UNIVERSITY OF THE WESTERN CAPE

DEPARTMENT OF BIOTECHNOLOGY

Evaluation of cytotoxic activity of gold nanoparticles naturally synthesised from South African indigenous medicinal plant extracts

By

Yamkela Mbandezi

A mini-thesis submitted in partial fulfilment of the requirements for the degree Magister Scientiae in the Department of Biotechnology, University of the Western Cape.

Supervisor: Prof Mervin Meyer
Co Supervisor: Prof Ahmed Mohammed

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Abstract

Nanotechnology has emerged as a promising field in the quest to address health conditions. Green nanotechnology is a fairly new branch of nanotechnology, which aims to produce and utilize nanomaterials in a way that is safe for living organisms and their environment. Plant extracts are increasingly used in the green synthesis of gold nanoparticles (AuNPs), which involves the reduction of sodium tetrachloroaurate (III) dehydrate by phytochemicals present in the plant extract. It is probable that the green synthesised AuNPs are more biocompatible than chemically synthesised AuNPs as biomolecules of plant origin are involved in the synthesis process. Therefore, this study aimed to explore various water extracts from indigenous South African plants, which included *Perlagonium capitatum*, *Otholobium bracteolatum*, *Gerbera linnae*, *Morrella quercifolia*, *Searsia lucida*, *Phylica bubescens*, *Euclea racemosa*, *Tetragonia fruticosa*, and *Searsia glauca* for their potential to synthesize AuNPs and to investigate their toxicity towards several microorganisms known to cause skin infections. These organisms play a significant role in delaying the healing of wounds. The antimicrobial properties of nanoparticles are increasingly exploited in the production of wound treatments. AuNPs were successfully synthesised from the selected plants. The AuNP synthesis was carried out at 25°C and 70°C. The UV-Vis data showed the reduction of the sodium tetrachloroaurate (III) dehydrate and formation of AuNPs by confirming the characteristic surface plasmon resonance (SPR) peaks of AuNPs. The Energy Dispersive X-ray spectroscopy (EDX) results indicated the presence of gold ions in the AuNPs. The Dynamic Light Scattering (DLS) data showed that AuNPs diameter were in a range of 41.52 - 84.67 nm at 25°C and 28.97-90.7 nm at 70°C. The DLS data also showed negative zeta potential values for the AuNPs suggesting their stability in solutions. The synthesised AuNPs were mostly quasi spherical and pentagonal in shape as shown by the High-resolution electron microscopy results. The AuNPs also showed excellent stability in different biological media. The antibacterial activity of *M. quercifolia*, *P. capitatum* and *S. glauca* plant extracts and their respective AuNPs were studied using the microtiter method. Both AuNPs and their respective plant extract showed significant antibacterial activity against *S. aureus*. *M. quercifolia* plant extract and its respective AuNPs showed antibacterial activity against both *S. aureus* and MRSA, while *P. capitatum* plant extract and its respective AuNPs showed antibacterial activity against *S. aureus*. These results show the great potential of these AuNPs and their respective plant extracts to be applied as antimicrobial agents.
Keywords

Nanotechnology
Green nanotechnology
South African indigenous plants
Phytochemicals
Gold nanoparticles
Characterization
Antibacterial activity
Antimicrobial agents
Bacterial wound infection
Cancer
Wound healing
Diabetes
Declaration

I declare that “Evaluation of cytotoxic activity of gold nanoparticles naturally synthesised from South African indigenous medicinal plant extracts” is my own work that has not been submitted for any degree or examination in any other university and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Signed: ……………………………………..

Date: 05.12.2018
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Abbreviations

AuNPs – Gold nanoparticles

OC – Optimum concentration

TEM- Transmission Electron Microscopy

UV-Vis- Ultra Violet-Visible Spectroscopy

EDX- Energy dispersive X- ray spectroscopy

DLS- Dynamic Light Scattering

ZP- Zeta Potential

PDI- Polydispersity Index

SPR- Surface Plasmon Resonance

LSPR- Localised Surface Plasmon Resonance

rhTNF- recombinant human tumour necrosis factor

EGFR- Epithelial Growth Factor Receptor

TiMPs- Tissue metalloprotease inhibitors

MPA- Mercaptopropionic acid

EDC- ethyl-3-[3-(dimethyl amino) propyl] carbodimide hydrochloride

PEG- Polyethylene glycol

DMEM- Dulbecco’s Modified Eagles Media

FBS- Fetal Bovine Serum

PAH- Poly- alllamine hydrochloride

DCP- Disease Control and Prevention

PSA- Prostate Specific Antigen
Acknowledgements

Firstly, I raise my “Hallelujah!” to my Lord and saviour Jesus Christ, without whom I would have lost myself and would have not been able to reach this point.

To my supervisor, Prof Mervin Meyer. I quote and agree with a statement from Lauren’s thesis “Simply put, you are the best”. Thank you for all the wise words, care, genuineness, outstanding patience and support even beyond the scope of what is required of you. Your time invested in me and encouraging words were truly not in vain. I have learnt the most valuable life lessons under your supervision and I’m grateful for that opportunity.

Thank you my co-supervisor, Prof Ahmed Mohammed, for your help, patience, support and willingness to help.

To both of you my supervisors a big thank you for allowing me the opportunity to pursue such an exciting study and for all your help and guidance throughout the process, even though I was not a very easy student to pull through, but you remained willing and supportive and for that I am forever grateful.

To Mr Abdulrahman M. Elbagory, my mentor in this study, I am so grateful of all your help and patience. For your persistent support and positive attitude, even in situations that were difficult to handle, which would have led you to quit and turn me away but instead you were always willing to help until the end. I am truly blessed to have had a mentor like you.

To Mr Salah Ramadan Alshibani, thank you for helping me during my lab work. You and Ephraim were the best lab mates I’ve ever had!!! Thank you for all the help and challenging me with questions while we do our experiments. Those moments brought out the scientist in me, I will forever treasure them.

To you my lovely Dr Nicole Sibuyi, who is Sis Nic to me, thank you for your tireless efforts in helping me in multiple ways, your care and support in difficult times is much appreciated.

To Dr Mustafa Drah, thank you for all your help and the sacrifices you made to help me with my experiments and for your assistance with lab supplies and equipment.
To Dr Christopher N. Cupido, thank you for helping me with the identification of the plants used in this study; without their identity, the study would have been meaningless.

To Miss Luveni Sonka, thank you for helping me with the plant collection and for your tremendous support.

Thank you to the DST/Mintek Nanotechnology Innovation Centre for allowing and welcoming me as a part of their research group; for every resource I used in this research project and financial assistance, thank you.

I would like to thank the whole NIC group for their valuable help, support and motivation. Your care, kind words and willingness to help has been outstanding and it is evident that we are truly like sisters and brothers, as Sis Nic always emphasises.

Thank you to the National Nanoscience Teaching and training Platform and the Department of Science and Technology for funding this project and particularly Valencia Jamalie for her help, kindness, support and words of wisdom as well as Chyril Abrahams.

To Dr Ashwil Klein, thank you for encouraging me to do my master’s degree and all your help with the necessary preparations. I will forever treasure that day we had a conversation while I was busy organising my documents applying for work and said “Do your Masters, Yamkela”. That moment changed the direction of my academic career.

To Prof Lullu Tshiwula, thank you for all your words of wisdom, love, care, kindness, genuineness and support. Your office is a space where I draw inspiration and courage. I always come out with the strength to bounce back from life’s setbacks. To the whole Resilience Network Institute, thank you for your contribution to me gaining the strength needed to bounce back from life’s setbacks.

To Mrs Laeticia Permall, thank you for everything, more especially for being you. Through you I found my “I”. To the Centre for Student Support Services (CSSS) thank you for your support in helping me with the tools to stay on my growing edge.

To Prof Abram Madiehe, thank you for all your guidance, encouragement and wise words (even those heard from a distance).
To my neighbour at the lab write up area, Miss Lauren Swarts, thank you so much for all your wise words and willingness to share your own journey to success with me. Your help in my journey of bouncing back was truly precious.

To Mr Ahmed Eldud and Mr Ephraim Maphasa, thank you for patiently and willingly helping me with my statistical analysis.

To my Mother, Mrs Zusakhe Malapi, my brothers and sister Mr Thabiso Tom, Miss Kamva Malapi, Miss Lutho Mbandezi and the whole family, thank you for all your support.

To my Pastor Adv. Sibulele Nkantsu and my Mamfundisi- Mrs Samkelisiwe Nkantsu, thank you for all the encouragement and support. Your wise words, love, care and most outstandingly your genuineness has made an outstanding contribution to my success. Your exemplary life made me see success feasible and victory achievable. To the Associates (Associates for Godly Operation on Campuses) family, thanks for all your love and support through it all. You all played unique and valuable roles in my life. I bless God for you.

To Miss Chwayita Ngewu, “Sis Chwayi”. Thank you Sis wam for the love, care and encouragement. I remember the day you said “Yamkela, UThixo akayiqali into angayiqibi”. God has really carried me through to the finish line.

I am truly grateful to God for every contribution each of the above people has made towards my success. The journey was not easy but it was one packed with valuable life lessons that I will carry with me to the next phase of life.
Chapter One: Literature review

1.1 Introduction

Huge investments have been made globally in the field of nanotechnology, which holds great potential to alleviate challenges faced in medicine and energy production (Cele et al, 2009). Gold nanoparticles (AuNPs), in particular, have desirable physico-chemical properties for various applications in this field. Various AuNPs have been synthesised by a variety of chemical and physical methods (Jain et al, 2008). The successful biological application of these nanoparticles is often hindered by their toxicity which is incurred by toxic reducing and stabilising agents used in their synthesis (Thakkah et al, 2010). Green synthesis of nanoparticles can overcome the limitation of using metal nanoparticles in biomedical applications (Wang et al, 2009).

Green nanotechnology is defined as the application of green chemistry which includes the 12 Principles of Green Chemistry framework for the production of new nanotechnologies which will benefit humans, the environment and ultimately the economy (Hutchison, 2008). Green nanotechnology includes the biogenic synthesis of metal nanoparticles using reduction capabilities of natural reactants obtained from various biological sources which include unicellular organisms and higher plants (Alam et al, 2013). These reactants can be any of the phytochemicals present in the plant extracts which can replace the toxic chemicals used in traditional chemical synthesis of the nanoparticles (Kannan et al, 2012). Developments in characterisation techniques allow for the investigation of various characteristics and possible interactions of these nanoparticles with other entities. This has opened new possibilities for their potential application. There is great potential for the application of these nanoparticles in medicine (Cele et al, 2009). Plants have emerged as more favourable sources of these reactants as they are easily available, possess rich biodiversity, and lead to eco-friendly synthesis procedures (Rajan et al, 2015).
South Africa possesses a wide variety of medicinal plants which have been used to treat various diseases (Gurib-fakim, 2006). These medicinal plants have been screened for their bioactive phytochemicals (Rybicki et al, 2012; Wiersum et al, 2006). A variety of plants have been used in the synthesis of AuNPs with various physico-chemical properties (Burygin et al, 2009). The biosynthesis process of these nanoparticles have several advantages over conventional synthesis methods, which include the ease of synthesis, environmentally friendly, energy efficient and cost effective process (Kannan et al, 2012). The possible therapeutic effect of the AuNPs is due to the presence of bioactive phytochemicals of the specific plant (Burygin et al, 2009).

