INTERACTIVE EFFICACIES BETWEEN FEVER-REDUCING PLANTS AND SILVER NANOPARTICLES

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Pharmacy,

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DECLARATION

I, Mahlatse Evah Lediga, hereby declare that this dissertation is my own work, with all other sources of information acknowledged by means of a complete reference list. This dissertation is being submitted in fulfilment of the degree of Master of Pharmacy, at the University of the Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination to this or any other university.

M.E. Lediga

06/03/2019

Date

ABSTRACT

Fever is a common symptom in infectious disease and one of the compelling factors for seeking healthcare. Traditional healthcare in South Africa is still widely relied on by developing communities. A literature review reveals that numerous medicinal plants are frequently used in the treatment of fever. Additionally, nanotechnology has received widespread attention in the 21st century in various fields of research. Of particular interest in this research is their antimicrobial properties. This study is aimed to investigate the antimicrobial properties of fever- reducing medicinal plants and whether these properties may be enhanced by the formation of Silver Nanoparticles (AgNPs) capped with compounds from these extracts.

A total of 80 medicinal plants (40 aqueous and 40 organic extracts) related to fever treatment were screened for their antimicrobial properties. The microdilution minimum inhibitory concentration (MIC) assay was applied and nine test pathogens were selected for further study based on their ability to cause fever through bloodstream infections (septicaemia). The lowest MIC value was displayed by the organic extracts of *Eucalyptus globulus* Labill. (MIC of 4.00 µg/mL) against *Clostridium perfringens* (ATCC 13124). High antimicrobial activity was also demonstrated by the organic extracts of *Helichrysum odoratissimum* (L.) Sweet against *C. perfringens* (MIC of 0.01 mg/mL) and *Listeria monocytogenes* (ATCC19111) (MIC of 0.13 mg/mL). With regards to the aqueous extracts, *Gunnera perpensa* L. proved to be the most active medicinal plant, displaying noteworthy activity against *Klebsiella pneumoniae* (ATCC 13883) (MIC of 0.5 mg/mL), *Staphylococcus aureus* (ATCC 25723) (MIC of 0.5 mg/mL) and against *Serratia marcescens* (ATCC 13880) (MIC of 0.13 mg/mL).

Ten well-known medicinal plant species (aqueous extracts) were selected for the synthesis of AgNPs. The AgNPs were synthesised using a one-pot green synthesis approach. Characterisation was done using the UV-Vis spectroscopy, Fourier-transform infrared spectroscopy (FTIR), Zeta sizer, and Transmission electron microscope (TEM). The antimicrobial properties of the AgNPs were tested using the MIC assay, against two Gram-positive and two Gram-negative pathogens.

A general increase in antimicrobial properties was observed. The AgNPs of three medicinal plants demonstrated noteworthy activity against all the four pathogens tested. *Gunnera perpensa* demonstrated MIC values of 0.63 mg/mL against *Enterococcus faecalis* (ATCC 29212) and *L.*

monocytogenes, and MIC values of 0.16 mg/mL against *Acinetobacter baumannii* (ATCC 19606) and *K. pneumoniae*. *Sclerocarya birrea* demonstrated MIC values of 0.31 mg/mL against *E. faecalis* and *L. monocytogenes*, MIC values of 0.16 mg/mL against *A. baumannii*, and 0.69 mg/mL against *K. pneumoniae*. *Eucomis autumnalis* demonstrated MIC values of 0.63 mg/mL against *L. monocytogenes*, *A. baumannii* and *K. pneumoniae*, and MIC values of 0.16 mg/mL against *E. faecalis*. The highest increase in antimicrobial activity was noted for the AgNPs of *T. violacea* and *P. sidoides* where > 100 fold increase was observed against *E. faecalis* and > 78.14 fold increase was observed against *A. baumannii* respectively.

Medicinal plants that were found to have noteworthy antimicrobial activity from MIC assays, together with the synthesised AgNPs, were further screened for toxicity using the Brine Shrimp Lethality Assay (BSLA). Only 28% of the organic medicinal plants were found to be toxic to the brine shrimp, and 21% of the aqueous extracts were toxic. With regards to AgNPs toxicity screening, only the AgNPs of *G. perpensa* were toxic, with a percentage mortality of 56.5%. The remaining AgNPs displayed a percentage mortality less than 50%.

The outcomes obtained from this study suggest that the potential of fever medicinal plants in antimicrobial studies should not be overlooked. Furthermore, it was evident that the incorporation of nanotechnology to enhance antimicrobial efficacy is a feasible avenue to explore in potentiating these extracts against bacterial infections.

DEDICATIONS

This dissertation is dedicated to my precious mother, **Mamathole Margaret Lediga Benjamin**.

Thank you, Mama, for working twice as hard to pillar my success. You have robbed yourself of many hours of sleep praying for me. You have spent your last penny investing in me. I am forever grateful to God for choosing you to be my mother. May I always make you proud.

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LIST OF PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS STUDY

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Lediga, M.E., van Vuuren, S.F., Olivier, D.K. Antimicrobial evaluation of fever-reducing medicinal plants and effects when combined with synthesized silver nanoparticles. Oral presentation for the School of Therapeutic Sciences Biennial Research Day, University of the Witwatersrand (Faculty of Health Sciences), Johannesburg, South Africa, September 12, 2017. (Refer to Appendix C for abstract).

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LIST OF ABBREVIATIONS

%	Percent
°C	Degrees celsius
µg	Microgram
µL	Microlitres
AD	Anno domini
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
Aq	Aqueous
ART-FTIR	Attenuated total reflectance-fourier-transform infrared spectroscopy
ATCC	American type culture collection
ATP	Adenosine tri-phosphate
BC	Before Christ
BSLA	Brine-shrimp lethality assay
CFU	Colony forming units
Cip	Ciprofloxacin
CO_2	Carbon dioxide
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
g	Grams
hrs	Hours
IL	Interleukin
INT	*p*-Iodonitrotetrazolium violet
LC_{50}	Concentration of test substance causing 50% brine-shrimp death

MIC	Minimum inhibitory concentration
Min	Minutes
mL	Millilitres
MTT	Methly thiazolyl tetrazolium
$NaBH_4$	Sodium borohydride
NAD	Nicotinamide adenine dinucleotide
NCCLS	National committee for clinical laboratory standards
nm	Nanometres
NSAID	Non-steroidal anti-inflammatory drug
POAH	Preoptic area of anterior hypothalamus
spp.	Species
SPR	Surface plasmon resonance
TEM	Transmission electron microscope
TGB	Thioglycollate broth
TNFα	Tumour necrosis factor
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UV-Vis	Ultraviolet-visible
WHO	World health organisation

CHAPTER 1

INTRODUCTION

1.1. Brief history of medicinal plants; a global perspective

For centuries, plants have been used as a source of medicine and have formed an enormous part of the ancient health care system. Amongst other benefits, such as food and shelter, numerous plants were known to treat and manage illness and were harvested for their medicinal properties.

The earliest records of medicinal plant use dates back to ancient Iraq (Mesopotamia) in 2600 BC (Borchardt, 2002). Famous plants in those days used for medicinal benefits included essential oils from *Papaver somniferum*, commonly known as Poppy juice, *Supressus sempervirens*, commonly known as cypress, and *Glycyrrhiza glabra*, commonly known as liquorice, *Cedrus* spp. (Cedar), *Commiphora* spp. (Myrrh), *Punica granatum* (Pomegranate), and *Senna alexandrina* (Senna). These plants are still used today to treat various ailments (Cragg and Newman, 2013).

In India, medicinal plants played an enormous role in the practice of Ayurveda. Translated from Indian, Ayurveda means *Science of Life*. It was a system of holistic approach to health that was believed to offer righteousness, life and wealth. Ayurveda is believed to be the most ancient system of medicinal tradition, extending its influence to ancient Greece. The principles of Ayurveda medicine were recorded in ancient texts known at that time as the Veda, as early as 2000 years BC (Gurib-Fakim, 2006).

Greece contributes substantially to the history of the use of traditional medicine. Historic figures such as Hippocrates (460–377 BC) and Aristotle (384–322 BC) greatly contributed to the development of traditional herbs in ancient Greece. Dioscorides, who is thought to have adopted some of his knowledge from India, revolutionised pharmacy in the early hundreds AD, with his complex prescriptions and compounding of medicinal herbs. Dioscorides later published numerous books, but his most famous book, entitled De Materia Medica¸ which contained a valuable amount of medicinal herbs, became the standard guideline in Europe for the next thousand years (Gurib-Fakim, 2006).

In China, traditional medicine use was based on a carefully compiled collection of recipes that involved mixtures of different herbs. These were used for acute and chronic conditions, alongside western medication. Chinese traditional medicines are still recognised today as a form of alternative medicine, influencing population deviations towards natural products (Gurib-Fakim, 2006).

1.2. Brief history of medicinal plants; an African perspective

In Africa, traditional medicine forms part of a holistic healthcare system that takes into account both the body and the mind. Most medicinal plants are obtained as prescriptions from a traditional healer following consultation, who would examine the physical and spiritual aspects of the patient during consultation. Although African traditional medicine is counted amongst the oldest existing forms of medicine, it was previously poorly documented, with indigenous knowledge being verbally passed from one generation to the other (Gurib-Fakim, 2006).

Medicinal plants still form an enormous part of the African healthcare system. To date, the vast majority of South Africans still rely on traditional medicine. In previous years, different researchers have reported a high amount of traditional medicines used by the African population to treat illnesses. In 1993, a study by Cunningham estimated that approximately 70 to 80 % of the African population is dependent on medicinal plants. One would expect a drop in this trend with the role of urbanisation of rural settlements; however, a more recent study conducted by Sultana et al. (2015) reveals that although there had previously been a dip in this trend, there is currently a renewed interest with more natural remedies. Ndhlala et al. (2011) states that although the African society is becoming more urbanised and western culture is adopted, African traditional medicine still remains a vital part of the urban South African culture.

1.3. Medicinal plants as a source of new pharmaceuticals

Traditional medicinal plants still play a vital role even in developed communities. A substantial amount of western medicine was developed from plant derived compounds or is directly synthesised from manipulation of plant compounds. A study described by Farnsworth et al. (1985), shows that 25% of dispensed pharmaceuticals in the USA between 1959 and 1980 contained plant extracts or active plant derivatives. This shows the impact of medicinal plants even in non-rural settlements. Furthermore, medicinal plants have great potential to be developed as suitable drugs.

Plants also contain an almost unlimited pool of chemical compounds, which have potential for further development. Throughout their lifespan, plants are subject to different environmental changes and harsh conditions around which they need to evolve and adapt. These factors lead to a change in chemical compounds. It is important to note that the presence and ratios of chemical compounds in plants may also be affected by factors such as geographical location, harvest time and storage of plants and understanding this may prove beneficial in utilising them to the maximum (Berg and Staaf, 1980; Jemâa et al., 2012).

However, studies show that over the years, interest in medicinal plant research and development was scarce. A review by Gurib-Fakim (2006), showed that only 1% of tropical plant species had been studied for their pharmaceutical benefit, with a yield of about 50 different drugs on the market today. This further magnifies the potential of medicinal plants as precursors for pharmaceutical agents. Moreover, it underlines the vastness of the area yet to be covered, and drugs yet to be discovered, as research so far seems to have only scratched the surface of the compositions of Mother Nature.

1.4. Pathogenesis of fever

Fever refers to a rise in body temperature above the normal physiological range, following an increase in thermal set point by the hypothalamus (Hughes et al., 1990; Aronoff and Neilson, 2001). The mechanism of fever is a complex one, involving multiple responses. Exogenous pyrogens, which typically comprise of microbes, are the most common causes of fevers. Certain physiological factors, such as hormone disorders, auto-immune disease, allergens, or trauma to the hypothalamus may also be contributing factors (Bannister et al., 2000). A breach of tissue integrity by exogenous pyrogens, or invasion thereof, stimulates the body's immune responses. This creates a cascade of events which leads to the development of a fever, as the host's defence mechanism (Kluger et al., 1998).

Microbial tissue invasion causes inflammatory responses, where leukocytes gather to the affected site. Monocytic cells mediate the release of cytokines (Marik, 2000) such as Interleukin 1β (IL 1β), Interleukin 6 (IL 6) and tumour necrosis factor (TNFα), which are known endogenous pyrogens (Aronoff and Neilson, 2001). These cytokines travel via the blood circulation to the preoptic region of the anterior hypothalamus (POAH), where they bind to specific neural receptors (Saper and Breder, 1994; Mackowiak and Plaisacne, 1998). The POAH is responsible for regulating and maintaining the mean body temperature at a set point. Activation of receptors at this region triggers the release of other modulators in the POAH,

particularly Prostaglandin E_2. The end result is an increased firing rate of the POAH neurons which evokes an increase in the thermal set point (Aronoff and Neilson, 2001). A rise in body temperature occurs to this new set point, resulting in a fever. Figure 1.1 illustrates the cascade of events leading to fever.

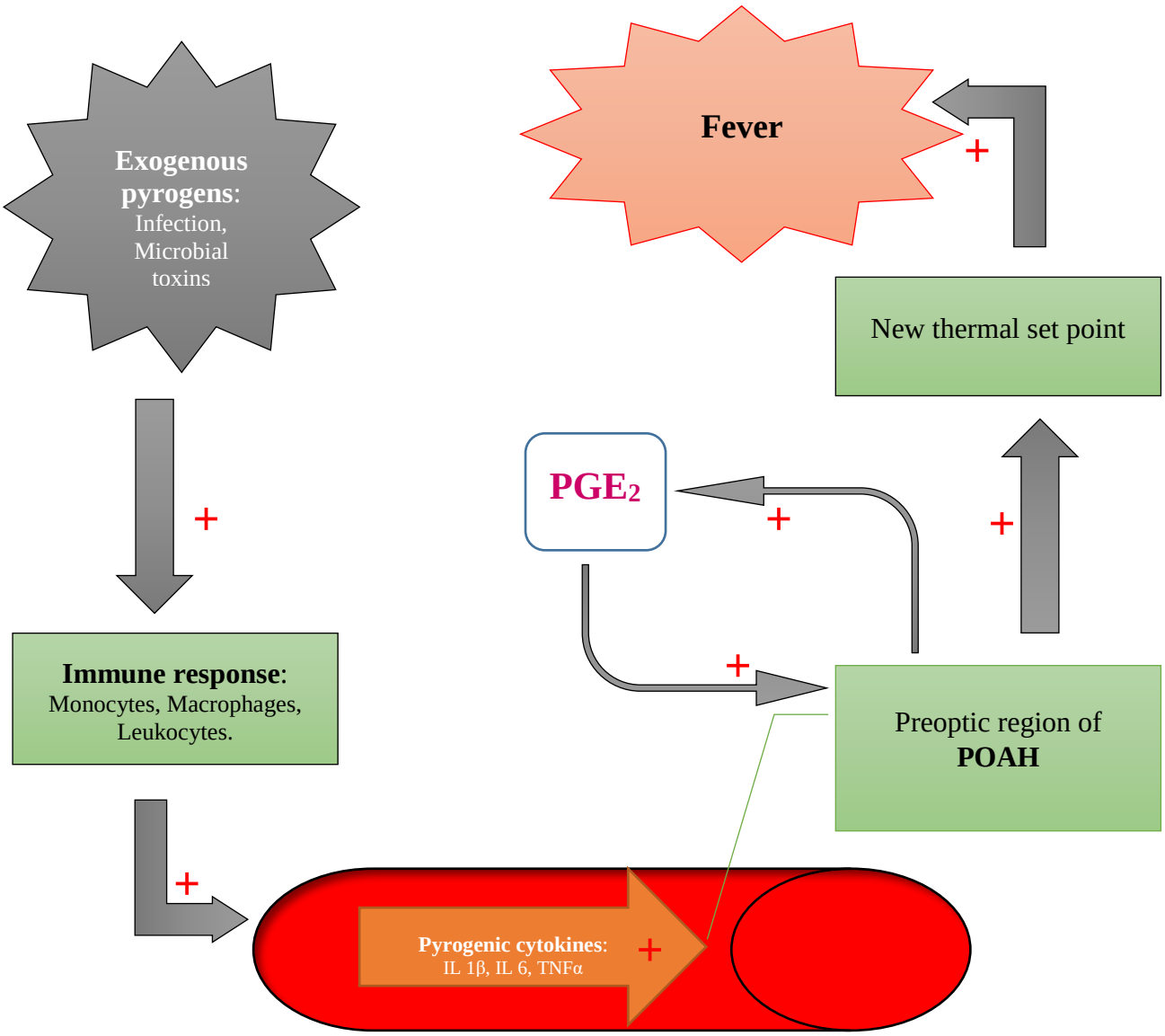


Figure 1. 1: Schematic diagram illustrating the physiological occurrences leading to fever.

1.5. Pathogens associated with fever

Because fever is a host defence mechanism against any foreign invasion of pathogenic species, amongst others, pathogens that are associated with fevers are numerous and broad. For the purpose of this study, pathogenic bacteria that are most commonly associated with septicaemia will be considered. Simply put, septicaemia refers to the presence of pathogenic micro-organisms in the blood circulatory system which cause a high fever. Pathogens may find their

way to the bloodstream through various localised infections such as skin infection, respiratory infections, and meningitis. Table 1.1 lists the common pathogens attributed to septicaemia

Table 1. 1: Common bacteria associated with septicaemia.

General category	Common pathogens	References
Coccal bacteraemia	*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* (less common)	Bannister et al. (2000); Naidoo et al. (2013)
Enterobacteriaceae	*Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Proteus* spp. and *Salmonella* spp.	Bannister et al. (2000); Clermont et al. (2007); Dubey et al. (2013)
Anaerobic and aerobic opportunists	*Pseudomonas aeruginosa*, *Acinetobacter* spp., *Serratia* spp. and *Clostridium* spp.	Bannister et al. (2000); Dubey et al. (2013)
Community-acquired bacteraemia	*Neisseria meningitidis*, *Haemophilus* spp. and *Listeria monocytogenes.*	Bannister et al. (2000)

1.6. The treatment of fever

In the words of the great historic physician, William Osler "Humanity has but three great enemies: fever, famine, and war; of these by far the greatest, by far the most terrible, is fever." (Cushing, 1925). Through this statement one can comprehend the grave reality of the perception of fever in ancient times. Fever in the 21st century, apart from trauma, is still a common case in the emergency room, being viewed by patients and parents of ill children as a noxious illness (El-Radhi and Wood, 2008). However, not only is fever a symptom of illness, but it also plays an important role as a host defence mechanism.

The benefits of reducing fever is an ongoing debate. Substantial evidence exists which shows that fever may reduce morbidity and mortality (Kluger et al., 1998). Fever has also been associated with decreased duration of illness. A study by Jiang et al. (2000) showed that generating an increase in core temperature in mice that had been infected with a lethal clinical

strain of *Klebsiella pneumoniae* peritonitis reduced bacterial growth, enhanced host defences and improved host survival. Several other studies with similar outcomes are described by Jiang et al. (2000).

Unlike in hyperthermia or during a heat stroke, the rise in core temperature during fever is regulated and seldom exceeds heights of 41°C (Mackowiak and Plaisance, 1998). Endogenous antipyretics, which antagonise endogenous pyrogen cytokines, modulate the rise in body temperature via negative feedback pathways to ensure that dangerous levels are not reached (Kluger et al., 1998). Despite this knowledge, a reduction of fever by using antipyretics is still popular and fever is still viewed as a cause for concern. Fever in children and the elderly, for example, may often result in undesirable outcomes such as seizures and mental complications. However, one has to understand the vulnerability of this group of individuals to hyperthermia and heat strokes, due to the underdeveloped or inadequate thermoregulatory mechanisms (Kluger et al., 1998). A low grade fever, however, is still considered to be beneficial in host immunity (Kluger et al., 1998; Jiang et al. 2000). One would reason that the concern with antipyretics should somehow be directed at the deliberate interference with the hosts defence mechanisms, particularly when the fever is due to microbial invasion or infectious disease, as is the common case. In this case it may be necessary to question whether therapeutic intervention is beneficial to the patient, or does it only provide a false sense of security, leaving the underlying true danger lurking in the body?

1.7. Medicinal plants used to treat fever

"Fever plants" are amongst the most widely used medicinal plants recorded in literature (Sultana et al., 2015). Over a hundred plant species are traditionally used for the treatment of different fevers. Table 1.2 presents a literature review of medicinal plants used for the treatment of fever.

Table 1. 2: Fever-reducing medicinal plants used in South Africa.

Scientific name	Family	Common name	Plant part used	References
Achyropsis leptostachya Hook. F	Amarantaceae	Isinama	Roots	Watt and Breyer-Brandwijk, 1962.
Adansonia digitate A L.	Malvaceae	kremetart, Baobab, Shimuwu, Movana, Muvhuyu	Bark	Van Wyk et al., 2009.
Adenia gummifera (Harv.) Harms var. *gummifera*	Passifloraceae	Impindamshaya, Monkey rope	Roots and leaves	uses.plantnet-project.org/en/Adenia_cissampeloides_(PROTA)
Adenium oleifolium Stapf	Apocynaceae	ouheip	Roots	Watt and Breyer-Brandwijk, 1962.
Agathosma betulina (P.J.Bergius) Pillans	Rutaceae	Boegoe, Buchu, ibuchu	Leaves	Van Wyk et al., 2009.
Albizia adianthifolia W. Wight	Leguminoseae	Platkroon, USolu	Bark, roots	McGaw et al., 2008.
Alepidea amatymbica Eckl. & Zeyh.	Apiaceae	Kalmoes, Lesoko, iqwili, ikhathazo	Roots and rhizomes	Van Wyk et al., 2009.
Alepidea cordifolia B.-E.Van Wyk	Apiaceae	Lesoko	Roots or rhizomes	Moffett, 2010.
Alepidea setifera N.E.Br.	Apiaceae	Lesokwana	Root or rhizomes	Moffett, 2010
Allium sativum L.	Amaryllidaceae	Knoffel, Garlic	Bulbs	Van Wyk, 2008.
Aloe maculata Thunb.	Xanthorrhoeaceae	icena, Soap aloe, Bontaalwyn.	Leaves	Felhaber, 1997.
Ambrosia hispida Pursh	Asteraceae	Sweet bay, Wormwood, Soap bush.	Bark	Moffett, 2010
Annona muricata L.	Annonaceae	Soursop	Bark and roots	Watt and Breyer-Brandwijk, 1962.

Scientific name	Family	Common name	Plant part used	References
Argemone ochroleuca Sweet	Papaveraceae	Hlabahlabane, Ntshwantshane	Roots.	Moffett, 2010
Artemisia afra Jacq.	Asteraceae	African wormwood, Lengana, Umhlonyane, Aslem, Wildeals.	Leaves	Van Wyk et al., 2009.
Arundinella nepalensis Trin.	Poaceae	Lehlakamane, Mahlakae, Modula	Roots	Moffett, 2010
Aster bakerianus Burtt Davy ex C.A.Sm.	Asteraceae	Phororwana, Phowa	Rhizomes	Moffett, 2010
Ballota africana Colmeiro	Lamiaceae	Kattekruid, Kattekruie	Leaves	Van Wyk et al., 2009.
Betula alba L.	Asteraceae	Downy birch, Mountain birch.	Bark	http://www.naturalmedicinefacts.info/plant/betula-alba.html
Chrysanthemum parthenium (L.) Pers.	Asteraceae	Feverdew	Leaves	http://www.naturalmedicinefacts.info/plant/chrysanthemum-parthenium.html
Cinnamomum camphora (L.) T.Nees & C.H.Eberm.	Lauraceae	Kanferboom, Camphor tree, Uroselina	Bark and leaves	Van Wyk et al., 2009.
cissampelos capensis Thunb.	Menispermaceae	Dawidjie	Root	Van Wyk et al., 2008.
Coleonema album E.Mey.	Rutaceae	koorsbos, White confetti bush.	Leaves	Van Wyk et al., 2009.
Combretum molle R.Br. ex G.Don	Combretaceae	Velvet bush willow, umBondwe-omhlope	Leaves	Grohaug et al., 2008.
*Commiphora Africana (*A.Rich.) Endl.	Burseraceae	African myrrh	Bark	Watt and Breyer-Brandwijk, 1962.
Commiphora zimmermannii Engl.	Burseraceae	Mukungugu	Leaves, bark and roots	Watt and Breyer-Brandwijk, 1962.

Scientific name	Family	Common name	Plant part used	References
Conopharyngia ventricosa stapf.	Apocynaceae	Umhlambamaas	Bark	Watt and Breyer-Brandwijk, 1962.
Conyza scabrida DC.	Asteraceae	Bakbos, Oven bush	Leaves	McGaw et al., 2008.
Crassula ericoides Haw.	Crassulaceae	Karkai	Plant	Van Wyk, 2008.
Crassula muscosa Roth.	Crassulaceae	Klein koorsbos	Plant	Van Wyk, 2008.
Croton gratissimus Burch.	Euphorbiaceae	Bergboegoe, laventelkoorsbessie, Lavender croton, Forest fever- berry, Maquassie, Umahlabekufeni	Bark	Van Wyk et al., 2009. Ngwenya et al., 2003
Cussonia spicata Thunb.	Araliaceae	Cabbage tree, Umsenge	Leaves	Felhaber, 1997.
Dicoma capensis Less.	Asteraceae	Koorsbossie, Wilde karmedik	Leaves	Watt and Breyer-Brandwijk, 1962.
Dodonaea viscosa Jacq.	Sapindaceae	Sandolien, Ysterhouttoppe, Sand olive, Mutepipuma, Mutata-vhana	Leaves	Van Wyk et al., 2009.
Elaeodendron transvaalense (Burtt Davy) R.H.Archer.	Celastraceae	Bushveld saffron, Umgugudo	Bark	Tshikalange et al., 2008.
Elephantorrhiza elephantine (Burch.) Skeels.	Fabaceae	Mositsane	Rhizomes	Kose et al., 2015.
Eragrostis plana Nees.	Poaceae	Selile	Root.	Kose et al., 2015.
Eucalyptus globulus Labill.	Myrtaceae	Gumtree, Blue gum, Impiskayihlangulwa, Umdlavusa	Leaves	Felhaber, 1997.
Eucalyptus sideroxylon A.Cunn.	Myrtaceae	Bloekom	Leaves	Nortje and Van Wyk, 2015.
Eucomis autumnalis (Mill.) Chitt.	Hyacinthaceae	Mathethebale	Bulbs, roots	Kose et al., 2015.

Scientific name	Family	Common name	Plant part used	References
Garuleum bipinnatum Less.	Asteraceae	Slanghoutjie	Root	Van Wyk et al., 2008.
Gunnera perpensa L.	Gunneraceae	Qobo	Rhizomes, Roots	Kose et al., 2015.
Harpagophytum procumbens DC.	Pedaliaceae	Sengaparele	Roots	Felhaber, 1997.
Helichrysum herbaceum Sweet	Asteraceae	Everlasting flower	Leaves	Verschaeve and Van Staden, 2008.
Helichrysum nudifolium Less.	Asteraceae	Hottentotseebossei, Everlasting, Isicwe, Icholocholo	leaves	Van Wyk et al., 2009.
Helichrysum odoratissimum Sweet	Asteraceae	Kooigoed, Hotnotskooigoed, Slagbos	Leaves	Nortje and Van Wyk, 2015.
Hermbstaedtia glauca Rchb. ex Steud.	Amaranthaceae	Bokhoutjie	Leaves	Nortje and Van Wyk, 2015.
Heteromorpha trifoliata Eckl. & Zeyh.	Apiaceae	Parsley tree, wildepietersielie	Roots	McGaw et al., 2008.
Hypoxis rooperii T.Moore	Amarantaceae	Inkomfe, Ilabatheka, Mooi karatsa, African potato	Root	Watt and Breyer-Brandwijk, 1962.
Indigofera dimidiata Vogel ex Walp.	Fabaceae	Mosapelo wa thaba	Root and bark	Moffett, 2010
Indigofera hedyantha Eckl. & Zeyh.	Fabaceae	Musapelo wa mafika	Root and bark	Moffett, 2010
Lannea edulis Engl.	Anacardiaceae	Rooiwaterbossie, Wildedruiwer, Wild grape, Pheho	Root and bark	Watt and Breyer-Brandwijk, 1962.
Leobordea lanceolata (E.Mey.) B.-E.Van Wyk & Boatwr.	Fabaceae	Khonathi	Roots	Kose et al., 2015.

Scientific name	Family	Common name	Plant part used	References
Lippia javanica Spreng.	Verbenaceae	Koorsbossie, Fever Tea, Musukudu, Inzinzniba, Umsuzwane	Leaves	Van Wyk et al., 2009.
Mentha longifolia L.	Lamiaceae	Ballerja, Wild mint, Koena-ya-thaba, Inixina, Ufuthane Lomhlange	Leaves	Van Wyk et al., 2009.
Mimulus gracilis R.Br.	Scrophulariaceae	Sehlapetsu, Slender Monkey-flower	Roots	Moffett, 2010
Momordica balsamina L.	Cucurbitaceae	Duwana	Root	Van Wyk et al., 2008.
Myrothamnus flabellifolius Welw.	Myrothamnaceae	Bergboegoe, Resurrection plan, Uvukwabafile	Leaves	Van Wyk et al., 2009.
Nidorella resedifolia DC.	Asteraceae	Kgotodia, Mokotedi moholo	Leaves	Moffett, 2010
Oenothera villosa Thunb.	Onagraceae	Mokankga, Monkga	Leaves	Moffett, 2010
Osmitopsis asteriscoides Cass.	Asteraceae	Bels, Belskruie	Leaves	Van Wyk et al., 2009.
Panax pseudoginseng Wall.	Araliaceae	Nepal gingseng	Root	https://www.naturalmedicinefacts.info/plant/panax-pseudoginseng.html
Pelargonium alchemilloides (L.) L'Hér.	Geraniaceae	Bodiba ba ditshwene, Makorotswane	Root	Moffett, 2010
Pelargonium sidoides DC.	Geraniaceae	Umckaloabo	Roots	Van Wyk, 2008.
Pentanisia prunelloides Walp.	Rubiaceae	Icimamilo, Setimamamollo	Root and leaves	Felhaber, 1997.
Pittosporum viridiflorum Sims.	Pittosporaceae	Cheesewood, Mofumo, Monkganku, Mosetlela, Phukgile, Phuku.	Bark	Moffett, 2010
Plumiera rubra L.	Apocynacea	Frangipani	Bark	Watt and Breyer-Brandwijk, 1962.

Scientific name	Family	Common name	Plant part used	References
Pseudognaphalium luteoalbum (L.) Hilliard & B.L.Burtt.	Asteraceae	Manku, Mosuwane, Thwala	Leaves	Moffett, 2010
Pterospermum acerifolium (L.) Willd.	Malvaceae	Mayang, Maple leaved bayur, Dinnerplate tree	Leaves	http://www.naturalmedicinefacts.info/plant/pterospermum-acerifolium.html
Ranunculus multifidus Pursh.	Ranunculaceae	Thlapi	Leaves	Watt and Breyer-Brandwijk, 1962.
Rauvolfia caffra Sond.	Apocynaceae	Kinaboom, Quinine tree, Umhlambamanzi	Bark, root, leaves	Van Wyk et al., 2009.
Rauvolfia vomitoria Wennberg.	Apocynacea	Munyasindi	Bark, root and leaves	Watt and Breyer-Brandwijk, 1962.
Rumex sagittatus Thunb.	Polygonaceae	Bodila bo boholo	Roots and leaves	Moffett, 2010
Ruta graveolens L.	Rutaceae	Wynruit	Leaves	Nortje and Van Wyk, 2015.
Salix babylonica L.	Salicaceae	Wildeboom bas	Bark	Van Wyk et al., 2008.
Salix mucronata Thunb.	Salicaceae	Wilde wilger, Rivierwilger, Wild willow	Leaves	Van Wyk et al., 2009.
Salvia dentata Aiton.	Lamiaceae	Toothed sage, Bergsalie, Bloublomsalie	Leaves	Nortje and Van Wyk, 2015.
Sceletium emarcidum (Thunb.) L.Bolus.	Mesembryanthemaceae	Kougoed	Leaves	Van Wyk et al., 2008.
Sclerocarya birrea Hochst.	Anacardiaceae	Marula plum, Murula	Bark	Felhaber, 1997.
Securidaca longipedunculata Fresen.	Polygalaceae	Violet tree, Fibre tree, Mmaba, Iphuphuma	Roots, bark and leaves	Watt and Breyer-Brandwijk, 1962.
Selaginella caffrorum (Milde) Hieron.	Selaginellaceae	Moriri oa matlapa	Whole plant	Kose et al., 2015.

