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Bioactivity of selected medicinal plants used for the treatment of sexually transmitted diseases

by

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Abstract

Background: Sexually transmitted diseases (STD's) have a major impact on sexual and reproductive health worldwide. Each year, the World Health Organization (WHO) estimates 448 million new cases of curable STD's are diagnosed. The emergence of drug resistance in STD related microorganisms and potential side effects demand the discovery of newer drugs. The exploration of newer anti-microbial substances from natural sources may serve as promising alternatives. In this study, twelve medicinal plant species used traditionally in the treatment of STD's are investigated in this regard.

Methods: Ethanol plant extracts and three flavonoids were evaluated for their antimicrobial properties against one fungi and three bacteria, through the micro-dilution assay. To determine the anti-inflammatory activities of the extracts and compounds, the inhibitory effect was measured on the pro-inflammatory enzyme lipoxygenase, 15-LOX. Extracts were further evaluated for their inhibitory effect on the supercoiling activity of bacterial DNA gyrase by using the DNA gyrase kit. The extracts and compounds were lastly investigated for their anti-HIV activities against recombinant HIV-1 enzyme using non-radioactive HIV-RT colorimetric assay.

Results: *Acacia karroo* and *Rhoicissus tridentata* extracts showed good antimicrobial activity with MIC values ranging between 0.4 and 3.1 mg/ml. Extracts of *Jasminum fluminense*, *Solanum tomentosum* and flavonoid 2 and 3 had good anti-inflammatory activity with IC_{50} less than the positive control quercetin (IC_{50} = 48.86 ug/ml). Extracts of *Diospyros mespiliformis*, *Peltophorum africanum*, *Rhoicissus tridentata* and flavonoids 1 and 2 showed the best inhibitory activity against the bacterial DNA gyrase. *A. karroo* and flavonoid 3 exhibited moderate HIV RT inhibition activity of 66.8 and 63.7 % respectively. *R. tridentata* and *Terminalia sericea* had the best RT inhibition activity (75.7 and 100 %) compared to the positive control doxorubicin (96.5%) at 100 ug/ml concentration.

Conclusion: The observed activities may lead to new multi-target drugs against sexually transmitted diseases.

Keywords: Sexually transmitted diseases, antimicrobial, anti-inflammatory, 15-LOX, DNA gyrase, HIV-1 RT

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ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral
ATP	Adenosine triphosphate
BV	Bacterial vaginosis
CFU	Colony forming units
COX	Cyclooxygenase
CO_2	Carbon dioxide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DIG- POD	Digoxigenin-POD
EB	Ethidium bromide
ELISA	Enzyme-linked immunosorbent assay
HAART	Highly active anti-retroviral therapy
HIV	Human immunodeficiency virus
IC_{50}	Inhibitory concentration at 50%
INSTI	Integrase inhibitor
LOX	Lipoxygenases
MIC	Minimum inhibitory concentration
NFκB	Nuclear factor kappa B
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside/ nucleotide reverse transcriptase inhibitor
NSAID	Non-steroidal anti-inflammatory drugs
pHOT1	Phototropins
PI	Protease inhibitor
RNA	Ribonucleic acid
RT	Reverse transcriptase
SD	Standard deviation
STD’s	Sexually transmitted diseases
UTI	Urinary tract infection
UV	Ultraviolet
WHO	World Health Organization

Chapter 1: Introduction

1.1 Problem statement

Microbial infections are a common public health concern, especially in most developing countries. Most of microbial infections include sexually transmitted diseases (STD's). Approximately 444 million cases of curable STD's (such as chlamydia, syphilis, trichomoniasis, and gonorrhoea) are reported every year, worldwide (WHO, 2011). In South Africa, it is estimated that 11 million STD cases are reported annually (Govender, 2012). The prevalence is even higher as the majority of the people with STD's do not present to health care providers due to social, moral, cultural or educational constraints (Shaukat, 2015). STD's rank among the top five diseases for which people seek clinical care and are a major cause of morbidity (Shaukat, 2015). Worldwide, more than one million people acquire STD's every day (Chen *et al*., 2016). STD's are clinically diverse; over 30 bacterial, viral and parasitic microorganisms. The increasing resistance and decreased susceptibility of these microorganisms to antimicrobial agents are emerging concerns (Hughes, 2015).

STD's are caused by infections that can be passed on from one infected individual to the other by any means of intimate sexual contact, such as vaginal, anal or oral sex. Other modes of transmission include maternofetal, via unsterilized needles and injections, as well as blood transfusions (Shaukat, 2015). The global prevalence of STD's is increasing and the majority of these infections occur in women of reproductive age. Most infections are present without any symptoms. Common symptoms of STD's

include vaginal discharge, urethral discharge or a burning sensation at the top of the penis in men, abdominal pain and genital ulcers. Precise diagnostic tests for STD's are commonly used in high-income countries. These are particularly useful for the diagnosis of asymptomatic infections. However, in low and middle-income countries, diagnostic tests are largely unavailable. When diagnostic tests are available, they are often expensive and geographically inaccessible (WHO, 2015).

STD's can impact the health of affected individuals with varying complications such as; infertility, cervical cancer, cardiovascular and neurological damage and adult mortality (Hughes, 2015). The presence of untreated infections may in addition lead to serious health complications, which may include male or female sterility, ectopic pregnancy, pelvic inflammatory disease, lower health quality of life, and may also lead to the increased transmission of human immunodeficiency virus (HIV) (Ruddock *et al*., 2011). Treatment or prevention methods are therefore highly escalating.

STD management includes preventive as well as medical treatment. There are three levels of prevention. Primary prevention is preventing the infection before it arises and involves advocacy and outreach programs (e.g. counselling and behavioural interventions). Secondary prevention is practiced after the acquisition of an infection and includes prevention of further transmission of STD's to their partners (e.g. safer sex/risk-reduction counselling, condom promotion). Tertiary prevention is the minimization of disability from infections in a patient and includes early treatment and avoiding complications. The most important component of syndromic management are the four Cs, i.e. compliance, counselling, condoms and contact (partner) management (Shaukat, 2015; WHO, 2015).

Untreated STD's increase the risk of acquisition and transmission of HIV by a factor of up to 10. Monitoring STD's is important for the prevention of HIV-1. Therefore; the control of STD's remains a priority for the WHO. The World Health Assembly recognized the global strategy for the prevention and control of STD's in May 2006 (Govender, 2012). When identified earlier, STD's can be treated with antibiotics. The drugs differ with each type of STD. The most common drugs currently used for STD treatment include cephalosporins (ceftriaxone, cefixime), macrolides (erythromycin, azithromycin), quinolones (ciprofloxacin, levofloxacin, ofloxacin), penicillins (benzylpenicillin, benzathine penicillin), tetracyclines (doxycycline, tetracycline), acyclovir, metronidazole, antifungals (fluconazole, tinidazole, terbinafine, itraconazole). Topical agents include metronidazole, clotrimazole, podophyllin (Shaukat, 2015). Male circumcision can also help reduce the transmission of STD's.

A person's chance of acquiring an STD depends on certain behaviours of their partners and themselves. Some members of society engage in activities and behaviours that place them at a higher risk for acquiring and transmitting STD's. These high-risk groups include male and female sex workers and drug users. People with a history of sexual contact with any of these high-risk groups, will have a higher risk of acquiring an STD (Shaukat, 2015). Earlier sexual debut, late-life marriages, more lifetime partners, population-level biological forces and more commercial sex may also vastly accelerate the spread of STD's (Muessig, 2013).

The use of herbal medicines is gaining popularity because of their long history of being used. Medicinal use of plants is also perceived to have several advantages like fewer side effects, better patient tolerance and its low expense (Maseve *et al*., 2015). Some plants can offer better prospects for the discovery of new pharmaceuticals and better

anti-infective agents. This results in the increased interest in medicinal plants used by medical practitioners for various diseases (Gail *et al*., 2015).

Taking into consideration the lack of scientific data validating the traditional use of plants for the treatment of STD's for some of the plants, a study was designed to address these issues of concern. Twelve medicinal plants were selected based on their traditional uses in the treatment of sexually transmitted related symptoms. This study aims to take an in-depth look into South African grown plants that are traditionally used for the treatment of STD's. Such research is particularly important in improving health care most likely to encounter those affected.

1.2 Hypotheses

- Ethnobotanical selected medicinal plants used traditionally in the treatment of STD's will exhibit significant *in vitro* antimicrobial and anti-inflammatory activities.
- These medicinal plants might contain secondary metabolites with anti-gyrase and HIV-1 reverse transcriptase activities as mechanisms of action.

1.3 Aims and Objectives of the study

The aim of the study was to investigate biological activities of twelve ethnobotanical selected medicinal plants, which are used traditionally for the treatment of STD's.

A breakdown of the specific objectives of the study is as follows:

- To determine the antimicrobial activity of selected medicinal plants and three flavonoids.
- To evaluate the anti-inflammatory activity of the plant extracts and previously isolated compounds.
- To determine the mechanism of action of plant extracts and compounds on DNA gyrase and reverse transcriptase enzymes.

1.4 Scope of dissertation

Chapter 1: A problem statement highlighting some of the impacts of STD's and complications resulting from untreated infections, are presented. The tested hypothesis and objectives are also stated in this chapter.

Chapter 2: This chapter outlines a review on the ethnobotanical use of medicinal plants in South Africa. The selected plants and their descriptions are detailed in this chapter.

Chapter 3: This chapter describes the antimicrobial activity of twelve medicinal plants and three isolated flavonoids, in detail.

Chapter 4: The anti-inflammatory activity of the medicinal plants and three isolated flavonoids is presented in this chapter.

Chapter 5: The determination of the mechanism of action of medicinal plants on DNA gyrase and HIV-1 reverse transcriptase enzymes is presented in this chapter.

Chapter 6: General discussion and conclusion based on the findings of the study are highlighted in this chapter. Future work activities are also stated.

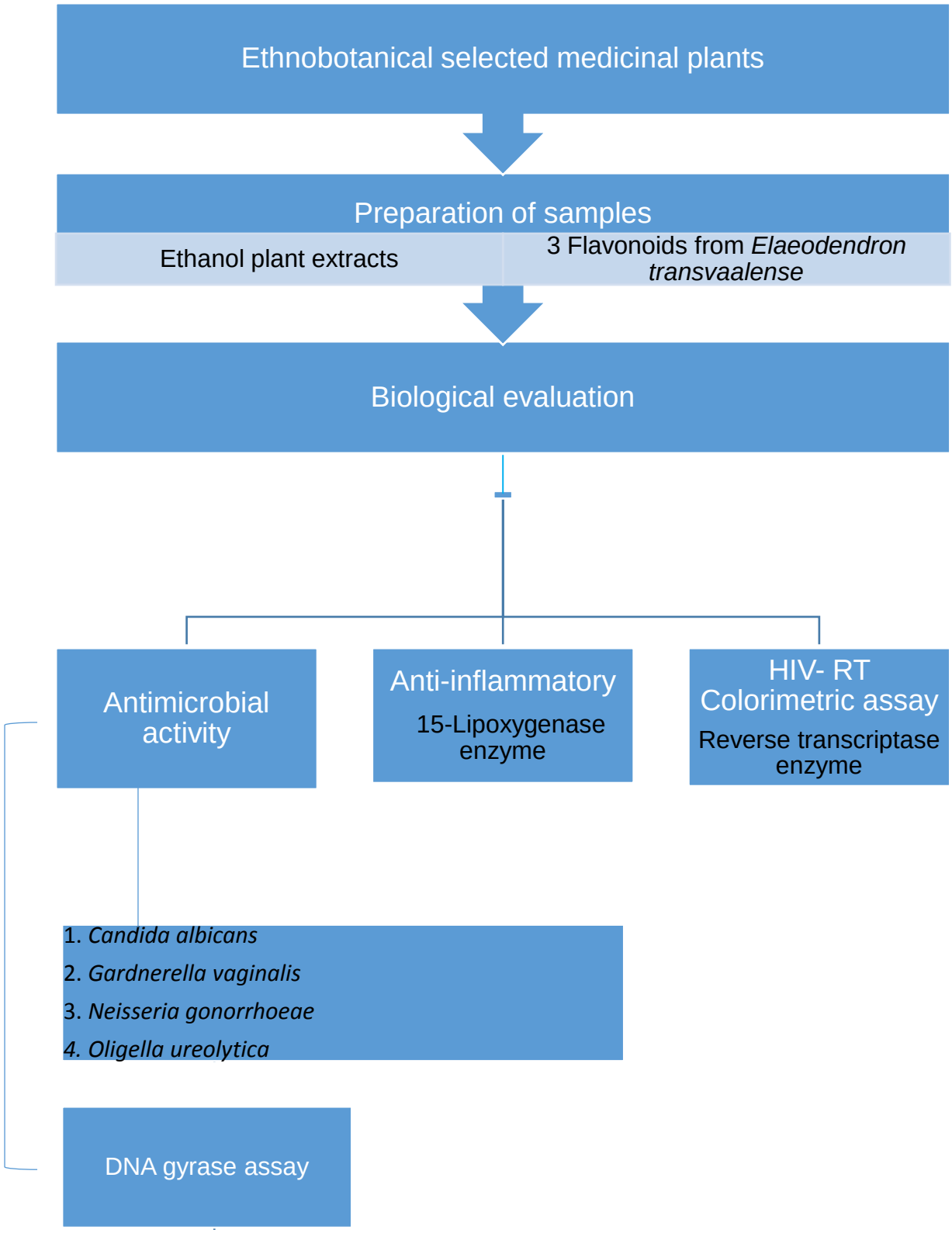

Figure 1.1 The work plan followed in this study.

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Chapter 2: Literature review

2.1 Background

The increased risk of HIV transmission continues to rise globally with estimates of more than 34.2 million people living with HIV and 2.5 million infections annually (Tucker *et al*., 2013). Sexually transmitted diseases (STD's) are one of the major risk factors for HIV infection. STD's facilitate HIV transmission by penetrating the protective mucosal barriers and recruiting susceptible immune cells (e.g. CD4 T-helper cells, macrophages) to the site of infection (Kalichman *et al*., 2011). Therefore, there still remains a need to accelerate STD/HIV prevention, control, and treatment methods.

STD's go beyond public health because they primarily result in a vast worldwide burden of sexual, reproductive and maternal-child health consequences (Gottlieb *et al*., 2014). Despite their common form of transmission, STD's are a group of diversified infections with multiple different causative agents. These can be characterized into viral, bacterial, fungal or protista infections (Fernandez-Romero *et al*., 2015).

Each year, the World Health Organization (WHO) estimates 448 million new cases of curable STD's are diagnosed (Tucker *et al*., 2013). Candidiasis, gonorrhoea, trichomoniasis, bacterial vaginosis and genital herpes, dominate the list of commonly encountered STD's. STD's have a major impact on sexual and reproductive health worldwide. They can cause genital symptoms that can affect the quality of life, serious morbidity and mortality. Symptoms may lead to pregnancy complications, chronic

infections, infertility, stillbirths, pre-term delivery, enhanced HIV transmission, and congenital abnormalities, thus, emphasizing the need to develop effective biomedical interventions for controlling STD's (Jadhav *et al*., 2014). The core aspect of the WHO's Global Strategy on Reproduction Health is controlling the transmission of STD's (Gottlieb *et al*., 2014).

