Cape Peninsula University of Technology

THE ANTIMICROBIAL AND IMMUNOMODULATORY EFFECTS OF COTYLEDON ORBICULATA EXTRACTS

By

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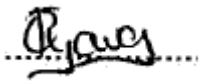
DECLARATION

I, Caroline Tyavambiza, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



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ABSTRACT

The challenge of antimicrobial resistance has increased drastically over the years as more microorganisms are becoming resistant to the available conventional treatments. The burden of antimicrobial resistant infections is intensified by the increase in immunocompromising conditions such as HIV/AIDS and cancer. Due to this challenge, pharmaceutical companies, health sectors and researches are in search of new antimicrobial agents that can solve the problem at hand. Medicinal plants are a reliable source for drug discovery as it is estimated that 25% of modern medicine originated from plants. They have also been used traditionally as sources of medicine in the treatment of many human ailments. Plants can also be applied in the field of nanotechnology. Nanotechnology is a promising field in medicine as it has the potential to offer improved methods for disease diagnostics and therapeutics. The use of plants in nanotechnology brings about biologically friendly nanomaterials. *Cotyledon orbiculata* is one of the well-known and common plants of South Africa that is used in traditional medicinal practices. The nanotechnology applications as well as the antimicrobial and immunomodulatory effects of this plant were evaluated.

The ability of *C. orbiculata* to synthesize silver nanoparticles was determined. Optimisation of silver nanoparticle synthesis using water extract of *C. orbiculata* was done at different conditions. The conditions evaluated include, reaction temperature (25 and 70°C), silver nitrate concentration (1 and 3mM), plant extract concentration (1.5, 3 and 6mg/ml) and reaction time. The synthesis of silver nanoparticles using this plant was successful. The optimal conditions for the synthesis of silver nanoparticles using *C. orbiculata* were 3mg/ml of the *C. orbiculata* extract, 3mM silver nitrate at a reaction temperature of 70°C for 2 hours. Under these conditions, spherical, crystalline nanoparticles with sizes of 20-40nm were produced.

The antimicrobial and immunomodulatory properties of *C. orbiculata* extracts and silver nanoparticles were evaluated. Antimicrobial activity was evaluated against *Staphylococcus aureus*, *Staphylococcus epidermidis*, Methicillin resistant *Staphylococcus aureus* (MR*SA*), *Pseudomonas aeruginosa* and *Candida albicans,* using the micro-dilution assay to determine the minimum inhibitory concentration (MIC). The results obtained revealed that all extracts of *C. orbiculata* have antimicrobial properties against all the microorganisms tested. The MICs

of the extracts ranged from 3.13 to 50mg/ml and the MBC/MFC from 6.25 to >100mg/ml. The methanol extract exhibited better antimicrobial activity in comparison to the others extracts whereas the water extract had better antifungal properties. The chloroform extract showed the lowest activity in both antibacterial and antifungal studies. Silver nanoparticles also exhibited antimicrobial activity against all the microorganisms tested. It's MICs against these microorganisms ranged from 5–80µg/ml and MBC/MFC from 20-160µg/ml. The silver nanoparticles were highly active than the water extract against both the bacteria and the fungi.

Immunomodulatory effects of the plant extracts and silver nanoparticles were determined by evaluating cytokine production using the enzyme linked immunoassay (ELISA) assay. All the extracts and silver nanoparticles of *C. orbiculata* were found to have anti-inflammatory properties. The water extracts showed more anti-inflammatory activity against the cytokines than the other extracts. However the silver nanoparticles were more active than the water extract. The findings from this study confirmed that *C. orbiculata* have antimicrobial and immunomodulatory effects. This provided scientific evidence of the traditional use of this plant in the treatment of skin infections and inflammatory conditions.

Keywords

Cotyledon orbiculata, Antimicrobial resistance, Immunomodulation, Silver nanoparticles, Medicinal plants.

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DEDICATION

To my mother, I will forever be grateful for your love and support

PREFACE

This thesis is written in an article based format and consists of a total of 6 chapters. Chapter 1 provides an introduction, aims, objectives and the significance of the research project. Chapter 2 is the literature review which gives a description of the basic concepts and an overview of the information related to this project. Chapters 3, 4 and 5 are the articles that will be submitted for publication. These chapters focus on different aspects of the research project therefore have separate abstracts, introductions, methods, results, discussions and conclusions. Chapter 6 gives a summary of the integrated discussions and conclusions of the whole thesis. Each of the chapters has separate references.

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CLARIFICATION OF TERMS

Antimicrobial agent: a substance that has the ability to kill, inhibit or slow the growth of microorganisms.

Bactericidal: an agent that can kill bacteria.

Bacteriostatic: an agent that inhibits the growth of bacteria but does not kill the bacteria.

***Candida albicans*:** a dimorphic fungus that resides on mucous membranes of the mouth, intestinal tract, and vagina of healthy people but can cause infection in immunocompromised individuals.

Commensal: a microorganism that does not cause harm to the host.

***Cotyledon orbiculata*:** a South African medicinal plant traditionally used to treat a variety of human skin ailments.

Immunocompromised: individual who has an impaired or weakened immune system.

Immunomodulation: adjustment of the immune system response to a desired level by the action of an immunomodulator.

Immunostimulator: an agent or drug that magnifies the response of the immune system by increasing the activity of one of its components.

Immunosupressor: an agent or drug that lowers or prevents the response of the immune system.

Infectious disease: disorders caused by microorganisms or their products such as toxins.

Nanotechnology: a field of science dealing with the synthesis, development and use of materials at the nanometer scale.

Nosocomial infection: infection or disease acquired whilst in a hospital where the patient was admitted for reasons other than the infection.

Phytochemicals: a biologically active chemical compound occurring naturally in plants.

***Pseudomonas aeruginosa*:** Gram-negative rod shaped bacteria that can cause a wide range of infections in immunocompromised individuals.

Silver: a valuable metallic element found in nature that is used for making jewellery and ornaments.

***Staphylococcus aureus*:** Gram-positive cocci bacteria that produce toxins and enzymes capable of causing a variety of infections.

***Staphylococcus epidermidis*:** a Gram positive coagulase negative coccus that is normally a commensal but can cause infections in immunocompromised individuals.

Wound: an injury to a living tissue leading to the disruption of its normal structure and function.