Scientific name	Family	Common name	Plant part used	References
Senecio cinerascens Aiton.	Asteraceae	Vieroulap, Oulap, Handjiesbos	Leaves	Nortje and Van Wyk, 2015.
Senna petersiana (Bolle) Lock.	Fabaceae	Senna, Monkey pod, Uhwabile.	Roots	Tshikalange et al., 2008.
Silene undulata Aiton.	Caryophyllaceae	kleinwildetabak, Dikomana, Kwaeqala, Lekoomana	Roots	Moffett, 2010
Siphonochilus aethiopicus (schweinf.) B.l.Burtt.	Zingiberaceae	African ginger, Isiphephetho, Indungulo	Roots and rhizomes	Van Wyk et al., 2009.
Stachys rugose Lam.	Lamiaceae	Koorsbos, Bergsalie	Leaves	Nortje and Van Wyk, 2015.
Strophanthus hispidus DC.	Apocynaceae	Brown strophanthus	Root and bark	Watt and Breyer-Brandwijk, 1962.
Strychnos henningsii Gilg.	Longaniaceae	Koffiehardepeer, Walking stick, Umqalothi	Bark and roots	Kuria et al., 2012
Strychnos spinosa Lam.	Longaniaceae	Spiny monkey Orange, Morapa, Nsala	Leaves	Isa et al., 2014. http://pza.sanbi.org/strychnos-spinosa
Sutherlandia frutescens (L.) R.Br. ex W.T.Aiton.	Fabaceae	Mmusapelo wa noka, Umnwele	Leaves	Moffett, 2010 . Aboyade et al., 2014.
Tarchonanthus camphoratus Houtt. ex DC.	Asteraceae	Wildekanferbos, Wild camphor bush, Sefahla, Igqeba-elimhlophe	Leaves	Van Wyk et al., 2009.
Tecomaria capensis (Thunb.) Spach.	Bombacaceae	Cape honeysuckle, Cape trumpet-flower, Bopa, Idywadi, Molaka	Leaves, Root and Bark	Watt and Breyer-Brandwijk, 1962.
*Tetradenia riparia (*Hochst.) Codd.	Lamiaceae	Watersallie, Ginger bush, Iboza	Leaves	Van Wyk et al., 2009.

Scientific name	Family	Common name	Plant part used	References
Thalictrum minus L	Ranunculaceae	Lefokotsane, Tloro	Root and leaves	Watt and Breyer-Brandwijk, 1962.
Thevetia peruviana Merr.	Apocynaceae	Yellow oleander	Bark	Watt and Breyer-Brandwijk, 1962.
Tulbaghia alliacea Ker Gawl.	Alliaceae	Wilde knoffel	Leaves	Van Wyk et al., 2008.
Tulbaghia violacea Harv.	Alliaceae	Wild garlic, Isihaqa	Leaves and bulbs	Van Wyk et al., 2009.
Warburgia salutaris (G.Bertol.) Chiov.	Canellaceae	Koorsboom, Fever tree, Pepper bark tree, Amazwecehlabayo, Sebaha,	Bark	Felhaber, 1997.
Withania somnifera (L.) Dunal.	Araceae	Mofera- ngope	Roots, leaves	Kose et al., 2015.
Xysmalobium reticulatum N.E.Br.	Asclepiadaceae	Disoke	Roots and rhizomes	Watt and Breyer-Brandwijk, 1962; Quattrocchi, 1999
Xysmalobium undulatum R.Br.	Asclepiadaceae	Milk bush, Ishinga, Ishongwe	Roots	Felhaber, 1997.
Zingiber officinale Roscoe.	*Zingiberaceae*	Gemmer, Ginger	Rhizomes	Van Wyk et al., 2009.

Despite the debate as to the benefits of reducing fever, traditional medicine has been used for centuries in this regard. A breakthrough study of these medicinal plants led to the discovery and isolation of salicylic acid from the English willow tree known as the "fever bark" (*Salix alba*). Further development of this compound led to its derivative acetylsalicylic acid (aspirin) and other nonsteroidal anti-inflammatory drugs (NSAIDS) (Aronoff and Neilson, 2001). Since then, several fever reducing medicinal plants were studied with the focus of achieving antipyresis via disruption of the pyrogenic pathways (Sultana et al., 2015). However, in addition to these properties, how much more beneficial would it be for these medicinal plants to contain an added advantage i.e. antimicrobial properties in order to reducing fever? In this manner, plant extracts would address the main cause of microbial induced fevers, which is microbial invasion and proliferation, rather than simply supressing the symptom and leaving the host unguarded.

Traditional medicinal plants prescribed to febrile patients may have antimicrobial properties, most of which remain unexplored. These antimicrobial properties may very well be subtly contributing to fever reduction by eliminating the primary perpetrator that is causing the fever. *Acacia albida*, for example, which is traditionally used exclusively against fever, was shown to possess antimicrobial properties against *Staphylococcus aureus* and *Shigella dysenteriae* in *in-vitro* studies (Ismail et al., 2016). Other previously studied plant species such as *Warburgia salutaris, Dicoma capensis*, *Lippia javanica, coleonema alba,* and *Croton gratissimus* which are named as "fever plants" in vernacular languages (Mitchell and Breyer-Brandwijk, 1962; Felhaber, 1997; Ngwenya et al., 2003; van Wyk et al., 2009) have also shown proficiencies in inhibiting the growth of certain pathogens.

Several research papers on fever-related medicinal plants are broad reviews, most of which do not focus entirely on fever, but rather the general traditional uses of the medicinal plants (Kose et al., 2015; Nortje and Van Wyk, 2015; Moffett, 2010). Many papers focus on the physiological effects of these plants, rather than the antimicrobial aspects (Sultana et al., 2015).

A review by Krettli et al. (2001) addressed the antimicrobial properties of some fever plants, in addition to randomly selected plants. Many studies have focus on single causative micro-organisms, such as malaria-related fever plants (Andrade-Neto et al., 2003). However, detailed studies exclusively on fever-related medicinal plants and their antimicrobial properties against common pathogens were found to be lacking.

This suggests that traditional medicines used to treat fever, should not be overlooked in the research for antimicrobial agents. Where the development of pharmaceutical agents is concerned, they may be potential precursors of the next wonder-drugs

1.8. Nanotechnology

The term "nano" is derived from the Greek word "*nanos*" which means "dwarf, tiny, or very small" (Chung et al., 2016). Nanotechnology refers to the discovery and development of nanoscale material. Nanoparticles are materials that range between 1-100 nm in size and exhibit completely different properties based on their size, morphology, and distribution (Praba et al., 2015).

1.9. Silver nanoparticles

1.9.1. Background (history and application)

The use of silver as an antimicrobial agent is dated back to ancient Greece. Silver in its pure form was used historically on numerous occasions as prophylaxis against microbial infections as well as for the treatment of pathogenic diseases. A study conducted by Barillo and Marx (2014) reviews some of the earliest incidents where silver was used therapeutically. The numerous uses of silver include: prophylaxis for neonatal eye infections in the early 1900s, as a treatment of burn wounds to prevent secondary infections, and as a purification system for water to prevent contamination (Barillo and Marx, 2014).

With such great potential displayed by this metal, transforming it into nanoparticles would have potential. In the nanoparticle form, the antimicrobial properties of silver are intensified, making it useful in effectively treating micro-organisms which are now developing resistance to current treatment regimens (Bharani et al., 2012).

Silver nanoparticles (AgNPs) possess unusual properties such as chemical, magnetic, optical, biological, photo-electronical and antimicrobial properties which make them desirable and applicable to a broad field of research. Uses vary from agriculture, drug delivery, food industries, cosmetics, anti-cancer to antimicrobial studies (Gopinath et al., 2013). This study focuses on the antimicrobial aspect of nano-silver and the potential it may carry when synthesised with aqueous extracts from antimicrobial-active plants that are used to treat fever.

1.9.2. Synthesising AgNPs

Two methods have been recognized in the green synthesis of nanoparticles, namely the physical and chemical approaches. These processes include complex procedures such as ion sputtering and sol-gel techniques. Ahmed et al. (2016) characterized the synthesis of nanoparticles in two methods; "top to bottom" approach or "bottom to top" approach. The "top to bottom" approach involves size reduction of a bulk material into smaller nanoscale particles. Various techniques such as milling, grinding or sputtering are used to break down a bulk material so as to decrease size. In the "bottom to top" approach chemical and biological means are employed for the synthesis of nanoparticles. It involves a reaction in which there is assembly of atoms to new nuclei which then grows into particles of nanoscale. The main components which are required in the synthesis of nanoparticles are:

1. Silver salt, e.g. silver nitrate ($AgNO_3$).
2. A reducing agent, such as sodium borohydride ($NaBH_4$).
3. A stabilizing or capping agent, such as a polyvinyl alcohol.

The reducing agent interacts with the silver nitrate, whereby Ag^+ ions are reduced to Ag^0 (elemental silver), while the capping agent serves to coat the AgNPs and prevent aggregation of the nanoparticles, thus providing stability to the nanoparticles (Chung et al., 2016).

The use of toxic chemicals such as $NaBH_4$, however, is unfavourable due to the hazardous effect on humans and the environment. The biological route of synthesis, also known as green synthesis of nanoparticles, has therefore gained popularity (Chung et al., 2016). Green synthesis is a branch of nanoparticle synthesis that utilizes natural biological systems to achieve nanoparticle production. This is advantageous as it is a safe and eco-friendly approach to synthesizing nanoparticles. This approach is also quicker and more cost effective. Plants and micro-organisms such as bacteria, yeast and fungi are two main resources used by this approach (Roy et. al., 2013; Praba et al., 2015). Using plants is advantageous as this eliminates the additional costs and demands of growing and maintaining cell cultures (Ahmed et al., 2016).

An added advantage to the green synthesis using plant extracts is that plants contain numerous biological compounds which have the ability to act as both reducing agents and stabilizing or capping agents. This eliminates the need to add a synthetic chemical which could serve as a stabilizing agent and reduces the use of chemicals which may be toxic.

In the synthesis of AgNPs, biological compounds within the plant extracts are responsible for the reduction of silver ions to elemental silver nanoparticles. These compounds, also known as phytochemicals, contain functional groups such as alcohols, ketones, aldehydes and carbonyl moieties e.g. flavonoids, proteins, such as terpenoids (Mallikarjuna et al., 2014). Glucose, fructose, alkaloids, and phenolic acids have also been identified as potential reducing agents for the synthesis of nanoparticles (Philip, 2010; Chung et al., 2016). Phytochemicals may act as both reducing and stabilizing or capping agents (Lee et al., 2014).

The complete synthesis of nanoparticles can be monitored by observing the visual colour change that occurs during the reduction. A dark brown-black colour change has been reported by several researchers as the first evidence of the presence of nanoparticles in the reaction solution. The colour change is attributed to the phenomenon known as surface plasmon resonance (SPR) (Chung et al., 2016). Surface plasmon resonance is a typical phenomenon in metallic nanoparticles which occurs when the conductive electrons in the metallic nanoparticles oscillate collectively. This causes a strong light scattering by an electric field at the wavelength where resonance occurs. This results in strong SPR bands that can be seen on a UV-Vis spectrum (Bharani et al., 2012).

Additional instruments, however, are required to ascertain whether the synthesis of AgNPs was successful. These include the Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) (Rajathi and Nambaru, 2014), the Fourier Transform Infrared spectrophotometer (FTIR) (Chung et al., 2016), and Zeta sizer (Ahmed et al., 2016) to assess the size, shape, and level of agglomeration of nanoparticles formed from different plant extracts.

1.9.3. Stability of nanoparticles

The stability of nanoparticles remains a concern. Due to their nanoscale nature, they may be subject to problems such as aggregation, agglomeration, dispersion, sedimentation, and dissolution. These factors pose a huge concern on the stability of nanoparticles and affect the antimicrobial properties (Islam et al., 2015). Temperature and pH are among the greatest parameters that influence the stability of nanoparticles. Silver nanoparticles have been shown to be very stable over long periods of time. Devaraj et al. (2013) observed stability with AgNPs for a six month period**.** However, stability profiles may vary from one formulation to another based on the plant extract used, as various plant species contain different phytochemicals. It

can be seen that various parameters influence AgNP formation in different ways due to the plant extract varying considerably in their compositions. AgNP synthesis has to be optimised with each plant species.

The unique pH of different plant extracts can affect the size and shape of nanoparticles. Larger nanoparticles form at lower pH values. An acidic environment almost retards the formation of nanoparticles. In a study where the pH of nanoparticles was monitored throughout the synthesis process it was found that the size of the nanoparticles decreased with increasing pH. In the biosynthesis of nanoparticles, a neutral pH of the plant extract provides the optimal condition for the formation of AgNPs synthesis (Chung et al., 2016).

Temperature not only affects the rate of reaction and formation of nanoparticles (Chung et al., 2016), but it also plays a role in influencing particle size. In a study conducted by Kumar et al. (2014), it was observed that a rise in temperature facilitated a reduction in particle size of nanoparticles and caused a blue shift of the SPR. This happens when small-size nanoparticles are formed and the absorption bands shift to a smaller wavelength (blue shift) on the UV-Vis spectra. Conversely, Islam et al. (2015) report a red shift (absorption band shifts to a larger wavelength due to increased size of the nanoparticles) of the SPR when there was increased temperature to 80˚C for 30 min. This was due to aggregation of nanoparticles. As the particle size of nanoparticles becomes smaller, stability becomes difficult to control as they become more predisposed to aggregation. An increase in diameter of nanoparticles or in size due to aggregation produces a red shift on the UV-Vis spectrum (Islam et al., 2015).

1.9.4. How silver nanoparticles work as antimicrobials

It was understood that the particle size of a nanoparticle plays a fundamental role. Smaller nanoparticles often exhibit a greater bactericidal activity. This is because a decrease in size of nanoparticles can improve the nanoparticles ability to invade the cell wall and cell membrane of the pathogen, bind to the necessary binding sites, and carry out its functions. The exact mechanism of action of silver nanoparticles, however, is not fully understood. A few methods have been proposed by previous researchers;

1.9.4.1. Release of silver ion into the cell

A proposed mechanism of action, applicable to AgNPs that are small enough to move through the bacterial cell wall, is the possible release of silver cations from the AgNPs capsule into the

bacterial cell. They then bind to oxygen, sulphur and nitrogen of essential biological molecules and inhibit cell growth and replication (Gopinath et al., 2013; Chung et al., 2016).

1.9.4.2. Disruption of cell wall and cell membrane

Silver nanoparticles are said to compromise the integrity of the bacterial cell wall and cell membrane by adhering to the outer cell membrane (Gopinath et al., 2013). When these structures have been disrupted, AgNPs can now accumulate inside the cell and cause further damage. Also, disruption of the cell wall and cell membrane causes a loss of potassium ions, which leads to a decrease in membrane potential. Provided the disruption is significant enough, a leakage of cytoplasm and related proteins may also result (Chung et al., 2016).

1.9.4.3. Interaction with thiol groups

Silver nanoparticles have a high affinity for sulphur and phosphorus- containing biomolecules. They bind to and inactivate intracellular proteins and DNA, as well as membrane proteins. This phenomenon interferes with critical functions of the cell such as respiration, ATP production, DNA replication and cell division. Eventually, when these functions are halted, cell death will occur (Gopinath et al., 2013; Chung et al., 2016).

1.9.4.4. Increase of reactive oxidant species.

Silver nanoparticles bind to the mesosome organelle and disrupt their functions, while increasing the generation of reactive oxidant species and free radicals. This leads to oxidative stress and inflammations which further damages protein and DNA (Gopinath et al., 2013; Roy et al., 2013)

It is important to note that over all the proposed mechanisms of action, the interaction with the bacterial cell wall and cell membrane is fundamental in order for all the other processes to continue. Pathogens that may be able to withstand this process therefore, are at an advantage to resist antimicrobial activity. Such is the case with Gram-positive bacteria; Gram-positive bacteria are thought to show greater resistance than Gram-negative strains due to their thicker cell wall, thus restricting entrance of AgNPs into Gram-positive pathogen cells (Kumar et al., 2014; Chung et al., 2016).

1.10. Cytotoxicity of nanoparticles

According to Bharani et al. (2012) silver is known to be safe to man and it produces little, if any, adverse reactions. Mollick et al. (2015) states that silver, when used in small concentrations, is safe to human cells, but lethal to bacterial cells and viruses. However, silver presenting in nanoscale size possesses different chemical and biological properties when compared to naturally occurring silver and its activity is reported to be intensified (Bharani et al., 2012). This raises the question on the safety of silver in its nanoparticle form when considering human cells.

The small sizes of nanoparticles are advantageous as they are able to evade immune responses and cross relatively impermeable barriers such as the blood brain barrier. However, from a toxicology point of view this may be a disadvantage. Nanoparticles are generally more reactive than silver ions, therefore administration through different routes such as the skin and lungs could bring about damage to body tissue (Roy et al., 2013). According to Roy et al. (2013) exposure to nanoparticles can result in DNA damage, mental cytotoxicity, growth retardation, cancer, and death of embryos. It is interesting to note that surface area to volume ratio also contributes to the level of cytotoxicity. It has been established that a decrease in particle size of nanoparticles increases the antimicrobial properties, which is desired. However, further decreasing the size of nanoparticles also increases the risk of cytotoxicity (Roy et al., 2013).

According to Lee et al. (2014), cytotoxicity of nanoparticles can be attributed to the reducing agent and stabilizing agent used in the synthesis of AgNPs as these form the organic shell of the nanoparticle. The green synthesis method shuns from using cytotoxic chemicals to synthesise nanoparticles and therefore results in a significant decrease in cytotoxicity as compared to nanoparticles synthesised using inorganic agents (Roy et al., 2013). Moreover, employing phytochemicals found in plant extracts as reducing and stabilizing agents may significantly reduce cytotoxicity. Phytochemicals are naturally occurring compounds found also in fruits and vegetables, which are consumed by humans. Further research, however, is required to ascertain the safety of AgNPs using plant extracts and the application of AgNPs in microbial infection related to fever, while also assessing their ability to enhance the antimicrobial properties of medicinal plants.

1.11. Aim

The aim of this research, therefore, was to investigate the antimicrobial properties of Southern African fever-reducing medicinal plants and to determine whether silver nanoparticles synthesised from the plant extracts would enhance the antimicrobial activity of the said extracts.

1.12. Objectives

The objectives of this study were as follows:

- To identify medicinal plants that are used to treat fever and source those plants that are readily available.
- To prepare aqueous and organic extracts from the collected plants.
- To determine the antimicrobial activity of the plant extracts against selected fever-causing bacteria using minimum inhibitory concentration (MIC) assays.
- To synthesise AgNPs from the aqueous plant extracts of commercial relevant plants.
- To characterise the synthesised AgNPs using the UV-Vis spectroscopy, Zeta sizer, the FTIR spectrophotometer and TEM.
- To determine the antimicrobial activity of the synthesised AgNPs against selected fever causing Gram-positive and Gram-negative bacteria by means of the MIC assays.
- To determine the cytotoxicity of the active plants extracts and the AgNPs using the Brine Shrimp Lethality assay.

Figure 1.2. is an illustrated flow diagram which provides an overview of the study.

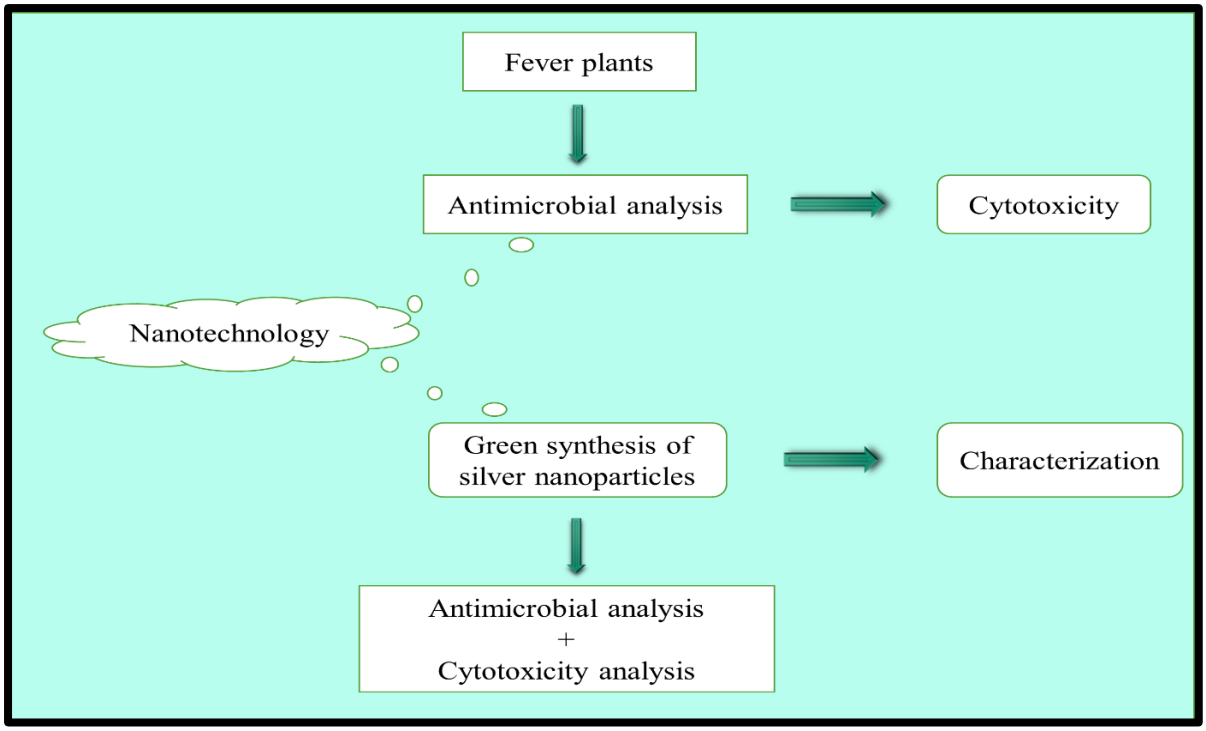


Figure 1. 2: Flow diagram demonstrating an outline of the study.

CHAPTER 2

ANTIMICROBIAL ANALYSIS OF MEDICINAL PLANT EXTRACTS

2.1. Introduction

While the benefits of treating fever have previously been debated, fever is still a significant cause of anxiety associated with illness. An ethnobotanical literature review revealed over 100 medicinal plants used for the treatment of fever in Southern Africa (Chapter 1, Table 1.1). Fever is not only a symptom of illness, but is also the host's defense mechanism against microbial invasion. This means that the deliberate treatment of fever may leave the patient defenseless against underlying pathogens. Therefore, a more beneficial pathway to alleviate fever, would be to counteract the cause of the fever by inhibiting microbial growth and invasion.

Medicinal plants used to treat fever may contain beneficial antimicrobial properties which remain unexplored. This is seen with the medicinal plant *Acacia albida*, which is used for fever, and shows antimicrobial activity against the pathogens *Staphylococcus aureus* and *Shigella dysenteriae* (Ismail et al., 2016). A study of the antimicrobial activity of fever-reducing medicinal plants is therefore imperative. In this chapter, the antimicrobial properties of 40 medicinal plants used traditionally for fever are explored against pathogens associated with fever in the minimum inhibitory concentration (MIC) assay.

The MIC assay is a widely accepted quantitative assay used in antimicrobial studies. The assay involves direct contact between the studied samples and test pathogens where the activities of the samples are evaluated at varying concentrations through serial dilution (Lambert and Pearson, 2000). The MIC is interpreted as the lowest concentration of a test sample that will inhibit the growth of the tested pathogen. Outcomes of the assay can be categorized according to the MIC values observed, i.e. noteworthy activity (MIC value of < 1.00 mg/mL), moderate activity (MIC value of ≥ 1.00 - < 4.00), weak activity (MIC value of ≥ 4.00 - ≤ 8.00) and very poor activity (MIC value > 8 mg/mL) (Fabry et al., 1998; Gibbons, 2004; Rios and Recio 2005;

van Vuuren, 2008). This criterion was used to assess the antimicrobial inhibition properties of fever-reducing medicinal plants in this chapter.

2.2. Methods and materials

2.2.1. Plant selection and collection

Fever reducing plants were identified from a variety of different literature sources, which included medicinal plant-based books such as the Sesotho Plant and Animal Names and Plants Used by the Basotho (Moffett, 2010), The Medicinal Plants of South Africa (Van Wyk et al., 2009), and the South African Traditional Healers' Primary Health Care Handbook (Felhaber, 1997). Databases such as ScienceDirect, Scopus, Google Scholar and PubMed were also used to access journal articles. Ethnobotanical reviews included, but were not limited to; A review of Khoi-San and Cape Dutch Medical Ethnobotany (Van Wyk, 2008), An Ethnobotanical Survey of Medicinal Plants in the South Karoo, South Africa (Van Wyk et al., 2008), The Ethnobotanical Survey of Medicinal Plants used in the Maseru District (Kose et al., 2015), Medicinal Plants of the Kamiesberg, Namaqualand, South Africa (Nortje and Van Wyk, 2015) etc. Search keywords included “fever”, “pyrexia”, “medicinal plants”, and “South Africa”. Over 100 medicinal plant species which are used traditionally to treat fever were identified (Table 1.2). Medicinal plant species which could be successfully harvested from various botanical gardens, with respect to cost, season and timely collection were selected for the study (Table 2.1). Furthermore, sustainable harvesting was considered.

The majority of the plants were sourced from the Walter Sisulu National Botanical garden in Gauteng. Plant identification and authentication was carried out under the guidance of Mr. Andrew Hankey, a specialist horticulturalist. Exceptions are plants which were sourced from various botanical gardens in Lesotho, and various areas in Cape Town. Table 2.1 provides further information on the various plants that were selected, collection sites from where they were obtained, and the relevant voucher specimen numbers.

The relevant plant part that is used traditionally were separated, cleansed thoroughly with sterile distilled water, and allowed to dry completely in an oven set at 25˚C. Thereafter, the plant material was ground to fine powder using a high-speed grinder, Fritsch Pulverisette (Labotec). The ground plant materials were stored in appropriately labelled plastic containers at room temperature, housed at the University of the Witwatersrand, Department of Pharmacy and Pharmacology.

2.2.2. Preparation of plant extracts

Two types of extracts were prepared for this study, i.e. organic and aqueous plant extracts. Organic extracts were prepared using a combination of dichloromethane and methanol in a 1:1 ratio in order to obtain polar and non-polar compounds from the plant material. An amount of 20 g of the macerated plant material was weighed and submerged in 200 mL of organic solvent. The mixture was left in a shaker incubator at 25˚C for 24 hrs. The resulting solution was subsequently filtered and evaporated leaving behind a sticky residue extract. This extract was stored at room temperature in appropriately labeled glass bottles for further analysis.

Aqueous plant extracts were prepared so as to mimic the traditional method of extraction where infusions are prepared for patient administration. This was accomplished using sterile distilled water. Following agitation of the plant:water mixture in a shaker incubator, which was left for 48 hrs, the liquid was filtered into sterile plastic containers and stored overnight in a freezer at -80˚C before undergoing lyophilization using the freeze dryer (Virtis) for 5-7 days. Finally, the lyophilized extracts were exposed to UV light in a sterile room for 24 hrs to ensure no microbial contamination, then stored at room temperature.

The % yield of each medicinal plant extract was calculated as follows:

$$\%\ Extract\ Yield = \frac{Weight\ of\ plant\ extract}{Weight\ of\ plant\ material} \times 100$$

2.2.3. Selection and culturing of pathogens

Study pathogens were selected based on their ability to cause septicaemia, a condition arising from a variety of primary localised infections and characterized by a high fever. *Staphylococcus aureus* (ATCC 25723) and *Streptococcus pneumoniae* (ATCC 49619) were selected representing coccal bacteraemia; *Enterococcus faecalis* (ATCC 29212) and *Klebsiella pneumoniae* (ATCC 13883) were selected representing the Enterobacteriaceae class; *Acinetobacter baumannii* (ATCC 19606), *Clostridium perfringens* (ATCC 13124) and *Serratia marcescens* (ATCC 13880), the opportunists; and finally, *Listeria monocytogenes* (ATCC 19111) and *Haemophilus influenzae* (ATCC 19418), the community-acquired pathogen category.

Table 2. 1: Investigated plants, their collection sites and voucher numbers.