1.2 Nanotechnology

A nanometer (nm) is one billionth of a meter, or \(10^{-9}\) m and a particle with at least one dimension in this nanometre range is termed a nanomaterial (Pokropivny, 2007). Nanotechnology involves nanoparticles that are able to form nano machines and other constructs or products through molecular manufacturing, atom by atom with total molecular control with an added advantage of atomic precision (Hunt, 2004). This involves the ability to study and manipulate matter at the nanoscale level (Pokropivny, 2007). The ability to work at an atomic level has the advantage of producing nanostructures with unique molecular organisation.

In this field, the study of material at their most basic form, using their inherent properties or takes advantage of certain processes to manipulate them in order to obtain a desired effect (Pokropivny, 2007). The application of nanotechnology, its processes, applications and impact revolve around the nanoparticle as the central concept which is defined as the smallest unit which has the ability to behave as a whole entity when properties are taken to consideration.
One nanometre is approximately 10 hydrogen atoms aligned in a row. Nanoparticles include but is not limited to carbon nanotubes, metal such as gold nanoparticles (Huang et al, 2007), polymer nanoparticles and quantum dots (Liu et al, 2007). The main reason for the versatility of nanoparticles is their small size. Nanoparticles are about one hundred to ten thousand times smaller than human cells. Nanoparticles are much smaller compared to biological macro-molecules which include proteins which function as enzymes and receptors (Xie et al, 2010).

Nanotechnology has showed great potential in different biomedical applications such as the diagnosis and the treatment of diseases (Kannan et al, 2012). Nanoparticles can also be used as carriers that deliver drugs to target tissues and maintain the drug at a therapeutic concentration in the body for a prolonged period of time (Duncan, 2006 and Kreuter, 2007). Various chemical methods have been used for the synthesis of nanostructures but their application in medicine has been limited by their cytotoxicity (Jana et al, 2001, Nikoobacht and Sayed, 2003). Nanotechnology can facilitate early disease detection, more accurate diagnosis, and personalized cancer treatment (Xie et al, 2010).

AuNPs are synthesized using chemical, physical and biological methods. Nevertheless, the most popular method used for the synthesis of AuNPs is chemical synthesis through the conversion of ionic gold to metallic nanoparticulate gold. Sodium citrate mediated chemical reduction described by Turkevich (1951) and Frens (1973) is used for the synthesis of size controlled and stable AuNPs.

The size of the nanoparticles is affected by the concentration of the precursor. Varying the concentration of the reducing agent (trisodium citrate) has shown to control the size of the resultant nanoparticles (Nguyen et al, 2009). It has been observed that delayed nanoparticle capping by citrate lead to larger nanoparticles whereas a faster capping reaction give rise to smaller nanoparticles. This makes the reducing agent (citrate) a determining factor of the size of the resultant nanoparticles (Nguyen et al, 2009). Due to the relationship between the application, size and shape of AuNPs. Controlling these characteristics of the AuNPs become crucial in obtaining the desired nanoparticles for a particular application in biomedicine.
AuNPs have an oscillation frequency which is in the visible region of the light spectrum which gives rise to a strengthened surface plasmon resonance (SPR) absorption. Their absorption coefficient is larger than that of other absorbing dyes which contributes favorably to their application in medicine (Nguyen et al, 2009). One of the advantages of anisotropy is that the anisotropic shaped nanoparticles have even stronger Plasmon absorption leading to enhanced sensitivity when used in diagnostic applications for the detection of biomolecules (Nguyen et al, 2009). Changes in the shape of the AUNPs lead to change in absorption spectra (Nguyen et al, 2009).

Another chemical synthesis method for AuNP that has been used is the sodium borohydride mediated reduction method (Schriffin, 1994). However, the most widely used method of chemical synthesis is the seed mediated growth method which was proposed by Schmid et al (1996). Although, a variety of chemical methods have been successfully developed and employed for the synthesis of various AuNPs, their application in medicine has been hampered by their toxicity (Jana et al, 2001 and Nikoobakht et al, 2003). Their versatile and modifiable surface chemistry are key to developing nanoparticles with the wide range of applications (Huang et al, 2007 and Jain et al, 2008). They can be easily modified and coated with a variety of molecules which include drugs, proteins, antibiotics nucleic acids, etc. Most applications require that the nanoparticles remain stable in biological fluids for applications in cell and animal imaging, drug delivery and diagnostics. To improve AuNPs stability, various compounds such as cyclodextrin, thiol compounds and surfactants are used (Gao et al, 2012).

These efforts have also been employed to assist dispersibility and biocompatibility of the nanoparticles in biological environment (Gao et al, 2012). The ligand modified AuNPs not only assists in obtaining stability but also forms the basis of biomolecule conjugation to the AuNPs. An example of this is the biligand- modified AuNPs which are modified with polyethylene glycol (SH-PEG) and mercaptopropionic acid (MPA) (Gao et al, 2012). This mixed ligand conjugated AuNPs were conjugated to an antibody of epidermal growth factor receptor (EGFR) using ethyl-3-[3-(dimethyl amino)propyl]carbodimide hydrochloride (EDC) as coupling reagent (Gao et al, 2012).
The properties of these AuNPs can be customised for different applications by varying the size or shape of the nanoparticles (Haung et al, 2008 and Jain et al, 2008). The unique properties of AuNPs have made them to be of great interest in medical research and these nanoparticles show promising applications in cancer treatment (Article, 2014).

1.3 Biomedical application in Cancer Diagnostics and treatment

1.3.1 AuNPs in Cancer Therapeutics

Brown et al (2010) had demonstrated that the active component of the anticancer drug oxaliplatin which is platinum-based showed improved drug delivery when conjugated to AuNPs. Gold nanoparticles were functionalized with a carboxylate group capped thiolated polyethylene glycol (PEG) monolayer (Brown et al, 2010). A platinum complex, [Pt (1R, 2R-diaminocyclohexane) (H_2O) 2]2NO_3, was added to the PEG to yield a supra-molecular complex with around 280 oxaliplatin drug molecules per nanoparticle (Brown et al, 2010). The nanoparticle complex was tested for cellular drug uptake, cytotoxicity and intracellular localisation in colon cancer cell lines (HCT116, HCT15, HT29, and RKO) as well as a lung epithelial cancer cell line (A549) (Brown et al, 2010). Significantly better cytotoxicity was found with the nano-construct than the oxaliplatin alone in all tested cell lines and nuclear localisation in lung cancer cells was also demonstrated (Brown et al, 2010).

CYT-6091, a recombinant human tumor necrosis factor (rhTNF) bound to colloidal gold underwent investigation in attempts to treat solid tumor and is currently undergoing clinical trials (Libutti, 2010). The rhTNF was conjugated to gold nanoparticles by use of a PEG linker which contributed as an antifouling layer. It was observed in the study that rhTNF’s dose when bound to AuNPs could be increased by 3 times the dose of native rhTNF without any toxic effects (Libutti, 2010). In another example, silica- coated AuNPs which were conjugated to iron oxide particles for controlling particle biodistribution via exterior
magnetic field modulation allowed localised ablation of blood vessel plaque and protein targeted microbubbles or stem cells to aid delivery (Kharlamor et al, 2015).

### 1.3.2 AuNPs in Cancer Diagnostics

AuNPs are the most suitable raw materials for rapid, robust diagnostic testing (Pellequer and Yann et al, 2008). The small quantities required make it inexpensive, whilst its sensitivity, stability and reproducibility give confidence towards large scale production for commercial application. Early cancer screening and respective diagnosis takes advantage of relevant biomarkers (Fang et al, 2009 and Yang et al, 2010). In one example, the transmembrane glycoprotein and the epithelial growth factor receptor (EGFR) was imaged using immunotargeted AuNPs in cervical epithelial cancer cells (SiHa cells) for early cervical cancer detection (Rahman, 2005). Another application of AuNPs includes the emergence of an immunoassay which was developed for detecting *Mycoplasma pneumonia*. In this assay an advantage of the red-shift property of AuNPs due to localised surface plasmon resonance (LSPR) is taken where a secondary antibody is labelled with alkaline peroxidase. This antibody catalyses chemical reactions leading to copper (I) formation which trigger an interaction between azido- and alkyne- functionalised AuNPs leading to a colour change from red to blue in the solution (Xianyu et al, 2014).

A similar approach was done to detect prostate specific antigen (PSA) and HIV- associated protein, p24. In this approach, a secondary antibody labelled with an enzyme that generate a compound that lead to the formation of AuNPs (De La Rica and Stevens, 2012). Cocaine is one of the small molecules where the red-shift property of AuNPs has been used for its detection based on dipole coupling (Wang et al, 2009). Diagnostic procedures which include fluorescence take advantage of a particular fluorophore emission which may be modulated by certain interactions with AuNPs with the particular fluorophore through the LSPR (Kang et al, 2011). Interference of AuNPs with radiative and non-radiative fluorophore excited state pathways lead to suppression or advancement of emitted light. This effect has been exploited in the development of “on/off” sensors which work through fluorophore removal from AuNP surface (Degliangeli et al, 2014).
Different functional moieties can be conjugated to AuNPs through their versatile surface chemistry which makes it possible to modulate AuNP affinity towards a wide range of different analytes (Conde et al, 2014).

Aptamers which are short nucleic acid sequences that can be functionalised to AuNPs have affinity towards different analytes (Veigas et al, 2012). An example of this application of AuNPs can be observed in a lateral flow strip designed for thrombin which was developed using aptamer- functionalised AuNPs. This apt-AuNPs lateral flow strip (LFS)’s analytical performance was higher and more advanced than the antibody equivalent. AuNPs can also be used in AuNP- based sensing of versatile bioreceptors such as peptides and enzymes. This technology was applied successfully to the detection of human IgG (Parolo, 2013).

1.3.3 AuNPs in Cancer Theranostics

Advancements in biomedical applications include the emergence of theranostics towards the development of personalised medicine. The combination of therapeutic and diagnostic capabilities in one nano-construct is called theranostics (Petrosa et al, 2015). Gold nanoparticles, in particular, possess unique features that make them to be excellent nanomaterials for theranostics, enabling the integration of imaging, targeting and therapeutics in one platform with proven ability to be applied in heterogeneous disease management such as cancer (Petrosa et al, 2015).

One property that makes AuNPs most suitable in cancer theranostics is their straightforward and versatile chemistry which makes it relatively easy for biomolecules to be attached to them (Petrosa et al, 2015). It was reported by Johnson that doxorubicin has the potential of being loaded onto hollow gold nanospheres due to the ability of the drug molecules to be adsorbed to both the outer and inner surfaces of the gold nanospheres. This was reported by a 60 % of doxorubicin payload which was assisted by electrostatic attraction (Johnson et al, 1998). It was recognised that NIR light could be used to activate drug release due to strong SPR absorption of AuNPs (Awada et al, 2014). Interestingly, AuNPs that were loaded with doxorubicin have also been observed to show ability of reversing resistance of cancer cells to the drug (Awada et al, 2014). AuNP-mediated photothermal
effect has shown potential to improve blood perfusion and lead to increased macromolecular tumour drug delivery (Paz-Ares et al, 2008).

