ANTIMICROBIAL, CYTOTOXIC AND PRELIMENARY PHYTOCHEMICAL ANALYSIS OF FOUR MEDICINAL PLANTS AND THEIR FORMULATION

A DISSERTATION SUBMITTED IN FULLFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY.

HLAYISANI FREDAH MBOWENI

(11531048)

Submitted to the Department of Microbiology

School of Mathematical and Natural Sciences

UNIVERSITY OF VENDA

PRIVATE BAG X5050

THOHOYANDOU

0950

SUPERVISOR : PROF SAMIE A

CO-SUPERVISOR: DR TSHIKALANGE T.E

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DECLARATION

I Hlayisani Fredah Mboweni declare that this dissertation is the result of my own research, that it does not incorporate without acknowledgement any material. It has not been submitted for a degree or diploma at any university and does not contain any materials previously published, written or produced by another person except where due references are made in the text.

Signed (Student)…….Date……

Signed (Supervisor)……...Date…….

Signed (Co-Supervisor)…….Date…….

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DEDICATION

This research work is dedicated to my Lord Jesus Christ. My siblings, my sisters Emeldah and Eseldah, my brothers Donald, Gerald, Decide and Leonard as well as my loving husband Hlamalani, may this work be an encouragement to you all.

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LIST OF ABBREVIATIONS

µl	Microliter
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
COX	Cyclooxygenase enzyme
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1, 1-diphenyl-2-picrylhydrazyl
ECGS	Endothelial cell growth supplement
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal bovine serum
g	Grams
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
HLEC	Human Lymphatic Endothelial cells
IC_{50}	50% Inhibitory Concentration

IL	Interleukin
MBC	Minimum Bactericidal Concentration
MCH	Multicomponent herbal concoction
MFC	Minimum Fungicidal concentration
mgGAE/g	Milligram gallic acid equivalent per gram
mgQE/g	Milligram equivalent of quercetin per gram
MIC	Minimum Inhibitory Concentration
mol/l	Mole per litre
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
Na_2CO_3	Sodium chloride
NSAID	Non-steroidal anti-inflammatory drugs
P/S	Penicillin/ streptomycin solution
RNA	Ribonucleic acid
RT-qPCR	Quantitative reverse real-time polymerase chain reaction
SA	South Africa
TNF-α	Tumor necrosis factor alpha
v/v	Volume per Volume
w/v	Weight per Volume
WHO	World health organization

PUBLICATIONS EMANATING FROM THE RESEARCH

The following manuscripts have been developed from the study and will be submitted to the appropriate journals shortly.

Mboweni F, Tshikalange T.E. Motaung S and Samie A. Evaluation of the antioxidant, cytotoxicity and anti – HIV activities of four commonly used Venda medicinal plants and their formulations. To be submitted to BMC complementary medicine.

Mboweni F and Samie A. Antimicrobial activities and preliminary phytochemical analysis of selected Venda medicinal plants and their combinations. To be submitted to the African Journal of Traditional Complementary and alternative medicine.

ABSTRACT

BACKGROUND: Medicinal plants form an important part of the Southern African cultural heritage. Indigenous populations, for example the Vha-Venda people, tend to use medicinal plants in formulations rather than western medicines for health and survival. In order to certify and give scientific credibility to the use of medicinal plants formulations used by Vha-Venda people for the treatment of diseases, several assays were carried out. The present study was aimed at assessing phytochemical content, antimicrobial, antioxidant and cytotoxic activities of four indigenous Venda medicinal plants in a formulation and compare their activity with each plant used individually.

METHODS: *Peltophorum africanum* (roots), *Pterocarpus angolensis* (bark), *Terminalia sericea* (roots) and *Ximenia caffra* (roots) were collected from the Thohoyandou area. The collected plant parts were extracted with methanol and water respectively. Individual plant extracts and Five designed formulations were tested for their antimicrobial activity against *Staphylococcus aureus* ATCC 25923 (Methicillin Resistant), *Staphylococcus aureus* ATCC 33591(Methicillin Susceptible), beta lactamase producing *Klebsiella pneumonia* (ATCC 700603) and extended spectrum beta lactamase producing *E. coli* (ATCC 35218), four clinical isolates of *Candida spp* and *Cryptococcus neoformans* using the Broth dilution method. Minimum bactericidal concentration (MBC) of the extracts was determined by culturing the contents of minimum inhibitory concentration (MIC) on nutrient agar. Similarly, minimum fungicidal concentration (MFC) was also determined by culturing contents of MIC in sabouraud dextrose agar (SDA). Extracts were further assessed for their total phenolic content, total flavonoid content and Qualitative phytochemical analysis. The antioxidant ability of the plants extracts and formulations to scavenge free radical DPPH was also determined. The plant formulations were assessed for their anti-HIV activity using the reverse transcriptase

colorimetric assay kit. Cytotoxicity against human lymphatic endothelial cells (HLEC) was determined using MTT assay.

RESULTS: Methanolic and aqueous extracts of *T. sericea* exhibited the best antifungal and antibacterial activities whilst *P. angolensis* and *X. caffra* showed poor activities. Methanolic plant formulations showed good activities compared to aqueous formulations. However, Fractional Inhibition Concentration Index showed that there was 1 synergistic interaction, 25 additive interactions and 14 antagonistic interactions between the plant extracts. The methanolic formulation 3 showed the best overall phenolic content at 11.85±0.109 mgGAE/g whilst aqueous *X. caffra* extract showed the least content at 4.546±0.104 mgGAE/g. Higher total flavonoid contents were seen in methanolic formulation 4 at 2.75±0.02 mgQE/g. Qualitative phytochemical analysis revealed the presence of flavonoids, phenolics, terpenoids, tannins, saponins and steroids in 80% of the tested plant extracts and formulations. All plant extracts and formulations exhibited good antioxidant activity against DPPH. The methanolic formulation showed the best antioxidant activity with IC_{50} of 0.094 ± 0.33µg/ml. For anti-HIV inhibition, all formulations at 200µg/ml exhibited higher percentage of HIV-1 reverse transcriptase inhibition with methanolic mixture 3 being the best overall at 97.5% activity whilst aqueous mixture5 was the least active with 63.03% inhibition activity. Moreover, the best anti-HIV activity at 100µg/ml was exhibited by methanolic mixture 3 at 71% inhibition. Furthermore, aqueous *X. caffra*, mixture 2 inhibited 26% and 51% at 12.5mg/ml and 3.125mg/ml respectively. *Peltophorum africanum* and mixture 5 inhibited 34%, 54% and 43% at 3.125mg/ml, 6.25mg/ml and 12.5 mg/ml respectively of Human Lymphatic Endothelial cells growth.

CONCLUSIONS: The results from the study indicated that most of the commonly used traditional medicinal Plants in the Venda region when mixed together have merit for use in traditional medical practice as they have shown good antimicrobial activities, good antioxidant

activities, good phytochemical activities and good cell proliferation activity. However some formulations showed antagonistic interaction against bacteria. Some Individual medicinal plants showed toxicity at higher concentrations against immune cells. Whereas formulations promoted cell proliferation, therefore, the use of such individual plants in the treatment of infections should be highly monitored as they may pose a health threat to normal immune cells. Generally, plants are potential pharmacological agents which needs to be preserved and harvested with care.

Keywords: *medicinal plants*, *formulations, phytochemicals, antioxidants, antimicrobial, anti-HIV, cytotoxicity.*

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Plants have always provided mankind with necessities such as food, clothing, perfumes and flavours etc. Other than these, plants have also provided people with treatments for different diseases. In fact, plant derived medicines have been part of the evolution of human healthcare for thousands of years. According to the World Health Organization (WHO), about three-quarter of the earth's population depend on plants to treat many diseases. At present, an extensive number of drugs which are active against several sicknesses are established from plants (Alam *et al*., 2011). In developed countries 25 percent of the medical treatments are based on plants and their derivatives and the use of medicinal plants is well known among indigenous people in rural areas of many emerging countries (Alam *et al*., 2011). In India, 9 500 herbal plants and 8000 higher plants have been used in traditional medicine (Sowjanya *et al.,* 2013). South Africa is also a home to over 30 000 species of higher plants and of these plant species, 3000 have been used throughout the country for medicinal purposes (Van Wyk *et al.,* 1997).

Plants used for traditional purpose have been found to have little side effects since ancient time. Furthermore, plant based drugs have been used for various ailments ranging from common colds to cancer (Sowjanya *et al.,* 2013). These plants are ingested as concoctions, decoctions, teas and juice preparations (Clarkson *et al.,* 2004; Njume *et al.*, 2009). Despite the therapeutic advantages, some plants are potentially toxic, carcinogenic and teratogenic, thus, it is

imperative to screen medicinal plants for their mutagenic and toxic effects on cells (Mulaudzi *et al.,* 2013).

The screening of medicinal plants used by different ethnic groups or communities has now become a widespread renaissance and a potential source for isolation of bioactive compounds (Sowjanya *et al.,* 2013). Plants naturally produce bioactive compounds (phytochemicals) as defence mechanism against bacteria, fungi and viruses, making them rich sources of different types of drugs (Yadav and Agarwala, 2011). These bioactive compounds can be derived from the roots, bark, leaves, flowers, seeds and fruits of the plant, and the knowledge of the chemical constituents of plants is desirable because it's important for the synthesis of complex chemical substances, as is the case with clinical drugs (Yadav and Agarwala, 2011).

Most of the clinical drugs that are used now are derived from plant constituents and are developed because of their use in traditional medicine (Salazar - Aranda *et al*, 2013). For decades, many plants that are of medicinal importance have been investigated by various researchers worldwide (Harborne, 1973; Xuan *et al*., 2005; Mulu *et al*., 2005; Samie *et al*., 2009a, Samie *et al.,* 2009b; Mulaudzi *et al*., 2013; Narr *et al*., 2013). These investigations have led to the isolation of drugs such as aspirin, cocaine, atropine, emetine, digitoxin, morphine and pilocarpine from medicinal plants (Fabricant and Farnsworth, 2001; Gilani and Attar-ur, 2005).

Moreover, some traditional healers and indigenous communities around the world have opted to multicomponent herbal (MCH) concoctions / plant formulations for the treatment of many conditions like functional dyspepsia, irritable bowel syndrome, acquired immunodeficiency syndrome (AIDS), cancer, pulmonary tuberculosis, diabetes and stroke (Rösha *et al*., 2006, Herman and von Ritchter, 2012). A plant formulation consisting of several species is

considered stronger and should be administered by someone with more experience such as traditional healers (Dahlberg and Trygger, 2009).

In plant formulations, extracts from either a combination of different plant parts of the same tree or a combination of different parts of different trees are used. A formulation that is beneficial in the treatment of diseases should contain the following types of herbs; the imperial herb which is the main component of the formulation, the ministerial herb which enhances and promotes the imperial herb action, the assistant herb which reduces the side effects that are caused by the imperial herb and lastly the servant herb harmonizing the action of the other herbs (Che *et al,* 2013).

Plant formulation remedies have also been documented to possess antifungal, antibacterial, antipyretic, analgesic and anti-inflammatory activity are used to broaden the spectrum of activity thereby increasing the medical effect of the remedies (Kwon *et al*., 2008). Currently, Multicomponent herbal concoction (MHC) has been used effectively in the treatment of many conditions like functional dyspepsia, irritable bowel syndrome, acquired immunodeficiency syndrome (AIDS), cancer and pulmonary tuberculosis (Rösha *et al*., 2006, Che *et al*., 2013). It is believed that the different phytochemicals present in many herbs interact enhancing the therapeutic effects of the herb and dilute its toxicity. However, the content of MHC therapy may only be beneficial when the individual plant or plant parts extracts in the concoction possesses different efficacies that will provide additive or synergistic effect (Che *et al*.,2013). This may also assist in the reduction of the dosage an individual must take as compared to individual plants (Oseni *et al*., 2012). Some examples of plant formulations and their uses are listed in (Table 1.1**)** below.

Table 1.1: Some examples of plant formulations and their uses

Plant formulation	**Uses**	**References**
Vanda tessellate leaves macerated with ginger slices *Zingiber officinale*.	Applied to affected areas of paralysis and rheumatic pain	Rahmatullah *et al*., 2012
Dutura metel wrapped in leaf of *Justicia adhatoda*	Smoked for asthma	Rahmatullah *et al*., 2012
Root combination of *Carissa edulis, Euclea natalensis, Harrisonia abyssinica* and *Ximenia caffra*	Treatment of Gonorrhoea, syphilis, internal swelling, chronic amoebic dysentery, skin infection and thyroid fever	Otieno *et al*., 2008
Hippocratea indica mixed with *Nauclea latifolia, Enatia sp, Citrus madica var acida* and the bark of *Mangifera indica*	Malaria treatment	Adeleye *et al*., 2005

1.2. STUDY RATIONALE

The use of medicinal plants has gained a lot of interest over the years worldwide. In South Africa, especially in rural areas which have limited excess to primary health care centres, people revert to traditional healers for treatment of infections and diseases. Medicinal plants

play a key role in the development of pharmaceuticals and there is a high demand in natural medicine for the global market. Although there are thousands of species listed as medicinal plants, only a small number are commercially used in traditional treatment. There are growing numbers of antibiotic resistant microorganisms and treatment failure using normal drug; in this respect, discovered active compounds of the plants have gained significance. The advantages of using these plant products to treat human diseases is that they are cheap to produce, environmental friendly and readily obtainable.

In South Africa (Venda region) as well as other African countries, people use *Pterocarpus angolensis*, *Ximenia caffra, Peltophorum africanum* and *Terminalia sericea* extracts to treat diseases such as diarrhoea, pain, headache and wounds (Mabogo, 1990 and Maroyi 2013). Although numerous research work have already been done on the biological activities of these plants (Steenkamp *et al*., 2004; Bizimenyera *et al.,* 2005; Samie *et al.,* 2005; Samie *et al.,* 2009b; Mulaudzi *et al*., 2013; Narr *et al*., 2013); in-depth scientific knowledge on the properties of the plants formulations as used by traditional healers in the Venda region to treat diseases is still lacking (Mabogo, 1990). In this respect, this study aimed to determine the biological activities of the plants formulations and compare the activities of each plant and in combination.

Peltophorum africanum (roots), *Pterocarpus angolensis* (bark), *Terminalia sericea* (roots) and *Ximenia caffra* (roots) and designed formulations from the plant extracts were evaluated for their antimicrobial activity against selected bacteria and fungi. The plant extracts and formulations were also investigated for their phytochemical content, their ability to scavenge free radical DPPH and their toxicity against Human Lymphatic cell lines in order to initiate safety precautions on using these plants extracts for the management of diseases and understanding their effects on the immune system.

1.3. HYPOTHESIS

In the present study, we hypothesize that a mixture of selected plant extracts will have greater antimicrobial, antioxidant, phytochemicals and less toxicity as compared to the plants used individually.

1.4. OBJECTIVES

1.4.1. Primary objective

- The main objective of this study was to determine antimicrobial, phytochemical and toxicity of different formulations of four Venda medicinal plants and to compare their activity with each plant used individually.

1.4.2. Secondary objectives

- To evaluate antimicrobial activity of individual plant extract and plant formulations using broth microdilution method.
- To determine the minimum inhibitory concentration of plant extracts and mixtures against test bacteria and fungi by broth microdilution method.
- To investigate the phytochemical content and antioxidant activities of mixtures of *Pterocarpus angolensis, Ximenia caffra, Peltophorum africanum* and *Terminalia sericea*
- To determine the anti-HIV activity of plant formulations against reverse transcriptase enzyme.
- To determine the *in-vitro* cytotoxic effects of the plant extracts and their formulations against Human Endothelial Lymphatic cells.

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CHAPTER 2: LITERATURE REVIEW

2.1. ACTIVE CONSTITUENTS OF PLANTS EXTRACTS

Plants have been documented to naturally produce compounds that contribute to their anti-microbial, anti-inflammatory, antioxidant and cytotoxicity activities because they possess phytochemicals such as flavonoids, phenolics, alkaloids, saponins, terpenoids and propolis (Harborne, 1973; Xuan *et al*., 2005; Mishra *et al*., 2011). These phytochemicals have been found to possess antioxidant activity against invading pathogens such as bacteria, fungi and parasites (Sparg *et al.,* 2000; Samie *et al*., 2009a). The activity of medicinal plants against invading pathogens has gained a lot of interest in different industries such as food industries, pharmaceutical companies and medicine. Researchers also found that these phytochemicals possess antioxidant activity against human pathogens causing cardiovascular diseases making them a rich source of different types of drugs (Sparg *et al.,* 2000; Sheejah and Kuttan, 2007; Yadav and Agarwala, 2011). They can be derived from the roots, bark, leaves, flowers, seeds and fruits of plants (Yadav and Agarwala, 2011; Dougari 2012).

2.1.1 Plant phytochemicals

Plant compounds are classified according to their biosynthetic pathway. Phenolic compounds are the most diverse groups that are also found in vegetables, nuts, honey, teas, seeds and wine and they constitute a high percentage in human diet (Yadav *et al.,* 2017). Phenolics are also divided into different polyphenol groups known as tannins, lignins and flavonoids and they are naturally involved in the growth and reproduction of plants as they are responsible for the

production of lignin. They are also responsible for the eradication of invading pathogens such as fungi, bacteria and parasites because they are effective antioxidants, metal chelators and free radical scavengers (Eldeen *et al*., 2007).

Polyphenols such as flavonoids are the most widely occurring and are present in every form of vegetation consumption. In the human body, flavonoids are known to be associated with the reduction of different cardiovascular diseases. Epidemiological studies have shown that flavonoids display different physiological activities such as anti-inflammatory, anticancer, anti-arthritic and anti-microbial activities (Gupta *et al.,* 2013). Curcumin, a polyphenol responsible for the bright yellow colour of the Indian spice turmeric derived from *Curcuma longa*, has been used for centuries within the Ayurvedic system of medicine for the treatment of many diseases, including inflammation (Kennedy and Wightman, 2011).

Terpenoids or essential oils are secondary metabolites highly enriched in compounds based on isopropene structure. They are also essential plant derived antimicrobials with activity against bacteria, viruses and protozoa. Their mechanism of action is not well understood but it is believed to involve membrane disruption by lipophilic compounds (Hamed, 2011).

Alkaloids are a structurally diverse group of over 12,000 cyclic nitrogen-containing compounds that are found in over 20% of all plant species. They are often distinguished on the basis of a structural similarity (e.g. indole alkaloids) or a common precursor (e.g. benzylisoquinoline, tropane, pyrrolizidine, or purine alkaloids). Alkaloids in plants acts as a feeding preventions and poisons to insects and other herbivores, in many cases by directly interacting with molecular targets within the nervous system such as the DNA (Shang *et al.,* 2017).

Saponins are secondary metabolite of glycosidic nature that are highly distributed in higher plants (Podalok *et al.,* 2010). They possess a wide range of pharmacological activities such as anti-inflammatory, vasoprotective, immunomodulatory and antimicrobial, most plants that are rich in saponins have been investigated since ancient times and are still useful for the manufacturing of drugs, cosmetics, food supplements and as adjuvant for in the production of vaccines (Sparg *et al*., 2004; Podalok *et al*., 2010).

2.1.2. Interaction between phytochemicals

The presence of phytochemicals in food and vegetables as well as herbs is important in the prevention of chronic diseases such as cancer and cardiovascular diseases (Snyder *et al*., 2011, Epps *et al*., 2013). It is believed that a mixture of phytochemicals that are complementing each other provides a better protective effect on health than a single phytochemical. Therefore, a plant formulation will have greater biological activities than one plant used individually only when there is synergy between the phytochemicals in the extracts. Some phytochemical synergy investigated by Liao and Yin (2000) demonstrated that combinations of alpha-tocopherol and/or ascorbic acid with catechin, epicatechin, caffeic acid, myricetin, quercetin, gallic acid, and rutin had greater antioxidant activity than any of the individual compounds in a Fe2+-induced lipid oxidation system. Another study by Parker *et al*., (2010) found synergistic interactions between mixtures of rutin, *p*-coumaric acid, abscisic acid, ascorbic acid, and a sugar mixture using oxygen radical absorbance capacity (ORAC) and electron paramagnetic resonance (EPR).