However, particularly in the low and middle-income countries where the health system infrastructure is least developed, the control of STD's remains a challenge (Gottlieb *et al*., 2014). As a result of these difficulties, traditional medicines remain the primary source of medical care to various health needs (Houngbeme *et al*., 2014). The use of plant-derived chemicals could provide alternative classes of antibiotics having different target sites than the already used antibiotics, which may be effective against drug resistant pathogens. As the emergence of drug-resistance in STD-related microorganisms and probable side effects demand the discovery of alternative drugs, the exploration of newer antimicrobial constituents from natural sources may serve as promising alternatives (Jadhav *et al*., 2014). The pharmaceutical industry is currently exploring phytochemicals which can overcome resistant pathogens without any or least toxic effects on the host (NandaKafle *et al*., 2015).

In an effort for an improved detection, effective treatments and control of STD's, a number of diagnostic assays using molecular techniques have been developed. A few selected pathogens have been identified for this study based on their prevalent infections worldwide, causing morbidity and socioeconomic problems.

Candida albicans are one of the parasitic fungi that can cause infections in the vagina, skin, mouth, and intestines or upon contraction (Mulaudzi *et al*., 2011). Rapid growths in diseases associated with fungal infection, such as candidiasis, are related to the

increased risk of HIV infection. The major concern with candidiasis is that it is associated with a mortality rate of 10–49% in immune compromised patients (Masevhe *et al*., 2015). The increase of urinary tract candidiasis has led to the emergence of antifungal-resistant *Candida* species (Behzadi *et al*., 2015). Thus, the search for alternative cures from traditional medicine is justified.

Gardnerella vaginalis infection is caused by a coccobacillus that is also sexually transmitted (Oyedeji *et al*., 2010). It is associated with bacterial vaginosis (BV), bacteraemia, preterm birth and pelvic inflammatory disease (Vick *et al*., 2013). BV is a common vaginal condition and symptoms often include vaginal discharge, pruritis, and odour (Eren *et al*., 2011). BV is characterized by a drastic reduction of the normal vaginal flora, particularly *Lactobacillus* species in the vaginal environment and an increase in the concentration of *G. vaginalis*. Although BV is a polymicrobial condition with no single causative agent, 95-100% of women with BV are colonised with *G. vaginalis* (Cox *et al*., 2015). It is associated with the increased risk of acquisition and transmission of STD's, including HIV (Muzny, 2015).

Inflammatory STD's, such as gonorrhoea, are associated with a three- to five-fold increase in the risk of acquiring HIV (Chin *et al*., 2012). These statistics imply that people of several African countries continue to increasingly take sexual risks. Gonorrhoea is an infection caused by the bacterium *Neisseria gonorrhoeae* (Ohnishi *et al*., 2011). Gonorrhoea is a sexually transmitted infection that can affect both males and females. Urethritis is the most common reproductive tract syndrome that is caused by *N. gonorrhoeae* (Manhart *et al*., 2013). It can be spread from one infected individual to another through unprotected vaginal, oral or anal sex. It can cause infections in the genitals, throat or rectum (Duncan *et al*., 2009). The study included the infectious

gonorrhoea, because it is one of the sexually transmitted infections that most clearly mirror trends in risky sexual behaviours.

Oligella caused infections are rare. *Oligella ureolytica* is more frequently recovered from urethral and respiratory tract specimens as a commensal organism. It is commonly isolated from urine of patients with indwelling urinary catheters (Demir, 2014). *Oligella* species have been implicated as a causative organism of urinary tract infection (UTI) in patients with chronic indwelling catheters (Baruah *et al*., 2014). Despite its low prevalence, *Oligella* infection generates high levels of inflammation in patients with immunosuppression (Rodríguez-Jiménez *et al*., 2015).

It is known that most of the STD's exist without symptoms (asymptomatic) and patients are at a higher risk of HIV infection if left untreated (Yadav *et al*., 2013). The symptomatic infections differ with each type of STD. Since many STD's have symptoms that are not easily recognized, sexually active individuals are advised to have regular medical check-ups. Most patients with such infections do not undergo treatment, and physicians sometimes have difficulty in making distinct diagnoses (Kweon *et al*., 2015). The treatment recommended depends on the diagnosed STD (Schneede *et al.*, 2003). Upon early detection, some of the infections can be cured with antibiotics (Smith, 2015).

The risk reduction practices of both STD/HIV rates include the reduction of sexual partners, protected sexual behaviours like the use of condoms and hormonal contraceptive use (Chin *et al*., 2012). Also, accessible and effective health care services, that is, prompt and appropriate diagnosis and treatment using effective and affordable drugs (Jadhav *et al*., 2014). These approaches may also include

behavioural interventions, improving the clinical management of STD's and syndromic management for preventing new infections (Jadhav *et al*., 2015).

2.2 Ethnobotany in South Africa

Plants are the reservoir of natural medicines which are still widely used for medicinal purposes in many African cultures. Natural products provide a source of new therapeutic drugs. About 60% of approved drugs are either directly isolated or derived from natural products (Kapewangolo *et al*., 2015). However, there is still a need to improve the quality of conventional medicines through the use of more natural plants.

The increasing importance of medicinal plants remains evident as many parts of South Africa still rely on the use of plant species for the treatment of various illnesses. Most of these illnesses include STD's. The use of medicinal plants in various areas of South Africa forms an integral part of the culture and may also be due to economic reasons (Mahomoodally, 2013; Maroyi, 2013). Urban residents also supplement the care they receive in clinics and hospitals with treatment from traditional healers. Due to the lack of proper health care facilities, traditional medicines will continue being the sources of medicines especially in rural areas (Fomogne-Fodjo *et al*., 2014; Gruca *et al*., 2015).

Many traditional healers reside in the communities, which makes it easier for patients to confide in them, especially concerning sexually transmitted illnesses, in fear of stigmatization (Vermani, 2002). The escalating dependence on herbal medicines is mostly due to the occurrence of increased microbial resistance to conventional antibiotics and prevalence of bacterial diseases that affect the human population.

Many traditional healers prefer plants that grow under natural environment because they regard them as having superior medicinal properties (Netshiluvhi, 2015).

It is estimated that around 70,000 plant species have been used for medicinal purposes (Guler *et al*., 2015). Because of the diversity of plant species, herbalists take that as an advantage to treat various infections. Although medicinal plants have been widely used in South Africa since ancient times as sources of medicines, the efficacy and safety of the remedies need to be scientifically evaluated (Naidoo *et al*., 2013). The research on the ethnopharmacological use associated to this subject can provide alternative approaches and novel solutions that can be given to pharmaceutical companies. This information about plants can lead to innovative drugs. It can also benefit local communities (Rigat *et al*., 2015).

Plants contain a variety of different phytochemicals, which are also known as secondary metabolites which may act individually or in synergy to improve human health. The combined effect of these metabolites tends to increase the activity and stability of the active compound(s) or phytochemical, thus minimizing the rate of undesired effects or additive effects (Mahomoodally, 2013).

Synthetic drugs are effective in managing different diseases but these drugs are out of reach to millions of people. The natural herbs provide the starting material for the synthesis of these conventional drugs (Kumar, 2010). Because of the long-term usage for the treatment of various ailments over the years, the natural growing medicinal plants are therefore considered to be safer than conventional synthetic pharmaceuticals (Kankara *et al*., 2015).

Medicinal plants have played important roles in the discovery of anti-STD drugs. The clinical research, analysis, and quality control are proficient in substantiating the

treatment value of herbal medicines which then allows the world of conventional medicines to highly accept the practice of herbal medicines (Soladoye *et al*., 2014). Thus, the objectives of studying African medicinal plants are to provide the scientific basis for their therapeutic and/or side effects which would, in turn, improve the accessibility of the populations to primary health care (Samba *et al*., 2015). The documentation of medicinal uses of African plants and traditional systems is becoming an urgent requirement because of the rapid loss of the natural habitats of some of these plants due to anthropogenic ventures and also due to an erosion of valuable traditional knowledge (Mahomoodally, 2013).

Information on the medicinal value of plants was gathered through questionnaires and word of mouth from traditional healers, as well as from the community members. A report was compiled on all the plants traditionally used for the treatment of STD's. These plant species were then scientifically investigated for their safety and efficacy.

2.3 Selected pathogens associated with STD's

STD's are a clinical syndrome caused by pathogens that can be acquired and transmitted through sexual activity. There are various pathogens known for causing STD's, in the form of bacterial, viral, fungal and protozoal and epizoal infections (Choi *et al*., 2013). This study takes into account one fungal (*Candida albicans* ATCC 10231) and three of the various bacterial (*Gardnerella vaginalis* ATCC 14018, *Neisseria gonorrhoeae* ATCC 19424 and *Oligella ureolytica* ATCC 43534) sexually transmitted associated microorganisms.

Candida albicans belongs to the Saccharomycetaceae family. This is a fungal strain that causes genital infections in humans. *C. albicans* is a diploid fungus that grows both as yeast and filamentous cells (Ohama, 1993). Thrush, oral thrush, fungal infection, yeast infection are diseases caused by *C. albicans.* Candidiasis is also caused by this yeast fungus (Ariyachet, *et al*., 2013). *C. albicans* normally infects the mucous membranes of the vagina (vaginitis), head of the penis (balanitis), the mouth, skin or rectum (Pizzorno *et al*., 1999; Van Vuuren, 2010).

Gardnerella is a genus of Gram-variable staining facultative anaerobic rod bacteria of which *Gardnerella vaginalis* is the only species (Baruah *et al*., 2014). This species is mostly isolated in women due to disruption in the normal vaginal microflora. Mostly, it can be isolated in genital cultures, but may also be detected in blood, urine, and pharynx samples. *G. vaginalis* urogenital biofilm is also increased in inflammatory bowel disease, and this observation suggests an epithelial barrier dysfunction of the genital tract (Oshima *et al.,* 2015).

Neisseria gonorrhoeae is the obligate human pathogen that causes the STD, gonorrhoea (Ohnishi *et al*., 2011). This means that *N. gonorrhoeae* is specific to causing the infection gonorrhoea. These bacteria belong to the family Neisseriaceae. This Gram-negative, coffee-bean shaped diplococci bacterium does not affect other animals, and does not survive freely in the environment but requires CO_2 for survival. The urethra, cervix, urinary tract, mouth, rectum and the throat are the main sites of infection (Pizzorno *et al.*, 1999). Once the gonococci gain entry into the mucous membranes of these regions, they target columnar non-ciliated epithelial cells. Transmission of gonorrhoea is common through vaginal, anal or oral sex (Edwards *et al.*, 2002).

Oligella ureolytica is an aerobic Gram-negative, motile bacterium which is mostly isolated from the urinary tract, cervical lymph node bloodstream infections or human urine (Demir, 2014). It is a small bacterium with nutritional properties limited (Rodríguez-Jiménez *et al*., 2015). Limitations in commonly available laboratory procedures make the identification of this bacterium difficult (Simmons *et al*., 2015).

2.4 Plant selection and description

Twelve medicinal plants used in the treatment of sexually transmitted diseases were selected from a database developed by Mophuting (2015). These plants have shown good antimicrobial activities in my previous studies. The plant material were collected villages under Jongilanga tribal council, in Mpumalanga. Voucher specimen of collected plant species were identified and deposited at the HGJW Schweickerdt Herbarium of the University of Pretoria (Table 2.1).

Table 2.1 The selected plants used in the study.

Samples	Voucher Number & Family	Part used	Medicinal uses	Biological activities	References
Acacia Cf karroo	BCM 119360 Fabaceae	Roots	STD's	Anti-bacterial, anti-inflammatory	Madureira *et al*., 2012; Mulaudzi *et al*., 2013.
Diospyros mespiliformis	BCM 117182 Ebenaceae	Roots and leaves	Urinary disorders, STD's	Anti-bacterial, anti-fungal	Dangoggo *et al*., 2012; Mabona *et al*., 2013.
Elaeodendron croceum	BC 11 Celastraceae	Bark	HIV	Anti-bacterial, anti-inflammatory	Eloff, 2000; Mbaveng *et al*., 2014.
Elaeodendron transvaalense	BCM 117182 Celastraceae	Bark	Inducing vomiting, STD's	Anti-microbial, anti-inflammatory	Tshikalange *et al*., 2005; Mbaveng *et al*., 2014.
Hilliardiella nudicaulis	BC 236 Asteraceae	Whole plant	STD sores	*	Mophuting, 2015.
Jasminum fluminense	BCM 119350 Oleaceae	Roots	STD's	Anti-microbial	
Peltophorum africanum	BC 40 Fabaceae	Roots	Body pain, TB, STD's	Anti-microbial, anti-inflammatory	Maregesi *et al*., 2008.
Rhoicissus tridentata subsp. *cuneifolia*	BC 119338 Vitaceae	Roots	Eye infections, STD's	Anti-microbial, anti-inflammatory	Steenkamp *et al*., 2007; Adebayo *et al*., 2015.
Schotia capitata	BC 99 Fabaceae	Roots	Pulmonary infections, STD's	Anti-bacterial	Lin *et al*., 1999.
Senna italica subsp. *arachoides*	BCM 117179 Fabaceae	Roots	STD's	Anti-bacterial	McGaw *et al*., 2002.
Solanum tomentosum	BCM 117177 Solanaceae	Roots	Eye infections, STD's	Anti-bacterial	Masoko *et al*., 2010.
Terminalia sericea	BCM 118704 Combretaceae	Roots	STD's, Tonsils	Anti-bacterial, anti-inflammatory	Aliero *et al*., 2006.

*No recorded information

Acacia Cf karroo

A. karroo (Figure 2.1) is very widespread throughout Southern Africa. It can be found in Angola, in the Western Cape in South Africa and also in Zambia. *A. karroo,* also known as the Sweet thorn because of its sweet tasting gum exuded from the wounds of the bark, belongs to the Fabaceae family (Joffe, 2001). It can vary in shape and in size, and can grow up to 12 m tall. The bark is red on young branches and darkens to coffee colour, becoming rough with age. The leaves are fine textured and are dark green in colour. The flowers emerge in a mass of yellow pompons. Seeds from this plant are flat with a crescent shape. It can be easily be identified by the presence of long white thorns (Kyalangalilwa *et al*., 2013).

The bark of the *A. karroo* is used to treat colds, diarrhoea and many venereal diseases (Mulaudzi *et al*., 2011).