LIST OF ABBREVIATIONS

ABBREVIATION	DEFINITION
AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
DLS	Dynamic Light Scattering
EDX	Energy Dispersive X-ray spectra
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
HIV	Human Immunodeficiency Virus
HR-TEM	High-resolution transmission electron microscopy
IL	Interleukin
LB	Luria-Bertani medium
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MHB	Müller-Hinton agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
PBS	Phosphate-buffered saline
PDI	Poly Dispersity Index
PMA	Phorbol 12-myristate 13-acetate
RPMI	Roswell Park Memorial Institute medium
SAED	Selected area electron diffraction
SPR	Surface plasmon resonance
SSTI	Skin and soft tissue infections
THP-1	Tamm-Horsfall Protein 1
TLC	Thin Layer Chromatography
TNF	Tumour necrosis factor
WHO	World Health Organisation
WST1	Water Soluble Tetrazolium Salts
YPB	Yeast Peptone Broth

CHAPTER ONE

1.1 Introduction

Antimicrobial resistance has emerged as a significant threat to the world's public health. It has been considered a global concern by the World Health Organisation (WHO) (WHO, 2014). Antimicrobial resistance challenges both well-resourced and developing countries such as South Africa. The ability of highly infectious microbes to develop resistance to conventional treatment has made it difficult to eradicate and control infections. An example of these microbes is Methicillin-resistant *Staphylococcus aureus* (MRSA) which is resistant to a numbers of antibiotics including all beta-lactam antibiotics (San sit *et al*., 2017). MRSA is highly prevalent in hospitals worldwide, however the number of MRSA infected patients vary according to country and region. High rates of more than 50% MRSA infected patients were reported in Asia, Malta, North and South America. In South Africa, Gauteng province, the prevalence of MRSA was reported to be 36% (Fortuin-de Smidt *et al*., 2015). *Pseudomonas aeruginosa* is another highly infectious microbe that is resistant to a number of drugs. This pathogen has both intrinsic and acquired resistance to many drug classes making it very difficult to treat (Cabot *et al*., 2016).

Exacerbating the detrimental effects associated with global antimicrobial resistance is the increase in the number of immunocompromised individuals, particularly those with HIV and AIDS, who are at greater risk of acquiring infections. Skin infections are one of the signs associated with HIV infections. The most prevalent skin infections in HIV/AIDS patients include cellulitis, folliculitis, psoriasis and dermatitis (Emadi *et al*., 2014). Antimicrobial resistant pathogens particularly those affecting immunocompromised individuals lead to elevated morbidity and mortality in South Africa and the world as a whole (Akova, 2016; Paruk *et al*., 2012). Pathological conditions of the immune system are also a threat to the wellbeing of many individuals in developing countries. They increase the risk of infections and reduce the body's ability to fight against infectious diseases (Bajaj and Tombach, 2017). Acquired immunological conditions have significantly increased over the years, due to the increased range of immunosuppressive conditions. Therefore, antimicrobial agents possessing immunomodulatory effects can be very effective in fighting infections.

Due to complications and limitations of conventional medicines, health sectors have come to consider African Traditional Medicines particularly plants as an important source for the development of new effective medicines (Oyebode *et al*., 2016). Traditional medicine is of great importance in the healthcare systems of developing countries. Traditional medicinal

plants have been widely used in Southern Africa as a source of medicine for a variety of human ailments. These ailments include wounds, coughs and colds, digestive problems, diabetes mellitus, ulcers, cancers as well as hypertension and asthma (Ncube *et al*., 2013). In 2013, it was estimated that more than 80% of the population in developing countries was using medicinal plants to treat various diseases (Maroyi, 2013). The populace in these countries greatly depend on herbal medicines as they are safer, cheaper and more accessible than conventional medicines. They are also environmentally friendly and are more acceptable since they are part of the communities various cultures (Mahima *et al*., 2012).

Some of these plants can be incorporated in the field of nanotechnology specifically green nanotechnology. Nanotechnology is a field of science which deals with the synthesis, development and use of materials ranging in nanometers (Kavitha *et al.*, 2013). This technology has the potential to revolutionise medicine by delivering improved methods for disease diagnostics and therapeutics. It can be applied in many fields inclusive of biology and biomedicine, electronics, physics, material science and agriculture. Green nanotechnology involves the synthesis of nanomaterials that are more bio-friendly, using methods that are safer for the environment. Nanomaterials produced in this manner are more suitable for applications in biological systems (Ahmed *et al*., 2016).

In South Africa, a country with a strong history of using traditional medicine, it is estimated that around 80% of the population consult one of the many traditional healers (Wintola *et al*., 2017). South Africa hosts a variety of around 30,000 flowering plant species most of which have medicinal benefits and have been used for the treatment of different ailments. One of these plants is *Cotyledon orbiculata* (*C*. *orbiculata*) commonly known as “Plakkie” in Afrikaans and “Imphewula” in Xhosa. This plant has been used traditionally to treat many human ailments including skin infections, acne, epilepsy, corns and warts, boils, earache and toothache acne (Kumari *et al*., 2016). Its use in the treatment of boils may suggest that it contains antimicrobial and possibly wound healing activity.

1.2 General objectives

To determine if the solvent extracts and silver nanoparticles produced from *C. orbiculata* have antimicrobial and immunomodulatory properties.

1.3 Specific objectives

i. To prepare water and organic solvent extracts of *C. orbiculata*.
ii. To determine if extracts of *C. orbiculata* can produce silver nanoparticles and to fully characterise these nanoparticles.
iii. To evaluate if the extracts and silver nanoparticles have antibacterial activity against *P. aeruginosa, S. aureus,* MRSA and *S. epidermidis.*
iv. To assess if the extracts and nanoparticles have antifungal activity against *C. albicans.*
v. To assess if the extracts and nanoparticles have immunomodulatory effects by determining their effects on cytokine responses in the human macrophage cell line, THP-1.

1.4 Significance of research

The WHO claims that 80% of the South African black population make use of African Traditional Medicine (ATM) (Wintola *et al*., 2017). However, very little scientific evidence is available to back up claims that these medicines are effective. The general perception is that traditional medicines are safe and effective because they are natural and have been used for a long time. In the absence of scientific evidence to back up claims about the therapeutic efficacy of ATM these claims will remain unsubstantiated. *C. orbiculata* is used in traditional medicine to treat skin infections. This plant is also claimed to have anti-inflammatory properties. However, little scientific evidence exists for the anti-inflammatory and antibacterial effects of this plant.

Antimicrobial resistance to existing antibiotics is a global health threat and new antimicrobial agents are urgently needed. It is estimated that 25% of modern drugs originated from plants, making plants a rich and reliable resource for drug discovery. An investigation into the antimicrobial and immunomodulatory effects of extracts prepared from *C. orbiculata* will lay bare claims about the medicinal uses of this plant.

The applications of this plant in the field of nanotechnology are yet to be explored. Silver nanoparticles are known for their antibacterial properties and have been used in the medical field to treat various bacterial infections. Plant derived silver nanoparticles with known antimicrobial activities may produce antimicrobial agents that are more toxic to microorganisms than the silver nanoparticles or the plants extracts alone. Silver

nanoparticles may also isolate the phytochemicals responsible for antimicrobial and immunomodulatory properties of this plant. This can lead to the development of new effective antimicrobial agents. This study can therefore lay the basis for future research activities aimed at discovering new chemical entities for the development of new antimicrobial agents.