Plant name	Plant part	Collection site	Voucher specimen no.	% Yield aqueous extracts	% Yield organic extracts
Adenia gummifera	Leaves	Walter Sisulu Botanical Garden	SVV-247	5.01	11.06
Agathosma betulina	Leaves	Commercial trader, S Chicken Naturals, Cape Town	SVV-1/13/2013	8.36	13.06
Aloe maculata	Leaves	Lesotho	KSKP-0031	3.17	5.10
Artemisia afra	Leaves	Walter Sisulu Botanical Garden	SVV-173	7.80	11.03
Aster bakerianus	Root	Mapakising, Lesotho.	MVSKK-011	10.26	13.24
Ballota africana	Leaves	ex. Jan Vlok Oudtshoorn	SVV-28	7.20	14.57
Cissampelos capensis	Leaves	Walter Sisulu Botanical Garden	SVV-27	2.03	3.02
Combretum molle	Leaves	Walter Sisulu Botanical Garden	SVV-01	3.28	9.09
Commiphora africana	Bark	Limpopo province	AV-1080	4.26	10.08
Croton gratissimus	leaves	Walter Sisulu Botanical Garden	SVV-11.1	6.98	12.28
Cussonia spicata	Leaves	Walter Sisulu Botanical Garden	SVV-02	2.99	3.76
Dicoma capensis	Leaves	North facing slope, near Mats'ela-beli, Lesotho	MVSKK-019	2.51	2.06
Dodonaea viscosa	Leaves	Walter Sisulu Botanical Garden	HS-223	8.31	11.29

Plant name	Plant part	Collection site	Voucher specimen no.	% Yield aqueous extracts	% Yield organic extracts
Elephantorrhiza elephantina	Root	Walter Sisulu Botanical Garden	UM-172	13.69	11.98
Eragrostis plana	Root	Mokema, mapoleseng, by the river, Lesotho.	KSKP-0050	7.53	9.01
Eucalyptus globulus	Leaves	Cresta, Gauteng	SFVV-34	4.49	16.02
Eucalyptus sideroxylon	Leaves	Walter Sisulu Botanical Garden	SVV-964	9.36	11.55
Eucomis autumnalis	Bulbs, Roots	Lekhalong la Maile, Thaba-putsoa, Lesotho.	MVSKK-002	5.79	7.88
Gunnera perpensa	Root	Sehlaba-thebe National Park, Lesotho.	MVSKK-005	12.23	23.09
Helichrysum herbaceum	Leaves	Northern Drakensburg	AL+AV-17	3.21	4.09
Helichrysum odoratissimum	Leaves	Mapakising, Lesotho	MVSKK-012	2.16	6.05
Hypoxis rooperii	Root	Walter Sisulu Botanical Garden	SVV-03	17.05	13.50
Lippia javanica	Leaves	Fairlands, Johannesburg	SVV-174	10.21	21.08
Mentha longifolia	Leaves	Walter Sisulu Botanical Garden	UM-148	4.04	7.21
Myrothamnus flabellifolius	Leaves	Leshiba, Limpopo	SVV-07	6.11	14.67
Osmitopsis asteriscoides	Leaves	Hermanus, Cape Town	SFVV-24	5.30	16.25

Plant name	Plant part	Collection site	Voucher specimen no.	% Yield aqueous extracts	% Yield organic extracts
Pelargonium sidoides	Root	Parceval (Pty) Ltd Pharmaceuticals, Cape Town	AV-212105	8.21	11.82
Pentanisia prunelloides	Root	Walter Sisulu Botanical Garden.	UM-183	10.04	16.04
Rauvolfia caffra	Leaves	Walter Sisulu Botanical Garden	UM-137	5.59	10.62
Salix mucronata	Leaves	Walter Sisulu Botanical Garden	SVV-04	6.21	8.02
Salvia dentata	Leaves	Walter Sisulu Botanical Garden	SVV-05	3.75	2.61
Sclerocarya birrea	Bark	Zululand, KwaZulu-Natal	NZ-22	7.67	8.03
Strychnos henningsii	Leaves	Walter Sisulu Botanical Garden	SVV-06	5.78	4.08
Sutherlandia frutescens	Leaves	Parceval (Pty) Ltd Pharmaceuticals, Cape Town	AV-312010	5.08	12.73
Tarchonanthus camphoratus	Leaves	Walter Sisulu Botanical Garden	SVV-26.1	4.01	10.35
Tecomaria capensis	Leaves	Walter Sisulu Botanical Garden	SVV-27.1	3.27	12.07
Tetradenia riparia	Leaves	Walter Sisulu Botanical Garden	SFVV-48	6.76	10.28
Tulbaghia violacea	Roots and bulbs	Walter Sisulu Botanical Garden	SVV-248	6.08	7.94
Withania somnifera	Leaves	Mats'ela-habeli, Lesotho	MVSKK-015	3.02	4.12
Zanthoxylum capense	Leaves	Walter Sisulu Botanical Garden	SFVV-49	7.42	9.25

Staphylococcus aureus, *E. faecalis*, *K. pneumoniae*, *A. baumannii*, *S. marcescens*, and *L. monocytogenes* were cultured in Tryptone Soya broth (TSB) (Oxoid) at 37˚C for 24 hrs and subsequently streaked out onto Tryptone Soya agar (TSA) (Oxoid) to confirm purity. *Clostridium perfringens* was cultured in Thioglycollate broth (TGB) and was incubated at 37˚C for 48 hrs using an anaerobic gas pack (Oxoid). *Streptococcus pneumoniae* was cultured in TSB supplemented with 5% sheep's blood and incubated at 5% CO_2 at 37˚C for 48 hrs. Both the latter pathogens were streaked out onto TSA supplemented with 5% sheep's blood. *Haemophilus influenzae* was cultured in Haemophilus Test Medium (Oxoid), which was supplemented with Haematin and Nicotinamide adenine dinucleotide (NAD) (Oxoid) according to manufacturer's recommendation (2.00 mL of sterile water was added to the vial containing the supplement powder. This was then agitated to achieve a homogenous mixture). The supplement was then added to 500 mL of the Haemophilus Test Medium (autoclaved and cooled to approximately 60˚C). *Haemophilus influenzae* was incubated in an anaerobic incubator set at 5% CO_2, 37˚C for 48 hrs.

2.2.4. Minimum inhibitory concentration (MIC) assay

Plant extracts were prepared at a concentration of 32 mg/mL in order to achieve a starting concentration of 8 mg/mL in the first well when dilution is accounted for. The organic plant extracts were dissolved in acetone and the aqueous plant extracts dissolved in sterile water. The Transsonic 540 sonicator (Labotec), run for 10 min, was used to aid in dissolving the organic extracts when solubility was problematic.

Procedures used for the MIC assay were adapted from Eloff (1998) and the NCCLS (2013) guidelines. Under sterile conditions, 100 µL of broth was introduced to each well of a 96 well micro-titre plate. This was followed by the introduction of 100 µL of prepared plant samples, placed in the first row of the micro-titre plate. Serial dilutions were then performed, moving down the rows of the plate, thus diluting the plant sample by half each time.

Thereafter, 100 µL of a 0.5 McFarland's standard inoculum, containing approximately $1x10^6$ colony forming units (CFU)/mL, of the pathogen was added to all the wells of the micro-titre plate. The plates were then sealed with a sterile adhesive seal and incubated as per incubation conditions required for each pathogen. Following incubation, 40 µL of 0.4% *p*-iodonitrotetrazolium violet (INT), an indicator dye, was added to each of the wells. *p*-Iodonitrotetrazolium violet is known to react with microbial living cells, and therefore changes to a pink colour in the presence of viability. Coloured wells therefore indicate bacterial growth

and depicts that the plant sample at that particular concentration did not inhibit microbial growth. The MIC was interpreted as the lowest concentration at which growth is inhibited (the first clear well) (Figure 2.1). Ciprofloxacin, a broad-spectrum antibiotic, was used as a positive control at 0.01 mg/mL to test susceptibility of micro-organism to an antibiotic. The organic solvent, acetone was used as the negative control to ensure that it did not contribute any antibiotic properties. A culture control ensured the broth's ability to support microbial growth.

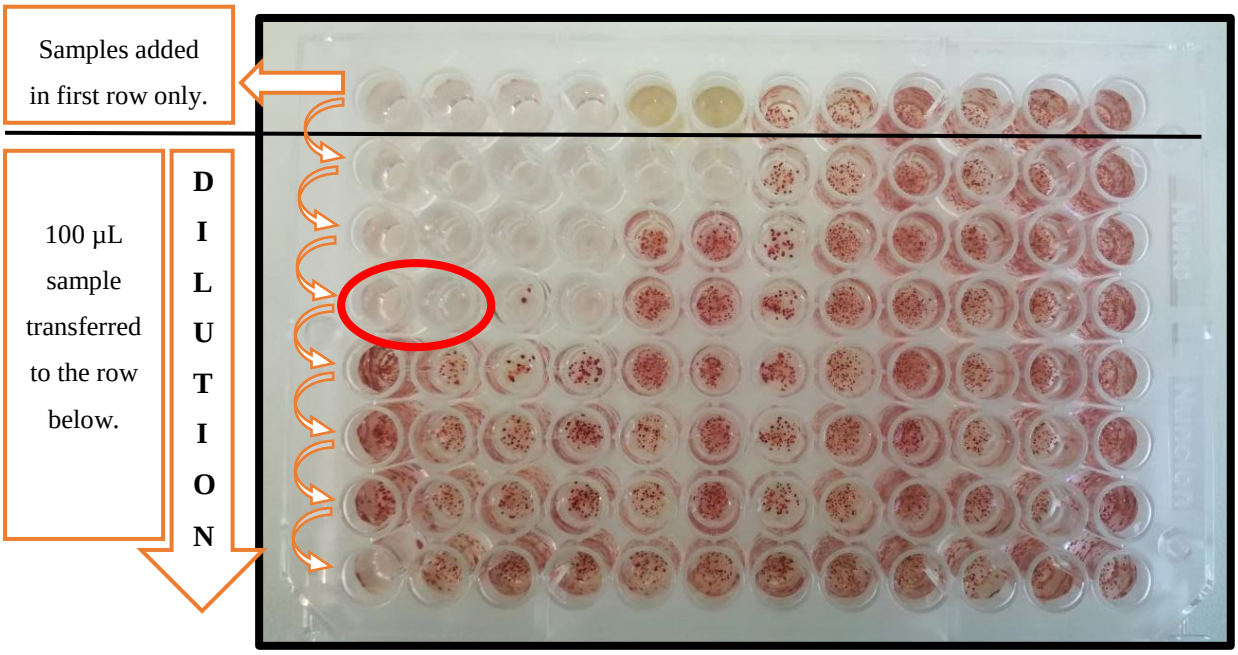


Figure 2. 1: Representation of a completed MIC plate after adding INT.
The MIC, which is the first clear well in each column, is circled in red.

2.3. Results

2.3.1. Extract yields

Prior to antimicrobial screening, the percentage yield of each extract was calculated. The yield of extracts attained in this study ranged from 2.03% to 17.05% for aqueous extracts and 2.06% to 23.09% for organic extracts. With regards to organic extracts, the highest percentage yield observed was with the root extract of *G. perpensa*, while the highest percentage yield of aqueous extracts was observed with the root extract of *H. rooperii*. Lower yields of extract were attained for extracts of *Salvia dentata, C. spicata* and *D. capensis*. The calculated percentage yields of all plants extracts can be found in Table 2.1.

2.3.2. Antibacterial MIC assay

The antibacterial activity of 40 fever-reducing medicinal plants (40 aqueous extracts and 40 organic extracts) were screened against nine pathogens associated with fever and septicaemia. Samples were tested at a starting concentration of 8 mg/mL (stock concentration of 32 mg/mL) therefore, those that did not display any activity were denoted as having an MIC that is greater than 8 (>8) (Fabry et al., 1998).

2.3.2.1. Minimum inhibitory concentration of the organic plant extracts

Table 2.2 contains results for the organic extracts. The values expressing noteworthy activity (MIC < 1 mg/mL) are marked in bold (Fabry et al., 1998; Gibbons, 2004; Rios and Recio 2005; van Vuuren, 2008).

Table 2. 2: MIC results for organic plant extracts.

Plant extract	**Mean MIC value (mg/mL) n=3**								
	Gram-positive species					**Gram-negative species**			
	C.p	***E.f***	***L.m***	***S.a***	***S.p***	***A.b***	***H.i***	***K.p***	***S.m***
A. afra	**0.06**	>8.00	2.00	1.00	1.00	2.00	>8.00	>8.00	4.00
A. gummifera	**0.06**	4.00	4.00	2.00	1.00	1.00	>8.00	8.00	2.00
A. butelina	**0.06**	4.00	8.00	2.00	4.00	1.00	>8.00	4.00	2.00
A. maculata	**0.06**	**0.50**	1.00	>8.00	1.00	1.00	>8.00	2.00	>8.00
A. bakerianus	1.00	>8.00	4.00	2.00	>8.00	1.00	>8.00	>8.00	>8.00
B. africana	**0.25**	>8.00	**0.25**	**0.50**	1.00	1.00	>8.00	8.00	1.00
C. capensis	**0.50**	2.00	2.00	4.00	1.00	1.00	>8.00	>8.00	>8.00
C. mole	**0.03**	8.00	1.00	2.00	4.00	2.00	>8.00	>8.00	2.00
C. africana	**0.25**	4.00	2.00	4.00	1.00	4.00	>8.00	>8.00	4.00
C. gratissimus	**0.06**	>8.00	2.00	8.00	4.00	2.00	>8.00	>8.00	4.00
C. spicata	2.00	2.00	2.00	4.00	4.00	**0.50**	>8.00	>8.00	4.00
D. capensis	4.00	2.00	1.00	4.00	4.00	1.00	>8.00	>8.00	4.00
D. viscosa	**0.06**	1.00	1.00	1.00	**0.50**	**0.50**	8.00	>8.00	1.00
E. elephantina	**0.06**	1.00	2.00	2.00	1.00	1.00	>8.00	>8.00	2.00
E. plana	>8.00	>8.00	2.00	>8.00	1.00	4.00	>8.00	>8.00	2.00
E. globulus	**0.004**	1.00	**0.13**	**0.13**	**0.13**	**0.50**	8.00	1.00	**0.02**
E. sideroxylon	**0.25**	2.00	4.00	4.00	1.00	1.00	>8.00	>8.00	2.00
E. autumnalis	>8.00	4.00	2.00	1.00	8.00	2.00	>8.00	>8.00	2.00
G. perpensa	**0.06**	1.00	2.00	2.00	1.00	**0.50**	4.00	1.00	2.00
H. herbaceum	**0.13**	>8.00	1.00	1.00	**0.5**	2.00	>8.00	>8.00	1.00
H. odoratissimum	**0.01**	1.00	**0.13**	**0.25**	**0.50**	1.00	4.00	1.00	**0.50**

Plant extract	Mean MIC value (mg/mL) n=3								
	Gram-positive species					Gram-negative species			
	C.p	***E.f***	***L.m***	***S.a***	***S.p***	***A.b***	***H.i***	***K.p***	***S.m***
H. rooperii	1.00	>8.00	4.00	>8.00	4.00	4.00	>8.00	>8.00	>8.00
L. javanica	**0.02**	>8.00	**0.06**	2.00	1.00	1.00	>8.00	>8.00	1.00
M. longifolia	**0.02**	1.00	2.00	1.00	4.00	1.00	>8.00	>8.00	4.00
M. flabellifolius	**0.13**	1.00	1.00	2.00	4.00	**0.50**	>8.00	8.00	1.00
O. asteriscoides	**0.06**	4.00	1.00	4.00	4.00	1.00	>8.00	8.00	1.00
P. sidoides	**0.25**	4.00	8.00	2.00	2.00	8.00	8.00	4.00	4.00
P. prunelloides	>8.00	>8.00	>8.00	2.00	>8.00	>8.00	>8.00	8.00	2.00
R. caffra	**0.50**	1.00	2.00	4.00	4.00	1.00	>8.00	2.00	4.00
S. mucronata	2.00	2.00	**0.25**	**0.50**	4.00	1.00	>8.00	2.00	**0.50**
S. dentata	**0.02**	1.00	4.00	4.00	2.00	**0.50**	>8.00	>8.00	>8.00
S. birrea	**0.02**	4.00	2.00	**0.50**	1.00	2.00	4.00	>8.00	2.00
S. henningsii	**0.50**	>8.00	1.00	8.00	4.00	1.00	>8.00	>8.00	>8.00
S. frutescens	**0.50**	>8.00	2.00	8.00	8.00	4.00	>8.00	>8.00	2.00
T. violacea	8.00	2.00	1.00	2.00	1.00	1.00	>8.00	>8.00	4.00
T. camphoratus	**0.03**	2.00	1.00	2.00	4.00	1.00	>8.00	2.00	4.00
T. capensis	2.00	2.00	4.00	>8.00	2.00	2.00	>8.00	>8.00	8.00
T. riparia	**0.13**	1.00	**0.50**	4.00	2.00	1.00	>8.00	8.00	2.00
W. somnifera	**0.25**	2.00	**0.02**	**0.50**	**0.25**	1.00	4.00	>8.00	1.00
Z. capense	**0.06**	2.00	1.00	2.00	1.00	2.00	>8.00	2.00	8.00
Controls									
Positive: Cip (µg/mL)	0.017	1.25	0.039	0.39	3.1	0.16	0.078	0.63	0.63
Negative: Acetone	>8	>8	>8	>8	>8	>8	>8	>8	>8
Culture control	+	+	+	+	+	+	+	+	+

Key: **C.p**: *Clostridium perfringens*; **E.f**: *Enterococcus faecalis*; **L.m**: *Listeria monocytogenes*; **S.a**: *Staphylococcus aureus*; **S.p**: *Streptococcus pneumoniae*; **A.b**: *Acinetobacter baumannii*; **H.i**: *Haemophilus influenza*e; **K.p**: *Klebsiella pneumoniae*; **S.m**: *Serratia marcescens*.

"+" denotes broth supports microbial growth.

*values in bold denote noteworthy activity.

** ">8": MIC falls above the concentration of sample that was tested, denoting very weak activity.

The majority of organic plant extracts (39%) displayed moderate activity against the tested pathogens. Noteworthy antimicrobial activity against a minimum of three pathogens was observed with *B. Africana, D. viscosa, E. globulus, H. odoratissimum, S. mucronata,* and *W. somnifera*. The plant species demonstrating the highest broad-spectrum activity was *H. odoratissimum* which showed noteworthy activity against five pathogens, and *E. globulus*

which showed the most noteworthy antimicrobial activities, inhibiting the growth of six of the nine pathogens tested.

Eucalyptus globulus demonstrated the lowest MIC value in the study against *C. perfringens* (MIC value of 0.004 mg/mL) and additionally demonstrated noteworthy activity against two Gram-negative pathogens; *S. marcescens* (MIC value of 0.02 mg/mL) and *A. baumannii* (MIC value of 0.50 mg/mL), where the activity of *E. globulus* against *S. marcescens* was also noted as the lowest MIC for all Gram-negative species. Figure 2.2 provides an overview of the observed antimicrobial activity.

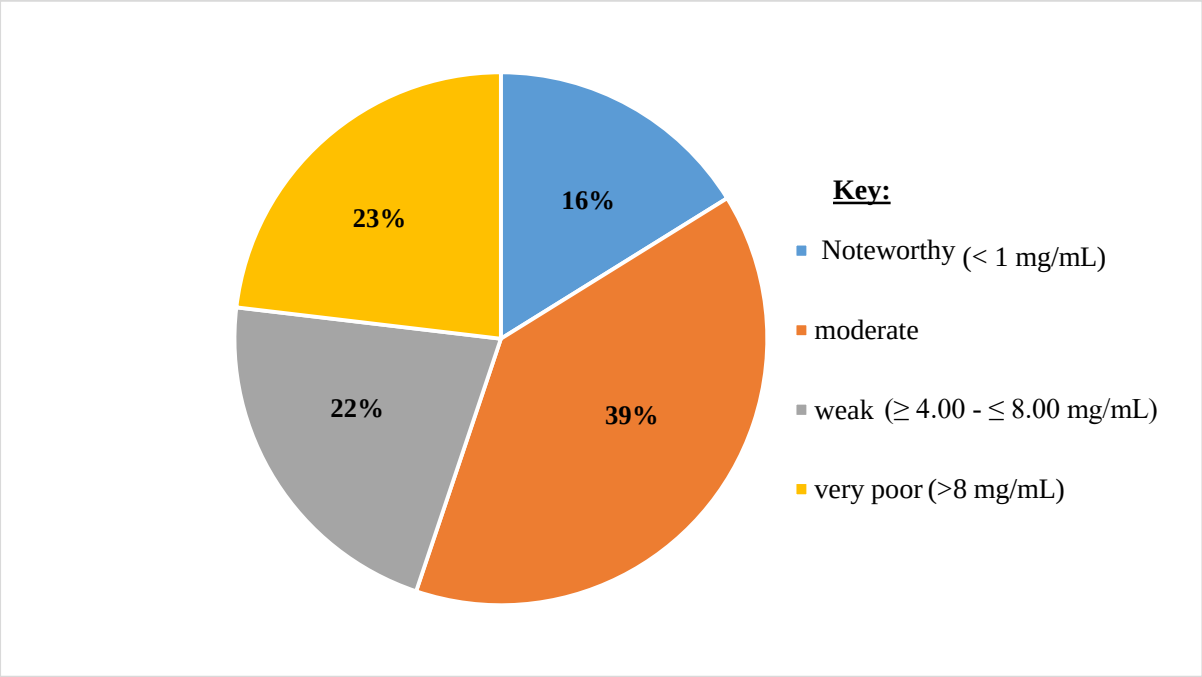


Figure 2. 2: Classification of antimicrobial activity observed with the organic plant extracts.

Some of the pathogens, particularly *K. pneumoniae* and *H. influenzae*, proved to be more resistant than others, leading to a low success rate in growth inhibition by the plant extracts. Interestingly, both *K. pneumoniae* and *H. influenzae* are associated with respiratory infections. None of the organic plant extracts, at the concentrations tested in this study, were able to inhibit the growth of these two pathogens to a noteworthy degree. Where *K. pneumoniae* is concerned, only 20% of plants showed moderate activity. The remaining seven pathogens, however, were notably inhibited by one or more of the organic plants extracts.

Worth mentioning is *C. perfringens*, which was inhibited by the majority of the medicinal plants tested. Approximately 75% of the medicinal plants studied demonstrated noteworthy

antimicrobial activity against this pathogen, followed by *L. monocytogenes* to which 17% of the medicinal plants demonstrated noteworthy activity. Moderate activity was most predominant against *A. baumannii*, with 70% of the organic extracts showing moderate antimicrobial activity. Additionally, the most noteworthy inhibitory activity against Gram-negative species was noted against *A. baumannii* where six of the organic extracts displayed activity. A summary of the antimicrobial activity of the organic extract against each pathogen tested can be seen in Figure 2.3.

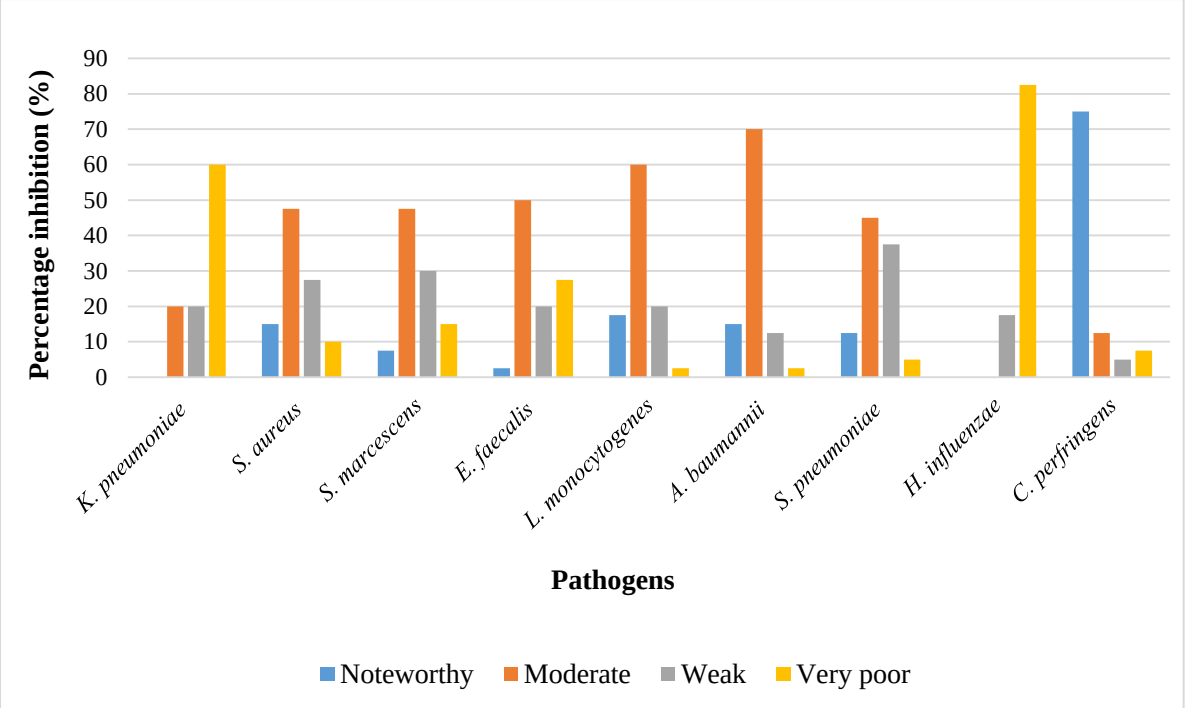


Figure 2. 3: Percentage inhibition of organic plant extracts against studied pathogens.

2.3.2.2. Minimum inhibitory concentration of the aqueous plant extracts

The aqueous plant extracts were tested against the five Gram-negative and four Gram-positive pathogens. Contrary to the organic extracts, the majority of the aqueous extracts presented with very poor antimicrobial properties against the tested pathogens. Table 2.3 is a breakdown of the antimicrobial activity displayed by these aqueous extracts.

Table 2. 3: MIC results for aqueous plant extracts.

Plant extract	Mean MIC value (mg/mL) n=3								
	Gram-positive species					Gram-negative species			
	C.p	***E.f***	***L.m***	***S.a***	***S.p***	***A.b***	***H.i***	***K.p***	***S.m***
A. afra	>8.00	>8.00	8.00	4.00	>8.00	8.00	>8.00	4.00	>8.00
A. gummifera	>8.00	>8.00	>8.00	2.00	>8.00	>8.00	>8.00	>8.00	>8.00
A. butelina	>8.00	8.00	8.00	>8.00	>8.00	8.00	>8.00	8.00	>8.00
A. maculata	>8.00	>8.00	>8.00	>8.00	8.00	>8.00	>8.00	>8.00	>8.00
A. bakerianus	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00
B. africana	>8.00	>8.00	>8.00	>8.00	>8.00	8.00	>8.00	2.00	2.00
C. capensis	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00
C. mole	>8.00	2.00	8.00	4.00	>8.00	8.00	8.00	2.00	4.00
C. africana	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00
C. gratissimus	>8.00	>8.00	>8.00	>8.00	>8.00	8.00	>8.00	8.00	>8.00
C. spicata	>8.00	4.00	8.00	4.00	>8.00	8.00	>8.00	4.00	4.00
D. capensis	>8.00	4.00	4.00	4.00	>8.00	8.00	>8.00	8.00	>8.00
D. viscosa	>8.00	>8.00	8.00	>8.00	>8.00	>8.00	>8.00	>8.00	4.00
E. elephantina	>8.00	>8.00	8.00	4.00	8.00	>8.00	>8.00	4.00	2.00
E. plana	>8.00	>8.00	8.00	8.00	>8.00	>8.00	>8.00	8.00	>8.00
E. globulus	8.00	1.00	1.00	2.00	8.00	4.00	8.00	1.00	4.00
E. sideroxylon	4.00	8.00	4.00	4.00	8.00	8.00	>8.00	4.00	4.00
E. autumnalis	>8.00	8.00	>8.00	>8.00	>8.00	8.00	>8.00	8.00	8.00
G. perpensa	8.00	1.00	2.00	**0.50**	2.00	1.00	8.00	**0.50**	**0.13**
H. herbaceum	**0.50**	8.00	8.00	4.00	4.00	>8.00	>8.00	4.00	>8.00
H. odoratissimum	**0.13**	4.00	**0.13**	4.00	4.00	>8.00	8.00	4.00	>8.00
H. rooperii	>8.00	>8.00	>8.00	8.00	>8.00	>8.00	>8.00	>8.00	>8.00
L. javanica	>8.00	8.00	>8.00	8.00	>8.00	8.00	>8.00	>8.00	>8.00
M. longifolia	4.00	>8.00	>8.00	8.00	4.00	>8.00	>8.00	>8.00	>8.00
M. flabellifolius	>8.00	4.00	8.00	4.00	>8.00	4.00	>8.00	4.00	8.00
O. asteriscoides	**0.13**	4.00	8.00	4.00	8.00	8.00	>8.00	4.00	4.00
P. sidoides	**0.25**	1.00	2.00	2.00	4.00	4.00	>8.00	8.00	2.00
P. prunelloides	8.00	4.00	>8.00	>8.00	>8.00	>8.00	>8.00	4.00	>8.00
R. caffra	>8.00	>8.00	>8.00	4.00	>8.00	>8.00	>8.00	>8.00	>8.00
S. mucronata	2.00	4.00	>8.00	4.00	>8.00	>8.00	>8.00	4.00	>8.00
S. dentata	**0.50**	>8.00	2.00	8.00	4.00	4.00	>8.00	8.00	4.00
S. birrea	**0.25**	4.00	4.00	4.00	4.00	4.00	8.00	4.00	4.00
S. henningsii	4.00	4.00	>8.00	4.00	>8.00	8.00	>8.00	4.00	>8.00
S. frutescens	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00
T. violacea	>8.00	>8.00	4.00	>8.00	>8.00	8.00	>8.00	>8.00	2.00
T. camphoratus	>8.00	4.00	>8.00	4.00	>8.00	4.00	>8.00	4.00	>8.00
T. capensis	>8.00	4.00	>8.00	4.00	>8.00	>8.00	>8.00	8.00	4.00
T. riparia	>8.00	>8.00	8.00	8.00	4.00	>8.00	>8.00	8.00	>8.00
W. somnifera	4.00	>8.00	1.00	4.00	4.00	>8.00	8.00	>8.00	>8.00

Plant extract	Mean MIC value (mg/mL) n=3								
	Gram-positive species					Gram-negative species			
	C.p	***E.f***	***L.m***	***S.a***	***S.p***	***A.b***	***H.i***	***K.p***	***S.m***
Z. capense	8.00	8.00	>8.00	>8.00	>8.00	>8.00	>8.00	8.00	>8.00
Controls									
Positive: Cip (μg/mL)	0.017	1.25	0.039	0.39	3.1	0.16	0.078	0.63	0.63
Negative: Acetone	>8	>8	>8	>8	>8	>8	>8	>8	>8
Culture control	+	+	+	+	+	+	+	+	+

Key: **C.p**: *Clostridium perfringens*; **E.f**: *Enterococcus faecalis*; **L.m**: *Listeria monocytogenes*; **S.a**: *Staphylococcus aureus*; **S.p**: *Streptococcus pneumoniae*; **A.b**: *Acinetobacter baumannii*; **H.i**: *Haemophilus influenza*e; **K.p**: *Klebsiella pneumoniae*; **S.m**: *Serratia marcescens*.

"+" denotes broth supports microbial growth

*values in bold denote noteworthy activity.

** ">8": MIC falls above the concentration of sample that was tested, denoting very weak activity.

Only seven out of 40 medicinal plants showed noteworthy antimicrobial activity (MIC value <1 mg/mL) against at least one of the tested pathogens. *Gunnera perpensa* was most active in this category with noteworthy antimicrobial activity against three pathogens; *S. aureus* (MIC value of 0.5 mg/mL), *K. pneumoniae* (MIC value of 0.5 mg/mL), and *S. marcescens* (MIC value of 0.13 mg/mL). Figure 2.4 is a graphical representation summarising the observed antimicrobial activity for the aqueous extracts.

Similar to the organic extracts, the most inhibited pathogen in the aqueous extract division was *C. perfringens*, although only 10% of plants achieved this inhibition. Noteworthy antimicrobial activity was observed against *K. pneumoniae* by one medicinal plant, *G. perpensa*, which interestingly was not found to be active when the organic extract was tested. Figure 2.5 is a graphical representation of the percentage inhibition activity displayed by the aqueous plant extracts against each pathogen tested.

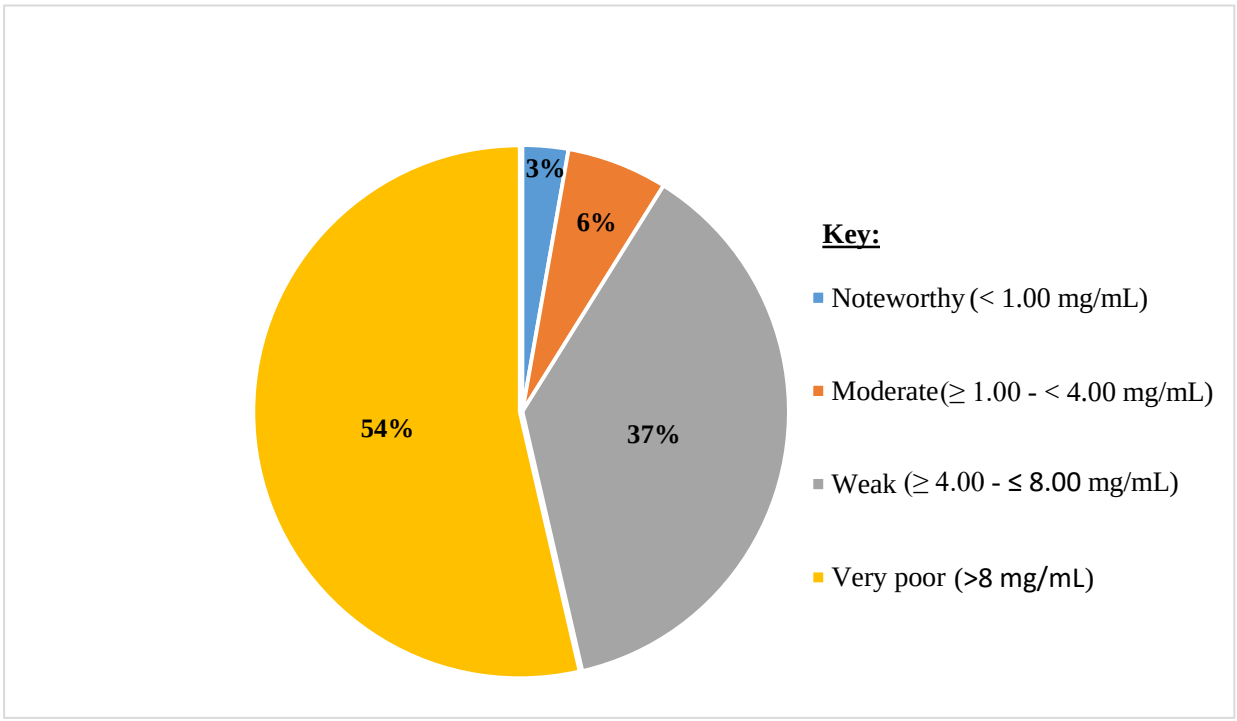


Figure 2. 4: Classification of antimicrobial activity observed with the aqueous plant extracts.

