Investigating the potential of *Gunnera perpensa* for the treatment of gonorrhoea

by

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Summary

Gonorrhoea, caused by *Neisseria gonorrhoeae*, is one of the most problematic sexually transmitted diseases (STDs) in the world as it negatively affects people's health and livelihoods. Antibiotic resistance of *N. gonorrhoeae* puts pressure on available therapeutics; thus there is a need to find alternative sources of bioactive compounds. In this study, antigonococcal screening was conducted on ethanolic extracts from roots of five South African indigenous plants, namely: *Gnidia kraussiana* Meisn, *Gunnera perpensa* L, *Pentanisia prunelloides* (Klotzsch) Walp, *Rhoicissus digitata* (L.f.) Gilg & M.Brandt and *Rhoicissus tridentata* (L.f.) Wild & R.B.Drumm, which exhibited minimum inhibitory concentrations (MICs) of 97.5 µg/ml, 48.7 µg/ml and 195 µg/ml, respectively while the *Rhoicissus* spp. had MICs of 780 µg/ml. *Gunnera perpensa* L. (GP) was selected as the lead plant as it had the best activity against *N. gonorrhoeae*. Furthermore, the plant was not found to be cytotoxic to human keratinocytes (HaCaT), cervical cancer cells (HeLa) and human monocytes (THP-1) (IC_{50} >400 µg/ml).

Gunnera perpensa was subjected to liquid-liquid partition chromatography which resulted in four semi-pure fractions. Butan-1-ol and water fractions showed the best antigonococcal activity (MIC=23.4 µg/ml) and showed no toxicity against the cell lines tested (IC_{50} >300 µg/ml). Five compounds were identified to be present in both bioactive fractions, namely: Z-venusol, ferulic acid glucoside, 4-*O*-β-D-glucopyranosyl-3,3'-tri-*O*-methylellagic acid, caffeic acid and 4-*O*-beta-D-glucosyl-trans-caffeate. While three compounds were classified as 1.4-benzoquinones. Z-venusol was identified as the most abundant compound present in both bioactive fractions.

Gold nanoparticles (AuNPs) were synthesized using the ethanolic crude extract of *G. perpensa*. Characterization of the GP-AuNPs was conducted using Zetasizer, X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM) and ultraviolet-visible spectrophotometry (UV-Vis). The nanoparticles had surface plasmon resonance (SPR) at 536 nm and a hydrodynamic size of 127.2±1.56 nm. At 24 h, 72 h and 1 week, the GP-AuNPs were found to be stable when subjected to various treatments (pH 4, pH 7, pH 10, 0.5 % cysteine, 0.5 % sodium chloride, 0.5 % phosphate-buffered saline (PBS), 0.5 % Bovine serum albumin (BSA), Dulbecco's Modified Eagle Medium (DMEM) and deionized water (control). The GP-AuNPs exhibited potent antigonococcal activity (MIC=10.4 µg/ml), which was found to be better than the crude extract (MIC=48.7 µg/ml). However, the nanoparticles were found to be

cytotoxic to HaCaT, HeLa and THP-1 cells with IC_{50} values of 22.12±0.52 µg/ml, 41.98±10.65 µg/ml and 27.53±6.02 µg/ml, respectively. This study revealed that *G. perpensa* should be considered in future, for pre-clinical and clinical studies for investigating its potential anti-gonococcal activity due to the good antibacterial effects and moderate toxicity observed in the present study. There is a possibility that individual compounds in the plant may exhibit better bioactivity. The nanoparticles of the plants showed superior antigonococcal activity however, the cytotoxicity effects are of concern. In future, avenues must be explored to optimize the NPs to reduce toxicity.

Submission declaration

I, Tanyaradzwa Tiandra Dembetembe declare that this dissertation, which I hereby submit for the degree MSc Medicinal Plant Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Signature: …… Date: ……08/12/21…….

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List of outputs from this research

The following manuscripts are under preparation:

1. Antibacterial and antiproliferative activity of medicinal plants traditionally used for the treatment of sexually transmitted diseases

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Proposed Journal: Journal of Ethnopharmacology **Impact factor:** 4.360

2. Evaluation of the anti-gonococcal and cytotoxicity activity of *Gunnera perpensa* and its's gold nanoparticles.

Tanyaradzwa T. Dembetembe[a], Danielle Twilley [a], Marco N. De Canha[a], Velaphi Thipe [b], Kattesh V. Katti [b],Vusani Madiwana [c], Lonji Kalombo [c],Rirhandzu Rikhotso [c], Suprakas Sinha Ray [d] , Namrita Lall[a,e,f,g] and Quenton Kritzinger[a]

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3. The use of South African medicinal plants in the pursuit to treat gonorrhoea and other sexually transmitted diseases

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Book Chapter in: Medicinal plants as anti-infectives: Current knowledge and new perspectives

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Abbreviations

AChE- acetylcholinesterase

Ag- silver

AMCSD- American Mineralogist Crystal Structure Database

APC- antigen-presenting cells

ART- antiretroviral therapy

ASGPR- asialoglycoprotein receptor

Au- gold

BSA- bovine serum albumin

BSS- β-sitosterol

BSSG- β-sitosterol glucoside

CAF- Central Analytical Facilities

CDC- Centers for Disease Control and Prevention

CEACAM- carcinoembryonic antigen-related cell adhesion molecule family

CFU- colony forming unit

CVM- cervicovaginal mucus

CPE- cytopathic effect

COX- cyclooxygenase enzyme

CR3- complement receptor 3

CSIR- Council for Scientific and Industrial Research

Cys- cysteine

DCM:MeOH- dichloromethane: methanol

DGI- disseminated gonococcal infection

DLS- dynamic light scattering

DMEM- Dulbecco's Modified Eagle Media

DMSO- dimethyl sulphur oxide

EGCG- epigallocatechin-3-o-gallate

ELISA- enzyme-linked immunosorbent assay

ESCs- extended-spectrum cephalosporins

ESI-LCMS- electrospray ionization-liquid chromatography-mass spectrometry

EtOAc- ethyl acetate

FDA- Food and Drug Administration

FIC- fractional inhibitory concentration

FTIR- Fourier-transform infrared spectroscopy

GA- gum Arabic

GC- gonococcal infections

GK- *Gnidia kraussiana*

GP- *Gunnera perpensa*

GP-AuNP- *Gunnera perpensa*- mediated gold nanoparticles with gum Arabic stabilizer

GPX-AuNP- *Gunnera perpensa*- mediated gold nanoparticles without gum Arabic stabilizer

HaCaT- human keratinocyte cell line

HeLa- cervical cancer cell line

HIV- human immunodeficiency virus

HIV/AIDS - human immunodeficiency virus/ acquired immunodeficiency syndrome

HPV- human papilloma virus

HSV- herpes simplex virus

IC_{50}- inhibitory concentration at 50 %

IUCN- International Union Conservation of Nature

IL- interleukin

IR- infred

ISOs- International standards organizations

JCPDS- Joint Committee Powder Diffraction Standards

Lbps- lactoferrin

LCMS- liquid chromatography-mass spectrometry

LD_{50}- the lethal dose at which 50 % of the population of animal models dies

LOS- lipooligosaccharide

LPS- lipopolysaccharide

LTR- long terminal repeats

n/a- not applicable

$[M]^+$- positive ionization in ESI-LCMS

$[M]^-$- negative ionization in ESI-LCMS

MDR- multi-drug resistant

MEM- minimum essential media

MH- Mueller-Hinton

MIC- minimum inhibitory concentration

m/z- mass: charge ratio

NaCl- sodium chloride

NF-κB - nuclear factor kappa light chain enhancer of activated B cells

NICD- National Institute for Communicable Diseases

^{1}H-NMR- proton nuclear magnetic resonance

NP- nanoparticles

Opa- Opacity-associated protein genes

PAMPs- pathogen-associated molecular patterns

PBP- penicillin-binding proteins

PBS- phosphate-buffered saline

PEE- plant extract equivalent

PEG- polyethylene glycol

PLGA- poly(lactic-co-glycolic acid)

PP- *Pentanisia prunelloides*

PVP- polyvinylpyrrolidone

RDDP- RNA-dependant DNA-polymerase

RNase H- ribonuclease

RPMI 1640- Roswell Park Memorial Institute Medium media

RT- reverse transcriptase

SDGs- sustainable development goals

SPR- dynamic light scattering

STDs- sexually transmitted diseases

Tbps- transferrin

TDr- traditional healer

TNF- α- tumour necrosis factor α

THP-1- monocyte like cell line

UV-Vis- ultraviolet-visible spectrophotometry

XDR- extensively drug-resistant

XRD- X-ray diffraction

WHO- World Health Organization

ZP- zeta potential

Chapter 1: Introduction

1.1. Introduction and motivation for the study

Sexually transmitted diseases (STDs) are a major problem internationally in that they have adverse effects on people's quality of life. According to the World Health Organization (WHO) (2016), there are about 1 million new infections of STDs recorded daily worldwide. The four most prevalent STDs, excluding human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), include chlamydia, gonorrhoea, syphilis and trichomoniasis. Consequently, gonorrhoea accounts for 21 % of the ~350 million cases reported yearly of these non-HIV/AIDS STDs (WHO Regional Office for Africa, 2018). Africa is the most affected region in the world with most gonococcal infections being recorded in sub-Saharan Africa (Rowley et al., 2019; WHO Regional Office for Africa, 2018). In South Africa, gonorrhoea is the most prevalent bacterial STD (Johnson et al., 2005). In 2017, it was estimated that of the 4.5 million South Africans infected with STDs, 10.1% suffered from gonorrhoea (Kularatne et al., 2018b). Of these cases, women were the most affected accounting for 6.6 % compared to the 3.5 % for males of the gonococcal infections.

Gonorrhoea is a disease transmitted through sexual contact in humans via bodily fluids or skin lesions in genitalia (Belcher and Dawson, 2017). The disease is caused by an obligate Gram-negative bacterium called *Neisseria gonorrhoeae.* Gonorrhoea is characterized by lower abdominal pains, vaginal or penile discharge, pain when urinating and ulcerations (lesions on the skin and mucosal tissue) (Achakazai et al., 2017; Centers for Disease Control and Prevention, 2021). If gonorrhoea is not treated, it may result in infertility, pregnancy complications and birth defects (Platt et al., 1983).

Currently, ceftriaxone/cefixime in combination with azithromycin is the current regimen prescribed for the treatment of gonorrhoea (Ryan, 2017; Wi et al., 2017). This dual therapy is also used in the treatment of other STDs such as chlamydia and chancroid that normally exists as co-infections with gonorrhoea (Belcher and Dawson, 2017). The high infection rate of gonorrhoea has been a great cause for concern as it results in the over usage of available first-line antibiotics (Crowther-Gibson et al., 2011). *Neisseria gonorrhoeae* has developed some resistance to well-known antibiotics like tetracycline, penicillin, amoxicillin and fluoroquinolones resulting in the occurrence of different antibiotic-resistant strains (Ohnishi et

al., 2011b; Unemo et al., 2012). Moreover, antibiotic-resistant strains to azithromycin, ceftriaxone and cefixime have been reported worldwide. Figure 1.1 shows a global representation of the prevalence of *N. gonorrhoeae* resistant strains against ceftriaxone and/or cefixime, with South Africa having 0.1-5 % of the drug-resistant bacteria (Wi et al., 2017). According to the South African National Institute for Communicable Diseases (NICD), cefixime-resistant strains have been detected in Cape Town, Gauteng and the Eastern Cape provinces (Kularatne et al., 2017). Antibiotic resistance results in a decline in the number of available efficient antibiotics for the treatment of gonorrhoea which has prompted the need for new drugs to be developed.

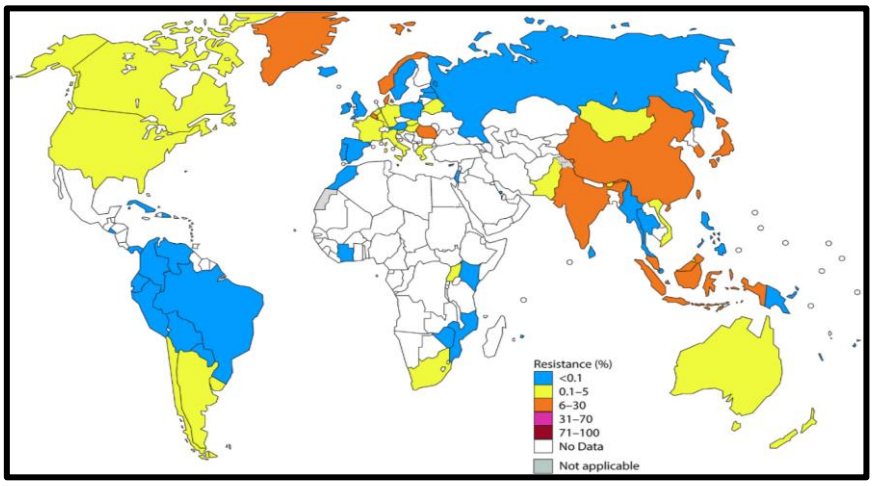

Figure 1.1: Geographical representation showing the percentage of *Neisseria gonorrhoeae* isolates resistant to cefixime and/or ceftriaxone (Ryan, 2017)

The problem of antibiotic resistance makes it important to look for other sources for the treatment of the disease. This brings medicinal plants into the limelight to develop new alternative therapeutics (Palmeira-de-Oliveira et al., 2013). Plants are renewable sources that have a variety of secondary compounds thus making them ideal sources for novel bioactive compounds against gonorrhoea (Palmeira-de-Oliveira et al., 2013). For centuries, medicinal plants have been used in the treatment of STDs by traditional healers in African traditional medicine, Ayurvedic medicine and Chinese medicine (de Wet et al., 2012; Yang et al., 2012).

In South Africa, various plants have been used traditionally for the treatment of STDs and they have been reported to exhibit antimicrobial activity (Fabricant and Farnsworth, 2001). *Gunnera perpensa* L., *Rhoicissus tridentata* (L.f.) Wild & R.B.Drumm, *Hypoxis hemerocallidea* Fisch., C.A.Mey. & Avé-Lall and *Peltophorum africanum* Sond are indigenous medicinal plants that are used in folk medicine for the treatment of STDs including gonorrhoea (Buwa and van

Staden, 2006; Samie et al., 2010; Tshikalange et al., 2016; van Wyk et al., 2017). These plants have been reported to have antibacterial activity against *N. gonorrhoeae* and other STD pathogens such as HIV and *Candida albicans. Gunnera perpensa* has shown antimicrobial activity against *Shigella flexneri*, which is a pathogen that causes shigellosis, an emerging STD seen mostly in homosexual individuals (Goulart and Wurcel, 2016; Nkomo and Kambizi, 2009). *Rhoicissus tridentata* has exhibited antimicrobial activity against *Candida albicans* whilst *H. hemerocallidea* and *P. africanum* are plants that have anti-HIV activity (Bessong et al., 2005; Hamza et al., 2006; Matyanga et al., 2020; Samie et al., 2010; Tshikalange et al., 2016). Medicinal plants also have antimicrobial activity against other non-STD pathogens. The traditional uses and antimicrobial activity reported in literature of medicinal plants shows that there is potential to develop new therapeutics for the treatment of diseases including gonorrhoea.

Indigenous plants have also been reported to contain chemical compounds that have the potential to be used for therapeutic purposes. *Gunnera perpensa* has been reported to contain Z-venusol which has uterotonic activity (Ndhlala et al., 2011a). Aloin, aloe-emodin and chrysophanol have been isolated from *Aloe ferox* Mill (Kambizi et al., 2005). Aloin has antigonococcal activity against *N. gonorrhoeae* (Kambizi et al., 2005). Hypoxoside, isolated from *H. hemerocallidea*, is converted to rooperol which enhances the immune system in HIV patients by maintaining the CD4 cells (Albrecht, 1996; Matyanga et al., 2020; van Wyk et al., 2017). This shows there is potential for discovering bioactive compounds for the treatment of gonorrhoea from plants.

Various drugs have been developed from plants for ailments such as cancer, malaria, pain, and STDs (Bonnez et al., 1994; CDC, 2015a; Desborough and Keeling, 2017). Praneem is a natural vaginal ointment formulated from purified leaf extracts of *Azadirachta indica* A. Juss., purified saponins from *Sapindus mukorossi* Gaertn and *Mentha citrata* Ehrh oil. The product has been reported to have a broad-spectrum activity on reproductive tract pathogens including *N. gonorrhoeae, Candida* spp. and *Chlamydia trachomatis* (causes chlamydia) (Talwar et al., 2000). In history, it has been seen that plants can provide compounds that can be used in medicine (Achan et al., 2011; Licciardi and Underwood, 2011). Podofilox is a gel used as a regimen for anogenital warts caused by the human papilloma virus (HPV) (Bonnez et al., 1994; CDC, 2015b). The active ingredient for this gel is podophyllotoxin, which has been isolated from the *Podophyllum* spp. The development of these plant-based vaginal products shows there

is potential to make new therapies for the treatment of STDs like gonorrhoea from medicinal plants.

1.2. Aims and objectives

This study aimed to investigate the potential of the five selected South African indigenous plants for the treatment of gonorrhoea. Moreover, gold nanoparticles were synthesized for the vaginal route of administration to investigate if this may increase the efficacy of the plant extracts to treat the disease.

To achieve these aims the following objectives were met:

1. Antigonococcal activity and synergistic testing of selected plants on *N. gonorrhoeae.*
2. Cytotoxicity testing of the lead plant/s based on the antigonococcal and synergistic assays on cervical cancer (HeLa), human keratinocyte (HaCaT) and monocyte-like (THP-1) cells.
3. Fractionation of the lead plant/s, using liquid-liquid partition chromatography, to obtain semi-pure fractions.
4. Antigonococcal testing and cytotoxicity testing of the semi-pure fractions to determine efficacy and the relative safety on HeLa, HaCaT and THP-1 cell lines.
5. Identification of secondary compounds from the bioactive semi-pure fractions using electrospray ionization-liquid column chromatography-mass spectrometry (ESI-LCMS), proton nuclear magnetic resonance (^{1}H-NMR) and Fourier-transform infrared spectroscopy (FTIR).
6. Synthesis of gold nanoparticles (AuNPs) mediated with the lead plant/s and stability testing of the AuNPs.
7. Characterization of the AuNPs using transmission electron microscopy (TEM), FTIR, X-ray diffraction (XRD), Zetasizer and ultraviolet-visible spectrophotometry (UV-Vis).
8. Antigonococcal testing of the AuNPs and the evaluation of the cytotoxic effects of the AuNPs on HeLa, HaCaT and THP-1 cells.

1.3. Structure of dissertation

Chapter 2: This chapter provides detailed background on gonorrhoea, which includes co-infections, transmission, and pathogenesis of *N. gonorrhoeae.* Further discussion on the current status of gonorrhoea treatments and mode of action of antibiotic-resistant *N. gonorrhoeae* is given. The chapter also provides an overview of South African plants used to treat STDs and polyherbal formulations available (locally and internationally) to treat STDs. In-depth background of the selected plants used in the study is given. Additionally, the vaginal delivery system and nanotechnology are discussed as potential ways to improve the efficacy of plants for gonococcal treatments.

Chapter 3: This chapter focuses on the antimicrobial and synergistic effects of the selected plants on *N. gonorrhoeae*. Moreover, the cytotoxic effects of the most bioactive plant extracts are evaluated.

Chapter 4: In this chapter, the isolation and identification of bioactive compounds from the lead plant (*Gunnera perpensa*) identified in chapter 3 are described. Additionally, the antigonococcal activity and cytotoxicity of these semi-pure fractions are investigated.

Chapter 5: This chapter reports on the **s**ynthesis and characterization of gold nanoparticles mediated with *Gunnera perpensa.* In addition, the antigonococcal and cytotoxicity properties were evaluated.

Chapter 6: An integrated discussion and conclusions based on the findings of this study is provided in this final chapter.

Chapter 2: Literature review

2.1. Background on gonorrhoea

Gonorrhoea is one of the most prevalent sexually transmitted diseases (STDs) in the world. There are approximately 350 million cases reported yearly of the four major STDs across the world: chlamydia (131 million), gonorrhoea (78 million), syphilis (6 million) and trichomoniasis (142 million) (World Health Organisation Regional Office for Africa, 2018). Global incidence rates for 2016 were estimated to be at 20 and 26 new reported gonococcal (GC) infections per 1000 women and men, respectively (Rowley et al., 2019). According to the World Health Organisation (WHO), sub-Saharan Africa is the most affected region in the world (Rowley et al., 2019; WHO Regional Office for Africa, 2018). In South Africa, gonorrhoea is the most prevalent bacterial STD affecting people (Kularatne et al., 2018a, 2018b). Gonorrhoea is prevalent in sexually active individuals mostly below 30 years (Shim, 2011). The high incidence of gonorrhoea has been due to several factors which include: low socioeconomic status, the onset of sexual activity, safe sex awareness and risky sexual behaviours (Barnes and Holmes, 1984; Oller et al., 1970).

Gonorrhoea is a disease transmitted via sexual contact in individuals through lesions and bodily fluids (Belcher and Dawson, 2017). The symptoms of the disease include: lower abdominal pains, genital discharge, pain when urinating, itching of genitalia and venereal sores (Achakazai et al., 2017; Centers for Disease Control and Prevention, 2021). In some cases, the disease may cause urethritis and pelvic inflammatory disease (PID). Gonorrhoea can also be asymptomatic in both males and females (Platt et al., 1983; Wallin, 1974). In women, most GC infections are asymptomatic and if symptoms are seen they are usually mild and nonspecific (Curran et al., 1975; McCormack et al., 1977). Untreated gonococcal infections could result in infertility, birth defects and life-threatening ectopic pregnancies (Platt et al., 1983). In some cases, the infection can spread to the blood resulting in disseminated gonococcal infection (DGI), which can lead to death (Centers for Disease Prevention and Control, 2021).

2.1.1. The causal agent: *Neisseria gonorrhoeae*

Gonorrhoea is caused by *N. gonorrhoeae*, a pathogen affecting humans, belonging to the proteobacteria group and the Neisseriaceae family. It is a non-spore-forming diplococcus bacterium, which means that when viewed under the light microscope the bacteria exist as two bacterial cells joined together (Figure 2.1) (Shim, 2011; Westling-Haggstrom et al., 1977). *Neisseria gonorrhoeae* mostly infects genital mucosa but has been found to affect rectal, pharyngeal, oral and conjunctiva mucosa (Quillin and Seifert, 2018). The pathogen cannot survive outside of the host as it relies on the host to acquire nutrients.

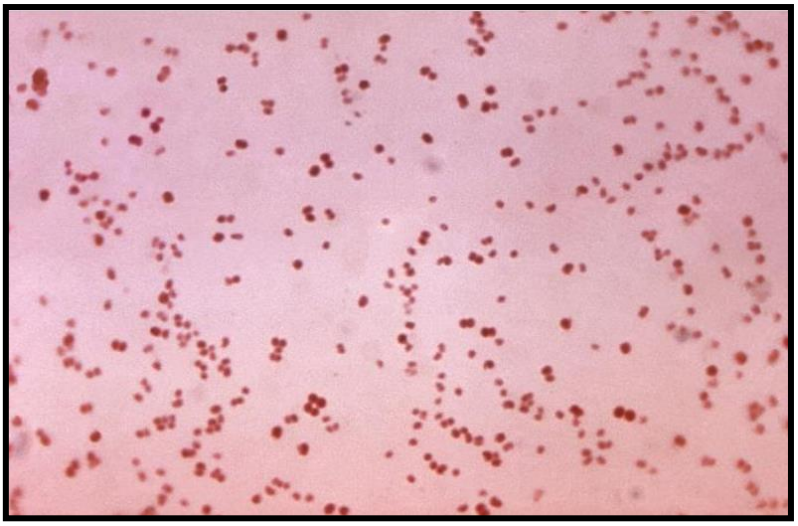

Figure 2.1: *Neisseria gonorrhoeae* viewed under the light microscope (CDC/ Woodall, 1969)

Neisseria gonorrhoeae is a Gram-negative obligate anaerobic bacterium therefore, it does not require oxygen to grow but rather carbon dioxide (Knapp and Clark, 1984). The pathogen has a lipooligosaccharide (LOS) as the major component of the outer membrane with a lipid A inner core (Arenas, 2012; Christodoulides, 2019). The LOS lacks the repetitive O- polysaccharide antigen, which is found in the lipopolysaccharide (LPS) of other Gram-negative bacteria (Figure 2.2) (McSheffrey and Gray-Owen, 2015). The gonococcal LOS is an immunostimulatory molecule that results in inflammation consequently increasing the pathogenicity of the bacteria (Zhou et al., 2014). Changes in the fatty acid chains of lipid A alters the pathogenesis and antibiotic susceptibility of *N. gonorrhoeae* (Lewis et al., 2009; Liu et al., 2010).

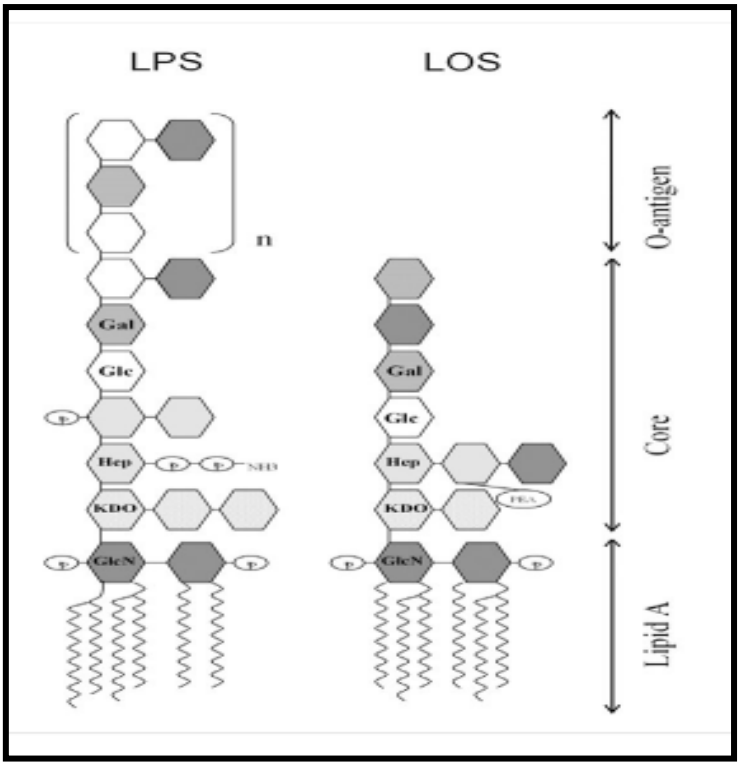

Figure 2.2: Difference between LOS and LPS membrane structures in Gram-negative bacteria showing the polysaccharide core and lipid A found in both molecules (Arenas, 2012).

Neisseria gonorrhoeae has a relatively small genome of ~2Mb compared to *Escherichia coli* with a genome of ~4Mb (Chung et al., 2008; McSheffrey and Gray-Owen, 2015). Gonococci express *pilin* and *Opa* (Opacity-associated protein) genes which translate to surface proteins that allow adhesion between bacterial and human cells thus increasing virulence of *N. gonorrhoeae* (Ball and Criss, 2013; Griffiss et al., 1999). The *Opa* and *pilin* genes are subject to phase variation, that is the switching on-and-off of genes triggered by DNA polymerase slippage while replicating tandem repeats. The slippage results in the addition or deletion of repeats which can cause reading frame shifts affecting the resultant proteins. Thus, *N. gonorrhoeae* has antigenic variation that allows the bacteria to evade the host immune systems enabling bacterial persistence and re-infection in a host (Murphy et al., 1989; Sadarangani et al., 2011; Yu et al., 2013).

2.1.1.1. Pathogenesis of *N. gonorrhoeae* and evasion of host immune system

Gonococci infections result in prolonged inflammation allowing for persistence and re-infection of the disease. The initial step in the infection of *N. gonorrhoeae* (Figure 2.3a) involves the tethering of the bacteria to the genital epithelial cells via pathogen-associated

molecular patterns (PAMPs). The PAMPs involved include pili, LOS and Opa proteins. The pili attach to host CD46/complement receptor 3 (CR3) whilst the LOS binds to asialoglycoprotein receptor (ASGPR) (McSheffrey and Gray-Owen, 2015; Sadarangani et al., 2011). Additionally, Opa proteins have an affinity for the carcinoembryonic antigen-related cell adhesion molecule family (CEACAM) (Lenz and Dillard, 2018; Sadarangani et al., 2011). The close interaction of any of these PAMPs and the epithelial cell receptors trigger the endocytosis of the bacterium, which elicits an immune response (Figure 2.3b) (McSheffrey and Gray-Owen, 2015; Wang et al., 1998). *Neisseria gonorrhoeae* triggers cytokine release which results in the overzealous recruitment of neutrophils to the site of infection (Criss and Seifert, 2012; Edwards and Butler, 2011). Cytokines such as NF-κB (nuclear factor kappa light chain enhancer of activated B cells) activate signal transduction pathways that induce IL-17 (interleukin-17) that drives the T_h17 response, which recruits neutrophils (Feinen et al., 2010). T_h17 response favours the production of IL-10 which suppresses T cells in adaptive immunity consequently, allowing persistence of the GC infection (Liu et al., 2014). The Opa proteins, within infected tissue, can bind to CEACAM 1 of dendritic cells which suppresses the maturation of T_1, T_2 and B cells (Yu et al., 2013; Zhu et al., 2012). The T_1 helper cells are responsible for the killing and clearing of infected cells whilst T_2 helper cells work in combination with B cells to produce memory cells and antibodies (Figure 2.3c). The inactivation of this adaptive immunity results in prolonged inflammation, thus gonococci infections are not cleared and the lack of antibodies permits re-infection in individuals (Hedges et al., 1999, 1998). Opa proteins can also interact with neutrophils via CEACAM 3 resulting in stimulation of an oxidative burst of the bacteria. The fragments of the bacteria can elicit immune responses in other non-infected cells within the tissue (Sadarangani et al., 2011; Sarantis and Gray-Owen, 2007).

2.1.1.2. Evasion of host immune system via nutrition immunity

The small genome of *N. gonorrhoeae* limits the metabolic capacity of the gonococci. Thus, the bacteria rely on host mucosal membranes for nutrition (Cornelissen, 2018; McSheffrey and Gray-Owen, 2015). Naturally, as a host defence mechanism, the body limits nutrients available to microbes. The available iron in the body is bound to transferrin and lactoferrin after which is transported and stored in the liver (Parrow et al., 2013). *Neisseria gonorrhoeae* has transferrin (Tbps) and lactoferrin (Lbps) receptors which interact with transferrin and lactoferrin that have bound iron (McSheffrey and Gray-Owen, 2015; Parrow et al., 2013). This

mechanism allows the gonococcal bacteria to acquire iron for metabolism. It has been shown that a defective gonococcal bacterium without both of these receptors eliminates virulence (Anderson et al., 2003).

2.1.1.3. Co-infections of *N. gonorrhoeae*

Sexually transmitted diseases are acquired via sexual contact hence there is a possibility of contracting two infections simultaneously. Gonorrhoea and chlamydia are the most common co-infections in individuals (Guy et al., 2015; Leonard et al., 2019; Seo et al., 2019). Approximately 50 % of GC infections are coupled with chlamydia (Creighton et al., 2003; McSheffrey and Gray-Owen, 2015). Individuals are usually treated for both diseases even though they have contracted one of the infections. The mechanistic interaction between the two is however unknown (Leonard et al., 2019).

It has been reported that there is a correlation between gonorrhoea and human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) in that prolonged exposure to gonorrhoea, increases the chances of HIV/AIDS (Cates and Wasserheit, 1991; Maier and Katsufrakis, 2015; Mlisana et al., 2012). *Neisseria gonorrhoeae* can interact with HIV by suppressing the expression of HIV memory response. The Opa_{CEA} protein of *N. gonorrhoeae* suppresses the maturation of dendritic cells, which are antigen-presenting cells (APC) (Yu et al., 2013). These cells are responsible for the activation of the humoral immune response, subsequently triggering the maturation of B lymphocytes into plasma cells that produce antibodies (Martin-Gayo and Yu, 2019; Steinman, 1991). *Neisseria gonorrhoeae* also increases replication of HIV-1 in T-cells by activation of the 5’ HIV long terminal repeats (LTR). This is because *N. gonorrhoeae* causes genital epithelial cells to increase the production of pro-inflammatory cytokines: tumour necrosis factor α (TNF- α) and interleukins (IL-6 & 8) (Ferreira et al., 2011). The gonococci pilin protein activates NF-κB, which is also known to escalate HIV replication (Acchioni et al., 2019; Dietrich et al., 2011). The occurrence of GC infections increases the likelihood of individuals to seroconvert to HIV positive (Bernstein et al., 2010; McSheffrey and Gray-Owen, 2015; Mlisana et al., 2012).