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CHAPTER TWO
LITERATURE REVIEW

2.1 Infectious diseases

Infectious diseases are a major cause of morbidity and mortality worldwide, particularly in developing countries. They were recorded to be the cause of approximately 15 million deaths (25.5%) of the estimated 58.8 million annual deaths worldwide in 2011 (Dye, 2015; WHO, 2011). In developing countries, studies revealed that more than 43% of all deaths are due to infectious diseases (Bryde and Waheed, 2013). These diseases inflict great damage and financial loss to the afflicted individuals, the society and the economy at large. The effects are exaggerated in developing countries by the prevalence of poverty and lack of resources. Despite great efforts to eradicate them, infectious diseases continue to be a great challenge (Naidu *et al*., 2014). This is because of the emergence of new pathogens, the ability of old pathogens to gain antimicrobial resistance, as well as the increase in the immunocompromised populace. The latter can be attributed to the increased range of immunosuppressive conditions such as cancers, transplants, autoimmune conditions and some congenital diseases (Young and Tambyah, 2012).

Infectious diseases are caused by microorganisms such as bacteria, fungi, virus and parasites. Various microorganisms live on the human skin as harmless commensals. However, loss of skin integrity associated with a weakened immune system may potentially result in these microbials penetrating the skin and proliferating in immunocompromised hosts, eventually causing life threatening infections. A number of factors such as skin abrasions, burns, immunosuppression and chemotherapy compromises the immune system and renders the individual highly susceptible to infection (Muluye *et al*., 2014).

2.1.1 Skin infections

Skin infections are one of the most common human conditions, reported to affect 30-70% of the world population in 2012 (Hay and Fuller, 2012). These infections have variable aetiology, presentation, and severity. Their severity can range from mild infections, such as pyoderma, to serious life-threatening conditions such as necrotizing fasciitis. Furthermore, these skin infections, if left untreated, can eventually lead to serious physical disabilities or death (Hay *et al*., 2014). Normally the skin is colonised with microbial flora which commonly consists of *Staphylococcus* species, *Corynbacterium* and yeast (Brugger *et al*., 2016). However these bacteria cannot invade the intact skin because of the protective function of

the skin.

The skin, which is an outer covering and the largest organ of the human body, plays a crucial role in the wellbeing of an individual. Its functions include protection, thermoregulation, secretory and sensory activities (Slominski *et al*., 2015). It offers protection against harmful pathogens by providing a physical barrier to microorganisms therefore bacteria colonising the skin cannot get through it unless it is broken. Skin can also secrete lactic and fatty acids, antimicrobial peptides and proteins such as human defensins and cathelicidins which inhibit the growth of microbes (Lee *et al*., 2006). Breaks in the skin such as leg ulcers, burns and surgical wounds compromise the physical barrier of the skin and allows invasion by microorganisms, resulting in infection. Microbial disease of the skin may also occur by the spread of bacteria through the blood; a condition known as septicaemia. The most frequently isolated skin pathogens include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Candida albicans* (Sanford and Gallo, 2013; Peleg and Hooper, 2010).

Skin and surgical site infection (SSI) are the most common types of nosocomial infections (Mohammed *et al*., 2014). Nosocomial infections are a major problem in hospitals worldwide. Their prevalence is two to threefold higher in developing countries than that recorded in the westernized countries (Naidu *et al*., 2014). Nosocomial infections are infections acquired by patients while under medical care in hospital settings. They are often caused by lack of hygiene in hospitals and health care facilities (Shahida *et al*., 2016). These infections lead to a prolonged hospital stay for infected patients, increased socio-economic disturbance, increased economic burden as well as increased mortality rate. Strict hygiene is essential in the prevention and control of nosocomial infections. Staff should practice personal hygiene and properly wash their hands before and after attending to patients. All medical equipment should be completely sterilized and health facilities should aim to always provide clean and sanitary environments to reduce these infections (Khan *et al*., 2017).

2.1.2 Conditions associated with skin infections

Numerous health conditions are associated with the development of certain skin infections. These include HIV/AIDS, diabetes, lupus, cancer, malnourishment and stress (Mendes *et al*., 2017). Most of these conditions result in the suppression of the patients' immune system. Due to the impairment of their immune system, immunocompromised individuals are at a greater risk of developing skin infections. This is because of their inability to eliminate the

colonizing skin microbes, which will eventually proliferate and cause infections. Skin infections are also frequently associated with HIV and AIDS, in most cases skin disease is the first sign of HIV infection (Dryden, 2010). Mendiratta *et al* (2010) stated that "skin is commonly involved in HIV infection and nearly 90% of patients with HIV infection have skin infection manifestations at some stage during the course of their disease". The association of HIV and skin infections contributes extensively to the increased prevalence of the skin infections. This is due to the high prevalence of HIV, it is estimated that there 38.8 million people infected with HIV, worldwide (Tobjörk *et al*., 2015). Skin problems frequently associated with HIV/AIDS include cellulitis, folliculitis, psoriasis and impetigo. However, according to Altman *et al* (2015), the most common skin manifestations in HIV patients are fungal infections, seborrheic dermatitis and eczema.

Patients with diabetes mellitus are more predisposed to skin infections than non-diabetic patients (Goboza *et al*., 2016). This is because of the poor blood flow and high levels of glucose in the patients' blood which decrease the ability of white blood cells to fight infections (De Macedo *et al*., 2016). Skin infections may be the first sign of diabetes mellitus presentation or may appear later during the course of the disease. Studies have shown that diabetic patients have an increased incidence of skin infections ranging from 20 to 50% (Gangawane *et al*., 2016). The most common skin infections associated with diabetes include foot infections, necrotizing fasciitis, folliculitis, furunculosis, subcutaneous abscesses, diabetic blisters, diabetic dermopathy and fungal infections, such as athlete's foot and ringworm (Gkogkolou and Böhm, 2014).

2.1.3 Antibiotic resistance

In spite of advances in the control of infections, skin infections remain a great problem because of antimicrobial resistance (Muluye *et al*., 2014). The increasing challenge to health care attributable to antimicrobial resistance, and the subsequent absence of access to effective antimicrobials, is of worldwide concern. The WHO noted AMR as a global public health challenge and a great threat to the lives of individuals worldwide. In Europe, it is estimated that 25 000 people die yearly as a result of multidrug resistant organisms (Blair *et al*., 2015) and in the United States approximately 23 000 deaths occur annually as a result of antimicrobial resistance (WHO, 2014). Developing countries are highly affected by antimicrobial resistance. In Africa, infectious diseases were reported to be responsible for 41.9% deaths (Morgan *et al*., 2011).