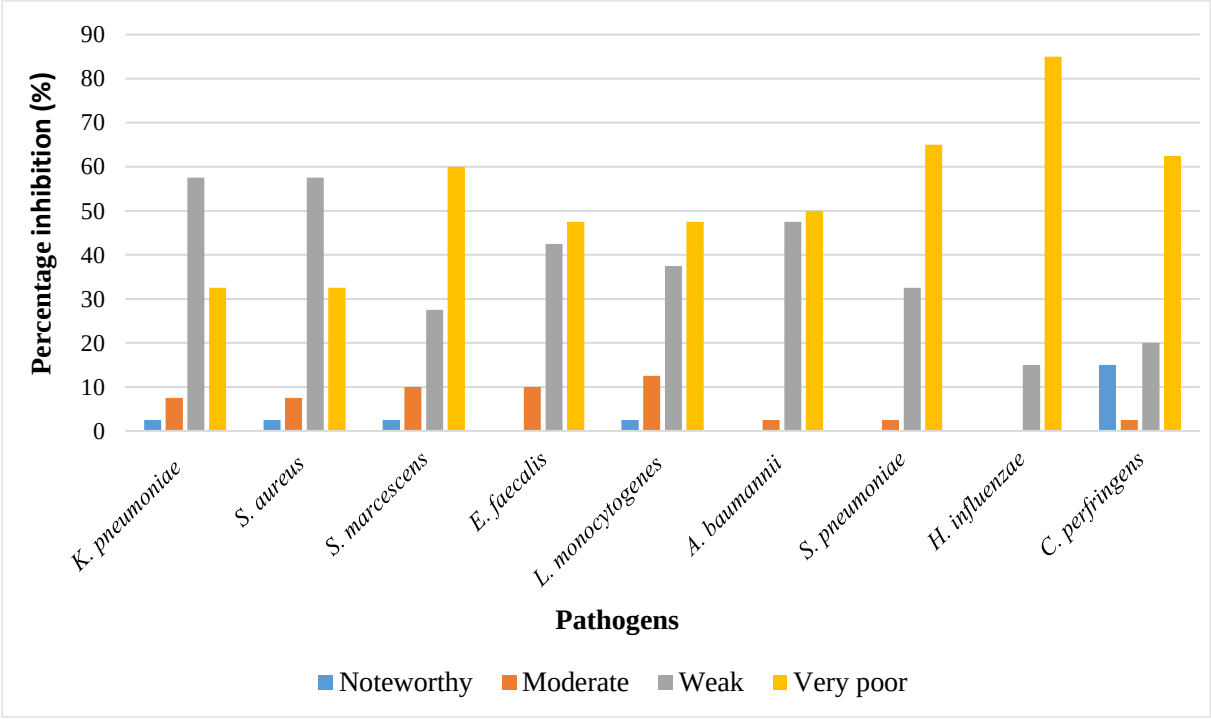


Figure 2. 5: Percentage inhibition of aqueous plant extracts against studied pathogens.

2.4. Discussion

2.4.1. Antimicrobial activity of organic plant extracts

Of the medicinal plants, 16% displayed noteworthy activity amongst the organic extracts, where certain medicinal plants displayed low inhibitory concentrations and broad-spectrum activity against Gram-positive and Gram-negative pathogens.

Ballota africana is one of the most popular and widely used medicinal plants in South Africa (Nortje and Van Wyk, 2015). Apart from fever, other traditional uses include lung infections, asthma and influenza (Watt and Breyer-Brandwijk, 1962; Van wyk et al., 2009). The organic extracts of *B. africana* showed noteworthy activity against *S. aureus* with an MIC value of 0.5 mg/mL, and against *L. monocytogenes* and *C. perfringens* MIC values of 0.25 mg/mL were obtained for both pathogens, demonstrating strong activity mainly against Gram-positive pathogens.

Salix mucronata is traditionally used in cases of rheumatic fevers, a disease affiliated to untreated coccal bacterial infections of the throat as well as scarlet fever (De Beer and Van Wyk, 2011). Leaf decoctions are administered orally to feverish patients (Watt and Breyer-Brandwijk, 1962; Van Wyk et al.,2008), headaches, backaches, and haemorrhoids are also treated traditionally with *S. mucronata* (Nortje and Van Wyk, 2015). The organic extract of *S. mucronata* showed noteworthy activity against *S. aureus* with an MIC value of 0.5 mg/mL. In a study by Eldeen et al. (2005), *S. mucronata* aqueous extract also displayed activity (0.5 mg/mL) against a different strain of *S. aureus* (ATCC 12600). *Salix mucronata* also showed noteworthy inhibitory activity against *S. marcescens* and *L. monocytogenes* with MIC values of 0.5 mg/mL and 0.25 mg/mL respectively.

Almost half of the identified species of *Helichrysum* occurs in South Africa (Hilliard, 1983). The genus is widely used by South African traditional healers to treat a variety of conditions such as influenza, kidney ailments, backache, stomach ailments, and fever (Nortje and Van Wyk, 2015). Both *Helichrysum* species included in this study, i.e. *H. odoratissimum* and *H. herbaceum*, showed noteworthy activity (organic extracts) against at least two pathogens. *Helichrysum odoratissimum* (organic extract) exhibited exceptional activity, showing noteworthy activity against five of the nine pathogens tested. Against *S. aureus* an MIC value of 0.25 mg/mL was observed, which is in close agreement with a study by Mathekga et al. (1998), where an MIC value of 0.1 mg/mL was observed. *Helichrysum odoratissimum* also

showed noteworthy activity against *S. pneumoniae* with an MIC value of 0.5 mg/mL. Comparative studies regarding *S. pneumoniae* could not be found, however, in a study undertaken by Ocheng et al. (2014), the hexane extract of *H. odoratissimum* was shown to have noteworthy activity against *Streptococcus mutans* (MIC value of 0.25 mg/mL) and *Streptococcus sobrinus* (MIC value of 0.125 mg/mL). The organic extract of *H. odoratissimum* also showed noteworthy activity against *L. monocytogenes* (MIC value of 0.13 mg/mL) and *C. perfringens* (MIC value of 0.01 mg/mL) demonstrating strong Gram-positive activity, but also against Gram-negative *S. marcescens*, with an MIC value of 0.5 mg/mL.

Eucalyptus globulus (organic extracts) appeared to be the most active medicinal plant in this study. The leaves of this plant displayed noteworthy broad-spectrum microbial inhibition against six of the nine pathogens tested. Among the three exceptions were *K. pneumoniae* and *E. faecalis*, which were moderately inhibited (both at 1 mg/mL). The essential oil from *E. globulus* is widely studied (Lis-Balchin and Deans, 1997; Ghalem and Mohamed, 2008; Tyagi and Malik, 2011; Bachir and Benali, 2012). In this study, the extracts were the focus and showed noteworthy antimicrobial activity against both *S. aureus* (MIC value of 0.13 mg/mL) and *S. pneumoniae* (MIC value of 0.13 mg/mL). This same activity regarding *S. aureus* was also reported by Dezsi et al. (2015), while similar activity against *S. pneumoniae* was reported by Salari et al. (2006). *Eucalyptus globulus* also showed inhibitory activity against *L. monocytogenes* (MIC value of 0.13 mg/mL), in agreement with a study by Dezsi et al. (2015), where *E. globulus* was shown to inhibit the growth of *L. monocytogenes* (MIC value of 0.03 mg/mL). *Eucalyptus globulus* demonstrated the lowest MIC value in the study against *C. perfringens* (MIC value of 0.004 mg/mL) which was previously not reported.

Withania somnifera is an important medicinal herb found in parts of Africa, including South Africa, Congo, and Egypt, as well as India, Sri Lanka and Pakistan, and is widely used as a home remedy for a wide array of complaints and illnesses (Owais et al., 2005). However, in India its medicinal properties were specially recognised and it was listed as an official drug in the Indian pharmacopoeia (Owais et al., 2005). In addition to fever, traditional uses of this plant include intestinal ailments, wounds and sores asthma, enema, and haemorrhoids (Van Wyk, 2008; Van Wyk et al., 2008; Kose et al., 2015). The organic extract of *W. somnifera* showed noteworthy activity against both *S. aureus* and *S. pneumoniae* with an MIC value of 0.5 mg/mL and 0.25 mg/mL respectively. The activity against *S. aureus* confirms previous findings where the methanol extracts of *W. somnifera* was tested in a disc diffusion assay (Mahesh and Satish, 2008). *Withania somnifera* further showed noteworthy activity against Gram-positive

L. monocytogenes (MIC value of 0.25 mg/mL) and *C. perfringens* (MIC value of 0.5 mg/mL) which corresponds with its traditional uses for intestinal ailments, wounds and fever. In this instance, the antimicrobial activity of *W. somnifera* leaves has been strongly attributed to the presence of withanolides and withaferin A, and which are extractable with organic solvents (Singh et al., 2010). In a study by Dhanani et al. (2017), the organic extract of *W. somnifera* was shown to contain more of these withanolides than the aqueous extract samples, which explains the difference in activity of the two extracts.

2.4.2. Antimicrobial activity of aqueous plant extracts

Compared to the organic extracts, the aqueous plant exhibited poor activity in the antimicrobial studies. Medicinal plant antimicrobial studies are generally expected to exhibit more prominent antimicrobial activity with organic extracts than with aqueous extracts of the plants. This is because water-based extraction may not be a suitable solvent for lipophilic or non-polar compounds. Methanol and dichloromethane therefore will extract a wider variety of chemical compounds from the plants than aqueous extracts, hence the pool of chemical compounds in organic extracts have a higher probability of containing compounds that possess antimicrobial activity (Cowan, 1999). This was true of all the plants tested in this study, with the exception of *Gunnera perpensa*.

The aqueous extracts of *G. perpensa* exhibited stronger inhibition compared to the organic extracts. It was interesting to observe that only the aqueous extract of *G. perpensa* showed noteworthy activity against *S. aureus* (MIC value of 0.50 mg/mL), which is agreement with the study by Buwa and Van Staden (2006) and McGaw et al. (2000). These studies also reported greater antimicrobial activity with the aqueous extracts of *G. perpensa* as opposed to the organic extracts. *Gunnera perpensa* also showed noteworthy antimicrobial activity against the Gram-negative pathogen *K. pneumoniae* (MIC value of 0.50 mg/mL). Activity against *K. pneumoniae* by *G. perpensa* aqueous extract was also reported by McGaw et al. (2000), and Buwa and Van Staden (2006).

Traditional dosage forms of *H. odoratissimum* vary depending on the ailment being treated: the leaves of the plant may be administered through smoke inhalation; plant material may be formed into a pulp for use as a wound dressing; and ashes of the plant may be rubbed onto the affected area or eaten. However, for treating fever, leaves are usually used in an infusion or administered as a tea (Watt and Breyer-Brandwijk, 1962; Hutchings, 1989; Lourens et al., 2008). The aqueous extracts used in this study closely resemble this dosage form, hence

noteworthy activity observed in this regard is of interest. Both *Helichrysum* species included in this study, *H. odoratissimum* and *H. herbaceum*, showed noteworthy activity against at least one pathogen in the aqueous extract form. *Helichrysum odoratissimum* showed noteworthy activity against *L. monocyogenes* and *C. perfringens* with an MIC value of 0.13 mg/mL against both pathogens, while *H. herbaceum* had noteworthy activity against *C. perfringens* (MIC 0.50 mg/mL).

2.4.3. Antimicrobial susceptibility of prevalent pathogens

All nine of the selected pathogens studied were found to be prevalent in cases of septicaemia, and therefore possibly the causative agent in the fever (Naidoo et al., 2013; Dubey et al., 2013; Clermont et al., 2007; Bannister et al., 2000). A study conducted by Edmond et al. (1999) ranked the most prevalent pathogens in septicaemia in 49 hospitals in the USA. Amongst the listed pathogens, *S. aureus* was found to have the highest correlation with cases of septicaemia and fever (15.7%), ranking second after coagulase-negative staphylococci (31.9%). This was followed by the *Enterococci* spp. (11.1%), of which *E. faecalis* constituted 55.2% cases of the *Enterococci* spp., and then *Klebsiella* spp (5.4%).

Both *E. faecalis* and *K. pneumoniae*, have been listed in a WHO report stressing the urgent need of research and intervention with regards to new treatment agents for these pathogens (WHO, 2017). The majority of organic medicinal plants studied here displayed very poor (60%), weak and moderate (20% each) antimicrobial activity against *K. pneumoniae*. The lowest MIC observed against *K. pneumoniae* was exhibited by the aqueous extracts of *G. perpensa* with an MIC value of 0.5 mg/mL, classified as noteworthy antimicrobial activity. This was also the only noteworthy activity observed against this pathogen in this study across both organic and aqueous categories. With *E. faecalis*, moderate antimicrobial activity was observed for several of the organic extracts (50 %), while the majority of aqueous extracts presented with very poor activity (MIC >8 mg/mL). For organic extracts, noteworthy activity was observed with the extract of *A. maculata* at MIC 0.5 mg/mL, and was the only noteworthy activity against *E. faecalis* observed in the study.

The *Serratia* spp. was not exempt from these statistics, ranking ninth out of ten pathogens listed as the important causative agents of fevers (Edmond et al., 1999). *Serratia marcescens*, a Gram-negative bacillus, was previously thought to be a non-pathogenic micro-organism, but increasing frequency of infections from this pathogen was observed since the 1950s (Dodson, 1968). It has since been implicated in the cause of septicaemia, meningitis as well as respiratory

and urinary tract infections. *Serratia marcescens* has the ability to survive under harsh environmental conditions and utilises a wide variety of nutrients for survival (Hejaz and Falkiner, 1997). The organic extracts of *E. globulus* showed noteworthy activity against this pathogen with an MIC value of 0.02 mg/mL, followed by the organic extracts of *S. mucronata* and *H. odoratissimum* (both with MIC values of 0.5 mg/mL).

Clostridium perfringens contributes significantly to skin infections which lead to sepsis and fever. However, *C. perfringens* is one of the most commonly neglected pathogens in antimicrobial research (van Vuuren and Holl, 2017). The species was also identified as problematic by the WHO with regards to antimicrobial intervention. It is usually affiliated to respiratory, gastrointestinal and systemic ailments, i.e. bacteraemia infections (WHO, 2017).

Over 70% of organic extracts displayed noteworthy antimicrobial activity against *C. perfringens*. The highest inhibition achieved against this pathogen was reported for the organic extract of *E. globulus* with an MIC value of 0.004 mg/mL. With regards to the aqueous extracts, *C. perfringens* was the most susceptible microbe in the category. The aqueous extracts of *O. asteriscoides* and *H. odoratissimum* presented with the lowest MIC values against this pathogen (MIC value of 0.13 mg/mL for both extracts), which is noteworthy activity.

Listeria monocytogenes is a Gram-positive, intracellular foodborne pathogen that multiplies readily in meat, poultry and fish. It has also been found to inhabit soil, contaminated water and decaying vegetables. Its ability to persist under stress conditions make it especially difficult to control and eradicate (Maurella et al., 2018). The pathogen has been implicated in several food outbreaks reported worldwide and is especially detrimental in pregnant or immunocompromised individuals (Farber and Peterkin, 1991). Neonates and elderly citizens also at high risk of immune-invasion. Pathogenic *L. monocytogenes* manifestations include listeriosis, septicaemia, and meningoencephalitis (Farber and Peterkin, 1991), all of which manifests with a high fever.

In 2016, *L. monocytogenes* was isolated from previously healthy individuals from two different schools in two different villages of the same province in Italy who presented with febrile gastroenteritis. *Listeria monocytogenes* was confirmed as the causative pathogen and traced back to a cold meat ham consumed in both schools (Maurella et al., 2018). More recently, between the period of 2017 and early 2018, *L. monocytogenes* was implicated in one of the greatest food outbreaks in South Africa. Between January 2017 and 17 July 2018, 1 060 laboratory-confirmed cases of Listeriosis were reported (NICD, 2018). As of December 2018,

L. monocytogenes has been included in the list of notifiable disease in South Africa (WHO, 2018). In this instance, organic extracts of *L. javanica* and *W. somnifera* exhibited the most noteworthy activity against this pathogen (MIC 0.06 mg/mL and 0.02 mg/mL respectively). Other organic extracts for which noteworthy activity was also observed against this pathogen included those of *E. globulus* (MIC value of 0.13 mg/mL), *B. africana* (MIC value of 0.25 mg/mL), *H. odoratissimum* (MIC value of 0.13 mg/mL), *S. mucronata* (MIC value of 0.25 mg/mL), *T. riparia* (MIC value of 0.5 mg/mL), as well as the aqueous extracts of *H. odoratissimum* (MIC value of 0.13 mg/mL).

Acinetobacter baumannii, a Gram-negative, aerobic pathogen, is an important opportunist in critically ill hospitalized patients, and causes bacteraemia in immunocompromised patients (Dubey et al., 2013; Dijkshoorn et al., 2007; Bannister et al., 2000). According to Logan et al. (2018), *A. baumannii* was noted in the Centre of Disease Control and Prevention (CDC) as a significant threat to antibiotic resistance, with 7300 Multi Drug Resistant (MDR) *Acinetobacter* infections resulting in 500 deaths in the USA each year. Despite this alarming reality, research in the field of medicinal plants to combat this pathogen is limited. In this study the organic extracts of *C. spicata, D. viscosa, E. globulus, G. perpensa, M. flabellifolius* and *S. dentata* showed noteworthy antimicrobial properties against *A. baumannii* with a MIC value of 0.5 mg/mL.

The antimicrobial inhibition of Gram-positive bacteria is generally known to be greater than that of Gram-negative bacteria (Takahashi et al., 2004). This is due to the high content of lipopolysaccharide layer of the Gram-negative cell wall which decreases permeability of the extract's compounds into the bacterial cell (Takahashi et al., 2004). This was also observed in the overall MIC assay where Gram-positive bacteria were generally more susceptible to medicinal plant extracts than Gram-negative pathogens. The results obtained from the MIC assays demonstrate the effectiveness of medicinal plants that are used traditionally as fever-reducing agents in inhibiting the growth of pathogens related to high fever.

2.5. Chapter Summary

- A total of 3% of the aqueous extracts demonstrated noteworthy activity, while 16% of the organic extracts had noteworthy activity.
- The best broad-spectrum organic plant extract activity was observed with *E. globulus* inhibiting the growth of six of the nine pathogens studied followed by *H. odoratissimum* inhibiting five pathogens

- The best broad-spectrum aqueous extract activity was observed with *G. perpensa* inhibiting growth of three of the nine pathogens studied.
- The lowest MIC was observed with the organic extract of *E. globulus*, with an MIC value of 0.004 mg/mL against *C. perfringens*.
- The most susceptible pathogen by both organic and aqueous extracts was *C. perfringens*, followed by *L. monocytogenes*, both of which are important Gram-positive pathogenic bacteria as according to the WHO.

CHAPTER 3

CYTOTOXICITY STUDIES OF MEDICINAL PLANT EXTRACTS

3.1. Introduction

More people are inclining towards natural therapeutic products in order to avoid the perceived side effects of synthetic drugs, and hence medicinal plants are still valued even in urbanised communities. A common misconception is that medicinal plants, being of natural origin, exhibit fewer side effects, are non-toxic, and generally safer than synthetic drugs (Fennell et al., 2004; Street et al., 2008). However, with the definition of cytotoxicity as an unpleasant manifestation resulting from the interaction between an exogenous chemical constituent with the host cell (Rozman and Doull, 2000), the safety of medicinal plants may not be necessarily justified based on their natural occurrence. Cytotoxicity studies are therefore imperative in any study that may present medicinal plants as promising therapeutic agents.

The concept of therapeutic plants and their potential cytotoxicity is thus not new amongst researchers. Several studies have reported on the cytotoxicity of numerous medicinal plants (Fennell et al., 2004; Reid et al., 2006; George, 2011; Nasri and Shirzad, 2013; Ndhlala et al., 2013). Unlike synthetic drugs, which consist of one or a few chemical constituents, medicinal plants used traditionally as crude extracts may contain up to 400 or more chemical constituents (George, 2011). Although this broadens the chemical pool from where new chemical compounds with therapeutic potential may be discovered, it also makes it much more difficult to analyse the therapeutic properties of the plant, i.e. in terms of synergy, antagonism, side effects and also cytotoxicity.

It is understood that a therapeutically active compound in a crude extract may be affected or modulated by several other compounds that coexist in the crude extract (George, 2011). Similarly, cytotoxicity of a medicinal plant may be expressed by a compound in the crude extract which is different from that which exhibited positive therapeutic properties. Because of this complex nature of crude extracts, the notion of cytotoxicity of medicinal plants, although they are natural products, still need to be considered.

The Brine shrimp lethality assay (BSLA) is a quick, yet efficient and cost effective bioassay that is commonly used as a preliminary screening tool for cytotoxicity of medicinal plants and natural products (Meyer et al., 1982; Bussmann et al., 2011). This assay allows for inexpensive, in-house screening of medicinal plant cytotoxicity using readily available *Artemia franciscanan* cysts. Combined with the effortless hatching of the cysts and rapid growth of the larvae, this has become one of the most popular assays in preliminary screening of cytotoxicity. It was therefore selected as the assay of choice for this study.

3.2. Methods and materials

3.2.1. Plant extract preparation

Aqueous plant extracts were prepared to a concentration of 2 mg/mL to achieve a concentration of 1 mg/mL when dilution is accounted for, i.e. after incorporation of the salt water containing the brine shrimp. If a plant requires a concentration greater than 1 mg/mL to exhibit cytotoxicity, it is considered non-toxic (Bussmann et al., 2011). Organic extracts were prepared at a sample concentration of 1 mg/mL to account for solubility challenges and hence testing concentration was 0.5 mg/mL (accounting for dilution).

3.2.2. Hatching the brine shrimp cysts

Artificial seawater was prepared by dissolving 16 g of Tropic Marine® salt in 500 mL of distilled water. Dried brine shrimp cysts (*Artemia franciscana*) (Ocean Nutrition), 0.5 g, were added to the artificial seawater and allowed to hatch at 25˚C for 48 hrs. A rotatory pump (Kiho) was used during this process for aeration of the artificial sea water and for dispersion of larvae, and a lamp (230 V) was positioned directly above the set up as a light source.

3.2.3. Brine shrimp lethality assay

Once the cysts had hatched, the salt water containing the shrimp was transferred to a rectangular container, where a light source was positioned to attract the shrimp to a particular area where they could be collected. An amount of 400 µL of seawater containing the hatched brine shrimp was aspirated and introduced to each of the wells of a 48 micro-titre well plate. This was followed by the introduction of 400 µL of aqueous plant extract (2 mg/mL) or organic extract (1 mg/mL) in each well. Artificial seawater, which provides an ideal environment for the growth of the brine shrimp cysts, was used as a negative control in this study, while 1.6 mg/mL potassium dichromate (Sigma Aldrich®), which is known to be toxic to brine shrimp larvae, was used as the positive control. The results were observed under a light microscope (Olympus)

at 4x magnification. This was performed initially after adding samples to rule out any brine shrimp that was already dead, after 24 hrs and again after 48 hrs. Thereafter, 50 µL of glacial acetic acid (Sigma Aldrich®), which is lethal to shrimp, was added to allow a total dead count to be performed. This allowed the percentage mortality after 24 and 48 hrs to be calculated. A percentage mortality of 50% or more was considered biologically toxic (Bussmann et al., 2011).

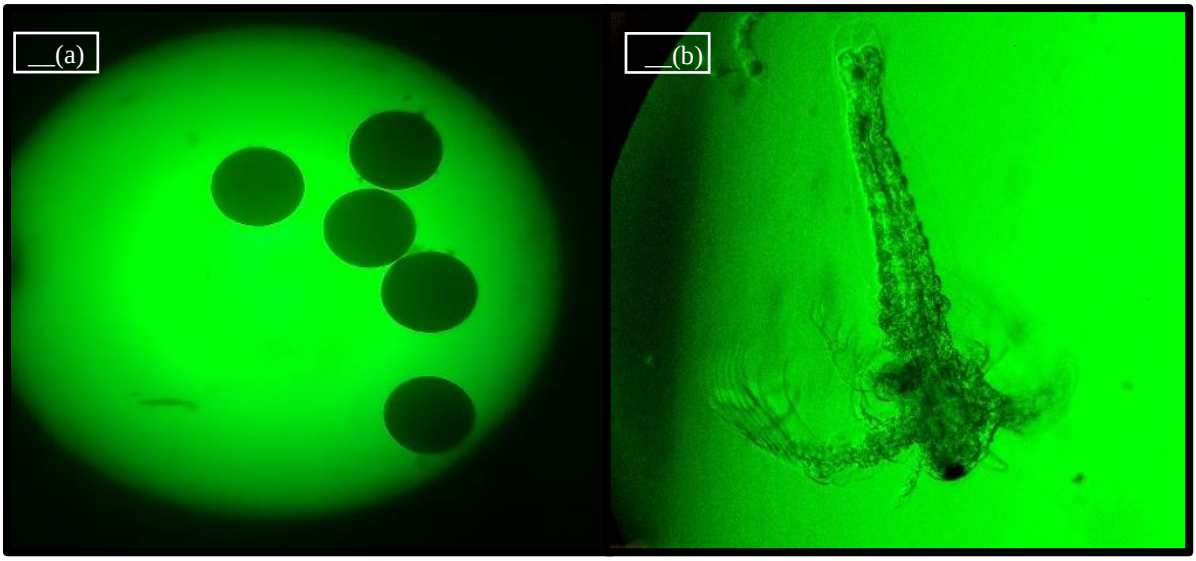


Figure 3. 1: Structure of the brine shrimp cysts (a) and adult larvae (b) as seen through the light microscope (Olympus) at 40x magnification.

3.3. Results

Of the 40 medicinal plants initially extracted, 28 of the plants were found to possess noteworthy antimicrobial properties (MIC value < 1 mg/mL, Chapter 2, Table 2.2). The potential cytotoxicity of both the organic and aqueous extracts of each of the 28 plants were consequently determined using the BSLA. The results of the assay, i.e. the average percentage mortality effected by each plant extract, is presented in Table 3.1 for organic plant extracts and Table 3.2 for aqueous plant extracts.

At first glance, it is noticeable that the organic extracts exhibited the highest cytotoxicity (percentage mortality of 17 of the 28 extracts were greater than 50% after 48 hrs). Two of the 17 plant extracts, *A. maculata* and *C. molle*, showed delayed cytotoxicity where cytotoxicity was exhibited after 48 hrs even though no cytotoxicity was exhibited after 24 hrs.

Organic extracts of *C. capensis*, *C. gratissimus*, *E. globulus*, *E. sideroxylon*, *H. herbaceum*, *H. odoratissimum*, *L. javanica*, *T. riparia*, and *W. somnifera* were found to be highly toxic to brine shrimp larvae, with percentage mortality exceeding 90%. In the case of *E. globulus*,

H. herbaceum, H. odoratissimum, L. javanica, and *W. somnifera* these extracts presented with a percentage mortality exceeding 90% at both 24 and 48 hrs.

Table 3. 1: Brine shrimp lethality assay of organic plant extracts.

Plant extract	**% Mortality n=3**	
	24 hrs	**48 hrs**
Aloe maculata	43.87	**54.55**
Ballota africana	35.79	38.85
Cissampelos capensis	**85.66**	**93.80**
Combretum molle	41.95	**61.44**
Commiphora africana	**57.35**	**64.34**
Croton gratissimus	**76.39**	**99.31**
Dodonaea viscosa	31.82	34.58
Elephantorrhiza elephantina	**72.89**	**86.14**
Eucalyptus globulus	**97.08**	**98.91**
Eucalyptus sideroxylon	**83.19**	**97.06**
Gunnera perpensa	36.06	39.96
Helichrysum herbaceum	**99.23**	**100.00**
Helichrysum odoratissimum	**93.19**	**96.73**
Lippia javanica	**97.36**	**100.00**
Mentha longifolia	**67.68**	**76.26**
Myrothamnus flabellifolius	40.56	42.40
Osmitopsis asteriscoides	43.96	46.33
Pelargonium sidoides	31.54	38.27
Rauvolfia caffra	**61.18**	**64.62**
Salix mucronata	20.16	22.33
Salvia dentata	**71.56**	**88.71**
Sclerocarya birrea	9.32	14.19
Strychnos henningsii	33.94	38.18
Sutherlandia frutescens	**75.54**	**86.14**
Tarchonanthus camphoratus	33.90	39.60
Tetradenia riparia	**97.38**	**100.00**
Withania somnifera	**98.26**	**99.71**
Zanthoxylum capense	33.27	42.89
Controls		

Plant extract	% Mortality n=3	
	24 hrs	48 hrs
Negative control: artificial sea water	0.00	0.00
Positive control: Potassium dichromate	100.00	100.00

+Values marked in bold denote a % Mortality greater than 50% and are considered toxic.

From Figure 3.2 it is evident that the majority of organic extracts were cytotoxic to brine shrimp larvae at 60.71%. However, 39.29% of the extracts did not display cytotoxicity. Eleven of the organic plant extracts, i.e. *B. africana*, *D. viscosa*, *G. perpensa*, *M. flabellifolius*, *O. asteriscoides*, *P. sidoides*, *S. mucronata*, *S. birrea*, *S. henningsii*, *T. camphoratus* and *Z. capense* demonstrated no cytotoxic activity against the brine shrimp at both 24 and 48 hrs. It is interesting to note that *B. africana* (% mortality of 38.85 at 48 hrs), *M. flabellifolius* (% mortality of 42.40 at 48 hrs) and *S. mucronata* (% mortality of 22.33 at 48 hrs) were among those that demonstrated strong antimicrobial properties (Chapter 2). The lowest % mortality was observed with *S. birrea*, with 9.2% mortality at 24 hrs and 14.19% at 48 hrs.