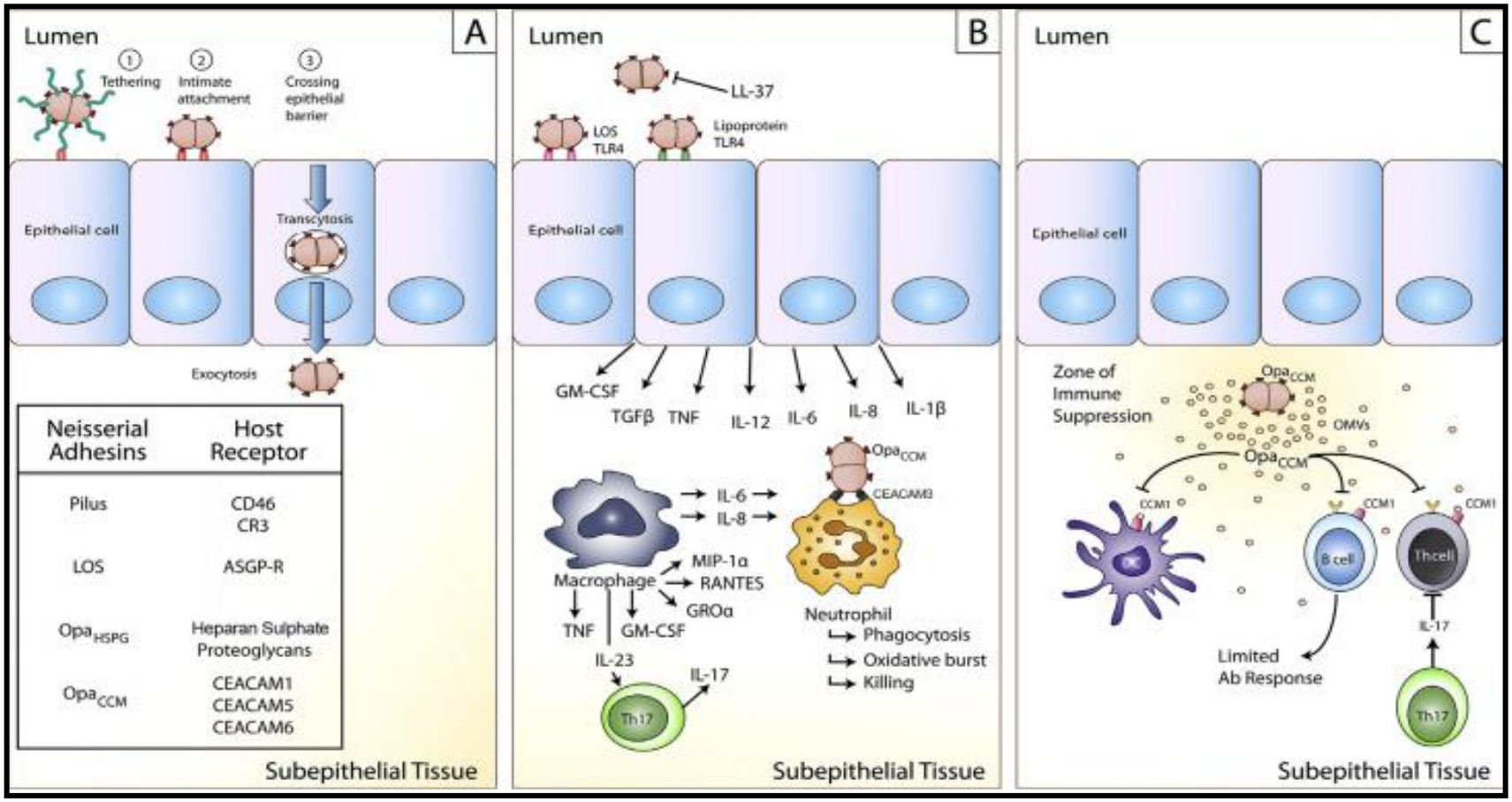

Figure 2.3: Overall pathogenesis of *Neisseria gonorrhoeae*. a) Initial step of infection. b) Immune response to gonococci infection. c) Mechanism of bacteria to avoid the immune system. Illustration created by Dr Prerna Patel © 2014 (McSheffrey and Gray-Owen, 2015).

2.2. Status of available treatments for gonorrhoea

At present, for the treatment of gonorrhoea a combination of first-line antibiotics azithromycin and ceftriaxone/cefixime is recommended (Ryan, 2017; Wi et al., 2017). The treatment is given as a single oral/intramuscular dose of ceftriaxone/cefixime in combination with a single oral dose of azithromycin (WHO, 2016). This therapy is also used in the treatment of other STDs such as chlamydia that normally exists as co-infections with gonorrhoea (Belcher and Dawson, 2017).

Ceftriaxone and cefixime (Figure 2.4 & Figure 2.5) are antibiotics classified as third-generation extended-spectrum cephalosporins (ESCs). These antibiotics inhibit *N. gonorrhoeae* by inhibiting the penicillin-binding proteins (PBP) which are used to make peptidoglycan cross-links in the bacterial cell wall (Unemo et al., 2016). Azithromycin (Figure 2.6) is a macrolide antibiotic that inhibits translation in *N. gonorrhoeae* by binding to the 50S ribosomal subunit (Douthwaite and Champney, 2001).

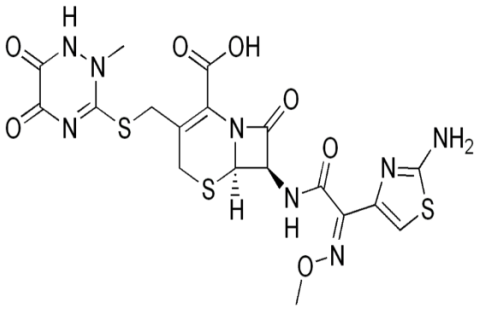

Figure 2.4: Structure of ceftriaxone

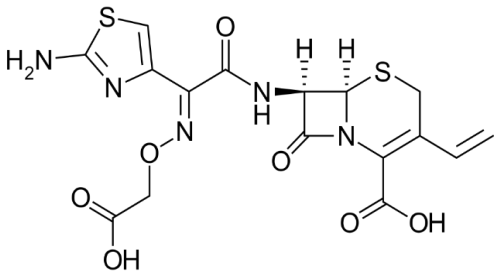

Figure 2.5: Structure of cefixime

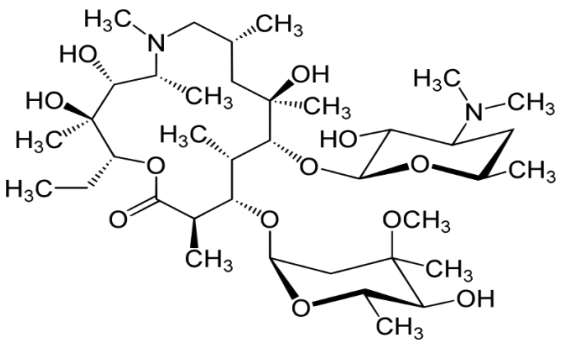

Figure 2.6: Structure of azithromycin

Azithromycin was first introduced for the treatment of gonorrhoea in the 1980s and the third-generation cephalosporins in the 1990s (Suay-García and Pérez-Gracia, 2018; Unemo et al., 2016). These antibiotics were introduced as *N. gonorrhoeae* had gained resistance to the then used tetracycline, penicillin, amoxicillin and fluoroquinolones like ciprofloxacin (Ohnishi et al., 2011b, 2011a; Unemo and Nicholas, 2012). In the 1980s ciprofloxacin and azithromycin were used as the first-line treatments however *N. gonorrhoeae* gained resistance (Lewis et al., 2008; Lynagh et al., 2015; Stevens et al., 2014; Unemo et al., 2016). The mode of action used by the ciprofloxacin-resistant strains is by reducing the affinity of DNA gyrase to the drug whilst azithromycin-resistant strains alter the bacterial 50S ribosomal subunit target (Unemo and Shafer, 2014, 2011).

The escalation in ciprofloxacin resistance resulted in the abandonment of the regimen for the treatment of gonorrhoea (Lewis et al., 2008; Unemo et al., 2016). As the years progressed azithromycin was discouraged as a monotherapy due to the resistance that was observed and it was therefore recommended to be used in combination with cefixime/ceftriaxone (Bignell et al., 2013). Azithromycin-resistant strains have been detected in many countries including Australia, Ireland and Canada (Kularatne et al., 2018a; Lynagh et al., 2015b; Martin et al., 2019; Stevens et al., 2014b; WHO, 2019). Even though ceftriaxone and cefixime are used as current regimens, antibiotic resistance has been reported in various parts of the world (Unemo et al., 2016; Wi et al., 2017). According to the National Institute for Communicable Diseases (NICD) of South Africa, cefixime-resistant isolates have been detected in Cape Town, Gauteng and the Eastern Cape province (Kularatne et al., 2017). Resistance of ESCs in *N. gonorrhoeae* is by mutations to the penicillin-binding proteins (PBP) and also increasing efflux of the ESCs in the bacterium (Unemo and Shafer, 2014). The rise of cephalosporin antibiotic resistance has resulted in some countries opting to alternatively use spectinomycin (Figure 2.7) or gentamicin (Figure 2.8) in combination with azithromycin (Australasia Sexual Alliance, 2019; Bignell et al., 2013; Public Health Canada, 2017; Suay-García and Pérez-Gracia, 2018b; WHO, 2019).

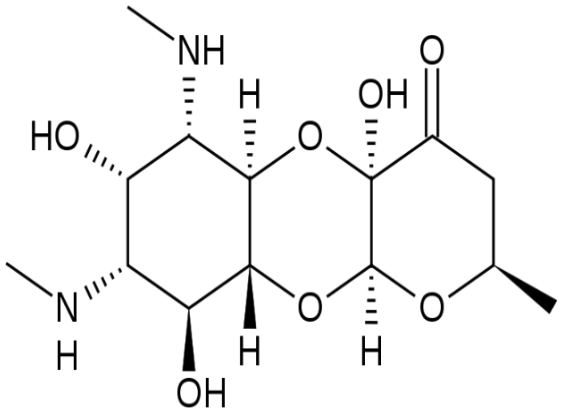

Figure 2.7: Structure of spectinomycin

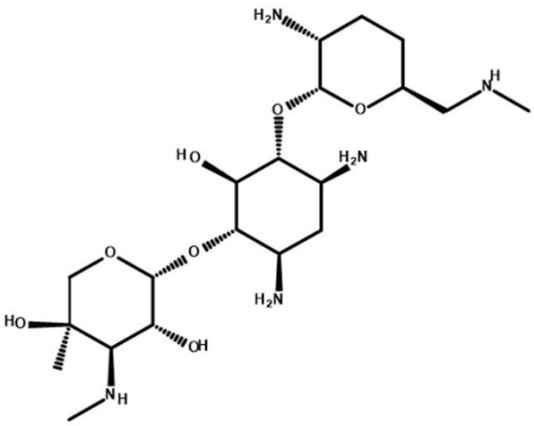

Figure 2.8: Structure of gentamicin

The upsurge of ESC resistance has been a concern as there is now an emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) *N. gonorrhoeae* (Alirol et al., 2017; Martin et al., 2019). Bacterial strains that have resistance to at least two of the currently recommended therapeutics, as well as resistance to at least two of penicillin, tetracycline, erythromycin or ciprofloxacin, are categorized as XDR strains (Martin et al., 2019). On the other hand, MDR bacterial strains have resistance to at least one of the current recommended antibiotics and resistance to at least two of the following: penicillin, tetracycline, erythromycin or ciprofloxacin (Martin et al., 2019). Treatment failures of both azithromycin and ESCs towards XDR *N. gonorrhoeae* have been reported (Martin et al., 2019). The first of such cases were reported in Australia and the United Kingdom (Australian Government Department of Health, 2018; European Centre for Disease Prevention and Control, 2018; Public Health England, 2018). Recently some XDR strains were detected in Canada as well (Martin et al., 2019). The emergence of XDR *N. gonorrhoeae* strains puts a burden on the available antibiotics by reducing their efficiency (Crowther-Gibson et al., 2011). At present, the WHO has published a global priority list that contains drug-resistant bacteria for urgent research and the development of new treatments (WHO, 2017). The list was categorized into three priority levels: critical (1), high (2) and medium (3) in which level one is top priority for developing novel treatments. The heightened antibiotic resistance observed in *N. gonorrhoeae* globally has resulted in the WHO placing it on the global priority list level 2 (WHO, 2017). This, therefore, prompts the need for drug discovery of new therapies for the treatment of gonorrhoea.

2.3. South African plants used in traditional medicine for the treatment of STDs and their bioactivity

The antibiotic resistance burden has consequently brought plants into the limelight for drug development (Palmeira-de-Oliveira et al., 2013). Plants have been reported to have a vast diversity in compounds and have been used throughout history in folk medicine by traditional healers worldwide (de Wet et al., 2012; Palmeira-de-Oliveira et al., 2013; Yang et al., 2012). Table 2.1 provides a summary of some of the South African medicinal plants used in traditional medicine for the treatment of venereal diseases including gonorrhoea. In the section below, selected plants are discussed further in detail with emphasis on their bioactivity and *in vitro* studies that have been conducted on STD pathogens. In general, for antimicrobial studies, a plant extract is considered to have noteworthy activity if the minimum inhibitory concentration (MIC) is below 1000 µg/ml (Ndhlala et al., 2013; van Vuuren, 2008).

2.3.1. *Aloe ferox*

Aloe ferox Mill, (bitter aloe) belongs to the Asphodelaceae family of plants. It is used in South African traditional medicine for the treatment of arthritis, conjunctivitis, eczema, hypertension, STDs, stress and venereal sores. Related species *Aloe marlothii* A. Berger and *Aloe vera* (L.) Burm.f are also used in phytomedicine to treat STDs including HIV (Semenya et al., 2013b). The fleshy leaves are made into a decoction for the treatment of STDs such as gonorrhoea and syphilis (van Wyk et al., 2017).

Aloe ferox has been reported to have antimicrobial activity against *Candida albicans*, *Gardnerella vaginalis*, *Shigella sonnei* and *N. gonorrhoeae* (Kambizi & Afolayan, 2008; van Vuuren & Naidoo, 2010). Kambizi and Afolayan (2008) found that methanol extracts of *A. ferox* inhibit *N. gonorrhoeae* at a MIC of 500 µg/ml.

Bioactive compounds including aloin, aloe-emodin and chrysophanol have been isolated from *A. ferox* extracts (Kambizi et al., 2005). Aloin exhibited antigonococcal activity with a MIC of 100 µg/ml, respectively (Kambizi and Afolayan, 2008). This bioactive compound has antiviral activity against the herpes simplex virus (HSV-1) by reducing the cytopathic effects (CPEs) of the virus (Kambizi et al., 2007). Cytopathic effects are structural changes to the host cell caused by a viral infection (Albrecht et al., 1996). Potent antiviral activity was seen at 63 µg/ml with 25-50 % CPEs being observed 120 hrs after infection with HSV-1. The positive control had 75

% CPEs noticed 24 hrs after infection. Aloin was, therefore, able to delay infection of HSV-1 in the Vero (monkey kidney) cells more than the positive control (Kambizi et al., 2007).

2.3.2. *Cassia abbreviata*

Cassia abbreviata Oliv, wild senna, is part of the Fabaceae family (Sobeh et al., 2018). In traditional medicine, it is used as a treatment for all STDs. The usual preparation of the prescription is a decoction of the stem or stem/bark of the plant. This decoction is taken orally by the patient (Chauke et al., 2015).

Water stem/bark extracts of *C. abbreviata* have antifungal activity against *C. albicans* with a MIC of 100 µg/ml (Mongalo et al., 2017). Ethanolic extracts of the plant have exhibited potent antigonococcal activity with a MIC of 46.88 µg/ml. *Cassia abbreviata* extracts have been seen to inhibit HIV-1 reverse transcriptase (RT) with an IC_{50} of 1250 µg/ml (Chauke et al., 2016). Moreover, antiviral activity against HSV has been reported (Viol et al., 2016).

Palmitic acid has been isolated from *C. abbreviata* (Dangarembizi et al., 2015). Palmitic acid is an inhibitor of HIV-1 by inhibiting entry and fusion of the virus (Lee et al., 2009; Lin et al., 2011). Palmitoleic acid, an unsaturated fatty acid, that can be biosynthesized from palmitic acid has activity against *N. gonorrhoeae* (Bergsson et al., 1999).

2.3.3. *Combretum molle*

Combretum molle R.Br. ex G.Don, the velvet bushwillow, is a member of the Combretaceae family (Hedberg et al., 1982). The whole plant is used traditionally in the treatment of syphilis and gonorrhoea (de Wet et al., 2012; Fyhrquist et al., 2002). Oral decoctions are preferred for the treatment of STDs (Bryant, 1966).

Methanol and acetone extracts of *C. molle* have the same activity with MICs of 40 µg/ml against *C. albicans.* Moreover, dichloromethane extracts inhibited the yeast at 320 µg/ml (Masoko et al., 2007). Methanol plant extracts are potent inhibitors of HIV-1 ribonuclease (RNase H) and RNA-dependant DNA-polymerase (RDDP) with IC_{50}s of 9.7 µg/ml and 9.5 µg/ml, respectively (Bessong et al., 2005). Thus, the plant is a potential candidate to develop novel anti-HIV drugs. Other plants in the Combretaceae family, namely *Combretum adenogonium* Steud. Ex A. Rich and *Terminalia sericea* Burch. ex DC have also exhibited anti-

HIV activity with 79 % and 98 % inhibition at 100 µg/ml of HIV-1 (Bessong et al., 2005; Mushi et al., 2012).

Terpenoids, combretene A and B, have been isolated from *C. molle* as well as punicalagin and sericoside (Ahmed et al., 2004; Asres et al., 2001). However, little is known about the antimicrobial properties of these compounds.

2.3.4. *Elaeodendron transvaalense*

Elaeodendron transvaalense (Burtt Davy) R.H.Archer, Transvaal saffron, is a medicinal plant in the Celastraceae family. It is used traditionally in the management of STDs (including gonorrhoea and herpes) and HIV infections (Mabogo, 1990; Maroyi and Semenya, 2019; Samie et al., 2010). It is used sometimes in combination with *Elephantorrhiza elephantina* (Burch.) Skeels in the treatment of these diseases (Semenya et al., 2013c). The root/bark decoctions of the plant are mostly preferred for the treatment of STDs (Maroyi and Semenya, 2019; Semenya et al., 2013c).

Mamba et al. (2016) found that the ethanolic plant extracts have antimicrobial activity against *C. albicans* and *G. vaginalis.* The extracts also had antigonococcal properties with MIC of 1600 µg/ml compared to the ciprofloxacin control (<10 µg/ml). Mamba et al. (2016) also found that *Elaeodendron croceum* (Thunb.) DC, a related species used in the treatment of HIV, has the same antigonococcal activity. The water and ethanol plant extracts of *E. transvaalense*, at 100 µg/ml, have moderate HIV RT inhibition of ~40 % when compared to nevirapine (~ 80%) and doxorubicin (100 %) controls (Mamba et al., 2016; Sigidi et al., 2017).

Three compounds, isolated from the plant, lup-20(30)-ene-3α,29-diol, lup-20(29)-ene-30-hydroxy-3-one and 4'-O-methyl-epigallocatechin have been tested on STD pathogens (Mamba et al., 2016). 4'-O-methyl-epigallocatechin inhibited *N. gonorrhoeae* at a MIC of 6300 µg /ml (Mamba et al., 2016). In the same study, it was seen that the active compound was an inhibitor of HIV-1 RT.

2.3.5. *Hypoxis hemerocallidea*

Hypoxis hemerocallidea Fisch., C.A.Mey. & Avé-Lall, previously known as *Hypoxis rooperi*, is part of the Hypoxidaceae family (van Wyk et al., 2017). It is known as the yellow star 'African potato' (van Wyk et al., 2017). It is a very popular plant used by traditional healers to

treat gonorrhoea and HIV. The plant tubers are usually made into a decoction and taken orally (de Wet et al., 2012; Semenya et al., 2013b). The plant extracts are commercially available in pharmacies as tonics, tinctures or capsules.

Naidoo et al. (2013) showed that aqueous extracts have antigonococcal properties with a MIC of 500 µg/ml compared to the 40 µg/ml of the ciprofloxacin control. The plant leaf aqueous, ethanolic and DCM extracts have exhibited activity against *C. albicans* all with MICs of 800 µg/ml (Ncube et al., 2011). *Hypoxis hemerocallidea* has been reported to have anti-HIV activity. The plant has been shown to maintain CD4 cells in HIV patients who were given capsules containing methanolic extracts. This validates the plant's use as a dietary supplement in individuals with HIV (Albrecht, 1996; Matyanga et al., 2020).

Hypoxoside is a compound that has been isolated from *H. hemerocallidea* (Drewes et al., 1984; Matyanga et al., 2020). This inactive compound is converted to rooperol, which improves the immune system (Drewes et al., 2008; Mills et al., 2005). Phytosterol, β-sitosterol (BSS), isolated from the plant has also been found to be an immune enhancer. Clinical trials have been done on humans and Bouic et al. (1996) showed that β-sitosterol glucoside (BSSG) can increase the proliferation of T cells by increasing the expression of CD25 in the cells thus boosting the immune system.

2.3.6. *Peltophorum africanum*

Peltophorum africanum Sond, weeping wattle, belonging to the Fabaceae family, is utilised traditionally to treat STDs such as HIV and gonorrhoea (Samie et al., 2010; Semenya et al., 2013c; Tshikalange et al., 2016). The roots and stem/bark of this plant are usually made into a decoction that is orally taken to treat STDs (Mongalo, 2013; Mongalo and Makhafola, 2018).

Ethanolic plant extracts of the plant have antigonococcal activity with a MIC of 1600 µg/ml compared to the ciprofloxacin control (<10 µg/ml) (Mamba et al., 2016). Furthermore, aqueous root extracts have been reported to have antibacterial activity against *G. vaginalis* and *N. gonorrhoeae* with MICs of 500 µg/ml (Naidoo et al., 2013). This plant has been studied for the potential treatment of HIV and has been reported to inhibit HIV-1 RT (Mamba et al., 2016; Tshikalange et al., 2008). The methanolic stem/bark extracts inhibit RDDP of RT with an IC_{50} of 3.5 µg/ml. It also inhibits RNase H of RT with an IC_{50} of 10.6 µg/ml (Bessong et al., 2005).

Bessong et al. (2005) have isolated gallotannin and catechin, from methanolic extracts of the stem/bark of the plant, which have anti-HIV properties. Epigallocatechin-3-O-gallate (EGCG) was isolated from methanolic extracts of the plant (Ebada et al., 2008). EGCG has antigonococcal activity with a MIC of 32 µg/ml whilst an EGCG derivate containing acyl group palmitoleate (C16) had a MIC of 16 µg/ml. The compounds had less activity than the positive controls amoxicillin (0.25 µg/ml) and cefazolin (0.5 µg/ml). The two compounds, C16 and EGCG, showed anti-*Candida* activity with a MIC of 16 µg/ml and 64 µg/ml, respectively. Fluconazole control had a MIC of 0.25 µg/ml (Matsumoto et al., 2012).

2.3.7. *Terminalia sericea*

Terminalia sericea Burch. ex DC, the silver cluster leaf, is a medicinal plant in the Combretaceae family (van Wyk et al., 2017). The stem/bark or roots are used as a decoction to treat STDs (syphilis and gonorrhoea) and other opportunistic illnesses related to HIV (Chinsembu, 2016; Hutchings, 1989; Mongalo and Makhafola, 2018; Watt and Breyer-Brandwijk, 1962).

Terminalia sericea has have been reported to have antimicrobial properties against *Trichomonas vaginalis*, *C. albicans* and *G. vaginalis* (van Vuuren & Naidoo, 2010). The DCM:MeOH extracts of the plant have antigonococcal effects with a MIC of 1000 µg/ml (van Vuuren & Naidoo, 2010). Chauke et al. (2016) revealed that the plant has anti-HIV properties, where acetone and water extracts had IC_{50} values of 80 µg/ml. Tshikalange et al. (2016) revealed that the ethanolic plant extracts of *T. sericea* can inhibit 100 % HIV-1 RT at 100 µg/ml. Bessong et al. (2005), showed that the methanolic leaf extracts of the plant inhibited the RDDP functions of HIV-1 RT by 98 % at 100µg/ml.

Resveratrol has been isolated from ethanol extracts of the plant and has antimicrobial activity (Joseph et al., 2007; Mongalo et al., 2016). The compound inhibits *N. gonorrhoeae* with a MIC of 25 µg/ml (Docherty et al., 2001). Furthermore, resveratrol has exhibited antifungal activity against *C. albicans* (Houillé et al., 2014; Weber et al., 2011). The bioactive compound has antibacterial properties against *Chlamydia trachomatis* with pathogenesis seen at concentrations below 75 µM (Petyaev et al., 2017). Resveratrol has antiviral activity against HSV with inhibition of 99 % seen at 100 µg/ml (Annunziata et al., 2018; Docherty et al., 1999).

2.3.8. *Tabernaemontana elegans*

Tabernaemontana elegans Stapf, the toad tree, is a South African medicinal plant belonging to the Apocynaceae family (de Wet et al., 2012). The roots and the leaves are commonly used to treat STDs. The plant is finely chopped and made into a decoction with either *H. hemerocallidea* corms or *Ipomoea batata* (L.) Lam (sweet potato) leaves to treat gonorrhoea (de Wet et al., 2012).

Tabernaemontana elegans has some pharmacological effects which include: antibacterial, antifungal and antiprotozoal activity. Naidoo et al. (2013) showed that the DCM:MeOH extract has antibacterial activity against *G. vaginalis* and *N. gonorrhoeae* with a MIC of 250 µg/ml and 1000 µg/ml, respectively. The extracts had lower activity than the ciprofloxacin positive control (0.39 µg/ml) against *G. vaginalis*. Similarly, the extract had lower activity than the positive control (0.04 µg/ml) for *N. gonorrhoeae*. The aqueous extracts had bioactivity against *C. albicans* with a MIC of 250 µg/ml compared to the 2.5 µg/ml amphotericin B control. The DCM:MeOH extracts had anti-protozoan properties inhibiting *T. vaginalis* at a MIC of 1 mg/ml.

Table 2.1: South African medicinal plants used traditionally to treat STDs

Plant species	**Family**	**Parts used**	**Traditional use**[a]	**References**
*Albizia adianthifolia**	Fabaceae	leaves	STDs, gonorrhoea	de Wet et al., 2012
*Aloe ferox**	Asphodelaceae	leaves	STDs	van Wyk et al., 2017
Aloe marlothii	Asphodelaceae	leaves	STDs	de Wet et al., 2012; Mongalo and Makhafola, 2018; Semenya et al., 2013b, 2013c
Burkea africana	Fabaceae	roots	HIV	Chinsembu, 2016; Semenya et al., 2013b, 2013c
*Cassia abbreviata**	Fabaceae	roots stem/bark	STDs, gonorrhoea, immune booster in HIV patients	Chinsembu, 2016; Mongalo and Makhafola, 2018
*Catharanthus roseus**	Apocynaceae	roots	STDs, gonorrhoea	Semenya et al., 2013c
*Combretum molle**	Combretaceae	leaves roots	STDs, gonorrhoea, syphilis	de Wet et al., 2012; Fyhrquist et al., 2002
*Dioscorea sylvatica**	Dioscoreaceae	bulb	STDs, gonorrhoea	Semenya et al., 2013b, 2013c
*Diospyros lycloides**	Ebenaceae	roots	STDs, gonorrhoea	Chinsembu, 2016
Elaeodendron transvaalense	Celastraceae	roots	STDs, herpes, HIV	Mabogo, 1990; Mongalo and Makhafola, 2018; Semenya et al., 2013a, 2013b, 2013c
Elephantorrhiza elephantina	Fabaceae	roots	STDs, HIV	Semenya et al., 2013a, 2013b, 2013c
*Helichrysum caespitutum**	Asteraceae	whole plant	STDs, gonorrhoea	Semenya et al., 2013a, 2013b

*Hypoxis hemerocallidea**	Hypoxidaceae	bulb	STDs, gonorrhoea, immune booster in HIV patients	de Wet et al., 2012; Mongalo and Makhafola, 2018; Semenya et al., 2013c)
*Hypoxis obtusa**	Hypoxidaceae	roots	Gonorrhoea, chylamydia	Semenya et al., 2013a, 2013c
*Jatropha zeyheri**	Euphorbiaceae	roots	STDs, gonorrhoea	Mongalo and Makhafola, 2018; Semenya et al., 2013a
Pelargonium spp	Geraniaceae	roots	HIV	Semenya et al., 2013c
*Peltophorum africanum**	Rhamnaceae	roots stem/bark	STDs, gonorrhoea, HIV	Chinsembu, 2016; de Wet et al., 2012; Mongalo and Makhafola, 2018; Semenya et al., 2013a, 2013c
*Senecio serratuloides**	Asteraceae	whole plant	STDs, gonorrhoea	de Wet et al., 2012
*Senna italica**	Fabaceae	root	STDs, gonorrhoea	Chauke et al., 2015; Semenya et al., 2013a
*Tabernaemontana elegans**	Apocynaceae	leaves roots	STDs, gonorrhoea	de Wet et al., 2012
Terminalia sericea	Combretaceae	roots stem/bark	STDs, opportunistic diseases associated with HIV	Chinsembu, 2016; de Wet et al., 2012; Mongalo and Makhafola, 2018
*Ximenia caffra**	Olacaceae	roots	STDs, gonorrhoea,	Chauke et al., 2015; Chinsembu, 2016; de Wet et al., 2012; Mongalo and Makhafola, 2018
*Ziziphus mucronata**	Rhamnaceae	roots	STDs, gonorrhoea, chlamydia	Chinsembu, 2016; Mongalo and Makhafola, 2018; Semenya et al., 2013c

[a] reported as generic treatments for any STDs, * *Plants used traditionally for the treatment of gonorrhoea*

2.4. Polyherbal formulations for the treatment of STDs

2.4.1. South African polyherbal formulations on the market

Currently, there are herbal mixtures available on the market which are prepared as tonics that are used to treat various ailments including STDs (Ndhlala et al., 2011b). However, there are currently no polyherbal mixtures being sold specifically formulated for action against gonorrhoea or any specific STD. The herbal mixtures available typically contain various plants mostly prepared as decoctions (Ndhlala et al., 2011b; Varga and Veale, 1997). The mixtures are sold on both the informal and formal markets (Ndhlala et al., 2011b).

In South African pharmacies, two herbal products are available namely, Ingwe Sejeso muthi mixture and Lion Izifonke herbal mixture. Ingwe Sejeso muthi mixture (Figure 2.9) is a water-based mixture that contains lesoko (*Alepidea amatymbica* Eckl & Zeyh), monnamalledu (*Hypoxis obtusa* Burch. ex Ker Gawl), an unknown ingredient, setimamollo (*Pentanisia prunelloides* (Klotzsch) Walp) and mositsane (*Elephantorrhiza elephantina* (Burch.) Skeels) (Nair et al., 2012). The herbal mixture is used for an array of ailments which include heartburn, constipation, stomach ache, STDs, stomach cramps and indigestion (Ndhlala et al., 2011b). Almost all plants with exception of *Hypoxis obtusa* in the mixture have been reported to inhibit *Candida albicans* that causes vaginal yeast infections in women (Mabona et al., 2013; Mulaudzi et al., 2009).

The Ingwe Sejeso muthi mixture is a potent inhibitor of the HIV-1 RT, which is essential for the proliferation of HIV in the host (Ndhlala et al., 2010). The plant constituents of the herbal product could have synergistic effects that give rise to the therapeutic action. The herbal product also has anti-*Candida* activity, MIC exhibited was 1560 µg/ml (Ndhlala et al., 2009).

The Izifonke herbal mixture (Figure 2.10) is a polyherbal mixture that contains mathunga (*Eucomis autumnalis* (Mill) Chitt), inguduza (*Merwilla plumbea* (Lindl.) Speta) and umhlaba (*Aloe ferox* Mill). *Eucomis autumnalis* and *Aloe ferox* have been reported to have anti-*Candida* activity (Kambizi and Afolayan, 2008; Muleya et al., 2014). As stated earlier, aloin isolated from *A. ferox* has shown antimicrobial activity against *N. gonorrhoeae* with a MIC of 100 µg/ml (Kambizi and Afolayan, 2008). The Izifonke herbal mixture has inhibitory effects against *C. albicans* (Ndhlala et al., 2009).

Figure 2. 9: Ingwe Sejeso herbal mixture sold on the formal market (https://clicks.co.za/ingwe_sejeso-herbal-mixture-500ml/p/223807)

Figure 2.10: Lion Izifonke herbal mixture sold on the formal market (http://www.maricosa.com/categories/ingwe/Lion-Izifozonke.html)

2.4.2. Polyherbal formulations from other countries

There are polyherbal formulations that have been developed in other countries for the treatment of STDs. Examples of these are the Indian herbal vaginal ointments namely, Basant and Praneem. Basant is made from purified extracts of *Emblica officinalis* Gaertn, purified saponins from *Sapindus mukerossi* Gaertn, *Aloe vera* and rose water. The polyherbal ointment has wide-spectrum antimicrobial activity and has shown inhibitory effects on *C. albicans, N. gonorrhoeae* and *C. trachomantis* (Bhengraj et al., 2008; Talwar et al., 2008). Praneem is formulated from purified saponins from *S. mukorossi*, leaf extracts of *Azadirachta indica* A. Juss, and *Mentha citrata* oil. The product inhibits *N. gonorrhoeae, Candida* spp. and *C. trachomatis* (Talwar et al., 2000). Podophyllotoxin, derived from *Podophyllum* spp., in the Berberidaceae family, has been used to develop podofilox (Li et al., 2012). This product is a topical gel that is used to treat genital warts caused by the human papilloma virus (HPV-6 &11) (Boxman et al., 1999; Vermani and Garg, 2002). Podophyllotoxin is highly cytotoxic, as it is an antimitotic agent that results in a hypersensitive response of the HPV infected cells leading to cell death (Kollipara et al., 2015). Podofilox has gone through clinical trials and is now a recommended treatment for genital warts (Bonnez et al., 1994; CDC, 2015b; Tyring et al., 1998).

2.5. Selected plants for the study

In this study, five plants were selected based on the traditional use for the treatment of STDs and other diseases by Traditional healer (TDr) Lulama Masinga from Hambanati village, Tongaat, KwaZulu-Natal, South Africa. Furthermore, the plants were selected as they have some traditional use for the treatment of gonorrhoea and STDs reported in the literature. The plants also have some bioactivity recorded from *in vitro* studies on STD pathogens and non-STD pathogens.