### 1.4. AuNPs as antimicrobial agents

Several studies have shown that AuNPs have antibacterial activity (Irshad et al, 2013 and Park et al, 2011). Recent findings have shown size and dose dependent antibacterial activity of AuNPs against four enteric pathogenic bacteria with high activity against gram-positive bacteria (Shahzadi et al, 2016). AuNPs were shown in other studies to have lower antibacterial activity when compared to silver nanoparticles (AgNPs) and insignificant Antibacterial activity when used in their native state (Majdalawieh, 2014). Regardless of this lower antibacterial activity, AuNPs are worth being explored for their antibacterial activity. Considering the that AuNPs are inert, have a versatile surface modifications and have polyvalent and photothermal effects (Boisselier and Astruc, 2009). Moreover, the nature of the capping agent has been observed to play a vital role in the antibacterial activity of the nanoparticle (Shah et al; 2014). This was observed from a comparison between citrate and poly-allylamine hydrochloride (PAH), which showed different mechanisms of action against the bacteria (Zhou et al, 2012). These benefits of AuNPs combined with potential benefits of a variety of plants in antibacterial activity can lead to the basis of research in the quest of finding the most effective antimicrobial agent. Various studies have already showed antibacterial activity of various South African plants (Elbagory, 2017).

In an effort to improve the antibacterial efficiency of AuNPs, conjugation of AuNPs with different antibiotics has been explored by scientists and evaluated for antibacterial activity. Citrate reduction method is among the methods used for antibiotic coating on AuNPs (Shah et al, 2014). A study showed that 14 nm AuNPs coated with kanamycin, ampicillin and streptomycin inhibit the growth of *E. coli, S. aureus* and *M. luteus* more effectively than free antibiotics. The results reveal an increased zone of inhibition against all three strains when compared to antibiotics alone (Saha et al, 2007). Another study modified the antibiotic vancomycin into bis (vancomycin) cystamide and then conjugated it to 4 nm-5 nm AuNPs. Higher antimicrobial activity was observed against *vancomycin-resistant enterococci* (Gu et al, 2003).
1.4.1 Mechanisms of the antibacterial activity of AuNPs

It has been observed that AuNPs exert antibacterial action through the use of transcriptomic and proteomic approaches (Cui et al, 2012). These AuNPs use mainly two ways in carrying out this antibacterial action, one is through the inhibition of the ATP synthase through the changing of membrane potential which leads to a decrease in the ATP levels. This decline in ATP levels leads to decreased metabolism levels which gives an indication of the general decline in metabolic mechanisms. The other mechanisms involve inhibiting the subunit of ribosomes responsible for tRNA binding, indicating a collapse of biological processes (Cui et al, 2012).

1.5 Wound infections

1.5.1 Effects of bacterial infections on wounds

The skin serves various crucial biological functions such as regulation of certain biological processes, excretion and synthesis of various important biological molecules and provides protection from harmful UV rays (Burns et al, 2004). It is made up of a structured system composed of various cell components, which contribute various roles in its essential functions (Burns et al, 2004). The disruption of the continuity of the skin is defined as a wound. This disruption allows the entry of microorganisms to the tissue causing infection (Burns et al, 2004).

Wounds are at risk of bacterial contamination from the surrounding skin, endogenous patient sources and the local environment, which is particularly true for hospitalised patients (Kingsley, 2003). The deposition and multiplication of the microorganisms in wounds can lead to death (Mengesha et al, 2014). A variety of microorganisms can cause wound infections, which include but are not limited to *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* (Mertz and Ovington, 1993). *S. aureus* and *S. epidermidis* are considered the most prevalent causative microorganisms of wound infections (Nicolau and Stein, 2010).
1.5.2 Consequences of wound infections

The cost of healthcare has escalated greatly due to chronic wound infections, which are responsible for considerable morbidity (Siddiqui, 2010). It is estimated that up to 2% of the population in developing countries will experience an episode of a chronic wound in their lifetime (Gottrup, 2004). An infection is a common and most prevalent problem associated with chronic wounds and can be fatal (Siddiqui et al, 2010). If a wound does not heal in 4-6 weeks, it is considered to be a chronic wound (Fowler, 1990; Singh et al, 2004). Another definition for a chronic wound is a wound that has failed to show a 20% to 40% area reduction 2-4 weeks after optimal therapy. However, standard surgical textbooks defined chronic wounds as those that have not healed in a period of 3 months or failure to restore an inadequate functional and anatomic state of the tissue (Bruicardi, 2004).

Diabetes Mellitus, venous stasis and peripheral vascular diseases are related to most common forms of chronic wounds (Siddiqui, 2010). Individuals suffering from Diabetes Mellitus have an increased frequency of wounds due to an elevated susceptibility, which is caused by a hyperglycaemic environment (Qasqueiro, 2012). This condition contributes to immune dysfunction, which leads to a variety of medical conditions (Qasqueiro, 2012). Diabetic foot is presented as deep tissue lesions associated with lower limb peripheral vascular disease and neurological disorders and it is a severe complication in DM patients (Apelqvist, 2012). It is a common and most prevalent problem associated with chronic wounds and results in serious morbidity and mortality of patients (Saddiqui, 2010). In the United States, Diabetic foot ulcers have imposed a substantial burden on private and public payer. A recent study examining the annual cost per patient of diabetic foot ulcers has reported around US $9 billion to $13 billion being added to yearly costs of diabetes management due to ulcer care (Rice et al 2014). The risk for developing foot ulcers could be 25% higher in patients with diabetes and it is also reported that every 30 seconds, one lower limb amputation in diabetes patients occurred around the world (Bakker et al, 2005).
1.5.3 Effect of MRSA infections

Wounds which have resulted from microbial infection have a significant impact on human health and the economic growth. *Staphylococcus aureus* is the most frequently isolated bacterial pathogen in humans and it is a key cause of soft-tissue and skin infections, pneumonia and endovascular infections among others (Lowy, 1998). These *S. aureus* strains have acquired resistance to all available penicillin and various other β-lactam antibiotics. These MRSA isolates were largely confined to hospitals, other various healthcare environments and patients that frequently visit these environments. In the 1990s, however the number of MRSA infections reported exploded for risk factor lacking populations which lack health care system exposure (Adam et al, 2007 and Baggett et al, 2003). This increase has emerged due to the recognition of new strains of MRSA. These new strains have led to an increase in the disease burden that has emerged in the last decade. MRSA infections population- based study done in San Francisco- 2004 to 2005 demonstrated an incidence rate of 316 cases. A 100,000 population with 90% of MRSA infections had onset in the community (Liu et al, 2008). Furthermore, a study by U.S. Centres for Disease Control and Prevention (CDC) estimated that in 2005, there were 31.8 invasive MRSA infections that have been culture-confirmed per 100,000 populations in the United States, giving a total of 94,360 cases in that year. When considering the invasive 7% of culture-confirmed MRSA (Klevens et al, 2007). It can be concluded that a total of greater than 1,300,000 MRSA infections occurred in the United States that year (Klevens et al, 2007).

1.6 Wound Healing

The multilayer structure of the skin, the different cell types and their complex organisation leads to a challenge of healthy and functional skin regeneration (Pereira et al, 2013). Wound healing includes the activation of multiple pathways that are synchronised to respond to the restoration and repair of the skin and its tensile strength (Burns et al, 2004). These processes involve several cell types, cytokines and various components of the extracellular matrix. The first process that occurs is haemostasis which occurs immediately upon wounding (within minutes) and involves platelets which act to seal off damaged blood
vessels by forming a stable clot, secrete vasoconstrictive substances and secrete growth factors which form the basis for the initiation of subsequent healing steps (Burns et al, 2004). The next phase is initiated by the recruitment of neutrophils and monocytes. Products of inflammation then trigger cell migration and proliferation (Burns et al, 2004). Extracellular matrix deposition is accomplished by fibroblasts. Remodelling and wound contraction take place upon closure of the wound. The central processes in wound healing are angiogenesis and re-epithelialisation. Different cells are activated in the process of wound healing and involve activation of keratinocytes, endothelial cells, fibroblasts, platelets and macrophages. Keratinocytes and fibroblasts play a central role in the wound healing process as they are respectively responsible for the production of a wide range of cytokines and growth factors as well as the secretion of extracellular matrix macromolecules (Wiegand and Hippler, 2008). Immune cells secrete metalloproteases which degrade necrotic tissue and these enzymes are regulated by their respective tissue inhibitors called metalloprotease inhibitors (TiMPs) which are produced by cells locally (Burns et al, 2004).

Failure of the normal process of wound healing to occur leads to specific conditions such as chronic wounds which lead to increased risk of infection affecting the patients’ health and overall quality of life (Burns et al, 2004). This failure could be due to extensive skin loss, non-healing ulcers or deep burns which are characterised by normal regeneration process disruption which is usually due a burden of bacterial colonisation, vascular deficiency and diabetes. This leads to a delayed and complicated healing process (Enoch and Leaper, 2007; van der Plas et al, 2010).

In chronic wounds healing is stalled due to suppression of cell division and migration, increased levels of metalloproteases and inflammatory cytokines (Burns et al, 2004). There is also a down regulation of TiMPs and growth factors. The main characteristic of a chronic inflammatory state is cell senescence and unresponsive state to growth factors (Burns et al, 2004). The occurrence of wound infections leads to complications in illnesses which include anxiety, increased discomfort of the patient which can ultimately lead to death. Surgical wounds can lead to increased hospitalisation by up to 10 days (Collier, 2004). The management and prevention of wounds has the potential to greatly impact the patient and health economics (Collier, 2004). An ideal wound healing product is one that can induce anti-inflammatory responses and can advance keratinocyte and fibroblast proliferation and migration, collagen synthesis, angiogenesis and possess anti-microbial activity.

http://etd.uwc.ac.za/
Plants have a variety of active compounds that could have anti-microbial activity or through activation of processes that promote cell proliferation and can reduce gold salt to form AuNPs which could have anti-microbial activity. Both the plant and the AuNP produced from the plant have the potential to produce an antimicrobial agent that can either speed up healing or kill infectious bacteria in the wound.

1.7 Antibacterial and wound healing properties of plants

There have been a lot of advances in wound care products that include medicated dressings and cellular tissue-engineered skin substitutes which lead to an increase in medical costs (Pereira et al, 2013). Although various developments have been made in wound management, there are still limited treatment options and the search for effective and affordable treatment options is still under investigation (Pereira et al, 2013).

An increased potential has been recognised in traditional therapies used for alternative clinical wound treatment (Pereira et al, 2013). Many plant derived biologically active constituents have shown potential to exert antimicrobial effects and activate cell proliferation which can be used in the treatment of wounds (Adetutu et al, 2011; Burns et al, 2004; Sharma, et al 2015). Several plant derived secondary metabolites have been identified and demonstrated to be responsible for the plant induced antimicrobial activity. Various plant derived compounds have been studied in the attempt to produce plant based potent antibiotics. These plant based antibiotics could be conjugated to AuNPs to enhance their specificity, accumulated concentration at target site leading to increased activity. An example is the plant alkaloid berberine which has been isolated in several extracts (Ball et al, 2006).