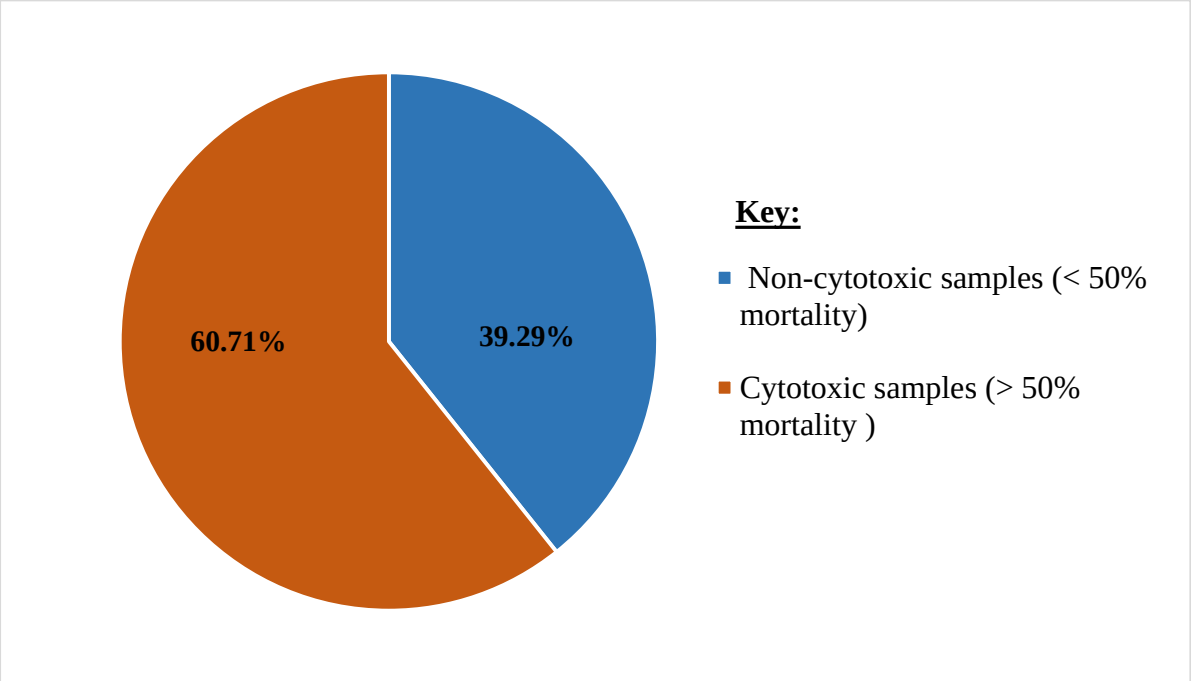


Figure 3. 2: Graphical representation of the observed cytotoxicity of organic extracts after a 48 hr period.

With regards to aqueous extracts (Table 3.2), very few plants exhibited toxic effects to the brine shrimp at 1 mg/mL. High mortality rates were seen with the extracts of *E. globulus*, *H. herbaceum*, *H. odoratissimum*, *S. dentata*, and *W. somnifera* both at 24 and 48 hrs. The lowest percentage mortality was noted with plant extracts *P. sidoides*, *S. birrea*, and *Z. capense*, where

all plant extracts had a percentage mortality of less than 10% at 24 hrs and not more than 20% mortality at 48 hrs. Although these plants did not exhibit strong antimicrobial inhibition against the pathogens tested in this study, they are still used widely in the practice of traditional medicine. It is therefore liberating to observe very low cytotoxicity profiles for these plants.

Table 3. 2: Brine shrimp lethality assay of aqueous plant extracts.

Plant extract	% Mortality n=3	
	24 hrs	48 hrs
Aloe maculata	12.76	21.62
Ballota africana	12.18	14.79
Cissampelos capensis	14.76	19.73
Combretum molle	17.04	35.21
Commiphora africana	16.67	40.48
Croton gratissimus	16.36	45.45
Dodonaea viscosa	5.36	30.36
Elephantorrhiza elephantina	14.16	36.28
Eucalyptus globulus	**52.78**	**89.29**
Eucalyptus sideroxylon	20.73	43.90
Gunnera perpensa	19.09	30.91
Helichrysum herbaceum	**51.60**	**96.00**
Helichrysum odoratissimum	**91.11**	**100.00**
Lippia javanica	22.43	47.80
Mentha longifolia	24.06	36.79
Myrothamnus flabellifolius	16.77	25.81
Osmitopsis asteriscoides	19.34	38.21
Pelargonium sidoides	7.41	9.88
Rauvolfia caffra	39.02	39.02
Salix mucronata	38.01	47.03
Salvia dentata	**84.04**	**85.92**
Sclerocarya birrea	2.11	14.10
Strychnos henningsii	35.67	43.05
Sutherlandia frutescens	20.70	30.93
Tarchonanthus camphoratus	13.04	21.20
Tetradenia riparia	36.26	**60.44**
Withania somnifera	**78.22**	**93.56**

Plant extract	% Mortality n=3	
	24 hrs	48 hrs
Zanthoxylum capense	7.69	18.77
Controls		
Negative control: artificial sea water	0.00	0.93
Positive control: Potassium dichromate	100	100

+Values marked in bold denote a % Mortality greater than 50% and are considered toxic.

In comparison to the organic extracts, the majority of the aqueous extracts were deemed safe, exhibiting less than 50% cytotoxicity (Figure 3.3). The large difference in cytotoxic profiles of the two types of extracts can easily be seen when studying Figure 3.2 and Figure 3.3. Although the aqueous extracts are the most frequently used in traditional practice, and despite the positive outcomes observed from the cytotoxic studies which deems a majority of them safe, the challenge that still needs to be addressed is the poor antimicrobial activity demonstrated by 54% of the plants studied (Chapter 2, Figure 2.4).

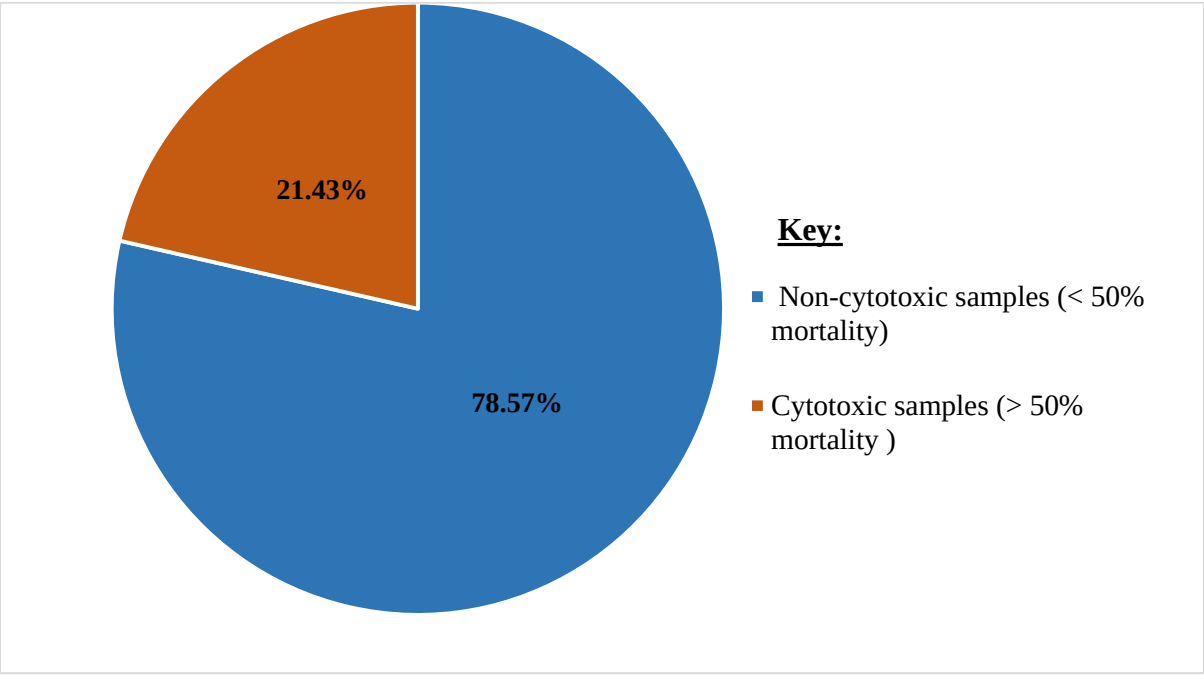


Figure 3. 3: Graphical representation of the observed cytotoxicity of aqueous extracts after a 48 hr period.

For medicinal plants with a percentage mortality of greater than 50%, i.e. toxic samples, LC_{50} values were further calculated. The LC_{50} value refers to the concentration of a substance which will result in 50% mortality rate of the test sample. A lower LC_{50} value indicates a higher toxic profile of a substance. The LC_{50} values that are below 249 µg/mL are therefore classified as

highly toxic, 250 µg/mL to 499 µg/mL are moderately toxic, and 500-1000 µg/mL are considered low in cytotoxicity (Bussmann et al., 2011). The LC_{50} values of the toxic samples can be seen in Table 3.3 for organic extracts and Table 3.4 for aqueous extracts.

Table 3. 3: LC_{50} values of toxic organic plant extracts at 24 and 48 hrs.

Plant extract	LC_{50} (µg/mL) n=3	
	24 hrs	48 hrs
Cissampelos capensis	**147**	**136**
Commiphora africana	387	346
Croton gratissimus	**227**	**178**
Elephantorrhiza elephantina	255	**236**
Eucalyptus globulus	**198**	**187**
Eucalyptus sideroxylon	253	**201**
Helichrysum herbaceum	**132**	**110**
Helichrysum odoratissimum	**132**	**127**
Lippia javanica	**210**	**203**
Mentha longifolia	287	270
Rauvolfia caffra	563	324
Salvia dentata	295	**236**
Sutherlandia frutescens	341	283
Tetradenia riparia	**216**	**216**
Withania somnifera	294	273

+Values marked in bold signify highly toxic medicinal plants (LC_{50} < 249 µg/mL).

The organic extracts of *C. capensis*, *E. globulus*, *H. herbaceum* and *H. odoratissimum* demonstrated high cytotoxicity to brine shrimp with LC_{50} under 200 µg/mL after 24 hrs, ranging from 110 µg/mL with *H. herbaceum* to 187 µg/mL for *E. globulus*. In total, seven medicinal plants demonstrated high cytotoxicity (LC_{50} < 249 µg/mL) after 24 hrs, while 10 of the medicinal plants showed high cytotoxicity after 48 hrs. With the exception to the organic extract of *R. caffra* at 24 hrs only (LC_{50} value of 563 µg/mL), the remaining organic extracts displayed moderate cytotoxicity with LC_{50} values between the range of 250 and 499 µg/mL.

With regards to the aqueous extracts (Table 3.4), all medicinal plants that displayed cytotoxicity to the brine shrimp were considered moderately cytotoxic, as the LC_{50} values fell

within the range that signifies moderate cytotoxicity, i.e. 250 to 499 µg/mL (Bussmann et al., 2011).

Table 3. 4: LC_{50} values of toxic aqueous plant extracts at 24 and 48 hrs.

Plant extract	LC_{50} (µg/mL) n=3	
	24 hrs	48 hrs
Eucalyptus globulus	372	362
Helichrysum herbaceum	286	273
Helichrysum odoratissimum	342	316
Salvia dentata	381	350
Withania somnifera	351	322

3.4. Discussion

The majority of the aqueous extracts were deemed non-toxic with the percentage mortality below 50%. Of the 28 aqueous plant extracts tested, only six were found to be toxic at 48 hrs. The opposite was true for organic extracts, where a larger number of plant extracts exhibited cytotoxic effects. The lowest cytotoxicity profile for both the organic and the aqueous extract category was seen with the extracts of *S. birrea*. A literature review undertaken by Ojewole et al. (2010) substantiates this data, as *S. birrea* was shown to exhibit low cytotoxicity in different *in vitro* and *in vivo* studies (studies include the BSLA, MTT cell viability assay, and the intraperitoneal delivery of *S. birrea* bark extracts to mice). All studies demonstrated no cytotoxicity, further supporting the safety of this medicinal plant. *Sclerocarya birrea*, vernacular name "marula", is a plant that has received international recognition, with all parts of the plant being valuable both as food and for medicinal purposes. In South Africa the bark of this plant, which is rich in tannins, is commonly used for medicinal purposes such as stomach ailments, toothaches, diabetes, arthritis, headaches and fevers (Felhaber, 1997; Ojewole, 2003; Street and Prinsloo, 2012).

Members of the *Cissampelos* genus are known for their toxic nature both in scientific literature and by traditional users. Traditionally, its toxic properties were put to use by application of the plant on hunting arrows. However, some species have also been used for their therapeutic properties to treat fever, asthma, dysentery, hypertension and various other complaints (Van Wyk et al., 2008; Semwal et al., 2014). *Cissampelos capensis* is the only species of

Cissampelos that is endemic to Southern Africa. In addition to fever, it is traditionally used to treat diabetes, tuberculosis, as well as stomach and skin cancers (de Wet et al., 2011). The cytotoxicity of the organic extract of *C. capensis* was also confirmed in a BSLA by Akhalwaya et al. (2018).

Croton gratissimus is sometimes referred to as 'koorsbessie', which makes reference to its traditional use in reducing fever. Additionally, *C. gratissimus* is also used traditionally to treat cold, coughs, sextually transmitted infections (STIs), and as a purgative for stomach ailments (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Van Wyk et al., 2002). The cytotoxicity of *Croton gratissimus* has, however, previously been noted (Bryant 1966, in Mulholland et al., 2010). In this instance, the stem bark of *C. gratissimus* was shown to have moderate cytotoxicity to ovarian cancerous cells in an MTT cell line assay (Mulholland et al., 2010). Additionally, the essential oil of *C. gratissimus*, as well as its organic dichloromethane:methanol extract, were also shown to exhibit toxic outcomes in studies undertaken by Lawal et al. (2017) and Akhalwaya et al. (2018).

Sutherlandia frutescens is referred to as the cancer bush, in relation to its traditional use against cancer (Skerman et al., 2011; Tai et al., 2004). There is a strong correlation which is often observed between cytotoxicity of medicinal plants and its anticancer properties (Itharat et al., 2004; Krishnaraju et al., 2006). The organic extracts of *S. frutescens* were found to be toxic to brine shrimp both at 24 and 48 hrs. However, the aqueous extract did not exhibit any cytotoxicity. In a study undertaken by Steenkamp and Gouws (2006), *in vitro* cytotoxicity studies of the aqueous extract of *S. frutescens* leaves against DU-145 prostate cancer cells, MDA-MB-321 and MCF-7 breast cancer cells, as well as MCF 12A non-malignant breast cells also did not show any profound cytotoxicity. *Sutherlandia frutescens* was able to inhibit the proliferation of the MCF-7 breast cancer cells, but encouraged proliferation of the other cells. Similar results were obtained by Hübsch et al. (2014), where the cell line assay of *S. frutescens*, tested at a concentration of 100 μg/mL, did not exhibit cytotoxicity against human kidney epithelial (Graham or HEK-293) cells. However, in a brine shrimp assay, Hübsch et al. (2014) observed cytotoxicity for the organic extracts of *S. frutescens* at 1 mg/mL. Similar results were obtained in this study, where only the organic extract of *S. frutescence* exhibited cytotoxicity. In a study undertaken by Tai et al. (2004), it was found that the cytotoxic effects of *S. frutescens* were dose dependent after testing different diluted concentrations of *S. frutescens* against a range of tumour cells, where 1/400 and 1/1200 dilutions from a 300 mg *S. frutescence* tablet did not significantly affect the cells.

Lippia javanica, which is also referred to as "koorsbossie" or "fever tea" due to its strong traditional use against fever, is amongst the frequently used medicinal plants which has previously been noted for its cytotoxicity (Hutchings and Van Staden 1994). In addition to the evidence of cytotoxicity observed in the aqueous and organic extracts of *L. javanica* in this study, a study undertaken by Hübsch et al. (2014) reached a similar conclusion when the organic extract of *L. javanica* presented with high percentage mortality (70.10%) in the BSLA, and the said extract caused cell death to human kidney epithelial cell (Graham or HEK-293) in an MTT cell line cytotoxicity assay. Although no cytotoxicity was observed for the aqueous extracts in this study, Madzimure et al. (2011) reports that administration of *L. javanica* aqueous extracts at 12.5-37.5% *v/v* to mice resulted in lethargy within 48 hrs of administration.

Cytotoxicity of *E. globulus* has been confirmed in several *in vitro* studies. Bussmann et al. (2011) reported the extracts of this plant to be highly cytotoxic to brine shrimp in a BSLA with LC_{50} values as low as 240 µg/mL for ethanol extracts, and 29 µg/mL for aqueous extracts. Even the essential oil of this plant, which is commonly used in aromatherapy, was also found to be highly toxic (Akolade et al., 2012).

According to Van Wyk (2011), *H. odoratissimum* is among the most underrated medicinal plants in terms of medicinal plant development, where the plant has previously shown activity. Lourens et al. (2008) noted that there is a lack of scientific cytotoxic data for *Helichrysum* species despite its frequent uses, which is most concerning. However, adjacent to this study, other studies which investigated the cytotoxicity of the *Helichrysum* species found the organic extract of *H. herbaceum* to have notable mutagenic activity in a Salmonella/microsome mutagenicity assay (Ames), where the whole plant was tested (Reid et al., 2006). The essential oil of *H. odoratissimum* also exhibited moderate cytotoxicity against brine shrimp larvae (Lawal et al., 2015).

Organic and aqueous extracts of *E. globulus*, *H. odoratissimum*, and *W. somnifera*, highlighted in Chapter 2 for their noteworthy antimicrobial activities, were found to be highly toxic to brine shrimp larvae. These plants, together with *S. dentata* and *H. herbaceum*, showed toxic effects with both aqueous and organic extracts tested. One would question at this stage whether the antimicrobial activities observed from these plants were indeed inhibition of the tested pathogens, or rather due to their cytotoxicity. A study undertaken by Cos et al. (2006) reveals a common error in research were false positive results are accepted, with the sample usually

exhibiting relatively high activity, without evaluating or considering the possibilities of cytotoxicity contributing to the observed outcomes.

It is important to note that cytotoxicity profiles of medicinal plants vary with varying chemical profiles which may be evident even in members of the same species. Factors such as the extraction procedure, part of the plant used, geographical location, environmental or climate conditions where the plants are cultivated, and seasonal variations may affect the chemical composition of plants.

Additionally, while the BSLA is a respectable preliminary assay in cytotoxicity screening, the effects observed on the larvae may not directly correlate with what is observed on mammalian cells (Meyer et al., 1982). However, outcomes may strongly warrant further cytotoxicity testing using more in-depth procedures, not only limited to *in vitro* studies, but also *in vivo* cytotoxicity assays.

Overall, it was observed that the majority of the plant extracts which demonstrated cytotoxicity were those that had previously demonstrated noteworthy antimicrobial activity (Chapter 2). Examples of this are the organic extracts of *E. globulus*, *H. odoratissimum*, *L. javanica*, and *W. somnifera*. In contrast, plant extracts which demonstrated the least cytotoxicity, such as the aqueous extract of *S. birrea*, did not display strong antimicrobial activity (Chapter 2). Further research into techniques that would enhance the antimicrobial activity of the plants without increasing cytotoxicity, such as the formation of silver nanoparticles, would prove to be most beneficial.

3.5. Chapter summary

- Cytotoxicity was observed more frequently with organic extracts rather than aqueous extracts. Aqueous extracts being the most traditionally used extracts.
- Aqueous extracts of *P. sidoides*, *S. birrea*, and *Z. capense* showed the lowest cytotoxicity in the study.
- Eleven organic extracts (*B. africana*, *D. viscosa*, *G. perpensa*, *M. flabellifolius*, *O. asteriscoides*, *P. sidoides*, *S. mucronata*, *S. birrea*, *S. henningsii*, *T. camphoratus*, and *Z. capense*) demonstrated less than 50% cytotoxicity at both 24 and 48 hrs.
- Plant species displaying strong antimicrobial activity with % mortality that is less than 50% were *B. africana*, *M. flabellifolius*, and *S. mucronata*.

- It was noted that organic and aqueous extracts of *E. globulus*, *H. odoratissimum*, *L. javanica*, and *W. somnifera*, which displayed promising antimicrobial profiles in the MIC studies exhibited high cytotoxicity to the brine shrimp larvae in this study.

CHAPTER 4

SILVER NANOPARTICLE SYNTHESIS FROM MEDICINAL PLANT EXTRACTS

4.1. Introduction

Nanotechnology is still a highly researched field, as it is expected to be a basis of many advancements, including antimicrobial studies. Green synthesis of nanoparticles has become more advantageous in the synthesis of nanoparticles compared to chemical and physical means as it is cost-effective, eco-friendly, and results in lower energy consumption (Roy and Das, 2015). To date, several researchers have reported on the green synthesis of nanoparticles for antimicrobial purposes (Sharma et al., 2009; Kumar et al., 2016; Vijayalakshmi et al, 2018; Yadav et al., 2018), where this type of green synthesis is done through a simple and quick one-pot synthesis procedure. The combination of the plant extracts with silver nitrate in this procedure is believed to result in increased antimicrobial effects, enhancing the antimicrobial properties of the individual plant extract (Durán et al., 2016). In this instance, AgNPs have been shown to exhibit noteworthy antimicrobial activity against a range of Gram-positive and Gram-negative bacteria (Ahmed et al., 2016; Kingslin and Ravikumar, 2016)

The size and morphology of nanoparticles is what is of interest in the antimicrobial application as they present with ideal properties such as a large surface to volume ratio applications. (Adavallan and Krishnakumar, 2014). The diverse phytochemicals in plants such as proteins, flavonoids, alkaloids, sugars and phenolic acids play a vital role in the formulation of nanoparticles, acting as both reducing and stabilizing agents (Mallikarjuna et al., 2014). Plant extracts vary considerably in their phytochemical composition which may influence the effectiveness of the reducing and stabilizing agents is an important parameter in synthesising ideal nanoparticles (Oliver et al., 2018).

The antimicrobial properties of 40 fever-reducing medicinal plants were tested in Chapter 2 against nine fever-causing pathogens. The toxicity profiles of 28 of the most antimicrobially active species were assessed in Chapter 3. The aim of this chapter was to assess whether the synthesis of silver nanoparticles from a selection of these plants can enhance their antimicrobial

properties against some of the fever-causing pathogens, without increasing cytotoxicity. Various techniques where applied to monitor the formation of the AgNPs, where the synthesis was accomplished through the reducing and stabilizing properties of the phytochemical compounds in the extracts. Minimum inhibitory concentration and BSLA assays followed after this to assess the antimicrobial and cytotoxic properties of the formed AgNPs.

4.2. Methods and materials

4.2.1. Selection and preparation of plant extracts

Ten medicinal plants were selected from the plants studied in Chapter 2 (Table 4.1) based on their commercial significance (Van Wyk, 2008). The relevant plant parts were extracted as follows: an amount of 5 g of ground plant material was weighed and submerged in 100 mL of sterile distilled water, the mixture was heated to approximately 80°C for 10 min to ensure optimal extraction of phytochemicals from the plants. The plant extract was then filtered off and stored at 2°C in appropriate glass bottles and covered with foil to protect from light. Table 4.1 shows the selected plants for AgNPs synthesis.

Table 4. 1: Fever- reducing medicinal plants used for AgNPs synthesis.

Plant samples	Family	Plant Part
Adenia gummifera Burtt Davy.	Passifloraceae	leaves
Artemisia afra Jacq.	Asteraceae	leaves
Dodonaea viscosa Jacq.	Sapindaceae	Leaves
Eucomis autumnalis (Mill.) Chitt.	Hyacinthaceae	Roots
Gunnera perpensa L.	Gunneraceae	Roots
Lippia javanica Spreng.	Verbenaceae	Leaves
Pelargonium sidoides DC.	Geraniaceae	Bark
Sclerocarya birrea Hochst.	Anacardiaceae	Bark
Sutherlandia frutescens (L.) R.Br.	Fabaceae	Leaves
Tulbaghia violacea Harv.	Alliaceae	Roots

4.2.2. Synthesis of silver nanoparticles

The synthesis of AgNPs was carried out by methods adopted from Amini et al. (2017). An amount of 10 mL of aqueous plant extract was added drop-wise (using a separatory funnel) to 90 mL of 1 mM ($AgNO_3$) (Sigma Aldrich), while rapidly stirring with the aid of a magnetic stirrer. The mixture was heated to approximately 80°C for 10 min and stirred for 20 to 30 min. A brown-black colour change was observed within the first few minutes of adding the plant extract, which demonstrated formation of AgNPs (Yadav et al., 2018). Figure 4.1 is a schematic diagram showing the synthesis of AgNPs using aqueous plant extract and $AgNO_3$ solutions.

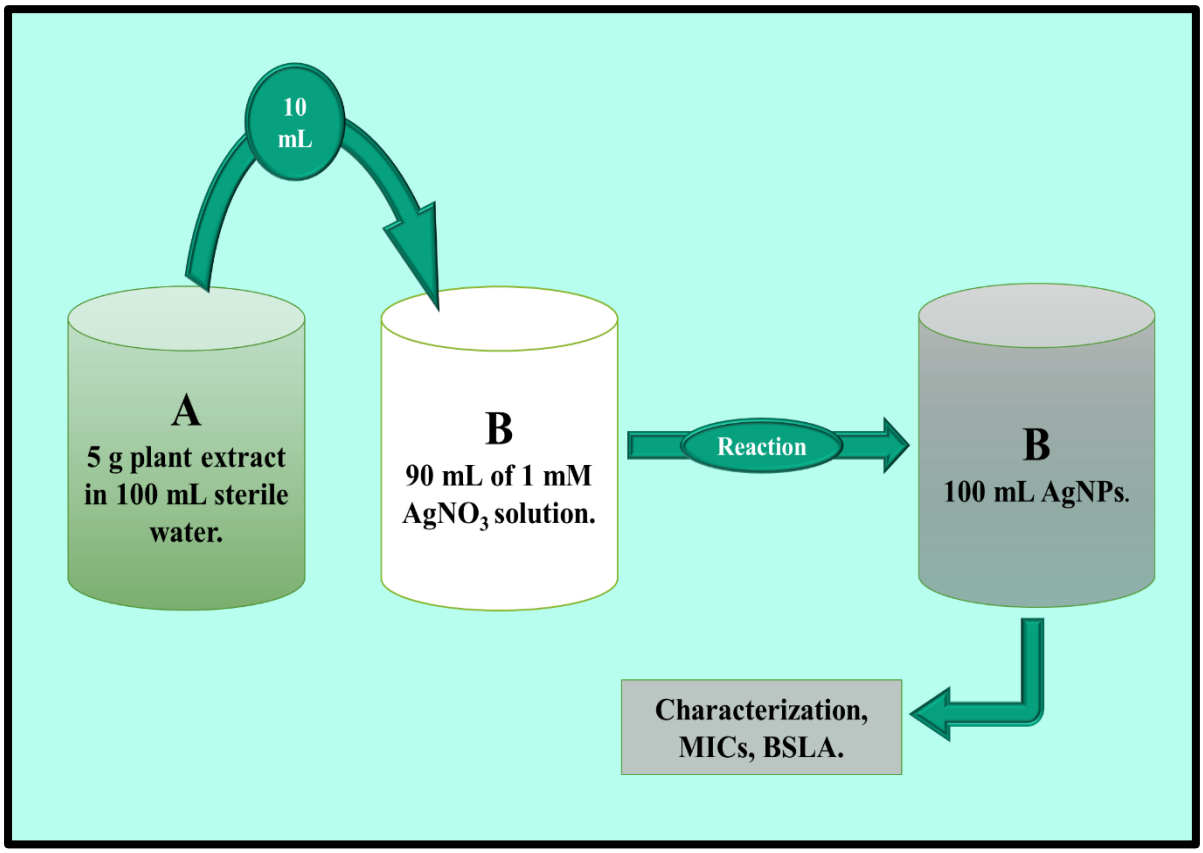


Figure 4. 1: Schematic diagram of the synthesis of AgNPs from aqueous plant extract and AgNO3.

4.2.3. Characterization of the synthesised silver nanoparticles

4.2.3.1. UV-VIS spectroscopy

The synthesis of AgNPs was monitored through UV-Vis spectroscopy at a wavelength range of 400–600 nm. The Perkin-Elmer Lambda-25 spectrophotometer was operated at a resolution of 1 nm using a scan speed of 930 nm/min. Distilled water was used as a blank and $AgNO_3$

was used as the control. The samples were diluted 1:10 with distilled water prior to analysis to avoid errors due to high absorbance of the solution. Data was recorded and analysed using the "UV Winlab" software and the obtained spectral data was plotted using Origin Pro software version 8.1.

4.2.3.2. Attenuated total reflectance-Fourier-transform infrared spectroscopy (ATR-FTIR).

The FTIR analysis was carried out to identify the functional groups of the biomolecules responsible for the reduction of $AgNO_3$ and capping of the AgNPs. The spectra were recorded on a FTIR spectrophotometer (Perkin-Elmer instruments Ltd, USA), at a resolution of 4 cm^{-1}, at a wavelength range of 4500 – 500 cm^{-1}. An aliquot of sample in solution form was placed on the sample platform and 25 scans per sample were taken. The sample was scanned in solution form. Origin Pro software version 8.1 was used to plot the spectra from the obtained data.

4.2.3.3. Zeta-sizer

The zeta-sizer (Zetasizer NanoZS, Malvern Instruments Ltd., Malvern, UK) was used to analyse the hydrodynamic size, the polydispersity index (PDI) and the zeta potential of the synthesised AgNPs. Hydrodynamic size refers to the diameter of the nanoparticles in liquid medium, while the PDI measures the size distribution of the nanoparticles. Zeta potential measures the surface charge of the AgNPs and the likelihood of particle interaction, which would lead to agglomeration of the AgNPs. An amount of 1 mL of AgNPs solution was filtered through a 2 µm syringe filter and into a quartz cuvette. This was then placed in the Zeta-sizer set to run 15 scans in triplicates at 25ºC. The results were obtained through the Malvern ZS nano software.

4.2.3.4. Transmission Electron Microscope (TEM)

Transmission Electron Microscopy (TEM) was used to investigate the shape of the synthesised nanoparticles. This was done in collaboration with Dr S. Noundou from the Department of Chemistry/Department of Biochemistry and Microbiology, Rhodes University. The AgNPs samples for TEM analysis were sonicated for 10 minutes prior to analysis. An aliquot of this solution was placed on a copper TEM grid. Excess solvent was removed using filter paper. The prepared TEM samples were then allowed to dry for 24 hrs at ambient temperature before

analysis. A Zeiss Libra TEM instrument was used, operated at an accelerating voltage of 120 KV.

4.2.4. Antimicrobial assay

The minimum inhibitory concentration (MIC) assay, as described in Chapter 2, Section 2.2.4, was used to test the antimicrobial properties of the synthesised AgNPs. Neat AgNPs samples of 100 µL, were tested against two Gram-positive pathogens *Listeria monocytogenes* (ATCC 19111) and *Enterococcus faecalis* (ATCC 29212), as well as two Gram-negative pathogens *Klebsiella pneumoniae* (ATCC 13883) and *Acinetobacter baumannii* (ATCC 19606). To allow for a broad selection, one pathogens from each category demonstrated susceptibility to plant extracts in Chapter 2 MIC assay (i.e. *L. monocytogenes* and *A. baumannii*), and one pathogen from each category demonstrated resistance (i.e. *E. faecalis* and *K. pneumoniae*). All pathogens were cultured In Tryptone Soya broth (TSB), incubated at 37°C for 24 hrs, and subsequently streaked out on Tryptone Soya agar (TSA) to confirm purity of culture.

4.2.5. Cytotoxicity assay

The Brine shrimp lethality assay, as detailed in Chapter 3, Sections 3.3.2 and 3.3.3, was used to test for the cytotoxicity of AgNPs. *Artemia franciscana* cysts were hatched in artificial seawater for 48 hrs under a constant light source. Once the cysts have hatched, an amount of 400 µL of the artificial seawater containing 30-60 brine shrimp larvae where introduced into each well of a 48 micro-titre plate. This was followed by 400 µL of the neat AgNPs solutions. The number of dead brine shrimp in each well was evaluated before adding the AgNPs samples, after 24 hrs, and after 48 hrs and after adding acetic acid, which kills off the brine shrimp so as to perform a total count of the brine shrimp. Samples were tested in triplicate, and a % mortality was calculated for each of the samples. A % mortality of ≥ 50% was considered biologically cytotoxic.