2.5.1. *Gnidia kraussiana*

Gnidia kraussiana Meisn, commonly known as yellow heads, have distinct yellow flowers (Figure 2.11). The species belongs to the Thymelaeaceae family (van Wyk et al., 2017). It is mostly found in grassland areas of Africa. In South Africa, it is found in the northern and eastern parts which include KwaZulu-Natal, Limpopo and Mpumalanga (van Wyk et al., 2017). *Gnidia kraussiana* grows into a shrub with a height of up to 0.3 m with perennial subterranean tubers (van Wyk et al., 2017).

Figure 2.11: *Gnidia kraussiana* (http://pza.sanbi.org)

Gnidia kraussiana is a toxic South African plant used in traditional medicine to treat various ailments. The plant is used in the treatment of burns, snake bites, stomach disorders, STDs and to facilitate childbirth (Hutchings, 1989; van Wyk et al., 2017; Zukulu et al., 2012). The leaves, rhizomes, stems and roots have vast medicinal properties. The underground parts are the

preferred choice for prescriptions (typically prepared as decoctions) by South African traditional healers (van Wyk et al., 2017). The plant is used to make imbiza ephuzwato, an herbal tonic, alongside *Gunnera perpensa*. The tonic is used to enhance sexual prowess and treat constipation, stress, hypertension, arthritis, kidney problems, and alleviate body pains (Maroyi, 2016).

Gnidia kraussiana has been tested against various microorganisms and has been found to have antimicrobial activity against *Bacillus subtilis*, *C. albicans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Ndhlala et al., 2011a; Saadabi and Moglad, 2011).

Compounds that have been derived from *G. kraussiana* are mostly daphnane type esters. These compounds are categorized as diterpenes and are found in all parts of the plant including roots, leaves, rhizomes and stems (Bhandurge et al., 2013). Daphnane esters are characteristic of the Thymelaeaceae family; being found in other *Gnidia* species such as *Gnidia lamprantha* Gilg and *Gnidia subcordata* Meisn (Bhandurge et al., 2013; Borris and Cordell, 1984; Kupchan et al., 1976a,b). The biological activity and toxicity of *Gnidia* spp. is attributed to the daphnane diterpenes that are present in the plants. These compounds have been reported to be tumour inhibitors (Bhandurge et al., 2013). Kraussianin (Figure 2.12) has been isolated from *G. kraussiana* and has been reported to have antileukemia properties (Bhandurge et al., 2013; Borris et al., 1988; Borris and Cordell, 1984). Gniditrin, gnidicin and gnidilatin are other daphnane diterpenes that have been isolated from *G. kraussiana* which all have been reported to have tumour inhibiting properties (Bhandurge et al., 2013; Borris and Cordell, 1984; Brooks et al., 1990; Fujita and Nagao, 1977). Gnidimacrin (Figure 2.13), a daphnane type diterpenoid, isolated from the same plant has anti-HIV properties (Bhandurge et al., 2013; Yan et al., 2015). A flavonoid compound, tiliroside, has also been isolated which has antioxidant, antiprotozoal, antibacterial and anticancer properties (Bhandurge et al., 2013; Calzada et al., 2017).

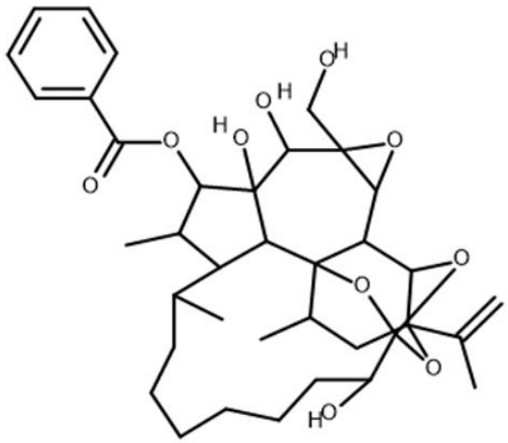

Figure 2.12: Kraussianin

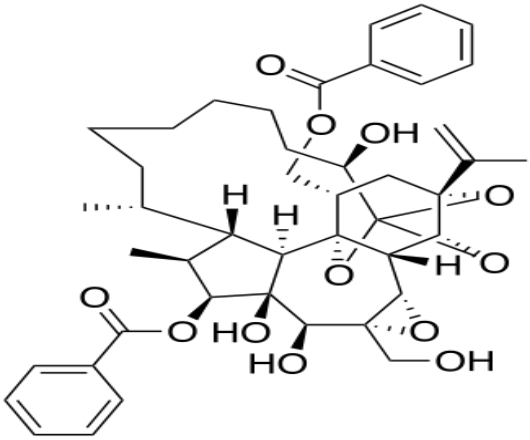

Figure 2.13: Gnidimacrin

2.5.2. *Gunnera perpensa*

Gunnera perpensa L. (Figure 2.14) is a member of the Gunneraceae family and is commonly known as wild rhubarb or river pumpkin (van Wyk et al., 2017). The name river pumpkin is due to the similarity of the leaves to pumpkin leaves (Khan et al., 2004). It is widely distributed throughout Africa with occurrences across South Africa except in the Northern Cape (Arnold and de Wet, 1993; Mammo et al., 2017). The plant grows in water-locked soils or near riversides which is because the plant has a symbiotic relationship with nitrogen-fixing cyanobacteria (Mammo et al., 2017; Silvester and Smith, 1969). *Gunnera perpensa* is a perennial shrub that has rhizomes in the subterranean parts of the plant (van Wyk et al., 2017).

Figure 2.14: *Gunnera perpensa* (http://pza.sanbi.org)

Gunnera perpensa is used traditionally to treat stomach disorders, antenatal health problems, rheumatic fevers, dysmenorrhoea, wounds, psoriasis, urinary tract infections and STDs (particularly, gonorrhoea and syphilis) (Brookes and Dutton, 2007; Buwa and van Staden, 2006; van Wyk et al., 2017). The leaves are used as an insect repellent for ticks and other parasites (Maroyi, 2016). In South African traditional medicine, the leaves, rhizomes and roots are used to make decoctions, infusions and tinctures (Drewes et al., 2005; Mammo et al., 2017; Maroyi, 2016). The plant is an ingredient in imbiza ephuzwato, inembe and isihlambezo, which are traditional concoctions used in traditional medicine. Inembe is a labour inducer whilst isihlambezo hastens childbirth and expulsion of the placenta (Maroyi, 2016; Varga and Veale, 1997). Imbiza ephuzwato is an herbal tonic made of 21 medicinal plants, which includes *G. kraussiana* (Maroyi, 2016).

This valuable medicinal plant has antimicrobial activity against *B. subtilis*, *C. albicans*, *E. coli*, *K. pneumoniae*, *Micrococcus kristinae*, *P. aeruginosa*, *Serratia marcescens*, *Shigella flexneri*, *Staphylococcus epidermidis*, *Streptococcus faecalis* and *S. aureus* (Buwa and van Staden, 2006; Goulart and Wurcel, 2016; Ndhlala et al., 2011a; Nkomo and Kambizi, 2009). Furthermore, Mabona et al. (2013) found that the plant can inhibit *Cutibacterium acnes*, formerly *Propionibacterium acnes*, which causes acne.

Gunnera perpensa has anti-inflammatory effects as it can inhibit cyclooxygenase enzymes (COX-1 & 2) (Maroyi, 2016; Muleya et al., 2014; Ndhlala et al., 2011a). Reports of anticancer, acetylcholinesterase (AChE) inhibition, antioxidant, anthelmintic and uterotonic properties have been reported (Maroyi, 2016; Mwale and Masika, 2015; Ndhlala et al., 2011a; Simelane et al., 2010). The AChE inhibition induces muscle contraction during labour.

Phenolic compounds, alkaloids, terpenoids and phytosterols have been isolated from *G. perpensa* (Brookes and Dutton, 2007; Chigor, 2014; Mammo et al., 2017). Several phenolic compounds have been isolated including ellagic acid, 3,3′,4′-tri-O-methyl ellagic acid, 4-O-β-D-glucopyranoside and punicalagin (Brookes and Dutton, 2007; Mammo et al., 2017). These compounds have been reported to have antioxidant, anti-inflammatory and anti-mutagenic properties. Two quinone derivatives have been isolated: 2-methyl-6-(3-methyl-2-butenyl)-benzo-1-4-quinone (Figure 2.15) and 3-hydroxy-2-methyl-5-(3-methyl-2-butenyl) benzo-1,4-quinone). The 2-methyl-6-(3-methyl-2-butenyl)-benzo-1-4-quinone has anticancer activity (Chavan et al., 2013). This compound also has antimicrobial activity against *Bacillus cereus*, *Cryptococcus neoformans*, *C. albicans*, *Enterococcus faecalis*, *S. aureus* and *S. epidermidis*

(Drewes et al., 2005; McGaw et al., 2005). Z-venusol (Figure 2.16) is a phytosterol that has been isolated that causes contraction of uterine muscles (Brookes and Dutton, 2007; Khan et al., 2004). It also can induce apoptosis in breast cancer cells (Mathibe et al., 2016).

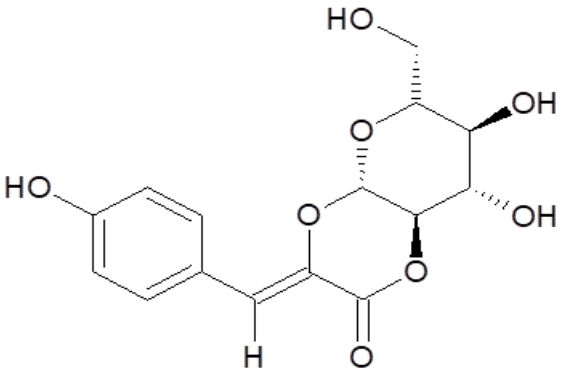

Figure 2.15: Structure of 2-methyl-6-(3-methyl-2-butenyl)-benzo-1-4-quinone

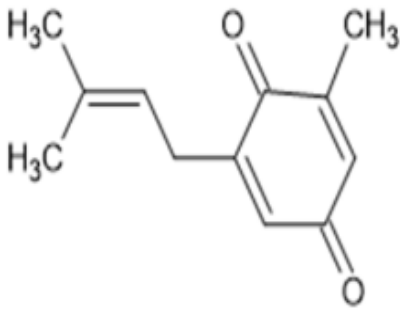

Figure 2. 16: Structure of Z-venusol

2.5.3. *Pentanisia prunelloides*

Pentanisia prunelloides (Klotzsch) Walp, is a member of the Rubiaceae family and is known as the wild verbena (van Wyk et al., 2017; Verdcourt, 1953). The plant is a perennial tuberous shrub that grows to about 30-60 cm (Maroyi, 2019; van Wyk et al., 2017). It bears blue-purple flowers at the end of each stem (van Wyk et al., 2017; Verdcourt, 1953) (Figure 2.17). *Pentanisia prunelloides* is a grassland plant that is widely spread throughout the southern parts of Africa (Maroyi, 2019). In South Africa, it is found in the Eastern Cape and KwaZulu-Natal (van Wyk et al., 2017).

Figure 2.17: *Pentanisia prunelloides* (http://pza.sanbi.org)

Pentanisia prunelloides has uterotonic, antioxidant, antidiabetic and analgesic activity (Kaido et al., 1997; Lindsey et al., 1999; Makhubu, 2017; Miya et al., 2016; Mpofu et al., 2014; Muleya et al., 2015). The plant has antiviral properties inhibiting replication of the influenza A virus (Yff et al., 2002). *Pentanisia prunelloides* has shown antibacterial activity against *B. subtilis, B. cereus, E. coli, E. faecalis K. pneumoniae, P. aeruginosa, P. acnes, S. aureus* and *S. epidermidis*, (Mabona et al., 2013; Yff et al., 2002; Mpofu et al., 2014). Anti-mycobacterial activity has been reported against *Mycobacterium aurum* and *M. tuberculosis* (Madikizela et al., 2014, 2013). Antifungal activity has been reported against *C. albicans* and *Microsporum canis* (causes skin infections) (Mabona et al., 2013). Furthermore, the plant inhibits COX resulting in anti-inflammatory activity (Mpofu et al., 2014; Yff et al., 2002).

Saponins, flavonoids, glycosides and steroids have been detected in the roots of *P. prunelloides* (Miya et al., 2016). Additionally, epigallocatechin-3-O-gallate (EGCG), epicatechin and catechin have been isolated from the plant (Maroyi, 2019). Palmitic acid (Figure 2.18) has been isolated from the plant by Yff et al. (2002). This fatty acid in combination with epicatechin has been reported to have antibacterial activity against *B. cereus, E. coli, E. faecalis* and *K. pneumoniae.* Palmitic acid is an inhibitor of HIV-1 by inhibiting entry and fusion of the virus (Lee et al., 2009; Lin et al., 2011). Palmitoleic acid (Figure 2.19), an unsaturated fatty acid, that can be biosynthesized from palmitic acid has activity against *N. gonorrhoeae* (Bergsson et al., 1999). However, the plant extract has not been tested on *N. gonorrhoeae*.

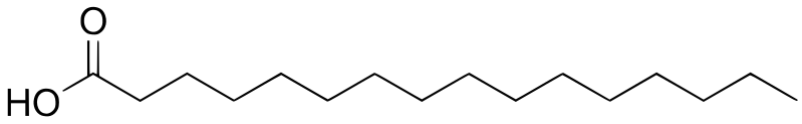

Figure 2.18: Palmitic acid

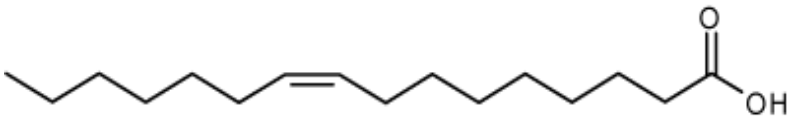

Figure 2.19: Palmitoleic acid

2.5.4. *Rhoicissus digitata*

Rhoicissus digitata (L.f.) Gilg & M.Brandt is part of the Vitaceae plant family and is known as the baboon grape (Figure 2.20) (Watt and Breyer-Brandwijk, 1962). It is a perennial creeper that can spread up to 15 m and can also grow into a loose shrub (van Wyk et al., 2017). It bears fleshy fruit that looks like grapes (Watt and Breyer-Brandwijk, 1962). This medicinal plant grows to have tuberous roots. The natural habitat of the plant includes coastal dunes, forest edges and grasslands (Boon, 2010; Pooley, 1998; Wild and Drummond, 1963). In South Africa, *R. digitata* grows in the Eastern and Western Cape, Mpumalanga as well as KwaZulu-Natal (Arnold and de Wet, 1993; van Wyk et al., 2017).

Figure 2.20: *Rhoicissus digitata* (http://pza.sanbi.org)

In Zimbabwe, the plant is used as a remedy for eye problems (Watt and Breyer-Brandwijk, 1962). In literature, there is little documented on the traditional uses of *R. digitata* compared to the closely related *Rhoicissus tridentata*, which is favoured by traditional healers.

Rhoicissus digitata inhibits the COX-1 enzyme and inhibits prostaglandin synthesis therefore it has anti-inflammatory activity (Lin et al., 1999). Antibacterial activity has been reported against *Alcaligenes faecalis*, *B. cereus*, *Bacillus coagulance*, *Bacillus megaterium*, *Bacillus pumilus*, *Micrococcus luteus*, *Mycobacterium phlei*, *Pseudomonas solanacearum*, *Pseudomonas syringae*, *Shigella boydii*, *S. epidermidis* and *S. aureus* (Lin et al., 1999). In the same preliminary study, the plant inhibited *C. albicans.*

There is little documentation of phytochemicals found in *R. digitata*, but more research has been conducted on *Rhoicissus tomentosa* (Lam.) Wild & R.B.Drumm and *Rhoicissus tridentata.*

2.5.5. *Rhoicissus tridentata*

Rhoicissus tridentata (L.f.) Wild & R.B.Drumm (wild grape) is a member of the Vitaceae family (Katsoulis et al., 2000). It is a tuberous perennial creeper that grows to 3 m high (Urton et al., 1986; van Wyk et al., 2017). This plant bears red-black edible fruit that resembles grapes and berries (Figure 2.21) (Smith, 1966). The leaves appear shiny which makes them different from *R. digitata*. *Rhoicissus tridentata* naturally occurs in sub-Saharan Africa (Urton et al., 1986). In South Africa occurrences of *R. tridentata* have been reported in all provinces except the Western Cape (Urton et al., 1986; van Wyk et al., 2017). It grows in various habitats which include savanna, woodlands, forest and rocky areas (Urton et al., 1986).

Figure 2.21: *Rhoicissus tridentata* (http://pza.sanbi.org)

In South African traditional medicine, the roots and tubers are mostly used for the treatment of ailments. The tubers are used to treat stomach, bladder and kidney disorders, dysmenorrhoea, infertility and to facilitate childbirth (van Wyk et al., 2017). The plant is also used by the Masai as a decoction to treat gonorrhoea (Watt and Breyer-Brandwijk, 1962). Women take the plant as part of isihlambezo and inembe to induce labour (Varga and Veale, 1997).

Rhoicissus tridentata has antifungal activity against *C. albicans* and it also inhibits *N. gonorrhoeae* (Tshikalange et al., 2016). It has antibacterial activity against *A. faecalis*, *B. cereus*, *B. coagulance*, *B. pumilus*, *E. coli*, *Klebsiella oxytoca*, *K. pneumoniae*, *M. luteus*, *Mycobacterium smegmatis*, *P. solanacearum*, *P. syringae*, *Salmonella* sp., *S. aureus* and *S. epidermidis* (Lin et al., 1999; Tshikalange et al., 2016). The plant has anti-inflammatory activity by inhibiting the COX-1 enzyme (Lin et al., 1999; Tshikalange et al., 2016). *Rhoicissus*

tridentata also has antidiabetic effects and antineoplastic activity (tumour formation inhibition) (Mukundi et al., 2015; Opoku et al., 2000). The plant has also inhibitory effects on HIV-1 RT (Mamba et al., 2016).

Catechin (Figure 2.22), a proanthocyanidin, has been isolated from *R. tridentata* as well as some of its derivatives which include gallocatechin, epicatechin and epicatechin-3-O-gallate (ECGC) (Brookes and Katsoulis, 2006). The aforementioned compounds have also been isolated from *P. prullenoides*. Epicatechin-3-O-gallate (Figure 2.23) has been reported to have antigonococcal activity when esterified to make an ECGC-palmitate fatty acid ester (Matsumoto et al., 2012). Sitosterol is another compound that has been isolated from *R. tridentata* (Brookes and Katsoulis, 2006). It is a phytosterol that has a similar structure to that of, the female hormone, estrogen. Sitosterol has exhibited estrogenic activity in uterine muscles during pregnancy (Brookes and Katsoulis, 2006).

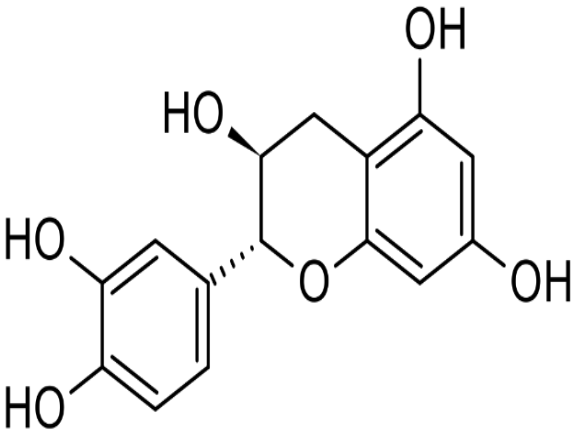

Figure 2.22: Catechin

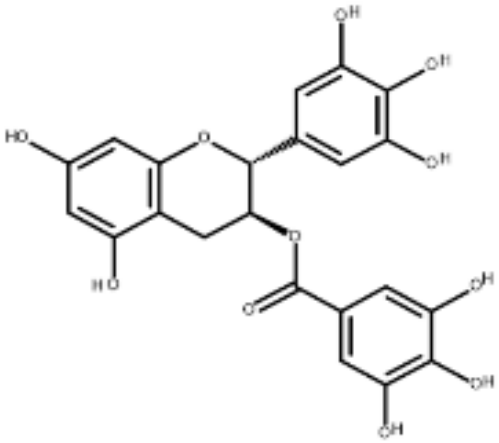

Figure 2.1: Epicatechin-3-O-gallate

2.6. Nanotechnology and vaginal delivery

Vaginal drug administration can be used in the treatment of local STD infections in women (Alexander et al., 2004; Ensign et al., 2014). Typically drugs administered via the vagina are formulated as creams, films, gels and ointments (Krishna et al., 2012; Ndesendo et al., 2008). The major advantage of using this drug delivery system is the increased bioavailability of the drug by avoiding the hepatic first-pass effect (Ensign et al., 2014). Thus, allowing for smaller doses as drugs are not metabolised by the liver. Vaginal delivery results in the avoidance of the harsh pH conditions in the gastrointestinal tract and reduces disturbances to the natural microflora (Ensign et al., 2014; Krishna et al., 2012). Furthermore, the vaginal delivery system allows for direct absorption to the bloodstream resulting in faster action as compared to the oral route (Hussain and Ahsan, 2005; Krishna et al., 2012). With vaginal administration, the drug acts locally which results in high concentrations of the drug at the target site which consequently increase efficacy (Ensign et al., 2014). The major drawback of vaginal delivery is the cervicovaginal mucus (CVM), which is the physical barrier of the vagina (Rossi et al., 2019). The mucus is made out of mucin, which forms a mesh with pores of about 340 nm (Cone, 2009; Rossi et al., 2019). The CVM has a high permeability for small molecules therefore it allows for low weight drugs to pass the epithelial cells of the vagina (Krishna et al., 2012).

In recent years, nanotechnology has been used for the development of nanoparticles used for therapeutic action. Nanoparticles (NP) are small molecules (10–1000 nm) consisting of the active ingredient and a polymer (organic or inorganic) (de Jong and Borm, 2008; Leyva-Gómez et al., 2018). The complex has functions related to treatment, prevention, or diagnosis of diseases. These nanoscale molecules can be absorbed and transported in the body for targeted release at specific sites of action (de Jong and Borm, 2008). The bioactive ingredient may be encapsulated in the polymer or be loaded on the surface. This complex then releases the loaded bioactive ingredient to target cells. For therapeutic action, NPs are usually between 50-600 nm in size (Leyva-Gómez et al., 2018). Nanoparticles less than 340nm can pass the CVM into the vagina making them ideal for vaginal administration (Ensign et al., 2014; Leyva-Gómez et al., 2018; Rossi et al., 2019).

There are several advantages to using NPs as there is increased surface area, enhanced solubility, lower doses and rapid onset of therapeutic action (Leyva-Gómez et al., 2018). The

minute nature of NPs increases the surface to which active ingredients is added. Thus, more of the bioactive molecules can be carried per one NP which decreases the dosage required for treatment. Consequently, NPs can enhance the therapeutic action and increase drug specificity to cells (Ensign et al., 2014; Leyva-Gómez et al., 2018).

Different types of NPs have been formulated for the treatment and prevention of sexually related diseases. Polymeric and metal-based NPs (gold and silver) have been developed.

2.6.1. Polymeric nanoparticles

Polymeric NPs have polymers such as poly (lactic-co-glycolic acid (PLGA) encapsulating the bioactive molecule (Leyva-Gómez et al., 2018). A vaginal film was made with PLGA coated nanoparticles loaded with known reverse transcriptase inhibitors, tenofovir and efavirenz (Cunha-Reis et al., 2016). The hybrid film was formulated for the treatment of HIV. This film did not exhibit any cytotoxic effects on the vaginal mucosa of mice (Cunha-Reis et al., 2016). Ariza-Saenz et al. (2017) developed polymeric NPs for HIV treatment using the HIV-1 inhibitor peptide E2, from the envelope protein of GB virus-C. The E2 peptide is known to inhibit the replication of HIV (Koedel et al., 2011; Mohr and Stapleton, 2009). The E2-loaded NPs can penetrate the vaginal mucosa of swine and could release the E2 to epithelial tissue (Ariza-Saenz et al., 2017). Das Neves and Sarmento (2015) have synthesized PLGA nanoparticles loaded with an antiviral agent, dapivirine, which were taken up by monocytes and genital cells of mice. The dapivirine NPs released the drug to the cells over 24hrs and there was higher drug retention in cells than with the drug alone. Nanoparticles coated with PLGA have also been developed for the treatment of HSV that causes herpes. These NPs are loaded with siRNA complexes against nectin-1 of HSV (Steinbach et al., 2012). Martínez-Pérez et al. (2018) synthesized polymeric PLGA nanoparticles loaded with clotrimazole, which have antifungal activity against *C. albicans.* The NPs increased the antifungal activity of clotrimazole. At a concentration of 10 μg/ml, there was no zone of inhibition in the clotrimazole control while zones of 15 mm were observed in the plates treated with clotrimazole-NPs.

2.6.2. Silver nanoparticles

Silver nanoparticles (AgNPs) have been the most favoured NPs as silver has been reported to increase the antimicrobial activity of the drugs loaded (Aderibigbe, 2017; Choi et al., 2008;

Ensign et al., 2014). The modes of action of silver NPs include inhibition of microbial enzymes, reducing the permeability of cells and cell death (Aderibigbe, 2017).

Antibacterial activity has been reported due to silver nanoparticles. Silver nanoparticles coated with polyvinylpyrrolidone (PVP) have been reported to have good anti-inflammatory effects that decrease the pathogenesis of *Chlamydia trachomantis* (Yilma et al., 2013). In a study by Li et al. (2013), AgNPs were synthesized using cefmetazole a broad-spectrum, second-generation cephalosporin antibiotic against *N. gonorrhoeae*. The cefmetazole-AgNPs inhibited the growth of MDR *N. gonorrhoeae* with a MIC of 12.5 µg/ml. Damelin et al. (2015) synthesized silver saccharinate-benzimidazole nanoparticles that exhibited antigonococcal activity showing 97.7 % inhibition of the bacteria at 10 µg/ml. The same NPs have antiviral activity against HIV-1 and HSV-2 (Damelin et al., 2015).

Silver nanoparticles have shown antiviral activity against HIV and HSV infections (Aderibigbe, 2017; Fayaz et al., 2012; Lara et al., 2010a). Lara et al. (2010b) showed that PVP-coated AgNPs had anti-HIV activity only requiring 0.15mg/min to kill the virus. The mode of action of these NPs is by binding to the gp120 protein of CD4 cells preventing entry of the virus (Elechiguerra et al., 2005). A silver nanoparticle coated condom was developed by Fayaz et al. (2012) as a prophylaxis measure against HIV and HSV infections. This AgNP coated condom prevented pathogenesis of HIV-1, HSV-1 and HSV-2. Baram-Pinto et al. (2009) showed that mercaptoethane sulfonate encapsulated AgNPs prevents the entry of HSV-1 into Vero cells as compared to the drug alone.

Some nanoparticles have been synthesized using plant extracts. Silver NPs have been synthesized with *Moringa oleifera* Lam. and exhibited antifungal activity against *Candida* species including *C. albicans* (Prasad and Elumalai, 2011). *Sargassum wightii* Greville ex J. Agardh (marine algae) AgNPs were found to reduce pathogenesis of HSV-1 & 2 by 70 % at a concentration of 2.5 µl per sample with higher concentrations leading to cytotoxicity (Dhanasezhian et al., 2019). Although AgNPs have good antimicrobial activity against STD pathogens, the major drawback is cytotoxicity to host cells at high concentrations, which is due to the silver (Dhanasezhian et al., 2019; Hu et al., 2014; Li et al., 2013).

2.6.3. Gold nanoparticles

Gold nanoparticles (AuNPs) have free thiols that are easily functionalized to enhance target specificity and better stability as compared to their silver counterparts (Ensign et al., 2014). The modes of action of AuNPs include reduction of ATP synthase activity, interference of metabolic processes and inhibition of microbial cell attachment (Aderibigbe, 2017).

The AuNPs have antimicrobial activity against bacteria and viruses (Aderibigbe, 2017; Bowman et al., 2008; Gu et al., 2003; Huang et al., 2007). Gold nanoparticles have been found to have antimicrobial activity against non-STD pathogens associated with urinary tract infections (Li et al., 2014). Li et al. (2014) found that decane-AuNPs were active against the resistant clinical isolates including *E. coli, Enterobacter cloacae* complex, *P. aeruginosa* and *S. aureus* (Li et al., 2014). Vancomycin-AuNPs have been reported to have antibacterial activity against resistant strains of *E. coli*, *E. faecalis, P. aeruginosa* and *S. aureus* (Gu et al., 2003; Huang et al., 2007).

Gold nanoparticles have been developed to increase the efficacy of HIV drugs. Nanoparticles loaded with azidothymidine, a reverse transcriptase inhibitor, that is conjugated with cysteine are active against HIV-1 (Kesarkar, 2015). Similarly, raltegravir, abacavir and lamivudine conjugated AuNPs have anti-HIV activity (Chiodo et al., 2014; Garrido et al., 2015). Multivalent AuNPs can be made due to the available thiol groups on the surface of the AuNPs enabling the NPs to have various ligands loaded increasing efficacy. Di Gianvincenzo et al. (2010) developed AuNPs that were coated with various sulfur ligands that bound to the gp120 protein thus inhibiting infection by HIV-1. Similarly, Bowman et al. (2008) synthesized AuNPs coated with multiple ligands of SDC- 1721 (HIV fusion inhibitor), which exhibited antiviral activity. Multivalent AuNPs capped with mercaptoethane sulfonate were seen to interfere with viral attachment and entry of HSV-1 (Baram-Pinto et al., 2010). Sarid et al. (2012) formulated an antiviral agent against HSV-1 preventing herpes. The active ingredient of the formulation consisted of sulfonate coated AuNPs.

Plant extracts have also been used in the synthesis of AuNPs (Patra et al., 2016). *Sargassum wightii* (brown algae) AuNPs were found to inhibit the cytopathic effects of HSV-1 & 2 by 70 % at concentrations of 2.5 µl and 10 µl per sample with no cytotoxic effects observed (Dhanasezhian et al., 2019).

Diagnostic methods have been developed using AuNPs for the detection of *C. albicans* and *G. vaginalis* (which causes bacterial vaginosis) (Hashemi et al., 2019). This diagnostic method has antibodies specific to the antigens of the microbes which are carried in gold NPs. These nanoparticles had high specificity and sensitivity when detecting vaginal infections.

2.6.4. Formulations with nanoparticles for treatment of STDs

There are a few nanoparticle products that have been formulated for the treatment of STDs. Viva Gel (SPL7013), developed by Starpharma Pty. Ltd, is a NP-containing vaginal gel that targets HIV and HSV attachment (Cojocaru et al., 2020; Rupp et al., 2007). The vaginal gel has been tested in animal models and is in phase I of clinical trials (Price et al., 2011). The same gel has undergone phase III clinical trials for the treatment of bacterial vaginosis and is available on the market in the United Kingdom, Asia and Australia (ClinicalTrials.gov, 2019; Starpharma, 2020). Some formulations that are not for vaginal delivery have been developed. Derma Vir, a nano-vaccine, has been developed as an immunostimulatory product for HIV patients. The vaccine contains plasmid DNA with the ability to express 15 HIV antigens. Derma Vir stimulates dendritic cells to induce B lymphocytes to produce HIV-specific memory cells (Rodriguez et al., 2013). The vaccine underwent phase I of clinical trials and was seen to be relatively safe (Price et al., 2011; Rodriguez et al., 2013). Doravirine (MK-1439) is another nano-based drug formulated by Merck Sharp & Dohme Corp. It is a tablet containing an HIV inhibitor that went through phase I clinical trials showing safety (ClinicalTrials.gov, 2015; Singh et al., 2017).

2.7. Conclusion

The increasing occurrence of multi-drug resistant and extensive drug-resistant *N. gonorrhoeae* has made it imperative to develop new therapeutics for the treatment of gonorrhoea. This brings plants into the limelight as sources for drug discovery and development. The plants selected for this study are of great interest based on their traditional uses in South African tradition for the treatment of STDs, and other ailments. The pharmacological and biological activity noted from literature also makes these plants ideal for further study in the quest to potentially produce a novel therapeutic for gonorrhoea. Novel bioactive compounds can also be isolated to develop formulations for better management of the disease. Furthermore, polyherbal therapeutics can

be enhanced by the use of nanotechnology for improved drug delivery via the vagina in women, as women are most prone to gonorrhoea. There is also evidence for the potential of formulating nano-based herbal products for the treatment of STDs.

Chapter 3: Antigonococcal, synergistic and cytotoxic activity of ethanolic extracts from selected South African indigenous plants

Abstract

Gonorrhoea, caused by *Neisseria gonorrhoeae,* accounts for most of the bacterial sexually transmitted diseases (STDs), second to chlamydia. It is most prevalent in Africa with approximately 78 million cases recorded yearly worldwide. This high incidence and the increase of antibiotic resistance make finding novel therapies for treatment important. The study aimed to determine the antigonococcal capabilities of selected ethanolic plant extracts, their synergistic and cytotoxic effects. The microdilution assay showed that three of the root extracts, *Gnidia kraussiana* Meisn, *Gunnera perpensa* L. and *Pentanisia prunelloides* (Klotzsch) Walp exhibited good activity (minimum inhibitory concentration (MIC) <390 µg/ml) against *N. gonorrhoeae*. The best antigonococcal activity was seen in *G. perpensa* (MIC=48.7 µg/ml). Synergistic testing using the five selected plants revealed that *G.kraussiana* and *P. prunelloides* exhibited additive effects (0.55<FIC<0.95) against the bacterium. All other combinations of the plants had neither antagonistic nor additive effects on *N. gonorrhoeae.* Cytotoxicity testing revealed that the most bioactive plants were not cytotoxic to HeLa and HaCaT cell lines (IC_{50} >400 µg/ml) however, *G. kraussiana* showed moderate cytotoxicity to HeLa cells (IC_{50}=194.1±6.2 µg/ml). *Gunnera perpensa* was further tested on THP-1 cells and was found to be non-cytotoxic to selected cell lines (IC_{50} >400 µg/ml). The results revealed that the ethanolic extract of *G. perpensa* was the most bioactive against *N. gonorrhoeae* and had limited cytotoxic effects. Thus, the plant extract may undergo further investigation to develop new therapies for the treatment of gonorrhoea.