2.2. ANTIOXIDANT ACTIVITIES OF PLANT EXTRACTS

Epidemiology and experimental studies have implicated oxidative cellular damage arising from an imbalance between free radical generating and scavenging systems as the primary cause of diseases (Govindappa *et al.,* 2011). Many researchers have also associated free radicals with aging and age - dependant diseases such as cardiovascular disease (Lakshmi *et al*., 2009), neurological diseases (Shukla *et al*., 2011), cancer (Jaganathan *et al.,* 2013; Sosa *et al*., 2013), inflammatory bowel disease (Pereira *et a*l., 2015) as well as infectious diseases such as AIDS and malaria (Bababunmi and Bewaji, 2002). Plants extracts have been documented to possess phytochemicals such as phenols and alkaloids with potent antioxidant activity against free radicals. Antioxidants protect the cell against the damaging effects of reactive oxygen species known as free radicals which results in oxidative stress leading to cellular damage (Mishra *et al.,* 2011). Primary antioxidants such as flavonoids, ascorbic acid and tocopherol, acts against free radicals by donating an electron to the free radical in the system forming a new radical, more stable than the initial one. Secondary antioxidants such as sulphides and sulphur dioxide act against ROS through the removal of ROS initiators by quenching chain-initiating catalysts (Harborne, 1973; Okwu, 2004). Many studies have reported the presence of phytochemicals with antioxidant activities in plants. Steenkamp *et al.,* (2004) reported that *Terminalia* spp contain tannins, saponins and the compound anolignan B. *Peltophorum africanum* contains tannins, as well as coumarins in *Ximenia caffra*.

Other studies have investigated the antioxidant activity of plant extracts against free radicals such as DPPH and ferric oxide. Karadeniz *et al.,* (2015) investigated the antioxidant activity of 9 plants in Burdur-Antalya provinces Turkey, and discovered that the highest DPPH radical scavenging was determined in *C. pestalozzae* extract with IC_{50} = 18.66µg/ml. in South Africa, a study by Chauke *et al*., (2012) found that the acetone extract of *Flueggea virosa* had the

highest antioxidant activity against DPPH with an IC_{50} value of 30µg/ml, closely matching the ascorbic acid standard with IC_{50} value of 25µg/ml.

2.3. PLANT EXTRACTS AS IMPORTANT ANTIMICROBIALS.

Humankind acknowledged the idea that plants are capable to cure various diseases even before the discovery of microbes. Many plants have been evaluated for their effectiveness in the treatment and the management of microbial infection (Ramalivhana *et al*., 2010). The effects these plant extracts on microbes have been studied by a very large number of researcher's worldwide for decades (Harborne, 1973; Xuan *et al*., 2005; Mulu *et al*., 2005; Samie *et al*., 2009a; Samie *et al*., 2009b; Green *et al.,* 2011; Doughari, 2012). Some examples of effective medicinal plants are marula tree (*Sclerocarya birrea*) and *Bridelia micrantha* which are mostly used for gastrointestinal disorders. Nwachukwu *et al.,* (2010) studied *Asmina triloba* (paw-paw) and *Psidium guajava* (Guava) and found them to have antimicrobial activity against malaria parasites. Moreover, species such as *Allium sativum* (garlic) have been documented as broad spectrum antimicrobial agents (Heinrich *et al.*, 2004).

2.4. INFECTIOUS DISEASE AND DRUG RESISTANT IN MICROBES

Many studies have demonstrated that prevalent pathogens have become greatly resistant to antibiotics leading to greater patient sickness and eventually death from nosocomial infections. Antimicrobial resistance threatens the effective prevention and treatment of infections caused by bacteria, fungi, parasites and viruses (Aleksun and Levy, 2007). The WHO records that the United States of America spends at about US$10 billion a year dealing with drug resistant problems. For example, in 2012, WHO reported a gradual increase in resistance to HIV drugs,

since then, further increases in resistance to first-line treatment drugs were reported requiring more expensive drugs for the future (WHO, 2015). The most resistant microbes amongst others includes Gram-positive *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Gram-negative beta–lactamases *Klebsiella pneumoniae*, *Acinetobacter* and *Escherichia coli* and fungal organisms such as *Candida* spp.

2.4.1. Antimicrobial resistant among bacterial organisms

2.4.1.1. Methicillin-resistant *Staphylococcus aureus (MRSA)*

Staphylococcus aureus is a facultative anaerobic Gram-positive bacterium that is usually found in the skin and the respiratory tract on humans. *S. aureus* is one of the major causes of community-acquired and hospital acquired infections causing a variety of symptoms such as food poisoning, toxic shock syndrome, atopic dermatitis, skin lesions, etc (Brosnahan and Schlievert, 2011). It is also responsible for aggressive infections which are life threatening such as bacteraemia, meningitis and pneumonia (Brosnahan and Schlievert, 2011). Patients with burnt wounds, sepsis is a major concern due to colonization by MRSA resulting in systematic infections and other major clinical complications that can be life threatening. A study by Naidoo *et al.,* (2013) reported that more than 50% of all hospital acquired *S. aureus* isolated from blood of South African sick patients in 2010 were MRSA, it accounted for three quarters of all hospital- acquired *S. aureus* infections in a large tertiary level paediatric hospital. This is a clear indication that MRSA is South Africa is of a major health concern and life threatening especially in hospital infections. Other that MRSA being resistant to β-Lactam antibiotics, it

has also been found to show resistance to other groups of antibiotics such as the Erythromycin and Aminoglycosides.

2.4.1.2. Beta-lactamase producing *Escherichia coli*

E. coli is part of a normal flora of the human gut in humans and warm-blooded animals, and it is distributed to the natural environment directly with feces or indirectly with treated wastewater. The bacterium is an important indicator of contamination in ecosystems, water, soil and food (Titilawo *et al*., 2015). *E.coli* cause a wide range of disease and infections such as urinary tract infection, wounds, bedsores, peritonitis, septic wounds, gastrointestinal infections, etc. Resistance in *E. coli* to one of the most widely used medicines for the treatment of urinary tract infections (fluoroquinolone antibiotics) is very widespread. There are countries in many parts of the world where this treatment is now ineffective in more than half of patients. Multidrug resistant Enterobactericeae *E.coli,* produces extended spectrum β-lactamases (ESLBs) such as CTX-M enzymes, these enzymes have greater activity against beta-lactam inhibitors such as cefotaxime.

2.4.1.3. *Klebsiella pneumoniae*

Klebsiella pneumoniae infections are also mostly observed in people with weakened immune system, it is responsible for destructive changes to human lungs through inflammation and haemorrhage with cell necrosis that produces a thick, bloody, mucoid sputum. In Southeast Asia, *K. pneumoniae* has been found to cause community acquired pneumonia accounting for significant death (Thamlikitkul and Hsueh, 2011). In South Africa studies from 2010 and 2012,

showed that up to 75% of *K. pneumoniae* isolated from the blood of hospitalised patients were ESBL(extended spectrum beta lactamases)- producing bacteria (Tau *et al.,* 2012). Common intestinal bacteria that can cause life-threatening infections – to a last resort treatment (carbapenem antibiotics) has spread to all regions of the world. *K. pneumoniae* is a major cause of hospital-acquired infections such as pneumonia, bloodstream infections, and infections in newborns and intensive-care unit patients. In some countries, because of resistance, carbapenem antibiotics do not work in more than half of people treated for *K. pneumoniae* infections.

2.4.2. Antimicrobial resistance among fungal organisms

2.4.2.1. *Candida spp*

Candida species such as *C. albicans,* C. *glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* can cause superficial infections of the oral and vaginal mucosa as well as disseminated bloodstream and deep-tissue infections (Whaley *et al.,* 2017). *Candida* infections are most often caused by *C. albicans* as reported by epidemiological studies in the United States (Clevelandetal, 2015), Europe (Klingsporetal, 2015), and the Middle East (Sharifzadehetal, 2013). Candida species have developed antibiotic resistance over the years with emerging new strains, reduced drug accumulation and the efflux pump (Tavoki *et al*., 2010). Drug resistance in fungal organisms is also overwhelming in Africa and South Africa especially in immunocompromised people with HIV /AIDS moreover, at about 90% of all HIV patients contract a fungal infection during the course of the disease and atmost 10% of these patients die as a direct cause of the fungal infection (Samie *et al.,* 2010). The most commonly found

fungal infections in HIV-positive patients are candidiasis caused by candida species and cryptooccosis caused by *Cryptococcus* species (Esebelahie *et al*., 2013).

The management of these infections in immune compromised individuals has become a problem because fungi can develop resistance without exposure to any drugs. For example, the resistance of *C. krusei* to fluconazole and *C. neoformans* to echinocandins have been recorded by Kanafani and Perfect, (2008). Furthermore, according to ARTEMIS global Antifungal Surveillance program, the incidence of fluconazole resistance in *Candida glabrata* has been found to have increased from 7% in 2001 to 12% in 2004 (Pfaller *et al.*, 2004).

2.4.1.2. *Cryptococcus neoformans*

Cryptococcus neoformans is a yeast that mainly cause cryptococcosis, an opportunistic infection that mainly affects immunocompromised individuals (Gago et al., 2016) meningitis is the most common *C. neoformans* infection is still highly affecting people with HIV/AIDS although they are using highly active antiretroviral therapy. Cryptococcus spp cause about 82% of cryptococcal infections worldwide, *C. gatti* have been reported for 218 cases in the Vancouver Island between 1999 and 2010 (Sloan and Parris, 2012). Cryptococcosis have been reported to be widespread in the world but Sub-Saharan Africa and south eastern USA have high endemic infections rates (Bratton et al, 2013). In this countries, mortality rate can reach up to greater than 30%. Cryptococcus can circulate from the lungs and cross the blood- brain barrier resulting in a central nervous system infection that is fatal when not treated (O'Meara and Alspaugh, 2012). Treatment for cryptococcosis requires the use of more than one antifungal agent. Amphotericin B is first administered, with or without flucytosine, and a 2 months to lifelong fluconazole maintenance therapy is recommended to prevent recurrence (Jarvis et al., 2012). Even though persistence of the original infecting organism is assumed to

occur in cryptococcal meningitis, it is possible that recurrences are the result of infection by a new isolate (Perfect and Bicanic, 2015).

2.5. THE USE OF ANTIMICROBIAL SUSCEPTIBILITY TESTING IN ANTIMICROBIAL TESTING

In the laboratory, microbiologists use antimicrobial susceptibility tests to test the degree of resistance of microbes to different antibiotics in order to prevent the use of ineffective treatment. Antimicrobial susceptibility test allow researchers to identify bacteria or fungi that can be treated with safe and effective antibiotics that are developed in the 20th century such as penicillin and clotrimazole and those that are caused by drug resistant microbes, which may require newer antibiotics such as cubicin or daptomycin. Moreover, antimicrobial susceptibility test can guide the physician in drug choice for difficult to treat infections. The increasing emerging resistance of microorganisms to antibiotics has led to finding alternative antimicrobial agents.

Antimicrobial susceptibility have also played a major role in assisting researchers to discover alternative medicine that can be used against emerging resistant microbial strains. Although these strains are emerging, medicinal plants have shown promising results in the eradication of drug resistant bacteria. Aqil *et al.,* (2005) reported that the ethanolic extracts of various Indian medicinal plants had significant inhibitory effects on both β-lactamase producing MRSA and methicillin-sensitive *S. aureus* (MSSA). Another study by Ganjewala *et al.,* (2009) evaluated the biochemical composition and antibacterial activities of *Lantana camara* plant, and found that of all microbes tested, *E.coli* was the most profound organism. Therefore, the use of medicinal plants to treat diseases caused by microbes can be highly recommended.

Moreover, plants have been widely explored for their therapeutic activities against most microbial infections using broth microdilutions. In South Africa, many efforts has been done especially in Kwazulu Natal (Mangale, 2013; Komolafe, 2014; Ndhlala *et al*., 2014) and Limpopo (Samie *et al.,* 2009a; Masoko and Nxumalo, 2013). In the Venda region of the Limpopo Province, the *in vitro* activities of 18 medicinal plants extracts were tested against certain gastrointestinal pathogens by Samie *et al.,* (2009a). The acetone and methanol extracts from the 18 plant species were tested against 110 clinical isolates of *Campylobacter* spp, and found that at least one extract of each plant had antimicrobial activity against some *Campylobacter* species and certain standard strain (HM-1:IMSS) of *Entamoeba histolytica.* In a similar study, Samie *et al*., (2009b) evaluated the extracts from eight plant species against a standard strain (HM-1: IMSS) of *Entamoeba histolytica* using the broth dilution method, out of the tested extracts only *P. angolensis* (MIC: 7.5mg/ml) was found to have inhibitory activities and that extracts of *P. angolensis* and *Lippia javanica* had the highest antibacterial activity, with a minimum inhibitory concentration (MIC) of 90ug/ml.

2.6. DRUG RESISTANCE AMONG VIRUSES

2.6.1. Human immunodeficiency virus (HIV)

HIV/ AIDS is a global concern that affects almost half of the population of people in the whole world. South Africa is also one of the countries that is greatly threatened by HIV/AIDS. Prior infection from HIV, the infected individual may acquire opportunistic infections such as candidiasis, cryptococcosis, and often suffer from tuberculosis (TB), diarrhoea, pneumonia and rarely cancers which sometimes lead to death (Tshikalange, 2007). Even though the human body produces antibodies and helper T-cells to fight the virus, ultimately the virus overcomes them leading to opportunistic infections occurring. In 2010, an estimated 7% of people starting

antiretroviral therapy (ART) in developing countries had drug-resistant HIV. In developed countries, the same figure was 10–20% (Freed, 2015). Some countries have recently reported levels at or above 15% amongst those starting HIV treatment, and up to 40% among people re-starting treatment. This requires urgent attention. Increasing levels of resistance have important overwhelming properties on the economy because second and third-line regimens are more expensive than first-line drugs (Mailler *et al.*, 2016).

2.6.2. Life cycle of HIV-1

Free virus binds to a CD-4 molecule and one of the two receptors which are either CC-Chemokine receptor 5 (CCR5) or CXC-Chemokine receptor 4 (CXCR4) which are found commonly in the surface of the cell, then the virus fuses with the cell (Olson *et al*., 2015). The infection occurs when the virus penetrates the cell and viral capsid is emptied into the cell. The released single strands of viral RNA are converted into double stranded DNA by an enzyme called reverse transcriptase. Then, the viral DNA is integrated into the cells own DNA by an intergrase enzyme in the nucleus of host cell. Transcription begins when the infected cell divides, the viral DNA is read and long chains of proteins are made. Sets of viral protein chains come together and the process of budding continues where immature virus pushes out of the cell, taking some cell membrane with it (Keana *et al.*, 2015). The protease enzyme begins processing the proteins in the newly forming virus. Immature viruses break free of the infected cell and the protease enzyme finishes cutting HIV protein chains into individual proteins that combine to make a new working environment (Mailler *et al*., 2016).

2.6.3. The HIV-1 therapy and management

The application of current antiretroviral chemotherapeutics such as antiHIV-1 RT drugs (nucleoside, nucleotide and non-nucleotide reverse transcriptase inhibitors) as well as antiproteases including those used in combinatorial therapy has caused significant reduction in the rate of mortality of HIV-1 infected individuals (Arts and Hazuda, 2012, Sharma 2014). It has allowed sufficient rise in CD4+ve lymphocyte counts into the HIV-1 infected individuals and imparted relatively longer and healthier lives. **Fig 2.1** below, shows the sites that are targeted by antiretroviral drugs. However, these drugs are extremely expensive, are not easily metabolised, not easily excreted out of the human body and they accumulate the cells (Laskey and Siliciano, 2014). There is therefore a great need for the search of anti-HIV treatment that is much safer, cheaper and widespread. Medicinal plants are now globally used to treat different diseases thus, they are also explored for natural compounds which may offer more opportunities to find anti-HIV drugs (Laskey and Siliciano, 2014).

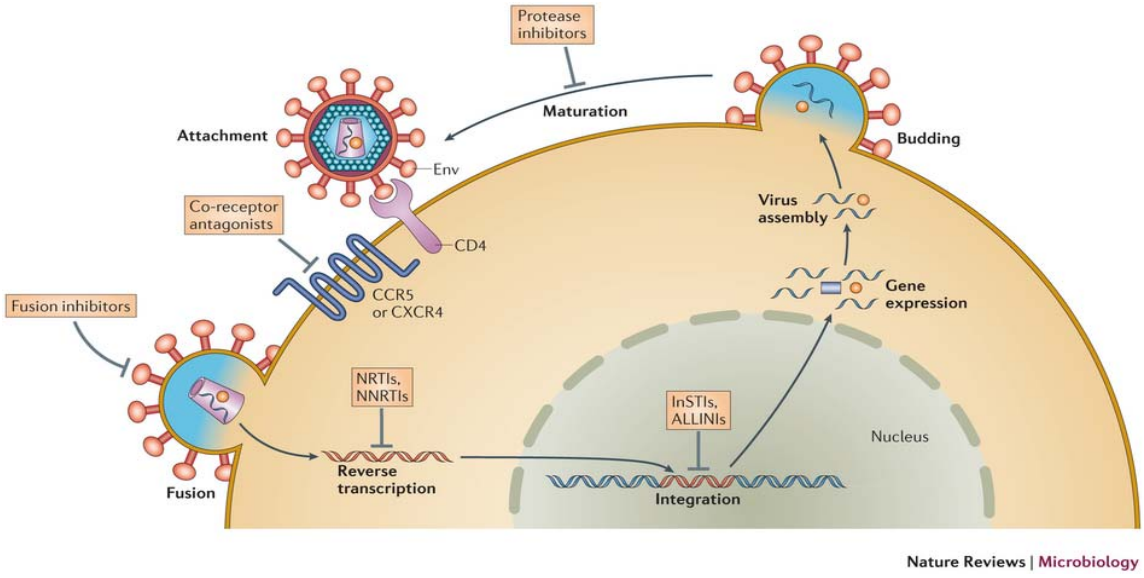

Figure 2.1: Stages of the life cycle of HIV-1 life cycle that are targeted by antiretroviral drugs (Laskey and Siliciano, 2014).

2.6.4. Natural products derived anti-HIV-1 treatment

Plant products have also drawn attention as possible anti-HIV drug by targeting a specific step on the viral life cycle such as the viral attachment and entry as well as enzymes and proteins that play an important role in viral transcription. Studies on medicinal plants have designated that presentation of plant based ideologies may prove to be highly useful, inexpensive and effective in order to arrest the HIV-1 progression (Tietjen *et al*., 2016). Besides of medicinal plants being able to arrest HIV-1 progression, Toxicity may be easily managed while treating AIDS patients with herbal preparations as these plant-ingredients are suitably metabolized and excreted out of body without much accumulation in human organs (Mthetwa *et al*., 2014). Research has shown that plants have phytochemicals that possess anti-HIV activity (Leteane *et al*., 2012). Some examples are plant extracts such as green tea containing ((-)-Epigallocatechin-3-gallate (EGCG)), Brazil nut containing immune modulators, grapes and red

wine containing plenty of antioxidants which mimic oxidative stress induced by intake of antiHIV-1 regimen (Nijveldt *et al.,* 2001).

2.7. TOXICITY OF MEDICINAL PLANTS

Medicinal plants have been used for centuries around the world to treat different diseases. Most traditional healers concentrate on treating the infection rather than the toxicity of the medicinal plant to the human cells. Although medicinal plants are widely used and assumed to be harmless, they can potentially be toxic especially in pregnancy (Nasri and Shirzad, 2013). Poisoning from medicinal plants is usually due to wrong identification of the plants in the form in which they are sold, or improper preparation and usage by people not well trained. There are plants which have important anticancer, antipyretic, antimicrobial and anti-inflammatory activity which can be toxic with the high dosage (Nasri and Shirzad, 2013). A study by Tamilselvan *et al*., (2014) showed that the ingestion of Abrin a toxalbumin from the seeds of *Abrus precatorius (Fabaceae)* as little as 3mg can be toxic to the human body, upon ingestion symptoms include vomiting, nausea, diarrhoea, severe dehydration and low blood pressure, after a few days the liver, kidney and spleen may stop working resulting in premature death. However, the same toxin is an important anticancer, antidiabetic as well as antifungal and antibacterial agent when used appropriately (Tamilselvan *et al*., 2014). Therefore, in order to meet the effectiveness, welfare and worth of medicinal plants for synthetic drug production, the pharmacological, toxicological and phytochemical profiles of the plant extracts have to be scientifically evaluated (Erharuyi *et al.,* 2014).