4.3. Results

4.3.1. Characterization of the synthesised silver nanoparticles

4.3.1.1. Visual analysis and UV-Vis spectroscopy

Visual observation of AgNPs revealed a brownish colour change in all the synthesised nanoparticles within the first 15 minutes of synthesis. This was the first indication of the formation of nanoparticles and can be attributed to the excitation of the SPR of the AgNPs in

solution. This occurs when electrons in the AgNPs oscillate collectively, resulting in absorption bands in the visible range of the electromagnetic spectrum. Figure 4.2 is a sample of the synthesised AgNPs and illustrates the observed colour change.

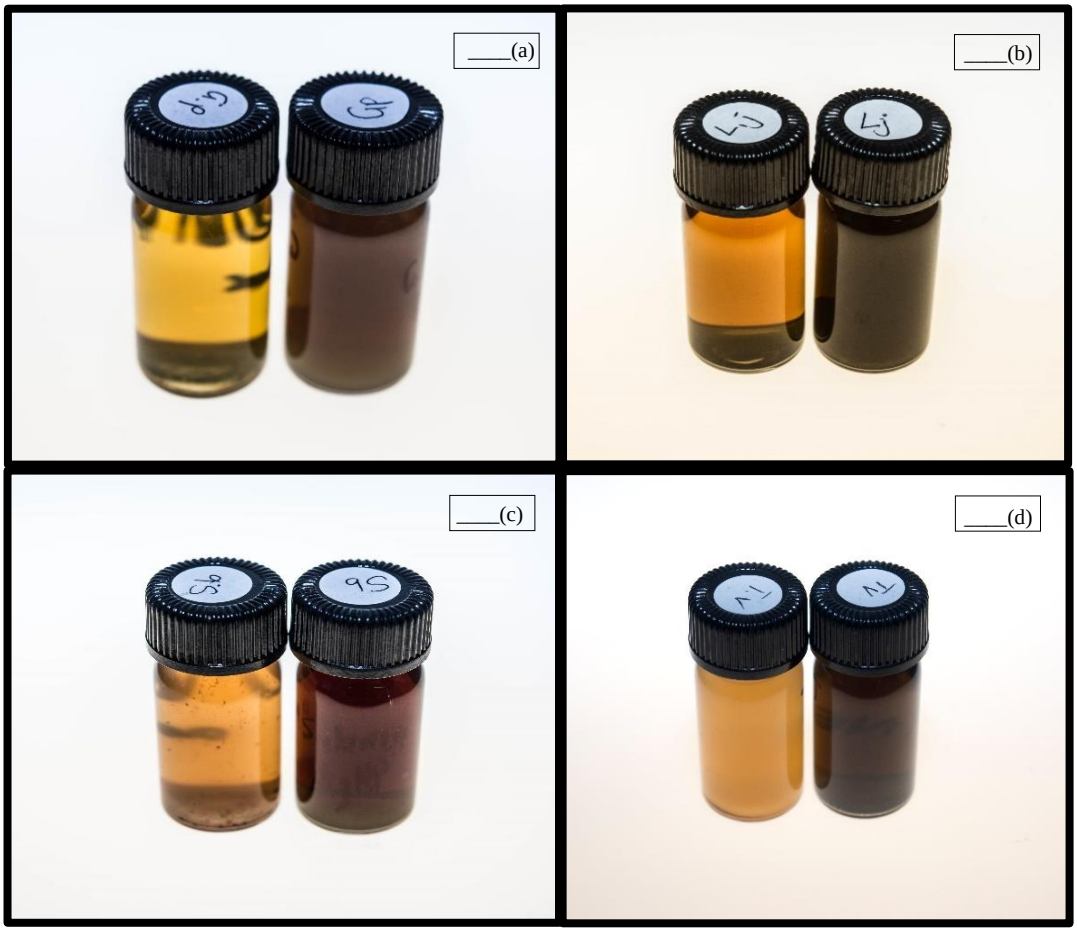
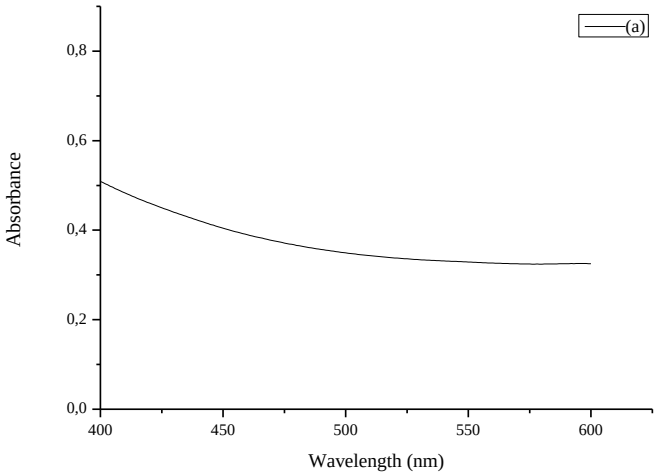
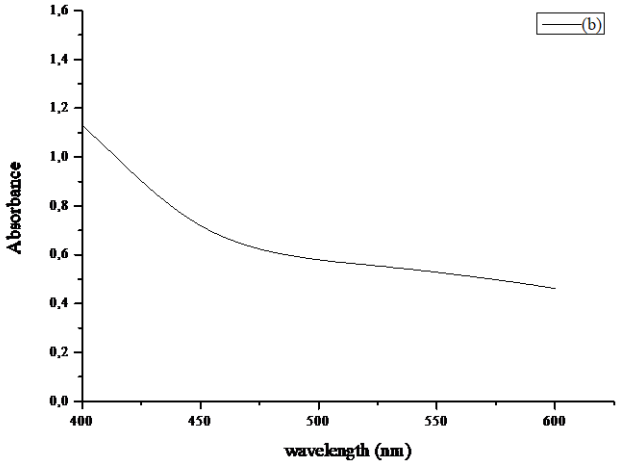
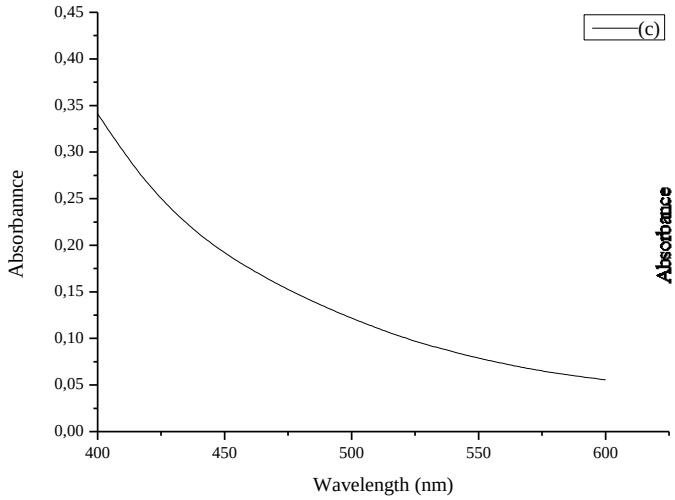


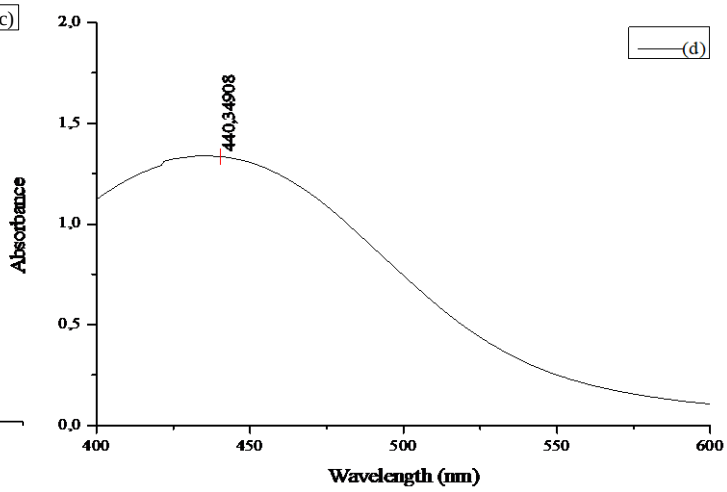
Figure 4. 2: Observed colour changes in AgNPs solutions: Aqueous extracts (to the left in each photo) and AgNPs in solution (to the right in each photo) of (a) *G. perpensa*; (b) *L. javanica*; (c) *S. birrea*; and (d) *T. violacea*.

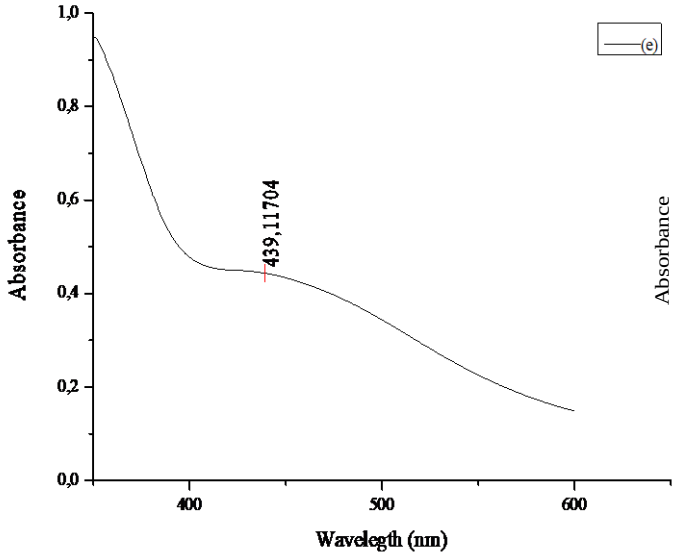
The AgNPs were further analysed using UV-Vis spectroscopy. Broad SPR peaks were observed with the AgNPs of *G. perpensa* and *S. birrea* which could be an indication of the wide size distribution of the particles in the liquid medium. The SPR peak was observed at 439 nm for *G. perpensa* and 423 nm for *S. birrea*. The UV-Vis spectra of *E. autumnalis* and *P. sidoides* also showed broad SPR peak, but these peaks were narrower and more intense than that of *S. birrea* and *G. perpensa*, which is an indication of a more monodispersed systems. Peaks appeared at 439 nm for *E. autumnalis* and 420 nm for *P. sidoides* (Fig 4.3).

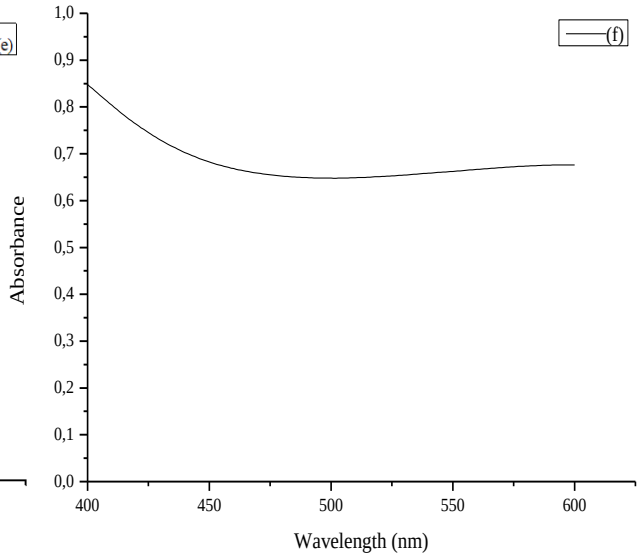

(a)
Absorbance
Wavelength (nm)


(b)
Absorbance
wavelength (nm)


(c)
Absorbannce
Wavelength (nm)


(d)
440,34908
Absorbance
Wavelength (nm)


(e)
439,11704
Absorbance
Wavelegth (nm)


(f)
Absorbance
Wavelength (nm)

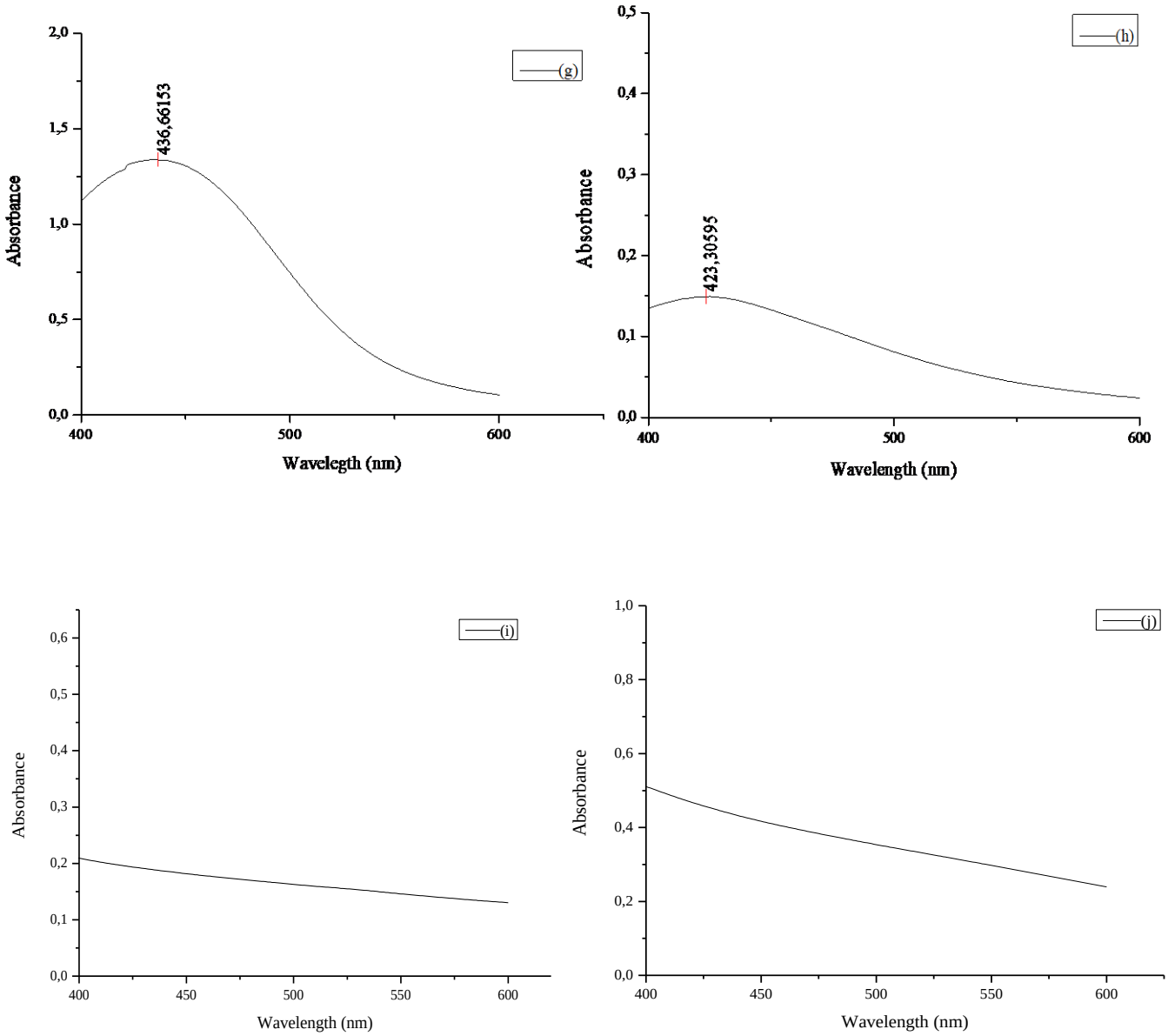


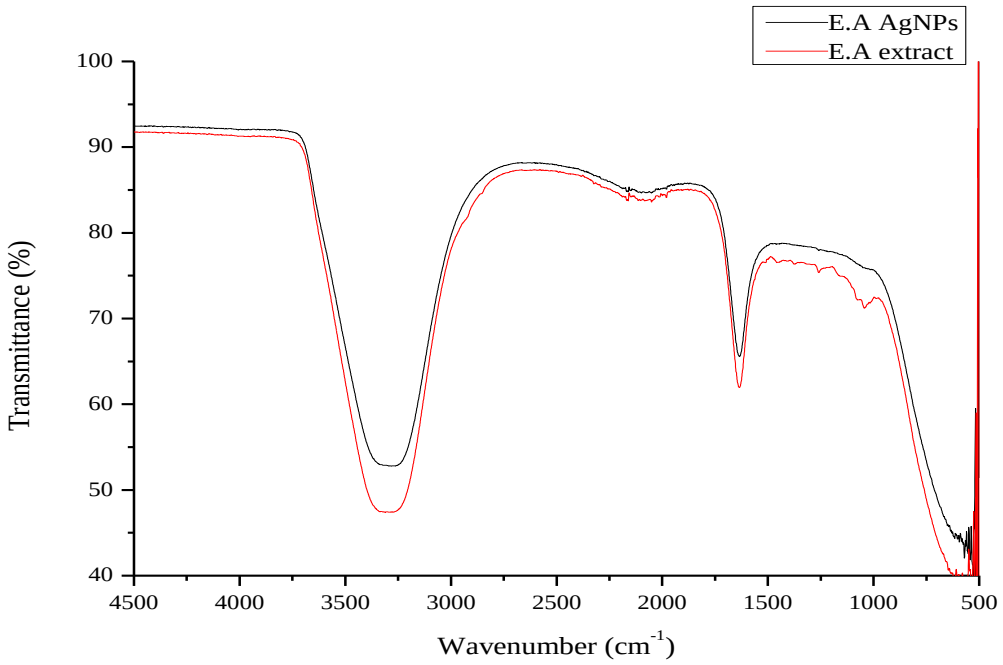
Figure 4. 3: The UV-Visspectroscopyof the synthesised AgNPs. (a) = A. afra ; (b) = *A. gummifera*; (c) = *D. viscosa*; (d) = *E. autumnalis*; (e) = *G. perpensa*; (f) = *L. javanica* ; (g) =*P. sidoides*; (h) = *S. birrea* ; (i) = *S. frutescens* ; (j) = *T. violacea*.

4.3.1.2. ATR-FTIR spectroscopy analysis.

The biomolecules responsible for the capping of AgNPs were analysed using the FTIR spectroscopy. A common spectral pattern was observed for all the synthesised AgNPs, which comprised of a broad absorption band at approximately 3300 cm^{-1}, characteristic for O-H stretching in molecules. This was followed by a prominent, narrow band at approximately 1600 cm^{-1}, which differs in intensity with each sample tested. This band falls in the double bond

region of the FTIR spectra, i.e. 1500- 2000 cm^{-1} and was attributed to the C=O carboxyl group of the molecules. The broad and less-intense band at 1900 cm^{-1} which can be seen with *E. autumnalis* (a), *P. sidoides* (b) and *S. birrea* (c) (Fig 4.4), is indicative of aromatic compounds. Smaller and less defined bands occur throughout the spectra which differentiate the aqueous extracts and their AgNPs counterparts, however, these were more pronounced with *E. autumnalis*, *P. sidoides* and *S. birrea*.

Multiple small bands occur in the fingerprint region of the FTIR spectra, 500-1500 cm^{-1}, which are due to skeletal vibrations of biomolecules in the extract. These were more pronounced with the aqueous extract of *S. birrea* and *E. autumnalis* which signify the complexity of biomolecules in the aqueous extract. With regards Figure 4.4 (c), *S. birrea*, clear absorption peaks can be seen at 1375, 1212, and 1054 cm^{-1} these can be attributed to the C-C stretch and C-O bonds in the molecule. The decrease in intensity of these peaks observed in the AgNPs signify the involvement of these biomolecules in the reduction process. Figure 4.4 shows the FTIR spectra of some aqueous extracts and their AgNPs.



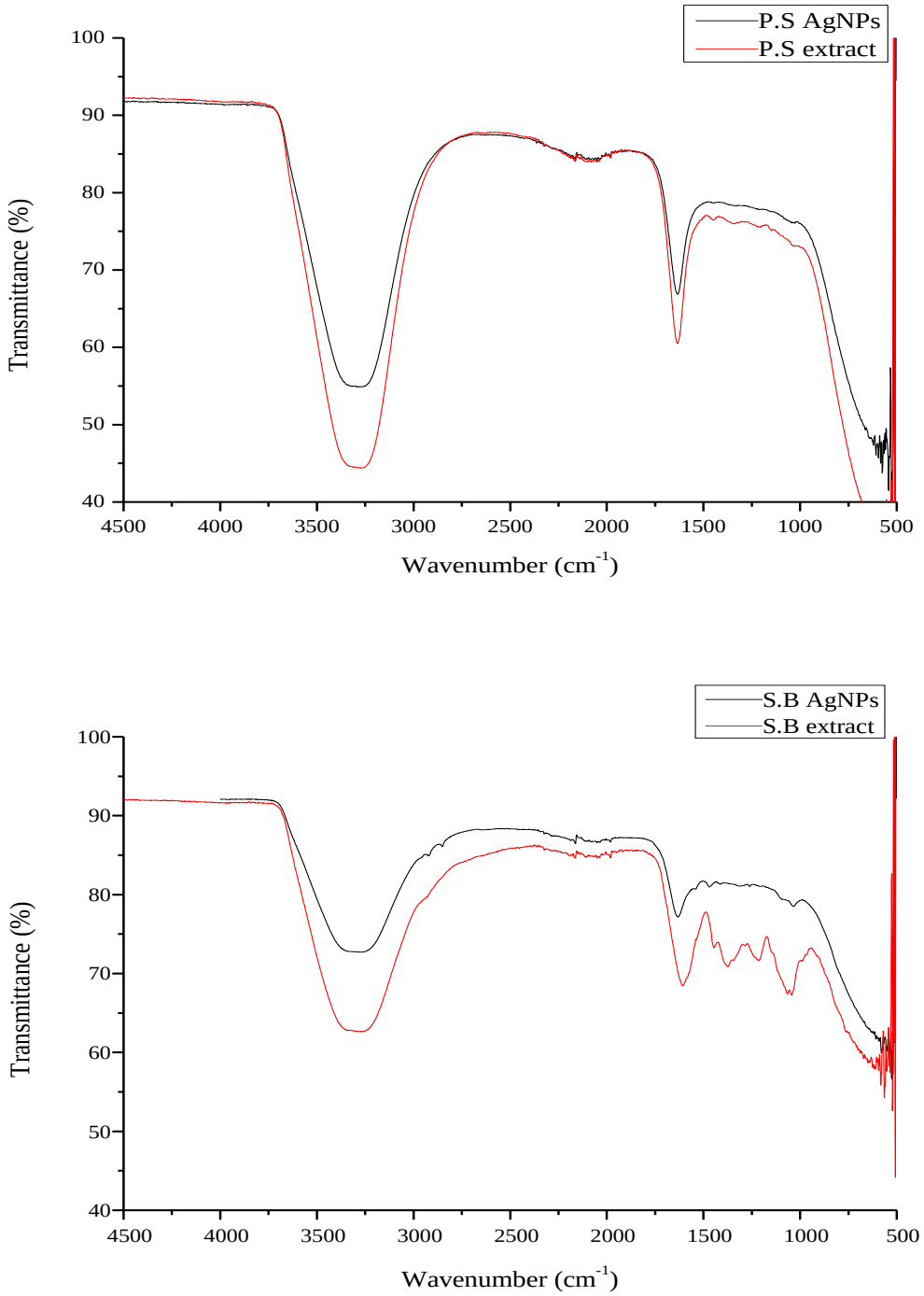


Figure 4. 4: The ART-FTIR spectra of aqueous extracts and their AgNPs.
E.A = *E. autumnalis*; P.S = *P. sidoides*; S.B = *S. birrea*.

Figure 4.5 shows the FTIR spectra of *G. perpensa*, which have been rescaled for observation of the biomolecules in the fingerprint region. Small and narrow bands occur in the aqueous extract spectra at approximately 1250 cm^{-1}, 1150 cm^{-1}, 1000 cm^{-1} and 1260 cm^{-1}. A broad and small band also occurs at 1250 cm^{-1}. The appearance of these bands is not observed in the

AgNPs spectra, which signify the involvement of these compounds in capping of the nanoparticles. These biomolecules demonstrate the characteristics of the chemical constituents of phytochemicals such as saponins, flavonoids, terpenoids and tannins.

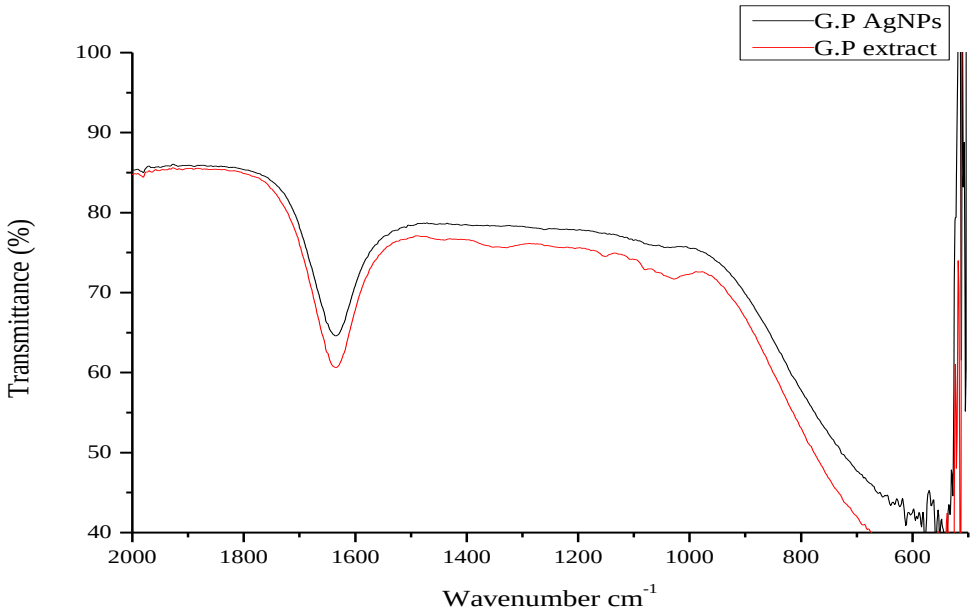


Figure 4. 5: The ART-FTIR analysis of *G. perpensa* re-scaled to show the fingerprint region.

4.3.1.3. Zeta sizer analysis.

The zeta-sizer analysis was carried out to investigate the particle size of the synthesised AgNPs, which was represented by the hydrodynamic size (nm) of particles when dispersed in a liquid medium. The polydispersity index (PDI) measures the dispersity of nanoparticles in liquid medium and was included in the assay. A high PDI (> 0.5) is generally undesired as it indicates the possible aggregation of AgNPs at the given time, while a low PDI refers to the monodispersity of the AgNPs with the least possibility of particle aggregation.

The zeta potential, which measures the surface charge of the AgNPs in liquid medium, was also analysed. By providing data on the mutual repulsive forces of the AgNPs, the zeta potential is an essential parameter in monitoring the aggregation of AgNPs and therefore can be used to predict the stability of the nanoparticles. Table 4.2 provides the full data on the hydrodynamic size, PDI, and Zeta potential of the synthesised AgNPs.

Table 4. 2: Particle size, PDI, and Zeta potential of the synthesised silver nanoparticles.

AgNPs samples	Hydrodynamic size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
A. afra	41.95	0.28	-23.00
A. gummifera	48.40	0.28	-18.80
D. viscosa	39.17	0.45	-18.20
E. autumnalis	112.02	0.19	-12.20
G. perpensa	39.32	0.39	-21.40
L. javanica	44.73	0.31	-19.40
P. sidoides	62.16	0.18	-15.60
S. birrea	56.01	0.10	-18.80
S. frutescens	59.03	0.24	-15.60
T. violacea	53.65	0.29	-16.20

The smallest particle size was observed with the AgNPs of *D. viscosa* (39.17 nm) and *G. perpensa* (39.32 nm), however, based on the previous results from the UV-Vis spectra (Figure 4.3) it would appear that this small size was not maintained for a long period and the AgNPs quickly became unstable. Of the remaining AgNPs tested, 40% of the AgNPs exhibited particle size in the range 40-55 nm. The largest AgNPs were that of *E. autumnalis* (112.02 nm) and *P. sidoides* (62.16 nm). With regards to the PDI of the nanoparticles, the lowest PDI value was observed with the AgNPs of *S. birrea* (0.10 units) while *D. viscosa* exhibited the highest PDI value (0.45 units). A relationship was observed between the particle size and PDI values of the synthesised AgNPs, where both parameters appeared to be inversely proportional. Silver nanoparticles with small particle size such as *D. viscosa* and *G. perpensa* showed the highest PDI values which suggest a tendency of aggregation of the small nanoparticles to form larger nanoparticles, whilst larger AgNPs such as those from *E. autumnalis, P. sidoides* and *S. birrea* showed low PDI values which refers to their monodispersity. This could further provide an explanation for the observed UV-Vis spectra of some of the synthesised AgNPs.

All nanoparticles were found to be negatively charged with zeta potentials in the range of -12.20 to -23.00 mV. This demonstrates that the nanoparticles repel each other. However,

considering that the ideal zeta potential range which signifies good stability are values greater than +30 mV and less than -30 mV, the zeta potentials of the nanoparticles were found to be lacking and no correlation could be found between PDI and zeta potential. Regardless of the particle sizes and PDI measurements, the AgNPs of *E. autumnalis* (-12.2 mV), *P. sidoides* (-15.60 mV), and *S. frutescence* (-15.60 mV) were found to have the lowest zeta potentials. The highest Zeta potentials was observed with the AgNPs of *G. perpensa* and *A. afra*.

4.3.1.4. TEM analysis

The size distribution and morphology of the AgNPs was observed using the TEM. Figure 4.6 shows the images that were obtained from the analysis. Figure 4.6 (a) shows the different sizes of the AgNPs synthesised from *D. viscosa* ranging from 18.30 nm to 54.54 nm, i.e. a size difference of 36.24 nm. This is further confirmed by the high PDI value of *D. viscosa* (0.45 units) observed in Table 4.2. Agglomeration of AgNPs was apparent as AgNPs appeared to be clustered together. Spherically shaped nanoparticles were predominant.

The TEM analysis of *E. autumnalis*, Figure 4.6 (b), also revealed nanoparticles that were predominately spherical. Small AgNPs were observed to "clump" together to produce bigger particles, hence the irregular shapes of some of the AgNPs. However, unlike *D. viscosa* the particles are well dispersed. When comparing the two images of *E. autumnalis* and *D. viscosa*, at 200 nm, *E. autumnalis* seemed to have smaller AgNPs. However, from the Zeta sizer, it was well established that *D. viscosa* possessed the smallest AgNPs size. Agglomeration of its small AgNPs to form large AgNPs may account for this observation.

Pelargonium sidoides AgNPs, Figure 4.6 (c), were observed to be the most monodispersed AgNPs in all the TEM images. Smaller AgNPs could still be seen "grouping" together as though forming aggregates, although not as readily as *E. autumnalis* and *D. viscosa*. Spherically-shaped AgNPs were again noted from the TEM image.

With *S. birrea* AgNPs, Figure 4.6 (d), triangular-shaped AgNPs were identified. This was the most predominant shape observed, followed by a minority of hexagonally-shaped AgNPs. Little aggregation of particles is noted, and the AgNPs are well dispersed without showing a strong tendency to "clump" together. The opposite was observed for *T. violacea* AgNPs [Figure 4.6 (e)]. A strong tendency of AgNPs to aggregate was observed and AgNPs appear to be clumped together. The shape of the AgNPs is not readily visible, as the aggregating system distracts this. However, some spherically- shaped AgNPs may be seen.