3.1. Introduction

Gonorrhoea is a sexually transmitted disease (STD) plaguing the world's population, recording 78 million cases yearly (World Health Organization (WHO) Regional Office for Africa, 2018). There is a high incidence of the disease in the African region (WHO Regional Office for Africa,

2018). In 2016, it was estimated there were ~22 million incidences of gonorrhoea on the continent (Rowley et al., 2019). In South Africa, gonorrhoea is the most prevalent bacterial STD, second to chlamydia, with 4.5 million active cases reported in 2017 (Kularatne et al., 2018b). Gonorrhoea is caused by *Neisseria gonorrhoeae*, a Gram-negative, diplococcus bacterium that thrives in anoxic conditions affecting mostly the genital mucosal membranes of humans (Quillin and Seifert, 2018; Shim, 2011). The bacteria causes genital lesions and dysuria in both females and males (Achakazai et al., 2017; Centers for Disease Control and Prevention, 2021).

Neisseria gonorrhoeae is highly infectious with a heightened likelihood of re-infection due to its ability to evade the host immune system (Murphy et al., 1989; Sadarangani et al., 2011; Yu et al., 2013). The persistence and high infection rates put pressure on the first-line drugs used for the treatment of gonorrhoea, resulting in the emergence of multi-drug resistant (MDR) and extensive drug-resistant strains (XDR) of *N. gonorrhoeae* (Alirol et al., 2017; Crowther-Gibson et al., 2011; Martin et al., 2019). Multi-drug resistant bacterial strains are defined as having resistance to at least one of the current recommended therapies and resistance to no less than two old antibiotic treatments: penicillin, tetracycline, erythromycin or ciprofloxacin (Martin et al., 2019). Strains with resistance to at least two of the current recommended therapies and resistance to no less than two of the old antibiotic treatments, are categorized as XDR strains. The upsurge of antibiotic resistance of gonococcal strains reduces the efficiency of the treatments, increasing the persistence of the disease.

The antibiotic resistance predicament intensifies the necessity of finding alternative novel therapeutics for use against gonorrhoea. Bioprospecting provides new avenues for drug discovery for treatment against the disease. Plants can be used to create therapeutics for the treatment of gonococcal infections including drug-resistant gonorrhoea. Why plants? Ethnobotany has shown that plants are used in traditional medicine globally for the treatment of STDs and other diseases (Chinsembu, 2016; de Wet et al., 2012; Mongalo et al., 2017; Palmeira-de-Oliveira et al., 2013; Yang et al., 2012). In addition, literature shows that plants have great variety in chemistry and can thus be used to develop novel therapeutics to kerb gonorrhoea and other STDs (Palmeira-de-Oliveira et al., 2013). *In vitro* studies have also indicated that plants do have antimicrobial activity including *N. gonorrhoeae* (Buwa and van Staden, 2006; Kambizi and Afolayan, 2008; Mabona et al., 2013; Mamba et al., 2016; Ndhlala et al., 2011a; Tshikalange et al., 2016).

In South Africa, traditional medicine is used in parallel to western medicine for the treatment of STDs, including gonorrhoea (Mothibe and Sibanda, 2019; Nchinda, 1976). Plants such as *Hypoxis hemerocallidea* Fisch., C.A.Mey. & Avé-Lall, *Ximenia caffra* Sond, *Ziziphus mucronate* Wild and *Tabernaemontana elegans* Stapf have been prescribed by traditional healers to treat gonorrhoea (Chinsembu, 2016; de Wet et al., 2012; Semenya et al., 2013). This study was aimed at evaluating the antibacterial and synergistic potential of selected plants against *N. gonorrhoeae.* The roots of *Gnidia kraussiana* Meisn, *Gunnera perpensa* L, *Pentanisia prunelloides* (Klotzsch) Walp, *Rhoicissus digitata* (L.f.) Gilg & M.Brandt and *Rhoicissus tridentata* (L.f.) Wild & R.B.Drumm were used in this study. *Gunnera perpensa and R. tridentata* roots have been reported to be used traditionally for the treatment of gonorrhoea (Maroyi, 2016; Watt and Breyer-Brandwijk, 1962). On the other hand, roots of *P. prunelloides* and *R. digitata* have been reported as non-specific treatments for STDs (Brookes and Dutton, 2007; Bryant, 1966; Kose et al., 2015; van Wyk et al., 2017; Watt and Breyer-Brandwijk, 1962). The herbal prescriptions are prepared as decoctions, which are then taken orally (Maroyi, 2016; van Wyk et al., 2017). There are no reports on STD treatments using *R. digitata*. however, it is used by Zimbabweans as an ophthalmic remedy (Watt and Breyer-Brandwijk, 1962). The five plants were selected for the study based on the antimicrobial activity and traditional medicinal uses reported in literature. In addition, these plants form part of the prescribed treatment of STDs by traditional healer (TDr) Lulama Masinga, from Hambanati village, Tongaat, KwaZulu-Natal, South Africa.

This study aimed to evaluate the antigonococcal testing activity of the five selected plants using the microdilution assay. Moreover, the synergistic effects of the plants on *N. gonorrhoeae* were investigated. Subsequently, cytotoxicity effects of the most bioactive plants, on cervical cancer cells (HeLa) and immortalized human keratinocyte cells (HaCaT), were evaluated. Additionally, cytotoxicity testing was performed on immortalized human monocyte (THP-1) cells on the lead plant. The THP-1 cell line was used on the lead plant, as it could be used in future cytokine studies to determine the effects of the plant on the human immune system and *N. gonorrhoeae*. This would be important as the bacterium is known to result in overzealous inflammation by upregulating cytokines such as interleukin-10 (IL-10) and tumour necrosis factor (TNF), which encourages persistence of the disease (Liu et al., 2014). The cytotoxicity testing was conducted to ensure the safety of the plant extracts which could be used to formulate a topical vaginal ointment for the treatment of gonorrhoea.

3.2. Materials and methods

3.2.1. Plant material

Shade dried root material of the five plants: *Gnidia kraussiana*, *Gunnera perpensa*, *Pentanisia prunelloides*, *Rhoicissus digitata* and *Rhoicissus tridentata* was obtained from Muthi Futhi nursery situated in the Edakeni Reserve, Uthungulu district, KwaZulu-Natal, South Africa (Appendix A1).

3.2.2. Preparation of extracts

The plant material (400g) was ground using a laboratory grinder (Janke & Kunkel, MF 10, Germany). Thereafter, ethanol (w/v of 1:5) was used for the extraction process. This solvent was selected as it has both polar and non-polar components that are capable of extracting polar and non-polar secondary metabolites. Each plant extract was shaken on a Labcon 3086 U shaker (South Africa) for 72 h at 140 rpm. The plant extracts were filtered under vacuum using a Büchner funnel with Whatman No. 1 filter paper. A vacuum rotary evaporator (Heidolph, Hei-Vap value digital HB/G3B, Germany) was used to remove excess solvent thus resulting in a concentrated extract which was then air-dried in a fume hood (Appendix A1). The dried plant extracts were then stored in a fridge at 4 ˚C until further use.

3.2.3. Microorganism

Primary cultures of *N. gonorrhoeae* (ATCC 19424, ThermoFisher Scientific, South Africa) were plated on GC chocolate agar (ThermoFisher, South Africa) at 37 ˚C in 5 % CO_2 (CO_2 Gen ThermoFisher Scientific, South Africa) for 48 h. Thereafter, aliquots were obtained and then maintained in Mueller-Hinton (MH) broth in Eppendorf tubes and stored at -80 ºC. Before the bioassays, inoculum suspensions were made using MH broth and incubated for 24 h at 37 ˚C in 5 % CO_2. To determine the amount of inoculum used for susceptibility testing the bacterial suspensions were spectrophotometrically standardized to 0.5 McFarland thereafter adjusted to 1.5 x10^5 CFU/ml (Cos et al., 2006; Tshikalange et al., 2016).

3.2.4. Antigonococcal assay: Microdilution method

The microdilution method was used to determine the minimum inhibition concentrations (MICs) of the plant extracts against *N. gonorrhoeae* (Eloff, 1998). The assay was conducted in a 96-well plate making it easier to test multiple samples at a time (Appendix A2). In all wells of the plate, 100 µl of Mueller-Hinton (MH) broth that supports the growth of *N. gonorrhoeae* was added. Plant extracts were re-dissolved in 10 % dimethyl sulfoxide (DMSO) to make a

final concentration of 12000 µg/ml. One hundred microlitres of the plant extracts were added in triplicate to the wells of the first row of the plate followed by two-fold serial dilutions to make a concentration range from 3000-23.4 µg/ml. Ciprofloxacin with a concentration range of 625-2.44 µg/ml was used as the positive control whilst 10 % DMSO and sterile MH broth as the negative controls. Additionally, a control with just the MH broth and the inoculum was included in the assay to ensure that *N. gonorrhoeae* was growing independently. After serial dilutions were done, 100 µl of the *N. gonorrhoeae* suspension was added to all wells with the plant extracts, 10 % DMSO control and the positive control. The plates were incubated for 24 h at 37 ˚C in 5 % CO_2. The MIC for each plant extract was determined by the addition of an indicator dye, PrestoBlue, to visualize the microbial inhibition (Lall et al., 2013). The dye changes from blue to pink to indicate microbial growth while the blue colour shows the bioactivity of the plant extract against *N. gonorrhoeae*. The MICs of the plant extracts were determined visually. Plant extracts that had good activity (MICs <390 µg/ml) were further tested for synergistic effects on *N. gonorrhoeae.* The entire assay was repeated three times.

3.2.5. Synergistic assay: Dilution method

The three most bioactive plant extracts (MICs <390 µg/ml) were tested to determine the synergistic (or antagonistic) effects of the plant extract combinations on *N. gonorrhoeae*. The dilution method was used as described by de Rapper et al. (2012). Plant mixtures of two plant extracts, both with a concentration of 6000 µg/ml, with varying ratios 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 were made. In a 96-well plate, 100 µl of MH broth was added to all wells and thereafter 100 µl of the plant mixture (ratio 9:1) was added to the first well of the plate. Subsequently, 100 µl of mixture ratios of 8:2, 7:3…. and 1:9 were used in the consecutive wells of the first row. Two-fold serial dilutions were conducted after which 100 µl of the inoculum was added to all wells (Appendix A3). Ciprofloxacin (625-2.44 µg/ml) was used as the positive control whilst sterile MH broth, 10 % DMSO and a combination of pathogen and MH broth were the negative controls. The plates were incubated for 24 h at 37 ˚C in 5 % CO_2. PrestoBlue was used to visually determine the MICs of the plant combinations. The experiment was carried out for all dual combinations of the three most bioactive plant extracts. Additionally, an assay was performed with all three bioactive plant extracts together in a ratio of 1:1:1. A further assay was conducted with all five selected plants at a ratio of 1:1:1:1:1. This was done as the plants are sometimes used traditionally together as a treatment. In each case, entire assays were repeated three times. For all synergistic assays, the fractional inhibitory concentration (FIC)

was calculated. The formula below was used to determine the FIC of the plant combinations (Mabona et al., 2013; van Vuuren and Viljoen, 2011, 2008).

- $FIC = \frac{MIC\ of\ plant\ extract\ \ A\ in\ combination}{MIC\ plant\ extract\ A\ only} + \frac{MIC\ of\ plant\ extract\ B\ in\ combination}{MIC\ plant\ extract\ \ B\ only}$

The resultant FIC values were defined as follows: FIC <0.5 (synergistic), 0.5 <FIC <1 (additive), 1 <FIC <4 (indifferent) and FIC >4 (antagonistic).

3.2.6. Cytotoxicity assay

Cytotoxicity testing was carried out using the three most bioactive ethanolic root extracts on cervical cancer cells (HeLa) and immortalized human keratinocytes (HaCaT) using the three bioactive plant extracts (Chen et al., 2013; Li et al., 2018; Seo et al., 2012; Wilson, 2013). Immortalized human monocytes (THP-1) were also used for testing the plant that exhibited superior antigonococcal activity of all plant extracts used (Bosshart and Heinzelmann, 2016; Chanput et al., 2014). All cytotoxicity testing followed the method as described by Lall et al. (2013). The THP-1 and HaCaT cell lines were donated by the Department of Human Biology, University of Cape Town (Cape Town, South Africa). The HeLa cell line was provided by the Department of Biochemistry, Genetics and Microbiology, University of Pretoria (Pretoria, South Africa).

The HeLa cells were grown in Gibco Minimum Essential Media (MEM) supplemented with a 1 % antibiotics mixture (amphotericin, penicillin and streptomycin) and 10 % foetal bovine serum. The cells were grown in a humidified incubator (ThermoFisher, Forma^{TM} 310, USA) at 37 ˚C and in 5 % CO_2. The cells (10000 cells /well) were seeded in the wells of a 96-well plate in MEM media. Stock solutions of 20 000 µg/ml in 100 % DMSO of the plant extracts were prepared. In a separate 24-well plate serial dilutions were carried out thereafter 100 µl of the diluted plant extracts were added, in triplicate, to the seeded 96-well plate. The test concentration range used was from 400-3.125 µg/ml. Actinomycin D was used as the positive control, with test concentrations ranging from and 0.5-3.91 × 10^{-3} µg/ml. Three negative controls were used; 2 % DMSO, media with cells and media with no cells embedded in wells. Subsequently, the plates were incubated in a humidified incubator at 37 ˚C and 5 % CO_2 for 72 h and thereafter PrestoBlue was added and further incubated for 1 h to determine the viability of the HeLa cells. For the HaCaT and THP-1 cell lines, the abovementioned steps were used. However, the HaCaT cells were cultured in Dulbecco's Modified Eagle Media

(DMEM) and the THP-1 cells were grown in Roswell Park Memorial Institute Medium (RPMI 1640) media. The cytotoxic effects were determined by measuring the fluorescence with an excitation/emission of 560 nm and 590 nm, respectively, using the Victor Nivo multimode plate reader (Perkin Elmer, USA). GraphPad Prism 7 (Version 7.04) software was used to analyse the data and obtain IC_{50} values. The IC_{50} value is defined as the concentration at which 50 % of the cells are killed. For all cell lines used cytotoxicity effects were defined as follows: IC_{50} <30 µg/ml (very cytotoxic), 30 µg/ml <IC_{50} <50 µg/ml (moderate cytotoxicity), 50 µg/ml <IC_{50} <200 µg/ml (low cytotoxicity) and IC_{50} >200 µg/ml (no cytotoxicity effect) (Kuete and Efferth, 2015; Steenkamp and Gouws, 2006; Suffiness and Pezzuto, 1990). The entire experiment was repeated three times.

3.3. Results and discussion

3.3.1. Antigonococcal activity

In the antigonococcal assay, ethanolic extracts of *G. kraussiana* (GK), *G. perpensa* (GP) and *P. prunelloides* (PP) showed good activity (<390 µg/ml) (Table 3.1). In bacterial susceptibility studies, agents with MICs <1000 µg/ml have significant antimicrobial activity (Ndhlala et al., 2013; van Vuuren, 2008). The best MIC was seen in *G. perpensa* extract (48.7 µg/ml) followed by *G. kraussiana* and *P. prunelloides* with MICs of 97.5 µg/ml and 195 µg/ml, respectively. Both *Rhoicissus* spp (MIC=780 µg/ml) were the least active plant extracts against *N. gonorrhoeae*. Even though *G. perpensa* was the most bioactive plant extract it had a higher MIC (lower activity) when compared to the ciprofloxacin positive control (MIC <5 µg/ml). Mamba et al. (2016) have reported a MIC <10 µg/ml for ciprofloxacin on the *N. gonorrhoeae* (ATCC 19424) strain. Ciprofloxacin has been reported to exhibit MICs <0.06 µg/ml on some clinical isolates (Melendez et al., 2018).

The antigonococcal activity of *G. kraussiana*, *G. perpensa*, *P. prunelloides* and *R. digitata* has not previously been assessed. However, the four plants have shown antibacterial and antifungal properties against other non-STD microbes (Buwa and van Staden, 2006; Lin et al., 1999; Mabona et al., 2013; Mabona and van Vuuren, 2013; Ndhlala et al., 2011a; Nkomo and Kambizi, 2009; Samie et al., 2010). *Gunnera perpensa* has shown antimicrobial activity against Gram-negative and Gram-positive bacteria (Buwa and van Staden, 2006; Mabona et al., 2013; Nkomo and Kambizi, 2009). Ndhlala et al. (2011a) reported that ethanolic root extracts of

G. perpensa showed the same antibacterial activity against *Bacillus subtilis, Escherichia coli,* and *Klebsiella pneumonia* with MICs of 390 µg/ml. Furthermore, it was reported that the ethanolic root extracts had a MIC of 195 µg/ml against *Staphylococcus aureus.* When the antibacterial activity of the abovementioned findings are compared to the findings in this study, *G. perpensa* had higher antibacterial activity against *N. gonorrhoeae* (MIC=48.7 µg/ml). All the selected plants, in this study, have shown bioactivity against *Candida albicans*, an opportunistic STD pathogen (Lin et al., 1999; Mabona et al., 2013; Mamba et al., 2016; Ndhlala et al., 2011a). The ethanolic root extract of *R. tridentata* has been reported to possess antigonococcal properties with a MIC of 400 µg/ml, which can be compared to the MIC of 780 µg/ml in this study (Mamba et al., 2016). Tshikalange et al. (2016) also reported a MIC of 6300 µg/ml for *R. tridentata* ethanolic root extract, which is higher than that of 780 µg/ml observed in this study. There is variation in the biological activity in the above-mentioned studies even though ethanol was used in the plant extraction process The differences seen could be due to seasonal variations, environmental factors (e.g. drought and flooding), developmental stage of plant or locality of the plant (Dey et al., 2017; Gololo et al., 2019; Refifa et al., 2015; Sampaio et al., 2016; Yang et al., 2018; Zribi et al., 2014).

Other South African medicinal plants such as *Aloe ferox* Mill, *Cassia abbreviata* Oliv, *Hypoxis hemerocallidea* Fisch., C.A.Mey. & Avé-Lall, *Terminalia sericea* Burch. ex DC and *Tabernaemontana elegans* Stapf have been reported to possess antigonococcal activity (Chauke et al., 2016; Kambizi and Afolayan, 2008; Mamba et al., 2016; Naidoo et al., 2013; van Vuuren and Naidoo, 2010). Chauke et al. (2016) reported that ethanolic extracts of *C. abbreviata* had a MIC of 46.88 µg/ml which was similar to the antigonococcal activity seen in *G. perpensa* (MIC=48.7 µg/ml). The similar bioactivities observed in these two plants could be because they have similar chemical constituents, as the same solvent was used in the extraction process (Maroyi, 2016; Mongalo and Mafoko, 2013). Dichloromethane: methanol (DCM:MeOH) extracts of *T. sericea* and *T. elegans* have exhibited MICs of >1000 µg/ml (Naidoo et al., 2013; van Vuuren and Naidoo, 2010). These extracts exhibited inferior antigonococcal activity compared to all plant extracts used in this study. Polar extracts of *A. ferox* (methanol) and *H. hemerocallidea* (water) have been reported to both have MICs of 500 µg/ml which was better than the bioactivity seen in the *Rhoicissus* spp. (Kambizi and Afolayan, 2008; Naidoo et al., 2013). However, the antigonococcal activity of these polar extracts was inferior to that of the three most bioactive extracts in this study. From literature, one can conclude that polar extracts have better activity than extracts containing non-polar

solvents such as DCM. The results in this study show that *G. perpensa* possesses good bioactivity against *N. gonorrhoeae* and can be further explored for the development of therapies to treat gonococcal infections.

Table 3.2: Antigonococcal activity of the five selected ethanolic plant extracts

Sample	MIC (µg/ml)[a]
Gnidia kraussiana	97.5[†]
Gunnera perpensa	48.7[†]
Pentanisia prunelloides	195[†]
Rhoicissus digitata	780
Rhoicissus tridentata	780
Ciprofloxacin[b]	<5

[a] Minimum inhibitory concentration, [b] Positive drug control, [†] Ethanolic plant extracts with MIC < 390 µg/ml defined as most bioactive

3.3.2. Synergistic activity

The synergistic effects were evaluated as plants are normally used in combination by traditional healers to treat diseases (Ndhlala et al., 2011b; Varga and Veale, 1997). Testing of the five selected plants using the dilution method showed that all plant combinations exhibited neither synergistic nor antagonistic effects except for the *G. kraussiana: P. prunelloides* (GK:PP) combination that had additive effects (FIC range of 0.55-0.95). Plant combinations with an indifferent relationship had FIC values ranging from 1.15-2.50 whilst the GK:PP combination had a FIC range of 0.55-0.95. The additive relationship between *P. prunelloides* and *G. kraussiana* could have been due to some compounds in both the extracts having some slight synergy against *N. gonorrhoeae.* In the experiment where all five plant extracts were combined in a ratio of 1:1:1:1:1, the FIC index was 1.42 showing that the samples had an indifferent relationship. The same relationship was noted in the 1:1:1 combination of the three most bioactive plants with a FIC index of 1.16. Indifference was seen in the GP:GK and GP:PP combinations as *P. prunelloides* (MIC=97.5 µg/ml) and *G. kraussiana* (MIC=195 µg/ml) had lower antigonococcal activity than *G. perpensa* (MIC=48.7 µg/ml). Additionally, there might be slight antagonism in the compounds present in the plant extracts but not enough to have negative antagonistic effects.

Mabona et al. (2013) conducted synergistic studies and revealed that the aqueous extract of *P. prunelloides* (root) mixed with *Dicoma anomala* Sond (tuber) exhibited some synergy

(FIC=0.31) against *C. albicans*. On the other hand, the same combination using DCM:MeOH extracts had indifferent effects (FIC=1.13) against the same pathogen. In the same study, aqueous extracts of *P. prunelloides* (root) and *Elephatorrhiza elephantina* (Burch.) Skeels (root) showed additive effects (FIC=1) against *C. albicans* There are no synergistic studies documented on the other plants in this study therefore there is a need for more research to include synergistic studies to better understand the plants' bioactivity.

Based on the results from this study, mixtures containing GP did not have improved activity due to the significant difference in antigonococcal activities. The GK:PP combination had additive effects as the combination of the two had a significant effect than the sum of their separate effects. Although, traditional healers use decocted plant mixtures to treat gonorrhoea, the indifferent effects seen in GP mixtures show that some plants have better activity when used individually. Consequently, it becomes important to test herbal prescriptions used in traditional medicine, which would help develop treatments for diseases.

3.3.3. Cytotoxicity activity

The cytotoxicity results of the three most bioactive plant extracts *G. kraussiana*, *G. perpensa* and *P. prunelloides* (GK, GP and PP) on Hela and HaCaT are shown in Table 3.2. Plant extracts were defined as cytotoxic if they had IC_{50} values <200 µg/ml. *Gunnera perpensa* and *P. prunelloides* were non-cytotoxic with IC_{50} values above the highest test concentration (400 µg/ml) used against HeLa and HaCaT cells. Furthermore, *G. perpensa* was not found to be cytotoxic to THP-1 cells (IC_{50} >400 µg/ml). *Gnidia kraussiana* had IC_{50} values of 194.1±6.2 µg/ml against HeLa and >400 µg/ml against HaCaT cells, respectively. Thus, the plant was not cytotoxic to HaCaT cells but had low cytotoxicity to HeLa cells. All plant extracts had IC_{50} values greater than the actinomycin D positive control in all cell lines. Actinomycin D recorded IC_{50}s of 0.0021±0.00055 µg/ml, 0.0019±0.00046 µg/ml and 0.084±0.0068 µg/ml against HeLa, HaCaT and THP-1 cells, respectively. The extracts were thus deemed relatively safe to cells compared to the positive control.

According to literature, the three most bioactive plants in this study have been tested for their cytotoxic effects. *Gnidia kraussiana* was reported to cause death in people after ingestion (Watt and Breyer-Brandwijk, 1962). Moreover, *in vitro* studies have shown that the plant has some cytotoxic effects. The methanolic root extract of *G. kraussiana* was tested on HeLa cells and it was reported that 100 µg/ml of the plant resulted in 75-100 % inhibition of the cells (Kamuhabwa et al., 2000). Thus, the IC_{50} was <100 µg/ml when compared to this study the

IC_{50} of 194.1 µg/ml. The variation in the IC_{50} values could be due to different compounds extracted by methanol and ethanol, respectively. There are no studies on HeLa and HaCaT studies on *G. perpensa* and *P. prunelloides* however there have been some cytotoxicity studies on other cell lines. *Pentanisia prunelloides* has been tested for cytotoxic effects on VK (endocervical) cells whereby ethanolic and ethyl acetate root extracts had no effects at concentrations below 125 µg/ml whilst cytotoxic effects were observed at concentrations greater than 250 µg/ml (Yff et al., 2002). *In vivo* studies on mice were conducted whereby varying concentrations of aqueous and ethanolic rhizome and leaf extracts of PP were administered orally. From this study, no mortality or acute cytotoxic effects were observed at doses of up to 5000 mg/kg. The lethal dose at which 50 % of the population of animal models dies (LD_{50}) was then determined to be >5000 mg/kg. This showed that *P. prunelloides* was not toxic when taken orally (Miya et al., 2016). Several studies have shown that *G. perpensa* does not exhibit cytotoxic effects. Mfengwana et al. (2019) reported that the methanol, DCM and aqueous root extracts of the plant were not toxic to RAW 264.7 cells (murine macrophage cells) with IC_{50} values >200 µg/ml. Additionally, root methanol *G. perpensa* extracts were not cytotoxic to human fibroblast (MRC5) cells since the IC_{50} was >1000 µg/ml (Steenkamp et al., 2004). Brookes and Smith (2003) showed that the IC_{50} of GP on Vero (monkey kidney epithelial) cells was >500 µg/ml. Furthermore, Mwale and Masika (2011) conducted an *in vivo* toxicity assay in Wistar rats which were dosed with *G. perpensa* aqueous leaf extracts. It was seen that the extracts did not cause mortality in rats when a dose of 400 mg/kg was used for 3 days. This also shows that the GP is relatively safe to use.

Based on the findings of this study and further supported by reports from literature, *G. perpensa* and *P. prunelloides* are eligible for use in formulations to be used for the treatment of gonorrhoea. In this study, *G. kraussiana* showed no toxic effects on the HaCaT cells but low cytotoxic effects on HeLa cells. The plant can be further investigated for cancer research with the possibility of isolation of pure compounds to treat cancers including cervical cancer.

Table 3.2: Cytotoxicity activity of the three most bioactive ethanolic plant extracts on cervical cancer cells, human keratinocytes and human monocytes

Sample	**IC_{50}[a]±SD[b] (µg/ml)**		
	HeLa[c]	HaCaT[d]	THP-1[e]
Gnidia kraussiana	194.1±6.2	>400	-
Gunnera perpensa	>400	>400	>400
Pentanisia prunelloides	>400	>400	-
Actinomycin D (Positive drug control)	$2.1\times10^{-3}\pm5.5\times10^{-4}$	$1.9\times10^{-3}\pm4.6\times10^{-4}$	$8.4\times10^{-2}\pm6.8\times10^{-3}$

[a]Fifty percent inhibitory concentration, [b]Standard deviation, [c] Human cervical cancer cells, [d] Human keratinocytes, [e] Human monocytes, - not tested

3.4. Conclusion

In this study, all ethanolic plant extracts exhibited some antigonococcal activity against *N. gonorrhoeae.* However, *G. perpensa* exhibited the best activity against the bacteria. There were neither synergistic nor antagonistic effects observed in mixtures containing GP however, additive effects were observed in the GK:PP combination. This showed that some plants have improved bioactivity in combination whilst others have better activity when used individually. Hence, this encourages more studies to be conducted on herbal mixtures. Plants used for drug development must ideally not have any cytotoxic effects to ensure safety for patients. This study confirmed that *G. perpensa* was non-cytotoxic to the HeLa, HaCaT and THP-1 cells thus, it can be further explored for development as a novel therapy for gonorrhoea treatment. With the results obtained bioactive compounds from *G. perpensa* can be isolated to determine whether a single component or the whole extract is responsible for the pharmacological activity. In future, the mode of action of the plant on *N. gonorrhoeae* and the immune system may be evaluated to decrease the persistence of gonorrhoea. *Gunnera perpensa* can be further explored for drug development as it is widely distributed in South Africa and is not on the International Union Conservation of Nature (IUCN) Red List. Moreover, further research may be conducted on the antigonococcal properties of the leaves of *G. perpensa*, as leaves are more

sustainable than roots. For more environmentally friendly production of *G. perpensa* cultivation may be considered for plant material supply. Additionally, bioactive compounds identified from the plant may be biosynthesized using molecular techniques such as biopharming. The superior antigonococcal properties of *G. perpensa*, from this study, make it a more suitable candidate to be a lead plant for further drug development studies for gonorrhoea.

Chapter 4: Isolation and characterization of bioactive fractions from *Gunnera perpensa*

Abstract

Antibiotic resistance places a heavy burden on available first-line drugs used to treat gonorrhoea. This increases the need to find alternative treatments for the disease. Plants are potential sources for developing new therapies as they have a variety of secondary compounds. For drug formulations, it is imperative to identify and characterize the known and unknown constituents of these plants to ensure the safety of these novel therapies. In this study, liquid-liquid partition chromatography was used to obtain fractions from the *Gunnera perpensa* (GP) ethanolic crude extract, which has shown promising activity against *Neisseria gonorrhoeae*. Hexane, ethyl acetate (EtOAc), butan-1-ol and water were used to separate the compounds in the plant extract and to further test the antigonococcal activity of the fractions obtained. Evaluation of cytotoxic effects was conducted on HeLa (cervical cancer), HaCaT (human keratinocytes) and THP-1 (human monocytes) cells. Subsequently, characterization of the two most bioactive fractions was performed using proton nuclear magnetic resonance (^{1}H-NMR), Fourier-transform infrared spectroscopy (FTIR) and electrospray ionization-liquid chromatography-mass spectrometry (ESI-LCMS). Results showed that the polar fractions (butan-1-ol and water) had the best antigonococcal activity, both with minimum inhibitory concentrations (MICs) of 23.4 µg/ml compared to the ethanolic crude extract of GP (MIC=48.7 µg/ml). Both bioactive fractions were not cytotoxic to all cell lines tested (IC_{50} >300 µg/ml). Characterization of the water and butan-1-ol bioactive fractions showed that the two fractions contain Z-venusol, 4-*O*-β-D-glucopyranosyl-3,3'-tri-*O*-methylellagic acid (ellagic acid derivative), caffeic acid, 4-*O*-beta-D-glucosyl-trans-caffeate, ferulic acid glucoside and simple benzoquinones. The presence of these compounds and the superior antigonococcal activity of the fractions from GP, shows that the fractions have the potential to be further investigated for use to treat gonorrhoea.

Introduction

In recent years, the rampant increase in multi-drug resistant (MDR) and the emergence of extensively drug-resistant (XDR) *Neisseria gonorrhoeae* strains have put additional pressure on available first-line antibiotics for the treatment of gonococcal (GC) infections (Alirol et al., 2017; Martin et al., 2019). This, thus calls for the production of new and effective therapies for the treatment of the disease. Plants are ideal candidates for the development of novel therapies for the treatment of gonorrhoea. These photosynthetic organisms possess biological properties and have been used for hundreds of years in phytomedicine by herbalists and traditional healers to treat various ailments (de Wet et al., 2012; Pan et al., 2014; Soyingbe et al., 2018). Plants are unique organisms that produce secondary compounds for adaptations to the environment, prevention of herbivory and protection from pathogens (Böttger et al., 2018; Yang et al., 2018). The great chemical diversity seen in plant species makes them perfect contenders for drug discovery (Bonnez et al., 1994; Mahdi, 2010; Maroyi, 2016; Saxena et al., 2013; Wang et al., 2019).

Traditional medicine, which uses plants extensively to treat diseases, continues to be used as a primary care service in some developing countries (Mothibe and Sibanda, 2019; Nchinda, 1976; Yuan et al., 2016). The main problem with herbal medicine is that there is no standardization of herbal products thus there is a lack of standards for dosages. Therefore, this can lead to overdoses or adverse reactions due to herb-herb interactions in the herbal mixtures (Kumari and Kotecha, 2016; Sachan et al., 2016). Hence it becomes crucial to determine the chemical constituents in the herbal products to ensure safety.

For the formulation of therapies used for the treatment of diseases, it is important to know the constituents before a drug is approved according to the United States Food and Drug Administration (FDA) (Kairuz et al., 2007; van Norman, 2016). This helps standardize herbal formulations for product development. It is imperative to identify compounds and targets for therapeutic action, which is to be done in the preclinical stages of drug development. Furthermore, *in vitro* tests have to be conducted to evaluate the efficacy and safety of the new formulations (van Norman, 2016). Therefore, bioactive compounds need to be identified in plant-based formulations.

Various compounds have been identified from plants that have bioactivity against pathogens associated with sexually transmitted diseases (STDs). *Aloe ferox* Mill, a South African medicinal plant, is used traditionally to treat STDs and venereal sores (Chen et al., 2012b; van

Wyk et al., 2017). Aloin, aloe-emodin and chrysophanol have been isolated from the plant where aloin was seen to inhibit *N. gonorrhoeae* (Kambizi et al., 2005). Furthermore, gallic acid derivatives such as epigallocatechin-3-O-gallate (EGCG) and gallotannin have been isolated from *Peltophorum africanum* Sond (Bessong et al., 2005; Ebada et al., 2008). Gallotannin was reported to inhibit reverse transcriptase of the human immunodeficiency virus (HIV) while EGCG exhibits antigonococcal activity (Bessong et al., 2005; Ebada et al., 2008; Matsumoto et al., 2012).