1.8 Drawbacks of conventional methods of AuNP synthesis

The conventional methods of synthesis of AuNPs are capital and energy intensive and involve various toxic chemicals which can hinder the biocompatibility of AuNPs and their efficacy in biomedical application (Mollick et al, 2015). The undesirable processes in the synthesis of nanoparticles, high energy consumption and the use of toxic solvents and hazardous by-products will end up in the production of nanoparticles with surface imperfections (Shanmugam, 2014 and Mollick et al, 2015). The safety of gold nanoparticles is still a controversial matter which may affect their potential application (Fratoddi et al, 2015).
Conflicting results have been obtained in this regard with some studies revealing AuNPs are toxic and others suggesting AuNPs are not toxic (Fratoddi et al, 2015). Many factors must be considered when studying the toxicity of a substance and with nanomaterials it is even more complicated. Nanoparticle accumulation in endocytic compartments has been observed by intracellular analysis which revealed undesirable cytokine secretion changes which confirms toxicity (Fratoddi et al, 2015).

The same physico-chemical properties that make nanoparticles suitable for biomedical applications such as size, surface charge and shape are the same properties that are responsible for their cytotoxicity (Rastogi et al, 2012; Fadeel and Garcia-Bennett, 2010). One of the most important parameters that plays a role in both the cellular uptake and function of the nanoparticle is the surface charge (Yah, 2013). Gold nanoparticles are either negatively or positively charged, which leads to their high reactive nature that influence their cytotoxicity (Frattoddi et al, 2014). Irreversible interaction of gold nanoparticles with DNA was found to cause cytotoxicity (Goodman et al, 2004). Various studies state that surface functionalisation is responsible for the cytotoxicity of gold nanoparticle (Fratoddi et al, 2014). Nanoparticle accumulation was found to alter cytokine secretion and selectively inhibit T-lymphocyte to stimulate cytokines for the induction of inflammation (Villiers et al, 2010). These observations show the importance of obtaining safer nanoparticles for biomedical applications. It is important for nanoscience researchers to obtain nanoparticles with desired structural properties for biomedical applications. The development of suitable synthetic routes for the design and synthesis of these nanoparticles is important for optimal functioning (Shah et al, 2014).

In order to safely use nanoparticles and benefit from their full potential in different applications we need to fully understand their properties, respective interactions and effects thereof in biological environments (Walczyk et al, 2010). The physiological conditions of the biological environment, colloidal stability and surface charge are some of the factors that strongly influence these interactions. This situation becomes even more complex in various compartments of cells and organisms as deviated by changes in factors such as ionic strength, temperature, pH and also considering biomolecule composition which when individual affects the nanoparticles differently than when combined (Bertoli et al, 2014). When nanoparticles enter the biological environment they immediately
encounter and are inevitably covered by a protein corona altering the physicochemical identity of the nanoparticle which can be lead to changes in the aggregation state, surface charge and hydrodynamic size. This newly acquired identity of the nanoparticles through interaction with surrounding biomolecules and membranes in the biological environment is what determines the fate of the nanoparticles in biological environments (Lynch and Dawson, 2008; Yan et al, 2013 and Bertoli et al, 2014).

An understanding of these dynamics will not only advance the applicability of nanoparticles in life sciences but will also give understanding of their long term impact in biology and environment through foreseeing their fate (Kelly et al, 2015). Studies that seek this understanding are already being undertaken in various research endeavors such as a study done by Dewald et al, (2015) which investigated these interactions of nanoparticles with proteins and therefore describe and explain the protein corona and their final physicochemical properties, in other words the final nanoparticle identity post exposure to the protein corona. Protein identity also plays a significant role in the study of the protein corona. Intrinsic properties of proteins differ from protein to protein depending on their biological function. The environmental conditions such as ionic strength, pH and temperature significantly affect the charge of both proteins and NPs, and therefore play a significant role in the NP and protein electrostatic interaction (Dewald, 2015).

1.9 Advantages of nanotechnology

Nanotechnology brings advantages such as obtaining nanoplatforms specifically designed to obtain an ideal therapeutic agent under ideal conditions for a specific condition (Vasir et al, 2005). This includes ideal conditions such as prolonged drug circulation time and maintaining the desired drug concentration at target site and no loss of activity and therapeutic efficacy during circulation (Vasir et al, 2005). The higher accumulation of nanodrugs at target site is largely due to their size compared to normal drugs, in addition to this, an enhanced permeability and retention effect of the nanoplatforms which is allowed by the impaired lymphatic drainage and increased vascular permeability in tumors or inflamed tissues also leads to increased concentrations at target sites (Maeda et al, 2000, Matsumura and Maeda; 1986).
This pathophysiological opportunity allows selective localization leading to increased activity and increased efficacy at target site of tumors and inflamed tissue (Allen and Cullis, 2004).

Passive targeting of nanosystems to macrophages in the spleen and liver can be achieved due to tendency of these nanosystems being trapped in the reticuloendothelial system presenting an excellent opportunity for the passive localization to the target sites which would be in this case the spleen and liver (Vasir et al, 2005). This natural system presents a great opportunity for targeting drugs of intracellular infections as well (Vasir et al, 2005). The blood-brain barrier is one limiting factor in the treatment of most neurological disorders and nanoplatforms. AuNPs present a way of overcoming this barrier through successful penetration of this brain segregation barrier from blood circulation. This allows successful delivery of many effective drugs to the target sites in the brain while reducing certain toxicity potential. This could be achieved by specific targeting and localization of loaded drugs and controlled accumulated concentration at target site (Calvo et al, 2001; Pardridge, 1999, Garcia-Garcia et al, 2005).

1.10 Green nanotechnology

Green nanotechnology is a relatively new research field directed at advancing nanotechnology by making it more eco-friendly through green chemistry which avoids the use of hazardous substances in the synthesis process through the use of materials that are less or non-toxic to humans and the environment (Walker, 2013). It has other advantages such as the use of less energy in the manufacturing process and an added advantage of decreased reaction time leading to a faster nanoparticle manufacturing process (Walker, 2013). The strong antioxidant capacity of phytochemicals can be utilised to convert gold precursors into their respective nanoparticles (Shukla et al, 2012). Plants are the most favourable source of reducing and stabilising agents in green nanotechnology due to characteristics such as the fact that they are easily available, inexpensive and hold great promise due to their diversity and potency (Nath and Benerjee, 2013).
Green chemistry however has been a widely considered alternative for the synthesis of nanoparticles giving rise to green nanotechnology (Faheem and Hussaina, 2015). Various plants and plant extracts have been used in the synthesis of these metallic nanoparticles due to the bioactivity and non-toxicity of their phytochemicals giving rise to green nanotechnology (Pantidos and Horsfall, 2014).

1.11 AuNPs synthesis from plants

Eco-friendly technologies have been under focus to develop herbal-based products in an effort to advance health care solutions (Chauhan et al, 2012). Stable dispersions of gold nanoparticles have been synthesised from various herbs and medicinal plant extracts (Sadowski, 2010). The resultant gold nanoparticles possess large surface to volume ratio and well-defined surface chemistry and chemical stability (Islam et al, 2015). These characteristics make it possible and favourable for a variety of drug moieties to be adsorbed on to the surface of the nanoparticles (Islam et al, 2015).

Willow plant is one example of a medicinal plant that has been used in green synthesis of gold nanoparticles. It has been used traditionally for its anti-inflammatory properties amongst its other medicinal properties (Mahdi et al, 2006). Gold nanoparticles synthesised from this plant have revealed the potential of this medicinal plant as a bio-reductant for gold nanoparticle synthesis, producing gold nanoparticles that have potential applications in the biomedical and pharmaceutical fields (Islam et al, 2015). An example of their potential use in the biomedical field has been reported in amino acid recognition and monitoring as colorimetric sensors (Islam et al, 2015).
Gold nanoparticles synthesised from various medicinal plants have the potential to replacing a variety of clinical constructs used in the management of various diseases. Benefits of using gold nanoparticles include increased therapeutic effect of the plant extract, a reduction in required dose and sustained release of the functional components. Versatility of the monolayer of AuNPs makes it appropriate in contributing to controlled release of the drug (Duncan, 2010). An example of this is photo responsive triggered release of a photo cleavable external stimulus (Nakanishi et al, 2009). This approach has been used for the regulation of a drug, engineering it in a manner that leads to activation of the drug only occurring when required (Nakanishi et al; 2009). An example of this approach is the use of amines in a photocleavable nanocarrier where near infrared UV irradiation is used to dissociate a carbamate linkage by the reaction of 2 –nitrobenzyl group (Nakanishi et al, 2009). This leads to increased activity and decreased side effects (Bonifa’cio and da Silva, 2014). Green nanotechnology contributes to the improvement of drug delivery system which will enhance the bioavailability of herbal drugs and contributes to the development of biological medicine (Bhadoriya et al, 2011).

Phenolic groups in the plant extract have been observed to play an important role in bio-reduction and the formation of metallic nanoparticles which are the ones that are most prevalent in the medicinal functioning of the plant (Islam et al, 2015). This has been observed in the Willow plant as its major phenolic content (salicin) which plays an important role in the production of aspirin as it is a precursor of salicylic acid has been shown to have an important role in the metal ion reduction and stabilisation of the nanoparticles (Harbourne et al, 2007, Islam et al, 2015). Salicin Electrostatic attraction between the nanoparticles and the drugs is possible through their amine groups and this leads to the drug molecules being adsorbed on the surface of the nanoparticles (Islam et al, 2015). The combination of the nanoparticle with the drug moieties has the potential of a combinatorial effect in the specific application (Burygin et al, 2009). It has been observed that bio-friendly nanoparticles have the comparable functional activity as the plant extract responsible for the bio-reduction. The advantage of nanoparticles is that it can do this at lower doses (Islam et al, 2015).
1.12 Plants as antimicrobial agents

Plants are one of the main sources of the natural products and are screened extensively for new antimicrobial agents (Berdy, 2005). A wide range of antimicrobial agents with differing complexity in structure and chemical constituents has been obtained from plants (Runyoro, 2016). South Africa has a wide variety of medicinal plant species, which form the basis of various support systems in the human health sector (Gurib-Fakim, 2010). Higher plant diversity of South Africa consists of approximately 3000 plant species, which have medicinal applications. Only about 350 plant species, which are traded as medicines have undergone chemical investigations (Van Wyk et al, 1997). South Africa’s plant diversity has attracted great interest in bio-prospecting as it contributes to approximately 10% of the world’s higher plant species. These plants have been actively explored for the presence of pharmacologically active compounds (Geldehuys and Mitchell, 2006, Rybicki et al, 2012). However, more research into their medicinal properties is needed to take full advantage of their potential (Eloff, 1998).

1.13 Phytochemical potential in South Africa: Cape Floral Kingdom

Many studies showed that South African medicinal plants possess a variety of complex compounds, which may be useful in the treatment of several diseases (Wiersum et al, 2006). This variety of complex compounds, which include, polyphenols, alkaloids, sugars, terpenoids and phenolic acids (Wiersum et al, 2006) can be ascribed to biodiversity of the plants. These phytochemical constituents differ from one plant to another and this diversity leads to a diversity of biological activities (Wiersum et al, 2006). The flora of the South Western Cape, also termed, The Cape Floral Kingdom is one of the richest regions in species and plant biodiversity in the world (Manning et al, 2012). This is one of the six floral kingdoms of the world, which reveals its sufficient distinctiveness. The Cape Floral Kingdom is the smallest floral kingdom, but contain about 4% of the world’s plant species. This flora
is sharply different from areas surrounding it, which has been an exciting feature to plant scientists (Manning et al, 2012).