Figure 2.1 The aerial parts of *Acacia karroo.*
(http://www.plantzafrica.com/plantab/acaciakar.htm)

Diospyros mespiliformis

D. mespiliformis (Figure 2.2) which is also known as the Jackalberry is a deciduous small or medium-sized tree that grows up to 25-40 m tall. *D. mespiliformis* has alternate, simple, leathery, dark green leaves, with a narrow blade. The rough-textured bark is black to grey in colour. The flowers are unisexual, and are white to greenish-yellow. The fruits, which many jackals feed on, are oval shaped and turn yellow to orange when ripe. Belonging to the Ebenaceae family, *D. mespiliformis* is very widespread, found in Ethiopia, Kenya, Namibia, northern South Africa and Swaziland (Janick, 2006; Orwa *et al*., 2009).

The roots of the *D. mespiliformis* plant are used to treat jaundice, malaria, bruises and to also to ease childbirth. The bark can also be prepared to treat coughs, syphilis, leprosy, bronchial diseases and ulcers. The leaf and fruit decoctions can be taken to treat fever, menorrhagia and diarrhoea (Maundu, 2005; Neuwinger, 2000).

Figure 2.2 The leaves and fruits of *Diospyros mespiliformis.*
(http://www.plantzafrica.com/plantcd/diospyrosmespil.htm)

Elaeodendron croceum

This is an evergreen, medium to tall tree, which belongs to the family Celastraceae. Its bark is greyish with greyish brown branches. The leaves of the *E. croceum* (Figure 2.3) tree are oppositely arranged, hard and leathery with large yellow berries. *E. croceum* is mostly found in KwaZulu-Natal, Limpopo and in some parts of Zimbabwe (Archer, 1995).

E. croceum is known for its medicinal properties for anti-HIV activities (Prinsloo *et al*., 2010).

Figure 2.3 The leaves and fruits of *Elaeodendron croceum.* (http://www.plantzafrica.com/plantefg/elaedendcroc.htm)

Elaeodendron transvaalense

Also belonging to the Celastraceae family, *E. transvaalense* (Figure 2.4) is a small to medium tree that can grow up to 8 m. It has a pale grey, smooth bark with branchlets having a cluster of leaves at the tips. The leaves are arranged in multiples of three or can be alternate. *E. transvaalense* is mostly distributed in the northern parts of South Africa, but can also be found growing in Swaziland, Mozambique, Zimbabwe and Zambia (Van Wyk, 1997).

The Bapedi traditional healers from the Limpopo Province use the roots of *E. transvaalense* to treat STD's as well as HIV/AIDS (Semenya *et al*., 2013; Tshisikhawe, 2013).

Figure 2.4 The flowers of *Elaeodendron transvaalense.* (http://www.plantzafrica.com/plantefg/elaeodentrans.htm)

Hilliardiella nudicaulis

H. nudicaulis (Figure 2.5) is a South African endemic plant that is found mostly growing in the Eastern Cape, KwaZulu-Natal and Mpumalanga provinces. It belongs to the Asteraceae family (Foden, 2005).

There is currently no reported data on the medicinal uses of this plant.

Figure 2.5 The flowering parts of *Hilliardiella nudicaulis.*

(http://www.ispotnature.org)

Jasminum fluminense

J. fluminense (Figure 2.6) is an evergreen, climbing woody vine with young densely hairy stems. It belongs to the Oleaceae family. *J. fluminense* has white coloured

flowers and leaves arranged oppositely. They are found growing in Limpopo, Mpumalanga and KwaZulu-Natal provinces (Foden, 2005).

Currently there are no reported data on the medicinal uses of this plant.

Figure 2.6 The leaves of *Jasminum fluminense.* (http://www.cabi.org/isc/datasheet/115014)

Peltophorum africanum

P. africanum (Figure 2.7) belongs to the Fabaceae family and is also known as the African wattle. It can be found natively growing in Africa, south of the equator in countries like DR Congo, South Africa and Swaziland. *P. africanum* is a small tree that grows up to 9–15 m tall. The bark is rough with young twigs that have rusty hairs. Leaves from this plant are hairy and alternate (bipinnate with 4–9 pairs of pinnae). Flowers are bisexual and contain yellow-coloured petals. The fruit is a flat indehiscent pod with compressed seeds (Bessong *et al*., 2005; Ellis, 2003).

P. africanum can be used to treat menorrhagia and infertility (Steenkamp, 2003). The roots and bark of *P. africanum* are used for the treatments of wounds, toothaches or sore throats and also for the treatments of venereal diseases in most southern African countries. The bark can also be used to treat stomach pains and fevers. The Zulu people boil the roots to cure infertility, and can also be applied as an enema to treat back pains (Bessong *et al*., 2005; Steenkamp, 2003).

Figure 2.7 The flowers of *Peltophorum africanum.*
(http://www.zimbabweflora.co.zw)

Rhoicissus tridentata subsp. *cuneifolia*

R. tridentata (Figure 2.8) belongs to the Vitaceae family and is commonly known as the Bushman's grape. It is a climber or scrambling shrub, very rarely a small tree, found throughout the eastern parts of South Africa. It can also be found growing in parts of Zimbabwe. The branches may grow into trees in grassy woodland to heights

of up to 10 m. Without support, it may grow amongst rocks. The leaf margins differ, depending on where the plant is located. It can vary from small or larger leaflets with four or more teeth on the leaf terminals (Van der Merwe *et al.*, 2001).

Decoctions and infusions of roots obtained from the *R. tridentata* plants are used mostly by women during pregnancies and also in infertility cases (Steenkamp, 2003). This traditional Zulu medicinal plant was also identified for its uses in the protection of liver damage or bladder complications, also known as hepatoprotective effects (Opoku *et al.*, 2007).

Figure 2.8 The leaves of *Rhoicissus tridentata* subsp. *cuneifolia.* (http://www.zimbabweflora.co.zw)

Schotia capitata

S. capitata (Figure 2.9) is a small, slender shrub that can grow up to 9 m tall and grows alongside other plant species in dense thickets. The bark is rough and pale-grey. Its

leaves are oppositely arranged. *S. capitata* belongs to the Fabaceae family. Its members are mostly found in the eastern Transvaal and in KwaZulu-Natal. They can also be found in Mozambique and in Swaziland (Setshogo, 2005).

No previously recorded uses were found in the literature surveyed.

Figure 2.9 The flower parts of *Schotia capitata.*

(http://witbos.co.za)

Senna italica* subsp. *arachoides

S. italica (Figure 2.10) is a member of the Fabaceae family. The plant is woody throughout and can be perennial herbs or shrubs that grow to heights of up to 60 cm tall. Its stems are hairy with hairy leaves on both sides, arranged alternately. Flowers are usually yellow or orange in colour, with green or black-coloured seeds. *S. italica*

can be found growing in the southern parts of South Africa, Mozambique, Swaziland and Zimbabwe (Irwin, 1982).

S. italica is widely used traditionally for the treatment of STD's and some forms of intestinal complications (Masoko *et al*., 2010). The Bapedi cultural group uses the roots for the treatment of gonorrhoea (Semenya *et al*., 2013).

Figure 2.10 The flowers of *Senna italica.*

(http://tropical.theferns.info)

Solanum tomentosum

S. tomentosum (Figure 2.11) is a shrub with yellowish stellate hairs with bright orange berries. It grows in grassy or rocky areas on hillsides, river beds or roadsides. The stems are densely covered with scattered golden brown spines that are sharp and usually straight. It belongs to the Solanaceae family (Aliero, 2006). *S. tomentosum* can be found in the Little Karoo to the Eastern Cape and Lesotho (Knapp, 2013).

The indigenous people of the Eastern Cape Province use this plant traditionally for the treatment of STD's like syphilis, sore throat or toothaches (Aliero, 2006).

Figure 2.11 The leaves and fruits of *Solanum tomentosum.* (http://www.operationwildflower.org.za /*Solanum tomentosum*)

Terminalia sericea

T. sericea (Figure 2.12) is a small to medium sized tree that can grow up to heights of 9 m. It has reddish- brown branches with crowded leaves at the end of its branches. The flowers are pale yellow to creamy white. *T. sericea* can be found growing in Angola, Botswana, Namibia, South Africa and Zimbabwe. *T. sericea* is a member of the Combretaceae family (Raimondo *et al*., 2009).

The root or bark of *T. sericea* is used for the treatment of STD's, including gonorrhoeae in the KwaZulu Natal province. This decoction is taken orally or is used as an enema (De Wet *et al*., 2012).

Figure 2.12 The leaves and flowers of *Terminalia sericea.* (http://www.plantzafrica.com/planttuv/terminaliasericea.htm)

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Chapter 3: Antimicrobial activity of selected plant extracts and compounds

3.1 Introduction

In many parts of the world, the use of plants remain the preferred form of health care. About 80% of the population in most developing countries use traditional medicine as their prime source of health care (Pratiwi *et al*., 2015). Medicinal plants have the potential to be of therapeutic advantage in the management of human diseases, because they can offer potential molecules and mixtures of bioactive compounds that can be exploited (Aumeeruddy-Elalfi *et al*., 2015).

Almost 50 000 people worldwide are dying daily because of infectious diseases. Infectious diseases are caused by microorganisms that are able to survive in warm, moist dark parts of the human body, which include the genitals, mouth and anus. Such diseases continue to be a major health problem worldwide (Mulaudzi *et al*., 2011). Genitourinary infections are the most common infectious diseases caused by abnormal overgrowth of commensal microorganisms and sexually transmitted diseases (STD's) (Kweon *et al*., 2015).

The impact of infectious diseases caused by microorganisms is increasing, particularly in most developing countries. The search for effective antimicrobial agents from natural products has attracted much attention in the health care sector because of their perceived fewer side effects, efficacy and safety (Aumeeruddy-Elalfi *et al*., 2015).

This is because the traditionally used medicinal plants have proven to be pharmacologically active (Pawar *et al*., 2015).

The major part of therapeutic therapy involves the use of plant extracts and their active constituents (Joshi *et al*., 2011). There are a number of reports that have been published on medicinal plants based on their antimicrobial activities. In recent years, the chemically identified compounds and biologically active molecules from plant extracts, and their diverse formulations, have been recommended for commercial use (Thanigaivel *et al*., 2015). This may lie in the structure of natural products which reacts with pathogens in a way that inhibits or reduces harm in other important molecules or the physiology of the host (Moorthy *et al*., 2015). As there is an increase in the occurrence of infectious diseases, there is also a need to synthesize and scientifically investigate new antimicrobial compounds with novel mechanisms of action (Pawar *et al.,* 2015).

Even though pharmaceutical industries have produced a number of antimicrobial drugs, the increase in resistance of microorganisms to these drugs is still a major problem. Antimicrobial resistance is a growing problem that impacts the treatment source of antibiotics to treat and improve the human well-being (Tekwu *et al*., 2012). One of the methods to reduce the resistance to these antibiotics is by the use of antibiotic resistance inhibitors from plants (Sen, 2012). Antimicrobials originating from plant extracts can be a choice of treatment, which has been reported to have therapeutic potential as treatment against infectious diseases (Pawar *et al*., 2015).

Resistance to the antimicrobial agents may be initiated through many factors including inadequate or inappropriate therapeutic therapy or a high occurrence of disease coupled with lack of ability to initiate prevention programs (Ruddock *et al*., 2011).

Antibiotic resistance can also be caused by the overuse of antibiotics. Plants are known to produce a range of compounds to protect themselves against pathogens. It is therefore expected that plant extracts showing target sites in addition to those used by antibiotics will therefore be active against drug resistant pathogens (Sen, 2012).

Those plants with compounds that can be considered as potential compounds for the development of new antimicrobial drugs, should either inhibit or kill the growth of microorganisms and should have no or least toxicity to host cells (Ahmad, 2001). The medicinal plants investigated in this study are those perceived to have antimicrobial properties based on the studied and local uses of the plants, in the community of Jongilanga.

The aim of this experiment was therefore to identify those plants that are able to inhibit the growth of selected microorganisms. The results of this screening may provide a powerful tool in the identification of a successful treatment for sexually transmitted diseases.

3.2 Materials and Methods

3.2.1 Plant collection

The medicinal plants collected in the Jongilanga community were organized into a database (Mophuting, 2015). It is in this database of plants used in the treatments of STD's that plants were selected. For each plant a specific plant part was used, as used traditionally.

3.2.2 Preparation of extracts

Different parts of each plant were dried and ground. The powdered plant material was dissolved in 70% ethanol and vigorously shaken for 72 hours using a Labcon 3086U machine at moderate speed. Filtration was then conducted using a vacuum system and Whatman filter paper and the remaining plant material was discarded. The filtrate was concentrated at 60°C using a Rotavapor (Buchi B-480) machine to evaporate the solvent from the samples. These extracts were stored in pre-weighed labelled polytops. The extracts were then placed in a fume hood to evaporate any remaining solvent. Polytops containing plant extracts were stored in a 4°C environment (Silva *et al*., 1998).

All extracts were prepared using ethanol as the extraction solvent which is able to extract both non-polar and polar solvents because of its medium polarity (Zhou, 2004). Extracts were then stored in polytops and placed in a 4°C environment for use in the different assays conducted (Figure 3.1). All assays were performed in a sterile environment under the laminar flow.

A. **B**.

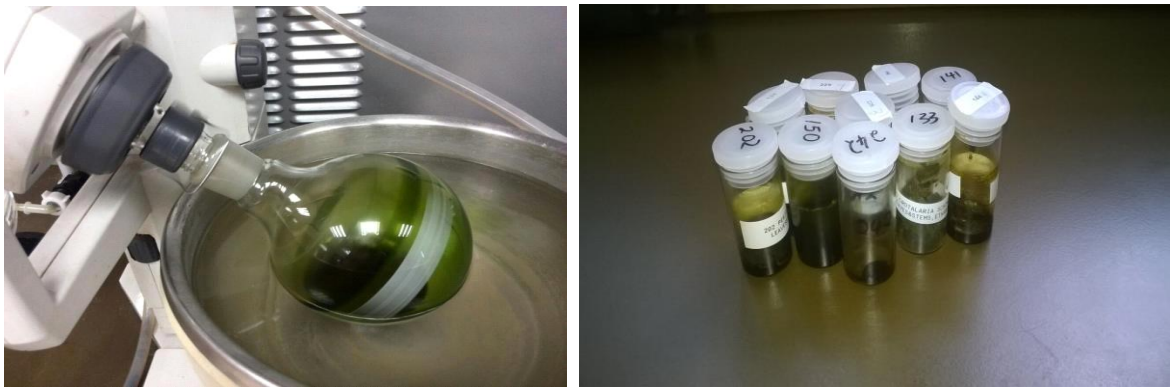

Figure 3.1 A) Plant extraction using a Buchi B-480 Rotavapor. **B**) Concentrated plant extracts stored in polytops.

3.2.3 Isolated compounds

Three pure compounds which were previously isolated from *Elaeodendron transvaalense* were also included in the study. This follows that this plant is traditionally used in the treatment of many STD's (Tshikalange, 2010). These compounds are shown in Figures 3.2, 3.3 and 3.4 respectively; lup-20(30)-ene-3,29-diol,(3α)-(9Cl) (flavonoid **1**), lup-20(29)-ene-30-hydroxy-3-one (flavonoid **2**) and 4'-O-methylepigallocatechin (flavonoid **3**).