There have been some commercial products that have been developed as analgesics and antimicrobials, using plant compounds (Anand et al., 2019). These include aspirin, podofilox and anti-malarial drugs (artemisinin and quinine) (Achan et al., 2011; Desborough and Keeling, 2017; Faurant, 2011). For the treatment of anogenital warts caused by the human papilloma virus (HPV), podofilox has been developed (Bonnez et al., 1994; Centers for Disease Control and Prevention, 2015b). This gel contains podophyllotoxin as the active ingredient, which is isolated from the *Podophyllum* species. The production of commercial plant-based drugs further proves that plants have great potential for drug development for STDs like gonorrhoea.

Gunnera perpensa L. was chosen as the lead plant as it exhibited potent antigonococcal activity and the absence of cytotoxic effects (Chapter 3). Thus, in this study, the chemical profile of the *G. perpensa* ethanolic extract was investigated. Firstly, liquid-liquid partitioning was employed to separate the compounds in the crude extract to obtain semi-pure fractions. Thereafter antigonococcal testing was performed on these fractions. The most bioactive fractions were tested for cytotoxicity effects on HeLa (cervical cancer), HaCaT (human keratinocytes) and THP-1 (human monocyte) cell lines. To identify the compounds in the bioactive fractions proton nuclear magnetic resonance (^{1}H-NMR), Fourier-transform infrared spectroscopy (FTIR) and electrospray ionization-liquid chromatography-mass spectrometry (ESI-LCMS) were used.

4.2. Material and methods

4.2.1. Fractionation using liquid-liquid partitioning

The ethanolic crude extract of *Gunnera perpensa* (GP) was prepared as described in section 3.2.2 (Chapter 3). Hexane, ethyl acetate (EtOAc), butan-1-ol and water were used as solvents to create a polarity gradient, to separate the compounds in the plant. Firstly, 13 g of the crude

plant extract was redissolved in 500 ml distilled water, which was then added to a separating funnel (Appendix B1). Five hundred millilitres of distilled hexane was added to the separating funnel resulting in a bilayer due to the polarity difference. The water fraction in the bottom layer was eluted into a 2 L conical flask; thereafter the hexane fraction was eluted into a separate 2 L conical flask. The water fraction (in the conical flask) was placed back into the separating funnel and the process was repeated using 500 ml of distilled ethyl acetate and then lastly 500 ml of distilled butan-1-ol. The four resultant fractions were concentrated using a vacuum rotary evaporator (Heidolph, Hei-Vap value digital HB/G3B, Germany) (section 3.2.2, Chapter 3). The water fraction was dried using a freeze dryer (SP VirTis, 4KBTZL, USA) while the other fractions were air-dried. All dried fractions were stored in glass vials at 4 ˚C in a refrigerator.

4.2.2. Antigonococcal activity of fractions

The antigonococcal activity of the four fractions was evaluated using the microdilution assay as described in section 3.2.4, Chapter 3 (Eloff, 1998). The fractions were dissolved in 10 % dimethyl sulphur oxide (DMSO) to make a final concentration of 6000 µg/ml. In a 96-well plate, 100 µl of the fractions were used for the serial dilutions resulting in a concentration range from 1500-11.57 µg/ml. The positive control for this assay was ciprofloxacin (625-2.44 µg/ml) whilst sterile Mueller-Hinton (MH) broth, distilled water and *N. gonorrhoeae* in broth served as negative controls. The plates were incubated at 37 ˚C in 5 % CO_2 for 24 h thereafter PrestoBlue was used to visualize the microbial growth and the entire experiment was repeated three times.

4.2.3. Cytotoxicity testing of the bioactive fractions on HaCaT, HeLa and THP-1 cell lines

Evaluation of the cytotoxic effects of the most bioactive fractions was conducted on HeLa (cervical cancer), HaCat (human keratinocytes) and THP-1 (human monocytes) as described in section 3.2.6, Chapter 3 (Lall et al., 2013). The test concentrations of the fractions ranged from 400-3.125 µg/ml. Actinomycin D was used as the positive control. Two negative controls were used namely media with cells and media with no cells embedded in wells. The entire experiment was repeated three times.

4.2.4. Characterization of bioactive fractions

The most bioactive fractions that had no cytotoxic effects were characterized using proton nuclear magnetic resonance (^{1}H-NMR), Fourier-transform infrared spectroscopy (FTIR) and electrospray ionization-liquid chromatography-mass spectrometry (ESI-LCMS). This was done to identify the secondary compounds that were potentially responsible for the good antigonococcal activity exhibited. The ^{1}H-NMR analysis was conducted at the Department of Chemistry, University of Pretoria while the FTIR was conducted at the South African Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa. Additionally, the ESI-LCMS was conducted at the Central Analytical Facility (CAF) at Stellenbosch University, Stellenbosch, South Africa.

4.2.4.1. Nuclear magnetic resonance (^{1}H-NMR)

Nuclear magnetic resonance is used to provide structural information (such as functional groups) of compounds present in plants and their fractions (Heyman and Meyer, 2012; Shi and Zhang, 2021). This aids in identifying compounds that can be used in formulations for the treatment of diseases such as gonorrhoea. Proton NMR spectroscopy (^{1}H-NMR) relies on the electromagnetic properties of hydrogen nuclei in compounds (García-Álvarez et al., 2016; Hammerath, 2012). Primarily, an external electromagnetic field is introduced to the sample which causes the hydrogen nuclei in compounds to spin in order to align with the external magnetic field (García-Álvarez et al., 2016; Hammerath, 2012). The resonance is due to the electron charge distribution of the hydrogen atoms in compounds which generate magnetic moments called chemical shifts (Koutcher and Burt, 1984). Chemical shifts are influenced by shielding and the electronegativity of adjacent atoms bonded to the hydrogen atoms hence, functional groups have specific chemical shifts (Diehl, 2008). This allows for the identification of specific functional groups in compounds.

In this study, the bioactive water and butan-1-ol fractions were compared to determine if there were variations in the compounds present in the fractions. ^{1}H-NMR was conducted using the Bruker AVIII-400 (Rheinstetten, Germany) and the experiment was run at 400MHz. For sample preparation, the two bioactive fractions were each dissolved in 600 µl of deuterated methanol.

4.2.4.2. Fourier-transform infrared spectroscopy (FTIR)

The Fourier-transform infrared spectroscopy (FTIR) was used to identify the functional groups present in the bioactive fractions. The technique uses the mid-infrared (IR) region (500-4000

cm^{-1}) of the electromagnetic spectrum to detect functional groups in samples which can aid in providing the structure of compounds (Coates, 2000; Mohamed et al., 2017). In a FTIR spectrophotometer, the IR beam is directed at the sample in which chemical bonds that are present selectively absorb the IR light at specific wavelengths at varying frequencies (Mohamed et al., 2017). Consequently, this results in optical signals due to changes in dipole moments in the sample (Bridle, 2020; Siesler, 2017). This produces IR spectra which can be interpreted using available reference databases (Coates, 2000; Nandiyanto et al., 2019).

In this study, the Perkin Elmer spectrum 100 FTIR spectrophotometer was used to obtain percentage transmittance for the wavenumber range of 500-4000 cm^{-1} (Patel et al., 2020). The data was analysed using Origin Pro software.

4.2.4.3. Electrospray ionization-liquid chromatography-mass spectrometry (ESI-LCMS)

Electrospray ionization-liquid chromatography-mass spectrometry (ESI-LCMS) was used to identify the chemical constituents of the bioactive fractions. ESI-LCMS is a technique that uses electrical energy to protonate or deprotonate compounds (in liquid form) to form positive $[M^+]$ or negative $[M^-]$ gaseous ions (Banerjee and Mazumdar, 2012; Ho et al., 2003). After ionization, the ions are detected based on mass/charge ratio (m/z) by a mass analyser. This generates mass spectra showing the m/z ratios and relative abundance of the ionic species present (Banerjee and Mazumdar, 2012). Finally, the m/z ratios from mass spectra are analysed using reference databases and computer software to obtain the molecular formulas of compounds present in the sample. Compounds have specific m/z ratios in the positive and negative ionization states which allows for easy identification of compounds (Ho et al., 2003). One major advantage of ESI-LCMS is that multiple compounds in a sample can be analysed and the resultant charged ions are intact (without fragmentation) making identification of compounds easier (Banerjee and Mazumdar, 2012).

In this study, ESI-LCMS was conducted using the Waters Synapt G2 qTOF (Milford, USA) (cone voltage 15V) system with an ESI probe that ran positive $[M^+]$ and negative $[M^-]$ modes. Two columns (Waters BEH C18, 2.1x100 mm) were used, each containing 0.1 % formic acid, to analyse the bioactive fractions. Data was analysed using mzMine (version 2.51). Web-based applications ChemCalc and massBank (version 2021.03) were used to determine the molecular formulas of the fragments obtained from the mass spectrometry. Additionally, the PubChem database was utilised to further identify the compounds in the samples.

4.3. Results and discussion

4.3.1. Fractionation using liquid-liquid partitioning

Four fractions were obtained, namely, hexane, EtOAc, butan-1-ol and water yielding 1 g, 3 g, 4 g and 8 g, respectively). It is known that ethanol (used in the extraction of the crude extract) is amphiphilic hence it separates both polar and non-polar compounds (Klemm, 1998). It has a polarity index of 5.2 (Sadek, 2002). Hexane and EtOAc are non-polar solvents with polarity indices of 0.1 and 4.4, respectively (Sadek, 2002). On the other hand, butan-1-ol and water are polar with indices of 4.0 and 10.2, respectively (Sadek, 2002). Butan-1-ol has a low polarity index but has the greater ability to form hydrogen bonds than EtOAc, making it very polar despite the relatively low index number (Barton, 1983; Burke, 1984). From the results obtained the water fraction had the highest dry mass showing that most compounds of the plant extract were eluted in this fraction compared to the other fractions. This showed that the *G. perpensa* crude extract had more polar compounds than non-polar ones.

4.3.2. Antigonococcal activity of *Gunnera perpensa* fractions

The antigonococcal assay revealed that the polar fractions had better activity than the non-polar fractions. The hexane fraction was least active against *N. gonorrhoeae* followed by EtOAc fraction with MICs of >1500 µg/ml and 187 µg/ml, respectively (Table 4.1). Butan-1-ol and water fractions had the best antigonococcal activity both exhibiting MICs of 23.4 µg/ml. The biological activity exhibited by the polar fractions was better than the crude ethanolic extract of *G. perpensa* (MIC=48.7 µg/ml; Chapter 3). The positive control had better activity (MIC <5 µg/ml) compared to GP and the fractions. The superior antigonococcal activity exhibited by the bioactive GP fractions compared to the crude extract could be due to some antagonistic compounds in the GP extract, which were removed during fractionation process (Caesar and Cech, 2019). There is no data on GP fractions being tested on *N. gonorrhoeae* however, some GP fractions possess antibacterial activity on non-STD pathogens. McGaw et al. (2005) obtained non-polar and polar fractions from *G. perpensa*. The authors found that hexane fractions from GP showed antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis* with MICs of 6.25 mg/ml and 12.5 mg/ml, respectively. The dichloromethane, acetone, methanol and ethanol/water fractions had bioactivity against *Escherichia coli*, *S. aureus* and *E. faecalis*, with MICs ranging from 12.5-2.61 mg/ml.

There are secondary compounds that have been isolated from other medicinal plants that have shown inhibitory activity against *N. gonorrhoeae* and other STD pathogens. An epigallic acid

derivative, epigallocatechin-3-O-gallate (EGCG), isolated from methanolic extracts of *Peltophorum africanum* exhibited lower antigonococcal activity (MIC=32 µg/ml) compared to that of the two GP bioactive fractions (MIC=23.4 µg/ml) (Ebada et al., 2008; Matsumoto et al., 2012). 4'-O-methyl-epigallocatechin isolated from *Elaeodendron transvaalense* (Burtt Davy) R.H.Archer, a South African plant, has antigonococcal activity with a MIC of 6300 µg/ml (Mamba et al., 2016). Aloin isolated from *A. ferox* has been reported to inhibit *N. gonorrhoeae* with a MIC of 100 µg/ml (Kambizi and Afolayan, 2008). Aloin and 4'-O-methyl-epigallocatechin have lower antigonococcal activity compared to the bioactive fractions in this study. It is therefore important to identify the pure compounds in the fractions as they may result in better antigonococcal activity. The results obtained show that GP and its semi-pure fractions have the potential to be used as treatments for gonorrhoea. The findings also suggest that individual components may have the potential to improve the efficacy of plant extracts. Consequently, the water and butan-1-ol fractions can be further separated by Sephadex column chromatography to obtain pure compounds for the development of formulations for the treatment of gonorrhoea.

Table 4.3: Antigonococcal activity of the four semi-pure fractions from *Gunnera perpensa*

Sample	MIC (µg/ml)[a]
Hexane[†]	>1500
Ethyle acetate[†]	187
Butan-1-ol[†]	23.4[*]
Water[†]	23.4[*]
Crude extract (GP)[b]	48.7
Ciprofloxacin[c]	<5

[a]Minimum inhibitory concentration, [b]Ethanolic crude extract of *Gunnera perpensa*, [c] Positive drug control, [*]Most bioactive fractions with MIC <100µg/ml, [†]Fractions of *Gunnera perpensa*,

4.3.3. Cytotoxicity of the *Gunnera perpensa* bioactive fractions

Cytotoxicity testing revealed that the water and butan-1-ol fractions along with the crude extract of GP had IC_{50} values >300 µg/ml (Table 4.2). For this study, cytotoxic effects were defined as IC_{50} <30 µg/ml (very cytotoxic), 30 µg/ml < IC_{50} <50 µg/ml (moderate cytotoxicity), 50 µg/ml < IC_{50} <200 µg/ml (low cytotoxicity) and IC_{50} >200 µg/ml (no cytotoxicity effect) (Kuete and Efferth, 2015; Steenkamp and Gouws, 2006; Suffiness and Pezzuto, 1990). Therefore, the bioactive fractions and the crude extract did not show any cytotoxic effects on

all cell lines. The positive control, actinomycin D, showed toxicity to the HaCaT, HeLa and THP-1 cells with IC_{50} values <0.2 µg/ml. McGaw et al. (2005) determined the cytotoxicity effects of hexane, dichloromethane, acetone, methanol and ethanol/water fractions from GP on brine shrimp. It was revealed that at 100 µg/ml of non-polar, hexane and dichloromethane fractions did not exhibit any cytotoxicity effects on brine shrimp. At the same concentration, the other more polar fractions caused some mortality in the brine shrimp (<24 %). This showed that the polar GP fractions at 100 µg/ml were not cytotoxic (McGaw et al., 2005). The cytotoxicity effects of the crude plant extract of GP have been investigated. It has been reported that GP is non-toxic to human fibroblast (MRC5) cells and Vero (monkey kidney epithelial) cells with IC_{50} values >500 µg/ml (Brookes and Smith, 2003; Steenkamp et al., 2004).

Evaluation of cytotoxic effects of fractions from other plants used to treat STDs has been conducted. *Codiaeum variegatum* (L.) Rumph. ex A.Juss has been used to treat gonorrhoea and syphilis (Ogunwenmo et al., 2007; Quattrochi, 2012). Njoya et al. (2014) reported that the EtOAc fraction from the decocted extract of *C. variegatum* does not exhibit any cytotoxic effects on the human colon carcinoma cell line (Caco-2) (IC_{50} >1000 µg/ml). Cunha et al. (2017) revealed that the dichloromethane fraction of the ethanol extract of *Cassia bakeriana* Craib (related to *Cassia abreviata* Oliv used to treat gonorrhoea) was not cytotoxic to Vero cells (IC_{50}=325 µg/ml). This shows that some fractions from plant extracts have the potential to be relatively safe to cells. Hence *G. perpensa* may be considered for drug discovery of gonorrhoea treatments due to the absence of cytotoxic effects.

Table 4.2: Cytotoxicity effects of *Gunnera perpensa* crude extract and the most bioactive *Gunnera perpensa* fractions on cervical cancer cells, human keratinocytes and human monocytes

Sample	**IC_{50}[a]±SD[b] (µg/ml)**		
	HeLa[c]	**HaCaT[d]**	**THP-1[e]**
Butan-1-ol[†]	321.7±8.1	342.0±3.1	>400
Water[†]	365.2±14.1	>400	>400
Crude extract (GP)[f]	>400	>400	>400
Actinomycin D (Positive drug control)	$2.1\times10^{-3}\pm5.5\times10^{-4}$	$1.9\times10^{-3}\pm4.6\times10^{-4}$	$8.4\times10^{-2}\pm6.8\times10^{-3}$

[a]Fifty percent inhibitory concentration, [b]Standard deviation, [c] Human cervical cancer cells, [d] Human keratinocytes, [e] Human monocytes, [f] Ethanolic crude extract of *Gunnera perpensa*, [†]Fractions of *Gunnera perpensa*

4.3.4. Characterization of *Gunnera perpensa* bioactive fractions

In general, characterization of the fractions revealed that the most bioactive fractions (water and butan-1-ol) contained the same compounds with varying intensity.

4.3.4.1. Nuclear magnetic resonance (^{1}H-NMR) and Fourier-transform infrared spectroscopy (FTIR) analysis of *Gunnera perpensa* bioactive fractions

The ^{1}H-NMR analysis was conducted to determine if the two bioactive fractions had the same constituents, as they had the same antigonococcal activity (MIC=23.4 µg/ml). This was done by looking at the functional groups present in the samples. ^{1}H-NMR can distinguish hydrogen-containing functional groups with each producing a specific chemical shift (Balci, 2005). Chemical shift is the frequency of the nuclear spin of a hydrogen atom due to neighbouring atoms bonded (Balci, 2005; Simpson, 2012). The spectra in Figure 4.1 show that the two fractions had similar chemical profiles. Signals were seen with chemical shift ranges at δ 4.4-4.9 ppm, δ 2.4-3.2 and δ 1.3-2.0 ppm. These indicated the presence of hydroxyl (-OH), carbonyl (C=O)/amino (N-H) and alkyl (C-H) functional groups, respectively (Balci, 2005). In the butan-1-ol fraction, there was a strong signal at δ 5.4-5.8 ppm (corresponding to the alkene group), which was weaker in the water fraction (Balci, 2005). This most likely means that the alkene compounds in the water fraction were less than in the butan-1-ol fraction.

The functional groups found using ^{1}H-NMR were further confirmed by FTIR analysis. The FTIR characterization of the two bioactive samples showed that they all had the same

compounds as the two samples had wavenumber peaks in the same regions (Figure 4.2). A summary of the FTIR results from the spectra is found in Table 4.3 and individual spectra are presented in Appendix B2. Generally, the water spectrum had weaker signals with lower % transmittance than the butan-1-ol fraction. Percentage transmittance is the amount of IR light that has not been absorbed by bonds in the compounds present (Subbramanian and Rodriguez-Saona, 2009). Thus, lower peaks show high absorption of IR light by compounds at specific wavelengths. The weak signals in the water fraction spectrum show that a lot of the IR radiation was absorbed showing that the fraction has more compounds than the butan-1-ol fraction. A broad peak was found in two fractions at ~3230 cm^{-1}, which correlates to a -OH group (Coates, 2000; Nandiyanto et al., 2019). There were prominent peaks at ~1700 and ~1600 cm^{-1} in the fractions. Peaks around 1600 cm^{-1} are indicative of double bonds (C=C) associated with alkenes or aromatic rings whilst the peaks at 1700 cm^{-1} often correlate with carbonyl groups (C=O) possibly associated with ketones, carboxylic acids or phenols (Coates, 2000; Nandiyanto et al., 2019). Peaks around ~1160 cm^{-1} and ~1035 cm^{-1} indicate that the compounds in the samples have some hydrocarbon (aliphatic) backbone. The signal at ~1320 cm^{-1} indicates a C-O group, possibly associated with a phenol (Coates, 2000; Nandiyanto et al., 2019). In this study, results from the FTIR and ^{1}H-NMR analysis revealed that the similar bioactivity of water and butan-1-ol fractions was due to the two fractions having similar compounds present.

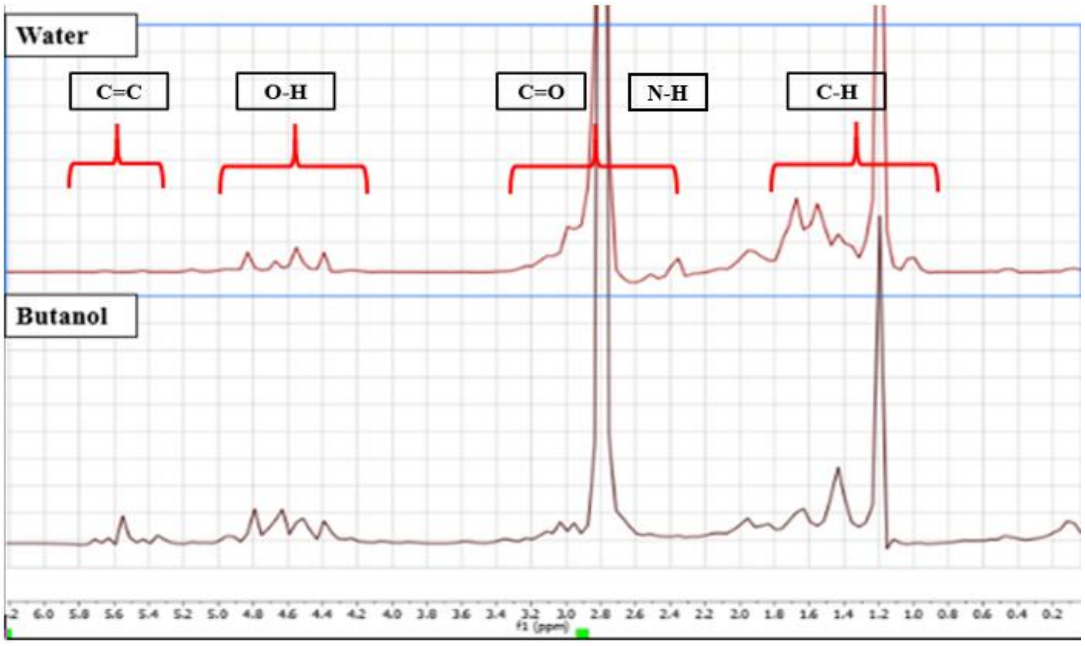

Figure 4.1: ^{1}H-NMR spectra of water and butan-1-ol fractions from *Gunnera perpensa*

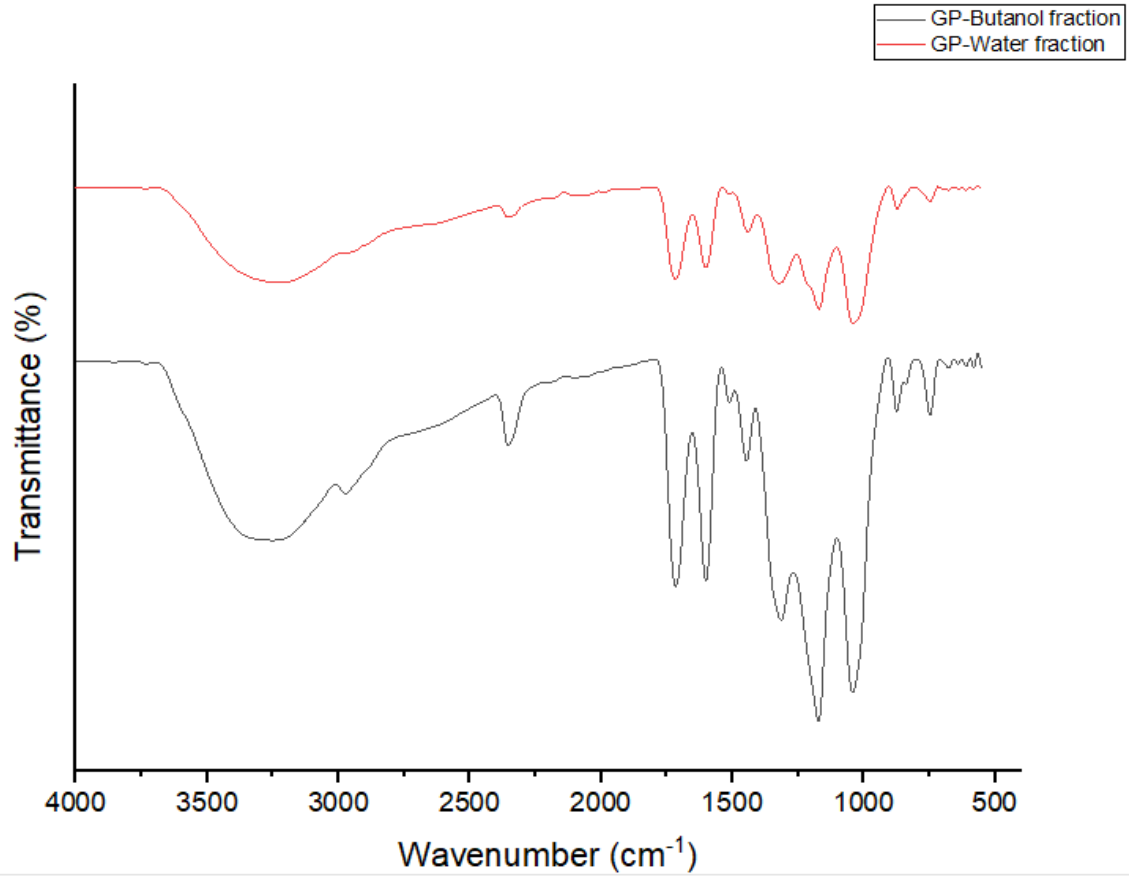

Figure 4.2: Infrared spectra of the bioactive water and butan-1-ol fractions from *Gunnera perpensa*

Table 4.3: Assignment of infrared absorption bands in the spectra of the water and butan-1-ol fractions from *Gunnera perpensa*

Peak wavenumber (cm^{-1})	**Functional groups**
~3278	-OH (hydroxyl group)
~2924	C-H (terminal methyne)
~2356	N-H (amino component)
~1714	C=O (associated with ketones or carboxylic acids)
~1602	C=C (alkene and aromatic groups)
~1442	CH_2 bend
~1320	C-O (possibly associated with a phenol)
~1166 & 1042	C-C (aliphatic alkane)
~872 & 754	C-H associated with disubstituted aromatics

4.3.4.2. Electrospray ionization-liquid chromatography-mass spectrometry (ESI-LCMS) analysis of *Gunnera perpensa* bioactive fractions

The ESI-LCMS was run in negative $[M]^-$ and positive $[M]^+$ modes using negative and positive ionization, respectively. The summary of compounds identified is found in Table 4.4. The spectra for the $[M]^-$ of the butan-1-ol and water are in Figure 4.3 & Figure 4.4 while the $[M]^+$ spectra are in Figure 4.5 & Figure 4.6. The water and butan-1-ol fractions had similar fragmentation patterns however, the butan-1-ol fraction had weaker signals in both modes. The butan-1-ol fraction in negative mode had intense peaks at a mass: charge ratio (m/z): 323.1(11.75) and 355.1(12.54) (Figure 4.3). While water had peaks$[M]^-$ m/z at 341.1 (8.01), 323.1 (10.23), 323.1 (11.84), 355.1(12.54), 369.1 (15.49) and 397.2 (22.14) (Figure 4.4). On the other hand, the butan-1-ol fraction in positive mode had prominent peaks at m/z 203.1 (1.03), 325.1 (11.84), 195.1(12.54) and 345.1 (19.29) (Figure 4.5). While water had peaks$[M]^+$ m/z at 325.1 (11.84), 209.1 (15.51) and 181.0 (22.13) (Figure 4.6). Generally, more compounds were detected in negative mode of ESI-LCMS.

Both fractions run in negative mode $[M]^-$ had a prominent peak with a mass: charge ratio (m/z) of ~323.1 at ~11.75 min and 11.84 min. The suggested molecular formula, after searching the databases (mzMine, ChemCalc and massBank), was $C_{15}H_{15}O_8$ (**1**). On the other hand, samples run under positive mode $[M]^+$ had an intense peak with m/z of 325.1 at ~11.79 min and 11.84 min. The suggested formula for the compound was $C_{15}H_{17}O_8$ (**2**). The suggested formulas of **1** and **2** were similar to that of Z-venusol that has been isolated from a decocted extract of *G. perpensa* (Khan et al., 2004). The Z-venusol had a formula $[M]^+$ m/z of 324.28 with a chemical formula of $C_{15}H_{16}O_8$. The compound has also been identified in *Umbilicus rupestris* (Salisb.) Dandy (Crassulaceae) with $[M]^-$ m/z 323 (Iydaa et al., 2019). Thus, compounds **1** and **2** were identified as Z-venusol, which was also the most abundant compound in the bioactive fractions.

Compound **3** had $[M]^+$ m/z of ~345.1 at 19.29 min, which was suggested to be $C_{17}H_{13}O_8$. This was similar to the ellagic acid derivative, 4-*O*-β-D-glucopyranosyl-3,3'-tri-*O*-methylellagic acid, which had $[M]^+$ m/z of 344.05434 and a formula of $C_{17}H_{12}O_8$ (Khac et al., 1990; Khan et al., 2004). Thus, compound **3** was identified as 4-*O*-β-D-glucopyranosyl-3,3'-tri-*O*-methylellagic acid.

At 12.54 min retention time, compound **4** with $[M]^+$ m/z of ~195.1 was identified to have a formula of $C_{10}H_{11}O_4$ while compound **5** with $[M]^+$ m/z of 203.1 at 1.08 min had a formula of $C_{12}H_{11}O_3$. Compound **6** had $[M]^+$ m/z of 209.1 at 15.49 min and the formula was determined to be $C_{11}H_{13}O_4$. Compounds **4-6** were classified as 1,4-benzoquinones. Drewes et al, (2005) isolated two simple 1.4 benzoquinones: 2-methyl-6-(3-methyl-2-butenyl) benzo-1,4-quinone and 3-hydroxy-2-methyl-5-(3-methyl-2-butenyl) benzo-1,4-quinone from *G. perpensa*. The two had $[M]^+$ m/z of 190.09867 and 206.9429, respectively. The formulas of the two benzoquinones isolated were $C_{12}H_{14}O_2$ and $C_{12}H_{14}O_3$, which were very similar to those from this study.

Compound **7** was identified to have $[M]^+$ m/z of 181.16 at 22.13 min with a formula of $C_9H_9O_4$. Similarly, Hebel-Gerber et al. (2020) detected caffeic acid that had $[M]^-$ m/z of 179.03 from *Gunnera tinctoria* (Molina) Mirb. Compound **7** was identified as caffeic acid. Caffeic acid has been isolated from *G. perpensa* and *G. tinctoria* (Doyle and Scogin, 1988; Hebel-Gerber et al., 2020; Maroyi, 2016).

Compound **8** was detected using negative ESI-LCMS with a peak at ~8.01 min with $[M]^-$ m/z 341.29 and had a formula of $C_{15}H_{17}O_9$. This compound was identified to be a glycoside, 4-*O*-

beta-D-Glucosyl-trans-caffeate. This compound is a derivative of caffeic acid. Caffeoyl hexoside has been identified from *Taraxacum formosanum* Kitam ($[M]^-$ m/z=341) (Chen et al., 2012a). Said et al. (2017) have also identified caffeoyl hexoside from *Phoenix dactylifera* L.

At 12.53 min under negative mode ionization, a peak with an m/z of 355.10 was detected and suggested to have a formula of $C_{16}H_{19}O_9$ (**9**). Compound **9** was identified as a glucoside of ferulic acid. Ferulic acid glucoside has been detected in, a related species, *G. tinctoria* and *Capsicum annuum* L with $[M]^-$ m/z of ~355.10 (Hebel-Gerber et al., 2020; Jeong et al., 2011).

Compounds **10** and **11** had peaks at $[M]^-$ m/z 369.12 (15.49) and 397.15 (22.14), which had suggested formulas of $C_{17}H_{21}O_9$ and $C_{19}H_{21}O_4$, respectively. These two compounds could not be identified, thus categorized as unknown.

The ESI-LCMS analysis revealed that butan-1-ol and water fractions from GP contained the same compounds with varying amounts. Nine compounds were identified with compounds **10** and **11** unknown. Z-Venusol was identified as the most abundant compound present in both bioactive fractions. Compounds identified included: 4-*O*- β-D-glucopyranosyl-3,3'-tri-*O*-methylellagic acid, 4-*O*-beta-D-Glucosyl-trans-caffeate, caffeic acid, ferulic acid glucoside and some 1.4 benzoquinones. Detection of these compounds in the bioactive fractions from GP showed that there is potential to isolate pure compounds that can be further evaluated for pharmaceutical development for gonorrhoea. In the future, it is suggested that the two bioactive fractions can be purified to isolate pure compounds to increase the efficacy of the GP against *N. gonorrhoeae* as the fractions have shown better antigonococcal activity than the crude extract. Additional, derivatization of compounds isolated from GP could also potentially improve bioactivity as was the case with aspirin (Desborough and Keeling, 2017).