Diversity of the habitats of the plants is influenced by the climatic variations which have its effects amplified by the steepness of the topography and its varied landscape. Due to this unique feature, the plants in the Cape Floral Kingdom are highly diverse and it can be expected that the phytochemistry of these plants are equally diverse. This holds great potential for the discovery of new chemical entities for exploitation in the development of new drugs. Additionally, these diverse phytochemicals can be exploited in green nanotechnology to produce a myriad of nanomaterials with useful applications in medicine.

### 1.14 Aims and Objectives of Study

This study investigated various indigenous South African plants randomly selected from the University of the Western Cape Nature Reserve, which also forms part of the impressive Cape Floral Kingdom. This study was aimed at determining the ability of the plants to synthesise AuNPs and evaluate their antibacterial activity. The plants used in this study were *Perlagonium capitatum*, *Otholobium bracteolatum*, *Gerbera linnae*, *Morrella quercifolia*, *Searsia lucida*, *Phylica bubescens*, *Euclea racemosa*, *Tetragonia fruticosa*, and *Searsia glauca*. The objectives of this study were to screen the plants for synthesis of AuNPs, characterise the AuNPs and evaluate the antibacterial activity of the AuNPs and plant extracts. Not much is known about the traditional uses of most of these plants, however, prior knowledge of traditional uses of these plants was not a factor that was considered in selecting the plants.
Chapter Two: Methods and materials

2.1 Materials and suppliers

Dulbecco’s Modified Eagles Media (DMEM)  
Whitehead Scientific

Fetal Bovine Serum (FBS)  
Biochrome

Penicillin/streptomycin  
Lonza - Germany

Gold salt (Sodium tetrachloroaurate (III) dehydrate (AuCl4H4NaO2))  
Sigma-Aldrich

Nutrient Broth (biolab)  
Merck

Mueller- Hinton Agar (biolab)  
Merck

Ampicillin  
Sigma

Kanamycin Sulfate  
Roche

2.2 Plant collection and extract preparation

Nine plants were randomly collected from the University of the Western Cape Nature reserve, Cape Town, South Africa in October 2015 and January, 2016. These plants were later identified through submitting a herbarium to a taxonomist (Dr C.N Cupido) as observed in figure 1. Upon collection the fresh whole plants were dried in the shade for two weeks and grounded whole. The ground whole plant material was sealed and stored at 4°C till further use. A fixed amount of 5g of dried plant sample was placed in 50ml boiled distilled water (dH2O) for 10min with occasional stirring. The solution was centrifuged at 3750 rpm for 1 hr to remove solids. The supernatant was frozen overnight at -80 °C and then lyophilised using a Free Zone 2.5L lyophilised (Labconco, USA) to obtain dry
mass of the plant extract. A working stock at a concentration of 10 mg/ml plant extract was prepared in dH$_2$O and stored at -20°C.

Figure 1: South African indigenous plants used for AuNP synthesis.

2.3 Screening plant extracts for AuNP synthesis

A 96 well plate was used to screen the plant extracts for their potential to reduce the Sodium tetrachloroaurate (III) dehydrate (AuCl$_4$H$_4$NaO$_2$) to AuNPs. AuCl$_4$H$_4$NaO$_2$ of a concentration of 1mM was added to different concentrations (2.5 mg/ml to 10μg/ml) of plant extract. This range of concentrations were obtained by performing a serial dilution in dH$_2$O. Nanoparticle formation was studied at two different temperatures (25°C and 70°C). Samples were incubated for 1 hour with constant mixing at 40 rpm. Colour change was observed as confirmation of synthesis of the nanoparticles. The measurement of the AuNP SPR made it possible for UV-Vis spectra (300-800 nm) to be determined using a POLAR star Omega microplate reader (BMG Labtech, Germany). AuNP synthesis was scaled up for further AuNP characterisation and evaluation of biological activity. The same concentration chosen for the AuNPs was maintained in all subsequent synthesis reactions.
2.4 Determining the optimum concentration of plant extract for AuNP synthesis

The same method used to synthesise nanoparticles was used to determine the optimum concentration (OC). The plant extract concentration with the most desirable UV-Vis spectra was chosen as the OC (chosen based on observation of the shape and position of maximum absorbance, generally a peak that is better defined and shows smallest size with maximum OD and preferably shows less anisotropy as observed in the shape of the tail near the infrared region of the UV-Vis spectra was chosen to represent the OC). This was obtained post AuNP synthesis through comparison to AuNPs synthesised at other concentrations of the same plant extract. Both temperature conditions (25°C and 70°C) were explored to obtain an OC at each temperature condition.

2.5 Analysis and Characterisation of AuNPs

2.5.1 Dynamic light scattering (DLS)

DLS was used to determine the nanoparticle hydrodynamic size and charge. This was done using a Zetasizer (Nano-ZS90 system, Malvern Inc.). After synthesis the nanoparticle pellet was centrifuged at 10 000 g for 10 min (repeated 3 times) to isolate and concentrate the nanoparticles. The nanoparticle pellet was suspended in 1 ml dH₂O in a DLS cuvette. The DLS (also known as photon correlation spectroscopy) was further used to determine Zeta Potential AuNPs. Water was the dispersant used and measurements were carried out in triplicates. The size was calculated using a Stokes–Einstein equation incorporated within the Zetasizer software version 7.11.

2.5.2 Transmission electron microscopy (TEM)

Surface morphology which includes crystallographic information, shape and core size of the bio-friendly AuNPs was performed using TEM analysis on a FEI Tecnai G² 20 field-emission gun emission transmission electron microscope (FEG-TEM). The carbon coated copper grid
was drop coated with the sample solution and further dried under a Xenon lamp for 10min. The TEM analysis of the sample coated grids was done with a TEM fitted with a CCD camera and micrographs operated in bright field mode at an accelerating voltage of 200kV. The SAED pattern of the nine AuNPs was also generated automatically using the FEI Tecnai G^2 20 field-emission gun. Analysis of elemental composition of the AuNPs was done by observation of EDX- spectra of the sample. EDX spectra were collected using EDAX liquid nitrogen cooled Lithium doped silicon detector.

2.5.3 Analysis of AuNP stability

The stability of the synthesised nanoparticles in biological media which include DMEM (Supplemented with 10% FBS) and Nutrient broth was determined by incubating the AuNPs in the biological media for a period of 24 hrs. The stability of the AuNPs was observed by evaluating UV-Vis spectra POLAR star Omega microplate reader (BMG Labtech, Germany) of different time intervals (after 2, 4, 16, 12, 24 hrs).

2.6 Determination of the concentration of nanoparticles

In order to determine nanoparticle concentration, a specific volume (e.g 1ml) of the nanoparticle solution obtained upon synthesis. The nanoparticle solution was centrifuged as stated in section 2.5.1 to remove all excess plant extract and unreacted gold ions. The concentrated nanoparticle solution was frozen by liquid nitrogen and then lyophilised with a Free Zone 2.5L lyophilised (Labconco, USA) to obtain dry mass of the AuNPs. The concentration of the AuNPs was then expressed as the weight per ml. This was done as an alternative to using spectrophotometric method due to the anisotropy of the AuNPs.
2.7 Antibacterial evaluation

Three most reactive plants in AuNP synthesis as observed in the rate of reaction of AuNP synthesis and potency were chosen which are *P. capitatum*, *M. quercifolia* and *S. glauca*. The antibacterial activity was studied against *Staphylococcus aureus* [ATCC-29213] and Methicillin resistant *Staphylococcus aureus* (MRSA) (ATCC-33591). The bacteria was grown overnight for 24 hours in a shaking incubator at 37°C. The bacterial culture of each strain was adjusted to 0.5 McFarland standards and further diluted to maintain a final bacterial population of $5 \times 10^{-6}$ CFU.

2.8 Determination of the bactericidal effect of AuNPs

The micro well plates were incubated at 37°C for 24 hrs and growth was evaluated by comparing the optical density (OD) of the untreated and treated samples. The OD was read using a POLAR star Omega microplate reader (BMG Labtech, Germany) at 450 nm. Indication of bacterial growth was a reduction in the OD value.

The bacterial cultures (*S.aureus* and MRSA) dispensed into a 96 well plate. Each well contained 50μl of the culture. The plant extract was serially (30 μg/μl to 1000 μg/μl) diluted in nutrient broth and 50μl of each dilution was added to the bacteria culture. All treatments were done in triplicate. The AuNPs were similarly diluted (6-400 μg/μl) in nutrient broth and 50μl was added to the bacterial culture. For AuNPs synthesized from *P. capitatum* however, the AuNPs serial dilution was 20-1280 μg/μl in nutrient broth. As positive controls for *S.aureus* and *S.aureus* (MRSA), the bacterial cultures were tested with 50μg/μl of ampicillin and 100 μg/μl of kanamycin, respectively.
2.9 Statistical analysis

Statistical analysis for dose- responses of each AuNP and respective plant extract against bacterial strains under investigation was carried out. The statistical comparisons among groups were done using GraphPad Prism 6 by means of a Dunnett’s multiple comparisons test. The P-value was calculated from three independent experiments using their means and standard deviation.
Chapter Three: Results and Discussion

3.1 Introduction

Nine plants namely; *P. capitatum*, *O. bracteolatum*, *G. linnae*, *M. quercifolia*, *S. lucida*, *P. bubescens*, *E. racemosa*, *T. fruticosa*, and *S. glauca* were collected from the UWC nature reserve. Water soluble compounds were extracted from these plants using water extraction as described in section 2.2. The plant extracts were used to synthesise AuNPs at two different temperature conditions namely 25°C and 70°C following the method stated in section 2.2. All the plant extracts were able to reduce Sodium tetrachloroaurate (III) dihydrate (NaAuCl$_4$. 2H$_2$O) to form AuNPs at various extract concentrations. Several studies reported the synthesis of AuNPs from several plant extracts using this procedure (Elbagory et al, 2016; Geethalakshmi et al, 2012 and Pasca et al, 2014). Nanoparticle formation depends on several factors, which include the nature of the plant extracts used in the synthesis of the nanoparticles. Plants differ in their reducing potential due to the presence of bioactive phytochemical content (Dzimatrowicks, 2016).