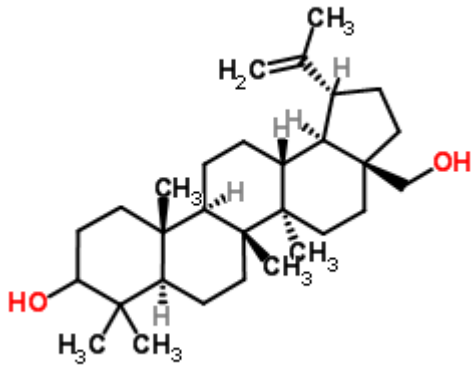

Figure 3.2 The structure of lup-20(30)-ene-3,29-diol,(3α)-(9Cl).

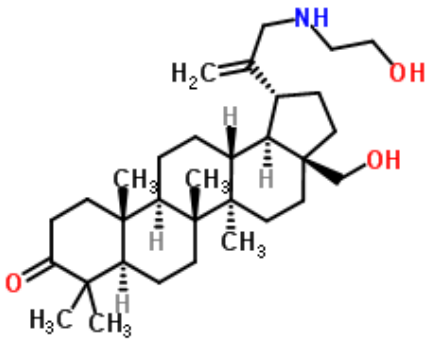

Figure 3.3 The structure of lup-20(29)-ene-30-hydroxy-3-one.

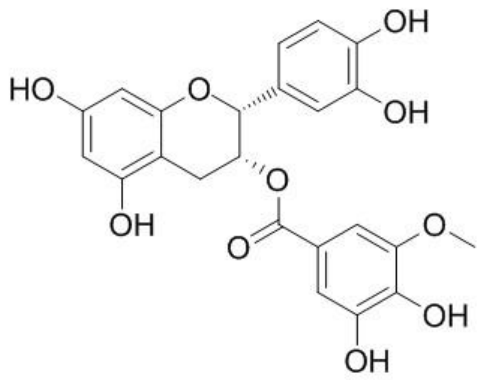

Figure 3.4 The structure of 4'-O-methyl epigallocatechin.

3.2.4 Culturing of pathogens

Fungal and bacterial test pathogens were selected based on conditions that the plants are reported to traditionally cure these STD related microorganisms. All cultures were grown in appropriate media and incubated accordingly. Standard protocols were adhered to for all the pathogens. All pathogens were cultured in a fume hood to ensure sterility.

Candida albicans was cultured on Nutrient agar and grown in Nutrient broth at incubation conditions of 37°C for 24 hours. Both *Neisseria gonorrhoeae* and *Oligella ureolytica* were grown on chocolate agar at 37° C for 48 hours and inoculated in Mueller-Hinton broth for 24 hours. *N. gonorrhoeae* was supplemented with CO_2. *Gardnerella vaginalis* grew optimally in Brain and Heart infusion Broth (HBI, Oxoid LTD) at 37°C for 24 hours.

3.2.5 Determination of minimum inhibitory concentration (MIC)

The determination of antimicrobial activity was determined using the broth micro dilution method in 96-well µl plates and performed as described by Eloff, (1998). Briefly, 50 mg of each plant extract was weighed in 200 ml Eppendorf tubes. Each plant extract was dissolved in 100 µl of 10% DMSO and 900 µl of Nutrient broth, to make a final concentration of 50 mg/ml. All samples were tested in a 96-ELISA well plate, in triplicate. Microorganisms were inoculated in sterile broth and prepared to a density of 1.5 x 10^{8} colony forming units (CFU) per ml (CFU/ml), corresponding with the 0.5 McFarland Standard. Serial 2-fold dilutions were made. Inoculated broth (100 µl) was added to the plates. Ciprofloxacin was used as a positive control for all extracts and 10% DMSO was used as a negative control to ascertain if any growth inhibition was attributed to the solvent and culture control (pathogen growing independently). Plates were incubated for 24 hours at 37ºC and the results were read based on the visual colour change of Presto blue dye; a pink colour change indicating microbial growth. The lowest concentration that showed no visible pathogen growth was recorded as the minimum inhibitory concentration (MIC) for each microorganism.

3.3 Results and discussion

3.3.1 MIC determination assay

The determination of antimicrobial susceptibility of plant extracts was performed using the serial broth micro-dilution assay. The broth micro-dilution assay is a simple and rapid method used to test plant extracts for antimicrobial agents. This assay is performed in a 96-well plate, making it easier to test various samples at once. Ciprofloxacin was used as the positive control and DMSO was used as the negative control. The minimum inhibitory concentration (MIC) is then determined with the addition of an indicator (dye) which visually indicates microbial inhibition. Pink colour indicates the growth of microorganisms (Figure 3.5) (Eloff, 1998).

MIC is defined as the lowest concentration of an antimicrobial agent or plant extract that will inhibit the visible growth of a microorganism upon addition of an indicator dye after overnight incubation (Andrews, 2001). Presto blue dye was added to observe the colour change; a pink change indicates microbial growth and no colour change (blue) indicates the MIC. MIC values were determined by the lowest concentration from the 96-well to remain blue.

The results of antimicrobial activity expressed in MIC values are shown in Table 3.1. The plant extract with the lowest MIC value observed is considered to have the best antimicrobial activity based on each microorganism. The highest tested concentration was 12.5 mg/ml and the lowest was 0.1 mg/ml/ (Figure 3.5). The aim of this assay was mainly to confirm resistance or *in vitro* activity of new antimicrobials.

Table 3.1 The MIC (mg/ml) values of ten plant species tested against different microorganisms.

Samples	*Candida albicans*	*Gardnerella vaginalis*	*Neisseria gonorrhoeae*	*Oligella ureolytica*
Ciprofloxacin	<0.01	<0.01	<0.01	<0.01
Acacia Cf karroo	0.8	6.3	0.8	1.6
Diospyros mespiliformis	3.1	6.3	6.3	3.1
Elaeodendron croceum	1.6	12.5	1.6	3.1
Elaeodendron transvaalense	3.1	12.5	1.6	3.1
Hilliardiella nudicaulis	1.6	12.5	0.8	12.5
Jasminum fluminense	3.1	<12.5	6.3	3.1
Peltophorum africanum	3.1	12.5	1.6	1.6
Rhoicissus tridentata* subsp. *cuneifolia	0.8	0.8	0.4	1.6
Schotia capitata	3.1	12.5	1.6	3.1
Senna italica* subsp. *arachoides	1.6	3.1	0.8	<12.5
Solanum tomentosum	<12.5	<12.5	12.5	3.1
Terminalia sericea	3.1	<12.5	1.6	<12.5
Lup-20(30)-ene-3,29-diol,(3α)-(9Cl) (F1)	<12.5	<12.5	<12.5	<12.5
Lup-20(29)-ene-30-hydroxy-3-one (F2)	<12.5	<12.5	<12.5	12.5
4'-O-methyl-epigallocatechin (F3)	3.1	<12.5	6.3	1.6

<0.01 = Activity is shown at all concentrations/lower.

>12.5 = Extracts can only show activity if tested at concentrations higher than 12.5 mg/ml.

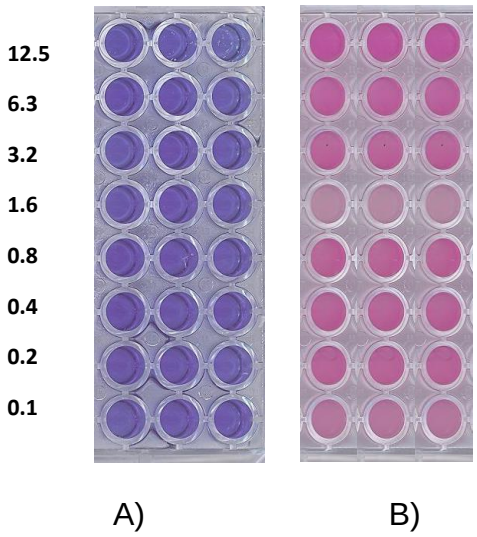

Figure 3.5: The micro-titre plates with positive (A) and negative control (B) in the presence of microorganisms

Ciprofloxacin is an antibiotic that is useful for the treatment of a number of bacterial infections (Carabineiro *et al*., 2012). It belongs to a group of drugs called fluoroquinolones, which are bactericidal agents that exhibit MIC dependant killing (Khan *et al*., 2015). Its range of activity includes most strains of bacterial pathogens which are responsible for respiratory, urinary tract, gastrointestinal, and abdominal infections, including Gram-negative and Gram-positive bacterial pathogens (Brunton *et al*., 2005).

Candida albicans

Acacia karroo and Rhoicissus tridentata showed the best antimicrobial activity by having the lowest MIC value of 0.8 mg/ ml (Figure 3.6). In a study conducted by Nielsen *et al*., (2012), the leaves and stem of *A. karroo* were tested for *C. albicans* and they showed MIC values of 0.6 mg/ml and 0.8 mg/ml, respectively. The anti-Candidal activity of *R. tridentata* was tested by Hamza *et al*., (2006) and later by Samie *et al* (2010), and these results also showed good inhibitory activities.

Also showing good activity of 1.6 mg/ ml (Figure 3.7) are *Elaeodendron croceum, Hilliardiella nudicaulis* and *Senna italica*. There are no previously conducted experiments on the antimicrobial activities of *H. Nudicaulis.* The antigonococcal and anti-candidal activities of *S. italica* were evaluated by Mulaudzi *et al* (2015), and were found to have a high inhibition activity and low anti-fungal activity against *C. albicans.* Plants that showed to have the least/no activity were *Solanum tomentosum* and the flavonoids 1 and 2 with MIC values higher than the tested concentrations (>/=12.5 mg/ml).

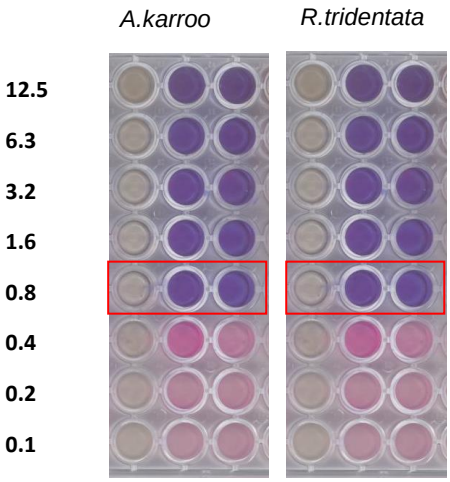

Figure 3.6 MIC determination of *Acacia karroo* and *Rhoicissus tridentata* in the presence of *Candida albicans.*

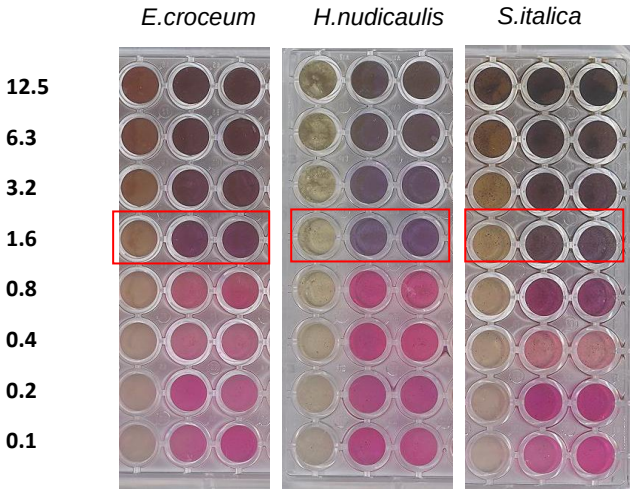

Figure 3.7 MIC determination of *Elaeodendron croceum, Hilliardiella nudicaulis* and *Senna italica* in the presence of *Candida albicans.*

Gardnerella vaginalis

When tested for *Gardnerella vaginalis*, *R. tridentata* also showed the best activity with an MIC value of 0.4 mg/ml (Figure 3.8). There are no previous reports on *R. tridentata.* Aqueous extracts of *Peltophorum africanum* exhibited an MIC value of 0.5 mg/ml when tested by Mongalo (2013) and Naidoo *et al* (2013). In contrast, it showed the lowest activity in this study (12.5 mg/ml). *Terminalia sericea* showed the least activity in the study with an MIC value of >12.5 mg/ml. However, Van Vuuren (2010), reported its aqueous extract to be active with MIC of 1.0 mg/ml. All the flavonoids showed no activity at the highest concentration tested (12.5 mg/ml).

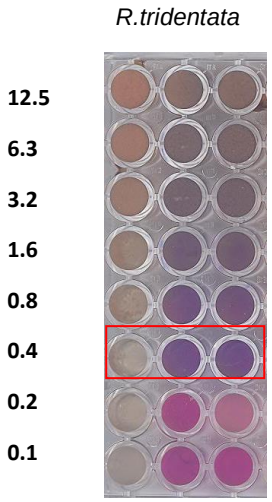

Figure 3.8 MIC determination of *Rhoicissus tridentata* in the presence of *Gardnerella vaginalis.*

Neisseria gonorrhoeae

R. tridentata showed the best activity with an MIC value of 0.4 mg/ ml (Figure 3.9). There are no previous reports on *Neisseria gonorrhoeae*. Also showing good activity with MIC values of 0.8 mg/ ml were *A. karroo, Hilliardiella nudicaulis* and *S. italica* (Figure 3.10). Mongalo (2013), reported notable activity on *Peltophorum africanum* on aqueous extracts and organic extracts with MIC values of 0.5 mg/ml and 0.25 mg/ml, respectively. Supporting reports from Van Vuuren (2010), reported *Terminalia sericea* to be active with MIC values of 1 mg/ml. In this study *Terminalia sericea* showed best activity with the pathogen *Neisseria gonorrhoeae,* with an MIC of 1.6 mg/ml. Only flavonoid 3 (epigallocatechin) showed some antibacterial activity with an MIC value of 6.3 mg/ml. Epigallocatechin has been shown previously to have many

pharmacological properties including antimicrobial, antioxidant and antiviral (Liang *et al*., 2015; Moreno-Vasquez *et al*., 2017; Xu *et al*., 2016).

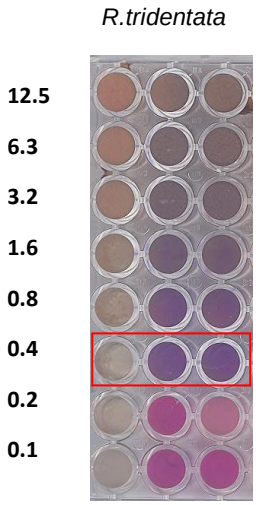

Figure 3.9 MIC determination of *Rhoicissus tridentata* in the presence of *Neisseria gonorrhoeae*.

