysfunction (Rakuambo *et al*., 2006), which serves as a springboard for further studies, including isolation of bioactive compounds in these respective plants.

9.2. Conclusion

The importance of scientific investigation into indigenous herbal medicines has been emphasised by the World Health Organization (WHO). Ethnopharmacology is not just a science of the past utilisation of an outmoded approach. It still constitutes a scientific backbone in the development of active therapeutics based upon the traditional medicine of various ethnic groups (Tshisikhawe, 2021). *E. burkei* fraction, which was isolated, has remarkable biological activities for treatment against pathogens related to male sexual health. This plant should be explored more, with better handling during assays. Pharmacological evaluation of herbal mixtures also serves to elucidate their biological activities and confirms their claimed efficacies towards ailments indicated (Matotoka, 2019). This was proven in this study.

9.3. Recommendation

Mineral analysis on the medicinal plants and herbal treatment to determine the concentration of essential minerals, namely; macro-elements, and micro-elements

(trace elements), which are important for cell functions at biological, chemical and molecular levels. There is also a need for mineral analysis of heavy metals to validate the safety of the medicinal plants and herbal treatment. Better extraction procedures that are more sensitive are recommended so that the biological activity is maintained and enhanced. The antimicrobial properties of extracts and purified compounds against microorganisms causing STIs is also deserving of further research (Mongalo *et al*., 2015). Clinical studies on human subjects to evaluate the potential of the pure compounds and side effects, contra-indications, effective dosage, supplementary and complementary additives, etc., is a dire need. There is also a need to investigate the cytotoxicity of these extracts against normal human cell lines. Any or all these criteria constitute ethnopharmacological research, whether conducted by individual or collaborating scientists. Thus, it is recommended that cutting edge research be conducted on the afore-mentioned aspects.

References

Barbehenn, R.V. and Constabel, C.P. 2011. Tannins in plant–herbivore interactions, *Phytochemistry*, *72*(13), pp.1551-1565.

Koçak, Y. Oto, G. Meydan, İ. and Şeçkin, H. 2020. Investigation of total flavonoid, DPPH radical scavenging, lipid peroxidation and antimicrobial activity of *Allium schoenoprasum L.* plant growing in Van Region. *YYU Journal of Agricultural Science, 30*(1), pp.147-155.

Matotoka, M.M. 2019. *In vitro* pharmacological and synergistic effects of herbal concoctions sold in Ga-Maja, Limpopo Province. MSc dissertation, University of Limpopo, Sovenga, South Africa.

Mongalo, N.I. McGaw, L.J. Finnie, J.F. and Van Staden, J. 2015. *Securidaca longipedunculata* fresen (Polygalaceae): A review of its ethnomedicinal uses, phytochemistry, pharmacological properties and toxicology. *Journal of Ethnopharmacology*, *165*, pp.215-226.

Newman, D.J. and Cragg, G.M. 2007. Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products*, *70*(3), pp.461-477.

Özyazici, A.P.D.G., 2021, New development on medicinal and aromatic plants. Iksad Publications, Turkey, pp.119.

Rakuambo, N.C. Meyer, J.J.M. Hussein, A. Huyser, C. Mdlalose, S.P. and Raidani, T.G. 2006, *In vitro* effect of medicinal plants used to treat erectile dysfunction on smooth muscle relaxation and human sperm. *Journal of Ethnopharmacology*, *105*(1-2), pp.84-88.

Samie, A., Obi, C.L., Bessong, P.O. and Namrita, L. 2005. Activity profiles of fourteen selected medicinal plants from rural Venda communities in South Africa against fifteen clinical bacterial species. *African Journal of Biotechnology*, *4*(12), pp. pp.1443-1451.

Tshisikhawe M.P. 2021, *BOT 2649 Ethnobotany I study guide,* University of Venda, Thohoyandou, South Africa.