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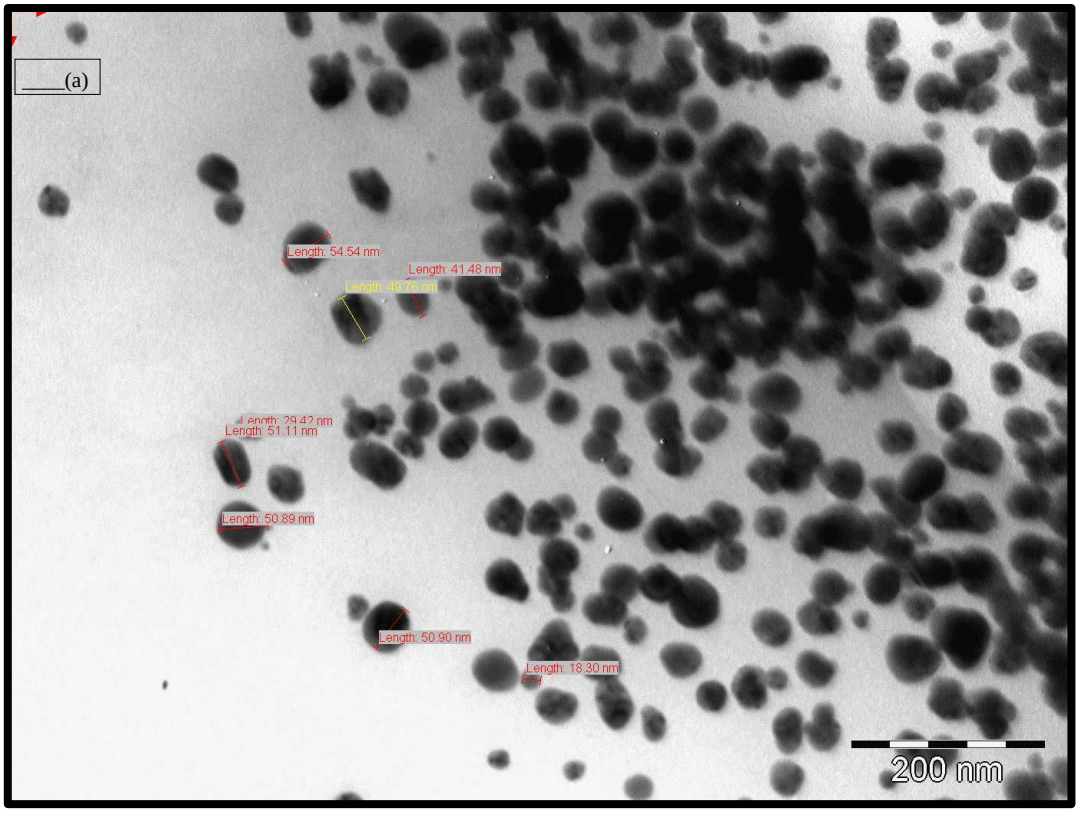
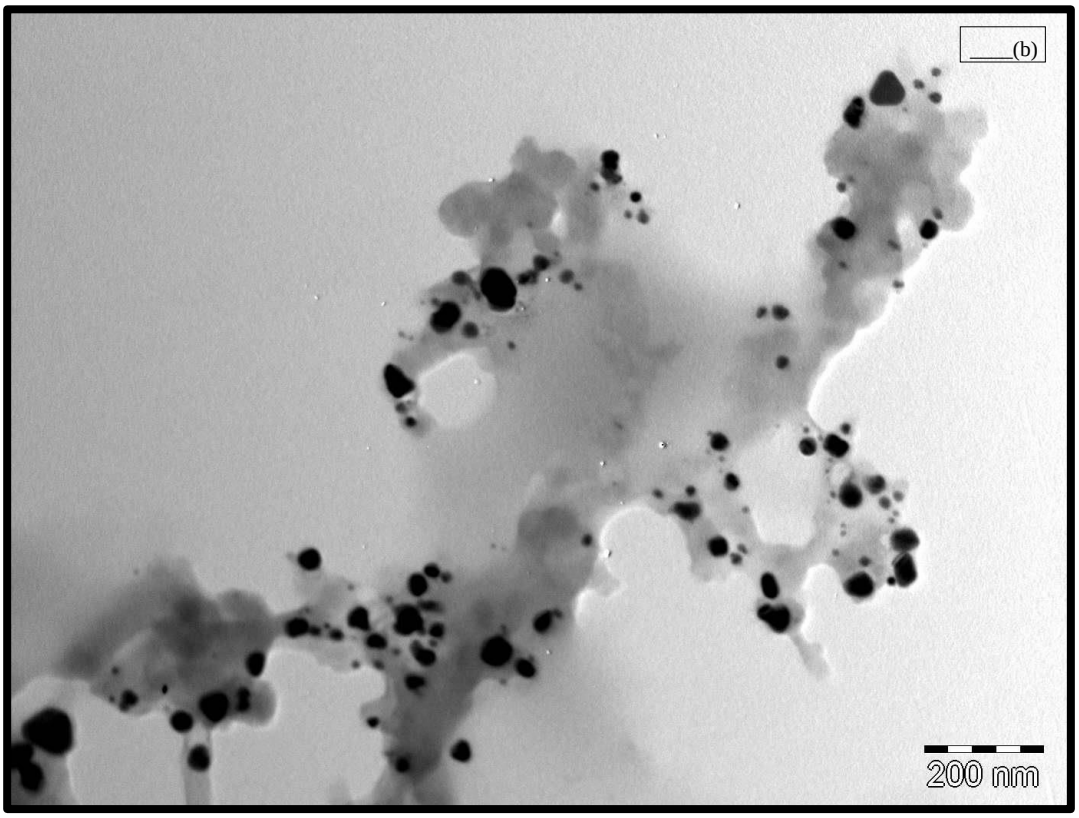
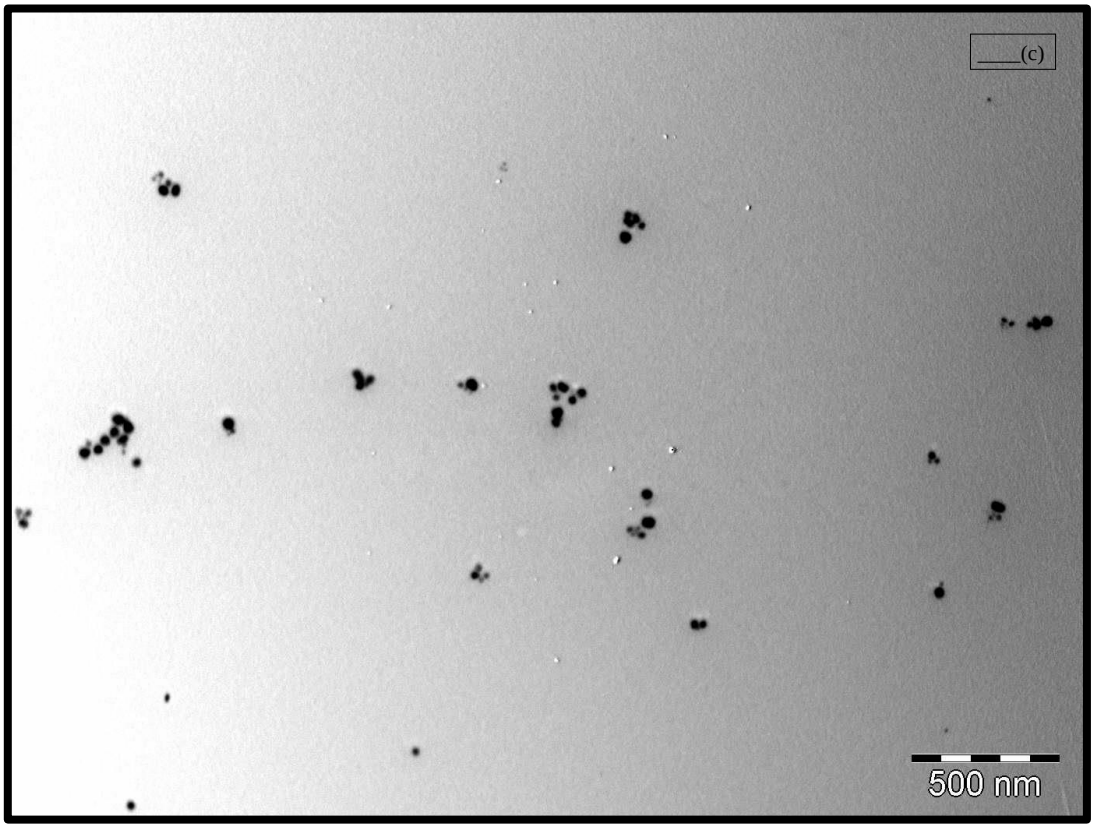
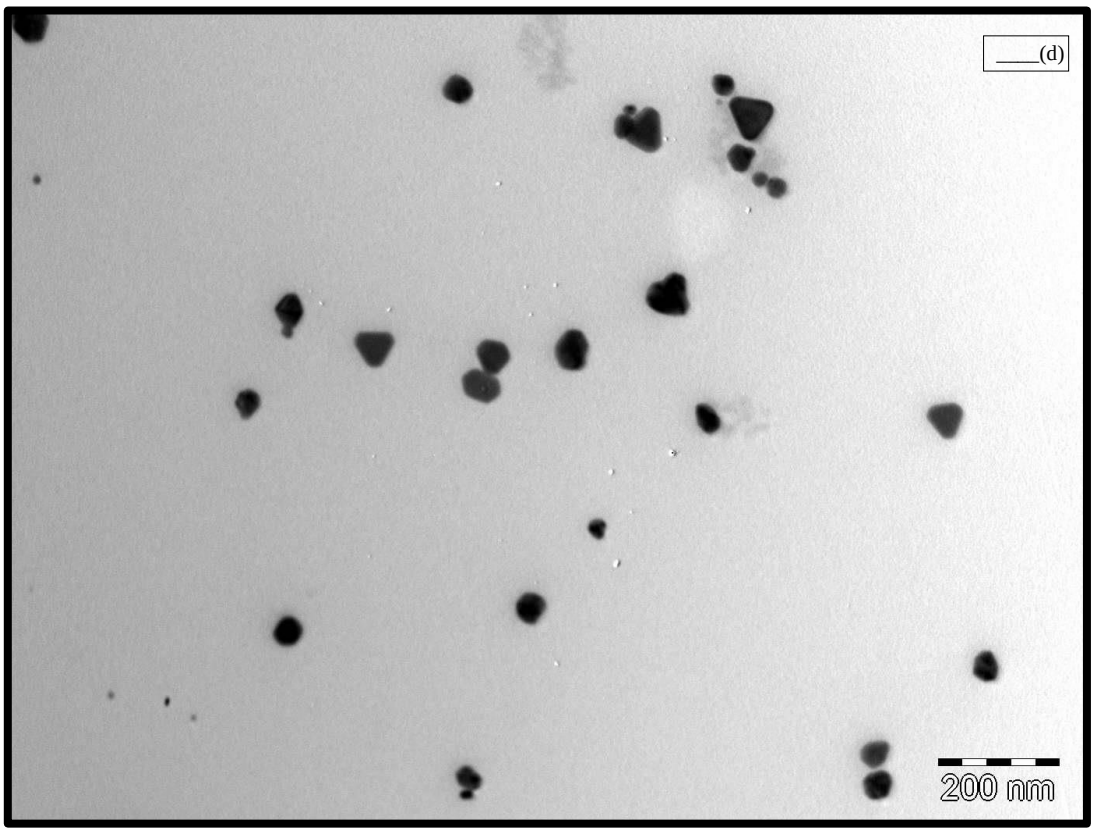

(a)
Length: 54.54 nm
Length: 41.48 nm
Length: 51.11 nm
Length: 50.89 nm
Length: 50.90 nm
Length: 18.30 nm
200 nm


(b)
200 nm





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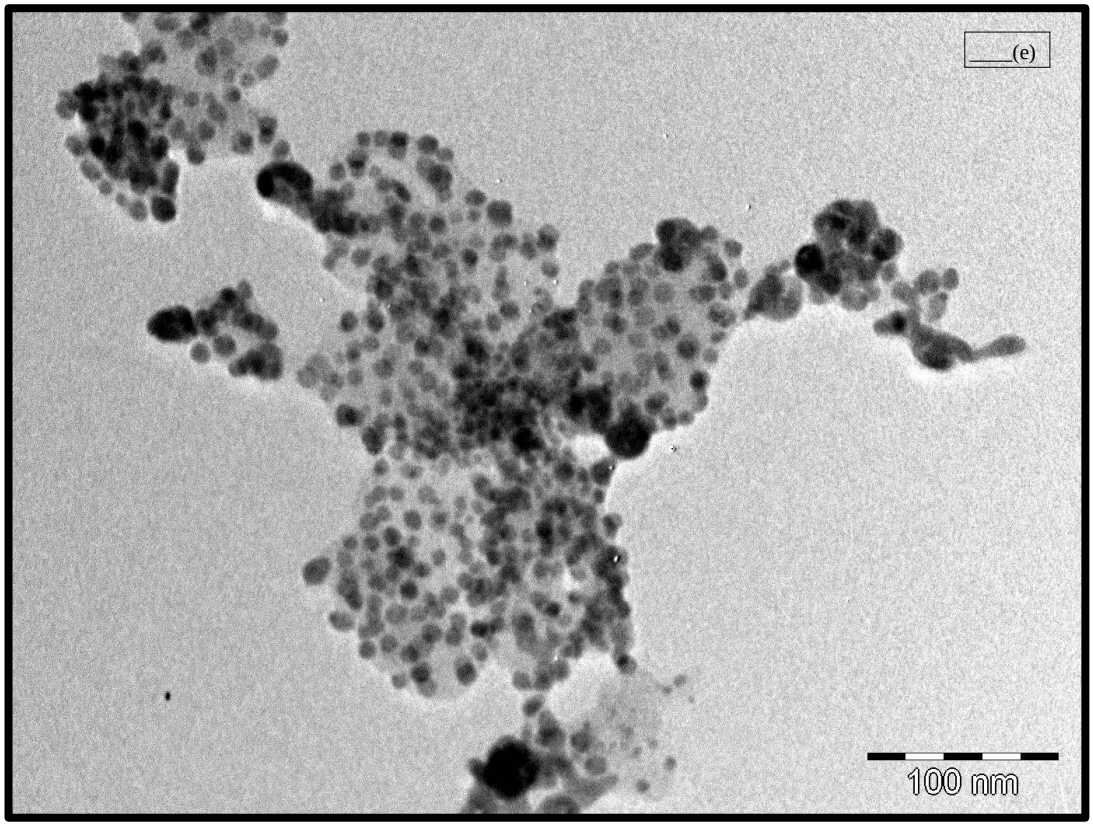

Figure 4. 6: The TEM illustrations of the synthesised nanoparticles from five AgNPs samples. (a) demonstrates the different particle sizes of AgNPs in liquid medium. Key: (a) = *D. viscosa*; (b) = *E. autumnalis*; (c) = *P. sidoides*; (d) = *S. birrea*; (e) = *T. violacea*.

4.3.2. Antimicrobial assay

The antimicrobial activity of all the synthesised AgNPs was investigated using the MIC assay. The results of this assay can be found in Table 4.3. The criteria used to categorise antimicrobial activity was discussed in Chapter 2, section 2.1, where noteworthy activity was regarded as MIC values < 1 mg/mL. In this instance, it was noted that the AgNPs of *S. birrea*, *E. autumnalis* and *G. perpensa* demonstrated the most noteworthy microbial inhibition against all four pathogens that were tested. The aqueous extracts of *S. birrea* and *E. autumnalis* had previously not demonstrated any antimicrobial properties against these four pathogens studied. Both *S. birrea* and *E. autumnalis* AgNPs reached an MIC value as low as 0.16 mg/mL against *A. baumannii* and *E. faecalis* respectively, from a value of 4 mg/mL that was observed with both aqueous extracts. *Gunnera perpensa* demonstrated MIC values of 0.16 mg/mL against *A. baumannii* and *K. pneumoniae*, and MIC values of 0.63 against both *L. monocytogenes* and *E. faecalis*.

The lowest MIC was observed for the AgNPs of *P. sidoides* and *T. violacea* (MIC value of 0.08 mg/mL) against *A. baumannii* and *E. faecalis* respectively. It is worth noting that *P. sidoides*, which had previously demonstrated moderate to poor activity with both its organic and aqueous extracts (Chapter 2, Tables 2.2 and 2.3), demonstrated noteworthy activity against three of the four pathogens when capping AgNPs.

Enterococcus faecalis was observed to be the most susceptible micro-organism to the AgNPs. All the nanoparticles tested demonstrated noteworthy activity against this pathogen. Although the aqueous extract of *G. perpensa* had demonstrated antimicrobial properties against *K. pneumoniae* these properties appeared to be greater with the synthesis of AgNPs, MIC values were reduced from 0.39 mg/mL for its aqueous extracts to 0.16 mg/mL for its AgNPs.

Table 4. 3: Minimum inhibitory concentration of plant samples combined with AgNPs compared to aqueous plant extracts without AgNPs.

AgNPs plant sample	**Mean MIC values (mg/mL) (n=3)**							
	L. m		***E. f***		***K. p***		***A. b***	
	AgNPs	**Aq**	**AgNPs**	**Aq**	**AgNPs**	**Aq**	**AgNPs**	**Aq**
A. gummifera	1.38	>8.00	**0.34**	>8.00	1.25	>8.00	1.38	>8.00
A. afra	1.25	>8.00	**0.63**	>8.00	1.25	>8.00	1.25	>8.00
D. viscosa	1.25	>8.00	**0.31**	>8.00	1.25	>8.00	1.25	>8.00
E. autumnalis	**0.63**	4.00	**0.16**	>8.00	**0.63**	>8.00	**0.63**	>8.00
G. perpensa	**0.63**	>8.00	**0.63**	>8.00	**0.16**	**0.39**	**0.16**	>8.00
L. javanica	1.25	>8.00	**0.63**	>8.00	1.25	>8.00	1.25	>8.00
P. sidoides	**0.31**	3.13	**0.31**	>8.00	1.25	>8.00	**0.08**	6.25
S. birrea	**0.31**	4.00	**0.31**	8.00	**0.69**	>8.00	**0.16**	4.00
S. frutescens	1.25	>8.00	**0.31**	>8.00	1.25	>8.00	1.25	>8.00
T. violacea	1.25	>8.00	**0.08**	>8.00	1.25	>8.00	**0.63**	>8.00
Controls								
Positive: Cip (μg/mL)	0.039		1.25		0.63		0.16	
$AgNO_3$	0.04		0.04		0.04		0.04	

AgNPs plant sample	Mean MIC values (mg/mL) (n=3)							
	L. m		***E. f***		***K. p***		***A. b***	
	AgNPs	**Aq**	**AgNPs**	**Aq**	**AgNPs**	**Aq**	**AgNPs**	**Aq**
Culture control	+		+		+		+	

Key: **L.m**: *L. monocytogenes* (ATCC 19111), **E.f**: *E. faecalis* (ATCC 29212), **K.p**: *K. pneumoniae* (ATCC 13883) **A.b**: *A. baumannii* (ATCC 19606).

"+" denotes positive culture control. Broth supports microbial growth.

*values in bold denote noteworthy activity.

** ">8" denotes very weak activity.

The increased fold of the MIC values was calculated for a more effective comparison of MIC values between aqueous extracts and AgNPs. The increased fold of each sample against each of the tested pathogens can be found in Table 4.4. A general increase in antimicrobial property was observed raging from tow to >100 fold. The highest increase in antimicrobial activity was observed with *T. violacea* (>100 fold when tested against *E. faecalis*), followed by *P. sidoides* with an increase fold of 78.13. A 50 fold increase in microbial inhibition was also noted for *E. autumnalis* against *E. faecalis* and *G. perpensa* against *A. baumannii*. These values are important as they signify that the interaction of aqueous plant extracts and AgNPs greatly increases their efficacy in microbial inhibition.

Table 4. 4: Increase fold of AgNPs MIC in comparison to MIC of aqueous extracts.

AgNPs samples	***L. m***	***E. f***	***K. p***	***A. b***
A. gummifera	>5.81	>23.53	>6.40	>5.81
A. afra	>6.40	>12.70	>6.40	>6.40
D. viscosa	>6.40	>25.81	>6.40	>6.40
E. autumnalis	6.00	**>50.00**	>12.70	>12.70
G. perpensa	>12.70	>12.70	2.44	**>50.00**
L. javanica	>6.40	>12.70	>6.40	>6.40
P. sidoides	10.10	>25.81	>6.40	**78.13**
S. birrea	12.90	25.81	>11.60	25.00
S. frutescens	>6.40	>25.81	>6.40	>6.40
T. violacea	>6.40	**>100**	>6.40	>12.70

Key**: L.m**: *L. monocytogenes* (ATCC 19111), **E.f**: *E. faecalis* (ATCC 29212), **K.p**: *K. pneumoniae* (ATCC 13883), **A.b**: *A. baumannii* (ATCC 19606).

4.3.3. Cytotoxicity assay

The cytotoxicity assay of all the AgNPs were done using BSLA. Results of the assay can be found in Table 4.5. Similar to the criteria used in Chapter 3, samples with a % mortality of greater than 50% were regarded as cytotoxic. The majority of the AgNPs did not exhibit toxic effects to the brine shrimp larvae. Percentage mortalities as high as 47.95% for *A. gummifera* and 42.39% for *T. violacea* were noted, however, they were still within the accepted range. *Gunnera perpensa* was noted as the only synthesised AgNPs which displayed some cytotoxic effects on the brine shrimp, with a % mortality of 56.50% at 48 hrs. No toxicity was observed at 24 hrs. Some AgNPs which displayed little to no toxicity were those of *D. viscosa*, *S. birrea*, and *P. sidoides* with a % mortality of < 20% both at 24 and 48 hrs.

Table 4. 5: Brine shrimp lethality assay of plant samples containing AgNPs.

AgNPs plant samples	**% Mortality**	
	24 hrs	**48 hrs**
A. gummifera	2.29	47.95
A. afra	2.30	38.97
D. viscosa	2.26	12.33
E. autumnalis	11.33	37.56
G. perpensa	24.95	**56.50**
L. javanica	1.19	20.83
P. sidoides	1.33	1.33
S. birrea	0.00	0.00
S. frutescens	1.15	30.91
T. violacea	4.75	42.39
Controls		
$AgNO_3$	54.00	76.10
Artificial seawater	0.00	0.95
Potassium dichromate	100.00	100.00

4.4. Discussion

4.4.1. Synthesis and characterization

The synthesis of all ten sets of AgNPs were successfully, with particle size ranging from 39.17 nm to 112.02 nm. Similar particle sizes were previously reported by Nune et al. (2009) where the green-synthesised gold nanoparticles they investigated had average particle size of 77 nm and 105 nm. Another study by Vijayalakshmi et al. (2018) observed particle sizes of 107 nm for green synthesised AgNPs using *Cressa cretica* plant extracts. According to Oliver et al. (2018) the ideal size of nanoparticles for antimicrobial application is less than 20 nm, as this is important for their intracellular antimicrobial activity. The AgNPs synthesised in this study were found to be lacking in that respect, however, it was interesting to note that enhanced noteworthy activity was still observed. The size in turn also depends on, among other factors, the effectiveness of the stabilizing agent (Oliver et al., 2018).

The AgNPs of *G. perpensa* and *D. viscosa* were the smallest in size. However, by studying their PDI values, in conjunction with the UV-Vis spectra showing broad peaks (Figure 4.3), this is evidence of wide distribution of AgNPs size. This may signify agglomeration of AgNPs. Similar outcomes were observed by Islam et al. (2015), where nanoparticles with a relatively small size were synthesised, but agglomeration became apparent which in turn lead to larger nanoparticles being formed. This caused a red shift on the UV-Vis spectra. With reference to the TEM analysis for *D. viscosa* [Figure 4.6 (a)], a wide distribution of AgNPs sizes was observed, which further confirms the PDI and UV-vis results thus providing visible evidence of agglomeration.

The tendency of small AgNPs to agglomerate may also be attributed to the loss of capping agent. As according to Dobias and Bernier-Latmani (2013) smaller nanoparticles more readily lose the encapsulated silver ions than larger nanoparticles. When the capping agent is lost, the particles agglomerate. Losing the organic layer capping agent of the nanoparticle may result in bare AgNPs which are free from stabilizing agents, and hence increase their instability in liquid medium.

The relatively high zeta potential of *G. perpensa* (21.40 mV; Table 4.2), however, provides some assurance on the current stability of these AgNPs. Other measures need to be researched and implemented to enhance future stability of the AgNPs. One such measure is to introduce additional organic stabilizing agents that may aid in maintaining stability. A study by An (2016)

investigated biopolymer Poly-L-Lysine, which contain amino acid functional groups, as a potential stabilizer to increase the stability of AgNPs.

4.4.2. Antimicrobial assay

Although the hydrodynamic size of the AgNPs did not fall within the specified criteria that is optimal for antimicrobial studies, enhanced antimicrobial properties where observed with the synthesis of AgNPs. Moreover, some aqueous extracts which previously displayed weak to very poor antimicrobial activity against the tested pathogens displayed some noteworthy activity when synthesised as AgNPs, such as in the case of *E. autumnalis* which, together with *G. perpensa* and *S. birr*ea demonstrated noteworthy activity against all four tested pathogens.

The *E. autumnalis* aqueous extract was one of the extracts which predominately displayed very poor antimicrobial properties against the four pathogens tested in this chapter as well as in Chapter 2 antimicrobial assay, with MIC values of 8 mg/mL against *K. pneumoniae*, *S. marcescens*, *E. faecalis*, *A. baumannii*, and >8 mg/mL against *S. aureus*, *L. monocytogenes*, *S. pneumoniae*, *H. influenza*e, and *C. perfringens*. Following synthesis of AgNPs, MIC values of 0.16 mg/mL were observed against *E. faecalis*, demonstrating an increase of 50.00 fold in microbial inhibition. Minimum inhibitory concentrations of 0.63 mg/mL against *L. monocytogenes*, *K. pneumoniae*, and *A. baumannii* were also obtained. Fourier-Transmission Infrared spectroscopy analysis of *E. autumnalis* showed evidence of polyphenols, carbonyl and hydroxyl groups which are characteristic functional groups of phytochemicals such as terpenoids and flavonoids. A study by Aremu et al. (2016), identified eight phenolic acids and three flavanols from *E. autumnalis*, signifying the rich phytochemical content in *E. autumnalis* which may be directly involved in the reduction of Ag^{+}-ions to form AgNPs. Eucomic acid was identified the major bioactive compound in *E. autumnalis* (Figure 4.7 is the chemical structure of eucomic acid) and is seen to contain hydroxyl groups, carboxylic acid groups and a phenol chain all which may be involved in the reduction reaction.

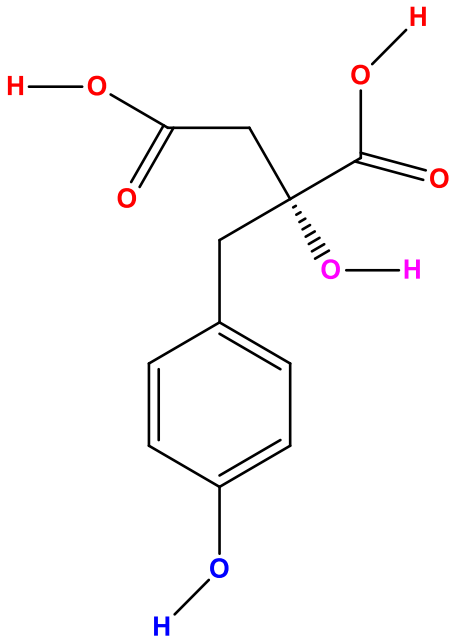

Figure 4. 7: Chemical structure of Eucomic acid.
Highlighted are the two carboxyl functional groups (red), the hydroxyl group (pink-purple) and the phenolic group (blue) present in the compound.

Sclerocarya birrea was also amongst aqueous extracts which demonstrated weak to poor antimicrobial activity against tested pathogens. However, with the synthesis of AgNPs, increased folds of 25.81 against *L. monocytogenes* and 25.00 against *A. baumannii* were observed. This plant is also called "the tree of life" which signifies its value to the South African community, both as food and medicine (Felhaber, 1997; Ojewole, 2003). The aqueous extract of the stem bark of *S. birrea* was reported to contain flavonoids and saponins, all of which are notable phytochemicals capable of reducing Ag^{+}-ions to form nanoparticles (Fotio et al., 2009). Procyanidins, such as epicatchin-3-*O*-gallate (Figure 4.8), a class of flavonoids found in plants, were the most common phenolic compounds identified in *S. birrea* (Russo et al., 2013). Their direct involvement in the reducing and capping of AgNPs may therefore be postulated. The stem bark of *S. birrea* was also reported to be rich in tannin compounds (Russo et al., 2013; Street and Prinsloo, 2012), which may also be key contributors in the formation of AgNPs with noteworthy activity. This rich pool of phytochemical compounds with varying functional groups in the stem bark of *S. birrea* resulted in predominantly triangular AgNPs, as observed from the TEM [Figure 4.6 (c)], with noteworthy antimicrobial activity.

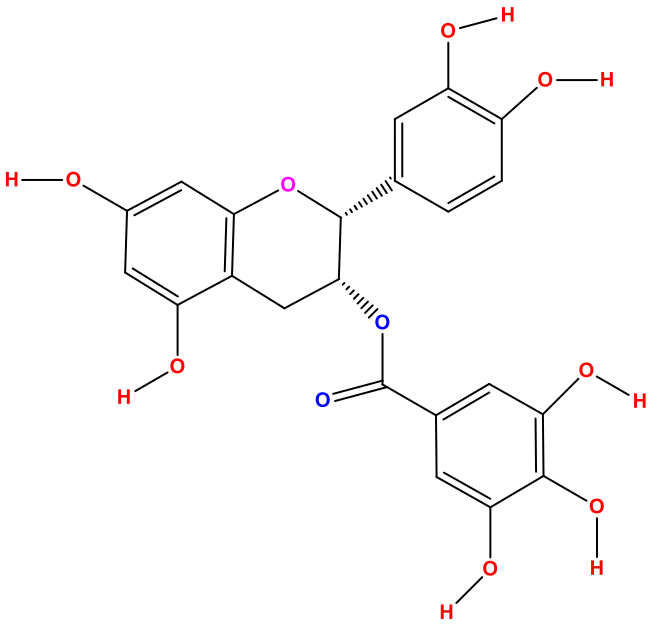

Figure 4. 8: Chemical structure of epicatchin-3-*O*-gallate, a procyanidins found in the stem bark of *S. birrea*.
Functional groups of phenols (red), an ether (pink-purple) and an ester group (blue)

Gunnera perpensa displayed some noteworthy antimicrobial properties in MIC assays in its aqueous form (Chapter 2, Table 2.3). With the synthesis of AgNPs, this activity was increased. The root of *G. perpensa* was reported to contain a minority of several acids which include 1,1'-biphenyl-4,4'-diacetic acid, *p*-hydroxybenzaldehyde, succinic acid, lactic acid and a trimethyl ether of ellagic acid glucoside (Khan et al., 2004; Brookes and Dutton, 2007). However, a phenylpropanoid glucoside, (Z)-venusol, has been isolated and identified as the major compound present in the roots of this plant, where biological activity of *G. perpensa* has been attributed to this compound (Khan et al., 2004). The chemical structure of this compound is provided in Figure 4.9, where ether, ester and hydroxyl moieties are evident. Several functional groups could be identified through the FTIR analysis of this extract (Figure 4.5), confirming the presence of organic acids and venusol as it showed peaks suggestive of phenolic groups and carboxylic groups which were involved in the synthesis of AgNPs with noteworthy antimicrobial properties.