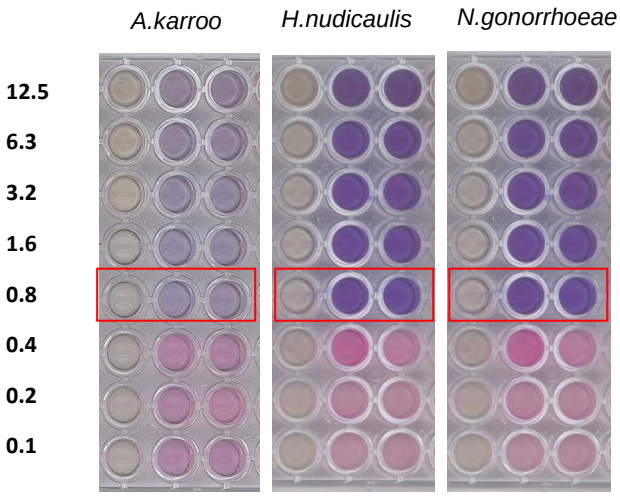

Figure 3.10 MIC determination of *Acacia karroo, Hilliardiella nudicaulis* and *Senna italica* in the presence of *Neisseria gonorrhoeae*.

Oligella ureolytica

The lowest MIC observed for *O. ureolytica* with MIC values of 0.8 mg/ ml and these were *A. karroo, Diospyros mespiliformis, R. tridentata* and *Schotia capitata* extracts (Figure 3.11). The broad spectrum of antimicrobial activities of *D. mespiliformis* has also been confirmed by Mabona *et al* (2013). *Solanum tomentosum* and the flavonoids 1 and 2 showed no activity at the tested concentrations. This study has reported for the first time the antimicrobial activity of epigallocatechin (F3) against *Oligella ureolytica,* which also showed to be active with an MIC value of 1.6 mg/ml.

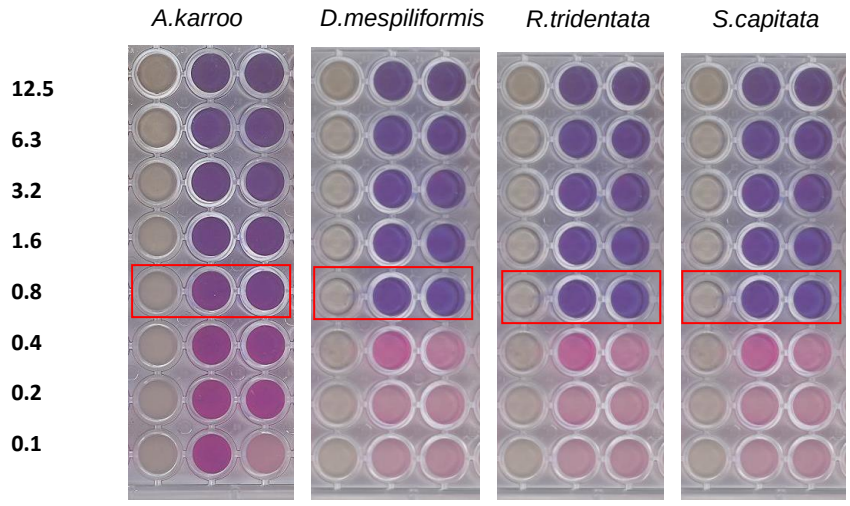

Figure 3.11 MIC determination of *Acacia karroo, Diospyros mespiliformis, Rhoicissus tridentata* and *Schotia capitata* in the presence of *Oligella ureolytica.*

The differences in MIC activities from previous reports may be due to locality or area of collection and environmental conditions, the age of the plant and the season of collection (Mongalo, 2013). This study is therefore a preliminary evaluation of antimicrobial activity of the plants. It indicates that some plants have the potential to

generate novel metabolites. The exhaustive use of antibiotics often results in the emergence of resistant strains. It is because of this drug resistance, that the exploration for new antibiotics remains unrestricted. In this connection, plants continue to be a rich source of therapeutic drugs. The active principles of many drugs originate from plants or are produced as secondary metabolites. The remarkable contribution of plants to the drug industries is made possible, because of the large number of the phytochemical and biological studies all over the world (Srinivasan *et al*., 2001).

3.4 Conclusion

After screening the selected plants for their inhibitory activities against selected sexually transmitted associated microorganisms, it can be concluded that all the extracts showed good antimicrobial activities for at least one of the microorganisms tested. For a plant to be rendered the best plant with best antimicrobial activities, it should have the lowest MIC value in each of the tested microorganisms. The extracts indicating antimicrobial activity could result in the discovery of novel antibacterial/ anticandidal agents. These extracts indicating wide spectra of activity, may help in discovering new chemical categories of antibiotics that could serve as selective agents for the maintenance of human health and provide biochemical tools for the study of infectious diseases.

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Chapter 4: Anti-inflammatory of selected plant extracts and compounds

4.1 Introduction

Pain and inflammation are one of the major conditions induced by multi-factorial causes and if sustained can be associated with various diseases (Verma *et al*., 2015). Inflammation is a physiological response of living tissues to infection, irritation and tissue injury against microorganisms but if uncontrolled may lead to the development and progression of various chronic diseases like cancer (Pinheiro *et al*., 2015). The mechanism of enzyme activation, cell migration, mediator release, tissue breakdown and repair, is a complex array which is involved, to maintain the equilibrium between the beneficial effects of inflammation (to restrict infection) and long term cell destruction (Sermakkani, 2013).

The initiation and progression of inflammatory processes involves the synthesis of effector proteins such as cell adhesion molecules and transcription factors such as the nuclear factor kappa B (NFκB) (Tran *et al*., 2015). NFκB is a homo- and hetero-dimeric transcription factor which plays a crucial role in the inflammatory process, and is responsible for the expression and regulation of genes that are involved in inflammation, apoptosis and immunity (Altmann *et al*., 2015). The inflammatory process is indicated by cellular and microvascular reactions that act by removing damaged tissues and generating new tissues. During the inflammatory process, there

is an activation of the innate immunity. There is also an escalated production of various mediators including pro-inflammatory eicosanoids and cytokines (Pinheiro *et al*., 2015).

During the inflammation process, arachidonic acid is broken down through the cyclooxygenase (COX) pathway to produce prostaglandins and thromboxane A_2, or via the lipoxygenases (LOX) pathway to produce hydroperoxy-eicosatetraenoic acids and leukotrienes (Figure 4.1). COX/LOX metabolites play a critical role in cell signalling and proliferation, which can increase eicosanoids levels leading to tumour growth (Adebayo *et al*., 2015).

LOXs are a family of non-heme oxidizing enzymes which catalyse the oxidation of polyunsaturated fatty acids such as arachidonic acid. These enzymes have received recognition due to their role in the advancement in several human diseases (Tehrani *et al*., 2014). The oxidation of substrates like arachidonic acid and linoleic acid by 15-LOX leads to the formation of pro-inflammatory and pro-thrombotic products. Therefore, the inhibitors of 15-LOX have been identified as possible agents for the treatment of chronic inflammation and several other human diseases like cancer (Radmark, 2007).

The lipoxygenase group of enzymes, such as 5, 8, 12 and 15-LOX, contribute significantly in various inflammatory disorders. The isomeric enzyme, 15-LOX is a key enzyme which functions in the synthesis of leukotrienes from arachidonic acids. Biologically active leukotrienes are mediators of various pro-inflammatory and allergic reactions, therefore the inhibition of the synthesis of leukotrienes by 15-LOX is regarded as one of the therapeutic strategies in managing inflammatory disorders (Adebayo *et al*., 2015).

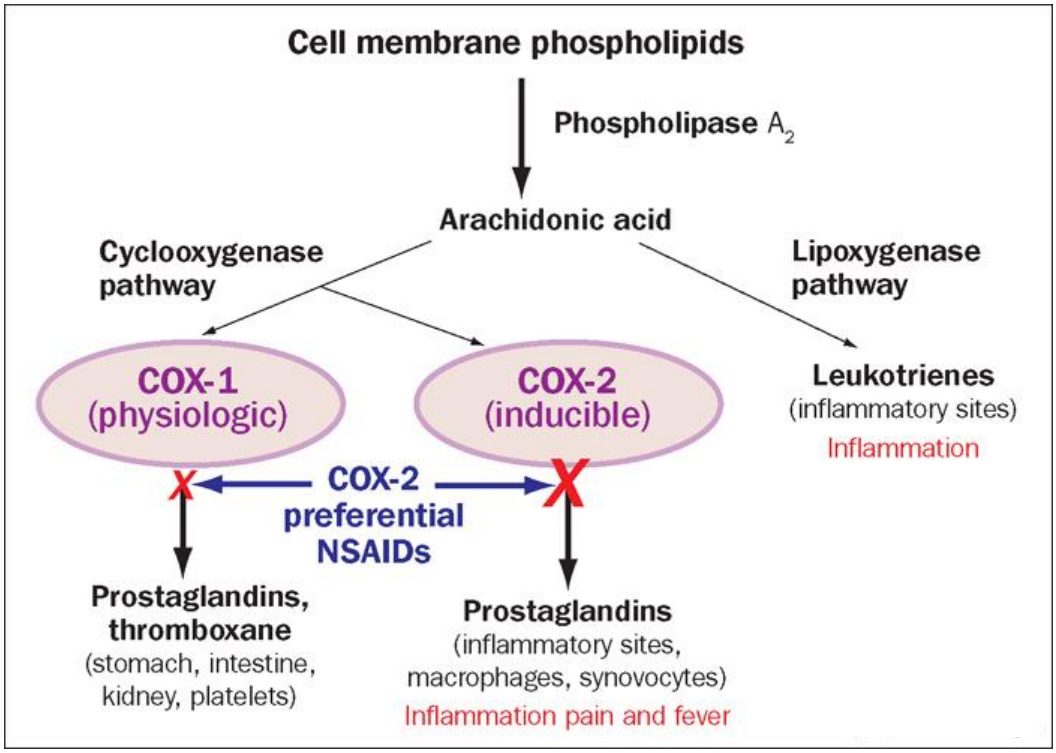

Figure 4.1 An illustration of the COX/LOX pathways during inflammation.

Inflammation is one of the major risk factors for various human diseases including venereal diseases and this may often lead to treatment complications (Mulaudzi *et al*., 2013). Sexually transmitted diseases (STD's) are presumed to be the major causes of inflammation in the genital mucosa. Diseases such as gonorrhoea and bacterial vaginosis (to a lesser extent) have also been implicated in causing inflammation through several mechanisms, including the disruption of the epithelial barrier (Shey *et al*., 2015). STD's ultimately occur at the mucosal surface of the genital tract, where inflammation from both ulcerative and non-ulcerative infections increase localized immune cell mobilization, which in turn enhances susceptibility to HIV infection (Ferreira *et al*., 2015).

Inflammation is a leading cause for symptoms in most STD's. Symptoms can vary from slight inflammation and discharge, to infertility and death. Others can include ectopic pregnancy, mild cervicitis, pelvic inflammatory disease and tuba pathology related infertility. In these diseases, inflammatory responses may not entirely have the positive result of triggering immune responses to eliminate the infection. Tissue scarring and failure to remove bacteria may often occur in these infections. Other processes, however, have ways of inducing inflammatory response to allow for better survival in the host. The activation of inflammatory responses rely on the ability of the host to detect the pathogen and initiate crucial inflammatory pathways (Singer *et al*., 2015).

At present, non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for managing both acute and chronic inflammation. However, these NSAIDs which are non-selective inhibitors for the cyclooxygenase enzymes (isoenzymes 1 and 2), have been reported to have a severe risk of gastrointestinal toxicity (Jeengar *et al*., 2014). Drugs currently used for inflammatory disorders are salicytes, corticosteroids but these have been associated with side effects like intestinal tract ulcers and erosion of the stomach lining (Verma *et al*., 2015). There is therefore a strong need for natural anti-inflammatory bioactive agents for the maintenance and reduction of risks of diseases without any side effects. This is because of the belief that there is a lower prevalence of adverse reactions of natural phytochemicals compared to synthetic pharmaceuticals (Cock, 2014).

The aim of this experiment was to screen 12 medicinal plants and isolated compounds for the potential anti-inflammatory activity through the inhibition of LOX enzymes.

4.2 Materials and Methods

4.2.1 Preparation of extracts

Plant extracts were prepared as previously described in Chapter 3.

4.2.2 Inhibition of 15-lipoxygenase (15-LOX) enzyme

The method followed was taken from Adebayo *et al* (2015). Briefly, plant extracts were weighed at 10 mg and compounds at 1 mg. Plant samples were dissolved in dimethyl sulphoxide (DMSO) to make a final concentration of 10 mg/ml and 1 mg/ml, respectively. The 15-LOX enzyme was prepared up to a final solution of 200 units/ml and placed on ice. A volume of 12.5 µl of each plant sample and control was added to 487.5 µl of 15-LOX in a 96-well micro-titre plate and incubated for 5 minutes at room temperature. Thereafter, 500 µl substrate solution (10 µl linoleic acid dissolved in 30 µl ethanol, made up to 120 ml with 2 M borate buffer at pH 9.0) was added to the solution and incubated for 5 minutes at room temperature. After incubation, the absorbance was measured with the micro-titre reader at 234 nm (Figure 4.2). Quercetin (1 mg/ml) was used as a positive control and DMSO was used as a negative control. The results were expressed as IC_{50}, i.e. concentration of the extracts and controls that resulted in 50 % of 15- LOX inhibited.

Figure 4.2 The anti-inflammatory experiment.

4.3 Results and discussion

4.3.1 Anti-inflammatory activity

The aim of this experiment was to assess the anti-inflammatory activity of selected plant extracts and compounds using the anti-15-LOX model of inhibition. The ability to inhibit the COX/LOX activity can be used to evaluate the anti-inflammatory activity of any given plant extract. Such plants with this ability would potentially have a therapeutic effect as an anti-inflammatory agent. A plant species identified to contain a known anti-inflammatory compound has the possibility of being generated into a product that can be used to manage inflammation. The anti-inflammatory activities of the extracts were expressed as IC_{50} and compared to those of Quercetin which showed an IC_{50} value of 48.86 µg/ml. The results for inhibition of 15-LOX enzyme are shown in Table 4.1

Table 4.1 The inhibition assay results for 15-LOX enzyme expressed as IC_{50}.