The frequent and extensive use of antibiotics has led to the emergence of drug-resistant microorganisms. Treating a patient with antibiotics causes microorganisms to either adapt or die, a phenomenon known as selective pressure (Tello *et al*., 2012). This is increased by the abuse of antibiotics in hospitals and also the non-compliance of patients to complete prescribed antibiotic treatment courses (Taddesse *et al*., 2014). The impacts of AMR are devastating; to the patient they include prolonged illness which means longer hospital stays resulting in increased mortality. These patients consume more healthcare resources which poses a great financial burden on the hospitals and the economy at large. Drug-resistant infections also compromise the success of treatments such as organ transplantation, cancer chemotherapy and major surgeries (Friedman *et al*., 2016).

2.1.4 Mechanisms of antibiotic resistance

Bacteria can either be intrinsically resistant to antibiotics or can acquire resistance through acquisition of mutated genes by horizontal gene transfer (Munita and Arias, 2016). Genetic material between microorganisms is usually transferred in three main ways namely conjugation, transformation and transduction (Blair *et al*., 2015). Conjugation is the transfer of DNA from one bacterium to another. The DNA is transferred in the form of plasmids which are defined as chromosomal genetic elements that can replicate independently in the cytoplasm (Holmes *et al*., 2016). During conjugation a small tube forms between the two bacteria and acts as a passage for the transfer of the plasmid from one bacterium to another. Conjugation is the main mechanism of gene transfer in antimicrobial resistance. It is attributable to most cases of resistance in clinical and hospital settings (Haaber *et al.*, 2017). Transformation involves the uptake of free DNA from the environment by bacteria. These bacteria incorporate the environment DNA into their own chromosome. An example of transformation is the uptake of free DNA from *Streptococcus mitis* by *Streptococcus pneumoniae*, which renders it resistant to penicillin (Tovpeko and Morrison, 2014). Transduction, a less common way of genetic transfer between bacteria, relies on bacteriophages for the transfer of the DNA across bacteria. Bacteriophages are viruses that infect bacteria; in the process of infections they transfer DNA from donor bacterium to the recipient bacterium (Adekunle, 2012).

Bacteria mediate their resistance to antibiotics by various mechanisms. The main mechanisms of resistance include alteration of drug target site, enzymatic inactivation of the drug, reduced permeability or uptake of the drug by the bacteria and lastly, increased efflux of the drug to the outside (Deng *et al*., 2013). For effective activity, most antibiotics bind with high affinity to their specific target sites, thus preventing the normal activity of that target.

Unfortunately bacteria have developed ways of altering these target sites. They achieve this by adding specific molecules to the target sites or by the expression of mutated genes encoding for the altered target sites (Kumar and Valera, 2013). Modifications to the target will prevent efficient binding of the antibiotic but the target will still carry out its normal function in the cell. A common example of this is the alteration of penicillin binding protein in *S. aureus* which renders it resistant to all beta-lactam antibiotics (Tang *et al*., 2014). Bacteria are also capable of directly degrading or inactivating antibiotics thus resisting their action. They achieve this by producing different enzymes, some enzymes directly degrade the antibiotic and some inactivates them by adding acetyl, adenyl or phosphryl groups (Davies and Davies, 2010). The enzyme beta-lactamase can cleave beta-lactam antibiotics rendering bacteria resistant to all antibiotics in this group. Some bacteria alter porin expression and restrict the influx of antibiotics therefore insufficient amounts of antibiotic will enter the cell (Petchiappan and Chatterji, 2017).

2.1.5 Common microorganisms causing human infectious diseases

2.1.5.1 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that is catalase and coagulase positive (Li *et al.*, 2015). It can live as a commensal on the skin, in the nose and throat, however it can also be pathogenic and cause diseases. It achieves this through the production of toxins and enzymes as well as direct tissue invasion (Otto, 2014). According to Ryu *et al* (2014), approximately 30% of healthy individuals are persistent carriers of *S. aureus* and 30% - 50% are intermittent carriers. Importantly, this carrier state is a great risk factor for *S. aureus* infection. *S. aureus* (Fig 2.1) is the primary cause of local skin and soft tissue infections (SSTI) such as impetigo, dermatitis, and cellulitis; however it can also cause deep seated infections such as endocarditis and bacteraemia (Taddesse *et al*., 2014). Furthermore, *S. aureus* is also one of the main causes of community and hospital acquired infections (nosocomial infections), which can result in life threatening conditions (Miller *et al.*, 2014). The prevalence of *S. aureus* is increasing with the increasing number of immunosuppressed individuals as well as the bacteria's ever-evolving antibiotic resistance.

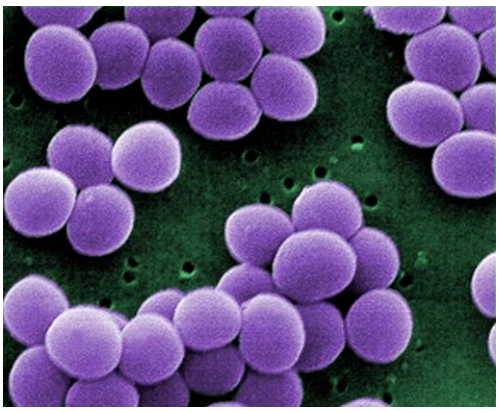

Figure 2.1: *Staphylococcus aureus* (Scanning electron microscopy)

(Source: https://www.popsci.com/why-staph-infections-are-so-hard-to-control)

For the treatment of methicillin sensitive *S. aureus* (MSSA), penicillinase-resistant penicillins and cephalosporins can be used. The most commonly used penicillinase-resistant penicillins include flucloxacillin and dicloxacillin. These are used in cases of serious MSSA infections such as endocarditis. In minor cases of *S. aureus* infections, first-generation cephalosporins can be used for treatment (Rayner and Munckhof, 2005).

S. aureus has the ability to acquire antimicrobial resistance mechanisms. Many *S. aureus* strains become resistant to penicillin by producing penicillinase. This enzyme breaks down the beta-lactam ring of the penicillin molecule and destroys the antibiotic (Antti *et al*., 2014). The acquisition of the *mecA* gene by *S. aureus* enables it to become resistant to methicillin, hence the name methicillin resistant *S. aureus* (MRSA). MRSA is currently among the bacteria of global concern (Marín *et al*., 2015). It is resistant to all penicillins, cephalosporins and carbapenems. Vancomycin is the preferred antibiotic for the treatment of MRSA infections. However recent studies have shown the emergence of Vancomycin-resistant *S. aureus*, this exacerbates the problem for the treatment of infections caused by this bacterium (Karmakar *et al*., 2016).