2.7.1. Toxicological screening of medicinal plants

Toxicology screening is important for the development of new drugs and the extension of already existing drugs (Parasuraman, 2011). The use of medicinal plants without an appropriate dosage has led to raising concerns on the efficacy of the plant and causing health problems for other individuals. A number of kidney failure has been reported in early 1993 resulting from the consumption of *Aristolochia fangchi* (Hasan *et al.,* 2014). Therefore, determining its toxicity helps to understand the dosage that is needed in the human body that will not be detrimental to life (Parasuraman, 2011). The toxicity of substances can be observed by studying the accidental exposures to a substance, the *in- vitro* studies using cells/ cell lines and *in vivo* exposure on experimental animals such as rats. However, there are some plants and plant formulation that promote cell proliferation, regeneration, repair and wound healing. A study by Krishnamoorthy *et al*., (2012) found that a polyherbal formulation consisting of extracts of *Wrightia tinctoria, Aloe vera, Curcuma longa* and *Terminalia chebula* promoted fibroblast cell migration and proliferation using scratch wound assay technique, consequently the formulation was considered that it may be useful in effective management of superficial wounds.

2.7.2. The importance of In-vitro cell proliferation assay in toxicological studies

Cell Proliferation assays are widely used in cell biology research in order to study growth factors, cytokines, nutrients and cytotoxic agents. There are numerous ways to regulate the quantity of cells in a proliferation assay. Cell number can be determined by using a microscope or an electronic particle counter and by measuring incorporation of radioactive precursors which employs the use of chromogenic dyes to quantitate total protein, or by measuring

metabolic activity of cellular enzymes. In toxicology studies, cell assays are used to determine the toxicity of a plant extracts against certain cell either obtained from humans or animals. Studies have shown that *In-vitro* cell proliferation assays have led to the discovery of many drugs that can be used for the management of diseases such as cancer, HIV, hypertension and diabetes. A study by Manosroi *et al*., (2006) showed that *Psidium guajava* leaf oil had anti-proliferation activity against epidermal carcinoma (KB) and murine leukemia (P388) cell lines.

Moreover, *in-vitro* cell proliferation assay in toxicology have also helped researchers avoid using poisonous plants which inhibit the proliferation of normal cell and also altering the immune system functioning. Even though some medicinal plants are poisonous, some have essential phytochemicals that are useful and when administered at appropriate concentrations are useful. Therefore, using cell proliferation assays also assist toxicologists to determine the concentration of the medicine that will be suitable for human consumption without any harmful effects. One of the methods used for toxicology screening is the CellTiter 96 Non-Radioactive Cell Proliferation Assay.

2.8. THE SELECTION AND DESCRIPTION OF THE PLANTS USED IN THIS STUDY

2.8.1. The selection of plants used in the study

Medicinal plants were selected during an ethnobotanical survey according to the information provided by traditional healers and literature. In literature, the plants used in the study were mentioned to cure diarrhea and other infections including those that are related to HIV/AIDS. Mabogo *et al*., (1990) also reported that a combination of *Pterocarpus angolensis* (bark), *Ximenia caffra* (roots), *Peltophorum africanum* (roots) and *Terminalia sericea* (roots) is also

used by different traditional healers in the Venda to treat different diseases. These plants were selected and collected in different areas around Thohoyandou.

Table 2.1: Selected plants used in the study, plant parts used and place of collection.

Plants	**Plants part**	**Place collected**
Pterocarpus angolensis	Bark	Thohoyandou
Peltophorum africanum	Roots	Thohoyandou
Ximenia caffra	Roots	Thohoyandou
Terminalia sericea	Roots	Thohoyandou

2.8.1. Description of *Pterocarpus angolensis* (mutondo)

Pterocarpus angolensis which is generally known as blood wood in English and Mutondo in Tshivenda is a deciduous, spreading and slightly flat-crowned tree with high covering. It also grows to reach about 15m in height and has a dark bark (Samie *et al*., 2009b). It is one of the well- known woods which are common in South Africa, Angola and other African countries (Moola *et al.,* 2009). It is also characterised by a durable heart wood which is resistant to fire, decay, wood rotting fungi, termite attack, terrestrial and marine borers (Mmoletsi *et al.,* 2012). It grows well together with other tree species such as *Brachystegia speciformis, Uapaca* species and *Isoberlinia angolensis*. All these species play a major role in the ecosystem by providing high value timber and non-timber forests that are core of local livelihoods, moreover, they constitute a habitat for wildlife (Ng'wane *et al.,* 2007).

Figure 1.2: Pterocarpus angolensis tree, and stem showing the sap

2.8.1.1. Uses of *Pterocarpus angolensis*

The heartwood of *P. angolensis* is used for timber for furniture and veneer because it is resistant to woodborer insects and termites. The wood is often used for woodcarving, implements and building canoes. The wood produces a rich, resonant sound and is used to make different musical instruments. The tree is valued also for several medicinal uses.

2.8.1.2. Medicinal uses of *Pterocarpus angolensis*

The extracts of *Pterocarpus angolensis* are used by traditional healers in the African continents. In central south-Zimbabwe, the bark extracts are dropped into the ear as earache medicine and drunk as remedy for menorrhagia, the roots extract is drunk by women as a remedy for

infertility whereas the sap of the tree is dropped into sore eyes (Maroyi 2013). In the Venda region in south Africa, traditional healers uses the extracts of this tree for the treatment of malaria, gonorrhoea, headaches, stomach aches, diarrhoea, mouth sores and rashes (Samie *et al*., 2009b) the sap is used for the treatment of ringworms, ulcer, malaria, skin inflammation and urinary schistosomiasis (Samie *et al*., 2009b).

2.8.1.3. Known research findings *on P. angolensis*

P. angolensis has been found to have anti-tumor activities and intense anti-HIV activity (Sigidi *et al*., 2015). A study by Samie *et al*., (2009a) showed that *P. angolensis* has antibacterial and cytotoxicity activities. *P. angolensis* also showed good anti-inflammatory activity against both COX-1 and COX-2 (Mulaudzi *et al*., 2012).

2.8.2. The description of *Peltophorum africanum* (Musese)

Peltophorum africanum (Musese) is a deciduous tree which is widely distributed in South Africa and other tropical countries (Tshisikhawe *et al*., 2012). In mature trees the bark is grooved and grey-brown; the bark of young branches is smooth and grey. The leaves are acacia-like and also silver-grey covered with fine hair; mature leaves yellowish at the tip of branches. The leaves are twice compound with a pair of leaflets at the tip; alternating up to nine pinnae each with 10-20 pairs of leaflets and the tree has no thorns (Mongale, 2013). The bright yellow flowers are bisexual with crinkled petals at the ends of the branches during November to February. The dark brown fruit is a flat, elliptical pod containing ovoid dark brown to black seeds, which is dispersed by birds, wild and domestic animals. *P. africanum* is successfully

propagated from seeds and grows fast during the summer season in well-drained soil types including sandy soils (Mongale, 2013).

Figure 2.2: Peltophorum africanum leaves and flowers

2.8.2.1. Uses of *Peltophorum africanum*

Peltophorum africanum tree has many uses. The flowers provide a high yield of nectar and pollen for bee- keeping. The timber can be used for furniture and the wood is good for fuel. It provides good shade for both livestock and humans. It is also used for various medicinal recorded for this plant.

2.8.2.3. Medicinal uses of *P. africanum*

The roots and bark are used to treat both human and livestock diseases (Bizimenyera *et al.,* 2007). In humans, the roots and bark decoction are used to treat eye infection, joints and back pains, toothache, ascites and abdominal pains, dysentery, infertility, skin rashes and blisters,

venereal diseases, depression, intestinal parasites, coughs, sore throat, human immunodeficiency virus (HIV/AIDS) and tuberculosis (Semenya *et al*., 2013; Okeleye *et al*, 2014) . The bark is used to cure fever, induce vomiting and cleanses the liver. (Mabogo 1990; Bizimenyera *et al.,* 2007). In livestock, the plant is used against diarrhea, dysentery, colic and as a general tonic (Motlhanka and Nthoiwa, 2013). The bark crushed and is used for the repel fleas and maggots as well as treating intestinal parasites and diarrhea (van der Merwe, 2000; Moreki, 2012).

2.8.2.4. Known research findings on *P. africanum*

P. africanum has been identified to possess antimicrobial activities (Okeleye *et al*., 2010). The aqueous and methanol extracts of the roots and stem bark were shown to inhibit RNA-dependent-DNA polymerase activity of HIV-1 reverse transcriptase and ribonuclease H activity of reverse transcriptase, which was ascribed to the gallotanin 45 (Kanta *et al*., 2011; Mazimba, 2014).

2.8.3. The description of *Ximenia caffra* (Mutshili)

Ximenia caffra (Mutshili) is a small semi-deciduous tree or shrub with straight, brown thorns. The leaves are simple, longitudinally folded, dark green, somewhat leathery; apex blunt and often irregular. The sapwood is white and the branchlets are spine-tipped with alternate leaves. The roots are non-aggressive and the flowers are quite small and creamy green. The tree flowers are greenish to creamy white in colour although sometimes they have been seen as tinged pink or red. The sourplum fruit itself is ellipsoidal in shape (Chivandi *et al.,* 2012). The skin of the fruit is smooth and starts green, and then ripens to an orange or red. Similarly the flesh is also orange or red in colour, and when ripe has a juicy pulp. The sourplum is 3.5 cm in length and

2.5 cm in diameter. The seed is smooth, ellipsoid, and yellow-brown to red in colour. It is also hard and around 2.5 cm in length. The tree is common in better rainfall areas and mixed woodlands.

Figure 2.3: Ximenia caffra leaves and fruits

2.8.3.1. Medicinal uses of *Ximenia caffra*

A decoction from the leaves is used as a wash to soothe inflamed eyes (Mulaudzi *et al*., 2011; De wet *et al.,* 2012). Infusions of the roots are used as a remedy for dysentery and diarrhoea and together with the leaves are taken for abdominal pain and bilharzia. Powdered roots are applied to sores to speed up healing (Narr *et al*., 2013). Powdered dried leaves are taken orally for fever and infertility, and extracts of the leaves are used as a mouthwash for tonsillitis (Mulaudzi *et al*., 2011). Porridge is made using a decoction of the roots, nausea in pregnancy; the root decoction is also taken for infertility (Narr *et al*., 2013).

2.8.3.2. Known research findings on *Ximenia caffra*

A study by Fabry *et al*., (1998) found that *X. caffra* have fungicidal activity. Another study showed that the plant possesses different phytochemicals such as Flavonoids, phenolic and tannins with antimicrobial activity (Mulaudzi *et al.*, 2012). Zhen *et al.,* (2015) found that a Cell-based assays showed that the leaf extract inhibits the mRNA expression of proinflammatory genes (IL-6, iNO, and TNF-α) by using RT-qPCR, implying its anti-inflammatory effects.

2.8.3. The description of *Terminalia sericea* (Mususu)

Terminalia sericea is common as a shrub or bush which grows up to 6-9 m tall, but individual trees may reach 23 m in height. The bark is dark grey or brownish often peeling off to expose a brownish under-bark. Young stems and branches often bear lengthy round galls often up to 2-3 cm in diameter frequently with leaves. They have an unpleasant smell and may be pollinated by flies. The fruit are winged nuts containing a single seed and turn a darker pink colour as they ripen. They may remain attached to the branch for a year and are dispersed by the wind and they sometimes become knobby and hairy as a result of the activities of opportunistic insect larvae.

Figure 2.4 : Terminalia sericea tree showing leaves and pods

2.8.3.1. Medicinal uses of *Terminalia sericea*

Roots of *Terminalia sericea* are used by African tribes to treat diarrhoea and indigestion as a decoction; it is also used to treat headaches and pains and the leaves are used against stomach disorders and as a cough remedy (Steenkamp *et al*., 2004).The bark is used to treat diabetes and topically for abrasions.

2.8.3.2. Known research findings on *T. sericea*

Tshikalange *et al*., (2008) showed that *T. sericea* had significant *in-vitro* anti-HIV-1 activity. *Terminalia sericea* extracts were the most active against the following tested fungal organisms *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Microsporum canis* and *Sporothrix schenkii* (Masoko *et al.,* 2005*)*. . Anolignan B isolated from *T.sericea* root inhibits *Bacillus subtilis* (Gram-positive) *at* 3.8 g/ml and *Escherichia coli* (Gram-negative) at 31 g/ml. In the anti-inflammatory assays, anolignan B showed activity against both COX-1 (IC_{50} = 1.5 mM) and COX-2 (IC_{50} = 7.5 mM) enzymes (Eldeen *et al*., 2006).

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CHAPTER 3: ANTIMICROBIAL ACTIVITIES AND PHYTOCHEMICAL SCREENING OF MEDICINAL PLANT FORMULATIONS.

3.1. ABSTRACT

Background: Medicinal plant and formulations have gained a lot of interest in the past decade amongst indigenous people to cure diseases. The objective of the study was to determine the antimicrobial activity and phytochemicals of four medicinal plants in a formulation as used by Venda people and compare their activity with each plant used individually.

Material and Methods: *Peltophorum africanum* (roots), *Pterocarpus angolensis* (bark), *Terminalia sericea* (roots) and *Ximenia caffra* (roots) were collected and extracted with methanol and water. Individual plant extracts and ten designed formulations were evaluated for their antibacterial activity against *Staphylococcus aureus* ATCC 25923 (Methicillin Resistant), *Staphylococcus aureus* ATCC 33591(Methicillin Susceptible), *klebsiella pneumonia* (ATCC 700603) , *E. coli* ATCC 35218, four clinical isolates of *Candida spp* and *Cryptococcus neoformans* using broth dilution method. Fractional inhibition index (FICI) was used to determine mutual relationship between the plant extracts. The phenolic content of the plant extracts and plant formulations was determined using Folin–Ciocalteu method. The total flavonoid content was determined using spectrophotometric methods. Preliminary phytochemical analysis were used to determine the presence of phenolics, flavonoids,tannins, saponins, terpernoids and steroids.

Results: Methanolic and aqueous extracts of *T. sericea* exhibited the best antifungal and antibacterial activities whilst *P. angolensis* and *X. caffra* showed poor activities. Methanolic plant formulations showed good activities compared to aqueous formulations. However, according to Fractional inhibition index (FICI) there was 1 synergistic interaction, 25 additive interactions and 14 antagonistic interactions between the plant extracts against tested bacteria. The methanolic formulation 3 showed the best overall phenolic content at 11.85±0.109 mgGAE/g whilst aqueous *X. caffra* extract showed the least content at 4.546±0.104 mgGAE/g. Higher total flavonoid contents were seen in methanolic formulation 4 at 2.75±0.02 mgQE/g. Qualitative phytochemical analysis revealed the presence of flavonoids, phenolics, terpenoids, tannins, saponins and steroids in 80% of the tested plant extracts and formulations.

Discussion and conclusion: The results indicated that the combination of the extracts has better antimicrobial activity, higher phenolic content compared to the individual plant extract, indicating a synergistic and additive effect of the phytochemicals. However, antagonistic interactions were also noted in the same formulations meaning that a combination can be synergistic and antagonistic at the same time depending on the organism tested. Therefore, caution should be taken when using medicinal plants formulations. Further in-vivo studies of these formulations needs to be conducted.

Key words: *antimicrobial, phenolic content, flavonoid content, phytochemical*

3.2. INTRODUCTION

Infectious diseases are leading causes of death in the whole world following cardiovascular diseases. People in countries that are still developing, experience extreme burden of infectious diseases resulting in death. However, the burden of infectious diseases is less in developed

countries as it only affects a minority. As previously reported by Samie *et al*., (2005) out of the approximately 57 million deaths annually worldwide, about 15 million are estimated to be caused by infectious diseases. Microorganisms that are responsible to these infectious diseases include viruses, fungi, bacteria and parasites causing diseases such as HIV/AIDS, hepatitis, food poisoning, Leshmaniasis and others. The use of drugs to eradicate these diseases has been seen useful from the twentieth century. However, some microorganisms were found to have developed resistance to such drugs (especially antibiotics) then leading to many deaths.

It is then imperative to find alternative methods to counteract the burden of antibiotic resistant strains and ease the burden of infectious disease the world is currently encountering. Traditional medicine has been used to treat infectious diseases since ancient times (Yim *et al*., 2013). Approximately 80% of the world's population still depends on traditional medicine for the treatment of common diseases (WHO, 2001; Yim *et al.,* 2013). Unlike western medicine which have causes side effects, traditional medicines derived from these plants are believed to be more effective and less toxic. Medicinal plants naturally produce phytochemicals such as flavonoids, terpenoids, tannins, phenolics and steroids for growth and development as well as for fighting invading pathogens. Therefore, exploring medicinal plants and herds as alternative therapy for infectious diseases could be a solution to the overwhelming burden of infectious diseases.

3.3. MATERIALS AND METHODS

3.3.1. Collection of plant material

Fresh bark of *Pterocarpus angolensis* and the roots of *Peltophorum africanum, Ximenia caffra* and *Terminalia sericea* were collected at different locations around Thohoyandou. Upon

collection, the plant parts were washed three times with running water and once with distilled water and air dried at room temperature for two weeks in the laboratory on a sterile blotter paper under a shade. After drying the plant parts were ground into powder using a Wiley grinder with a 2mm wire mesh.

3.3.2. Plant extraction

Fifty grams (50g) of each ground plant material was soaked in 500ml water, methanol and ethyl acetate respectively with frequent shaking for 72hrs followed by suction filtering through a Whattman no. 1 filter paper under sterile environment. The methanol filtrates were evaporated to dryness under reduced pressure at 40ºC using a rotary evaporator (Rotavapor-R, Buchi, Switzerland) whereas the water filtrates were frozen to dryness at -80ºC until completely dry using a nitrogen freeze dryer. A stock solution of 0.1 g/ml in dimethyl sulfoxide (DMSO) was prepared for each extract (Samie *et al.*, 2005). After dissolving the extracts in DMSO, 10 mixtures were formulated from the extracts where the water extracts were mixed together, methanol extracts were mixed together as described in **Table 3.1**below:

Table 3.1: Percentage of plant extracts in different formulations

Plants	**Mixture 1**	**Mixture 2**	**Mixture 3**	**Mixture 4**	**Mixture 5**
P. angolensis	100µl=25%	60µl=15%	100µl=25%	180µl=45%	60µl=15%
P. africanum	100µl=25%	100µl=25%	60µl=15%	60µl=15%	180µl=45%
X. caffra	100µl=25%	180µl=45%	60µl=15%	60µl=15%	100µl=25%
T. sericea	100µl=25%	60µl=15%	180µl=45%	100µl=25%	60µl=15%

3.3.3. Phytochemical evaluation

3.3.3.1. Determination of total phenolic content (TPC)

Total phenolic content of the plant extracts and formulations was determined according to the Folin-Ciocalteu method as previously explained by Stankovic', (2011) with slight modification. The 96 well plate was filled with 80 µl of water then 20 µl of each sample (5mg/ml of plant extracts and formulations) was added in triplicate. Twenty microliters of 10 % Folin-Ciocalteu reagent and 60 µl of 7 % sodium carbonate (Na_2CO_3) were added to the mixture respectively. Prior to reading, 120 µl of distilled water was added to the mixture and allowed to stand for 30 min at room temperature. The absorbance was read at 760 nm using VersaMax Microplate Reader (Molecular Devices, Sunnyvale, USA). The same procedure was repeated for gallic acid and a calibration curve was constructed using linear regression. The

concentration of phenolics was read in (mg/ml) based on absorbance and gallic acid equivalent for the content (mgGAE/g of plant extract).

3.3.3.2. Determination of total flavonoid content (TFC)

The total flavonoid content of the extracts and formulations was determined by spectrophotometric method (Quittier *et al.,* 2000, Hossain and Shah, 2015) with slight medication. Briefly, About 100 µl (1mg/ml of each extract and formulations in ethanol) was mixed with 2 % aluminium chloride ($AlCl_3$) in ethanol in a 96 well plate and was allowed to stand for 1 hour then the absorbance was measured using a Versa max microplate reader (Molecular Devices, Sunnyvale, USA) at 420 nm. Tests were carried out in triplicate. The same procedure was repeated for quercetin and a calibration curve was plotted using linear regression, the concentration of flavonoids was read in (mg/ml) and the content was read as quercetin equivalent per gram of plant extract (mgQE/g).

3.3.3.3. Statistical analysis

All experiments were carried out in triplicate and are expressed as average of three analyses ± standard deviation. Statistical software package (SPSS for Windows, version 21.0) was used for analysis. One –way ANOVA (Tukey Test) was used to calculate the correlation between variables.

3.3.4. Preliminary phytochemical analysis

The preliminary phytochemical analysis of the medicinal plants was evaluated to detect the presence of various classes of phytochemicals such as alkaloids, tannins, terpenoids and saponins and to confirm the total flavonoids and phenolics tests in accordance with the methods of Aiyegoro and Okoh, (2010) and Kazeem *et al*.,(2013). For all the prepared samples in each test, formulations were designed as illustrated in **Table 3.1** above and also tested for phytochemicals**.**

1. Test for Alkaloids

To test for alkaloids, exactly 5mg of the extract was dissolved in 5ml of dilute hydrochloric acid (1%), then stirred and filtered. About 1ml of the filtrates were tested for the presence of alkaloids by adding a few drops of Wagner's reagent. Turbidity and precipitation confirmed a positive result.