Samples	**IC_{50} (µg/ml)**
Quercetin	48.86 ±
Acacia karroo	62.24
Diospyros mespiliformis	188.1
Elaeodendron croceum	82.51
Elaeodendron transvaalense	80.17
Hilliardiella nudicaulis	51.32
Jasminum fluminense	35.22
Peltophorum africanum	88.69
Rhoicissus tridentata subsp. *cuneifolia*	87.39
Schotia capitata	83.13
Senna italica subsp. *arachoides*	77.89
Solanum tomentosum	37.16
Terminalia sericea	122.82
Lup-20(30)-ene-3,29-diol,(3α)-(9Cl) (F1)	69.77
Lup-20(29)-ene-30-hydroxy-3-one (F2)	39.06
4'-O-methyl-epigallocatechin (F3)	31.38

Quercetin is a widely used polyphenol that has a wide spectrum of beneficial pharmacological effects such as free radical scavenging, antioxidant and anti-inflammatory activities (Bi *et al*., 2013). Quercetin was used as a positive control and DMSO was used as a negative control, i.e. 100% enzyme activity or no enzyme inhibition. The percentage enzyme inhibition of each extract compared with negative

control as 100% enzyme activity was calculated and the results were expressed as IC_{50} (concentration of the extracts and controls that resulted in 50% 15-LOX inhibition). Good inhibitors were based on their low IC_{50} values when compared to quercetin.

The lipoxygenase enzyme assay is based on the ability for lipoxygenase enzymes which catalyze the oxygen-dependent oxidation of fatty acid substrates (linoleic acid and arachidonic acid are common examples) to form hydroperoxy-fatty acid products. This method is useful for detecting inhibitors of such enzymes (Burnett *et al.*, 2007).

The mode of action of many phenolic compounds such as tannins, curcumins and flavonoids are believed to be via their free radical scavenging activities or via the inhibition of pro-inflammatory enzymes such as cyclo-oxygenases (COX) and lipoxygenases (LOX) in the inflammatory pathways (Adebayo *et al*., 2015).

From previous literature, it has been reported that *Acacia karroo* is very rich in proanthocyanidins and flavonols. Flavonoids are known for their significant scavenging properties on oxygen radicals *in vitro* and *in vivo*, affecting various steps in the arachidonate cascade via cyclooxygenase or lipoxygenase. The anti-inflammatory activities exhibited by *A. karroo* may be due to the presence of the above mentioned phytochemicals (Mulaudzi *et al*., 2013). In this study, *A. karroo* showed to have good 15-LOX inhibition activity. The IC_{50} value was higher than that of quercetin 48.86 µg/ml, however, it can be comparable. The calculated IC_{50} value was 62.24 µg/ml. It has also been reported that *Elaeodendron croceum* contains a naturally occurring flavonoid (naringenin) that enables it to exhibit anti-inflammatory activities (Mbaveng *et al*., 2014). However, in this study, *E. croceum* showed low inflammation inhibitory activity, showing an IC_{50} value of 82.51 µg/ml.

Isolates of *Diospyros mespiliformis* include tannins, steroids, anthocyanins and flavonoids (Belemtougri *et al*., 2006). Flavonoids can regulate cellular activities of inflammatory related cells such as macrophages and lymphocytes (Sharifi-Rad *et al*., 2015). *Terminalia sericea* showed an IC_{50} value of 122.82 µg/ml. However, Cock (2014), reported that *T. sericea* is known to contain a phenolic anti-inflammatory compound, resverstrol. *T. sericea* was able to inhibit cyclooxygenase enzyme COX-2 in that study. Cock (2015), later reported a compound, triterpenoidal saponin sericoside to be responsible for the anti-inflammatory activities in the inhibition of COX-2 and 5-LOX enzymes.

Jasminum fluminense, the root extract of *Solanum tomentosum* and flavonoids 2 and 3 showed to be the best inhibitor for inflammation against 15-LOX enzymes with IC_{50} values of 35.22 µg/ml, 37.16 µg/ml, 39.06 µg/ml and 31.38 µg/ml, respectively.

Figure 4.3 shows the 96-well test plate for the 15-LOX inhibition activity for all plant extracts. The colour changes can also be used to detect the activity of the extracts when compared to the colour change of the negative control; which indicates no enzyme inhibition.

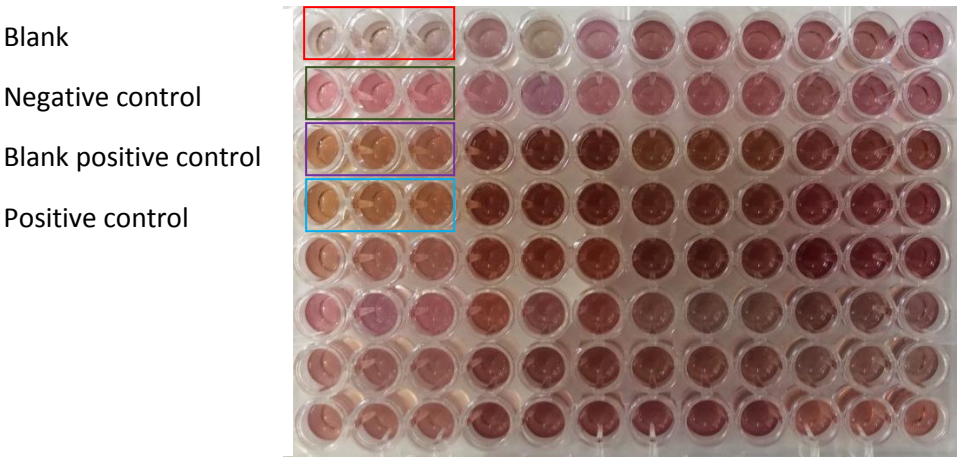

Figure 4.3 The test plate for inhibition of 15-lipoxygenase.

These findings imply that these plants may contain compounds with higher and more significant activity than that of quercetin. However, additional work should be done in the isolations of these active compounds.

Lipoxygenases are lipid-peroxidising enzymes, which are part of the biosynthesis of leukotriene from arachidonic acid. They are also mediators of inflammatory cascades and are responsible for allergic reactions. Furthermore, these LOX enzymes also catalyse the introduction of molecular oxygen to unsaturated fatty acids such as linoleic and arachidonic acids, which are the common substrates for LOX. There are four main iso-enzymes already mentioned, namely, 5, 8, 12 and 15-LOX, and are determined by the position of oxidation in the unsaturated fatty acids (Adebayo *et al*., 2015). To explain the role of each LOX enzyme, it is particularly important to develop inhibitors that are enzyme specific. To date, very few anti-inflammatory drugs originating from herbal constitutes have been found, and a number of plants are under laboratory investigations across the world (Sermakkani, 2013).

4.4 Conclusion

The plant extracts or compounds which are able to inhibit the pro-inflammatory activities of the 15-LOX enzymes may involve the discovery of possible leads for the development of effective anti-inflammatory drugs. However, more work is required to accurately characterize the compound(s) which are responsible for the anti-inflammatory principles in these plant species and further comprehend their mechanisms of action.

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Chapter 5: DNA gyrase and HIV-1 Reverse Transcriptase activity of plant extracts and compounds

5.1 Introduction

The emergence of antimicrobial resistance amongst sexually transmitted diseases (STD's) is a major global concern that has resulted to human mortality (Ngocu *et al*., 2012; WHO, 2003). Molecular methods have the potential to enhance the management of antimicrobial resistance, more especially for organisms that cannot be easily characterized, which is the case for most STD's. However, there are challenges in the monitoring of antimicrobial resistance and these include; the many or unknown mechanisms of resistance from bacteria, the overlooking of novel mutations or that their specificity can be undermined where target sequences are shared across different species (Whiley *et al*., 2013). Thus, the search for new methods to maintain these resistances are still at their highest peak.

After decades of research driven by genomics and proteomics, there are strategies that have been identified for developing new antimicrobials such as seeking new target sites (Karkare *et al*., 2013). Most antibiotics act on a few of these bacterial targets such as ribosomes, cell wall biosynthesis and DNA gyrase. DNA gyrase is essential for bacterial DNA replication, repair and decatenation and found in all bacteria and plants, but is deficient in higher eukaryotes (Jacobsson *et al*., 2014). Thus, because

of its essential nature and its mechanism of action, DNA gyrase is considered as one of the best-validated targets in anti-microbial drug development (Chiriac *et al*., 2015).

DNA gyrase is a DNA topoisomerase which manipulates and catalyse changes in the structure of DNA (Karkare *et al*., 2013). Amongst topoisomerases is this unique DNA gyrase, a type II bacterial topoisomerase which is the only enzyme capable of the introduction of negative supercoils (Gunn *et al*., 2014). This means that they are able to interconvert relaxed and supercoiled forms of DNA and introduce and remove catenanes and knots (Collin *et al*., 2011). To generate negative supercoils, DNA gyrase forms a double stranded break in DNA and passes a second strand of DNA through the resulting break, a process directly linked to ATP hydrolysis (Gunn *et al*., 2014).

The exploitation of DNA metabolism can be used as a target for the identification of novel antibacterial agents. DNA replication targets such as type II topoisomerases/DNA gyrase remain a successful approach for the discovery of novel antibiotics with selective antibacterial activity in a desired range of organisms (Fan *et al*., 2014). The quinolone class of antibiotics has been used as treatment for various bacterial infections over the past decades. They target DNA gyrase and topoisomerase IV causing a rapid decrease in viable cells, ultimately resulting in the diminished rate of resistant development (Pucci, 2014). This process is known as dual-targeting, and can also result in low frequencies for drug resistance (Ehmann, 2014).

According to the World Health Organization (WHO, 2003), about 100 million acts of sexual intercourse take place each day. Approximately 356 000 cases of STD's are reported in each year, including both bacterial and viral infections. Thus, resulting in an increase in the numbers of people seeking for HIV-1 testing and care. In 2013, it

was reported that more than 40 million people are living with the human immunodeficiency virus (HIV) worldwide (Shah *et al*., 2005; Valadao *et al*., 2015). It can be clearly deduced from these studies that it is important to implement strategies that will reduce sexually transmitted associated infections, especially HIV-1.

The HIV-1 is a causative agent of acquired immunodeficiency syndrome (AIDS) (Valadao *et al*., 2015). As a human virus, once internalized, HIV-1 reverse transcriptase (RT) is reversed transcribed from the viral genomic RNA into a double stranded DNA intermediate which is then integrated into the host cell DNA. The HIV-1 RT is a DNA-dependent polymerase, which is essential for the life cycle of HIV, ultimately leading to the pandemic disease, AIDS (Chukwujekwu *et al*., 2014).

The HIV-1 is an enclosed virus, with a single stranded RNA genome which releases an RT enzyme during replication. The attachment of the virus to cells of the immune system is followed by a series of events which then lead to huge numbers of new viral particles, thereby resulting in the death of infected cells, ultimately weakening the immune system (Yu *et al*., 2007). This RT enzyme is multifunctional with RNA and DNA-dependent DNA polymerase, strand transfer, RNase H and strand displacement activities. However, some plant extracts are able to inhibit the HIV-1 virus replication and control its essential enzymes (Matamoros *et al*., 2005).

HIV-1 RT enzyme is one of the prime targets (Figure 5.1) for the treatment of HIV/AIDS, in identifying new inhibitors possibly active on drug resistant strains (Kumar, 2014; Xu *et al*., 2015). The most common route of infection of HIV-1 is through sexual transmission (Pasetto *et al*., 2014). Studies in HIV-1 infected patients have revealed that bacterial STD's like gonorrhoea are associated with higher concentrations of HIV-1 in genital mucosal secretions. Strategies were then implemented, that targeted

STD's which would in turn reduce the HIV-1 transmission risk. The treatment of these STD-related conditions reduces genital HIV-1 shedding, thus it provides evidence of reduced infections (McClelland, 2006).

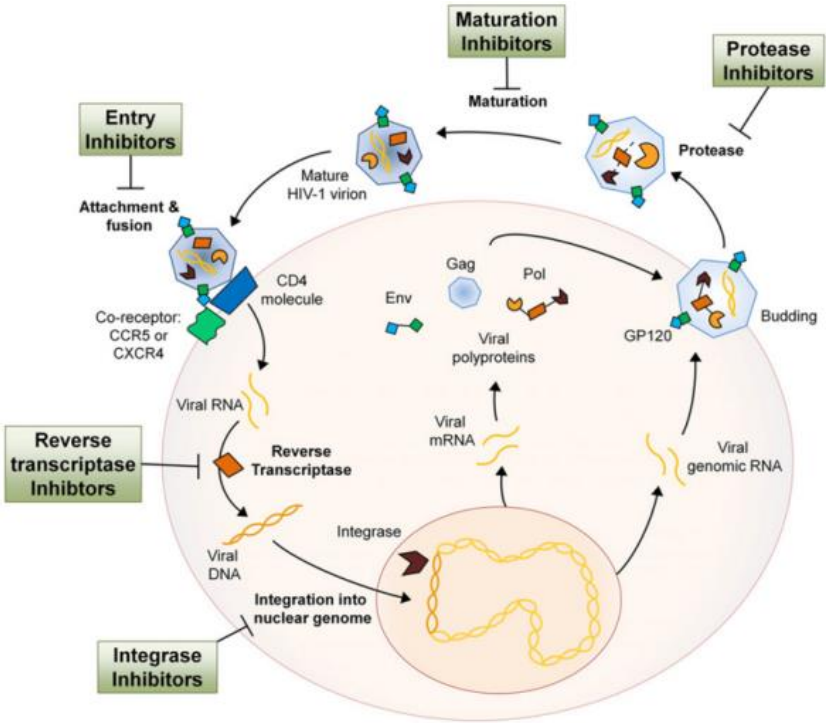

Figure 5.1 An illustration of HIV life cycle and inhibition stages (Smith *et al*., 2013).

According to their different mechanisms of action, the ultimate function of the RT inhibitors is to inhibit the enzyme reverse transcriptase thus resulting in the inhibition of transcription of viral single strand RNA into double strand DNA (Narayan, 2013; Xu *et al*., 2015). Present RT inhibitors comprise of nucleoside/ nucleotide RT inhibitors (NRTIs), the non-nucleoside reverse RT inhibitors (NNRTIs), protease inhibitors (PIs) and integrase inhibitors (INSTIs) (Singh *et al*., 2014). The highly active anti-retroviral therapy (HAART) as a current therapy to suppress viral replication, combines drugs from at least two of the different classes of the anti-retroviral agents available

(O'Rourke *et al*., 2016). However, because of the occurrence of drug resistant retroviruses, the effectiveness of HIV-1 therapy depends on the discovery and approval of novel therapeutics that are able to fight against several viral replication phases of HIV-1 (Bashyal *et al*., 2014). These are often found in herbal products.

Herbal medicines and natural products are a reassuring source of new therapeutic agents and for the development of complementary and alternative medicines to conventional drug systems. This is because of their rich source of chemical diversity (Xu *et al*., 2015). Herbal preparations have several possible benefits for anti-HIV-1 treatments, including the supplementation of already existing drug therapies, improvement of anti-HIV-1 treatment in resource limited backgrounds and the reduction of the risk of emergence of viral resistance. Moreover, they may also demonstrate new mechanisms of action which are different from the current single molecule drugs (Helfer *et al*., 2014). However, no natural compound has been developed up to clinical approval for HIV-1 treatment (Xu *et al*., 2015). Thus, it is meaningful to do various experimental investigations to evaluate anti-HIV-1 activities of herbal extracts.