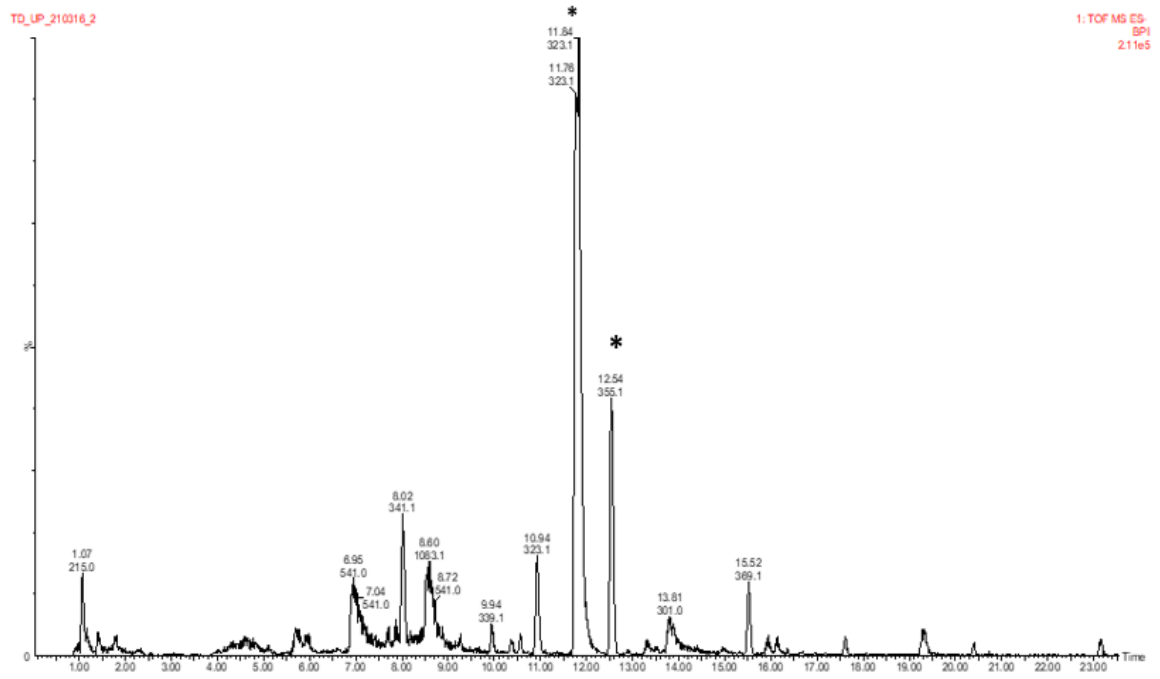

Figure 4.3: Negative ionization $[M]^-$ ESI-LCMS chromatogram for the butan-1-ol fraction from *Gunnera perpensa*

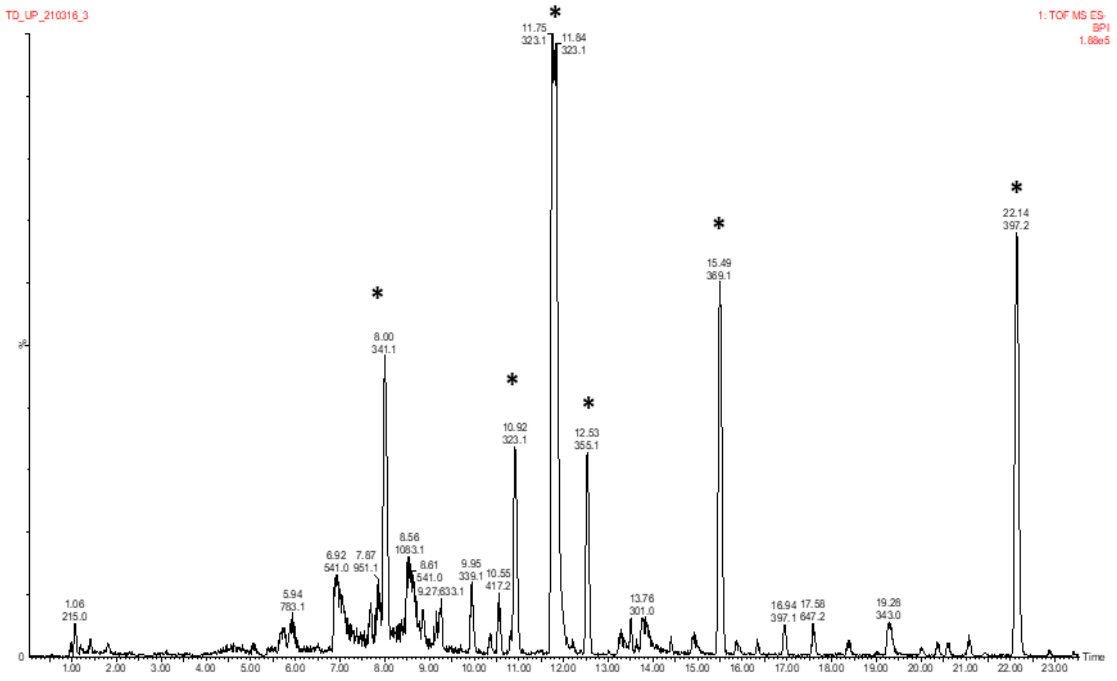

Figure 4.4: Negative ionization $[M]^-$ ESI-LCMS chromatogram for the water fraction from *Gunnera perpensa*

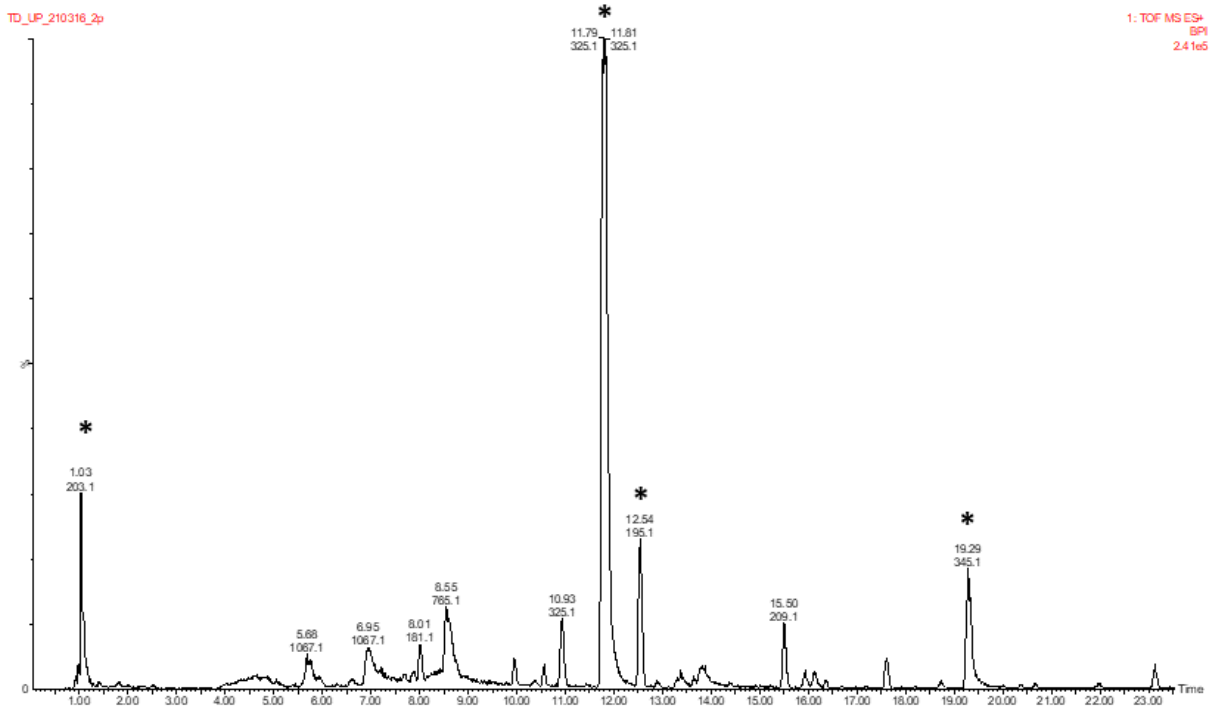

Figure 4.5: Positive ionization $[M]^+$ ESI-LCMS chromatogram for the butan-1-ol fraction from *Gunnera perpensa*

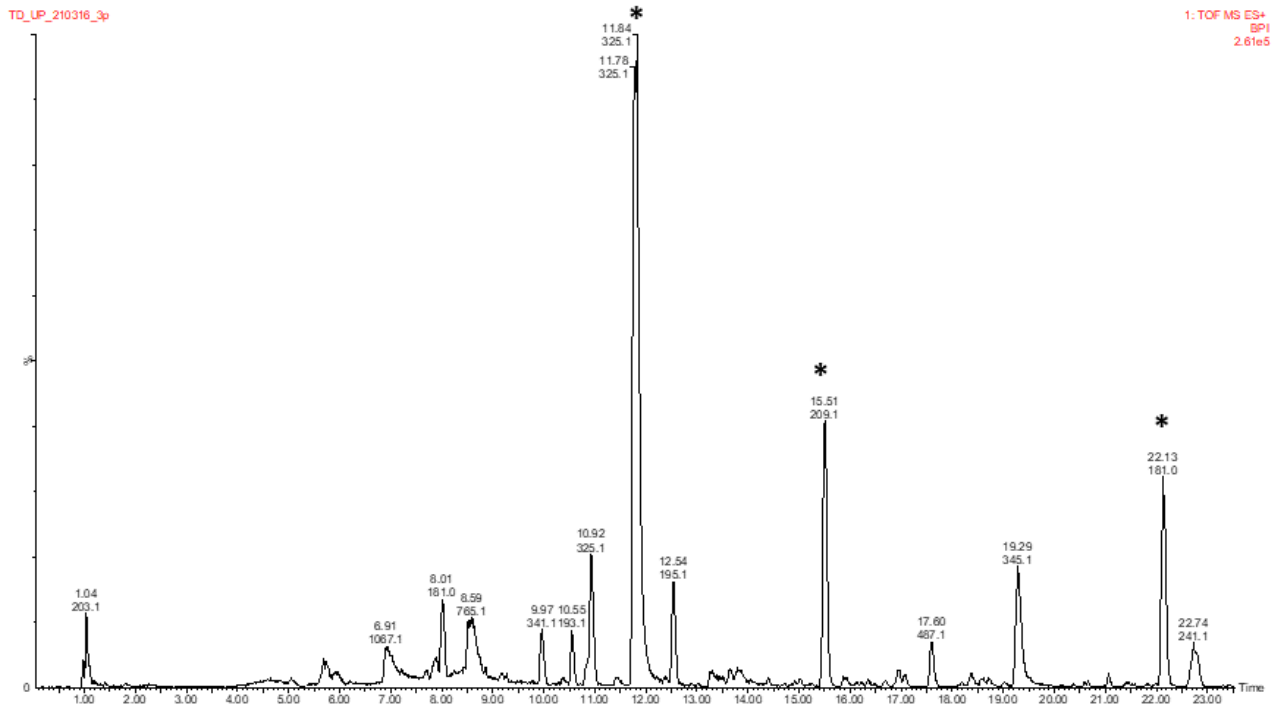

Figure 4.6: Positive ionization $[M]^+$ ESI-LCMS chromatogram for the water fraction from *Gunnera perpensa*

Table 4.4: Qualitative characterization of compounds in the water and butan-1-ol fractions from *Gunnera perpensa* using ESI-LCMS .

Compounds	Retention time (T_R)/min	Ionization	Experimental mass	Theoretical mass	Suggested formula	Accuracy (ppm)
1	~11.75 & 11.84	$[M]^-$	323.0768	323.28	$C_{15}H_{15}O_8$	2.03
2	~11.79 & 11.84	$[M]^+$	325.0923	325.29	$C_{15}H_{17}O_8$	-0.13
3	~19.29	$[M]^+$	345.0607	345.28	$C_{17}H_{13}O_8$	-0.99
4	~12.54	$[M]^+$	195.0652	195.19	$C_{10}H_{11}O_4$	-2.74
5	~1.08	$[M]^+$	203.0693	203.21	$C_{12}H_{11}O_3$	-7.48
6	~15.52	$[M]^+$	209.0811	209.22	$C_{11}H_{13}O_4$	-0.28
8	~22.13	$[M]^+$	181.0498	181.16	$C_9H_9O_4$	2.46
7	~8.01	$[M]^-$	341.0867	341.29	$C_{15}H_{17}O_9$	-1.63
9	~12.53	$[M]^-$	355.1022	355.32	$C_{16}H_{19}O_9$	-1.99
10	~22.14	$[M]^-$	397.1504	397.4	$C_{19}H_{25}O_9$	1.37
11	~15.49	$[M]^-$	369.1189	369.3	$C_{17}H_{21}O_9$	0.93

4.4. Conclusion

In drug development, it is essential to identify the bioactive ingredients in formulations. In this study, an attempt was made to identify compounds in the *G. perpensa* crude plant extract that are responsible for the good antigonococcal activity. Liquid-liquid partition chromatography resulted in four semi-pure fractions being obtained. The findings of the study showed that the polar (water and butan-1-ol) fractions had better bioactivity than the crude extract and the non-polar fractions. Additionally, ESI-LCMS showed that the bioactive fractions contained nine known compounds and two unknown compounds with Z-venusol being the most abundant. It is suggested for future prospects that separative techniques such as Sephadex column chromatography be employed to the polar fractions to obtain pure compounds to identify the compounds that are potentially responsible for the antigonococcal effects observed. Moreover, pure compounds isolated may exhibit more potent antigonococcal activity. The non-toxic nature of the bioactive fractions on the cell lines used encourages further exploration of GP to develop novel formulations to treat GC infections.

Chapter 5: Synthesis, characterization, antigonococcal activity and cytotoxicity of *Gunnera perpensa*-mediated gold nanoparticles

Abstract

Gonorrhoea is the second most problematic bacterial sexually transmitted disease (STD) worldwide. The prevalence of antibiotic-resistant strains of the causal agent, *Neisseria gonorrhoeae*, necessitates the development of new therapies and the use of efficient drug delivery systems to clear gonococcal (GC) infections. The vaginal route of administration is an ideal drug delivery system that delivers drugs directly into the bloodstream, thus increasing the bioavailability of drugs. The vaginal route requires small weight molecules to pass the cervicovaginal mucus (CVM) barrier; thus nanoparticles (NPs) may be explored to develop vaginal products to treat GC infections. In this study, gold nanoparticles (AuNPs) were synthesized using the ethanolic crude extract of *Gunnera perpensa*. L (GP). The GP-AuNPs were characterized to determine the nature and stability of the NPs. Furthermore, antigonococcal activity and cytotoxic effects were evaluated. Ultraviolet-visible spectrophotometry (UV-Vis) revealed that the GP-AuNPs had a surface plasmon resonance (SPR) at 536 nm while the Zetasizer showed that GP-AuNPs had a hydrodynamic size of 127.2±1.56 nm. Transmission electron microscopy (TEM) revealed that the GP-AuNPs were mostly spherical while X-ray diffraction (XRD) showed that the GP-AuNPs were indexed at (111), (200), (220) and (311) cubic face-centred (FCC) using the Bragg's law. Furthermore, the GP-AuNPs were moderately stable when subjected to nine treatments (pH 4, pH 7, pH 10, 0.5 % cysteine (Cys), 0.5 % sodium chloride (NaCl), 0.5 % phosphate-buffered saline (PBS), 0.5 % Bovine serum albumin (BSA), Dulbecco's Modified Eagle Medium (DMEM) and deionized water) for 24 h, 72 h and 1 week. The antigonococcal screening of the GP-AuNPs showed that the nanoparticles had good antigonococcal activity (MIC=10.4 µg/ml). However, the nanoparticles were cytotoxic to HaCaT, HeLa and THP-1 cells with IC_{50} values of 22.12±0.52 µg/ml, 41.98±10.65 µg/ml and 27.53±6.02 µg/ml, respectively. This study revealed that GP-AuNPs may not be ideal to use for the treatment of gonorrhoea due to the potential cytotoxic effects on human cells. However, the GP-AuNPs can be investigated further for the treatment of cervical cancer caused by the human papilloma virus.

5.1. Introduction

Gonorrhoea is more prominent in females, with studies in 2016, indicating that women accounted for 0.9 % of the worldwide gonococcal cases when compared to males which made up 0.7 % of cases (Kirkcaldy et al., 2019; Rowley et al., 2019). A study focusing on South African gonococcal infections in 2017 reported that 6.6 % of cases were attributed to females while males contributed 3.5 % of the cases (Kularatne et al., 2018a). The high incidence of infections has also increased the occurrences of multi-drug resistant (MDR) *Neisseria gonorrhoeae* (Martin et al., 2019). The antibiotic resistance and high prevalence of gonorrhoea dictate the need to use an effective drug delivery system for the treatment of the disease. The vaginal route of administration is a good delivery system to treat both local and systemic GC infections (Ensign et al., 2014). This is mainly because the drug is not affected by the hepatic first-pass as drugs are delivered directly into the bloodstream (Ensign et al., 2014; Krishna et al., 2012). This results in faster delivery of therapeutics to target sites than via the oral route (Hussain and Ahsan, 2005; Krishna et al., 2012).

Nanotechnology is rapidly becoming a means of increasing drug delivery for the treatment of sexually transmitted diseases (STDs) such as gonorrhoea (Li et al., 2013; Yilma et al., 2013). This technology has been incorporated in vaginal formulations as the nanoscale of the delivery system is ideal to pass the vaginal barrier called the cervicovaginal mucus (CVM) (Ensign et al., 2014; Rossi et al., 2019; Vermani and Garg, 2000). The CVM only allows molecules <340 nm to pass through, indicating that nanoparticles (NPs) below this threshold can be synthesized allowing for potential clearing of local and/or systemic GC infections (Cone, 2009; Krishna et al., 2012; Rossi et al., 2019).

Inorganic nanoparticles prepared using silver and gold have been developed for the treatment of STDs (Ensign et al., 2014; Kesarkar, 2015; Lara et al., 2010a). The NPs have the bioactive agent loaded onto the surface or encapsulated in the inorganic polymer. These NPs can be further capped with a stabilizer to increase shelf-life (Javed et al., 2020).

Gold nanoparticles (AuNPs) have been reported to have antimicrobial activity as they disrupt cell membranes and the metabolic processes of microbes (Aderibigbe, 2017). Furthermore, AuNPs are easily taken up by cells thus improving therapeutic action (Thipe et al., 2019). Gold nanoparticles have mostly been developed for vaginal delivery to treat the human immunodeficiency virus (HIV) and herpes simplex virus (HSV) infections (Baram-Pinto et al., 2010; Chiodo et al., 2014; Ensign et al., 2014; Garrido et al., 2015). Baram-Pinto et al., (2010)

have synthesized multivalent AuNPs mediated with mercaptoethane sulfonate, which affects viral entry and attachment of HSV-1. On the other hand, Di Gianvincenzo et al. (2010) produced AuNPs with several sulfur ligands that can bind to the gp120 protein of HIV thus inhibiting infection. Nanoparticles have been employed to increase the bioavailability and efficacy of plant actives. Plant-mediated AuNPs have been reported to have activity against STD pathogens (Patel et al., 2020; Patra et al., 2016). *Sargassum wightii* Greville ex J. Agardh (brown algae) AuNPs have been found to inhibit the cytopathic effects of HSV-1 & 2 by 70 % at concentrations of 2.5 µl and 10 µl per sample with no cytotoxic effects observed on Vero (monkey kidney) cells (Dhanasezhian et al., 2019). Additionally, AuNPs have been reported to have antibacterial activity against non-STDathogens (Gu et al., 2003; Huang et al., 2007; Li et al., 2014). This shows that AuNPs can potentially be used to treat STDs.

In this study, gold nanoparticles were synthesized from the lead plant, *Gunnera perpensa*. L. *Gunnera perpensa* (GP) was chosen as the lead plant as it displayed good antigonococcal activity (MIC=48.7 µg/ml) and no cytotoxic effects (Chapter 3). Furthermore, the bioactive fractions from GP, obtained in Chapter 4, exhibited better bioactivity (MIC= 23.4 µg/ml) compared to the crude plant extract. The characterization of the GP-AuNPs was done using Fourier-transform infrared spectroscopy (FTIR), Zetasizer, X-ray diffraction (XRD), transmission electron microscopy (TEM) and ultraviolet-visible spectrophotometry (UV-Vis). Stability testing was conducted to ensure that the GP-AuNPs would have a longer shelf-life without agglomeration. In addition, antigonococcal activity and potential cytotoxic effects of the gold nanoparticles were evaluated.

5.2. Material and methods

5.2.1. Gold nanoparticle synthesis and stability testing

5.2.1.1. Gold nanoparticle synthesis

The AuNPs were synthesized using a method described by Thipe et al. (2019) which was optimized for the AuNPs made in this study. For the synthesis of the AuNPs, 60 mg of ethanolic plant extract of *G. perpensa* (preparation as described in section 3.2.2, Chapter 3) was added to 30 ml of deionized water to make a final concentration of 2 mg/ml. In a glass beaker, 14 ml of the plant extract was added to 46.7 mg of gum Arabic (stabilizer). The mixture was then heated using a magnetic hot plate to 60 ˚C and stirred continuously using magnetic stirrer bars

to mix the solution. Subsequently, 117 µl of 0.1M gold ($HAuCl_4$) salt was added dropwise to the solution. The mixture with formed nanoparticles was then filtered using Whatman No. 1 filter paper and was labelled GP-AuNPs (Appendix C1). Another set of nanoparticles were synthesized without gum Arabic (GPX-AuNP) using the aforementioned steps. This was to determine whether gum Arabic affected AuNPs synthesis and stability. Lastly, AuNPs were made with gum Arabic (GA), under the same conditions as the GP-AuNPs and GPX-AuNPs, and these served as the negative control (GA). Confirmation of nanoparticle formation was characterized by the formation of a pink-red wine aqueous solution as the gold ions (Au^{3+}) are reduced to(Au^0) by the plant extract (Shnoudeh et al., 2019). A yellow solution without any precipitate would be a negative result. The filtrates of GP-AuNPs and GPX-AuNPs were collected and stored in a fridge at 4 ˚C in glass vials until further use. These solutions were used for the characterization and bioactivity experiments.

5.2.1.2. Surface plasmon resonance of *Gunnera perpensa*-mediated gold nanoparticles using ultraviolet-visible spectrophotometry

The surface plasmon resonance (SPR) is a measure of the refractive index of light on the surface of the AuNPs where the plant extract binds (Shnoudeh et al., 2019). This optical effect results in a distinctive absorption peak when using ultraviolet-visible spectrophotometry (UV-Vis). Therefore, SPR can be used to confirm the formation of NPs. For this study, a 96-well plate was used where 100 µl of GP-AuNPs was added in three separate wells and the assay was repeated using the GPX-AuNPs. The plate was then read under UV-Vis, using a Victor Nivo multimode microplate reader (Perkin Elmer, USA), where absorbance spectra were generated for each sample for the wavelength range 200-800 nm. To confirm the synthesis of gold nanoparticles SPR peaks (λ_{max}) are observed between 530-540 nm of the absorbance spectra generated.

5.2.1.3. Stability testing of the *Gunnera perpensa*-mediated gold nanoparticles

The stability profiles of the two sets of AuNPs were compared using a modified method described by Thipe et al (2019). This was performed to determine the possible agglomeration of the AuNPs over time under several conditions. The nanoparticles were placed under nine treatment solutions: pH 4, pH 7, pH 10, 0.5 % cysteine (Cys), 0.5 % sodium chloride (NaCl), 0.5 % phosphate-buffered saline (PBS), 0.5 % bovine serum albumin (BSA), Dulbecco's Modified Eagle Medium (DMEM) and deionized water (control). In separate 1.5 ml Eppendorf

tubes, a ratio of 1:2 of GP-AuNPs solution to treatment was used for all nine treatments. These steps were repeated using the GPX-AuNPs. In total there were 18 tubes, which included two controls, for both AuNPs and all tubes were incubated in a humidified incubator (ThermoFisher, Forma^{TM} 310, USA) at 37 ˚C and in 5 % CO_2 for 24 h, 72 h and 1 week. At each interval, the SPR was determined using UV-Vis (Victor Nivo, Perkin Elmer, USA).

5.2.2. Characterization of the *Gunnera perpensa*-mediated gold nanoparticles

Characterization of the GP-AuNPs was conducted to determine the hydrodynamic size, core size, shape, zeta potential, crystalline lattice nature, functional group conjugation and phenolic content of the GP-AuNPs. The transmission electron microscopy (TEM) was conducted at the University of Johannesburg, Johannesburg, South Africa while all other characterization techniques were performed at the South African Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa. The phenolic content quantification was evaluated at the Department of Plant and Soil Sciences, University of Pretoria.

5.2.2.1. Core size analysis of GP-AuNPs using transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was used to determine the core size and shape of the GP-AuNPs. The aqueous GP-AuNPs (section 5.2.1.1) were analysed using the JEM-2100 transmission electron microscope (JEOL, Japan) equipped with X-MaxN 80T EDS. The micrographs obtained were analysed using ImageJ 1.50i software to determine the core size of the GP-AuNPs. The software measures the diameter of AuNPs in the micrograph by using a ratio of the number of pixels in the image to the known scale on the TEM micrograph.

5.2.2.2. Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) analysis of GP-AuNPs

Samples used for Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) analysis were fine powder GP-AuNPs obtained from freeze-drying (SP VirTis, 4KBTZL, USA) the GP-AuNPs. Fourier-transform infrared spectroscopy was used to determine the surface conjugation of functional groups of the GP-AuNPs using the Perkin Elmer spectrum 100 FTIR spectrophotometer. Measurements for percentage transmittance were obtained for the wavenumber range of 500-4000 cm^{-1} (Patel et al., 2020). The data was analysed using Origin Pro and e-FTIR software. The crystalline nature of the GP-AuNPs was determined by X-ray diffraction (XRD). The PAnalytical XPERT-PRO (Malvern Instruments, UK) with a

diffractometer using NI filtered CuKα radiation (λ= 1.5406 Å) at 45kV/40 mA was used. The data was analysed using Origin Pro software. To determine the crystalline size of GP-AuNPs, the equation from Scherrer's law was used:

$$D = \frac{k\lambda}{\beta cos\theta}$$

where: D- crystallite size (nm), k- Scherrer constant =0.9, λ- wavelength of the X-ray source, θ- peak position (radians), β- full width at half maximum (FWHM)

5.2.2.3. Zeta potential and hydrodynamic size measurement of GP-AuNPs

The Zetasizer Nano ZS (Malvern Instruments, UK) was used to determine the zeta potential (ZP) of the NPs. This was used to determine the surface charge on the AuNPs. Additionally, electrophoretic light scattering was used to ascertain the hydrodynamic size of the GP-AuNPs. The sample used was the solution of GP-AuNPs synthesized in section 5.2.1.1. Eight hundred microliters of the AuNPs was loaded into the DTS1070 folded capillary cell and then placed in the Zetasizer Nano ZS and analysis was carried out at 25 ˚C.

5.2.2.4. Phenolic content of *Gunnera perpensa*-mediated gold nanoparticles using the Folin-Ciocalteu test

The Folin-Ciocalteu test was used to quantify the phenolic content of the GP-AuNPs (Thipe et al., 2019). Firstly, a standard curve of known concentrations of the *G. perpensa* was generated. To make the stock solution (1 mg/ml) 2mg of the dried crude extract of GP (prepared in section 3.2.2, Chapter 3) was dissolved in 2 ml of deionized water. Thereafter, 1 ml of the stock solution was added to 500 µl of 7.5 % sodium carbonate solution mixed with 1 ml of 10 % Folin-Ciocalteu reagent in a 5 ml Eppendorf tube. The resultant concentration of the plant extract in the reaction tube was 400 µg/ml. This reaction tube was incubated in a water bath at 30 ˚C for 30 mins. After incubation, 400 µl of deionized water was added to eight (1 ml) Eppendorf tubes. In the first tube, 400 µl of the reaction mixture was added thereafter the remaining tubes were serially diluted ensuing plants extract concentration ranging from 400-1.56 µg/ml. In a 96-well plate, 100 µl of each concentration was added to separate wells and the absorbencies were read using UV-Vis at a wavelength of 760 nm (Victor Nivo, Perkin Elmer, USA). For the nanoparticles, the Folin-Ciocalteu test was carried out as mentioned above using 1 ml of the GP-AuNPs solution. After incubation, 100 µl of the GP-AuNPs reaction mixture was added to a well in the 96-well plate and the absorbance was read using UV-Vis as mentioned above. The plant phenolic content standard curve was generated to extrapolate the concentration of plant extract in the GP-AuNPs. Consequently, this

concentration was used to determine the antigonococcal activity and cytotoxicity of the GP-AuNPs. Therefore, the minimum inhibitory concentration (MIC) and IC_{50} values were given as plant extract equivalents (PEE µg/ml).

5.2.3. Antigonococcal testing of GP-AuNPs on *Neisseria gonorrhoeae*

The antigonococcal activity of the GP-AuNPs was determined using the microdilution method as described in section 3.2.4, Chapter 3 (Eloff, 1998). One hundred microlitres of the aqueous GP-AuNPs solution (section 5.2.1.1) were added to three wells in a 96-well plate. Subsequently, serial dilutions were conducted with concentrations ranging from 83.3-0.6 µg/ml. The negative controls used were distilled water, sterile Mueller-Hinton (MH) broth and bacteria with MH broth whilst ciprofloxacin (625-2.44 µg/ml) was used as the positive control. The plate was then incubated at 37 ˚C for 24 h. The MIC was determined visually using the PrestoBlue indicator.

5.2.4. Cytotoxicity testing of GP-AuNPs on HaCaT, HeLa and THP-1 cell lines

The cytotoxic effects were evaluated used in the PrestoBlue method as described in section 3.2.6, Chapter 3 (Lall et al., 2013). To determine the relative safety of the GP-AuNPs, testing was carried out on HeLa, HaCaT, and THP-1 cell lines. Aqueous GP-AuNPs with concentrations ranging from 38.96-1.22 µg/ml were used. Actinomycin D was used as the positive control whilst GA solution was used as the negative control.

5.3. Results and discussion

5.3.1. Synthesis, surface plasmon resonance and stability testing of GP-AuNPs and GPX-AuNPs

Two sets of gold nanoparticles were synthesized: GPX-AuNPs (without gum Arabic) and GP-AuNPs (with gum Arabic), while the GA control did not form AuNPs. The formation of red-wine coloured solutions of GPX-AuNPs and GP-AuNPs confirmed the successful synthesis of the NPs (Figure 5.1). The colour observed was due to the surface plasmon resonance (SPR) of the synthesized AuNPs (Shnoudeh et al., 2019). This is because the electromagnetic field of light cause the free electrons in the gold metal to oscillate at the same frequency as visible light causing the colour change (Badi et al., 2020; Huang and El-Sayeda, 2010). The GA control

remained a yellow solution showing that the gold ions (Au^{3+}) were not reduced (Shnoudeh et al., 2019).

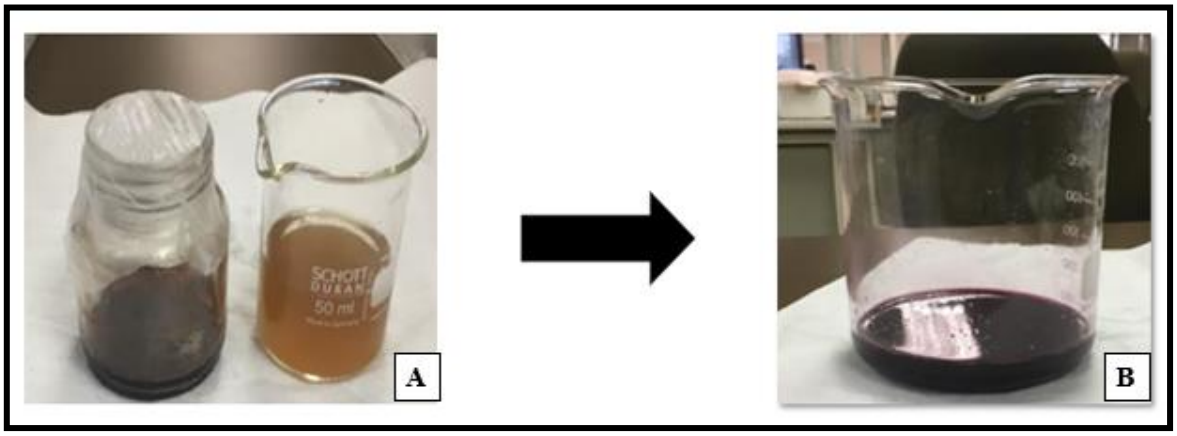

Figure 5.1: a) *Gunnera perpensa* plant extract solution used to make nanoparticles and the resultant b) GP-AuNPs solution.

The SPR (λ_{max}) of the successfully synthesized AuNPs was determined (Figure 5.2) whereby the λ_{max} of the GP-AuNPs and GPX-AuNPs were 536 nm and 542 nm, respectively. The λ_{max} of the GP-AuNPs were in the normal range of λ_{max} for AuNPs (530-540 nm) while that of the GPX-AuNPs was out of the normal range, thus further characterization studies were performed on the GP-AuNPs. Similarly, other plant-based AuNPs have been synthesized with λ_{max} within this SPR range (Ghosh et al., 2012; Patra et al., 2016; Thipe et al., 2019). Gold nanoparticles biosynthesized from *Aspalathus linearis* (Burm.f.) R. Dahlgren and *Allium cepa* L were reported to both have a λ_{max} of 535nm (Blom van Staden et al., 2021; Patra et al., 2016). The SPR influences the shape and size of NPs, narrower sharper peaks indicate monodispersed NPs of similar size and shape, hence the GP-AuNPs were more monodispersed than the GPX-AuNPs as they had a narrower λ_{max} peak (Ashkarran and Bayat, 2013; Patra et al., 2016; Srinath and Ravishankar Rai, 2015). The GP-AuNPs peak was narrower and sharper than that of the GPX-AuNPs, which could be due to the gum Arabic stabilizer in GP-AuNPs.