2. Test for Flavonoids

Briefly, dried plant extracts were treated with dilute NaOH followed by the addition of dilute HCl. The formation of a yellow solution with NaOH turning colourless with a dilute HCl showed the presence of flavonoids.

3. Test for Phenolics

About 5mg of the plant extract was treated with 3-4 drops of ferric chloride solution. The formation of the bluish black colour indicated the presence of phenols.

4. Test for Saponins

About 2ml of distilled water was added into 5mg of the plant extract and shaken vigorously for ten seconds and allowed to stand. The formation of persistent honeycomb like froth showed a positive test for saponins.

5. Test for Tannins

Briefly, exactly 1g of powdered extract was boiled with 10ml distilled water for five minutes in a waterbath and filtered (using Whattman No 1 filter paper). A few drops of 10% ferric chloride were added. The formation of a bluish black precipitate indicated the presence of tannins.

6. Test for Terpenoids

In order to test for terpenoids, 5 ml of extract (0.5 mg/ml) was mixed with 2 ml chloroform and 3 ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface indicated a positive test (Kazeem *et al*., 2013).

7. Test for Steroids

In this test, 5mg of the plant extract was treated with chloroform and filtered, a few drops of conc. HCL was added, the appearance of a golden yellow colour indicated the presence of triterpenes (Kazeem *et al.*, 2013).

3.3.5. Antibacterial activity test

3.3.5.1. The growth and maintenance of test bacteria for antimicrobial studies

Staphylococcus aureus ATCC 25923 (Methicillin Resistant), *Staphylococcus aureus* ATCC 33591 (Methicillin Susceptible), *Klebsiella pneumonia* (ATCC 700603) and *Escherichia coli* ATCC 35218 were obtained from the ATCC. The microorganisms were cultured on a plate count agar. Before testing, a McFarland standard of 0.5 was prepared for each organism and was used in the microdilution assay.

3.3.5.2. Broth microdilution method

The microdilution method was used to determine the minimum inhibitory concentration (MIC) as previously described (Eloff, 1998). Briefly, A 96 well plate was used for antimicrobial activity, in the first raw, 160µl of Mueller Hinton broth was added followed by 100µl in the remaining raws. In the first raw, 40µl of plant extracts or formulations were added into the first column as well as positive control (ciproflaxin) and the negative control (distilled water). A series of dilutions of the plant extracts or formulation with reduced, concentrations was performed to cover all the wells and the control wells. Serial dilution was performed by mixing the controls, plant extracts and formulations with the media in the first raw, 100µl of the each column in first raw was taken and mixed with the media in the second column to give a serial two fold dilution in the last raw. 100µl of the test microbial cultures were added to each well and the plates were incubated overnight at 35ºC. In the following day, 50µl of 0.2mg/ml INT (iodo-nitro-tetrazolium) was added in each well and incubated for 10 min. after 10min; results were read observing the colour change determining the MIC (minimum dilution concentration). Those wells which showed no colour change were considered as active meaning that the plant

inhibited the growth of the bacteria whereas those which were pink indicated bacterial growth. The clear wells were incubated in agar plate overnight to determine the MBC (minimum bactericidal concentration).

3.3.5.3. Determination of minimum bactericidal concentration (MBC)

The MBC was determined by inoculating the contents of the MIC plate into a nutrient agar plate and the results were observed after 24h incubation at 35ºC. The formation of bacterial colonies on agar plate was an indication that the plant extract only inhibit the growth of the bacteria without killing them and the absence of colonies shows that plant extract was able to kill the bacteria. The smallest concentration of the plant extract that was able to kill the bacteria was considered as the minimum bacterial concentration (Samie *et al*., 2010).

3.3.5.4. Determination of fractional inhibition index (FICI)

In order to determine the mutual influence of the four medicinal plants in the designed mixtures against the four test bacteria. Fractional inhibition index (FICI) was determined using the following formula:

$$FICI = \frac{MIC\ of\ the\ combination}{MIC\ of\ EOA} + \frac{MIC\ of\ the\ combination}{MIC\ of\ EOB}$$

Where EOA and EOB are the individual tested plants, results were interpreted as follows: FICI≤ 0.5 synergy, 0.5 < FICI ≤ 4 additive, FICI > 4 Antagonistic (Odds, 2003).

3.3.6. Antifungal activity test

3.3.6.1. The growth and maintenance of fungal organisms for antimicrobial activity

Clinical isolates of *Candida ablicans, Cryptococcus neoformans, Candida krusei, Candida tropicalis and Candida grabrata* isolated from AIDS patients with oro-pharyngeal thrush and cryptococcal meningitis obtained in the Department of Microbiology were used in this study. Sabouraud dextrose broth (SDS) was used for the preparation of fungal cultures.

3.3.6.2. Broth dilution method

Similarly to the antibacterial activity, antifungal activity was determined as previously described by Samie and Mashau (2013). Briefly, Positive control used in the test was clotrimazole. After adding INT, all the clear wells which were considered as active were cultured again in Sabouraud dextrose agar in order to determine the MFC (minimum fungicidal concentration).

3.3.6.3. Determination of MFC (minimum fungicidal concentration)

Similar procedure used in MBC was followed, however, Sabouraud dextrose agar was used instead of nutrient agar**.**

3.4. RESULTS

3.4.1. Total phenolic content (TPC) of the plant extracts

The folin-Ciocalteu's reagent was conveyed in terms of the Standard Gallic acid equivalent with the curve equation y =0.006x + 0.083, r^2 = 0.9997. Phenolic content in the plant extract and the formulations were expressed as gallic acid equivalent per gram of extract (**Table 3.2).** Methanol extracts and their combinations displayed the greatest amount of phenolics, and they showed greater amount of phenolic content than water samples. All extracts and combinations that contained total phenolic concentration greater than 5mg/g were considered most active. The aqueous *X. caffra* extract water extract was the only one which gave a value less than 5mg/ml**.** The highest amount of phenolics was showed in the methanolic mixtures 3 and 4 with 11.85±0.10 mg GAE/ g and 10.03±0.04 mg GAE/ g respectively**.** Values showing the same letter are not significance differences ($p < 0.05$) were marked with different letters.

Table 3.2: Total phenolic content of methanol extracts and their formulations and water extracts and their formulations. Values are expressed as Gallic acid equivalent (mgGAE) per gram of plant extract. Each value is the average of three variables ± standard variation.

PLANT EXTRACTS AND MIXTURES	**METHANOL**	**WATER**
Pterocarpus angolensis	6.33±0.07^{c}	5.87±0.02 ^{b}
Peltophorum africanum	6.59±0.31^{c,d}	5.04±0.06 ^{a}
Ximenia caffra	5.21±0.07^{a}	4.54± 0.10 ^{j}
Terminalia sericea	6.99±0.09^{d,e}	7.73±0.03 ^{g}
Mixture 1	7.80±0.08^{g}	7.43±0.08 ^{f}
Mixture 2	6.30±0.03^{c}	7.72±0.20 ^{g}
Mixture 3	11.85±0.10^{i}	7.66±0.10 ^{g}
Mixture 4	10.03±0.04^{h}	6.68±0.07 ^{d,e}
Mixture 5	7.21±0.064^{e,f,g}	6.61±0.06 ^{c,d}

Values marked with the same letters showed no statistical significance between the means ($p < 0,05$)

3.4.2. Total flavonoid content (TFC)

The flavonoid content of the tested plant extracts and formulations are expressed in terms of quercetin equivalent with a standard curve equation y =0.0244x + 0.046, r^2 = 0.9994. Flavonoid content in the plant extract and the formulations were expressed as quercetin equivalent per gram of extract. Similarly to total phenolic test, each value is the average of three variables ± standard variation (Figure 3.3). Moreover, all samples which showed small differences or insignificant differences ($p>0.05$) were marked with the same letters.

Table 3.3: Total flavonoid content of water extracts and formulations and methanol extracts and formulations. Values are expressed as quercetin equivalent (mgQE) per gram of plant extract. Each value is the average of three variables ± standard variation.

PLANT EXTRACTS AND MIXTURES	**WATER**	**METHANOL**
Pterocarpus angolensis	0.81 ± 0.125^{a}	0.99 ± 0.065^{c}
Peltophorum africanum	0.943 ± 0.010^{d}	0.45 ± 0.045^{b}
Ximenia caffra	1.045 ± 0.06^{k}	2.24 ± 0.036^{h}
Terminalia sericea	2.59 ± 0.0179^{I}	2.77 ± 0.066^{J}
Mixture 1	1.23 ± 0.018^{e}	2.18 ± 0.058^{h}
Mixture 2	0.763 ± 0.010^{c}	1.46 ± 0.053^{f}
Mixture 3	1.82 ± 0.028^{g}	2.73 ± 0.045^{J}
Mixture 4	$1.36 \pm 0.045^{e,f}$	$2.742 \pm 0.025^{I,J}$
Mixture 5	$1.33 \pm 0.091^{e,f}$	0.752 ± 0.034^{c}
Values marked with the same letters showed no statistical significance between the means ($p < 0.05$)		

3.4.3. Preliminary phytochemical analysis

3.4.3.1. Qualitative phytochemical analysis for methanolic extracts and formulations

The phytochemical composition of the tested plant extracts and formulations of methanol indicated that most plants contained more phenolics such as tannins, flavonoids and terpernoids, saponins and steroids (**Table 3.4**) were found in all the extracts tested while alkaloids were only slightly detected in *T. sericea*. Flavonoids were also detected in all extracts and mixtures except for *P. angolensis.*

Table 3.4: Qualitative phytochemical analysis results for methanol extracts and their formulations.

Plant (Part used)	alk	flav	terp	tan	sap	ster	phen
P. angolensis **(B)**	-	-	++	++	+	+	++
P. africanum **(R)**	-	+++	++	++	+++	++	+++
X. caffra **(R)**	-	+	++	+++	+	++	++
T. sericea **(R)**	-	+++	+++	+++	+++	++	+++
Mixture 1	-	+++	++	+++	+++	++	+++
Mixture 2	-	+++	++	+++	+++	++	+++
Mixture 3	-	+++	++	+++	+++	++	+++
Mixture 4	-	+++	+++	+++	+++	++	+++
Mixture 5	-	+++	++	+++	+++	++	+++

Key: - = absent, + = slightly positive, ++ = positive, +++ = highly positive, Alk = alkaloids, flav = flavonoids, tan= tannins, sap = saponnins, ster = steroids, phen = phenolics, B=Bark, R= Root.

3.4.3.2. Qualitative phytochemical analysis for aqueous samples

Aqueous samples did not contain as much phytochemicals as methanolic extracts. But water samples were also a rich source of phenolic compounds, flavonoids, terpenoids and they were also slightly positive for steroids and saponins, alkaloids were only slightly detected in *T. sericea.*

Table 3.5: qualitative phytochemical analysis results for aqueous extracts and their formulations.

Plant (Part used)	**alk**	**flav**	**terp**	**tan**	**sap**	**Ster**	**phen**
***P. angolensis* (B)**	-	-	++	++	+	+	++
***P. africanum* (R)**	-	++	++	++	+	+	++
***X. caffra* (R)**	-	+	-	+	+	+	++
***T. sericea* (R)**	-	+++	+++	++	+++	+	+++
Mixture 1	-	++	++	++	++	+	+++
Mixture 2	-	+	++	++	++	+	+++
Mixture 3	-	+++	++	++	++	+	+++
Mixture 4	-	++	++	++	++	+	+++
Mixture 5	-	++	++	+	++	+	++

Key: - = absent, + = slightly positive, ++ = positive, +++ = highly positive, Alk = alkaloids, flav = flavonoids, tan= tannins, sap = saponnins, ster = steroids, phen = phenolics, B=Bark, R= Root.

3.4.4. Antibacterial activity

Ciproflaxin was used as positive control and DMSO as a negative control for all tests, and tests were run in triplicates and the results were recorded as mean MIC. The highest concentration tested was 10mg/ml and the lowest concentration was 0.078mg/ml of all the plant extracts, formulations and the positive control. Mixture 1 had the same concentration of all plants, mixture 2 the highest concentration of *X. caffra*. Mixture 3 had the highest concentration of *T. sericea*. Mixture 4 had a higher concentration *of P. angolensis* and mixture 5 had the highest concentration of *P. africanum*. Most tested extracts and combination showed activity against all test bacteria. An MIC value of less than 0.1mg/ml was considered the most active.

3.4.4.1. Antimicrobial activity of plant extracts and their combinations against *Staphylococcus aureus* (methicillin resistant)

All the methanol extracts and their combinations showed better antimicrobial activity with the lowest MIC value of 5mg/ml. The water mixtures 2 and 3 showed the best antimicrobial activity with MIC value of 1.25mg/ml and as indicated in (**figure 3.1)** below:

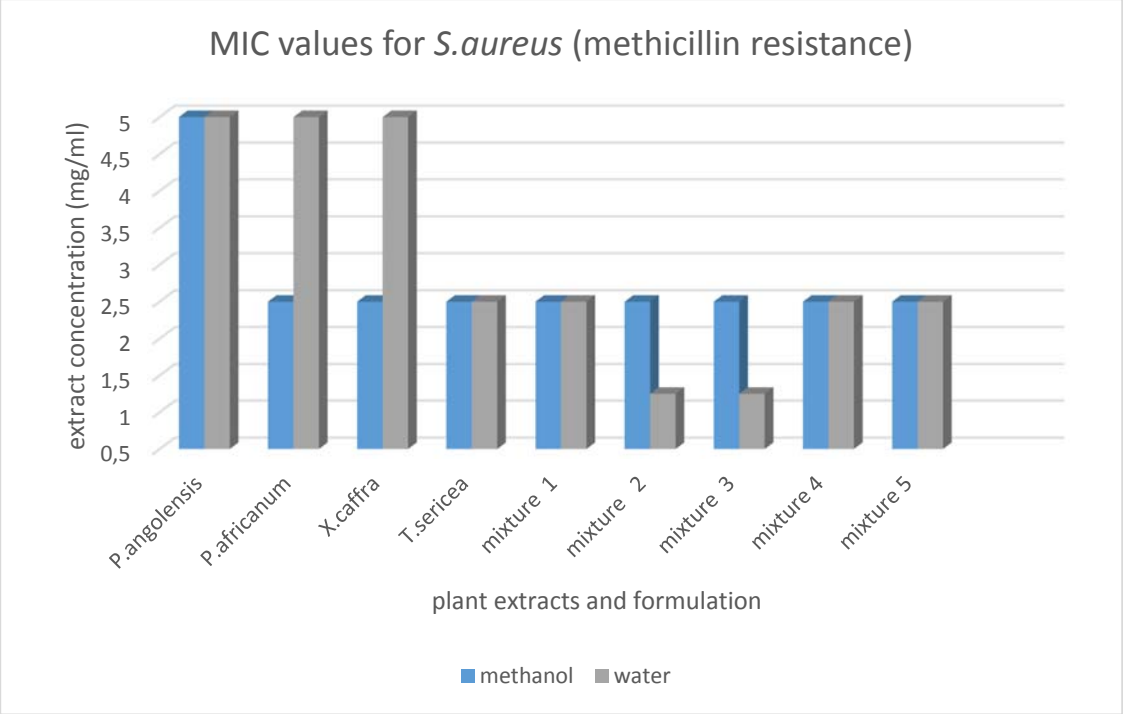

Figure 3.1: Mean MIC values of plant extracts and their formulations against *Staphylococcus aureus* (methicillin resistant). Positive control was ciprofloxacin with MIC value = 0.008mg/ml

3.4.4.2. Antimicrobial activity of plant extracts and their combinations against *Staphylococcus aureus* (methicillin susceptible)

All methanol extracts and their formulations as well as all water extracts and their combinations showed the best antimicrobial activity against test organism with MIC values ranging between (2.5mg/ml to 0.078mg/ml) and (5mg/ml to 0.078mg/ml) respectively. Moreover, both T. sericea methanol and water extracts showed the best antimicrobial activity against the tested bacterium as compare to the other tested individual extracts **(figure 3.2).**

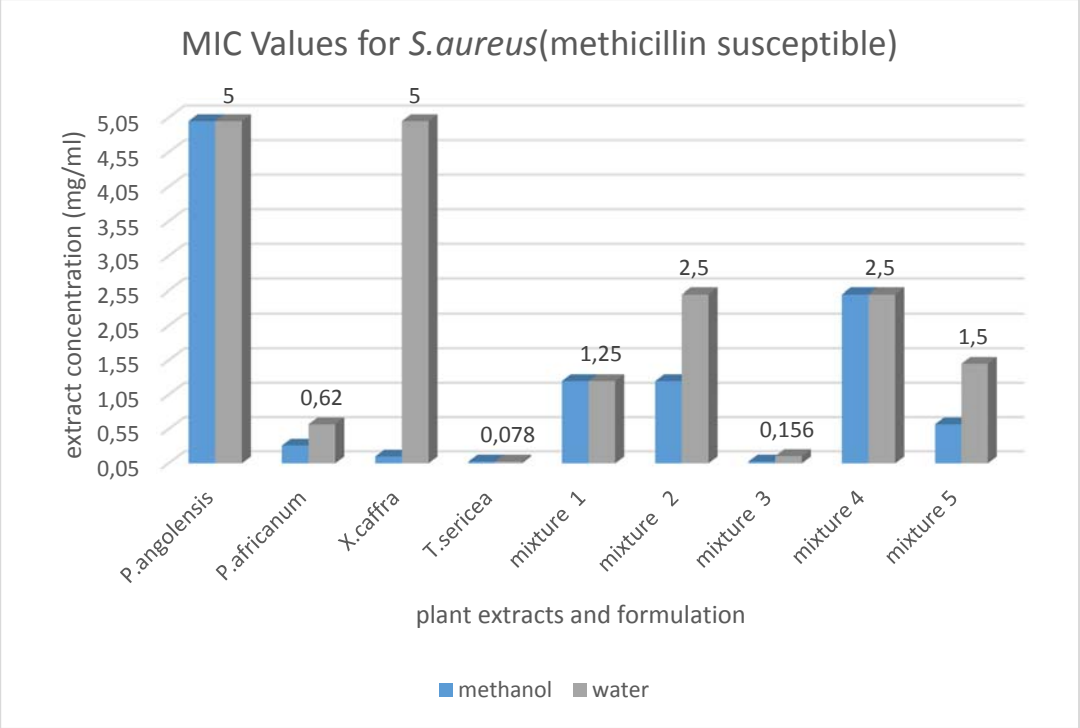

Figure 3.2: Mean MIC values of plant extracts and their formulations against Staphylococcus aureus (methicillin susceptible). Positive control used was ciprofloxacin with MIC value = 0.008mg/ml.

3.4.4.3. Antimicrobial activity of plant extracts and their combinations against beta lactamase producing *Escherichia coli*

Individual methanol extracts and their combinations showed the best antibacterial activity against *E. coli* with MIC values ranging between (2.5mg/ml – 0.156mg/ml) with methanolic mixture 3 having the best antimicrobial activity followed by water extracts and their combination except for *P. africanum* which showed little activity (**figure 3.3**).