The aim of this experiment was to determine the mechanism of action of the selected medicinal plant extracts and compounds against DNA gyrase and reverse transcriptase enzymes. These are important enzymes in the inhibition of bacterial and viral replication.

5.2 Materials and Methods

5.2.1 Preparation of extracts

Plant extracts for all experiments were prepared as described in Chapter 3.

5.2.2 DNA gyrase assay

Ethanol extracts were evaluated for their inhibitory effect on the supercoiling activity of bacterial DNA gyrase by using the DNA gyrase kit from TopoGEN according to the manufacturer's instructions. Briefly, extracts (0.2 g) were dissolved in 1 ml methanol to make a final concentration of 0.2 g/ml. A reaction mixture was prepared to a volume of 20 µl and contained; 2 µl ethanol extract, 4 µl assay buffer, 1 µl of pHOT1 relaxed DNA, 1 µl gyrase and sterile distilled water (vary as needed to bring volume up to 20 µl). The reaction mixture was incubated at 37°C for an hour. Thereafter, 4 µl of stop buffer/loading dye was added, with 20 µl of chloroform and vortexed briefly. The resulting blue aqueous phase was then loaded onto a 1% agarose gel and electrophoresis was run for 30 minutes. The gel was stained with 0.5 µg/ml ethidium bromide for 30 minutes and distained for 30 minutes. The gel was then visualized using a Labcon UV trans-illuminator. This non-ethidium bromide gel is ideal for detecting gyrase activity which is demonstrated by the resolution between supercoiled and relaxed DNA forms (Figure 5.2). As negative controls; (1) DNA gyrase was omitted and linear DNA was run independently, (2) ethanol extracts were substituted with

ciprofloxacin (0.025 g/ml), also with no DNA gyrase. As positive controls; (1) DNA gyrase was added onto linear DNA, (2) ciprofloxacin was added to the DNA gyrase.

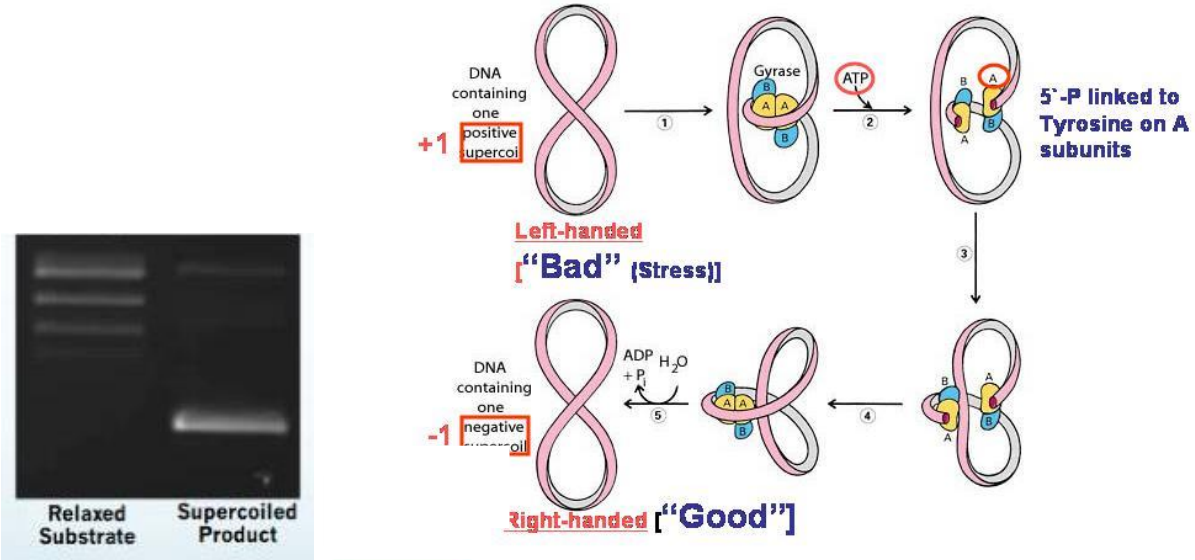

Figure 5.2 An illustration of DNA gyrase activity (www.topogen.com).

5.2.3 HIV-1 RT assay

The method followed was taken from Nair (2012). Briefly, the activity of plant extract on RT activity was determined with recombinant HIV-1 enzyme, by making use of a non-radioactive HIV-1 RT colorimetric ELISA kit (Roche). All plant materials were weighed up to 3 mg and were dissolved in 1 ml DMSO to make a final concentration of 3 mg/ml stock solution. 10 µl of stock solution was added to 90 µl of lysis buffer making a final concentration of 0.3 mg/ml. The enzyme was prepared to a stock solution of 0.764 mg/ml and 0.327 µl was added to 1000 µl lysis buffer. Onto wells of the plates, 20 µl of enzyme, diluted extract and reaction mixture were added together.

For positive control; ABTS tablets at 100 µg/ml was used; (1) lysis buffer was added with DMSO and (2) lysis buffer was added with no DMSO. For negative control; only the Lysis buffer and reaction mixture were added. The plates were incubated for an hour at 37°C. The plates were washed 5 times with 250 µl of the Washing buffer. 200 µl of Anti-Dig-POD working solution was added in each well. Thereafter, the plates were incubated at 37°C, for an hour. The plates were washed 5 times with 250 µl Washing buffer. 200 µl of ABTS substrate solution was added in each well and allowed a 10 minute stand. The absorbance was read on a micro-titre plate reader at 405 nm with a reference wavelength of 490 nm. An illustration of the HIV-RT test principle is shown in Figure 5.3. The mean of the duplicate absorbance was analysed using the formula:

$$\textbf{Percentage (\%) inhibition: } 100 - \frac{\textbf{Mean}_{\textbf{sample absorbance}} \times 100}{\textbf{Mean}_{\textbf{control -2 absorbance}}}$$

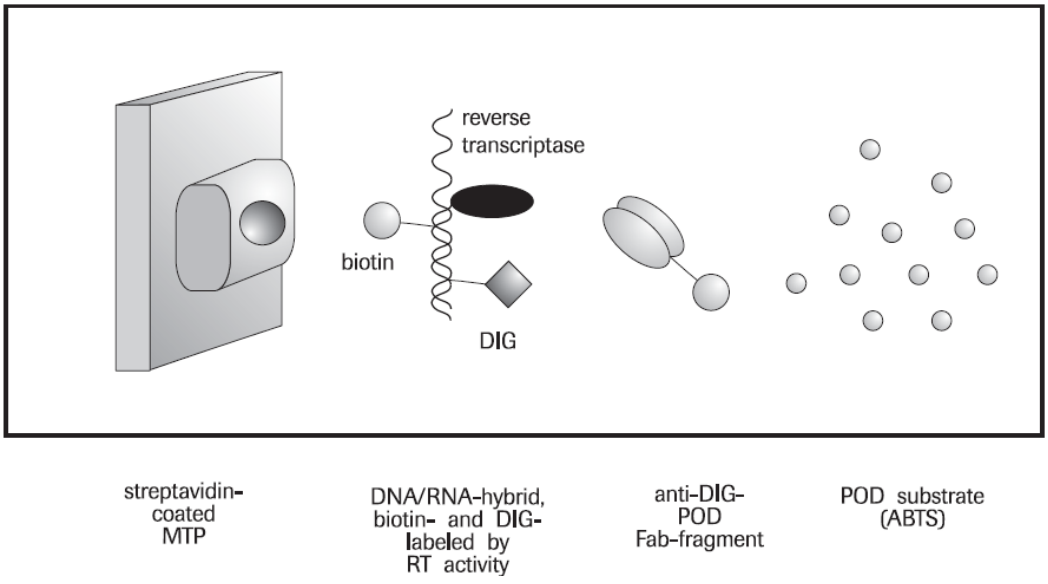

Figure 5.3 Reverse Transcriptase Assay test principle (Roche).

5.3 Results and discussion

5.3.1 DNA gyrase assay

This experiment was conducted to determine if the plants in study had inhibitory activities against the DNA gyrase enzyme, using the DNA supercoiling assay. Extracts were tested at a concentration of 0.2 g/ml. The obtained results are shown in Figures 5.3 and 5.4. None of the plant samples have been previously tested for their inhibitory activity against the DNA gyrase enzyme.

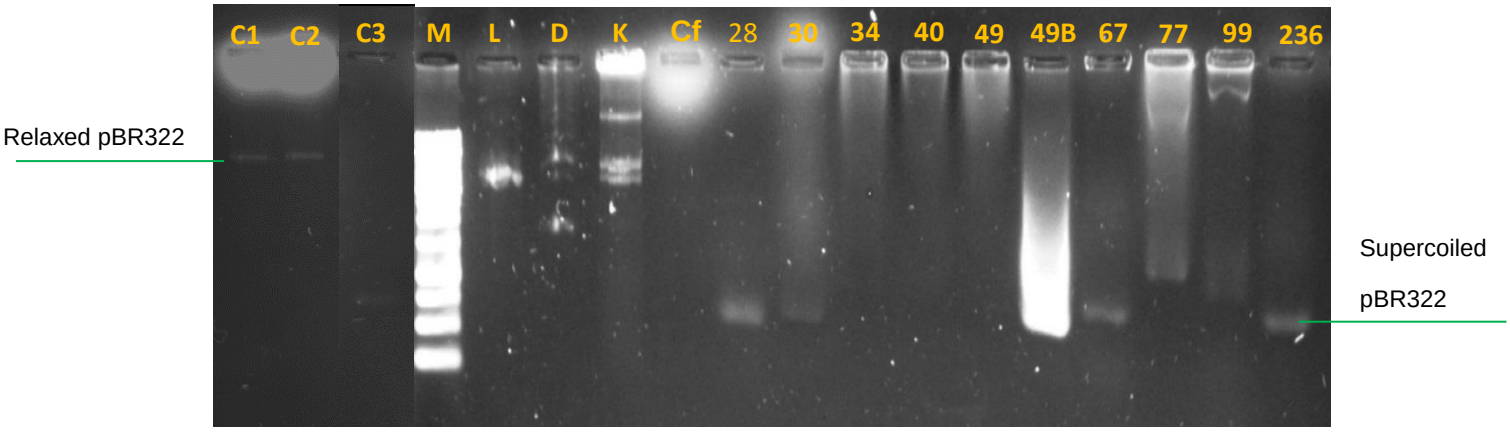

Figure 5.4 DNA gels with extracts and DNA gyrase.

M= Marker. **L**= linear DNA with no DNA gyrase. **D**= Decatenated DNA. **K**= KDNA. **Cf**=Ciprofloxacin. **C1**= Linear DNA with Ciprofloxacin. **C2**= Linear DNA with DNA gyrase and Ciprofloxacin. **C3**= Linear DNA with DNA gyrase.

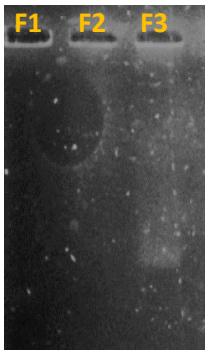

Figure 5.5 DNA gel containing the flavonoids 1-3 and DNA gyrase.

The results shown in Figure 5.4 and 5.5 either show supercoiled bands (indicating the activity of DNA gyrase) or show no bands at all (indicating the inhibitory effects of extracts against the DNA gyrase enzyme). Supporting results are shown in Table 5.1.

Table 5.1 The activity of extracts tested on their *in-vitro* inhibitory activity of bacterial DNA gyrase.

No.	Sample	Inhibitory activity
77	*Acacia Cf karroo*	-
34	*Diospyros mespiliformis*	+
11	*Elaeodendron croceum*	0
33	*Elaeodendron transvaalense*	0
236	*Hilliardiella nudicaulis*	-
67	*Jasminum fluminense*	-
40	*Peltophorum africanum*	+
49	*Rhoicissus tridentata* subsp. *cuneifolia*	+
99	*Schotia capitata*	-
30	*Senna italica* subsp. *arachoides*	-
28	*Solanum tomentosum*	-
12	*Terminalia sericea*	0
F1	lup-20(30)-ene-3,29-diol,(3α)-(9Cl)	+
F2	lup-20(29)-ene-30-hydroxy-3-one	+
F3	4'-O-methyl-epigallocatechin	-

Inhibitory activity: '**+**' : inhibitory effect, '**-**' : no inhibitory effect, '**0**' : Not tested

The plant extracts that showed supercoiled bands suggested that the bacterial gyrase enzyme is active are not of interest. Those extracts that showed no supercoiled bands/relaxed DNA bands possess strong inhibitory effect against the enzyme, DNA gyrase. The plant extracts of 34, 40, 49, F1 and F2 were the most effective in DNA gyrase inhibitory activity.

Ciprofloxacin was used as a positive control. Ciprofloxacin, an antibiotic belonging to the class of fluoroquinolones, has wide clinical applications because they have a better safety profile and good *in vitro* effectiveness against resistant pathogenic organisms (Khan *et al*., 2015). These fluoroquinolones develop into a complex with the topoisomerase and the DNA, in which both strands of the DNA backbone are cleaved, resulting in rapid bacterial cell death (Mitscher, 2005) and covalently linked to the protein thus inhibiting the supercoiling of the DNA. This cleaved complex stabilization is believed to be responsible for the cytotoxic effect of the inhibitors (Shapiro, 2012).

The DNA gyrase assay is based on the supercoiling ability on a relaxed plasmid DNA substrate (pHOT1, which is a derivative of pBR322). The enzyme inserts negative supercoils into a relaxed pHOT1 plasmid substrate via a sign inversion model (Chiriac *et al*., 2015). An energy co-factor (ATP) is essential for the complete supercoiling reaction. Samples are loaded on an agarose gel that lacks ethidium bromide (EB). These non-EB gels are ideal for detecting gyrase activity as indicated by the excellent resolution between supercoiled and relaxed DNA forms (Dutta *et al*., 1995). The treatment of relaxed plasmid DNA substrate (pHOT1, which is a derivative of pBR322) with DNA gyrase converts the relaxed DNA to the supercoiled form of the plasmid which migrates faster on an agarose gel (Zechiedrich *et al*., 2000). From the agarose gel, bands of relaxed DNA were of interest.

Infections caused by multidrug-resistant bacteria pose a significant threat to human health (Mitton-Fry *et al*., 2013). DNA gyrase is an important enzyme which is required for the viability of bacterial cells and has shown to be a promising target for many antibiotics, including the quinolones. Quinolone-mediated cell killing however, is complex and involves the formation of a gyrase-DNA-drug complex, which ultimately leads to the release of double-stranded DNA and, as a result, fragmentation of the chromosome and cell death (Webber *et al*., 2013). Bacterial DNA gyrase and topoisomerase IV are responsible for the maintenance of the topology of the DNA duplex during the replication process (Mesleh *et al*., 2016). Therefore, the disruption of bacterial DNA replication, especially by inhibition of type II topoisomerases, creates a powerful and well-precedented antibacterial drug development approach (Mitton-Fry *et al*., 2013).