2.1.5.2 *Staphylococcus epidermidis*

Staphylococcus epidermidis (*S. epidermidis*) is a Gram-positive coagulase-negative coccus, which is diagnostically distinguished from *S. aureus* by its inability to produce coagulase (Pinheiro *et al.*, 2015; Otto, 2009). This microorganism is the most common commensal of the skin and mucous membranes. Although originally regarded as a commensal, *S. epidermidis* has recently been identified as an important opportunistic pathogen. This is due to the increased occurrence of infections caused by this microorganism in

immunocompromised individuals, in particular those with indwelling foreign bodies such as heart valves and intravenous catheters (Buttner *et al.*, 2015). *S. epidermidis* (Fig 2.2) was reported to causes approximately 20% of all orthopaedic device-related infections, with increase in prevalence up to 50% in late-developing infections (Bresco *et al*., 2017; Moriarty *et al*., 2016).

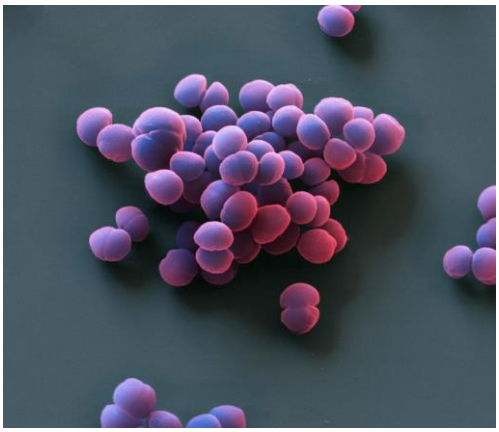

Figure 2.2: *Staphylococcus epidermidis* (Scanning electron microscopy)

(Source: https://tinyurl.com/yc62oad4)

S. epidermidis is one of the most important opportunistic pathogens in nosocomial infections. The pathogenicity of *S. epidermidis* is mainly attributed to its ability to form biofilms (Xu *et al*., 2017). The biofilm it possesses shields it from attack by both the host's immune system and antibiotic treatment, making its infections difficult to treat (Khodaparast *et al.*, 2016). Because of the biofilm, *S. epidermidis* shows resistance to different classes of antibiotics including beta-lactams, macrolides and aminoglycosides. It has developed resistance to many common antibiotics such as methicillin, novobiocin and clindamycin (Vandecandelaere *et al*., 2017).

The majority of *S. epidermidis* strains remain susceptible to newer antibiotics such as daptomycin, tigecycline and linezolid. However for more resistant microorganisms, the effective treatment for the biofilm infections is to remove or replace the implanted devices (Lv *et al*., 2017).

2.1.5.3 *Pseudomonas aeruginosa*

The Gram-negative bacillus, *Pseudomonas aeruginosa* (*P. aeruginosa*) is a great cause of concern in the medical field. Driscoll *et al* (2007) stated that infections caused by *P.*

aeruginosa are associated with higher morbidity and mortality rates in comparison to other bacterial pathogens*. P. aeruginosa* (Fig 2.3) is an opportunistic pathogen; hence nearly all the infections caused by this microorganism occur in immunocompromised individuals (Mowat *et al.*, 2011). Individuals with cystic fibrosis, immunocompromising conditions such as HIV/AIDS and patients with foreign body such as mechanical ventilator or catheters are predisposed to *P. aeruginosa* infections. This pathogen is also an important cause of both community and hospital-acquired infections. It accounts for 11-13.8% of all nosocomial infections, with even higher rates of 13-22.6% in ICU (Khosravi et al., 2016; Mayank *et al*., 2009). The community-acquired infections include skin and soft tissue infections, ulcerative keratitis and otitis externa. The life-threatening nosocomial infections associated with *P. aeruginosa* include pneumonia, urinary tract infections, bacteraemia, cystic fibrosis, bone and joint infections, gastrointestinal infections and other systemic infections (Gellatly and Hancock, 2013).

Figure 2.3: *Pseudomonas aeruginosa* (Scanning electron microscopy)

(Source: https://za.pinterest.com/pin/96897829458108615/)

Although it causes numerous complications, therapeutic options are greatly limited due to the ability of *P. aeruginosa* to acquire mechanisms of resistance to multiple groups of antimicrobial agents (Yayan *et al.*, 2015). *P. aeruginosa* is naturally resistant to many antibiotics due to the barrier provided by its impermeable outer membrane. The bacterium alters protein binders of penicillin (PBP) and causes porin mutations, plasmid enzymatic modification and DNA-gyrase mutation (Ochoa *et al*., 2013). *P. aeruginosa* can also form biofilms which allows it to survive harsh conditions such as very high temperatures and different chemicals. The protection by the biofilms renders most antibiotic treatments inefficient therefore promoting chronic infectious diseases (Rasamiravaka *et al.*, 2015).

A few antibiotics are therefore effective against *P. aeruginosa* and these include fluoroquinolones, gentamicin and imipenem (Meletis and Bagkeri, 2013). For serious infections and for cases where the risk of antibiotic resistance is high, a combination treatment is given (Rizvi *et al*., 2015). Synergistic effects of double and triple antibiotic combinations including an aminoglycoside, an anti-pseudomonal beta-lactam, colistin, a fluoroquinolone, a macrolide, or rifampin have been demonstrated (Tangden, 2014).

2.1.5.4 *Candida albicans*

Candida albicans (*C. albicans*) is a dimorphic fungus that grows as both yeast and filamentous cells. It resides as a harmless commensal on human skin and in mucous membranes such as the vagina and mouth (Mayer *et al*., 2013). *C. albicans* (Fig 2.4) can however take advantage of an impaired immune system and cause infections ranging from superficial infections of the skin to life-threatening systemic infections. The superficial diseases are termed candidiasis and they represent the fourth leading cause of nosocomial infections at 8% to 10% (Papon *et al*., 2013). Increase in the prevalence of HIV/AIDS and diabetes consequently increases the prevalence of superficial candidiasis as it is often observed in these patients (Martinez *et al*., 2013; Pfaller and Diekema, 2007).