3.2 Screening of plant extracts for AuNP synthesis

The formation of the AuNPs is indicated by a colour change from yellow to ruby red. The phenomenon that is responsible for this observation is called SPR, which is unique to metal nanoparticles and gives a characteristic band in the UV spectrum (Jensen et al, 1999). In this study different concentrations of the plant extracts were mixed with 1mM of NaAuCl$_4$. 2H$_2$O. A colour change was observed suggesting the formation of AuNPs synthesised from all the plant extracts after 1 hr of incubation at 25°C and 70°C using microtiter plate method described by Elbagory et al, 2016.
3.2.1 Determining the optimum concentration (OC) of the plant extracts for AuNP synthesis

UV-Vis was used to confirm the presence of AuNPs at the two temperature conditions (25°C and 70°C). All the plants were able to produce AuNPs at both temperature conditions. Following the screening step, an optical concentration (OC) for each plant extract was determined based on the characteristics of the UV-Vis spectra such as the shape (narrow or broad), sharpness, the wavelength of the absorption band and absorption in the near infrared region (NIR) region. Based on this, the desire characteristics for uniform AuNPs include a sharp absorption band at a wavelength between 500 and 600, (with an absorption peak between 500 and 550 nm) and the absence of an absorption band in the NIR region of the spectrum. The selection of the OC was also determined based on the Dynamic Light Scattering (DLS) analysis. The desired OC should produce AuNPs with an average diameter below 100 nm. Fig 1 shows the UV-Vis absorption spectra for AuNPs synthesised from *P. capitatum* using increasing concentrations of the extract. In this example 6.4 μg/μl was selected as the OC since the absorption band meeets best the desired characteristics. The absorption band appears to be sharp and no absorption bands are present in the NIR region. In contrast, at a concentration of 51.2 mg/ml produced a broad absorption band between 600 and 775 nm which indicates a probability of high anisotropy in shape and/or size. At concentrations of 12.8 mg/ml and 25.6 mg/ml absorption bands were produced between 525 and 700nm also suggesting a probability of larger and anisotropic AuNPs when compared to the selected concentration of 6.4 mg/ml.
The OC differed from one plant to another. This could be due to differences in chemical composition of the plants as suggested by Dzimatrowicks, 2016. It is worth noting that the conditions of the AuNP synthesis reaction and prior extract production method was standardised and kept constant for all nine plants. Colour change of the synthesis reaction for AuNP AuNPs synthesised from *P. capitatum* was observed within 2 minutes, while those synthesised from *S. glauca* and *M. Quercifolia* showed instant colour change of the AuNP synthesis reaction upon addition of reagents in the reaction mixture. The overlays of the UV-Vis spectra of the other plants are not shown but the same analysis was performed and is summarised in table 1. The UV-Vis spectra of the OC for all plants are shown in fig 2. It is possible that synthesis is much faster at 70°C and that reactions at 25°C requires more time. It is also possible that temperature dependant modification of certain phytochemicals occurred at 70°C which favoured NP formation. It is therefore also possible the extract of *O. bracteolatum* contain compounds that are more sensitive to temperature and therefore requires higher conditions of the extract. It was observed that the OC was generally lower at the higher temperature (70°C) compared to the lower temperature (25°C) as can be observed in Table 1 with one exception in the case of *O. bracteolatum*. This exception could be due to the presence of temperature sensitive phytochemicals in *O. bracteolatum* giving rise to low synthesis efficiency at the higher temperature (70°C) than at...
the lower temperature (25°C). Also, the synthesis of AuNPs from *S. glauca* was done at low concentration (as shown in Table 1), which indicate the strong reactivity of this plant’s phytochemical contents.

Table 1: Optimum concentrations (OC) of the plant extracts used for AuNP synthesis.

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Conc.(mg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>25°C</td>
</tr>
<tr>
<td><em>Peragonium capitatum</em></td>
<td>6.4</td>
</tr>
<tr>
<td><em>Searsia glauca</em></td>
<td>1.6</td>
</tr>
<tr>
<td><em>Morrella quercifolia</em></td>
<td>3.2</td>
</tr>
<tr>
<td><em>Euclea racemosa</em></td>
<td>1.6</td>
</tr>
<tr>
<td><em>Otholobium bracteleobium</em></td>
<td>3.2</td>
</tr>
<tr>
<td><em>Phyllica bubescens</em></td>
<td>12.8</td>
</tr>
<tr>
<td><em>Gerbera linnae</em></td>
<td>1.6</td>
</tr>
<tr>
<td><em>Searisa lucida</em></td>
<td>6.4</td>
</tr>
<tr>
<td><em>Tertragonia fruticosa</em></td>
<td>12.8</td>
</tr>
</tbody>
</table>
3.3 Characterisation of the AuNPs

3.3.1 UV-Vis spectroscopy analysis

UV-Vis is used to verify the presence of AuNPs as shown above but it can also be used to estimate the size, shape and concentration of the AuNPs by observing the optical intensity. This observation is made possible by the SPR effect mentioned above giving rise to an absorption band at a wavelength ranging from 500 to 600 nm (Rastogi et al, 2012 and Oldenburg et al, 1998). UV spectroscopy works based on the the Beer-Lambert law, which states: when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution.
The resultant AuNPs from the OC of each plant extract were chosen for further study and their UV-vis spectra results are discussed above (Figure 2). The UV-Vis spectra of the AuNPs are affected by various factors including, the size, shape and concentration of the nanoparticle (Guo et al, 2015). The synthesis at higher temperature (70°C) was observed to increase reaction efficiency as sharper Plasmon bands are observed with a blue shift (indicated by decrease in λ max) compared to the synthesis at room temperature (25°C). A few exceptions were observed with the AuNPs synthesized from *M. quercifolia* and *S. lucida* (Figure 3: D and E, respectively) that show a red shift at room temperature (indicated by increase in λ max), which could be due to the presence of heat sensitive capping agents in those extracts that were ineffective at higher temperature and led to formation of less anisotropic AuNPs at room temperature (Figure 3). This temperature dependant correlation was also reported in various other studies (Elbagory, 2016; Abdelhalim et al, 2012; Saifuddin et al, 2009). This observation may suggest the generation of smaller and more uniform nanoparticles at higher synthesis temperature (Elbagory et al, 2016 and Mountrichas et al, 2016). AuNPs synthesised from *P. capitatum* and *M. quercifolia* (Figure 3: A and E) are more anisotropic at synthesis temperature condition of 70°C compared to 25°C based on the appearance of absorption band at the NIR region. AuNPs synthesised from *S. lucida* (Figure 2: I) are more anisotropic at 70°C based on the broadness of the absorption band at 70°C compared to 25°C.

Moreover, the reaction at 70°C led to a higher yield of the AuNPs as demonstrated by a higher optical intensity (Figure 3). This correlation to a higher yield is according to Beer Lambert’s Law, which states that optical intensity is directly proportional to concentration of the AuNPs. The lower yield at 25°C could be due to the lower reaction efficiency at this temperature as the chemical reaction could be slower resulting in decreased yield. Elbagory et al (2016) suggested that the reaction may need a higher temperature for the initiation of the reduction process and/or a longer incubation time depending on the type of the phytochemicals present in each extract.

Exceptions to the above were *E. racemosa*, *S. glauca* and *T. fruticosa* extracts that produced AuNPs with higher yield at lower temperature (25°C). This could be due to
the unique properties of the phytochemicals present in these plants, which react differently with the gold salt under the specific conditions (Figure 3). It could also be postulated that the destruction of some heat sensitive phytochemicals occurs at the higher temperature (70°C) which affects the AuNP yield for the aforementioned plants (Dzimatrowicks, 2016).

Further, the UV-Vis spectra, of some AuNPs, showed the presence of an absorption tail in the NIR at higher temperature (70°C) such as in the case of AuNPs synthesised from *P. capitatum*, *P. bubescens* and *E. racemosa* (Figure 3: A, E and F). This is attributed to the in-plane SPR excitation giving rise to this absorption tail in the longer wavelength region (Elbagory et al, 2016). This may indicate the anisotropic nature of the resultant AuNPs synthesised during this reaction condition (Narayanan and Sakthivel, 2008), or the formation of nanoparticle aggregates as shown in Figure 3: A and E (Shipway et al, 2000). The diversity of the chemical composition and their respective concentrations included in each plant extract may explain the production of AuNPs with different physico-chemical properties.

### 3.3.2 Dynamic Light Scattering (DLS) analysis of the AuNPs

The size of the nanoparticle remains the most important parameter as the chemical and physical characteristics of the nanoparticle associated with their function are dependent on it (Lim et al, 2013). One of the important features of AuNPs is their surface to volume ratio, which is inversely proportional to the diameter of the nanoparticle. The smaller the nanoparticle, the larger its surface area and therefore the more the binding sites for various applications such as drug and gene delivery (Lim et al, 2013).

DLS was used to determine the characteristics of the synthesised AuNPs such as size (average diameter), polydispersity index (PDI) and Zeta potential (ZP), using a Nano-ZS90 Zetasizer system as stated in section 2.5.2. The DLS analyses temporal fluctuations through a photon or intensity or photon auto-correlation function. Table 2 below shows the DLS results of the AuNPs synthesised from the selected plant extracts. The hydrodynamic diameter of the AuNPs was calculated by the Stokes-Einstein equation where water was used as a continuous phase.
The effects caused by the different reaction conditions selected in this study were assessed by DLS to observe the influence of these effects on the size of the resultant AuNPs. The changes were observed to be unique to each plant extract from which the AuNPs have been produced.

In this study, *E. racemosa* gave the smallest average diameter (41.52 nm) at 25°C, while *S. glauca* gave the smallest average nanoparticle diameter at 70°C with 28.97 nm. This could be due to the higher content of capping agents in *E. racemosa* that leads to sufficient reaction efficiency at room temperature. The reaction efficiency is highly increased at the elevated temperature of 70°C for *S. glauca*, which could indicate that the high temperature favours the reaction conditions of AuNP synthesis in the case of this plant. The DLS data show that AuNPs had an overall average diameter of 63.8 nm at 25°C and 56.9 at 70°C. Similar results were observed in a study by Elbagory et al (2016). Nanoparticle aggregates contributes less to this average size as DLS is very sensitive towards the formation of aggregates. This feature decreases the occurrence of erroneous measurements (Lim et al; 2013).

On the other hand, *S. lucida* gave the largest nanoparticles compared to the other plants for both temperature conditions (25°C and 70°C) under study. This could be due to the presence of less active phytochemicals for AuNP synthesis or that the phytochemicals present in the extract require longer period of synthesis for efficient capping to occur. Another reason could be that the higher temperature (70°C) negatively affecting the capping reaction of the resultant AuNPs.
Table 2: Dynamic light scattering (DLS) of AuNPs synthesised from the tested plants.