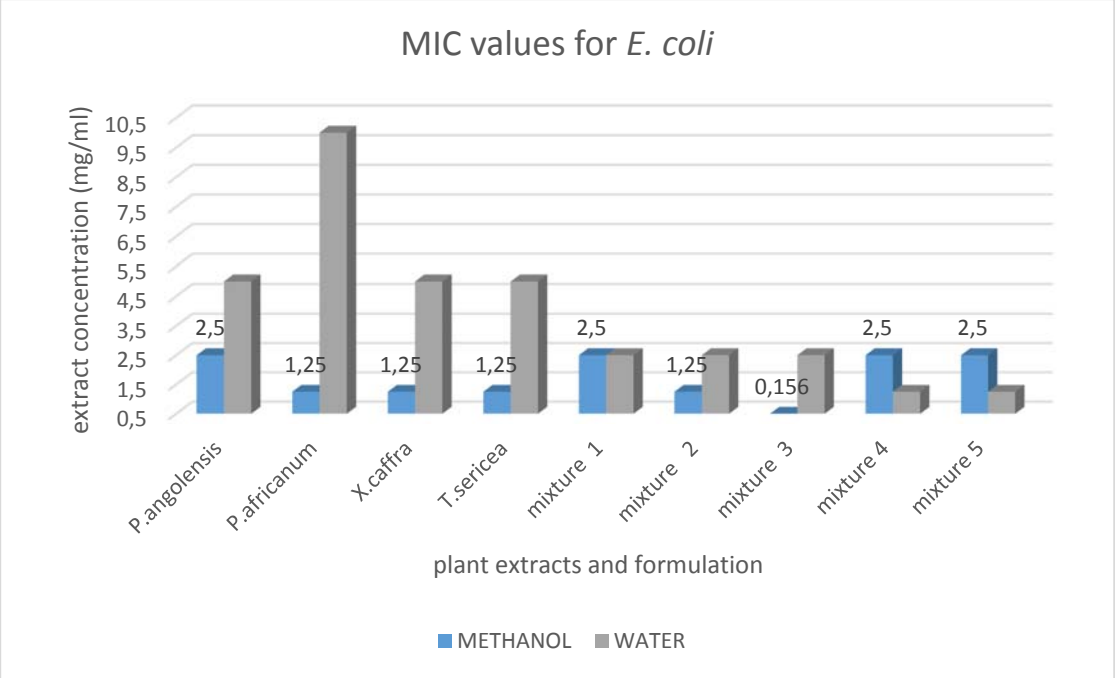

Figure 3.3: Mean MIC values of plant extracts and their formulations against *E. coli.* Positive control used was ciproflaxin with MIC value =0.008mg/ml

3.4.4.4. Antimicrobial activity of plant extracts and their combinations against *Klebsiella pneumoniae*

Methanolic extract of *T. sericea* showed the best antimicrobial activity against *K. pneumoniae* with MIC value of 0.65mg/ml, methanolic mixture 3 was the most active with MIC 0.65mg/ml amongst all the mixtures. Whilst methanolic *P. angolensis* extract was the least active with MIC = 5mg/ml **(figure 3.4)** below.

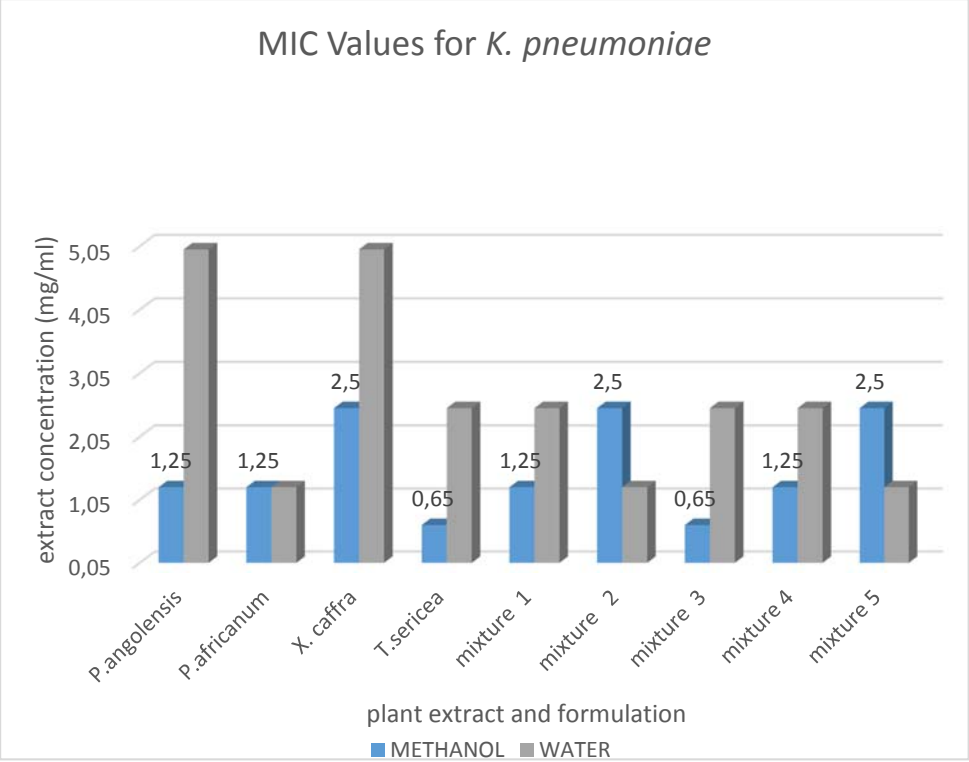

Figure 3.4: Mean MIC values of plant extracts and their formulations against *Klebsiella pneumoniae*. Positive control used was ciproflaxin with MIC value =0.008mg/ml

3.4.5. Minimum bactericidal concentration

The MBC was determined by inoculating the contents of the MIC plate into a nutrient agar plate and the results were observed after 24h incubation at 35ºC. No plant extract or formulation exhibited an MBC less than 1mg/ml **(Table 3.6)**. However, the highest MBC were observed at methanolic mixture 3 with MBC = 1.25mg/ml against MRSA and MSSA and *P. africanum* at MBC =1.25mg/ml against *E. coli.* Most aqueous extracts and formulations did not show any bactericidal activities.

Table 3.6: Minimum bactericidal concentration of plant extracts and formulations against test bacteria.

Extract and plant part used	**MBC methanolic extracts (mg/ml)**				**MBC aqueous extracts (mg/ml)**			
	MRSA	MSSA	Kp	E.c	MRSA	MSSA	Kp	E.c
P. angolensis (B)	10	10	-	2.5	-	-	-	-
P. africanum (R)	10	2.5	5	1.25	-	5	10	-
X. caffra (R)	-	1.25	2.5	2.5	-	10	2.5	-
T. sericea (R)	-	1.25	1.25	2.5	10	5	1.25	-
Mixture 1	2.5	2.5	2.5	2.5	-	5	2.5	-
Mixture 2	2.5	2.5	2.5	2.5	-	5	2.5	-
Mixture 3	1.25	1.25	2.5	2.5	-	5	2.5	-
Mixture 4	2.5	2.5	2.5	2.5	-	5	2.5	-
Mixture 5	-	2.5	2.5	2.5	-	5	2.5	-

Key: B=Bark, R=Root, MRSA= Methicillin resistant *Staphylococcus aureus*, MSSA=methicillin susceptible *Staphylococcus aureus*, Kp= *Klebsiella pneumoniae*, E.c= *Escherichia coli.*

3.4.6 Fractional inhibition index (FICI)

Out of all the ten formulations that were tested, 1 synergy was seen in mixture 3 against *E. coli*, 25 additive interactions between the mixtures were obtained and only 14 antagonistic interactions were detected mixtures. Both the methanolic mixtures and water mixture were mostly antagonistic against MSSA and *E. coli* as indicated in table 3.7 below:

Table 3.7: Combination effects between four medicinal plants formulations.

Test Bacterium	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Mixture 5
Methanol samples					
MRSA	2.5(A)	3.5(A)	3.5(A)	3.5(A)	3.5(A)
MSSA	8.33(D)	28.63(D)	1.78(A)	8.8(D)	14.20(D)
E.coli	7 (D)	5(D)	0.42 (S)	7(D)	7(D)
K. pneumoniae	4.48(A)	8,8(D)	2.56(A)	3.52(A)	8.8(D)
Water samples					
MRSA	2.5(A)	1.5(A)	1.25(A)	2.5(A)	2.5(A)
MSSA	28.63(D)	37(D)	2.22(A)	37.09(D)	21.66(D)
E.coli	1.75(A)	1.75(A)	1.75(A)	0.87(A)	0.87(A)
K.pneumoniae	4(A)	2.25(A)	4(A)	3.5(A)	1.75(A)

Key: MSSA= methicillin susceptible *S. aureus,* MRSA= Methicillin resistant *S. aureus,* A=Additive, S = Synergy, D= Antagonistic

3.4.7. Antifungal activity

Clotrimazole was used as positive control for all antifungal tests and DMSO as a negative control, tests were run in triplicates and the results were recorded as mean MIC. Similarly to the antibacterial tests, the highest concentration tested was 10mg/ml and the lowest concentration was 0.078mg/ml. Mixture 1 had the same concentration of all plants, mixture 2 the highest concentration of *X. caffra*. Mixture 3 had the highest concentration of *Terminalia sericea*. Mixture 4 had a higher concentration of *P. angolensis* and mixture 5 had the highest concentration of *P. africanum.* Most tested extracts and combination showed low activity against the tested fungal organisms except for *T. sericea* water and methanol extracts as well as mixture 3 which showed high activity against *C. ablicans*. An MIC value of less than 0.1mg/ml was considered the most active. Although some of the extracts and combinations were fungistatic against tested fungal organisms, none of them were fungicidal against the tested fungi.

Table 3.8: Minimum inhibitory concentration of plant extracts and formulations tested against four *Candida spp* and one *Cryptococcus neoformans.*

Methanol samples	**Fungal organisms**				
Plant extracts and formulation	*C. abl*	*C. par*	*C. kru*	*C. neo*	*C. trop*
P. angolensis (B)	_	-	_	-	-
P. africanum(R)	_	-	_	10	-
X. caffra (R)	10	-	_	-	10
T. sericea(R)	5	-	5	10	5
Mixture 1	_	-	10	-	-
Mixture 2	_	-	10	5	-
Mixture 3	5	-	5	5	5
Mixture 4	_	-	10	-	5
mixture 5	_	-	10	-	5
Water samples					
P. angolensis(B)	-	-	-	-	-
P. africanum (R)	-	-	-	-	-
X. caffra (R)	-	-	10	-	-
T. sericea (R)	-	5	-	5	-
Mixture 1	-	5	10	10	10
Mixture 2	-	-	10	10	-
Mixture 3	5	5	10	10	10
Mixture 4	-	-	10	10	-
mixture 5	-	-	-	-	-

Key: B=bark, R= Root, C. abl=*C. ablicans*, C. par= *C. parapsilosis*, C. kru = *C. krusei*, C. neo = *Cryptococcus neoformans*, C. trop=*C. tropicalis.*

3.5. DISCUSSION

There are many studies which have been conducted over the years by researchers worldwide on the antimicrobial activity of different medicinal plants. Such research has led to the discovery of different drugs that are used to cure different diseases, for example, morphine was isolated from opium which was produced from cut seed pods of *Papaver somniferum* commonly known as the poppy plant (Katiyar *et al*., 2012). However, most research work focusses mostly on individual plants than when they are mixed. As previously suggested by Fabry *et al*., (1998) the useful potential activity for crude extracts is when MIC < 8mg/ml. however, this study considered the suggestion by Gibbons, (2005) and Steenkamp *et al*., (2007) which outlined that a phytochemical with MIC < 1mg/ml has good antimicrobial activity was followed.

As indicated, water mixtures 2 and 3 showed the best antimicrobial activity against the MRSA both with MIC=1.25mg/ml and both mixtures were additive, this is due to the fact that the mixtures had highest concentration of *X. caffra* (R) and *T. sericea* (R) respectively. These plants have been documented to have the best antimicrobial activities. Ndhlala *et al.,* (2011) showed that the MIC for *X. caffra* roots against *S. aureus* was 3.125mg/ml for water extract and 0.195mg/ml for ethanol extract. In this, study, however, aqueous *X. caffra* roots exhibited an MIC value of 5mg/ml and aqueous *T. sericea* roots MIC was 2.5mg/ml against *S. aureus*. Even though the individual extracts showed low activity, mixture 2 and 3 showed better results and thus additive interactions between the plants was observed. This shows good additive interactions between the phytochemicals in both mixtures. These results are also confirmed by the presence of all tested phytochemicals known to exhibit excellent antimicrobial activity, in both mixtures except for alkaloids.

In this study, most water samples showed very good activity against *S. aureus* (Methicillin Susceptible). Methanolic mixture 3 showed excellent results against the test bacterium with MIC of 0.039mg/ml less than the positive control ciprofloxacin with MIC of 0.078mg/ml. Moreover, Even though it has been reported that water extracts do not work, (Luseba *et al*., 2007) water mixture 3 also showed the best results equal to the positive control. However, methanol mixture 3 showed the best overall, this can be due to the fact that mixture higher concentration of *T. sericea* which is known to have triterpenoids with antibacterial activity (Masoko *et al.,* 2005). Furthermore, methanol mixture 3 showed MBC of 5mg/ml and 1.25mg/ml respectively against both MRSA and MSSA, this is mainly because *S. aureus* is a gram- positive bacteria which have a protective outer membrane acting as a barrier against the surrounding environment, therefore, it is not easy for the extract to completely kill the bacterium (Munckhof *et al.,* 2003). Methanol *P. africanum* also showed excellent MIC of 0.31mg/ml against MSSA. Methanolic mixture 3 showed additive interaction against MSSA while the others were antagonistic.

Escherichia coli is a Gram negative bacteria, part of typical gut microbiota in humans and warm-blooded animals, and it is disseminated to the natural environment directly by faeces (Osinska *et al.,* 2017). Gram negative bacteria are considered to be more resistant to antibiotics, however, in this study all tested medicinal plant or formulation showed good antimicrobial activity against *E. coli* with methanolic mixture 3 showing an MIC of 0.156mg/ml, this was the only synergistic interaction found in this study. Although no extracts or formulation was bactericidal against *E. coli*. Nevertheless results obtained for *K. pneumoniae* which is also a gram negative bacterium were different from *E. coli*. Methanol mixture 3 exhibited the best antimicrobial activity with MIC of 0.65mg/ml with MBC of 2.5mg/ml and methanolic *T. sericea* with the same MIC, this was the best for all tested extracts and formulations for this

test bacterium. All tested extracts showed some activity against all tested bacterial strains, however, not all showed excellent antimicrobial activity.

Besides the burden of bacterial infections, fungal infections also pose a health hazard on humans especially those with compromised immune system such as those living with HIV/AIDS (Steenkamp *et al*., 2007). Fungal organisms such as *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. parapsilosis* causing candidiasis and *Cryptococcus spp* C. *neoformans* causing cryptococcosis have been emerging over the years causing fungal opportunistic infections that are usually resistant to drugs (Samie *et al*., 2010). In this study, most tested extracts showed no activity against the fungal organisms with MIC >1mg/ml. Methanolic extract of *T. sericea* showed better antifungal activity against *C. krusei, C. ablicans* and *C. tropicalis* (5mg/ml). *Candida Spp* also emerged as important causative agents of oropharyngeal candidiasis and candidemia and have shown to be the most resistant to many antifungal drugs (Mulaudzi *et al*., 2012). A study by Samie and Mashau, (2013) discovered that acetone extracts *T. sericea* bark were fungicidal to at least one of the *Fusarium spp*. tested at concentrations between 0.06 and 7.5mg/ml. Even though most tested extracts in this study showed no activity between 5mg/ml to 0.078mg/ml. Some formulations showed activity against some *Candida spp* and *Cryptococcus spp*, however, the MIC values were considered not active. This observations also suggested synergy between the phytochemicals in the formulations. Of all the tested extracts, those that showed promising results were Methanolic *T. sericea and P. africanum.* This is similar to the findings of Samie *et al*., (2010) who discovered that the acetone extract of *T. sericea* inhibited the growth of *C. ablicans* and *C. krusei* and didn't inhibit *C. neoformans.*

Methanolic *P. africanum* root extract exhibited a MIC value of 10mg/ml against *C. neoformans.* This is in agreement with the findings of (Steenkamp *et al*., 2007; Okeleye *et al*., 2013). However, Mulaudzi *et al*., (2012) found that *P. africanum* bark water and ethanol extract exhibited a MIC 1.56mg/ml and 3.125mg/ml against *C. ablicans* respectively with MFC

12.5mg/ml and 3.125mg/ml respectively. In this study, both the water and methanol root extracts of *P. africanum* showed no activity against *C. ablicans, C. krusei*, *C. parapsilosis, C. tropicalis. P. angolensis* bark extract also showed no activity against all tested fungal species, but, a study by Mulaudzi *et al*., (2012) showed water extract had MIC of 3.125mg/ml and MFC of 12.5mg/ml, the differences in the results could be due to the season of plant collection and the environment where plants were collected. Methanolic *X. caffra* root extract showed inhibition of fungi *C. ablicans* and *C. tropicalis* at 10mg/ml. A study by Samie *et al*., (2010) showed that the acetone root extract was active against *C. ablicans* and *C. krusei* and *C. neoformans* with MIC of 0.94mg/ml for *Candida* spp and 3mg/ml for *Cryptococcus* spp.

The results observed in the present study suggests that the solvents methanol and water did not extract all the phytochemicals that are of high antifungal properties. Lastly, mixing the extracts together increased the therapeutic effects of the medicinal plants, even those that were not active individually, showed some activity in the mixtures with MIC ranging between 2.5mg/ml to 10mg/ml. Overall, the tested extracts and mixtures were fungistatic against the fungal organisms and none were fungicidal.

The antimicrobial activity of the plant extracts and formulations was confirmed by the high presence of total phenolics and flavonoids. In this study, phenolic content that are greater than 5mg/ml of gallic acid equivalent were considered as most active. As previously reported, it was observed that the solvent of extraction played a major role in the total phenolic content of the plants (Malualem *et al.,* 2011; Ogunmoyole *et al.,* 2013). Solvent difference in the extraction of plants photochemical is more obvious when the plant is supposed to be used for therapeutic purposes. However, many traditional healers have little knowledge on this aspect and they usually use water for the extraction of medicinal plants.

Methanolic samples in the study displayed the best total phenolic content than most water samples. Specifically, the highest total phenolic content was observed in the methanolic mixture 3 and methanolic mixture 4 with 11.85mgGAE/g and 10mgGAE/g respectively, this showed the good interactions between the extracts phenolics in this mixtures. One - way ANOVA revealed significant differences among methanolic extracts and their mixtures as well as aqueous samples and their mixtures ($p < 0.05$). Also, there were significant differences between most aqueous and methanol samples ($p < 0.05$), however, there were samples which showed no differences between different species, the methanolic *P. angolensis* extracts and *P. africanum* had little differences. Some factors that could result in similarities of the phenolic content of the different plants could be factors such as season, cultivation, physiological and the environment where the plants were collected (Machu *et al*., 2016).

Lastly, it was also observed that in some mixtures with higher yield of a particular plant extract, no statistical significance was observed between the plant extract and the mixture ($p < 0.05$). This was seen in aqueous mixture 3 (7.52mgGAE/g) and aqueous *T. sericea* (7.73mgGAE/g) extract. Furthermore, aqueous mixtures 1, 2 and 3 also showed no differences amongst themselves and they had little differences to methanolic mixture 3. This could be due to the fact that they contained the same type of extracts which possessed similar amounts of phenolics. Overall it was observed in this study that solvent variation amongst samples extraction had no impact on the amount of total phenolics found. However, mixing plant extracts yielded better phenolic content.

Flavonoids have been recorded as important phenolics occurring in fruits, vegetables, and plants foods (Manach *et al*., 2004) flavonoids are known to be highly potent antioxidant compounds that help reduce the risk of cardiovascular diseases such as strokes, heart failure, cancer and diabetes (Ghasemzadeh and Ghasemzadeh, 2011). Furthermore, they also possess antimicrobial, anti-allergic anti-ulcer anti-inflammatory, enzyme inhibition, and vasodilator

properties (Shohaib *et al*., 2011). a flavonoid Quercertin used as a standard in this study was isolated from whole plant ethanolic extract of *W. indica* and independently inhibited the production of inflammatory mediator nitric oxide (NO), cytokines (TNF)-α and interleukin (IL)-12, in lipopolysaccharide and interferon activated murine peritoneal macrophages, without any cytotoxicity (Rao *et al*., 2005).

Similar to the results obtained with phenolic content, methanol samples showed the best flavonoid content with as compared to water extracts. Moreover, methanol mixture 4 showed the best overall flavonoid content. One-way ANOVA Turkey test showed that there were significant differences between all tested samples ($p < 0.05$), although there were samples which showed little variances. Similarly to total phenolic content, the different concentrations of the plant extracts used in the mixtures all yielded positive results. The methanolic *X. caffra* extract behaved similarly to methanolic mixture 1, even though, mixture 1 contained the same concentration of all extracts. This could be due to the fact that *X. caffra* is the additive herb in mixture 1. *T. sericea* showed the highest flavonoid content 2.59±0.0179mgQE/g, and the sample with lower flavonoid content was mixture 2 with total flavonoid content of 0.763±0.0100mgQE/g. One – way ANOVA in this study pointed out that even though the extracts were mixed at different concentrations, they yielded similar amounts of flavonoids. Furthermore, a good interactions between flavonoids was seen in aqueous mixture 3 which yielded higher results than all mixtures suggesting good interactions of flavonoids in the mixture, because flavonoids are known for their good antimicrobial activity, the results obtained in this study are in agreement with this statement. Lastly, the lower contents of flavonoids in aqueous mixture 2 could be due to the fact that the ratio of the extracts in this mixture is not synergistic, thus, the higher contents of flavonoids were reduced through mixing the extracts together.