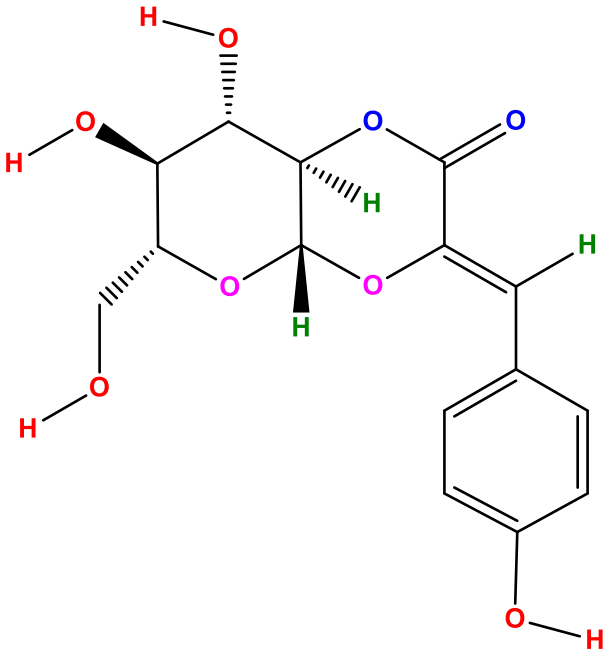

Figure 4. 9: Chemical structure of (Z)-venusol, the major chemical compound isolated from the roots of *G. perpensa*.
Functional groups observed are phenol and alcohols (red), ethers (pink-purple), an ester group (blue), and alkane groups (green).

Both aqueous extracts of *P. sidoides* and *T. violacea* were recognised not only as having the highest increase in antimicrobial activity (78.13 and >100.00 fold against pathogens *A. baumannii* and *E. faecalis* respectively), but also achieved the lowest MIC for AgNPs in the study (*P. sidoides* exhibited an MIC value of 0.08 mg/mL against *A. baumannii*, and *T. violacea* exhibited the same MIC against *E. faecalis*).

Pelargonium sidoides is one of the most well-known medicinal plants in South Africa of which the pharmacological properties have been recognised and incorporated into western pharmaceutical medicine for commercialization (Hübsch, 2014; Moyo and Van Staden, 2014). *Pelargonium sidoides* is rich in phytochemicals from various classes: the root of *P. sidoides* contains four coumarins, i.e. umckalin, scopoletin, 6,8-dihydroxy-5.7-dimethoxy-2H-benzopyran-2-one, and 6,8-dihydroxy-7-methoxy-2Hbenzopyran-2-one) which have been isolated by Mativandlela et al. (2007), together with two flavonoids, i.e. catechin and epigallocatechin. A comprehensively summarised review detailing the phytochemicals present in *P. sidoides* shows the high coumarin content, where coumarin sulphate, e.g. 5,6-dimethoxycoumarin 7-sulfate (Figure 4.10), and coumarin glycosides are the major chemical constituents in the root of this plant, followed by flavan-3-ols or proanthocyanidins content (Kolodziej, 2007).

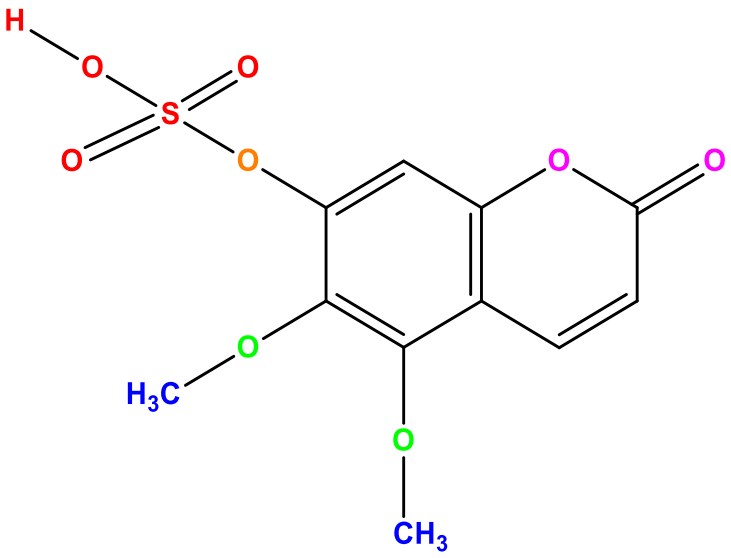

Figure 4. 10: Chemical structure of 5,6-dimethoxycoumarin-7-sulfate, a coumarin sulphate found in the roots of *P. sidoides*.
Functional groups observed are the sulphate group (red), a ketone (orange), an ester group (pink-purple), and alkane groups (blue) and ethers (green).

With regards to *T. violacea*, volatile sulphur-containing compounds have been reported as the primary phytochemicals responsible for the characteristic smell and taste of this medicinal plant (Kubec et al., 2002; Fritsch and Keusgen, 2006). The chemical compound marasmin (Figure 4.11), which has the potential to further decompose into marasmicin and several other sulphur- containing compounds, was identified as the main chemical constituents of *Tulbaghia* species (Kubec et al., 2002; Kubec et al., 2013). Steroidal saponins have also been isolated in large quantities from *T. violacea*, to which biological activity of *T. violacea* has been partly attributed (Ncube et al., 2011). Several sugars such as glucose, fructose, and maltose have also been identified in the aqueous extract of *T. violacea* (Burton, 1990), all of which are capable as acting as reducing agents (Philip, 2010; Chung et al., 2016), thus demonstrating the possibility of these phytochemicals to have been directly involved in the synthesis of AgNPs.

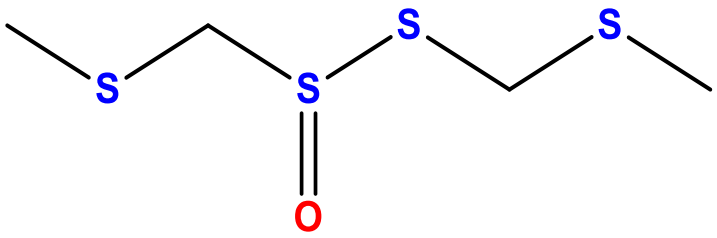

Figure 4. 11: Chemical structure of marasmin, the main chemical compound in *T. violacea*.

Functional groups observed are the sulphure groups (blue), a double bonded oxygen (red).

In the current study noteworthy antimicrobial activity was benchmarked with MIC values of <1 mg/mL in accordance to previous researchers. More recently, current MIC noteworthy values were re-evaluated, and new criteria were introduced by van Vuuren and Holl (2017). These stipulated medicinal plants antimicrobial activity be accepted as significant activity at concentrations of 0.10 mg/mL. When this stricter criterion is implemented, *T. violacea* and *P. sidoides* are seen to fall within this range.

4.4.3. Cytotoxicity assay

With the advancement of nanotechnology, the potential of toxicity of nanoparticles has raised considerable concern. Of the 10 synthesised sets of nanoparticles in the current study, only the AgNPs synthesised from *G. Perpensa* displayed cytotoxicity, with a % mortality of 56.50% after 48 hours of contact with brine shrimp larvae. According to Lee et al. (2014), cytotoxicity of nanoparticles may be attributed to the capping agent used. Bare nanoparticles, without capping agents, were found to be highly cytotoxic (Ahn et al., 2014), therefore adding a capping agent is essential not only for stability purposes, but to reduce the cytotoxicity of the metal nanoparticles as well. In this study, it was thus shown that the phytochemicals in the aqueous extracts not only reduced the Ag^{+} ions to elemental Ag^{0} nanoparticles, but also stabilized or capped the nanoparticles. These molecules are not cytotoxic and hence reduce the cytotoxicity of the AgNPs so that their use in NP synthesis becomes important when considering toxicity.

Chemicals such as polyvinylpyrrolidone (PVP) that were previously used to stabilize nanoparticles were found to contribute significantly to the cytotoxicity of nanoparticles (Vazquez-Munoz et al., 2017). Green-synthesised nanoparticles, as apparent from the results observed in this study, results in AgNPs with reduced cytotoxicity. This can be due to the organic layer capping of nanoparticles from plant phytochemicals (Roy et al., 2013) rather than

the use of synthetic chemicals. A similar study were AgNPs synthesised from plants had yielded little cytotoxic effects was done by Jacob et al. (2017). In this study AgNPs were synthesised from dried fig extract (*Ficus carica*) and no cytotoxicity was observed. In a study by Mohan et al. (2016), cytotoxicity was observed with the AgNPs synthesised from starch, although less so than $AgNO_3$.

Another critical element which has been attributed to an increase in toxicity is the decrease of particle size of nanoparticles (Carlson et al., 2008). Consequently, the relatively small particle size of the AgNPs of *G. perpensa* may be a contributing factor, coupled with the biological nature of its capping agents, to the observed cytotoxicity. Over and above the fact that smaller sized AgNPs are more capable of evading immune responses, they may also be more readily able to cross relatively impermeable membranes (Roy et al., 2013), where the predisposition of these nanoparticles to lose their capping agent more readily may account for this (Dobias and Bernier-Latmani, 2013). Readily losing the capping agent will inevitably result in bare nanoparticles which have marked toxicity as stated above. The balance, therefore, is to obtain AgNPs which are at optimal size for maximum antimicrobial properties, but yet remain stable with low toxicity is needs to be addressed.

In the present study it was observed that the synthesis of AgNPs by a simple, cost effective, and non-toxic of green chemistry allowed for improved antimicrobial efficacy of the plant extracts. Furthermore, AgNPs functionalised with extracts of *G. perpensa*, *S. birrea* and *E. autumnalis* were found to exhibit noteworthy antimicrobial properties against all the tested pathogens in this Chapter, where such noteworthy activity had previously not been demonstrated by the aqueous extracts of the plants. With exception to *G. perpensa*, the synthesised AgNPs were found to have negligible and low toxicity respectively.

4.5. Chapter summary

- Silver nanoparticles were successfully synthesised with particle size range of 39.17-112.02 nm sizes.
- The smallest size AgNPs were those of *D. viscosa* (39.17 nm), whilst *S. birrea* AgNPs showed the smallest PDI value (0.10), and the highest zeta potential for AgNPs were those of exhibited for *A. afra* (-23.00 mV).
- Triangular-shaped NPs were observed with the AgNPs *S. birrea* extracts as well as some hexagonal AgNPs. Silver nanoparticles for *D. viscosa*, *E. autumnalis*, *P. sidoides* and *T. violacea* were predominantly spherical.

- Silver nanoparticles of *G. perpensa*, *S. birrea*, and *E, autumnalis* extracts displayed the best broad-spectrum activity, inhibiting all four pathogens tested.
- The lowest MIC was 0.08 mg/mL, observed with the AgNPs of *T. violacea* and *P. sidoides* against *E. faecalis* and *A. baumannii* respectively.
- The highest increase in antimicrobial activity from aqueous extracts to their AgNPs counterparts was observed with *T. violacea* (> 100 fold) against *E. faecalis*, followed by *P. sidoides* against *A. baumannii* (78.14) fold, *G. perpensa* (> 50 fold) against *A. baumannii* and *E. autumnalis* (> 50 fold) against *E. faecalis*.
- Cytotoxicity of AgNPs was only observed with the AgNPs of *G. perpensa* (56.50 %) at 48 hrs.

CHAPTER 5

SUMMARY AND CONCLUSION

5.1. Summary

The review by Van Wyk (2008) and Van Wyk (2015) lists well-known medicinal plants in South Africa which are recognised for their considerable commercialisation potential but have not received significant attention. Amongst these are medicinal plants that have shown noteworthy antimicrobial activity in this study, viz.; *B. africana, D.viscosa, G. perpensa, H. odoratissimum, M. flabellifolius* and *S. birrea.* A common factor that exists between these plants is their traditional use for the treatment and reduction of fever. Validation for this study is found not only in this argument, but also in the frantic need for new antimicrobial agents.

The objectives of this study were to firstly investigate the antimicrobial properties of South African medicinal plants used in the treatment of fever. From the minimum inhibitory concentration (MIC) assays were conducted using nine different pathogens where only 3% of the aqueous extracts showed noteworthy microbial inhibition, as opposed to the organic extracts which demonstrated 16% with noteworthy inhibition. *Gunnera perpensa* was found to be the most active with noteworthy activity exhibited by the aqueous extracts against *K. pneumoniae* (0.5 mg/mL). *S. aureus* (0.5 mg/mL) and *S. marcescens* (0.13 mg/mL). *Eucalyptus globulus, H. odoratissimum,* and *W. somnifera* organic extracts were noted to be most active and inhibited four or more of the studied pathogens. The lowest MIC value was observed with the organic extract of *E. globulus* against *C. perfringens* with an MIC value of 0.04 mg/mL.

The cytotoxicity of the medicinal plants displaying noteworthy antimicrobial activity was determined. The findings showed that cytotoxicity was observed more frequently with the organic extracts than with the aqueous extracts. Moreover, it was noted that medicinal plant extracts which exhibited high cytotoxicity were mostly those that demonstrated strong antimicrobial activity. This was seen with the organic extracts of *E. globulus* (% mortality of 97.08% at 24 hrs), *H. odoratissimum* (% mortality of 93.19% at 24 hrs), and *W. somnifera* (% mortality of 98.26% at 24 hrs). In contrast, the majority of medicinal plants which demonstrated no cytotoxicity were those that did not demonstrate strong antimicrobial properties. In this

instance, the improvement of antimicrobial properties of these medicinal plants without increasing cytotoxicity would prove beneficial.

The enhancement of antimicrobial properties by synthesis of silver nanoparticles (AgNPs) was demonstrated in this study, with minimal cytotoxicity for nine out of the ten aqueous extracts used. Table 5.1 comprises of a summary of all synthesised AgNPs; the quality of synthesised AgNPs, toxicity profiles and antimicrobial properties.

Table 5. 1: Summary of the properties exhibited by the 10 selected fever-reducing plant extracts.

Plant samples	**Synthesis of AgNPs[a]**	**Toxicity (BSLA)[b]**	**Antimicrobial assay (MIC)[c]**			
			A. b	***E. f***	***L. m***	***K. p***
A. gummifera	++	+	-	+	-	-
A. afra	+	+	-	+	-	-
D. viscosa	+	+	-	+	-	-
[d]*E. autumnalis*	++	+	+	+	+	+
G. perpensa	++	-	+	+	+	+
L. javanica	++	+	-	+	-	-
P. sidoides	++	+	+	+	+	-
S. birrea	++	+	+	+	+	+
S. frutescens	+	+	-	+	-	-
T. violacea	+	+	+	+	-	-

A.b = *A. baumannii* (ATCC 19606); ***E. f*** = *E. faecalis* (ATCC 29212); ***L. m*** = *L. monocytogenes* (ATCC 19111); ***K. p*** = *K. pneumoniae* (ATCC 13883).

[a] Poor (-), Moderate (+), Good (++);

[b] > 50% mortality (-), < 50% mortality (+) (Bussmann et al., 2011);

[c] < 1 mg/mL (+), > 1 mg/mL (-) (Van Vuuren, 2008);

[d] Shaded area indicates plant species demonstrating the most favourable properties.

Medicinal plant extracts which were inactive in the MIC assays were able to exhibit enhanced antimicrobial properties against the tested pathogens. With the exception to *G. perpensa*, all the aqueous extracts used prior to AgNP synthesis did not exhibit noteworthy antimicrobial inhibition against the two Gram-negative and two Gram-positive pathogens tested. However, after synthesis of AgNPs, there was at least one noteworthy inhibition displayed by each plant. The highest increase factor was seen with the AgNPs of *T. violacea* and *P. sidoides* with > 100 and 78.13 fold respectively. The AgNPs of *S. birrea*, *E. autumnalis* and *G. perpensa* were specially noted to demonstrate noteworthy antimicrobial activity against all four pathogens tested, with a good quality synthesis of AgNPs and, with exception to *G. perpensa* AgNPs, less than 50% mortality of brine shrimp larvae.

5.2. Limitations of the study

5.2.1. Silver nanoparticles synthesis

Initially, an attempt to recover pure AgNPs pellets from the reaction solution was made with the aid of centrifugation. The recovered AgNPs pellets would be washed out and reconstituted into desired concentrations for testing. However, after several trials, the yield of AgNP pellets recovered proved too minimal to continue with AgNPs characterization, antimicrobial studies, and BSLA. To overcome this limitation, the aqueous extract solutions containing the synthesised AgNPs were used as they were without centrifuging in this study.

5.2.2. Brine shrimp lethality assay

A substantial amount of organic extracts proved problematic with the BSLA in the sense of solubility in the solvents used, especially considering the existing criteria that not more than 2% of organic solvent may be used for the BSLA. Due to this, sample concentration had to be manipulated to achieve solubility, yet maintaining the organic solvent at below 2%. This resulted in a lower testing concentration of the organic samples. Another limitation was encountered from the aqueous extracts, where these extracts tended to form a layer on top of the liquid in the well, making it difficult to read results. This could be overcome to some degree by increasing the brightness of the light microscope in the eyepiece. However, more effectively, it is recommended that the aqueous extracts be completely dissolved in liquid medium prior to analysis and be used fresh to avoid this limitation.

5.3. Future recommendations

In this study, the antimicrobial and cytotoxic properties of aqueous and Dichloromethane: Methanol extracts were investigated. In future studies, further consideration can be given to the combination of hydro-alcoholic extracts (Eguale et al., 2007). Hydro-alcoholic solvents are not commonly studied in plant based antimicrobial research, but their extracts may yield alternative compounds which are worth investigating.

More recently, medicinal plant studies have included cytotoxicity assays as part of the antimicrobial and biological analysis. This indicates the importance of cytotoxicity of medicinal plants which has been acknowledged by scholars. However, the degree to which the research outputs have affected the traditional use and prescribing of these medicinal plants in traditional healthcare practice remains questionable. A majority of medicinal plants which were found to be cytotoxic to brine shrimp in this study are surprisingly widely used and popular in traditional medicine. Since standard regulatory guidelines do not extend to traditional healthcare practices, the use of these plants, including prescription and indication, is at the discretion of the traditional prescriber. Effective ways to ensure that scientific research and traditional healthcare interact for the benefit the communities in South Africa, especially where cytotoxicity and safety is concerned need to be considered.

For the purpose of this study, the BSLA was used as a basic screening tool to obtain a general idea of toxicity of plants and nanoparticles. From here on, it is recommended that further in-depth cytotoxicity studies should be considered, especially pertaining to the AgNPs. Cytotoxicity assays such as the *in vitro* cell line Methly thiazolyl tetrazolium (MTT) assay (Hübsch, 2014; Sarkar and Kotteeswaran, 2018) and the *in vivo* Zebra fish assay (Asharani et al., 2008; Barros et al., 2008) may provide an in-depth analysis of the effects of these AgNPs on mammalian cells.

The medicinal plant compounds in the crude extract were responsible for the reduction and stabilization of silver ions (Ag^{+}) into AgNPs (Ag^{0}). It would be interesting to observe whether the isolation of active compounds in the plant extracts prior to AgNPs synthesis, and then using the active compound in its pure, concentrated form (as opposed to in its traceable amount in a crude extract) would result in even greater antimicrobial properties. An example of this is a recent article in which the antimicrobial properties of green-synthesised NPs, synthesised from catechins, are investigated (Oliver et al., 2018). Therefore, it is recommended to experiment

with AgNPs synthesis using pure isolated active compounds from medicinal plants is of interest.

This study design was an *in vitro* focused study and it is recommended that research in this field be extended into *in vivo* studies to fully comprehend the outcomes observed. One of the avenues in which this can be accomplished is to consider medicinal plants which displayed antimicrobial activity and induce into rat models, thereafter a comparison of the observed effects with AgNPs can be drawn up. In this manner the physiological effects of the medicinal plants and AgNPs capped with these medicinal plants' extracts on fever can also be monitored.

Another instance in which *in vivo* studies could prove to be beneficial is regarding formulation studies of green synthesised AgNPs. Previous studies have looked at AgNPs in wound dressings and topical gels (Nair and Laurencin, 2007; Maneerung et al., 2008; Jain et al., 2009), however, formulation studies regarding plant mediated AgNP synthesis, such as the study by Lakkakula et al. (2017) where *Desmodium adscendens* mediated AgNP synthesis and subsequent incorporation in wound dressing is investigated, are lacking. This, in part, can be attributed to the limited *in vivo* studies which focus on the physiological effects of green synthesised AgNPs. Fully grasping this may provide a greater insight on the interaction of these AgNPs with mammalian tissue, which in turn can curb the path for formulation studies.

5.4. Final conclusion

Fever is a complex phenomenon that arises from different biological and physiological factors, where microbial infection remains the root cause. Therefore, the study was aimed at analysing whether the therapeutic properties of these plants experienced when used traditionally to treat fever may be attributed to the microbial inhibition of fever-causing pathogens. The organic extracts of the medicinal plants were found to be more active than the aqueous extracts, but the aqueous extracts are used more often in traditional preparations. Gram-positive pathogens were more susceptible than Gram-negative pathogens. It was also found that *C. perfringens*, which is commonly affiliated with soft tissue infections that strongly leads to septicaemia and fever, was the most susceptible pathogen to both organic and aqueous extracts.

The second objective of this study was to investigate whether incorporating AgNPs with the studied aqueous plant extracts would enhance their antimicrobial properties. From the ten medicinal plants studied, a positive correlation of enhanced antimicrobial activity was observed, while low levels of cytotoxicity were exhibited. The outcome of this study supports

the use of nanotechnology to enhance antimicrobial activity of medicinal plant extracts, and furthermore enlightens future researchers on the broad area that holds promise for development. The future of green nanotechnology coupled with the promising antimicrobial activity of the unique South African flora that exists may certainly provide leads for novel antimicrobials, desperately needed in the current climate of antimicrobial resistance.

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APPENDIX A

ABSTRACT FOR PUBLICATION ACCEPTED IN THE *SOUTH AFRICAN JOURNAL OF BOTANY*

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Biosynthesis and characterization of antimicrobial silver nanoparticles from a selection of fever-reducing medicinal plants of South Africa.

Most developing countries, including South Africa, depend strongly on traditional medicine for a therapeutic outcome and have therefore employed numerous medicinal plants to treat fevers. Therefore, it is imperative that fever-reducing medicinal plants are investigated to establish their efficacy and to determine their potential as sources of new antimicrobials. The incorporation of nanotechnology in antimicrobial research with reference to medicinal plants is a growing domain. The interest in silver nanoparticles (AgNPs) encompasses the tested hypothesis that the chemical combination of silver with medicinal plant extracts results in nanoparticles with enhanced antimicrobial properties in comparison with plant extracts alone. This study investigated the antimicrobial properties from ten medicinal plants of commercial significance used traditionally for the treatment of fever in South Africa and their potential for enhanced antimicrobial efficacy when incorporated within silver nanoparticles (AgNPs). Plant extracts and AgNPs were tested against fever-related pathogens, i.e. two Gram-positive pathogens; *Listeria monocytogenes* (ATCC 19111) and *Enterococcus faecalis* (ATCC 29212) as well as two Gram-negative pathogens; *Klebsiella pneumoniae* (ATCC 13883) and *Acinetobacter baumannii* (ATCC 19606) using the broth microdilution method. Chemical characterization and AgNPs monitoring included Ultraviolet–visible (UV-Vis) spectroscopy, Dynamic light scattering (DLS), Fourier-transform infrared spectroscopy (FTIR), and Transmission electron microscopy (TEM). The toxicity profiles of the AgNPs were evaluated using the brine-shrimp lethality assay (BSLA). Both nanoparticles of *E. autumnalis* and *S. birrea* display a dramatic increase in antimicrobial activity against the four test pathogens compared to their aqueous plant extracts. The greatest difference in antimicrobial activity was observed against *E. faecalis* where an increase in antimicrobial activity of at least 50 fold when *E. autumnalis* aqueous samples were compared with the AgNPs counterparts. Toxicity of both AgNPs samples from the BSLA emerged at less than 50% mortality, which denotes non-toxicity. The results obtained in this study justify the use of selected fever-reducing plant extracts with the biosynthesis of AgNPs as promising antibacterial agents with low toxicity.

APPENDIX B

ABSTRACT FOR ORAL PRESENTATION AT THE INDEGINOUS PLANT USE FORUM (IPUF) (PRETORIA)

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Antimicrobial evaluation of South African medicinal plants to treat fever and effects in combination with synthesized nanoparticles.

Fever is a characteristic symptom in many infectious diseases that plague South African communities. Various medicinal plants have been employed in the treatment of fever, and the medicinal properties of these plants are often linked to infectious diseases. Green synthesis of silver nanoparticles has previously demonstrated promising antimicrobial properties which may be useful in combating fever related ailments. The aim of this study was to investigate the antimicrobial properties of medicinal plants used in the treatment of fevers and to investigate whether the antimicrobial properties of these medicinal plants could be enhanced by incorporating them in the synthesis of silver nanoparticles (AgNPs). As AgNPs have the potential to exhibit toxicity, this was also investigated. Medicinal plants that presented with noteworthy antimicrobial activity included extracts of *Gunnera perpensa* L. (Gunneraceae) with an MIC value of 125 µg/mL against both *Serratia marcescens* (ATCC 13880) and *Listeria monocytogenes* (ATCC 19111). *Eucalyptus globulus* Labill. (Myrtaceae) and *Withania somnifera* (L.) Dunal (Solanaceae) also showed exceptional activity with an MIC value of 16 µg/mL against *Serratia marcescens* (ATCC 13880) and *Listeria monocytogenes* (ATCC 19111) respectively. Results generally displayed an increase in antimicrobial efficacy with the synthesis of AgNPs. *Sclerocarya birrea* Hochst. (Anacardiaceae) and *Eucomis autumnalis* (Mill.) Chitt. (Hyacinthaceae) AgNPs were found to possess excellent antimicrobial properties with minimal toxicity. The results obtained in this study justify the use of these medicinal plants traditionally, and the using nanotechnology to enhance the antimicrobial effects of fever medicinal plants.

APPENDIX C

ABSTRACT FOR ORAL PRESENTATION AT THE SCHOOL OF THERAPEUTIC SCIENCES BIENNIAL RESEARCH DAY (UNIVERSITY OF THE WITWATERSRAND)

Mahlatse E. Lediga[1], Sandy F. van Vuuren[1] and Denise K. Olivier[2].

[1]Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, 2193, Johannesburg, South Africa. [2]Research and Development, Seobi, PO Box 906482, Magalieskruin, 0150, 19 Mountain street, Derdepoort, 0186, South Africa.

Antimicrobial evaluation of fever-reducing medicinal plants and effects when combined with synthesized silver nanoparticles.

Introduction: South African medicinal plants and silver nanoparticles have previously demonstrated antimicrobial properties. Thus, it was investigated whether green synthesis of Silver nanoparticles from these plants would result in an increase in their antimicrobial properties and thus result in a greener approach to antimicrobial therapy. As nanoparticles have a potential to be toxic, toxicity of the Silver nanoparticles was also investigated.
Methods: The Minimum inhibitory concentration (MIC) method was used to perform antimicrobial assays. Silver nanoparticles were synthesized through a reaction involving a combination of plant extract with silver nitrate. The Brine shrimp lethality assay was used to assess toxicity of the synthesized silver nanoparticles.
Results: Noteworthy antimicrobial activity was noted for *Gunnera perpensa*, which showed activity against *Serratia marcescens* (ATCC 13880) (125 µg/mL) and *Listeria monocytogenes* (ATCC 19111) (125 µg/mL). *Eucalyptus globulus* and *Withania somnifera* also showed exceptional activity with an MIC value of 16 µg/mL against *S. marcescens* and *L. monocytogenes* respectively. There was a general increase in antimicrobial activity with synthesis of Silver nanoparticles. Key results include silver nanoparticles of *Sclerocarya birrea* and *Eucomis autumnalis* which were found to possess increased antimicrobial activity, with minimal toxicity.
Discussion: The antimicrobial results obtained justify the use of these medicinal plants traditionally. Incorporation of silver nanoparticles was shown to enhance the antimicrobial effects of fever medicinal plants in this study.

APPENDIX D

ETHICS WAIVER CERITICATE FOR USE OF MICROBIAL CULTURE

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Tobias Health Sciences Building, 2nd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-160720-1 20/07/2016

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Prof S van Vuuren, M Lediga (Student no 671432).**

Project title: Interactive efficacy between fever-reducing plants and silver nanoparticles.

Reason: This is a laboratory study using bacterial cultures. The bacterial strains include:
Staphylococcus aureus (ATCC 25723) *Listeria monocytogenes* (ATCC 19111), *Streptococcus pneumoniae* (ATCC 49619), *Enterococcus faecalis* (ATCC 29212), *Clostridium perfringens* (ATCC 13124), *Klebsiella pneumoniae* (ATCC 13883), *Acinetobacter baumannii* (ATCC 19606), *Serratia marcescens* (ATCC 13880) and *Haemophilus influenza* (ATCC 19418).
There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2016 -07- 20
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi.

APPENDIX E

ORIGINALITY REPORT

Thesis

ORIGINALITY REPORT

13%	5%	13%	1%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	I.E. Cock, M.I. Selesho, S.F. van Vuuren. "A review of the traditional use of southern African medicinal plants for the treatment of selected parasite infections affecting humans", Journal of Ethnopharmacology, 2018 Publication	2%
2	U. Mabona, S.F. Van Vuuren. "Southern African medicinal plants used to treat skin diseases", South African Journal of Botany, 2013 Publication	2%
3	Lerato Seleteng Kose, Annah Moteetee, Sandy Van Vuuren. "Ethnobotanical survey of medicinal plants used in the Maseru district of Lesotho", Journal of Ethnopharmacology, 2015 Publication	1%
4	M.E. Lediga, T.S. Malatjie, D.K. Olivier, D.T. Ndinteh, S.F. van Vuuren. "Biosynthesis and characterisation of antimicrobial silver nanoparticles from a selection of fever-reducing medicinal plants of South Africa", South African Journal of Botany, 2018	1%

	Publication	
5	Namrita Lall, Navneet Kishore. "Are plants used for skin care in South Africa fully explored?", Journal of Ethnopharmacology, 2014 Publication	1%
6	Katerere, David, and Jacobus Eloff. "Management of Diabetes in African Traditional Medicine", Traditional Herbal Medicines for Modern Times, 2005. Publication	1%
7	A. Moteetee, R.O. Moffett, L. Seleteng-Kose. "A review of the ethnobotany of the Basotho of Lesotho and the Free State Province of South Africa (South Sotho)", South African Journal of Botany, 2018 Publication	<1%
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10	dspace.stir.ac.uk Internet Source	<1%
11	V. Steenkamp. "Traditional herbal remedies used by South African women for gynaecological complaints", Journal of Ethnopharmacology, 2003	<1%

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12	Andreas Lardos. "The botanical materia medica of the latrosophikon—A collection of prescriptions from a monastery in Cyprus", Journal of Ethnopharmacology, 2006 Publication	<1%
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14	B.-E. Van Wyk. "A review of commercially important African medicinal plants", Journal of Ethnopharmacology, 2015 Publication	<1%
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16	www.reproduction-online.org Internet Source	<1%
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19	S. Akhalwaya, S. van Vuuren, M. Patel. "An in vitro investigation of indigenous South African medicinal plants used to treat oral infections", Journal of Ethnopharmacology, 2018 Publication	<1%

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21	Z. Hübsch, R.L. Van Zyl, I.E. Cock, S.F. Van Vuuren. "Interactive antimicrobial and toxicity profiles of conventional antimicrobials with Southern African medicinal plants", South African Journal of Botany, 2014 Publication	<1%
22	S. Van Vuuren, D. Holl. "Antimicrobial natural product research: A review from a South African perspective for the years 2009–2016", Journal of Ethnopharmacology, 2017 Publication	<1%
23	B.-E. Van Wyk. "The potential of South African plants in the development of new medicinal products", South African Journal of Botany, 2011 Publication	<1%
24	H. De Wet, V.N. Nzama, S.F. Van Vuuren. "Medicinal plants used for the treatment of sexually transmitted infections by lay people in northern Maputaland, KwaZulu–Natal Province, South Africa", South African Journal of Botany, 2012 Publication	<1%
25	link.springer.com Internet Source	<1%

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*A majority of the similarities is attributed to the literature review table (Table 1.2, Chapter 1) i.e. the plant species, common names and references.

27/09/2018

Prof. S.F van Vuuren — Date