The stability of the two sets of the NPs was evaluated to ensure that the AuNPs would not agglomerate or flocculate resulting in clumps that may affect the bioactivity (Cartwright et al., 2020; Halamoda-Kenzaoui et al., 2017). Agglomeration results in changes in the refractive index (SPR) thus affecting the optical effects of the NPs (Kaur et al., 2021; Thilagam and Gnanamani, 2020). These optical changes result in SPR peaks flattening out. Figures 5.3-5.8 show the effects of the different treatments on the two sets of NPs after 24 h, 72 h and 1 week. Generally, the GPX-AuNPs peaks flattened out at each time interval compared to the GP-

AuNPs which had sharper peaks. Thus, GPX-AuNPs were more unstable than the GP-AuNPs. The GPX-AuNPs were most unstable in the 0.5 % NaCl after 24 h incubation while they were unstable in 0.5 % cysteine and pH 4 conditions after a week. In these three treatments, there was precipitation of the GPX-AuNPs observed. On the other hand, the GP-AuNPs were moderately stable in all conditions for all intervals tested as the SPR peaks did not flatten out. Furthermore, there was no agglomeration observed in all treatments at all intervals. The GP-AuNPs were relatively stable due to the gum Arabic added in the biosynthesis step.

Gum Arabic is a non-toxic stabilizer used to cap NPs during biosynthesis, preventing agglomeration (Thipe et al., 2019). Previously, Thipe et al. (2019) had synthesized resveratrol AuNPs stabilized with gum Arabic. These NPs had a similar stability profile as the GP-AuNPs. It has been shown that low pH and high salt conditions reduce the stability of colloidal nanoparticles (Fuller and Köper, 2018; Tseng et al., 2015). High NaCl concentrations decrease the Debye length of the NPs thus decreasing the repulsive force of the NPs (Fuller and Köper, 2018; Smith et al., 2016). Debye length is the distance at which separation between ions can occur (Bryant, 1996; Stenson et al., 2017). Subsequently, there is an increase in the Van der Waals forces between NPs which reduces monodispersity and promotes agglomeration (Fuller and Köper, 2018). Capping polymers such as gum Arabic maintain monodispersity by providing steric protection, preventing clumping up of the NPs (Musa et al., 2019; Randall et al., 1988; Williams et al., 2006).

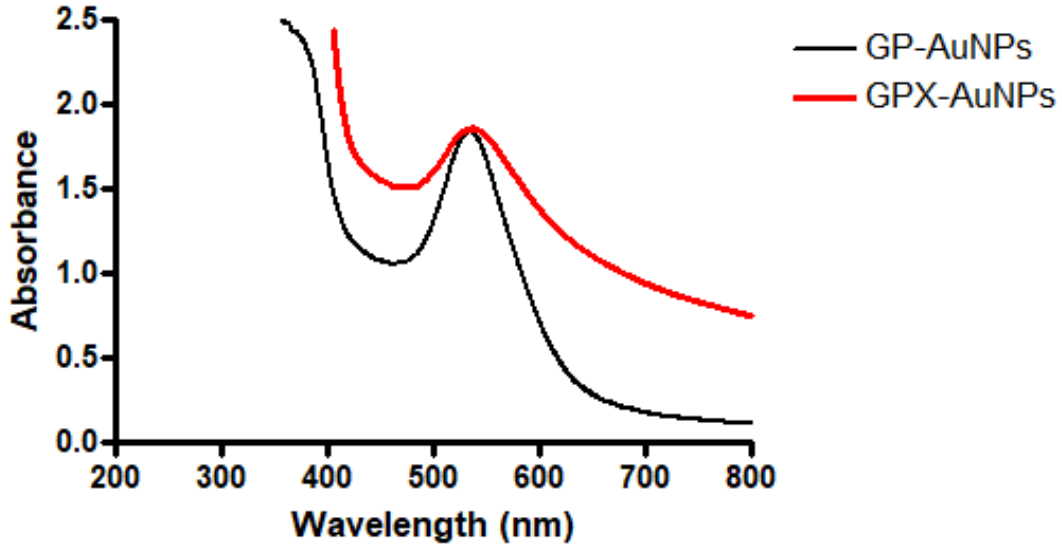

Figure 5.2: Surface plasmon resonance (SPR) of GP-AuNPs and GPX-AuNPs

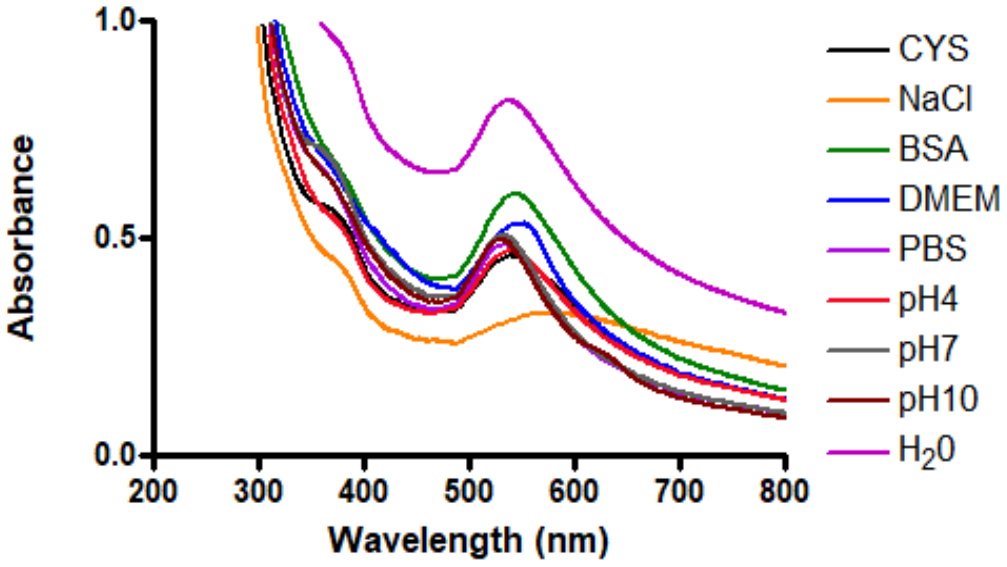

Figure 5.3: Surface plasmon resonance (SPR) of GPX-AuNPs in different biological media after 24 h

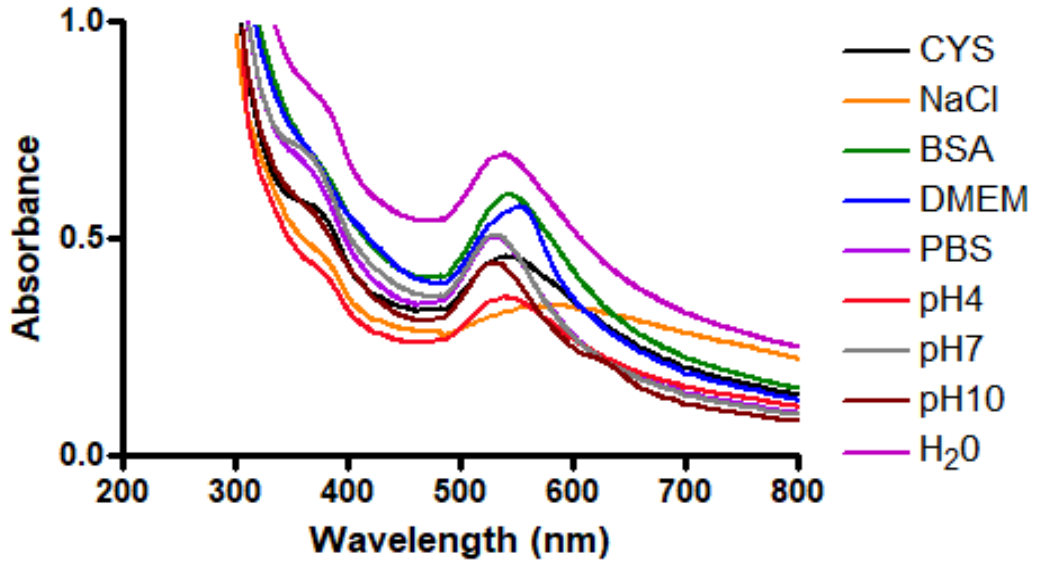

Figure 5.4: Surface plasmon resonance (SPR) of GPX-AuNPs in different biological media after 72 h

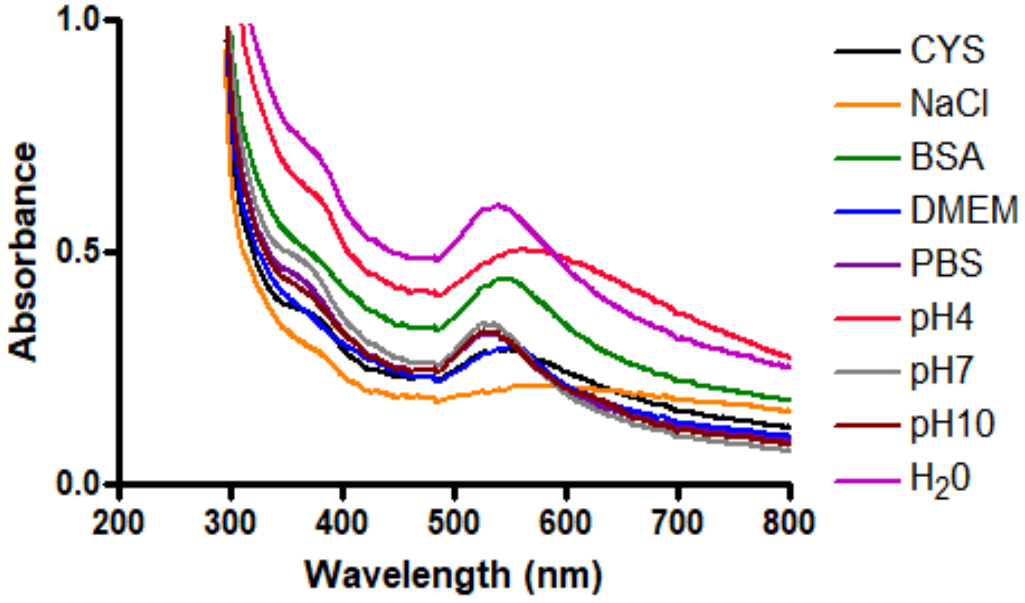

Figure 5.5: Surface plasmon resonance (SPR) of GPX-AuNPs in different biological media after 1 week

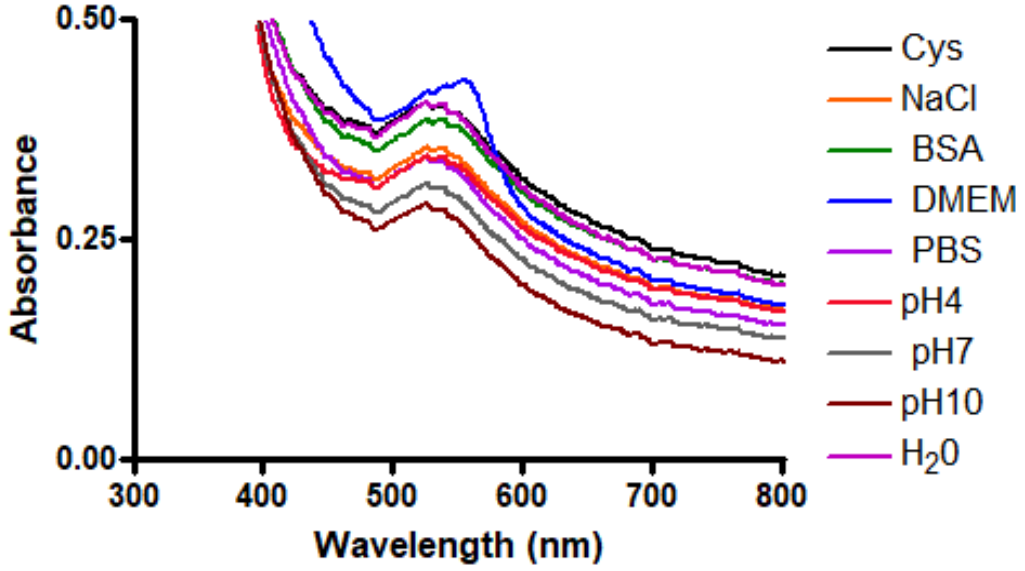

Figure 5.6: Surface plasmon resonance (SPR) of GP-AuNPs in different biological media after 24 h

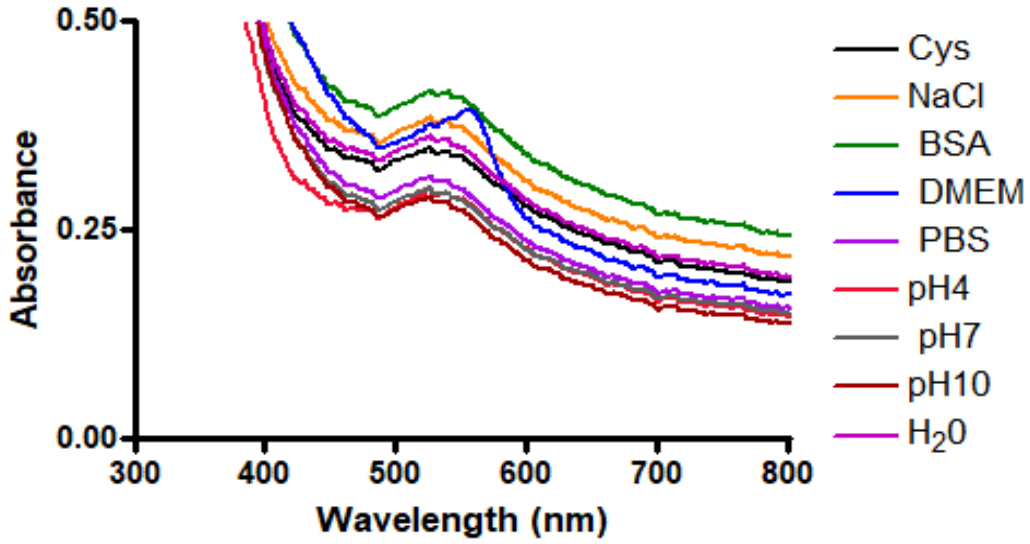

Figure 5.7: Surface plasmon resonance (SPR) of GP-AuNPs in different biological media after 72 h

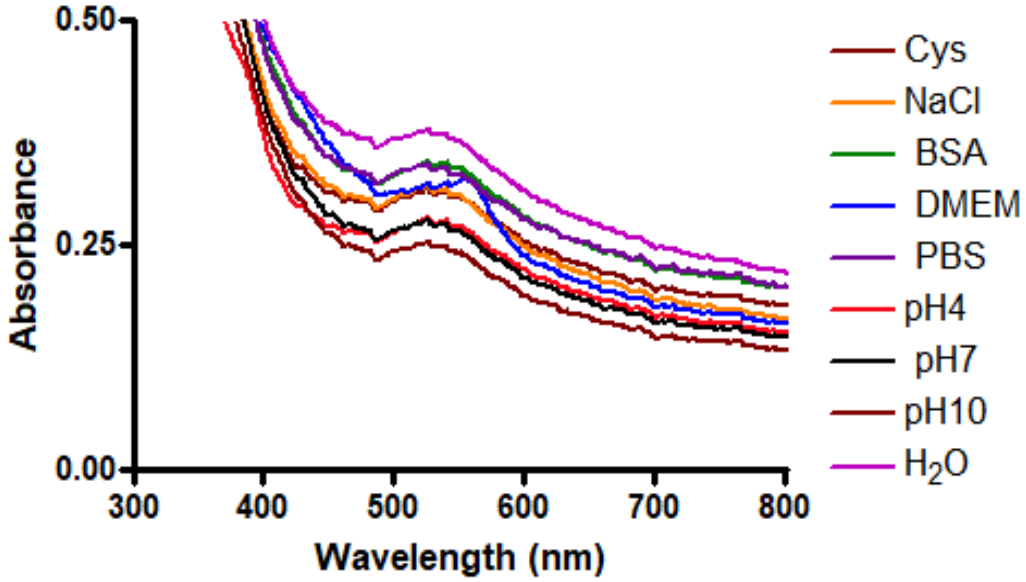

Figure 5.8: Surface plasmon resonance (SPR) of GP-AuNPs in different biological media after 1 week

5.3.2. Characterization of GP-AuNPs

5.3.2.1. Fourier-transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM) and X-ray diffraction (XRD) analysis

Further characterization was conducted on the moderately stable GP-AuNPs using TEM, FTIR and XRD. The TEM analysis of the GP-AuNPs determined the core size and shape of the NPs. The results revealed that most of the NPs were spherical while a few were triangular and hexagonal (Figure 5.9) and the NPs were dispersed. The GP-AuNPs had shapes consistent with other AuNPs that have been synthesized (De Canha et al., 2021; Dikshit et al., 2021; Khan et al., 2014). The average core size of the GP-AuNPs was 39.51±6.46 nm (Figure 5.10). Gold nanoparticles of 40 nm usually have SPR peaks around 530 nm however, the SPR of GP-AuNPs was 536 nm (Dalal et al., 2019). This difference was because the GP-AuNPs had diverse core sizes (Ashkarran and Bayat, 2013; Patra et al., 2016).

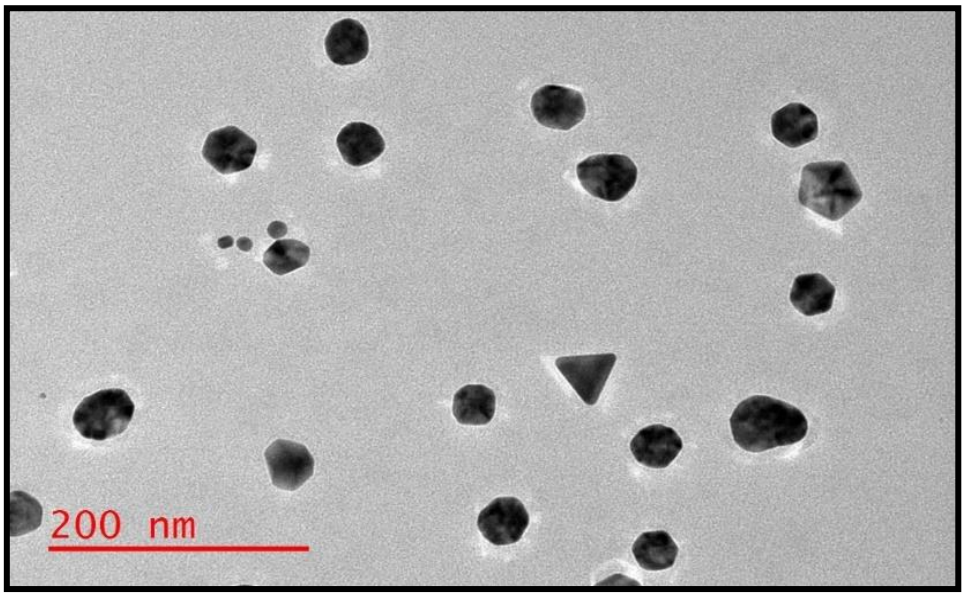

Figure 5.9: Transmission electron micrograph showing the morphology of the GP-AuNPs

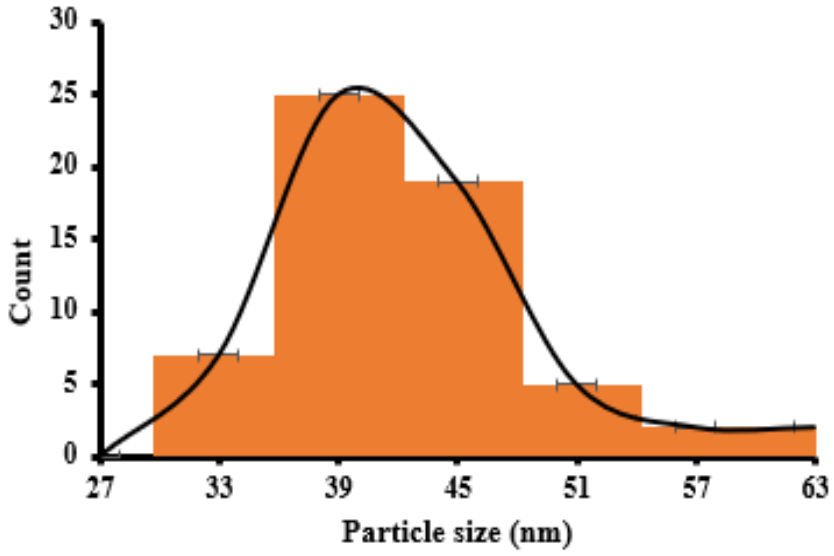

Figure 5.10: The distribution of core sizes of the GP-AuNPs

The characterization of possible functional groups, in the plant extract, which were potentially involved in the reduction of the gold to make GP-AuNPs was investigated using FTIR. The FTIR spectrum generated from the analysis showed three distinct peaks at 3312 cm^{-1}, 2352 cm^{-1} and 1642 cm^{-1} (Figure 5.11). The broad and strong peaks at 3312 cm^{-1} are characteristic of the hydrogen-bonded -OH functional group found in alcohols/phenolic compounds such as venusol that has been isolated from *G. perpensa* (Coates, 2000; Doyle and Scogin, 1988; Khan et al., 2004; Nandiyanto et al., 2019). The weak peak at 2352 cm^{-1} indicates there was an amino component (-NH) interacting with the surface of the gold of the GP-AuNPs (Nandiyanto et al., 2020, 2019). It is known that proteins (e.g enzymes) can bind to gold in NP capping to prevent agglomeration which stabilizes the NPs (Rao et al., 2002; Srinath and Ravishankar Rai, 2015). The sharp peak at 1642 cm^{-1}, in the double bond region of the FTIR spectrum, correlates to a carbonyl group (C=O) in ketone compounds such as the 1,4 benzoquinones present in *G. perpensa* (Coates, 2000; Drewes et al., 2005; Nandiyanto et al., 2019). Patel et al. (2020) had similar results where they identified the same functional groups as the major components involved in the capping of *G. perpensa* in the silver nanoparticles. The functional groups identified by FTIR are from the compounds in the ethanolic extract used to synthesize GP-AuNPs. The FTIR spectra of the plant extract and the GP-AuNPs were different (Figure 5.11) with the GP crude plant extract having more peaks, in the 500-1700 cm^{-1} region, than the GP-AuNPs. This could be because not all functional groups of the compounds in the crude extract were involved in the capping of the GP-AuNPs.

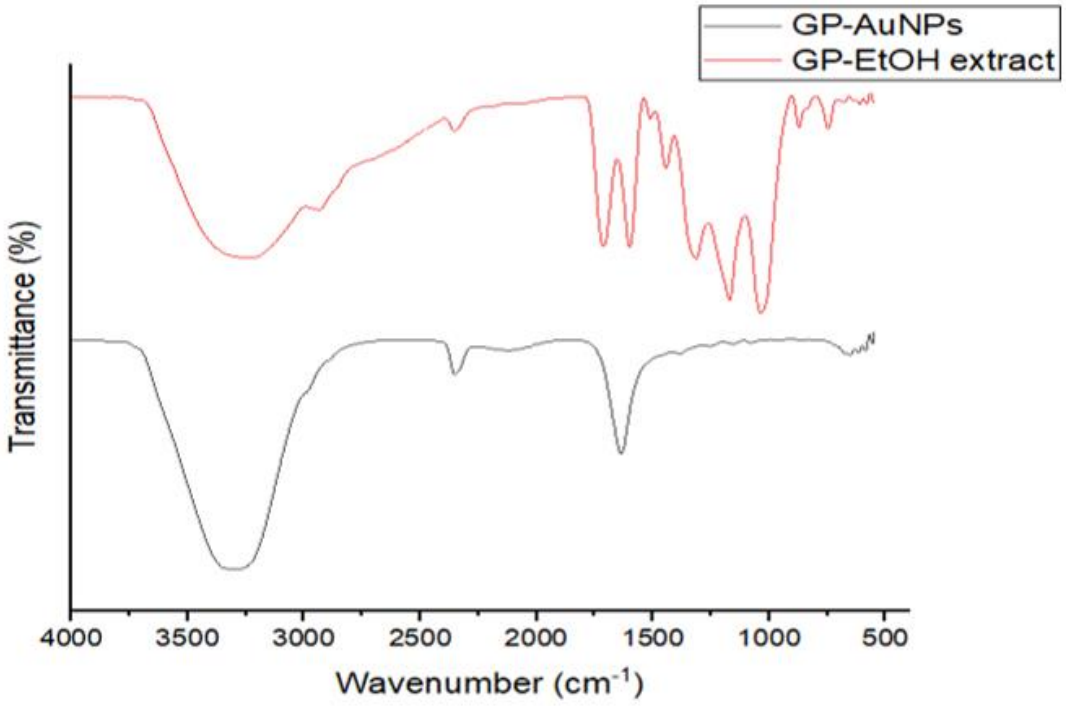

Figure 5.11: Fourier-transform infrared spectra of *Gunnera perpens*a extract and GP-AuNPs

To determine the crystalline nature of the GP-AuNPs XRD was used (Figure 5.12). Four distinct peaks at 38.58˚, 44.80˚, 64.53˚ and 76.98˚ were observed with planes indexed at (111), (200), (220) and (311), respectively, using Bragg's law. These diffraction indices are consistent with cubic face-centred (FCC) gold particles according to the American Mineralogist Crystal Structure Database (AMCSD, No. 0015133) and Joint Committee Powder Diffraction Standards USA (JCPDS, No. 00-004-0784) confirming the synthesis of the gold nanoparticles of *G. perpensa*. Similar results have been recorded in other studies of plant-mediated AuNPs (Patra et al., 2016). Additionally, the crystallite size of the AuNPs and *d*-spacing (interplanar spacing) between crystals were determined (Table 5.1). Most of the crystals were ~ 6.4 nm, which corresponds to the highest peak at 38.58˚ in Figure 5.12. The *d*-spacing lattice parameter is defined as the distance between crystals in parallel planes (Cullity, 1978). The most abundant crystals had a *d*-spacing of ~2.34 nm. Furthermore, the *d*-spacings for all gold crystal species confirmed the synthesis of AuNPs as every mineral has a unique set of *d*-spacing parameters (Davey, 1925).

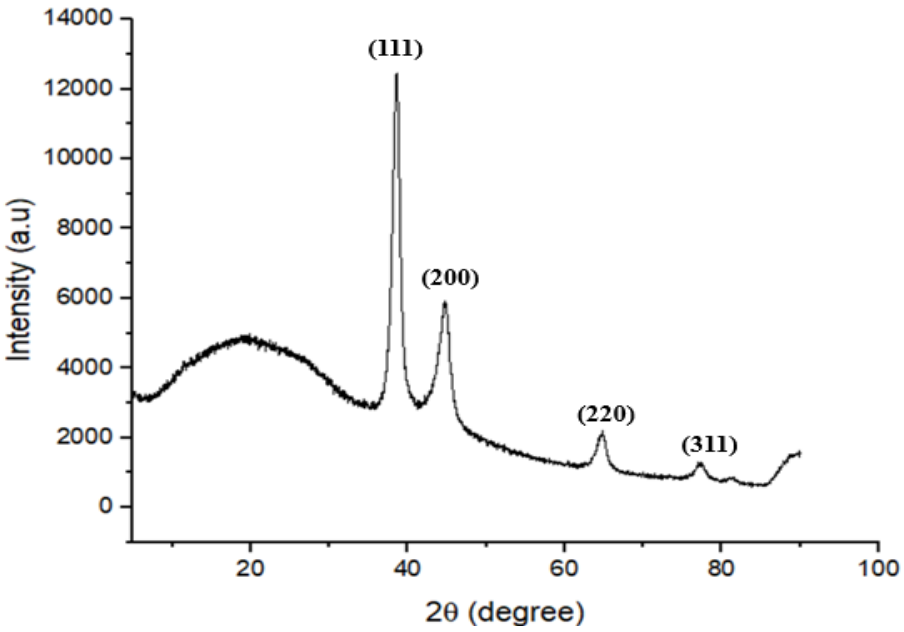

Figure 5.12: The X-ray diffraction pattern of the GP-AuNPs

Table 5.1: Crystallite size of GP-AuNPs and *d*- spacing between the crystals

Peak position (2θ)	Crystallite size (nm)	*d*- spacing (nm)[a]
38.58	6.414122389	2.335726
44.80	4.118781208	2.030632
64.53	3.155814723	1.440449
73.98	0.11133364	1.281334

[a] distance between crystals in parallel planes of the gold nanoparticles

5.3.2.2. Zeta potential and hydrodynamic size

The hydrodynamic size (Z-average) of GP-AuNPs and zeta potential (ZP) were determined. The electrophoretic light scattering/dynamic light scattering (DLS) showed that the GP-AuNPs were 127.2±1.56 nm. The DLS was also used to determine the size distribution of the NPs. The GP-AuNPs were relatively monodispersed due to the low polydispersity index (PDI) of 0.192. According to the International standards organizations (ISOs), highly monodispersed NPs have PDI <0.05 while PDI >0.7 show great size variations (Danaei et al., 2018; Mudalige et al., 2019). For therapeutic action NPs with PDI <0.2 are acceptable (Danaei et al., 2018). It is interesting to note that the hydrodynamic size of the GP-AuNPs (127.2 nm) was larger than the core size (39.51 nm) obtained from TEM. This is because the hydrodynamic size of NPs

includes the nanoparticle core and the capping agent. Gum Arabic is a high molecular weight polymer consisting of a combination of glycoproteins and polysaccharides (Ahmed, 2018; Gamal-Eldeen et al., 2016). The highly branched nature of the molecule increases the hydrodynamic size of NPs (Ahmed, 2018; Thipe et al., 2019). Likewise, Thipe et al. (2019) have synthesized gum Arabic resveratrol AuNPs with a Z-average of 187.7 nm. The overall size of the GP-AuNPs makes them an ideal size for vaginal delivery (<340 nm) (Ensign et al., 2014; Leyva-Gómez et al., 2018; Rossi et al., 2019). Lastly, the ZP is the measure of surface charge on the NPs which indicates stability. Usually ZP of > +30 mV and < -30 mV is used to predict the stability of the NPs (Geraldes et al., 2016). The GP-AuNPs had a ZP of -5.34±0.25 mV which was in the unstable range however, the GP-AuNPs solution did not show any agglomeration under the various treatments in section 5.2.1.3. The GP-AuNPs were thus regarded to be moderately stable. The absence of agglomeration and separation in the GP-AuNPs could have been due to the gum Arabic stabilizer. The capping agent used in synthesis is known to bulk up NPs providing steric protection, eliciting stability (Musa et al., 2019; Randall et al., 1988).

5.3.2.3. Phenolic quantification of GP-AuNPs

To quantify the amount of plant extract in the GP-AuNPs, the phenolic content was determined using the Folin-Ciocalteu test (Thipe et al., 2019). This was to give an arbitrary value of the amount of plant extract present in the gold nanoparticles, which would be used to determine the bioactivity of the GP-AuNPs. The phenolic content of the GP-AuNPs was extrapolated from the standard curve of the *G. perpensa* crude extract (Figure 5.13). The GP-AuNPs had 332.86 µg/ml of phenolics using the equation y=0.0015x + 0.0457 where y is 0.545 nm and x is 332.86 µg/ml. This was then used for calculations to determine MIC and IC_{50} values from the antigonococcal and cytotoxicity assays.

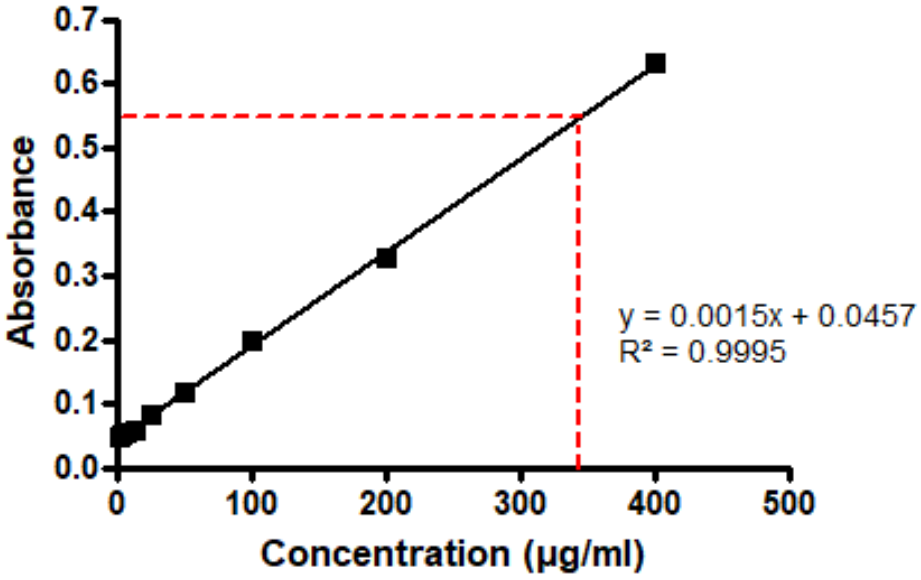

Figure 5.13: Phenolic content standard curve of *Gunnera perpensa* extract

5.3.3. Antigonococcal activity of GP-AuNPs against *Neisseria gonorrhoeae*

The antigonococcal assay of the GP-AuNPs showed that the MIC was 10.4 µg/ml (Table 5.1). This was lower than the 48.7 µg/ml from the crude extract. Thus, the results indicated that the GP-AuNPs has better antigonococcal activity than *G. perpensa* ethanolic extract. Metal NPs are known to disrupt cell walls, induce reactive oxygen species and disrupt the metabolic processes of bacteria (Aderibigbe, 2017; Hajipour et al., 2012; Sánchez-López et al., 2020). Nanoparticles also form strong interactions with N, O and S atoms of bacterial molecules thus having a broad spectrum of activity (Cui et al., 2012; Sánchez-López et al., 2020; Shamaila et al., 2016). There have not been any AuNPs of *G. perpensa* that have been synthesized or tested on *N. gonorrhoeae.* However, silver (Ag)NPs have been synthesized using GP and have been reported to exhibit antibacterial activity on non-STD pathogens (Patel et al., 2020). Silver NPs prepared from methanolic extracts of GP exhibited MICs of 6.3 µg/ml on *Escherichia coli* (P4055) and *Staphylococcus aureus* (S5878) while aqueous AgNPs prepared from GP had MICs of 3.2 µg/ml against both bacteria (Patel et al., 2020). Other AgNPs that have antigonococcal properties have been developed but cytotoxicity has been noted at high concentrations due to the silver (Damelin et al., 2015; Dhanasezhian et al., 2019; Li et al., 2013). Li et al. (2013) synthesized cefmetazole-AgNPs with a MIC of 12.5 µg/ml against an MDR strain of *N. gonorrhoeae* (ATCC 49226) however these NPs had relatively inferior antigonococcal activity compared to the GP-AuNPs (MIC=10.4 µg/ml). The difference in bioactivity observed could be due to different strains of *N. gonorrhoeae* that were used.