The qualitative results for the phytochemicals screened for methanolic extracts and mixtures are being reported in the present study. Besides the fact that all mixture showed antibacterial activity. They also showed best phytochemical content during the analysis. Tested plant extracts exhibited 90 % flavonoids, 80% of alkaloids, 100% saponins, terpenoids, tannins and steroids. Methanol solvent showed the best phytochemical content**.** Methanolic *T. sericea* showed the presence of all tested phytochemicals. Methanolic *T. sericea* high antimicrobial activity is suggested by the high presence of saponins, tannins and terpernoids as previously reported (Bombardelli *et al*., 1974; Clark and Hufford, 1993; Bruneton, 1995; Steenkamp *et al*., 2004; Tshikalange *et al*., 2008), this is in line with our findings in this study. All methanolic extracts and mixtures contained higher presence of tannins. Tannins are known to selectively inhibit HIV replication. A study by Kurapati *et al.,* (2016) discovered that the oleanolic acid from the methanolic extracts of *Xanthoceras sorbifolia* showed inhibition of HIV-1.

Furthermore, tannins are important antimicrobials because they may prevent the development of microorganisms by triggering microbial protein and depleting nutritional proteins (Kurapati *et al*., 2016). This is confirmed by the good antibacterial activity of the extracts and mixtures with higher tannin contents.

Saponins present in the plants are also important therapeutically as they are shown to have hypolipidemic and anticancer activity are also necessary for activity of cardiac glycosides (Just *et al*., 1998; Fang *et al*., 2017). Alkaloids were not detected in this study. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity (Nobori *et al*., 1994; Tosun *et al*., 2009), and their absence in the plants used in this study lower the risk of poisoning by the plants. Alkaloids are known to be more soluble in alcohol and they are sparingly soluble in water, however, they were not detected in both aqueous samples and methanolic samples, their absence was good because it lowered the toxicity of the plants (Moslemizadeh *et al*., 2017).

3.6 CONCLUSION

The broad spectrum antimicrobial activity of some plants justifies the use of these medicinal plants in traditional medicine. *T. sericea* followed by *P. africanum* showed the best antibacterial activities. Even though *P. angolensis* and *X. caffra* were the least they exhibited their activity when they were in formulations resulting in synergistic effects with other plants.

It was also interesting to note that the activity of some of the plants was influenced by others although only one synergy was observed from the tested mixtures. Most mixtures showed additive interaction against test bacteria. The increased phytochemical content in formulations was seen to have a great impact in the effectiveness of the formulations against tested microorganisms. therefore the antimicrobial activity, phenolic content, flavonoid content, preliminary phytochemical analysis in this part of the study are in support with the hypothesis of the study.

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CHAPTER 4: ANTIOXIDANT ACTIVITY, ANTI-HIV ACTIVITY AND CYTOTOXICITY SCREENING OF PLANT EXTRACTS AND FORMULATIONS.

4.1. ABSTRACT

BACKGROUND: Medicinal plants form an important part of the Southern African cultural heritage. Indigenous populations, for example the Vha-Venda people, tend to use medicinal plants in formulations rather than western medicines for health and survival. The present study was aimed at assessing the antioxidant and cytotoxicity activities of four Venda medicinal plants and their formulations and to assess the anti – HIV activity of the medicinal plant formulations.

MATERIAL AND METHODS: *Peltophorum africanum* (roots), *Pterocarpus angolensis* (bark), *Terminalia sericea* (roots) and *Ximenia caffra* (roots) were collected all from the Thohoyandou area. The collected plant parts were extracted with methanol and water respectively. From the Individual plant extracts, five formulations were designed per solvent used. All the plant extracts and their formulations (a total of 18) were assessed for their antioxidant ability scavenge free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) using a spectophotometer. The toxicity of the 18 plant samples against human lymphatic endothelial cells (HLEC) was determined using the CellTiter 96 Non-Radioactive Cell Proliferation Assay.

All 10 plant formulations were assessed for their anti-HIV activity using the Reverse Transcriptase Colorimetric Assay kit.

RESULTS: All plant extracts and formulations exhibited good antioxidant activity against DPPH, methanolic formulation showed the best antioxidant activity with IC_{50} 0.094 ± 0.33µg/ml. Some extracts showed toxicity, aqueous *X. caffra*, mixture 2 inhibited 26% and 51% at 12.5mg/ml and 3.125mg/ml respectively. Methanolic *P. africanum* and mixture 5 inhibited 34%, 54% and 43% at 3.125mg/ml, 6.25mg/ml and 12.5 mg/ml respectively of Human Lymphatic Endothelial cells growth. For anti- HIV inhibition, all formulations at 200µg/ml exhibited higher percentage of HIV-1 reverse transcriptase inhibition with methanolic mixture 3 being the best overall at 97.5% activity whilst aqueous mixture 5 was the least active with 63.03% inhibition activity. Moreover, the best anti-HIV activity at 100µg/ml was exhibited by methanolic mixture 3 at 71% inhibition.

DISCUSSION AND CONCLUSION: The results from the study indicated that most of the commonly used traditional medicinal Plants in the Venda region when mixed together have merit for use in traditional medical practice as they have shown good antioxidant activities, good cell proliferation activities and good anti-HIV activities. However, some formulations showed toxicity against HLEC, therefore, an experienced personnel is needed for preparation and the administration of such plants since they pose a health threat to immune cells. Moreover, mixing plant extracts resulted in reduced toxicity of individual plants.

***Key words**: antioxidant, toxicity, anti –HIV, formulation*

4.2. INTRODUCTION

An antioxidant is a molecule that inhibits the oxidation of other molecules (Govindappa *et al*., 2011). Epidemiology and experimental studies have implicated oxidative cellular damage arising from an imbalance between free radical generating and scavenging systems as the primary cause of cardiovascular diseases, aging , cancer, DNA damage ,etc. (Govindappa *et al*., 2011). Free radicals like reactive oxygen species (ROS) and nitrogen reactive species (RNS) are produced within the human body due to different biochemical processes such as metabolism, they are also introduced into the body from external environmental sources such as UV light, radiation, smoking, microbes, stress and unhealthy food leading to diseases (Mangalo, 2013). In vivo antioxidants protect the cell against the damaging effects of reactive oxygen species (Cheng, 2006).

Antioxidants such as enzymatic antioxidant glutathione and non-enzymatic antioxidant such as nitric oxide (NO) exist within the human body and are responsible for scavenging free radicals within the body. However, ROS have the ability to counteract the action of these free radicals leading to diseases. Therefore, there is a need to be supplemented by making use of natural exogenous antioxidants such as vitamin C and E which are derived from dietary sources like fruits, vegetables and teas (Li *et al*., 2002; Mishra *et al*., 2011). Phytochemicals such as flavonoids, alkaloids, cardiac glycosides, saponins, tannins, etc. are known to contribute towards the antioxidative effects of the medicinal plants. They have the ability to delay or prevent an antioxidative reaction that is catalysed by free radicals (Biapa *et al*., 2007).

For decades, HIV/ AIDS has been a global concern that affects almost half of the population of people in the whole world. It was recently reported that the global situation shows that HIV/AIDS is the leading cause of death in Sub - Saharan Africa and the fourth leading cause

of mortality worldwide (Hall and Strang, 2017). HIV infections increases the production of free radicals (RIOs) leading to oxidative stress and subsequently AIDS (Morris *et al*., 2012). Some medicinal plants have been documented to maintain the health and vitality of individuals and also cure diseases because of the phytochemicals which they naturally produce that possess antioxidant activity (Chigayo *et al*., 2012). Phytochemicals such as flavonoids, phenolic acids, polyphenols and condensed tannins are documented as essential antioxidants (Diem Do *et al*., 2014). To date, the search for plant produced alternative medicine that can ease the burden of HIV/ AIDS worldwide is still of a great interest.

The use of medicinal plants to treat different diseases has gained a lot of interest worldwide. Besides being extremely expensive pharmaceutical drugs are only designed to elicit specific reactions however their side effects are said to be risk factors against the benefits of the primary effect (Nasri and Shirzad, 2013). Unlike pharmaceuticals, medicinal plants are believed be easily accessible, affordable, and harmless (Nasri and Shirzad, 2013). However, toxicity of medicinal plants needs to be studied.

Although medicinal plants are regarded as harmless, some medicinal plants needs to be taken with caution since some cause adverse reaction especially when taken in adverse concentrations, or concurrently with pharmaceutical drugs (Lakmichi *et al*., 2011). Research shows that the presence of alkaloids, saponnins and tannins in medicinal plants have produced excellent cytotoxic effects against many cancer cells, however the same plants can be toxic to immune cells (Tamilsevan *et al*., 2014). Poly-herbal combinations of medicinal plants are also believed to have better therapeutic effects than individual plants. However, can also have damaging effects on the immune system. There is therefore still a great need to screen such medicinal plants for toxicity activities against normal cells.

4.3. MATERIALS AND METHODS

4.3.1. Free radical scavenging activity (DPPH)

In order to test the free radical scavenging ability of the plant extracts and formulations, samples were prepared as illustrated in chapter 3 **(table 3.1)**. The study adopted the method described by Du Toit et al., (2001). Free radical ascorbic acid was used as a control in order to compare the activity of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) which is a free radical that can be reduced by an antioxidant, DPPH was prepared by dissolving 5 mg in 50 ml ethanol to give a stock concentration of 0.05 mg/ml. Briefly, The 96 well plate was filed with 100 µl of distilled water. In all the well in first row 100 µl of each extract and formulations were added. The concentration of the first row was 2.5mg/ml and the last row was 0.078mg/ml after serial dilution. Blank controls were prepared by adding ethanol to distilled water and negative controls also had test samples and distilled water. These were serially diluted from the first row to the last row. A volume of 200 µl of DPPH was added in all the wells except the negative control. The mixture was allowed to stand in dark for 30 minutes. All the samples were tested in triplicate. The absorbance were read in a Versa max microplate reader at 517 nm. The percentage (%) of radical scavenging was calculated by the following formula:

$$\%\ \text{free radical scaven.} = \frac{\text{Absorbance of DPPH} - \text{Absorbance of sample}}{\text{Absorbance of DPPH}}\ \text{X}\ 100$$

4.3.1.1. Statistical analysis

All experiments were carried out in triplicate and are expressed as average of three analyses ± standard deviation. Statistical software package (SPSS for Windows, Version 21.0). One –way

ANOVA (Duncan's Test) was used to calculate the correlation between variables. Differences between groups were considered significant when $p < 0.05$.

4.3.2. Anti - HIV activity

4.3.2.1. Preparation of extract

About 6mg of each of the extracts was dissolved in 1ml of DMSO to make a stock solution of 6mg/ml. from the stock solution, five methanol extracts mixtures and five water extracts mixtures were prepared the same way as illustrated in **chapter 3 (Table 2.1)**. A volume of 10µl of the prepared mixtures was added to 90µl of lysis buffer to make a final concentration of 0.6mg/ml which was used in the test.

4.3.2.2. Preparation of reagents

1. **Enzyme preparation**

About 250µl of distilled water was added into 500ng enzyme solution and the solution was divided into four equal aliquots. The three aliquots were stored for later use and one aliquot was diluted with 1ml of lysis buffer and mixed thoroughly.

2. Preparation of the reaction mixture

The template [primer hybrid poly (A). Oligo $(dT)_{15}$ ($9A_{260nm}$/ml), lyophillizate] was reconstituted in 430µl of autoclaved redistilled water. 1ml of incubation buffer was added to a nucleotide vial and then 100µl of reconstituted template was added to the diluted nucleotide solution to obtain a reaction mixture.

4.3.2.3. HIV-1 reverse transcriptase inhibitory bioassay

The effect of both methanol and water plant mixtures against RT was assessed using a non-radioactive HIV-1 RT colorimetric ELISA kit obtained from Roche Diagnostics (Germany) following the manufacturers protocol. The principle of the kit is as illustrated on **Figure** 4.1 below:

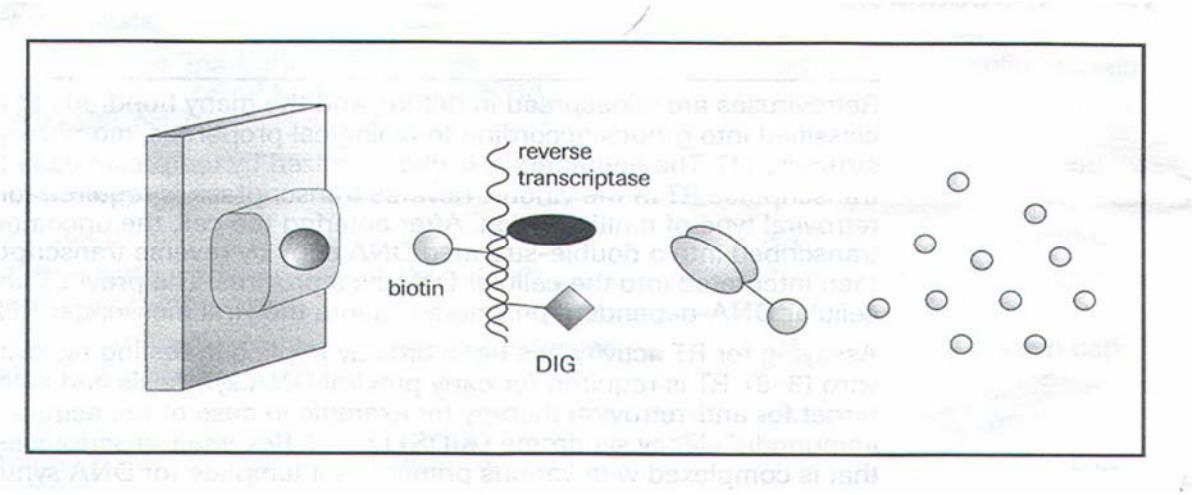

Figure 4.1: reverse transcriptase colorimetric assay principle (Roche, 2002; Tshikalange, 2007).

Briefly, A 96 microtitre well plate was used for the experiment, in which 20µl of the enzyme and 20µl of the reaction mixture and 20µl of the plant mixtures were added to one well for each sample (making the final concentration of the plant formulations to be 200µg/ml. For control

A, 40µl of the lysis buffer and 20µl of the reaction mixture was added to another well and the control B well contained 20µl of the enzyme, the same volume of lysis buffer and reaction mixture for the reaction to occur. The plate was incubated for 1 hour at 37ºC. Following incubation period, the samples were transferred from the microtitre plate to a microtitre module (precoated with streptavidin and postcoated with blocking reagents). The plate was incubated for 1 hour at 37ºC. After the incubation period, the solution was completely removed from the plate and the plate was washed 5 times with 250µl of wash buffer. Approximately 200µl of anti-DIG-POD (a combination of the antibody and the conjugate dilution buffer) working solution was added to each well followed by one hour incubation at 37 ºC. After the incubation period, the negative control was treated with 40 µl of lysis buffer and 20µl of the reaction mixture. The solution was removed completely and the plate was rinsed with washing buffer 5 times. ABTS substrate solution (2 ABTS tablets were dissolved in 10ml substrate buffer to prepare the solution) was then added to all the wells and the absorbance was measured @405nm with a reference wavelength of 490nm after 10 minutes. All tests were run in triplicates**.** The results were analysed using formula**:**

$$\text{Percent inhibition} = 100 - \frac{\text{Mean sample Absorbance}}{\text{Mean control Absorbance}} \text{ X } 100$$

4.3.3. Toxicity assay

4.3.3.1. Preparation of complete medium for Human Lymphatic Endothelial Cell culture

Media was prepared according to ScienCell^{TM} Research Laboratories manufacturer's protocol. Briefly, the vials containing Endothelial Cell Growth Supplement (ECGS), fetal bovine serum (FBS) and penicillin/ streptomycin solution (P/S) were thawed at 37ºC. The tubes were gently

inverted several times to ensure that the contents are completely mixed before adding into the medium. The medium bottle and the vials were then sprayed with 70% ethanol and transferred to a laminar flow. The cap of the medium bottle was removed and ECGS, FBS and P/S were added into the medium and mixed well using a glass pipette. The medium was covered with foil to avoid light and stored at 4ºC.

4.3.3.2. Initiation and maintenance of culture

In a laminar flow, a culture was initiated by adding 500µl of the cells into 5ml of complete medium in a sterile cell culture flask to make a desired seeding density of 5000 cells/cm^2. The flask was observed under a microscope at 10X magnification and a picture was taken (f**igure 4.2 below).** The culture was incubated overnight without any disturbance at 37ºC with 5% CO_2. The following day, the supplemented medium was aspirated out, and the cells were supplied with 5ml fresh Endothelial Cell Medium (ECM). This process was repeated until the cells reached 70% confluency at day 7, then medium was changed every day until cells reached 90% confluency at day 10.

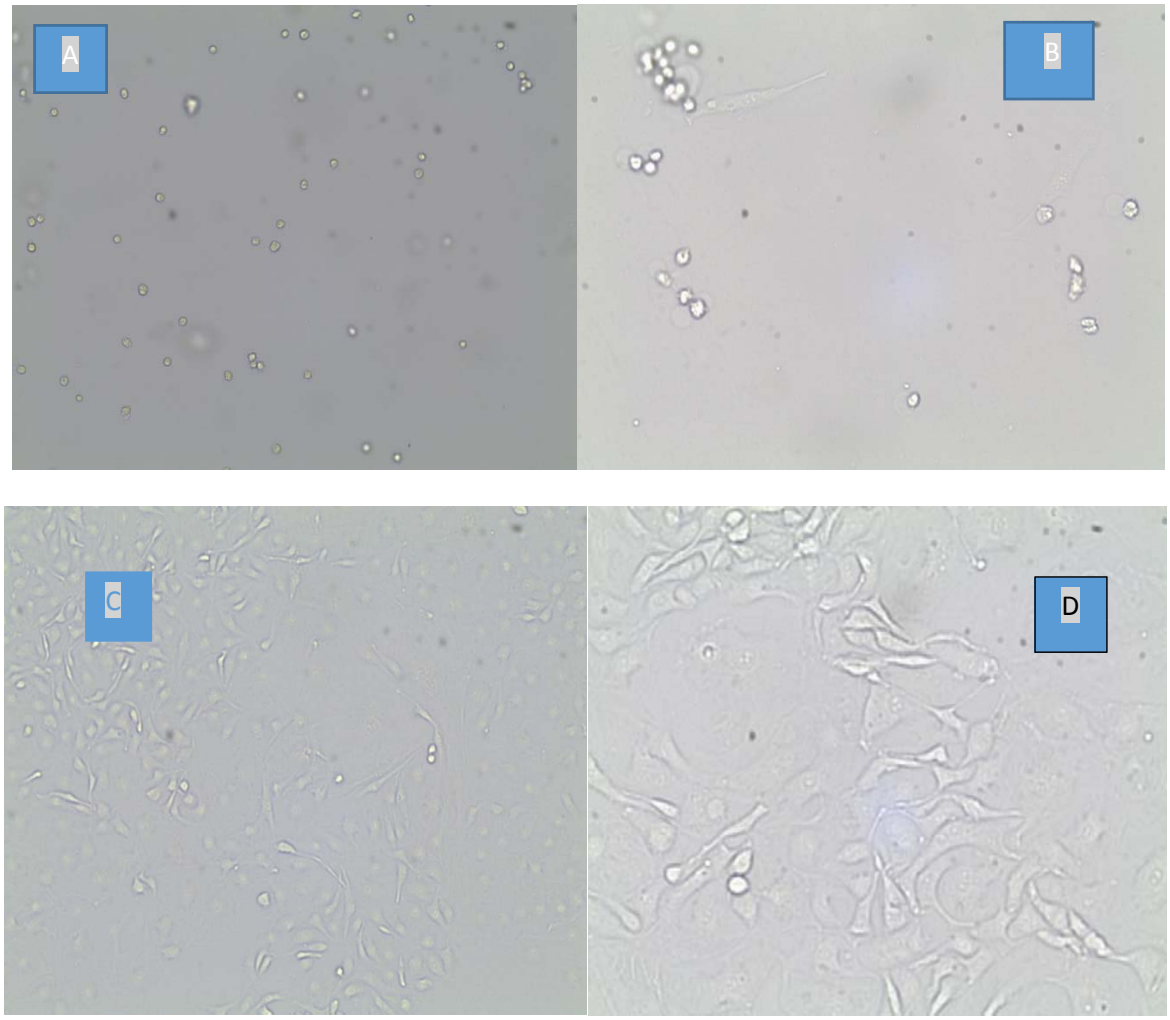

Figure 4.2: An image of the HLEC at 10x magnification at A= day 0, B= day 5, C =day 7 and D = day 10.