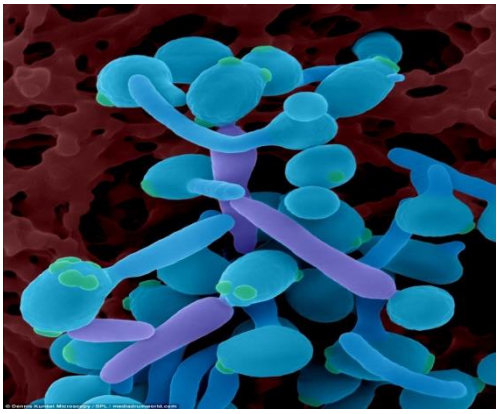

Figure 2.4: *Candida albicans* (Scanning electron microscopy)

(Source: https://tinyurl.com/y8tmkmke)

There are different types of candidiasis, the most common type in HIV patients being oropharyngeal candidiasis. Oropharyngeal candidiasis is so common among AIDS patients that its appearance used to be considered a marker of the development of AIDS in HIV-

positive individuals (Papon *et al*., 2013). Infants younger than a month are also at a higher risk of acquiring oropharyngeal candidiasis, the risk increases for premature babies and for HIV infected babies. A study done by Gaitan-Cepeada *et al* (2014) showed a prevalence of 79.1% for HIV infected children with oropharyngeal in Africa.

Other types of candidiasis include vulvovaginal, cutaneous and invasive candidiasis (Patil *et al*., 2015). Vulvovaginal candidiasis is frequently seen in adult women. It has a high prevalence of 75% meaning every 3 out of 4 women are likely to encounter an event of vulvovaginal candidiasis at least once in their lifetime (Armstrong *et al*., 2016). Cutaneous candidiasis usually affects the skin and nail and is commonly found in diabetics and obese people (Dabas, 2013). Invasive candidiasis is a highly lethal infection and is associated with mortality rates between 40 and 60 % (Calandra *et al*., 2016). Deep invasive infections may remain localised or may become systemic by spreading throughout the entire body. Mortality due to systemic candidiasis can be very high (Papon *et al*., 2013).

Antifungal compounds commonly used to treat candidiasis include polyenes, azoles and echinocandins (Mathe and Van Dijck, 2013). Due to the prolonged and frequent use of these drugs, resistance to the drugs have been reported, rendering *C. albicans* a challenging pathogen to health professionals (Vila *et al*., 2017).

2.2 Immunomodulation

2.2.1 Innate immune system

The immune system is a sophisticated defence system that is designed to protect the host from invading agents and pathogenic microorganisms. This is achieved through the different immune responses that occur in the human body namely the innate and adaptive immune response mechanisms (Rachh *et al*., 2014). The innate immune response is the first line of defence against physical, biochemical and cellular components invading the host. It is nonspecific and comes into play immediately or within hours of invasion (Iwasaki and Medzhitov, 2015). It comprises of host defences such as barrier function, cytokines and complement secretion, phagocytes and natural killer cells activation. Innate immune response is activated by the binding of pathogen-associated molecular pattern (PAMP) receptors found on the organism to pathogen recognition receptors (PRRs) on the host cells (Nagarathna *et al*., 2013). Cells belonging to this type of immunity include monocytes, macrophages, nuetrophils, dendritic cells and natural killer cells. Phagocytes (neutrophils, monocytes and macrophages), are important cells in the innate immune system as they are

the earliest cell types to respond to invasion by pathogenic organisms (Wynn *et al*., 2013).

2.2.2 Role of macrophages in the innate immune system

Monocytes and macrophages originate from a myeloid progenitor cell in the bone marrow. The monocytes differentiate into macrophages in response to differentiation factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and colony-stimulating factor-1 (CSF-1) (Ohradanova-Repic *et al*., 2016). Unlike monocytes which circulate for a short period of time in the bloodstream, macrophages are found in almost every organ and have a longer life span (Italiani and Boraschi, 2014). Macrophages are responsible for the recognition and clearance of pathogens and dead cells in the body. They achieve this through their three primary functions namely phagocytosis, antigen presentation and cytokine secretion (Yu, 2012). These cells are equipped with a broad range of pathogen recognition receptors such as the Toll like receptors (TLR) which help them recognize pathogens. Pathogens are recognised by PAMP receptors such as lipopolysaccharide (LPS). TLR-2 recognises Gram-positive bacteria and yeast whereas TLR-4 recognises gram negative bacteria (Parihar *et al.*, 2010).

Upon recognition of the pathogen by the macrophage, the pathogen is phagocytised. In this process, macrophages ingest the pathogen and trap it in a phagosome (Esteban *et al*., 2015). Lysosomes within the macrophage will fuse with the phagosome to form a phagolysosome. In the phagolysosome, the pathogen will be exposed to the toxic enzymes of the lysosome and will be digested into small molecules or antigens (Gordon, 2016). The antigens will be presented to helper T cells (CD4) by the macrophages. They are displayed on the surface of the macrophage next to a major histocompatibility complex (MHC) class II molecule. Antigen presentation triggers the adaptive immune response and will eventually result in the production of antibodies targeted against the specific pathogens leading to their destruction (Hume, 2008).

2.2.3 Cytokine secretion by macrophages

Macrophages activated in the presence of microbial products such as LPS are classified as M1 (Classically activated macrophages) whereas those activated by signals such as IL-4, IL-13, glucocorticoids and IL-10 are called M2 (alternatively activated macrophages) (Akinrinmade *et al.*, 2017). M1 macrophages have enhanced antimicrobial and inflammatory properties; they can produce abundant pro-inflammatory cytokines. M2 macrophages on the other hand have anti-inflammatory properties and also function in wound healing and tissue

repair (Gordon and Plüddemann, 2017). M1 macrophages secrete cytokines during an infection to induce inflammation and protect the body against the pathogens. The pro-inflammatory cytokines produced as mediators and inducers of inflammation include IL-6, IL-1β and TNF-α. These cytokines are involved in a broad range of biological processes which include cell proliferation, differentiation, apoptosis and coagulation (Rider *et al*., 2016).

IL-6 induces inflammation by interacting with IL-6 receptor alpha which is found on normal T-lymphocytes in the resting phase, normal activated B-cells and hepatic cells (Mauer *et al*., 2015). It stimulates the production of acute phase reactants such as C-reactive protein by the liver (Rider *et al*., 2016). This causes the onset of fever which can destroy pathogens since many pathogens cannot withstand high temperatures. IL-6 is also important in the maturation and differentiation of B-cells into plasma cells and promotes the production of antibodies particularly IgA and IgG (Duque and Descoteaux, 2014). Just like IL-6, small levels of IL-1β can evoke high fevers by enhancing the synthesis of prostaglandin E2 (PGE2) by the hypothalamus. IL-1β can also stimulate T cell proliferation and promote the release of histamine from mast cells at the site of inflammation. Histamine will increase the permeability of the capillaries to white blood cells allow them to engage pathogens in the infected tissues (Shaikh, 2011). TNF-α is one of the important cytokines secreted by activated macrophages. It shares some pro-inflammatory properties with IL-1β. This cytokine is able to induce fever, either directly or indirectly by stimulating secretion of IL-1β. In the liver, it stimulates the production of acute phase reactants. TNF-α also regulate the release of chemokines thus help in the recruitment of white blood cells to the inflammation site (Boshtam *et al.*, 2017).