Plant-based AuNPs with activity against other STD pathogens have been synthesized. Dhanasezhian et al. (2019) synthesized *Sargassum wightii* AuNPs which had antiviral activity against HSV-1, which causes herpes. Folorunso et al. (2019) produced *Annona muricata* L AuNPs, which were tested on *Candida albicans*, which resulted in 42 % inhibition at 4 µg/ml. Many AuNPs have been synthesized for HIV treatment with antiviral drugs being used as bioactive ingredients (Bowman et al., 2008; Chiodo et al., 2014; Garrido et al., 2015; Kesarkar, 2015). Other AuNPs have been synthesized against non-STD pathogens (Aljabali et al., 2018; Dubey et al., 2010; Patra et al., 2016; Senthilkumar et al., 2017).

5.3.4. Cytotoxicity effects of GP-AuNPs on HaCaT, HeLa and THP-1 cell lines

HaCaT cells are immortalized human keratinocytes, while THP-1 cells are immortalized human monocyte cells (Bosshart and Heinzelmann, 2016; Chanput et al., 2014; Seo et al., 2012; Wilson, 2013). HeLa cells are derived from cervical cancer cells (Chen et al., 2013; Li et al., 2018). These cell lines were used to evaluate the potential cytotoxic effects of GP-AuNPs. The GP-AuNPs had IC_{50} values of 22.12±0.52 µg/ml, 41.98±10.65 µg/ml and 27.53±6.02 µg/ml, on HaCaT, HeLa and THP-1 cell lines, respectively (Table 5.2). The GP-AuNPs were very cytotoxic to HaCaT and THP-1 cells as the IC_{50} was <30 µg/ml. While the GP-AuNPs were moderately cytotoxic to HeLa cell lines as the IC_{50} was between 30 µg/ml and 50 µg/ml (Kuete and Efferth, 2015; Steenkamp and Gouws, 2006; Suffiness and Pezzuto, 1990). The GP-AuNPs were more cytotoxic to the selected cell lines than the GP plant extract (IC_{50} >400 µg/ml; Chapter 3) on the three cell lines used. Currently, no *in vitro* studies have been conducted on HaCaT, HeLa and THP-1 cytotoxicity using *G. perpensa* or GP-AuNPs. Several studies have shown that *G. perpensa* does not exhibit cytotoxic effects (Brookes and Smith, 2003; Mwale and Masika, 2011). Methanolic root extracts of *G. perpensa* were not cytotoxic to human fibroblast (MRC5) cells since the IC_{50} was >1000 µg/ml (Steenkamp et al., 2004). Additionally, aqueous root extracts of GP have also been reported to be non-toxic to human embryonic kidney (HEK293) cells with IC_{50} values of 279.43 µg/ml (Simelane et al., 2012). Presently, there are limited studies on the toxic effects of AuNPs on non-cancerous cell lines. Thipe et al. (2019) showed that resveratrol AuNPs had IC_{50}s of 72 µg/ml on pancreatic (PANC-1) and breast cancer (MDAMB-231) cells while having an IC_{50} of 59 µg/ml on prostate (PC-3) cells. Gold nanoparticles have selective cytotoxic effects on cell lines based on their surface charge due to the functional groups present in the NPs (Boisselier and Astruc, 2009; Butterworth et al., 2010; Patra et al., 2007). It has been noted that very anionic AuNPs (≤ -

30mV) are less cytotoxic than cationic ones (Goodman et al., 2004; Jeon et al., 2018; Murphy et al., 2008). The zeta potential of GP-AuNPs was closer to zero at -5.34 mV hence the GP-AuNPs were slightly anionic which could explain the cytotoxicity effects observed. From this study, the cytotoxicity effects noted show that GP-AuNPs may not be ideal to treat gonorrhoea however, they may be used to treat cervical cancers caused by the human papilloma virus (HPV). This is because the NPs was relatively cytotoxic to HeLa cells with an IC_{50} of 48.88 µg/ml. In future, the GP-AuNPs synthesis may be optimized to produce more anionic NPs which may reduce the cytotoxicity of the NPs. Additionally, other polymers such as poly(lactic-co-glycolic acid) (PLGA) or polyethylene glycol (PEG) may be considered as carriers for the *Gunnera perpensa* extract and could potentially result in the synthesis of non-toxic NPs

Table 5.2: Antigonococal and cytotoxicity effects of *Gunnera perpensa*-mediated gold nanoparticles and the *G. perpensa* crude extract

Sample	**MIC[a]**	**IC_{50}[b]±SD[c] (µg/ml)**		
		HeLa[d]	**HaCaT[e]**	**THP-1[f]**
GP-AuNPs[g]	10.4	41.98±10.65	22.12±0.52	27.53±6.02
GP extract[h]	48.7	>400	>400	>400
Ciprofloxacin[i]	<5	-	-	-
Actinomycin D[j]	-	$2.1\times10^{-3}\pm5.5\times10^{-4}$	$1.9\times10^{-3}\pm4.6\times10^{-4}$	$8.4\times10^{-2}\pm6.8\times10^{-3}$

[a]Minimum inhibitory concentration, [b] Fifty percent inhibitory concentration, [c] Standard deviation, [d] Human cervical cancer cells, [e]Human keratinocytes, [f] Human monocytes, [g]*Gunnera perpensa*-mediated gold nanoparticles, [h] Ethanolic crude extract of *Gunnera perpensa*, [i] Positive control for the antigonococcal assays, [j] Positive control for the cytotoxicity assays, - not tested

5.4. Conclusion

The findings of this study indicate *G. perpensa* may be used to make gold nanoparticles. The particles were of ideal size (<340 nm) to be used for vaginal delivery and were relatively stable. The GP-AuNPs had superior antigonococcal activity compared to the crude plant extract thus supporting claims that NPs can increase the efficacy of bioactive agents. However, the cytotoxicity testing showed that the GP-AuNPs were toxic thus they may not be ideal for the treatment of GC infections. It is recommended that the GP-AuNPs from this study be further

explored for the treatment of cervical cancer as the NPs were toxic to HeLa cells. In future, to improve the safety of the GP-AuNPs for the treatment of gonorrhoea the AuNP synthesis may be optimized to reduce the cytotoxicity. Furthermore, other non-toxic organic polymers may be used to make *Gunnera perpensa* -mediated nanoparticles that can be used in therapeutics to treat gonorrhoea.

Chapter 6: General discussion and conclusion

6.1. Discussion

In this study, the focus was on the plant-based treatment for gonorrhoea. Gonorrhoea is the second most prevalent bacterial sexually transmitted disease (STD) worldwide (WHO Regional Office for Africa, 2018; World Health Organization, 2019). The disease predominately affects the African region due to various factors which include: low socioeconomic status, the onset of sexual activity and general risky sexual behaviours (Barnes and Holmes, 1984; Oller et al., 1970). Similarly, in South Africa gonorrhoea is the most prevailing bacterial STD alongside chlamydia (Kularatne et al., 2018a, 2018b). The high infection rates burden the available first-line antibiotics resulting in antibiotic-resistant *Neisseria gonorrhoeae.* The obstacle of antibiotic resistance calls for developing new therapies for the treatment of gonorrhoea. Plants thus offer avenues that can be explored for drug discovery.

Usage of traditional medicine has aided in the treatment of diseases for centuries and continues to be used as a main primary care service in some developing countries (Mothibe and Sibanda, 2019; Nchinda, 1976; Yuan et al., 2016). Thus, it is important to evaluate the safety of these prescriptions to ensure that patients are not harmed. The validation process is important as the herbal medicine sector is not regulated thus there is a lack of standardized dosages (Kumari and Kotecha, 2016; Sachan et al., 2016). In this study, it was seen that *Gunnera perpensa* had superior antigonococcal activity than the other selected plants and did not exhibit any cytotoxic effects. Thus, validating its traditional use for STD treatment.

In history, it has been seen that plants can provide compounds that can be used in medicine (Achan et al., 2011; Licciardi and Underwood, 2011). Various drugs have been developed from plants for ailments such as cancer, malaria, pain, and STDs (Bonnez et al., 1994; Centers for Disease Control and Prevention, 2015b; Desborough and Keeling, 2017). In this study, attempts were made to separate compounds from *G. perpensa* crude extract using liquid-liquid partitioning. There was evidence that the water and butan-1-ol fractions had better antigonococcal activity than the crude plant extract. This suggests that pure compounds maybe isolated in future studies. Additionally, derivatization may be done to improve bioactivity as was the case with aspirin (Desborough and Keeling, 2017).

In the midst of high prevalence of gonorrhoea and antibiotic-resistant *N. gonorrhoeae* there is a need to improve bioactivity. In this study, one of the aims was to look at possibly targeting the vaginal delivery system to improve the bioavailability of the plant-based medicine. This was so because women are mostly affected by the disease. The study aimed at improving the antigonococcal activity of plant extracts by synthesis of gold nanoparticles (AuNPs). It is known that the miniature nature of AuNPs increases the surface-to-volume ratio and thus decreases the amount of bioactive ingredients required to give a therapeutic action (Leyva-Gómez et al., 2018). The *G. perpensa*-mediated gold nanoparticles (GP-AuNPs) synthesized were 127.2 nm thus, they have the ability to pass the cervicovaginal mucus (CVM) barrier of the vagina as they were less than 340 nm (Cone, 2009; Rossi et al., 2019). The GP-AuNPs synthesized were stable and had improved antigonococcal activity than the crude plant extract. However, the GP-AuNPs were cytotoxic to all cell lines used which is a cause for concern. Thus, they are not viable for use in the treatment of gonorrhoea but may be further explored for cancer treatments. To address the cytotoxicity concerns, the biosynthesis of the GP-AuNPs will have to be optimized and organic polymers used to reduce cytotoxicity ultimately improving the safety of the NPs for treatment of gonorrhoea. This study is the first to report the antigonococcal activity of *G. perpensa* ethanolic root extract (GP) and the synthesized gold nanoparticles (GP-AuNPs).

6.2. Take home message

In conclusion, the study successfully addressed the main aim, which was to evaluate the potential of medicinal plants for the treatment of gonorrhoea. The study was able to validate the use of the five selected plants for the treatment of STDs by traditional healer (TDr) Lulama. *Gunnera perpensa* was identified as a good bioactive agent that can be used for further drug development research. This research has great impact since it provides additional knowledge to the scientific community to enable collaborative research between scientists to better understand the treatment of gonorrhoea. This will allow for the development of novel efficient therapeutics as alternatives to the current regimens in light of the recent surge in antibiotic resistance of *N. gonorrhoeae*. Furthermore, the study is well aligned with goal 3 of the United Nations Sustainable Development Goals (SDGs) aiming to ensure healthy lives and promote wellbeing for all at all ages. This is crucial to ensure that the world population, especially South Africans, do not suffer economic losses due to people being sick with gonorrhoea and further

improve the health of individuals affected by this disease. Lastly, the study used indigenous knowledge systems to find bioactive plant extracts to treat gonococcal infections, thus linking social sciences with natural applied sciences.

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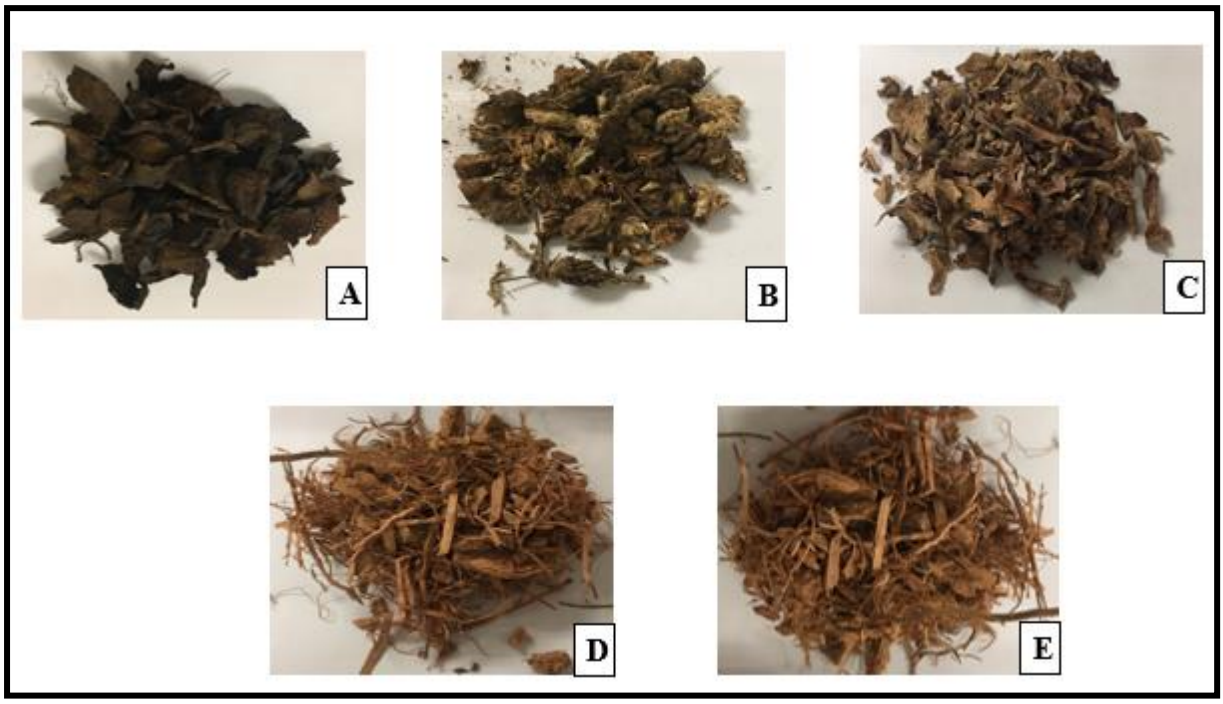
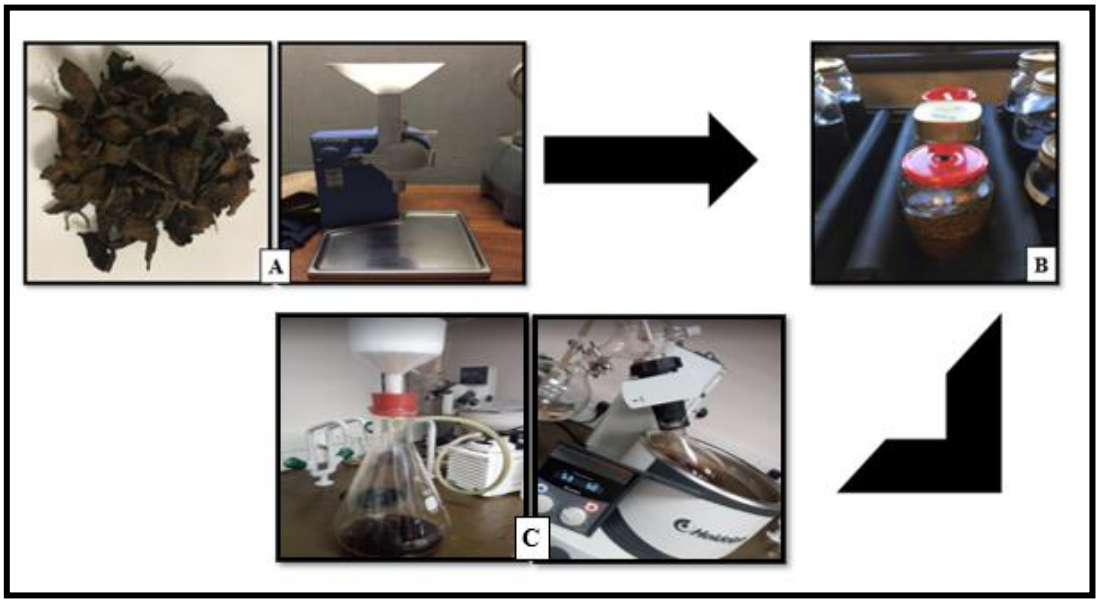
Appendix A

A1. Plant material and extraction

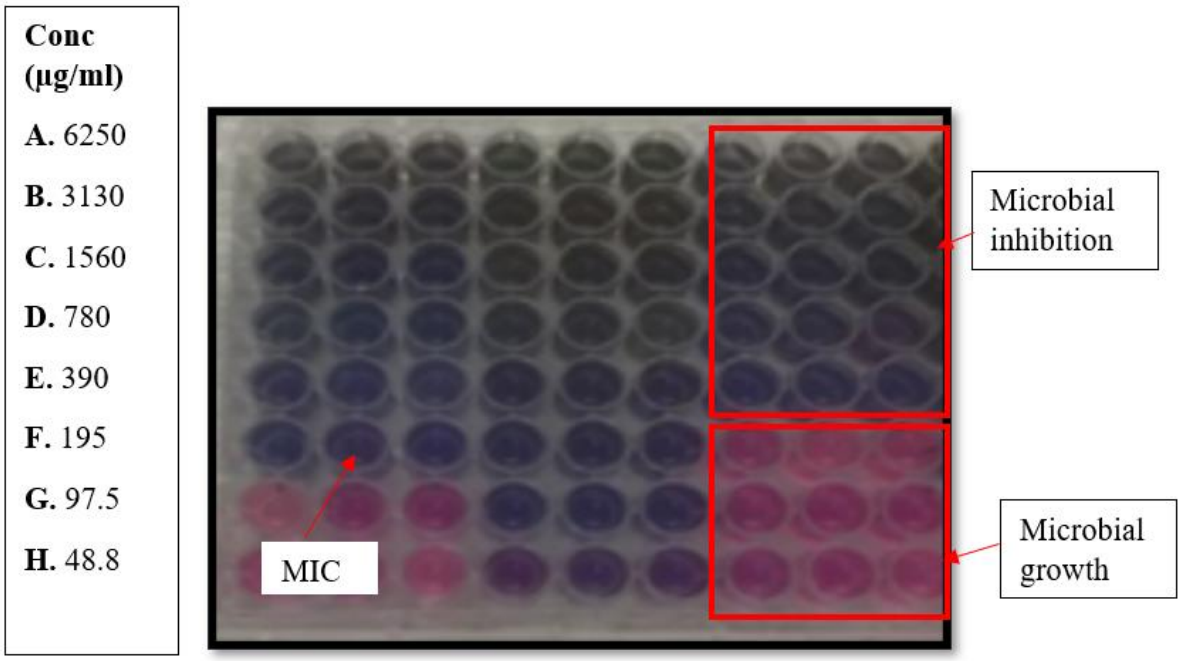


Shade dried plant material used in the study a) *Gunnera perpensa* b) *Gnidia kraussiana* c) *Pentanisa prunelloides* d) *Rhoicissus digitata* e) *Rhoicissus tridentanta*



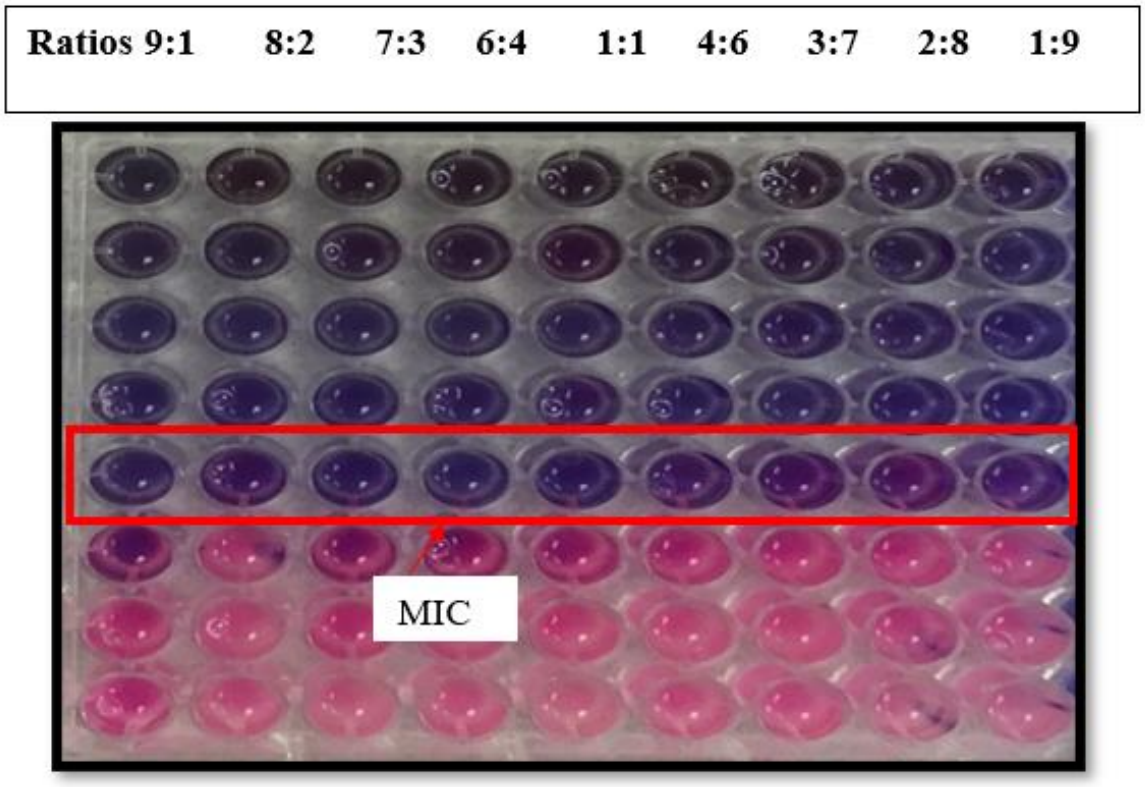
The extraction process whereby a) the dried plant material is ground then b) macerated and finally c) filtered and concentrated using a rotary evaporator.

A2. Antigonococcal assay



The serial dilution concentrations on the 96-well plate used in the microdilution assay.

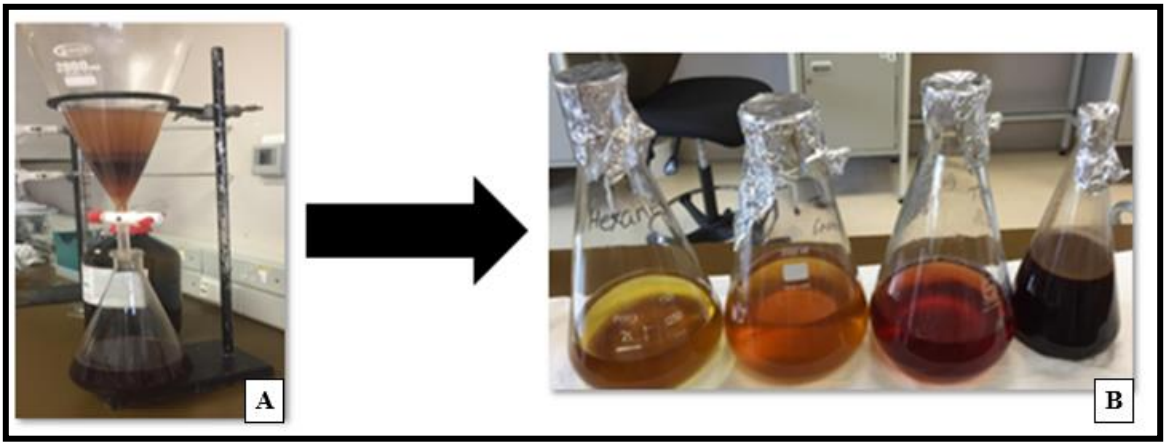
A3. Synergistic assay



Dilution method showing synergistic assay for *Gunnera perpensa*: *Gnidia kraussiana*.

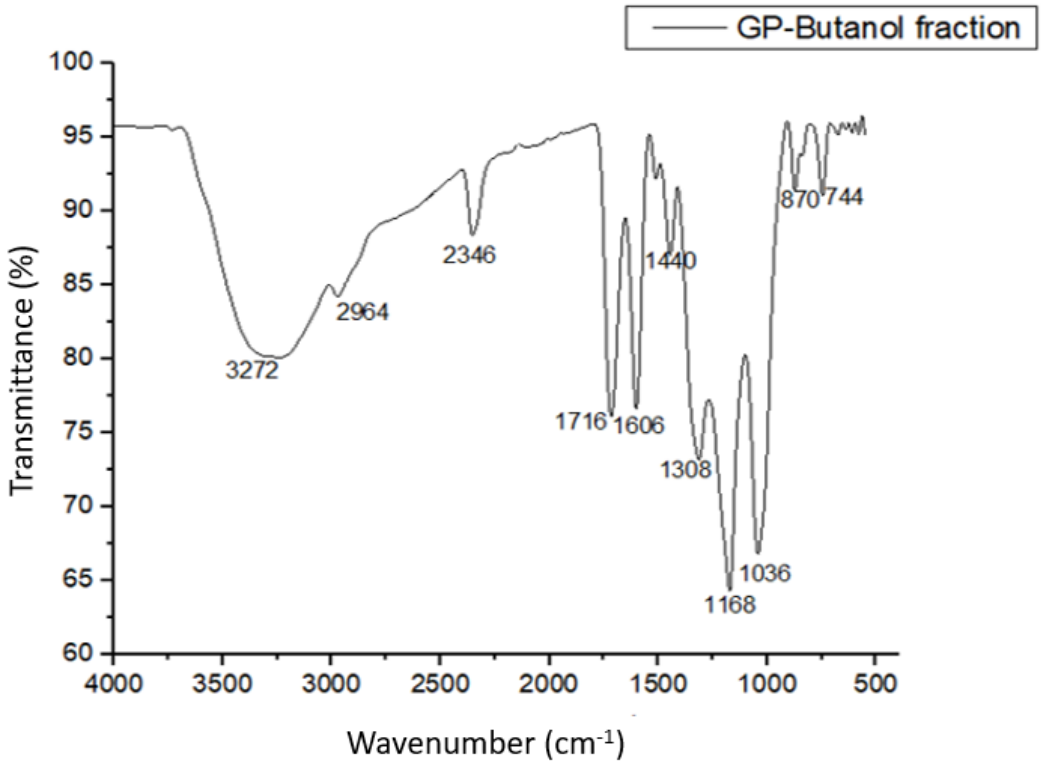
Appendix B

B1. Fractionation using liquid-liquid partitioning

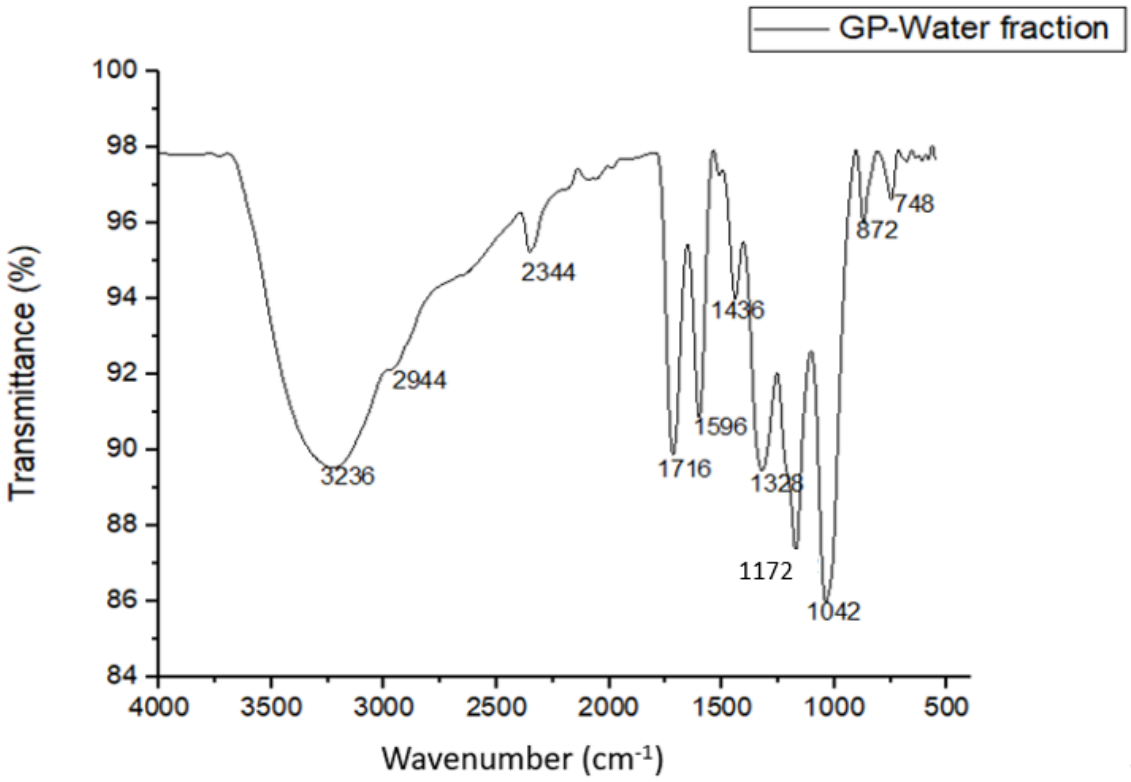


a) Fractionation by liquid-liquid partitioning and b) the resultant fractions (hexane, ethyl acetate, butanol and water)

B2. Fourier-transform infrared spectra for the bioactive fractions from *Gunnera perpensa*



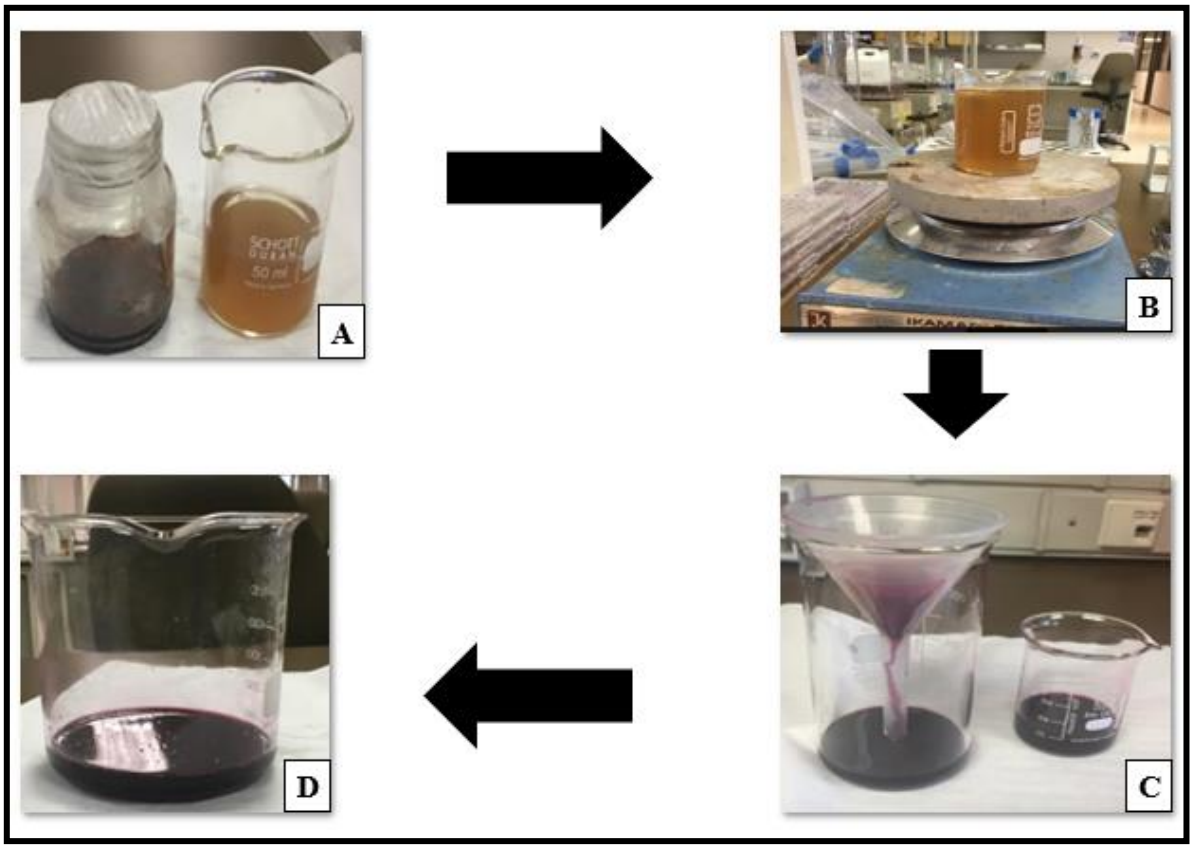
FTIR spectrum for the butan-1-ol bioactive fraction from *Gunnera perpensa*



FTIR spectrum for the water bioactive fraction from *Gunnera perpensa*

Appendix C

C1. Gold nanoparticle synthesis



a) Dissolve *Gunnera perpensa* plant extract in deionized water. b) Stir the mixture on a magnetic hot plate with magnetic stirrer bars and add gold salt solution at 60˚C. c) Solution is filtered. d) Filtrate with red wine aqueous solution of NPs.