4.3.3.3. Cell count

The media were aspirated out of the flask and the cells were washed two times with 1X PBS-pH 7. About 2ml of trypsin was added into the flask to detach cells and incubated for 5 minutes at 37ºC with 5% CO_2. After cells have detached, 2ml of medium was added into the flask and the cell solution transferred into a tube and centrifuged (Heraeus Megafuge 16R Centrifuge,

Thermoscientific) at 1500rpm for 5 minutes. After centrifugation the supernatant was aspirated out and 1ml of medium was added to the pellet and mixed well. A TC10 automated cell counter (Bio-rad) was used to count the cells. Briefly, about 10µl of trypan blue dye was mixed with 10µl of cells. A drop from the mixture was placed in the chambers of the counting slides and automatically counted. Total cell counted was 1.05×10^6 cells/ml with the live cell count was 9.46×10^5cells/ ml. Then, 1ml of cell suspension was diluted with 19ml of complete medium to give a desired concentration of 5×10^4cells/ml which was used for viability assay.

4.3.4. Assay for Cytotoxicity

Toxicity of the extracts was evaluated on human lymphatic endothelial cells using CellTiter 96® Non-Radioactive cell proliferation assay (Promega, USA) following the manufacturer's protocol. The principle of the Cell viability assay is that viable cells produces formazan from a tetrazolium salt that is easily detected using a 96 well plate reader. Briefly, in a 96 - well plate, 50µl of the complete medium was added to all the wells. About 50µl of the plant extracts or formulations, hydrogen peroxide (1% H_2O_2) which was the negative control and cells only which was the positive control were added to the first raw wells with medium to make a final volume of 100µl. A series of dilutions of the plant extracts or formulation with reduced concentrations was performed to cover all the wells and the control wells. Serial dilution was performed by mixing the controls, plant extracts and formulations with the media in the first raw, 50µl of the solution from each column in first raw was taken and mixed with the media in the second column to give a concentration that is half the concentration of the first raw. The volume of all the wells was 50µl with reduced concentration from well to well in all the columns. Then 50µl of 5×10^4cells HLEC was added to each well with the treatment and controls and the plate was observed under a microscope at 10X magnification. The plate was

incubated for 48hrs at 35ºC with 5% CO_2. After 48hrs incubation, 15µl of the dye solution was added to all the wells and incubated again for 4hrs. After 4hrs, a stop solution was added to all the wells and the plate was incubated overnight at -4ºC. The following day, the plate was read at 570nm wavelength using an ELISA plate reader (Multiskan GO, Themoscientific). All tests were run in triplicates. The average absorbance values of plant extracts only at the same concentration as used in the test were used as blanks. The percentage of cell viability was calculated using the formula.

$$\%\ \text{cell viability} = \frac{\text{absorbance of sample} - \text{absorbance of blank}}{\text{absorbance of control} - \text{absorbance of blank}} \text{x}100$$

4.3.5. Statistical analysis

All data were captured in Microsoft® Excel spreadsheets, the average, standard deviation, and coefficient of variance were performed and the graphs were drawn using Microsoft Excel. Student t-test was used for comparison of results obtained. Differences in the data were considered significant when $P < 0.05$.

4.4. RESULTS

4.4.1. DPPH scavenging activity

Table 4.1 shows the 50% Inhibitory Concentration of the plant extracts and formulations needed for scavenging free radical DPPH **(** 1, 1-diphenyl-2-picrylhydrazyl) by donating a hydrogen or an electron and reducing DPPH to a more stable diamagnetic molecule.

Apparently, all methanolic extracts and their formulations and water extracts and their formulations exhibited potent DPPH free radical scavenging activity which was significant ($P < 0.05$). However, there was no significant differences between other water and methanol samples as indicated by One - way ANOVA followed by Duncans variance analysis (**Table 4.1**) where all values marked with the same letters showed little differences. The standard deviations were calculated to 3 significant figures and it measured the variability of the data sets which in this case were not that dispersed as shown by the small differences. Methanolic mixture 4 was showed the best overall antioxidant activity with IC_{50} value of 0.094 ± 0.33 µg/ml and the least activity was seen in aqueous *X. caffra* extract with IC_{50} value of 0.7077 ± 0.12 µg/ml.

Table 4.1: IC_{50} values (µg/ml) values of DPPH scavenging activity of both water and methanol extracts and their formulations. Results are expressed as mean and standard deviation of three different tests.

Plant extracts and formulation	**Methanol extracts**	**Water extracts**
Ascorbic acid (positive control)	0.225 ± 0.01	
P. angolensis	$0.367 \pm 0.46^{b,d,e}$	0.457 ± 0.21^{c}
P. africanum	$0.33 \pm 0.44^{b,d,e}$	$0.264 \pm 0.26^{a,b,d}$
X. caffra	$0.622 \pm 0.05^{f,g}$	0.7077 ± 0.12^{g}
T. sericea	0.161 ± 0.06^{a}	$0.192 \pm 0.004^{b,a}$
Mixture 1	0.167 ± 0.14^{a}	0.113 ± 0.18^{a}
Mixture 2	0.182 ± 0.03^{a}	$0.277 \pm 0.04^{b,d}$
Mixture 3	0.097 ± 0.31^{a}	0.205 ± 0.01^{b}
Mixture 4	0.094 ± 0.33^{a}	$0.515 \pm 0.20^{e,f}$
mixture 5	$0.475 \pm 0.08^{e,f,g}$	$0.606 \pm 0.08^{e,f,g}$

Values marked with the same letters show insignificant differences between the means ($P < 0.05$).

4.4.2. Anti-HIV activity

The results for anti-HIV activity of 10 water and methanol formulations are presented in **Table 4.2**. Mean absorbance values of all tested mixtures were used to calculate the percentage of inhibition. The ARV Combivir® (GlaxoSmithKline) at 3mg/ml was used as a positive control. All the samples that showed percentage inhibition of greater than 50% were considered as active (Tshikalange *et al*., 2007). However, Ndhala *et al*., (2010) considered samples with inhibition activity of greater than 70% to be the highly active against RT-inhibitory enzyme. In this study, sample with percent inhibition >70% were considered the most active. All the tested mixtures showed excellent HIV-1 reverse transcriptase enzyme inhibition at 200µg/ml.

Table 4.2: Percentage HIV-1 RT inhibitory activity of 10 medicinal plant formulations at 200 µg/ml concentration of plant formulation

sample name	**Methanol mixtures (%)**	**Water mixtures (%)**
mixture 1	97.4	94.8
mixture 2	96.16	88.3
mixture 3	97.5	97
mixture 4	97.2	95
mixture 5	95.8	63.03
Control A	97.37	
Control B	0	
Combivir (positive control)	30.11	

All samples that showed a percentage that was greater than 70% were tested again at a much lower concentration in order to validate the activity of the mixtures at low concentration of 100µg/ml. most mixtures showed higher inhibition activity that was greater 50% which is great according to Tshikalange *et al*., (2007). Methanolic mixture 3 showed the best results at 100µg/ml than all mixtures tested.

Table 4.3: Percentage HIV-1 RT inhibitory activity of 10 medicinal plants plant formulations at 100 µg/ml concentration of plant formulation.

sample name	Methanol mixtures at 100µg/ml	Water mixtures at 100µg/ml
mixture 1	64	57.69
mixture 2	55.5	69.23
mixture 3	71	59.4
mixture 4	14.95	23.07
mixture 5	29.27	44
Control A	97%	
Control B	0	
Combivir (positive control)	65%	

4.4.3 Cell viability assay

Cells only were used as a positive control and 1% H_2O_2 was used as a negative control during the experiment, most tested extracts and formulation promoted cell proliferation at many concentration tested. However, lower concentrations between 12.5µg/ml to 100µg/ml showed greater cell proliferation activities than higher concentration between 3.125mg/ml to 100mg/ml. Moreover, the promotion of cell viability by the treatment was concentration

dependant, some low concentrations promoted less HLEC proliferation than higher concentration for the same plant extract.

4.4.3.1. Percentage cellular viability of water extracts, methanol extracts and their formulations at concentration at lower concentrations (12.5µg/ml to 100µg/ml).

All the tested extracts and formulations were above the positive (100±3.039) and negative control (11±1.51) in percentage of cellular viability. Aqueous *P. angolensis* bark extracts promoted cell proliferation the most at 25µg/ml with percentage cellular viability (894±2.43) this was the highest for the extract. Furthermore, aqueous mixture 4 with higher concentration of *P. angolensis* also exhibited the highest percentage cellular viability of 2997±6.58 at 25µg/ml, however, methanolic *P. angolensis* extract showed a high cell viability at 12.5µg/ml at 666±0.60 and mixture 4 was also very active at 50µg/ml at 769±0.103 cellular viability.

Aqueous and methanolic *X. caffra* root extract were most active at 12.5µg/ml with cellular viability 851±14.59 and 667±1.31 respectively. The aqueous mixture 2 and methanolic mixture 2 with high concentration of *X. caffra* exhibited higher cellular viability of 883±12.46 and 794±14.48 at 25µg/ml. Aqueous and methanolic *T. sericea* exhibited the highest cellular viability of 1004±6.38 and 566±0.70 at 25µg/ml, however the aqueous mixture 3 formulation with high *T. sericea* content was less effective in cell proliferation. Methanolic mixture 3 showed good results at 50µg/ml with 830±71.52.

Aqueous *P. africanum* was more effective at 50µg/ml with cellular viability of 825±2.53 and the mixture 5 with high *P. africanum* content was more active at the same concentration with 2268±3.04. Methanol extract and mixture 5 was less active. Mixture 1, the formulation with

the same content of all the four plant extracts behaved similarly with Cellular viability 842±5.77 at the same concentration.

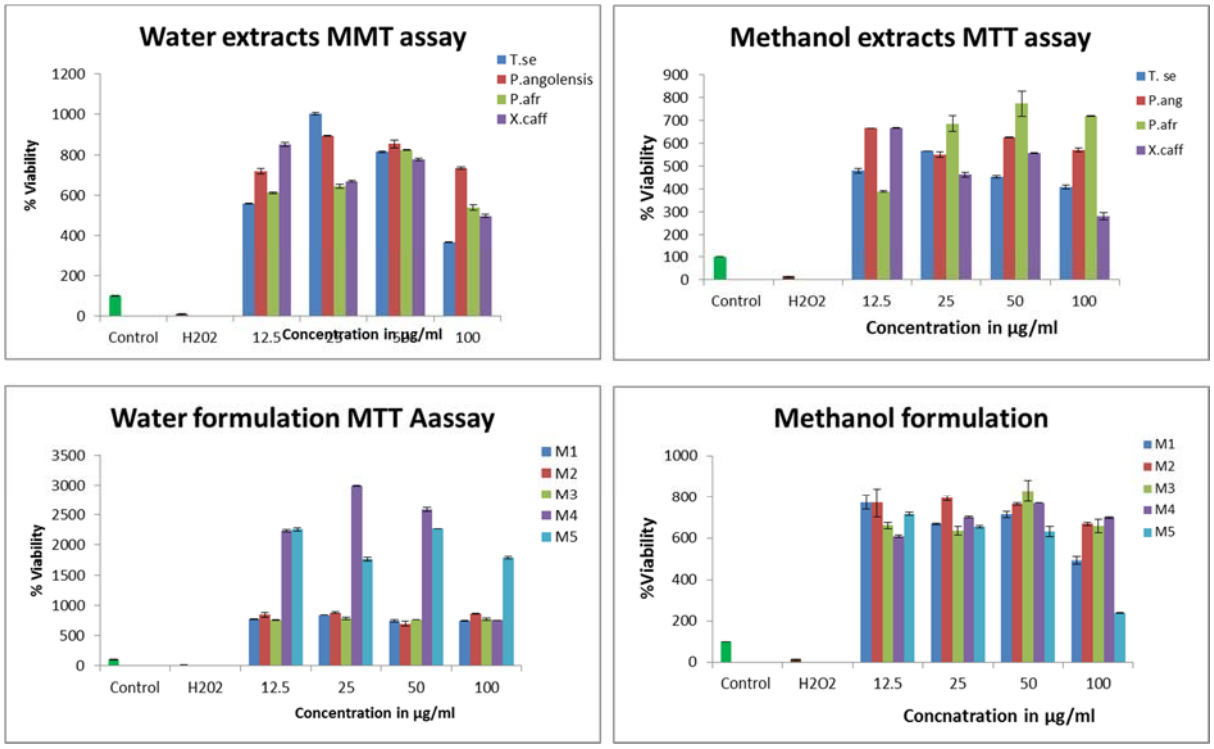

Figure 4.3: Cell viability assay for low concentration of plant extracts and their formulations the data was significant ($P<0.05$) for all tested extracts and formulations compared to positive control.

4.4.3.2. Percentage Cell viability for water extracts, methanol extracts and their formulation at higher concentrations (25mg/ml to 3.125mg/ml).

At higher concentrations, aqueous *P. angolensis* and mixture 4 extract promoted cell proliferation the best at 25mg/ml with cellular viability of 147±3.31 and 218±9.62 respectively.

Methanolic *P. angolensis* promoted cellular viability at 196±3.19 at 12.5mg/ml and mixture 4 155±8.12. Aqueous *X. caffra* root extract was toxic at 12.5mg/ml with 74±2.96 and mixture 2 was toxic at 6.25mg/ml with 49±0.61. Unlike the aqueous sample, methanolic *X. caffra* promoted cell proliferation all concentration tested and mixture 2 was also non-toxic.

Aqueous *T. sericea* promoted viability and cell proliferation the most at 6.25mg/ml with 212±12.30 and Mixture 3 with 185±4.36. Methanolic *T. sericea* and mixture 3 promoted cell proliferation with the highest viability of 245±0.32 and 175±16.53 at 25mg/ml Aqueous *P. africanum* promoted proliferation at 147± 5.44 but, mixture 5 was toxic at 12.5mg/ml with 57± 2.58 and at 6.25mg/ml with 44 ± 1.34. Methanolic *P. africanum* extract promoted cell proliferation with percentage cell viability of 213±11.89 at 25mg/ml and it was toxic at 3.125mg/ml.

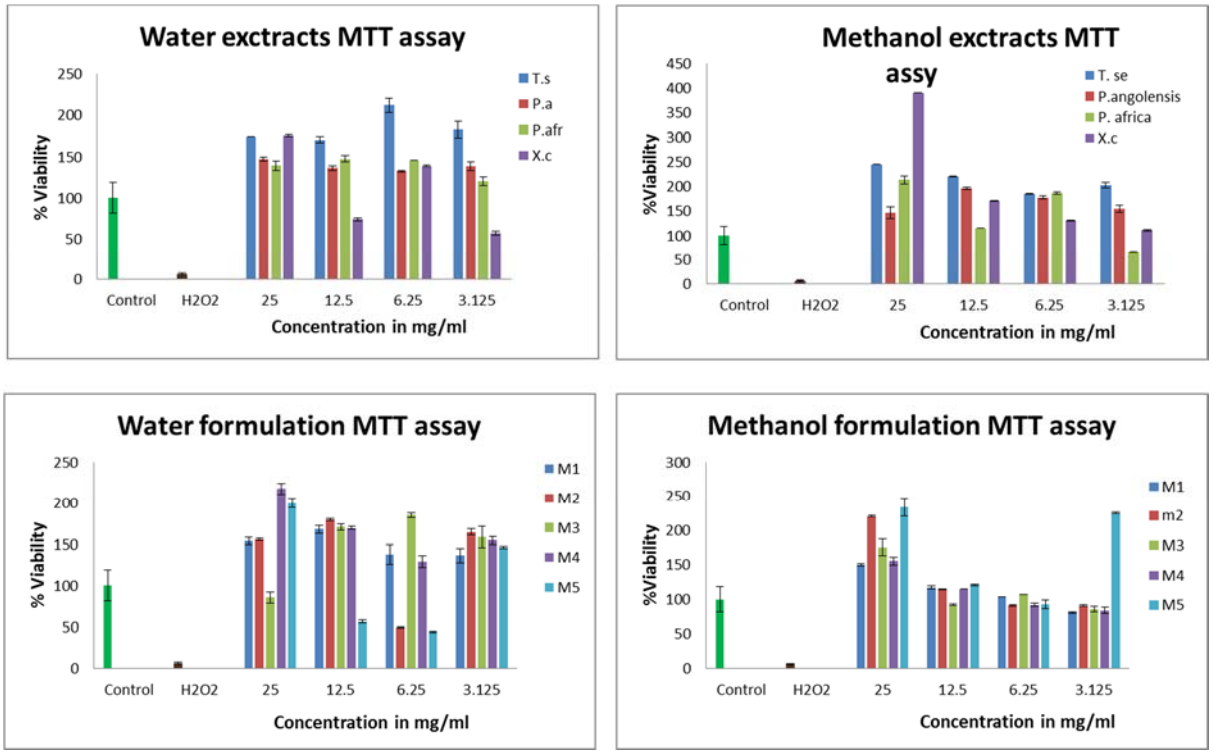

Figure 4.4: Cell viability assay for high concentration of plant extracts and their formulations ($p < 0.05$).

4.4.3.3. Cell viability for water extracts, methanol extracts and their formulation at higher concentrations (50mg/ml and 100mg/ml)

Most tested plant extract at 50mg/ml and 100mg/ml were not toxic, except for aqueous *P. africanum* extract that showed toxicity at 100mg/ml.

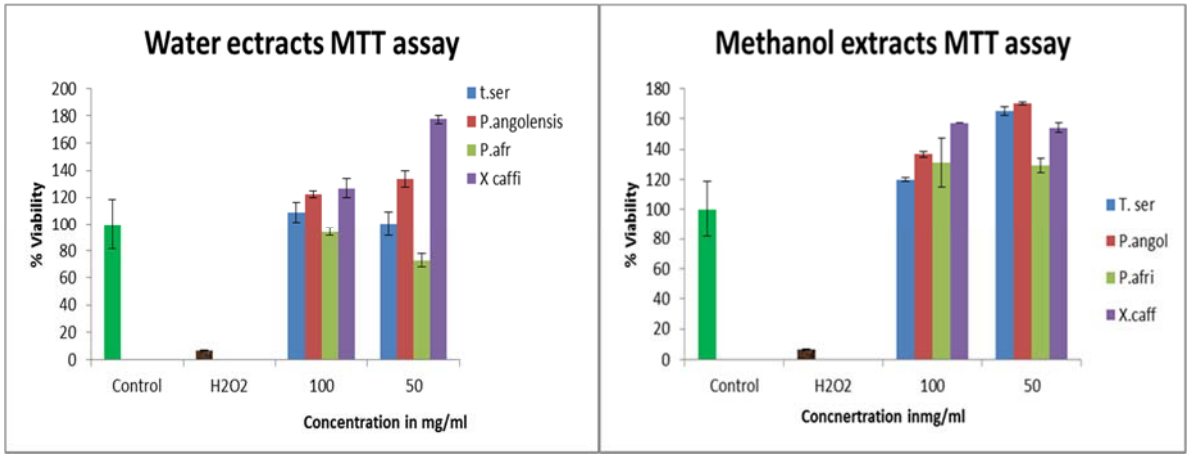

Figure 4.5: cell viability assay of plant extracts at 50mg/ml and 100mg/ml of plant extracts ($p < 0.05$)

4.5. DISCUSSION

In this study, all extracts and formulations exhibited potent DPPH free radical scavenging activity which was significant ($P < 0.05$), however, this was not the case for some samples. DPPH is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic antioxidant. Antioxidants are known to induce a reduction of diphenylpicrylhydrazyl (DPPH) radicals causing a decrease in the absorbance as a result of a visual discoloration from purple to yellow. Hence, DPPH is usually used as a substrate, to evaluate antioxidative activity of antioxidants since such antioxidant have the ability to readily donate their hydrogen to DPPH (Ogunmoyole *et al.*, 2013). In this study, both methanolic and

aqueous extracts and all formulations are potent radical scavengers suggesting that they could act as chain breaking antioxidants. However, it was observed that there were similarities between methanolic extract and formulations as well as water extracts and formulations ($P > 0.05$).