Although pro-inflammatory cytokines are essential for inflammation (the response of tissue to injury), unregulated secretion of these cytokines is associated with harmful effects (Gulati *et al*., 2016). This can lead to organ dysfunction and complications such as rheumatoid arthritis, glomerulonephritis and some cancers. Therefore anti-inflammatory cytokines are secreted to inhibit the synthesis of pro-inflammatory cytokines and reduce inflammation (Wojdasiewicz *et al.*, 2014). However a balance must be kept between the secretion of pro and anti-inflammatory cytokines. Major anti-inflammatory cytokines include IL-4, IL-10, IL-11, and IL-13 (Malutan *et al*., 2014).

2.2.4 Adaptive immune system

The adaptive immune response, which is more complex than the innate system is more specific and has an element of memory. It is further divided into humoral and cell mediated immunity (Das *et al*., 2014). The humoral immunity involves the production of antibodies by

B-lymphocytes; this involves the killing of extracellular organisms. The cell-mediated immunity is mediated by T lymphocytes, which directly kill infected cells and abnormal tumour cells (Kumar *et al*., 2011). Failure of the immune system to function well leads to complications in the human body. The state in which one or more components of the immune system are inactive or not functioning optimally is known as immunodeficiency (Osingada *et al*., 2016). There are numerous factors that can contribute to immunodeficiency; these include malnutrition, viral infections (e.g. HIV), cancer, as well as use of particular medications such as chemotherapy and immunosuppressive drugs (McCusker and Warrington, 2011). All these conditions, especially HIV/AIDS that has a very high prevalence, can lead to immunosuppression thereby worsening the condition of the patients (Dube *et al*., 2017). Immunosuppression increases patients' risk of infection, delays wound healing and causes delayed recovery from a wide range of disease (Osingada *et al*., 2016).

2.2.5 Plants extracts as Immunomodulators

A large number of plant extracts have been shown to potentiate immunity. Research has been done proving that some medicinal plants exert anti-inflammatory, anti-stress and anti-cancer effects by modulating the immune system (Shukla, 2014). The immunomodulation of medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases. Immunomodulation refers to a process in which an immune response is altered to a desired level. Mukherjee *et al*. (2014) defined immunomodulation as the alteration of an immune response, which may increase or decrease the immune responsiveness. The magnification in the immune responsiveness is known as immunostimulation and the reduction in the immune responsiveness is known as immunosupression. According to Vaseeharan and Thaya (2014), immunostimulators are substances that stimulate the immune system by inducing activation or increasing activity of any of its components. Immunostimulators are used to boost the immune system under conditions of impaired immune responsiveness. Immunosupressors may be used in conditions such as autoimmunity and hypersensitivity (Mahima *et al*., 2012).

Although synthetic immunostimulators and immunosuppresors are available, they are a great disadvantage when it comes to their negative side effects and the associated complications such as increased risk of infections and toxicity (Arreola *et al*., 2015). Herbal medications are being preferred as they are believed to be free from side effects and toxicity and to be safer, since they are more natural. They are also more cost-effective, hence would be preferred over the synthetic drugs. It is therefore important to continuously find natural substances that can help increase the activity of the immune system when needed (Nagarathna *et al*., 2013).

For plants extracts that show immunomodulation activity, specific phytochemicals can be identified, extracted and can be developed into new drugs.

2.3 Medicinal plants

The history of traditional medicinal plant practice in Southern Africa is as old as the people who first settled in the region (Shumba *et al*., 2009). Medicinal plants and their derived medicines have been widely used in traditional cultures as a source of medicine for a variety of human ailments. Maroyi (2013) stated that 80% of the population in developing countries is still using medicinal plants to treat diseases. These ailments include wounds, coughs and colds, digestive problems, diabetes mellitus, ulcers, cancers as well as hypertension and asthma (Ncube *et al*., 2013).

Medicinal plants were found to possess many beneficial biological activities which include antimicrobial, anticancer, antioxidant, antihelminth, analgesic and wound healing activity (Sasidharan *et al*., 2011). Many medicinal plants have been reported to have wound healing activity and have been found useful in the treatment of wounds and skin infections (Nagori and Solanki, 2011). Due to the complications of drug resistance, toxicity and side effects encountered due to the use of modern synthetic drugs, the pharmaceutical industry has come to consider traditional medicine as an invaluable source for the identification of new bioactive agents that can be used as leads in the preparation of synthetic medicine (Gurib-Fakim and Kasilo, 2010). South Africa has a rich floral biodiversity encompassing approximately 4000 plants species with medicinal properties (Amabeoku *et al*., 2007; Van Wyk and Gericke, 2000). A lot of research is therefore being done worldwide on medicinal plants and their activities. Although a number of medicinal plants have been scientifically validated, there are still many medicinal plants to be studied.

Herbal medicines are advantageous over the conventional medicines in that they are safer, cheaper, environmental friendly and more acceptable since they are part of peoples' own culture (Mahima *et al*., 2012).

Table 2.1: Some South African plants used for bacterial skin infections

Plant name	Traditional use	Bacteria tested	References
Carpobrotus edulis	• soothing lotion for burns, bruises, ringworm and eczema	*S. aureus* *E. coli* *P. aeruginosa*	(Ordway *et al*., 2003).
Elephantorrhiza elephantina	• treat acne and other skin diseases	*S. aureus* *P. aeruginosa* *C. albicans* *S. epidermidis*	(Mabona *et al*., 2013).
Glycyrrhiza glabra	• eczema • relieves itching and swelling.	*S. aureus* *E. coli* *P. aeruginosa* *C. albicans*	(Dinesh *et al.*, 2012).
Leonotis leonurus	• treating snakebites • soothe diseases such as itchy skin • wound healing	*B. cereus* *S. aureus* *E. coli* *P. aeruginosa* *S. epidermidis*	(Hurinanthan, 2009).
Hypericum perforatum	• psoriasis • first degree burns • wounds	*S. aureus* *E. coli* *P. aeruginosa* *E. faecalis*	(Prakash *et al*., 2010). (Zeb-Saddiqe *et al*., 2010).
Withania somnifera	• inflamed wounds • abscesses		(Girish *et al*., 2006).
Cissampelos capensis	• Heal wounds and sores, including venereal lesions and snakebite.	*S.aureus*, *S. pyogenes*, *E. coli*, *P. vulgaris* *C. albicans*	(Semwal *et al*., 2014).
Ziziphus mucronata	• boils • swollen glands • wounds and sores	*S. aureus* *E. coli* *C. albicans* *C. neoformans*	(Olajuyigbe and Afolayan 2012).
Kigelia africana	• infections • wounds and sores • ulcers	*E. coli* *P. aeruginosa* *S. aureus* *B. subtilis*	(Agyare *et al*., 2013).
Hypoxis hemerocallidea	• dizziness • burns • wounds • diabetes mellitus • cancer • hypertension • asthma	*S. aureus* *P. aeruginosa* *C. albicans* *E. faecalis* *E. coli*	(Katerere and Eloff, 2008). (Ncube *et al*., 2013).
Aspalathus linearis (Rooibos)	• stomach, and digestive problems • skin problems		(McKay and Blumberg, 2007).
Galenia africana	• asthma • coughs • wounds • eye infections • TB • skin diseases • relieves toothache	*M. tuberculosis* *E. feacalis* *C. neoformans*	(Pool *et al*., 2009). (Mativandlela *et al*., 2009).