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Average diameter (nm)</th>
<th>PDI 25°C</th>
<th>PDI 70°C</th>
<th>Zeta Potential (mV) 25°C</th>
<th>Zeta Potential (mV) 70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Perlagonium capitatum</em></td>
<td>62.19</td>
<td>0.397</td>
<td>0.446</td>
<td>-17.6</td>
<td>-3.63</td>
</tr>
<tr>
<td><em>Seasia glauca</em></td>
<td>48.14</td>
<td>0.315</td>
<td>0.581</td>
<td>-3.88</td>
<td>-14.5</td>
</tr>
<tr>
<td><em>Morrelia quercifolia</em></td>
<td>49.21</td>
<td>0.448</td>
<td>0.447</td>
<td>-4.95</td>
<td>-12.9</td>
</tr>
<tr>
<td><em>Euclea racemosa</em></td>
<td>41.52</td>
<td>0.397</td>
<td>0.321</td>
<td>-8.58</td>
<td>-6.85</td>
</tr>
<tr>
<td><em>Otholobium bracteolabium</em></td>
<td>54.0</td>
<td>0.539</td>
<td>0.598</td>
<td>-20.7</td>
<td>-0.885</td>
</tr>
<tr>
<td><em>Physica bubescens</em></td>
<td>76.60</td>
<td>0.567</td>
<td>0.375</td>
<td>-9.26</td>
<td>-10.5</td>
</tr>
<tr>
<td><em>Gerbera linnae</em></td>
<td>78.9</td>
<td>0.391</td>
<td>0.407</td>
<td>-14.1</td>
<td>-13.5</td>
</tr>
<tr>
<td><em>Searisa lucida</em></td>
<td>84.67</td>
<td>0.487</td>
<td>0.493</td>
<td>-18.7</td>
<td>-16.6</td>
</tr>
<tr>
<td><em>Tertragonia fruticosa</em></td>
<td>78.64</td>
<td>0.669</td>
<td>0.3</td>
<td>-18</td>
<td>-9.11</td>
</tr>
</tbody>
</table>

The Polydispersity index (PDI) measures the molecular mass distribution of the synthesised nanoparticles. No specific pattern was observed for the PDI values in Table 2 as some AuNPs had higher PDI at room temperature, while other AuNPs had higher PDI at 70°C. Moreover, some AuNPs had insignificant differences at the different temperature conditions (Table 2). This could also be due to the heterogeneity of the chemical components of the plants hence their sensitivity to temperature changes.

The ZP, which is a measurement of the average net surface charge of the nanoparticles, was also variable among the AuNPs synthesised from the tested plants, which could also be due to the same reasons as stated for the PDI above. ZP was observed to be negative for all the synthesised AuNPs, which corresponds to a negative surface charge of the nanoparticles. A negative surface charge leads to increased stability of the nanoparticles through columbia repulsion forces that prevent the aggregation of the nanoparticles (Gannimani et al, 2014; Rao et al, 2013).
3.3.3 Transmission Electron Microscopy (TEM) and Energy Dispersive X-ray spectroscopy (EDX) analysis of the AuNPs

Transmission electron microscopy (TEM) was used to determine the morphology of the synthesised AuNPs. This was carried out using TEM analysis on a FEI Tecnai G² 20 field emission transmission electron microscope (FEG-TEM) as stated in section 2.5.3. TEM is a microscopy technique which uses a beam of electrons that is transmitted through a specimen forming an image. The TEM images of AuNPs synthesised from the 9 plants were of various shapes and sizes (Figure 3). Shapes which included spheres, rods, pentagons, hexagons and triangles were observed among the synthesised AuNPs. Spheres and pentagons were more dominant (Table 3). This is in line with other studies that also reported this diversity in the geometrical shapes of green synthesised AuNPs (Chen et al, 2010 and Elbagory et al, 2016). This anisotropic nature of the nanoparticles as determined by TEM, confirms the analysis done by UV-Vis which suggested that AuNPs synthesised from *P.Capitatum*, *S. glauca* and *S.lucida* are likely to be anisotropic in shape and/or size. AuNPs synthesised from *P.Capitatum*, *M.quecifolia* at 25°C and those synthesised from *E.racemosa* at 70°C were more uniform in shape and size and this observation is in agreement with the UV-Vis analysis (Figure 2).

The TEM analysis can give an insight on the polydispersity of the synthesised nanoparticles. For instance, AuNPs from *S. glauca* showed more uniform nanoparticles at 25°C, while the AuNPs of *T. fruticosa* were more uniform at 70°C. This was also in agreement to the PDI results shown in Table 2. The AuNPs synthesised from *S. glauca* extract gave a PDI of 0.315 and 0.581 at 25°C and 70°C respectively while AuNPs from *T. fruticosa* gave PDI of 0.669 and 0.3 at the same temperatures, respectively. These results are also observed in the UV-Vis spectra which confirm higher diversity in shape or size represented by the higher absorbance at NIR region (Figure 2: H and I). The difference in polydispersity among the different plants can also be traced to variations in chemical composition among the plants which leads to different reaction outcomes. It should be noted that the conclusions on the polydispersity of the AuNPs from the TEM technique should be taken with caution, since small population of total sample can only be detected in each TEM frame. Further, a halo around some of the nanoparticles was observed in some TEM images (Figure 4), which could be showing the biological layer surrounding the nanoparticles (Elbagory et al, 2016).
Figure 4: Transmission Electron Microscopy (TEM) images of AuNPs synthesised from the tested plants at different temperatures. A scale bar is shown in the images.
Table 3: Summary of TEM analysis of AuNPs synthesised from the tested plants.

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Dominant AuNP shape/ Uniformity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
</tr>
<tr>
<td>1. Euclia racemosa</td>
<td>Spherical like/non-uniform</td>
</tr>
<tr>
<td>2. Ortholobum bracteolatum</td>
<td>Asinotropic</td>
</tr>
<tr>
<td>3. Phyllox lurus</td>
<td>Asinotropic</td>
</tr>
<tr>
<td>4. Peragonium capitatum</td>
<td>Spherical like/uniform</td>
</tr>
<tr>
<td>5. Gerbera linnae</td>
<td>Spherical like/non-uniform</td>
</tr>
<tr>
<td>6. Rhus Glauca</td>
<td>Anisotropic</td>
</tr>
<tr>
<td>7. Myrica quercifolia</td>
<td>Spherical like/non uniform</td>
</tr>
<tr>
<td>8. Searisa Lucida</td>
<td>Spherical like/Uniform</td>
</tr>
<tr>
<td>9. Tetragonia fruticosa</td>
<td>Spherical like/non-uniform</td>
</tr>
</tbody>
</table>

The EDX spectra show the chemical composition of the colloidal AuNPs. The EDX is a technique for elemental analysis of a sample and uses interactions between the sample and X-ray excitations. All nine plants were confirmed to contain gold ions as evidenced in their EDX spectra at both temperature conditions (25°C and 70°C). The Figure 6 shows the Au peaks from the AuNPs synthesised from M. quercifolia and P. capitatum. The EDX spectra of the other 7 plants are not shown but the same analysis was performed. Other peaks for elements such as calcium and oxygen can be attributed to components present on the AuNPs which are suggested to be the...
phytochemical(s) involved in the reduction and stabilisation of the AuNPs (Rajathi et al, 2014). Cu peaks can be attributed to the copper grid used to analyse the sample (Rodríguez-León et al, 2013).

Figure 6: EDX spectra of AuNPs synthesised from two plant extract. EDX spectra from *M. quercifolia* and *P. capitatum* at 25°C (A, C) and 70°C (B, D) are shown.

The Selected area diffraction pattern (SAED) showed the crystallinity of the nanoparticles (Bar et al, 2009). All the synthesised AuNPs were found to be polycrystalline in nature as observed by the small spots making up multiple rings and spots characteristic of polynanocrystallinity in the SAED patterns of the nanoparticle. Figure 7 shows the SAED pattern of the AuNPs synthesised from *G. linnae* and *S. lucida*, and the lattice fringes on the nanoparticle surface at high magnification (2 nm scale)
(Figure 7C). An amorphous (diffuse rings) and crystalline (bright spots) sample is observed for G. linnae AuNPs (Figure 6A) while S. lucida and polynanocrystalline (small spots making up a rings) (Figure 7B). The presence of five Bragg’s reflection orientations namely 111, 200, 220, 311 and 422 also confirm the presence of AuNPs in the sample with each spot arising due to Bragg reflection of an individual crystallite (Ahmad et al, 2003; Sayed et al, 2013). The Scherrer ring pattern which is characteristic of face centred cubic (fcc) structure of gold confirms the nanocrystallinity of the nanoparticles observed in the TEM images (Sayed et al, 2013). The SAED patterns of the other 7 plants are not shown but the same analysis was performed. The crystalline nature of AuNPs synthesised from plant extract has also been observed previously by various studies (Elbagory et al, 2017 and Mollick et al, 2015). Crystallinity of these resultant AuNPs could be further explored for future applications in electronics due to their effect on ion/electron transport properties, in catalysis where the crystallinity can enhance catalytic activity and in molecular sensing devices where they function for the optimisation of performance (Fujita et al, 2012; Tang and Ouyang, 2007).

Figure 7: SAED pattern of AuNPs synthesised from two plant extracts showing their Scherrer ring patterns with five Bragg's reflection orientations. A shows the results for G. linnae while B shows results for S. lucida. The high resolution image at 2nm shows the lattice fringes of AuNPs synthesised from S. lucida.
3.4 Stability evaluation of the AuNPs

The stability of the synthesised AuNPs is an important aspect as it contributes greatly to the biological function of the resultant nanoparticles (Gupta and Gupta, 2005). The colloidal stability of the nanoparticle determines the fate of the nanoparticle, directly affecting the potential application of the nanoparticles (Islam et al, 2015). Once the physico-chemical properties of nanoparticles have been established it is critical to measure the stability of the nanoparticles in buffer conditions that simulate the environment in which the nanoparticles will be applied in or stored in. In this study, the potential antibacterial effect of the AuNPs was explored and for this reason the stability of the AuNPs was tested in Nutrient Both. Additionally the AuNPs were also tested in DMEM.

Aggregation of the nanoparticles due to their instability influences their nanoscale properties. These changes can alter cellular uptake and the bioavailability of the nanoparticles (Zhu et al, 2012). It is therefore important to understand the state of the stability of the nanoparticles synthesised prior to biological application. Proteins have been observed to contribute greatly to the stability of the AuNPs by possibly forming a coat around the nanoparticles inhibiting the inter-particle interaction (Nel et al, 2009).

The stability of all synthesised AuNPs were studied by incubating the AuNPs in DMEM containing 10% FBS and Nutrient broth for a period of 24 hr at 37°C and monitoring the changes in their UV-Vis spectra as stated in section 2.5.5. This approach allows monitoring of spectra shift due to aggregation forces that could be caused by loss of stability caused by buffering conditions (Kanjanawarut, 2013). All the AuNPs showed stability in DMEM with 10% FBS as only minimal changes in the UV-Vis spectra of the AuNP can be observed (Figure 7). There appears to be a moderate reduction in the λ-max of the UV-Vis spectrum, but no flattening or shifting of the UV-vis adsorption spectra was observed. When the UV-Vis absorption spectra of the AuNPs in water without incubation (Figure 3) is compared to the spectra in the stability studies (Figure 8) before incubation (0 hrs), shows a red shift in the absorption spectra which could indicate an increase in size of the AuNPs due to interaction with DMEM proteins. However, the DLS was not performed to observe and confirm the hydrodynamic size changes due to the suspected protein and AuNP interactions.
The stability of the AUNPs could have been further enhanced or attributed by the DMEM media sufficient concentration of FBS in the DMEM through the formation of a protein corona as this has been observed to play a role in the stability of the nanoparticles (Von White et al, 2012). A study done with varying concentrations of FBS in DMEM media showed that increasing FBS concentration led to enhanced stability (Sabuncu et al, 2012). A different study comparing citrate silver nanoparticles (AgNPs) and garlic capped AgNPs showed that the stability remains unaffected after 18 hr as observed by the Plasmon band of the UV-Vis spectrum at different ratios of the DMEM to nanoparticle dispersion. On the other hand, citrate capped nanoparticles without the inclusion of DMEM agglomerated within 30 min and to an irreversible state after 15 hr revealing the stability conferring factor that could be attributed by DMEM (Von White et al, 2012). It is concluded that the proteins present in the medium coat the nanoparticles and stabilise them through the favourable interaction between the natural corona of the AuNPs and the medium’s components.
The AuNPs selected for the antibacterial evaluation were further evaluated for their stability in appropriate media used in the antibacterial experiments (nutrient broth). Figure 8 shows the UV-Vis spectra of AuNPs synthesised from *P. capitatum*, *M. quercifolia* and *S. glauca*. These AuNPs were chosen based on their rapid activity observed in the AuNP synthesis reaction. This rapid activity can be correlated to higher antioxidant activity of the plant which has previously been shown to be associated with high reducing potential of the plant in AuNP synthesis reaction as stated by Lee et al, 2013. The genus of *P. Capitatum* among the chosen plants, from which the AuNPs were synthesised from, has shown antibacterial activity as reported by Street et al 2013. The UV-Vis spectra revealed that there is no shift in the absorption band indication no change in the size of the nanoparticles upon incubation with nutrient broth for a 24hr period. However, AuNPs from *M. quercifolia* (Figure 8B) showed a significant decrease in the absorption peak intensity as a result of the interaction between the medium’s components and the AuNPs. These results suggest that the AuNP retain their physico-chemical characteristics in terms of their size and level of anisotropy as revealed in the shape of the spectrum.