In the current study, the IC_{50} of methanolic and aqueous *P. africanum* root extract were 0.33±0.44 µg/ml and 0.264±0.26 µg/ml respectively giving values that are much closer to the ascorbic acid standard with IC_{50} value of 0.225 ± 0.01 µg/ml. The Potential of this plant as a neuroprotective antioxidant based therapeutics has already been reported, so the results of this current study are not unique but conform to the literature available (Biziminyera *et al*., 2007). previously Similar studies found that the DPPH EC_{50} measures for the acetone extracts of the leaf, bark and root were 6.54, 4.37 and 3.82 µg/mL respectively, compared to EC_{50} = 5.04 µg/mL for the standard ascorbic acid (Biziminyera *et al*., 2005; Biziminyera *et al*., 2007). .

Another study on *Pterocarpus angolensis* by Monadawafa, (2007) reported that the methanolic bark extract inhibited 96.5±0.141% of DPPH closer to Beta carotene antioxidant which was used as a standard which inhibited 98.6±0.100%. In this study, the half minimum concentrations of methanolic and aqueous *P. angolensis* root extracts needed to scavenge free radical DPPH were 0.367±0.46 µg/ml and 0.457±0.21 µg/ml respectively. This shows that *P. angolensis* is a good antioxidant, and these results could be due to the good phenolic content of *P. angolensis* as seen in Chapter 3 because phenolics are suggested to be the major bioactive compounds for health benefits and they are also good antioxidants.

For decades, reports have flourish on the multiple biological effects of phenolics, flavonoids, including their antimicrobial and antioxidant activity (Ejechi *et al*., 1998; Angioni *et al*., 2004). This was seen in this study, *T. sericea* root extracts showed the best phytochemical content in chapter 3 and the best antimicrobial activity, moreover, it also showed the best antioxidant

activity in this chapter with the lowest DPPH scavenging IC_{50} values of 0.161±0.06 µg/ml for the methanolic extract and 0.192±0.004 µg/ml for the aqueous extract lower than ascorbic acid with IC_{50} value of 0.225 ± 0.01 µg/ml. These findings are similar to the previous report of Adewusi and Steenkamp, (2011) who reported that the antioxidant activity of the methanolic *T. sericea* root extract exhibited an IC_{50} value of 0.0147±0.004 mg/ml compared to Troxol which was used as a positive control with IC_{50} value of 9.6 × 10-6 mg/ml.

As indicated both the methanolic and the aqueous *X. caffra* root extracts showed the highest antioxidant activity, these results were similar to the findings of Tshikalange *et al*., (2016) who reported that the IC_{50} of the ethanolic root extracts of X. caffra was 11.77µg/ml, while Vitamin C (positive control) had an IC_{50} value of 1.44 µg/ml. according to Duncan's analysis, there were little differences between methanolic Mixtures 1, 2, 3, 4 and water mixtures 1, 2, 3, methanolic extracts of *T. sericea, P. angolensis, P. africanum* and water extracts of the same samples, there were also no significant differences between water mixtures 4, 5 and methanolic mixture 5, methanolic extract of *X. caffra* and aqueous *X. caffra* extract. This observation could be due to the fact that all the formulations contained the same type of extracts although at different yields, they acted similarly when scavenging DPPH.

However, the best overall lowest antioxidant activity was found in methanolic mixture 4, suggesting that the phenolic and flavonoid compounds in this mixture are more synergistic than the other mixtures. Naidoo *et al*., (2013) reported that a combination of different plant herbs results in synergism, antagonistic, additive and non-interactive of the extracts. Moreover, flavonoids and phenolics are known to exhibit good antioxidant activities and most plant extracts showed good phytochemical content, therefore some insignificant differences in water and methanol samples in chapter 3 during the determination of the total phenolics content and Total flavonoid content may be the cause of the observations this chapter. Furthermore,

Moreover, the results obtained in this part of the study suggested that polar solvents are excellent for the extraction of antioxidant compound.

Almost all HIV infected people rely on antiretroviral drugs to improve the quality and prolong their lives (Mulaudzi *et al.,* 2012). However, ARVs have a lot of disadvantages such as resistance, limited availability, high cost and lack of any curative effects (Klos *et al*., 2009; Mulaudzi *et al.,* 2012). Medicinal plants can be an excellent source of anti-HIV agents as they were seen to inhibit microbes that cause opportunistic infections related to HIV/AIDS in the results in chapter 3. In this part of the study ten aqueous and methanolic formulations from four Venda medicinal plants which are used to treat diarrhoea were tested for Reverse transcriptase enzyme inhibition. Although the use of a single plant have been seen to have therapeutic effects on the treatment of HIV, plant formulation have also gained popularity in the treatment of HIV. A plant formulation of importance in treatment of infections in the Phalaborwa region in Limpopo Province was reported by Chauke *et al.,* (2015), a mixture of *Cassia abbreviata, Terminalia sericea* and *Ozoroa paniculosa* is used to treat antibacterial infections that are mostly associated with HIV/AIDS and other sexually transmitted viruses.

In this study, the obtained results showed that both Methanol and water mixtures containing *Pterocarpus angolensis bark, P. africanum roots* and *X. caffra* roots and *T. sericea* roots exhibited the best inhibition percentages (>75%) at 200µg/ml. The mixtures showed inhibition of reverse transcriptase enzyme much greater than the Combivir which inhibited only 30, 11% of the enzyme. Reverse transcriptase enzyme is very important in the life cycle of HIV-1 virus as it is responsible for converting the viral RNA are into double stranded DNA after the virus has penetrated the cell and viral capsid is emptied into the cell (Leteane *et al*., 2012). Therefore,

disruption of this process lends to the destruction of the life cycle of the virus and the eradication of the virus.

Although most studies are done *in- vitro*, medicinal plants used in the mixtures in this study have shown promising results in the disruption of different stages of the life cycle of HIV. A study by Sigidi *et al*., (2016) found that the aqueous extracts of *P. angolensis* roots inhibited HIV replication activity at moderate percentage (60%). In this study, mixture 4 had higher concentration of *P. angolensis* bark extract, and showed excellent enzyme inhibition at 97.2% for methanolic sample and 95% for the aqueous sample at 200µg/ml, however, mixture 4 did not show good results at 100µg/ml. This can be due to the fact that the enzyme was able to survive the toxicity of the mixture at lower concentration. This also implies that although medicinal plants are good alternative medicine, the dosage is of outmost significant.

Another study by Tshikalange, (2007) reported that ethyl acetate extract of *T. sericea* roots showed an outstanding inhibition of reverse transcriptase enzyme at 94.0%, and Bessong *et al.*, (2004) showed that methanolic *T. sericea* leaves strongly inhibited RNA-dependant-DNA polymerase (98%) and ribonuclease H (RNase) at (99.3%) activities of HIV-1 reverse transcriptase. During the study, the methanolic mixture 3 containing higher concentration of *T. sericea* root extract was evaluated and showed the best overall activity against HIV-RT at 97.5% with the aqueous sample at 97% inhibition at 200µg/ml, moreover methanolic mixture 3 also showed the best inhibition of enzyme at 100µg/ml. This implies that *T. sericea* phytochemicals such as terpenoids, saponins, tannins, steroids, flavonoids and phenolics which were qualitatively determined in chapter 3 have significant anti-HIV activity and together with the phytochemicals of the other plant extracts yielded greater inhibition activity. It was also reported previously that biological activities of *Terminalia sericea* were mainly attributed to triterpenoids, saponins and tannins (Tshikalange *et al*., 2007, Mulaudzi *et al*., 2012, Chinsembu 2016) and this was confirmed in our study**.** In the previous chapter, methanolic mixture 3 also

showed greater antimicrobial activity, good phenolic and flavonoid contents and also good antioxidant activity.

Both the methanolic and aqueous Mixture 2 which contained higher concentration of *X. caffra* root extract also showed high inhibition of reverse transcriptase enzyme with percentage inhibition of 96.16 and 88.3 at 200µg/ml respectively. *X. caffra* has been reported to be used for the treatment of venereal diseases in Zimbabwe (Maroyi, 2011), this correlated with the findings of Naidoo *et al*., (2013) in Maputuland. The inhibition of mixture 2 against the enzyme validates the use of this plant against different STIs since HIV is also an STI. Although *X. caffra* had low antioxidant activity against DPPH, mixture 2 showed greater antioxidant activity possibly resulting in good anti-HIV activity.

Peltophorum africanum that was also used in the mixtures was also previously reported to have anti-HIV activities (Mazimba, 2014; Okeleye *et al*., 2013). The aqueous and methanol extracts of the roots and stem bark were shown to inhibit RNA-dependent-DNA polymerase activity of HIV-1 reverse transcriptase and ribonuclease-H activity of reverse transcriptase (Ebada *et al*., 2008). *P. africanum* root extract was highly concentrated in mixture 5. However, in this study, only the methanolic mixture showed good inhibition of reverse transcriptase enzyme, with percentage of 95.8%. The aqueous extract showed moderate inhibition activity against HIV-1 reverse transcriptase. This is associated possibly with the antioxidant activity of the plant extracts, when looking at Mixture 5, both the aqueous and the methanolic extracts showed higher DPPH scavenging activity as compared to the other mixtures. Also, there was some kind of an antagonistic relationship between the extracts in mixture 5.

Of all tested plant extracts used to treat diarrhoea, the cell viability assay against human lymphatic endothelial cells (HLEC) showed that all the plants promoted cell growth and are not toxic at low concentration. The Human Lymphatic endothelial cells are cells which their

function is to maintain homeostasis in the body through protein transport, tissue fluid balance, and the development of cellular immunity (Kaldjian *et al*., 2001). During infection by microbes in the gastrointestinal system, the lymph nodes become swollen, sometimes the drainage lymphatics also become infected by bacteria (Alexander *et al*., 2010). HLEC produce chemokines, including CCL21 that in turn draw cells such as Antigen loaded dendritic cells into the draining lymphatic vessel, which they transverse to reach secondary lymphoid organs where they simulate immune response (Alexander *et al*., 2010). Therefore, the promotion of these cells' growth by medicinal plants and formulation *in –vitro* can play major role in the stimulation of immune response in-vivo during infections. The use of the medicinal plants at such low concentrations reduces toxicity to normal cells by the plant extract and using them as formulations results in higher cell growth, thus, good stimulation of the immune response.

However, some extracts displayed level of toxicity against human lymphatic endothelial cells. When comparing the activity of the positive control which yielded 100% of cell growth with Aqueous *X. caffra* root and aqueous mixture 2 with high concentration of *X.caffra*, they inhibited 26% and 51% of cell growth at 12.5mg/ml and 3.125mg/ml respectively. Naidoo *et al*., (2013) did not find any toxicity from *X. caffra* extracts, however, *K. africana* aqueous and organic leaf extracts inhibited 22% and 16% of human kidney epithelial cell line at 100µg/ml respectively when compared to the positive control (100% cell growth). Aqueous mixture 5 with higher concentration of *P. africanum* inhibited 43% of cell growth at 12.5mg/ml and 54% of cell growth at 6.25mg/ml. Methanolic *P. africanum* showed some degree of toxicity at 3.125mg/ml by inhibiting 34% of cell growth. According to a study by Okeleye *et al.*, (2013)the ethyl acetate extract of *P. africanum* at exactly 25 µg/ml reduced human breast (MCF – 7) cancer cell growth from 100% to 48.38± 1.56% after just 24 hours of exposure.

Therefore, it was not surprising to observe that aqueous *P. africanum* root extracts and mixture 5 with high content of *P. africanum* showing toxicity against HLEC even though it always has

been reported as a good antifungal and antibacterial medicinal plant. In previous studies Okeleye *et al.,* (2013) reported that the ethyl acetate stem bark extracts of *P. africanum* were toxic to the human Chang liver cells with LD_{50} = 82.64 ± 1.40 µg/ml after 24 hours. The acetone leaf, bark and root extracts did not show toxicity on the Vero monkey cell line and the brine shrimp larval mortality assays (Biziminyera, 2007). The root and leaf non-toxicity were also indicated by inhibition of HelaP4 cell growth at a concentration of 400 µg/mL (EL-Sherbeiny *et al*., 1977). Although *P. africanum* root extract showed toxicity, the toxicity was dose dependent. At lower concentrations, there was no toxicity identified. Toxicity was only seen at higher concentrations were at 100mg/ml, it inhibited 23% of cell growth. Therefore, caution should be taken when administering this extract.

X. caffra root extract was non-toxic at 100µg/ml which is in line with the findings of the study by Naidoo *et al*., (2013) which showed that *X. caffra* root extract were not toxic to human kidney epithelial cell line at 100µg/ml. These variations in toxicity could be due the different cell lines being used, the concentration of the sample and the environment were plants were collected therefore, high dosage of *X. caffra* roots are to be taken with caution. Furthermore, aqueous mixture 2 inhibited most of the cell growth, therefore, formulating this plant extracts should be done by a person who is well trained.

Although *P. angolensis* showed no harmful effects on the cells in this study, Sigidi *et al*., (2016) reported that *P. angolensis* bark extract was more toxic to Vero and MeWo normal cells. *T. sericea* also showed no toxicity against the cells. Although some mixtures were not toxic (methanolic mixture 2 and methanolic mixture 5), most formulated extracts increased the activity of individual plants. The most synergistic activity of the formulations were observed in aqueous Mixture 4 and 5.

4.6. CONCLUSION

Traditional medicine offers important healthcare solutions to different ethnic groups worldwide including the treatment of HIV/AIDS and opportunistic infections associated with HIV infections. Medicinal plants with higher antioxidant activities are believed to have greater anti-HIV activity. Moreover, medicinal plants are believed to have no or fewer side effects, less expensive and readily available. Therefore, the use of the medicinal plant extract in formulations cannot be disregarded, since the compounds might act synergistically to produce better activity. All the mixtures showed good antioxidant activities resulting in better anti-HIV activity. Moreover, when examining toxicity, most plants extracts and formulations were not toxic to human lymphatic endothelial cells except for aqueous *P. africanum* and *X. caffra*. Moreover, toxicity of plant formulations was only observed at mixtures with high concentration of these plants. Toxicity from medicinal plants has been increasingly reported in South Africa (Naidoo *et al*., 2013). It was also noted that toxicity of the extracts was dosage dependent. Therefore, precautionary measures should be taken when preparing plant extract and formulating.

4.7. REFERENCES

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CHAPTER 5: OVERALL DISCUSSION, CONCLUSION AND RECOMMENDATIONS.

5.1 OVERALL DISCUSSION AND CONCLUSION

Medicinal plants have been used by mankind for the treatment of diseases for decades especially in rural areas. They are known as cheap, easily accessible, with no side effects. They have also been the sole suppliers of chemical compounds that are used by different pharmaceutical companies to produce drugs and medicine. They also gained a lot of interest in cosmetic production. Moreover, over the years, medicinal plant formulations have also gained popularity .Therefore, there is an overwhelming interest in research to screening plants and formulations in order to identify as well as to give scientific credibility to plant extracts that will lead in drug discovery and ease the burden of resistant microbial infections.

Research on traditional medicine have already indicated that about 80% of rural communities in South Africa consult traditional healers before going to western doctors in clinics and hospitals (Mbelekani *et al*., 2017). Furthermore, it is believed that a combination of different plants have better therapeutic effect than each plant used individually (Parasuraman *et al.,* 2014). In the Venda region, traditional healers are consulted for several conditions which can be either spiritual healing, to appease the ancestors and sicknesses such as diarrhoea and HIV related infections.

Infectious diseases such as diarrheoa and HIV/ AIDS and are amongst the most common spreading diseases in the world. Diarrheoa caused by pathogens such as MRSA *Staphylococcus*

aureus, MSSA Beta – lactamase producing *E.coli* and Beta-lactamase producing *Klebsiella pneumoniae* have been found in HIV/ AIDS patients (Hidron *et al*., 2010, Hel *et al*., 2017). Candidiasis is also a common yeast opportunistic infection found in HIV/ AIDS infected individuals (Anwar *et al*.,2012). There are antibiotics than are already available for the treatment of diarrhoeal infections and candidiasis, however, resistance of these microbes to antibiotics is of a great concern and the management of such infections have become a great challenge. Therefore there is a great need to search new alternative that can counteract the resistance of the resistance of these microbes. Medicinal plants have been reported to poses phytochemicals which they use as defence against invading microbes.

This study investigated the antimicrobial, antioxidant, phytochemical content and the toxicity of four plant extracts and their formulations, as well as the HIV-1 inhibitory activities of the methanolic and water formulations. Four medicinal plants that are commonly used by the Venda community and their formulations (a total of 18) were tested for the total phenolic and flavonoid content. The preliminary phytochemical composition of the medicinal plants was also detected to determine the presence of steroids, phenolics, flavonoids, saponins, terpenoids, tannins and alkaloids. Their antimicrobial activity against MRSA, MSSA, beta lactamase producing *E.coli*, beta lactamase producing *K. pneumoniae* and four clinical isolates of *candida spp* and *C.neoformans* was also determined. The ability of the plant extracts and their formulation to scavenge free radical DPPH was also evaluated. The plant formulations were screened for their ability to inhibit HIV-1 reverse transcriptase enzyme and lastly the toxicity of all the plant extracts and formulation against HLEC was determined using MTT.

The study demonstrated that all plant extracts contained phenolics and flavonoids. The best phenolic content was detected in methanolic mixture 3 with higher content of *T.sericea*, furthermore, the best flavonoid content was detected in aqueous *T.sericea* root extract. Preliminary phytochemical analysis revealed that all methanolic mixtures contained the highest

flavonoids, tannins, saponins, steroids and phenolics. Methanolic *T.sericea* had the highest phytochemicals of all the extracts tested. It was very interesting to note that T.sericea showed good pharmacological activities. No alkaloids were detected in all the plant extracts and formulations which is good because alkaloids are known to be toxic.

Both the aqueous and methanolic extract and their formulations were active against all the test bacteria. The plant extract with the best MIC less than 0.1mg/ml was both methanolic and aqueous *T.sericea* extract against MSSA, even though no mixture had MIC less than 0.1mg/ml most formulations showed additive interactions against all test bacteria yielding good MBC results. FICI showed that mixtures 1, 2, 4 and 5 showed some antagonistic interactions between the plant extracts against MSSA, *E.coli* and *K.pnuemoniae* . However, they were also showed interactions additive against MRSA.

The plant extracts and their formulations were also tested against four *candida spp* and *C. neoformans* which usually affect immunocompromised individuals. All plant extracts and formulations did not show good antifungal activity against the tested fungal organisms however, mixing the plant extracts together resulted in an improved therapeutic effect of the plant extracts and none of the formulations were fungicidal. The best antimicrobial activity overall was exhibited by methanolic mixture 3 and methanolic *T.sericea* root extract.

All the plant extracts and formulations were also tested for their antioxidant activity, both the methanolic and aqueous samples are potent DPPH free radical scavengers. The best overall antioxidant activity was exhibited by methanolic mixture 4 with the lowest IC_{50} and the least potent antioxidant was detected in aqueous *X.caffra* root extract.

Reverse transcriptase is an important enzyme in the life cycle of the human immunodeficiency virus and its inhibition result in the disruption of the multiplication of the virus. The aqueous and methanol formulations exhibited high HIV-1 reverse transcriptase inhibition at 200µg/ml.

However, only methanolic mixture 3 with higher concentration of *T. sericea* inhibited more than 50% of the enzyme showed at 100µg/ml. the obtained results showed that plant formulations can be excellent for alternative medicine for slowing down HIV/AIDS progression.

In order to deduce the safety of ingestion of these medicinal plants and their formulations, immune Human lymphatic endothelial cells were treated with the extracts and the mixtures. Both the water and methanol extracts and their formulations showed good cell proliferation activity for HLEC. This was seen in lower dosage. However, toxicity was detected at high dosage for aqueous *X. caffra* root extract, aqueous mixture 2, aqueous mixture 5 and methanolic *P. africanum* root extract, these mixtures were also antagonistic during the FICI determination. It was also interesting to note that the same extracts and formulations exhibiting good percentage of cell viability at a much higher concentration except for aqueous *P. africanum* which remained toxic.

This study has shown that medicinal plants commonly used in the Venda region in the management of diarrhoea, HIV and other opportunistic infections related to HIV/AIDS can improve human health and that formulating the plant extracts results in better therapeutic effect of the individual plant, however, caution should be taken when preparing the plant extract and formulation. Moreover, the dosage administered play a vital role in the activity of the medicinal plant and formulations. This study will also serve as a platform to which plant formulations from plant extracts used can be further explored.

5.2. RECOMMENDATIONS

Further research needs to be carried out especially on the formulations to isolate and identify the active compound(s) responsible for their antimicrobial, antioxidant and low cytotoxic properties.

The chemistry behind the interaction of the phytochemicals in the plants used in the study need to be studied in order to identify chemical reactions resulting in synergistic effects of the plant extracts.

In -vivo cytotoxicity studies are needed on the formulations to confirm cell proliferation activities found in this study.

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