These results show that DNA supercoiling assay is a successful method to investigate the inhibitory effect of plant extracts on bacterial DNA gyrase. Some extracts do not contain any inhibitors against DNA gyrase and some do. The extracts that showed strong inhibitory effect in this experiment were; *Diospyros mespiliformis*, *Peltophorum africanum*, *Rhoicissus tridentata* and flavonoids 1 and 2. Further investigations need to be carried out to classify which compound(s) is/are responsible for the inhibition of DNA gyrase. As DNA gyrase is only found in the prokaryotic kingdom and is important for the survival of bacteria, it seems to be an ideal target for antibacterial drugs. The discovery of new gyrase inhibitors from extracts may contribute to the establishment of new antibacterial agents and overcome the existing resistance to the already available DNA gyrase inhibitors. However, further investigations will assist in revealing the inhibition mechanism of these extracts against DNA gyrase (Xu *et al*., 2013).

5.3.2 HIV-1 RT assay

The experiment was aimed at determining those plants that are able to inhibit viral replication of HIV-1 RT enzyme by using a non-radioactive HIV-1 RT colorimetric ELISA kit. The results are expressed as % inhibition of the RT enzyme and also confirmed with the calculated standard deviation (SD) as shown in Table 5.2.

The HIV-1 RT colorimetric assay manipulates the potential of reverse transcriptase to synthesize DNA using the hybrid poly (A) x oligo (dT) 15 as a template and primer. The detection and quantification of the synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol, biotin-labelled DNA. The DNA is bound to the surface of streptavidin-coated microplate modules. An antibody, digoxigenin, conjugated to peroxidase (anti-DIG- POD), is added and bound to the digoxigenin-labelled nucleotides (Fonteh *et al*., 2009). The peroxidase enzyme substrate ABTS is added to catalyse the cleavage of the substrate to produce a coloured reaction product (which can sometimes be unstable and quickly change colour). The absorbance of the samples is determined by using a microplate (ELISA) reader, which is directly correlated to the level of RT activity in the sample (Li *et al*., 2009).

Doxorubicin was used as one of positive controls. Doxorubicin's cellular target is topoisomerase II. Doxorubicin joins both DNA and Topoisomerase II (Top2) to form the ternary Top2-doxorubicin-DNA cleavage complex, which activates cell death (Zhang *et al*., 2012). Most of the plants under study showed to have significant anti-viral activity and therefore were all tested for activity against the HI virus and inhibition of the RT enzyme, which is vital in the lifecycle of HIV-1.

Table 5.2 The RT inhibition activity of tested samples expressed in percentage (%) inhibition and standard deviation.

Sample	Percentage (%) inhibition	Standard deviation (SD)
Negative control	100	3.29
Positive control	0	3.29
Doxorubicin control	96.5	4.83
Acacia Cf karroo	66.8	12.35
Diospyros mespiliformis	17.4	14.96
Elaeodendron croceum	30.2	16.05
Elaeodendron transvaalense	30.6	2.70
Hilliardiella nudicaulis	36.9	3.97
Jasminum fluminense	55.1	6.76
Peltophorum africanum	44.1	10.52
Rhoicissus tridentata subsp. *cuneifolia*	75.7	16.97
Schotia capitata	37.9	3.40
Senna italica subsp. *arachoides*	38.5	2.20
Solanum tomentosum	37.4	12.88
Terminalia sericea	102.8	13.91
4’-O-methyl-epigallocatechin (F3)	63.7	2.10

The sample results were compared to the positive control Doxorubicin which showed to have 96.5% inhibitory activity and a standard deviation of 4.83. Samples with activity below 20% inhibition will be considered as being insignificant, 20–40% low, 40–70% moderate, and 70–100% high inhibition activity (Mulaudzi *et al*., 2015).

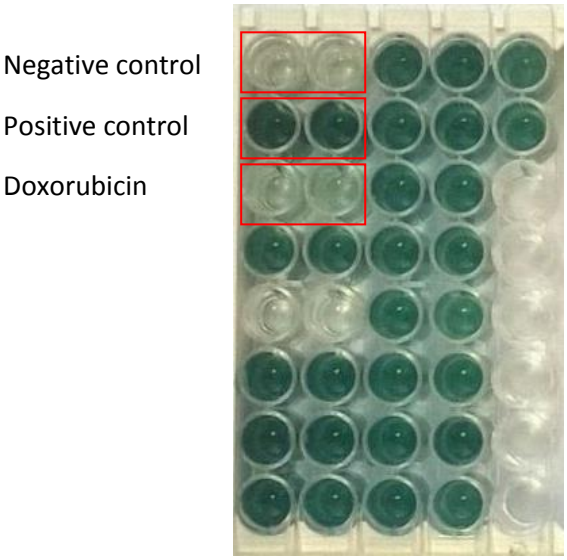

Figure 5.6 The test plate for the HIV-1 RT assay.

Terminalia sericea showed the highest inhibition of RT activity, having 102.8% inhibition. This implies the low RT activity in the assay conducted. The standard deviation was 13.91. A study conducted by Krishnaveni (2012), agrees with the use of *T. sericea* as an effective inhibitor of RT. *Rhoicissus tridentata* also showed that it is an effective inhibitor against HIV-1 RT. It showed to have high inhibitory activity of 75.7%. The standard deviation showed to be 16.97. *Acacia karroo* showed good inhibitory activity with an inhibitory percentage of 66.8%. The standard deviation was 12.35. In a study conducted by Moll *et al* (2013) *A. karroo* also showed to have good activity against the RT enzyme in HIV-1. The plants in the study were extracted with

methanol. It can then be concluded that *A. karroo* can be used as a treatment or possibly a cure against HIV-1 RT.

The inhibition activity of the pure compound F3 (4'-O-methyl-epigallocatechin) was moderate, with a percentage inhibition of 63.7%. The standard deviation was calculated to be 2.10. The obtained results, however, contradict those reported by Maragesi *et al* (2010) which state that this isolated phenolic compound did not show any anti-HIV activity. *Jasminum fluminense* showed moderate activity with 55.1% inhibition. The standard deviation is 6.76. There are no previous experiments reported on RT inhibition of *J. fluminense.*

Diospyros mespiliformis showed insignificant inhibitory activity showing 17.4% inhibition of the RT enzyme. *D. mespiliformis* also showed a standard deviation of 14.96. This is also shown in a study conducted by Hedimbi (2015), which shows *D. mespiliformis* leaves extract at 0.1 mg/ml exhibiting high RT activity (78.7%). This indicated that *D. mespiliformis* is a less effective inhibitor. Both these results agree that *D. mespiliformis* has insignificant inhibitory activity against RT enzyme.

The ongoing requirement for the development of new therapeutic anti-HIV-1 agents is due to the rapid emergence of viruses resistant to these drugs and also the need of novel drugs with fewer adverse effects (Helfer *et al*., 2014). Medicinal plants are a good source for the discovery of novel antimicrobial therapeutic agents. Several plant extracts have been shown to possess activity against HIV-1 by inhibiting various viral enzymes. Many plant products are being used by patients with HIV-1 without any scientific proof that they contain anti-HIV-1 activity. Traditional healers are now offering their remedies for scientific evaluation (Mamidala, 2014).

HIV-1 RT contributes to the increase in resistance to all anti-AIDS drugs by introducing mutations into the viral genome (Das, 2013). Reverse transcription is an essential step in the progression of HIV-1 infection (Figure 5.2), and therefore, HIV-1 RT is the target of many anti-AIDs therapeutic drugs and the ongoing efforts are to help identify new RT inhibitors (Pant *et al*., 2014).

5.4 Conclusion

The increase of drug resistance continues to be a challenging problem, and also the fact that many drugs have encountered substantial dose-limiting and long-term toxicities. This therefore, results in the need for the discovery and development of new drugs that have unique or different structures or modes of action from the currently approved drugs. Lately, there has been renewed interest in natural products as sources for drug improvement. These natural products derived from medicinal plants have great potential as sources of anti-HIV-1 drugs that might possess activity against drug-resistant HIV-1 strains. The uniqueness of DNA gyrase has made it a successful target for antibacterial agents. Positive effects observed in these experiments for these medicinal plants, give some validation to their reported traditional uses.

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Chapter 6: General discussion and conclusion

6.1 General discussion

In South Africa, it was established that 26% of all deaths that occurred during the year 2000 were as a result of STD's (including HIV) (De Wet *et al*., 2012). However, with the progression in Science and Technology, outstanding progress has been made in the field of medicine with the discoveries of various natural and synthetic drugs (Sen, 2012).

Plants do not only serve as crude drugs or over the counter drugs, but also as natural resources for the identification and development of novel drugs. The medicinal properties and health benefits of plants are attained from their bioactive constituents, also known as phytochemicals. Many phytochemicals are known to contain, but not limited to, antimicrobial (antibacterial, antifungal), anti-inflammatory, antitumor and antioxidant activities. These diverse bioactivities would present plants with disease-preventive and therapeutic potentials (Tsuchiya, 2015). Phytochemicals which are derived from plant products, assist in the development of less toxic and more effective medicines used to control the growth of microorganisms. These compounds have significant therapeutic implementation against human pathogens including bacteria, fungi or virus (Sen, 2012). Herbal products from medicinal plants are favoured because of their efficiency, lesser side effects, less testing time, higher safety and cultural acceptability (Prasad *et al*., 2012).

In order to avoid complications which may arise from the indiscriminate use of poisonous plants which can be mistaken for medicinal plants, there is therefore a need to research these plants and experimentally analyse and evaluate them. This will help in promoting the pharmaceutical industry and preserving valuable information that could be lost to non-documentation. The chemical evaluation of herbal medicine has made it possible to transform traditional medicine from an almost invisible trade into modern industrial enterprises. This evaluation is capable of making significant contribution to both healthcare delivery and the economic growth, especially in developing countries (Chuku *et al*., 2016).

Traditional herbal medicines have remained in use since ancient times, and many of them have been shown to be effective by means of laboratory and clinical studies. Therefore, it may be postulated that there is a higher possibility of discovering bioactive constituents in natural products from traditional herbal medicines based on their therapeutic properties, rather than from natural sources by means of random screening. This study will therefore validate the use of some traditional herbs (Ji *et al*., 2016).

The sustained attention in traditional medicine in the African healthcare system can be justified by two main reasons. The first one is the deficiency of effective modern medical treatment for some illnesses such as malaria or HIV/AIDS. Although these treatments sometimes may be distributed globally; Africa is more affected than other areas in the world. Second, the majority of people in Africa cannot afford access to allopathic medicines and modern forms of treatments, because it is either too expensive or because of the lack of medical service providers (Mahomoodally, 2013). Other factors for seeking traditional medicines include traditional beliefs, cultural

barriers, low socio-economic status, stigma, lack of confidentiality, and inadequate user-friendly facilities (Chinsembu, 2016).

It is projected that plant extracts showing alternative target sites than those used by current antibiotics, will be active against drug resistant pathogens. Medicinal plants have been used as a means of traditional therapy for many human diseases in the past years and in several parts of the world. Therefore, researchers have recently given attention to safer phytomedicines and biologically active compounds. These compounds are isolated from plant species and are used in herbal medicines, with an acceptable therapeutic index for the improvement of newer drugs (Sen, 2012).

Infections that are caused by multidrug-resistant bacteria present a developing threat to human health. Therefore, antibacterial agents that can overcome this increasing medical problem are urgently required. Nonetheless, much energy has been dedicated to recognizing new antibacterial targets as a method to overcome resistance. DNA replication targets such as type II topoisomerases remain a steady successful procedure to discover novel antibiotics with selective antibacterial activity, in a desired range of microorganisms (Fan *et al*., 2014). Inflammation is a known mechanism which facilitates the acquisition and the transmission of HIV -1 infection. The female genital tract is lined by genital epithelial cells which are one of the first cells to encounter any infection during sexual transmission (Ferreira *et al*., 2015). It is therefore of vital importance that management or control of these infections are treated at the point of entry, which is sexual/intimate activity, before they can spread resulting in chronic diseases.

For this study, twelve medicinal plants and three flavonoids were identified as possible remedies for the treatment of STD's. These plants were extensively analyzed for the

scientific justification of their traditional use. Plants were investigated for their antimicrobial activities against 4 known microorganisms associated with sexually transmitted diseases, their anti-inflammatory activities, their DNA gyrase inhibition activities and anti HIV-1 reverse transcriptase inhibition. Positive outcomes were achieved for most of the tested samples and most plants showed to have activity on the tested microorganisms.

For the *in vitro* antimicrobial activity, extracts were tested for four different pathogens; *Candida albicans*, *Gardnerella vaginalis*, *Neisseria gonorrhoeae* and *Oligella ureolytica*, through the broth micro dilution assay. The plant extracts that showed the lowest MIC in all the microorganisms were *Acacia karroo* and *Rhoicissus tridentata*. These extracts also showed little or no toxicity in our previous studies, when tested against Vero cells. This study has reported for the first time the antimicrobial activity of epigallocatechin against *Oligella ureolytica.* The antimicrobial activity of the extracts from this investigation supports the ethnomedicinal claim of the plants being used in the treatment of STD's, thereby unlocks new usage of the plants in ethnomedicinal and possible new drug formulation.

The anti-inflammatory activity of extracts was tested on the inhibition of 15-lipoxygenase (15-LOX) enzyme. The extracts of *Jasminum fluminense*, *Solanum tomentosum* and flavonoids 2 and 3 had good anti-inflammatory activities. Inhibitors of the 15-LOX enzymes may involve the discovery of possible leads for the development of effective anti-inflammatory drugs.

The selected medicinal plants were further tested for their inhibitory effect on the supercoiling activity of bacterial DNA gyrase. Good inhibitory activities were obtained from *Diospyros mespiliformis*, *Peltophorum africanum*, *Rhoicissus tridentata* and

flavonoids 1 and 2. The finding of novel gyrase inhibitors from extracts through the DNA gyrase kit, may contribute to the development of new antibacterial agents and overcome the existing resistance to available DNA gyrase inhibitors.

The HIV-1 reverse transcriptase (RT) enzyme is the target of many antiretroviral (ARV) therapeutic drugs and the ongoing efforts are to identify new natural RT inhibitors. The HIV-RT colorimetric assay was used to identify potential inhibitors from the selected medicinal plants in this study. *Rhoicissus tridentata* and *Terminalia sericea* exhibited the most promising anti-HIV-1 RT activity when compared to the positive control (doxorubicin).

6.2 Conclusion

The fact that a plant extract displays activity is of interest, but it is only a preliminary piece of data which should be followed by the identification of the active compounds by means of a bio-guided assay. After chemical evaluation of twelve medicinal plants and three flavonoids, positive outcomes were obtained for most of the tested samples. Most of the plants showed activity for all experiments conducted. These results further validate the use of these medicinal plants in the treatment of sexually transmitted diseases (STD's). The observed activities may lead to new multi-target drugs against STD's. However, additional work is required to isolate and identify the bioactive compound(s) that is/are responsible for the activities.

6.3 References

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