![Figure 9: UV-Vis spectra of nanoparticles synthesised from 3 plant extracts after incubation in nutrient broth for 24 hrs. *P. capitatum* (A), *M. quercifolia* (B), *S. glauca* (C).](http://etd.uwc.ac.za/)
### 3.5 Evaluation of Antimicrobial activity of the AuNPs

A bacterial infection is a common problem associated with chronic wounds (Siddiqui, 2010). *S. aureus* is considered the most prevalent causative microorganism of wound infections (Nicolau and Stein, 2010). Methicillin-resistant *S. aureus* (MRSA) are strains that have acquired resistance to all available penicillin and various other β-lactam antibiotics. MRSA can cause a variety of problems ranging from skin infections, sepsis and pneumonia to bloodstream infections. The incidence of bacterial strains with antibiotic resistance has led to an urgent quest for alternative antimicrobial agents to combat this challenge. Ancient civilisations have been using various fungal and plant extracts to treat infections. The search for alternative antimicrobial agents has again sparked research interests in the antimicrobial activities natural products. Plants are frequently used in African traditional medicine to treat microbial infections and may very well contain new chemical entities that can be developed into new antimicrobial agents.

In this study the antibacterial activity of *M. quercifolia* and *P. capitatum* plant extracts and their respective AuNPs were studied on an antibiotic sensitive strains of *S. aureus*. *M. quercifolia* plant extract and its respective AuNPs was also testing on the antibiotic resistant *S. aureus* (MRSA). The effects of the plant extracts and AuNPs were assessed by measuring changes in the optical density of the bacterial culture as described in section 2.6. Optical density is directly proportional to the number of bacteria in a culture and can therefore be used as an indication of bacterial growth (Hall et al, 2013).

Figure 10 represents the outcome of antibacterial evaluation of AuNPs synthesised from *P. capitatum* and *M. quercifolia* as well as the antibacterial activity of the crude plant extract from which the nanoparticles were synthesised. Both AuNPs and their respective crude plant extract showed dose dependent antibacterial activity against *S. aureus* (Figure 10).
*S. aureus* cultures were treated for 24hrs with increasing concentrations of the AuNPs (3.75 – 120 µg/ml) and plant extracts (15.12 – 500µg/ml).

Figure 10 shows that *M. quercifolia* AuNPs and plant extract were able to significantly reduce the viability of *S. aureus* at the lowest concentrations tested (3.75 µg/ml for AuNPs and 15.12 µg/ml for the plant extract). It is possible that lower concentrations can still have significant antibacterial activity, but this will need to be tested in the future. It is not clear at this stage if the *M. quercifolia* AuNPs have more antibacterial activity than the extract. Antibacterial testing at lower doses and the determination of Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) can be used to make this assessment. The antibacterial properties of *M. quercifolia* have not been reported before. In fact, very few scientific studies have been conducted on this plant.

The antibacterial activity of the *P. capitatum* extract and its respective AuNPs was significantly low compared to *M. quercifolia*. Although *P. capitatum* extract and AuNPs was able to induce a significant reduction in the growth of *S. aureus*, it was only able to do this at much higher doses, 125 and 320 µg/ml, respectively.
Figure 10: Dose dependent bactericidal effects of *M. quercifolia* and *P. capitatum* plant extracts and their AuNPs against *S. aureus*. *S. aureus* was cultured in 96 well plates and treated for 24hrs with *M. quercifolia* and *P. capitatum* plant extracts and their AuNPs. Ampicillin was used as positive control. Cell growth was assessed measuring optical density at 450nm. Results are expressed in means ± SD. *Different from control group. P < 0.05, *(P ≤ 0.05),**(P ≤ 0.01),***((P ≤ 0.001),****(P ≤ 0.0001).

Figure 11 shows that the *M. quercifolia* extract has significant antibacterial activity against the antibiotic resistant *S. aureus* (MRSA). At the lowest dose of 15.12 µg/ml the plant extract induced a significant reduction in the growth of *S. aureus* (MRSA). The AuNP produced from this plant extract was less effective in inhibiting the growth of this microorganism. A higher concentration (240 µg/ml) was required to significantly inhibit the growth of *S. aureus* (MRSA). The antibacterial activity of the *M. quercifolia* extract against *S. aureus* (MRSA) is a very significant finding since this bacterial strain is resistant to several antibiotics.
One study on plants collected from the Cape Floral Kingdom shows that the bioactivity of the AuNPs is even higher than the activity of the extract (Elbagory et al. 2017). AuNPs synthesised from leaves of *Eucalyptus macrocarpa* have antibacterial activity against *E. subtilis* and *E. coli* (Cui et al 2012 and Shah et al, 2014). Similarly to the study by Shahzadi et al (2016), this study also show a dose dependent increase in the activity of the AuNPs. These results show AuNPs synthesised from these two plants and the respective plant extracts as potential antimicrobial agents against *S. aureus*. These antimicrobial AuNPs can be formulated into ointments and wound dressing to treat skin infections with microorganisms such as *S. aureus* (MRSA).

![Graph](http://etd.uwc.ac.za/)

**Figure 11:** Dose dependent bactericidal effects of *M. quercifolia* plant extract and its respective AuNPs against *S. aureus* (MRSA). *S. aureus* (MRSA) was cultured in 96 well plates and treated for 24hrs with *M. quercifolia* plant extract and its respective AuNPs. Kanamycin was used as positive control. Cell growth was assessed was measuring optical density at 450nm. Results are expressed in means ± SD. *Different from control group. P < 0.05, * (P ≤ 0.05), **( P ≤ 0.01), *** (P ≤ 0.001), **** (P ≤ 0.0001).
Chapter Four: Conclusion and Future prospects

Extracts prepared from *P. capitatum*, *O. bracteolatum*, *G. linnae*, *M. quercifolia*, *S. lucida*, *P. bubescens*, *E. racemosa*, *T. fruticosa*, and *S. glauca* were successfully screened for their potential to synthesise AuNPs through the reduction of sodium tetrachloroaurate (III) dehydrate. The formation of AuNPs was observed at both temperatures (25°C and 70°C) tested in this study. The optimal concentration of plant extract was determined for all 9 plants at both temperatures.

The resultant AuNPs were characterized using Ultra Violet-Visible Spectroscopy (UV-Vis), Dynamic Light Scattering (DLS), High Resolution Transmission Electron Microscopy (HR-TEM) and Energy-Dispersive X-ray Spectroscopy (EDX). All four of these techniques confirmed the successful synthesis of colloidal AuNPs. AuNP formation and the physico-chemical properties (size, charge and shape) of the nanoparticles were dependent on several factors, which includes the plant, the concentration of the plant extract, and the temperature.

The DLS data show that the AuNPs had an average diameter of 63.8 nm at 25°C and 56.9 at 70°C. The Zeta Potential was observed to be negative for all the synthesised AuNPs, which corresponded to a negative surface charge of the nanoparticles contributing to the stability of the AuNPs, which prevents aggregation. The HR-TEM showed the morphology and polydispersity, which reveals the uniformity of the synthesised AuNPs. The AuNPs had shapes observed to be dominantly quasi spherical and pentagonal and their crystalline nature was also observed. A halo surrounding the nanoparticle surface as also observed which possibly reveals the presence of a layer of biological material surrounding the nanoparticle.

The stability of all synthesised AuNPs was studied by incubating the AuNPs in DMEM containing 10% FBS and nutrient broth prior to biological application. All AuNPs under study showed stability in DMEM (supplemented with 10% FBS) as only minimal changes in the UV-Vis spectra of the AuNP can be observed. The antibacterial activity of *M. quercifolia*, *P. capitatum* plant extracts and their respective AuNPs were studied using the microtiter method. The growth inhibition as demonstrated by the changes in the turbidity of the...
sample observed through optical density analysis was determined for each sample. Both AuNPs and their respective crude plant extracts showed significant dose dependent antibacterial activity against *S. aureus*. *P. quercifolia* extract and the AuNPs produced from this plant also showed antibacterial activity against *S. aureus* (MRSA).

It is probable that these nanoparticles possess an added pharmacological effect due to the phytochemicals capping the metallic nanoparticles. These nanoparticles have revealed a promising bioactivity for developments of new antibacterial agents against *S. aureus* strains. It was observed that as the concentration of all the AuNPs tested in this study increased, the antibacterial activity also increased demonstrating a dose-dependent activity of AuNPs. These results show that these two plant extracts and their respective AuNPs are potential antimicrobial agents against *S. aureus*. Their activity against other gram negative and gram positive bacterial strains and fungal species should be evaluated further, as well as its mechanisms of action. For applications in the development of antimicrobial agents its toxicity must be investigated further.

The green chemistry branch of nanotechnology holds great potential in contributing to future developments in various fields. This field has opened greater opportunities and possibilities, which bring hope to medicine worldwide. Further optimisation of synthesis and an in depth characterisation of these nanoparticles will lead to knowledge based advancement in the field of green nanotechnology. Different experimental conditions yield different nanoparticles in varying yields. Therefore, in line with the effort is being done towards advancement of desired physico-chemical properties which are towards establishing desired characteristics for various applications, optimal experimental conditions are crucial for obtaining desired characteristics and nanoparticle yield.

Different phytochemicals are involved in synthesis. The synergistic effects of plant phytochemicals may play a key role in the bioactivity. More stable and potent formulations can also be explored by developing conjugates with the identified active phytochemicals. This will probably confer increased stability of the active compounds or currently available antibiotics and increase their availability in the target site. These interactions and benefits are already expected from the synthesised nanoparticles and can be further enhanced to fit desired applications.
The analysis of the effect of these nanoparticles on various cells and the evaluation of their genotoxicity should be done to increase the understanding of their safety and increase their potential for biomedical applications. This will also give clear results when the nanoparticles are used in combination with other drugs for delivery or as part of combinatorial medicine as their effects will be fully understood. These studies will lead to a clearer understanding of their sole therapeutic effects and mechanisms.
Chapter five: References


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