2.3.1 *Cotyledon orbiculata*

2.3.1.1 History

Cotyledon orbiculata is one of the important medicinal plants of South Africa. It is a small shrub with fleshy leaves and is locally known as “Seredile” in Sotho and Tswana, “Plakkie” in Afrikaans and “Imphewula” in Xhosa. It is often referred to as pig’s ears because of its oval shaped grey-green leaves, which are very variable with red or pale margins (Rowley, 2007). The genus name *Cotyledon* was derived from the Greek word *kotyledon,* which means “cup-shaped hollow” referring to the shape of the plant leaves. The specie name *orbiculata* was from a Latin word meaning, round. This succulent plant under the *Crassulaceae* family is native to South Africa in which it is mostly distributed in the Western, Eastern and Northern Cape Provinces. *Cotyledons* commonly colonise rocky grounds, indicating that they highly favour well-drained conditions in cultivation (Mort *et al*., 2005).

Cotyledon is a small genus consisting of 10 species that are distributed primarily in South Africa. The species of *Cotyledon* include *C. orbiculata, C. velutina, C. woodii, C. barbeyi, C. adscendens, C. tomentosa, C. cuneata, C.papillaris, C. elisseae, C. campanulata* (Mort *et al*., 2005). Amongst these species *C. orbiculata* is notable for its wide distribution and diversity. It was divided into five varieties based on distribution, growth form, flower size, shape, and colour as well as leaf morphology. These varieties are var. *orbiculata,* var. *oblonga,* var. *dactylopsis,* var. *flanaganii* and var. *spuria.* The varieties *C. orbiculata* var. *orbiculata* and *C. orbiculata* var. *oblonga*, are widely distributed in southern Africa in countries such as Namibia. The other three *C. orbiculata* varieties are more narrowly distributed and are present only in South Africa (Nowel, 2008; Rowley, 2007).

Figure 2.5: *Cotyledon orbiculata*

(Source: https://it.pinterest.com/pin/322922235757396119/)

2.3.1.2 Medicinal uses

C. orbiculata has been used traditionally to treat various ailments in different parts of South Africa. The fleshy leaves have been applied to corns and warts and have been said to soften the hard tissue if left in place (Terblanche *et al*., 2017). The heated leaves were used as a poultice for boils and other accessible inflammations (Aremu *et al*., 2010). The leaf juice was used to treat earache, toothache and acne (when applied as a lotion). The leaves could also be taken orally for the treatment of epilepsy (Kumari *et al*., 2016).

2.3.1.3 Bioactivity and chemical composition

C. orbiculata comprises of a number of compounds. The most common ones being bufanolides (Iwalewa *et al*., 2007), however according to Molefe *et al* (2013), alkaloids, saponins, cardiac glycosides, tannins, reducing sugars, anthraquinones, triterpene steroids and flavonoids are also found in the plant. These compounds are responsible for the characteristics and the bioactivity of the plant.

C. orbiculata is known for its anticonvulsant activity (Abubakar *et al*., 2013). It is possible that the saponin, which may be of triterpenoid type and the triterpene steroid present in *C. orbiculata,* might contribute to the anticonvulsant activity of the plant (Amabeoku *et al*., 2007). The plant also has antinociceptive and anti-inflammatory activities, which may be attributed to the plant compounds inhibiting various chemical inflammatory mediators such as

prostaglandins and bradykinin. Saponins have been reported to have analgesic and anti-inflammatory properties; hence they contribute to the antinociceptive and anti-inflammatory activities of *C. orbiculata* (Amabeoku and Kabatende, 2012).

C. orbiculata has been shown to have antioxidant activities. The methanol extract of *C. orbiculata* has high antioxidant effects through a wide concentration range (Roux, 2012). *C. orbiculata* also have some antibacterial activities. Studies done by Kumari *et al* (2016) show that the ethanolic extracts of the plants have antibacterial activity against the bacteria, *E. faecalis, M. luteus, S. aureus, E. coli, K. pneumonia* and *P. aeruginosa.* A literature search has indicated that not much research has been done on this plant; therefore there is still little known about the antifungal and immunomodulatory properties of this plant.

C. orbiculata may however be poisonous to animals due to chronic bufadienolide poisoning. The bufadienolides have a neurotoxic effect that produces a syndrome known as 'krimpsiekte' or shrinking disease (Botha, 2013). This syndrome usually affects livestock predominantly in goats and sheep. The severity of the syndrome depends on a number of factors, which include dose, duration of exposure and the bufadienolides involved. Consumption of large amounts of the plants in a short period of time by the animals may lead to sudden death or an acute syndrome including paralysis of the tongue and depression may develop (Roux, 2012). The chronic form of poisoning characterised by low but continuous plant intake causes affected animals to lag behind the flock. Affected animals tire quickly and they assume the characteristic 'shrinking' posture hence the name shrinking disease (Botha and Penrith, 2008).

2.4 Green nanotechnology

2.4.1 Introduction

The growth of research and applications in the area of nanoscience and nanotechnology has markedly increased in recent years. Nanotechnology is a field of science which deals with the synthesis, development and use of materials of nanometer in size (Kavitha *et al.*, 2013). It is defined as the engineering of functional systems at the molecular scale that is less than 100 nanometres (Heera and Shanmugam, 2015). This technology has the potential to revolutionise medicine by delivering improved methods for disease diagnostics and therapeutics. Due to their small size, nanoparticles have a very high drug loading capacity, which implies that materials can deliver drugs more efficiently (Athar and Das, 2014). These particles have diverse applications in different fields including the biology and biomedicine,

electronics, physics, material science, agriculture and environmental remediation. The popularity of nanoparticles is attributable to their unique properties such as their large surface to mass ratio which increase their functionality and ability to adsorb and carry other compounds (Banerjee *et al*., 2014). Green nanotechnology is a fairly new branch of nanotechnology, which aims to synthesis nanomaterials that are more bio-friendly, using methods that are safer for the environment. Aside from the fact that the development of such synthesis methods can be beneficial to the environment, nanomaterials produced in this fashion can also be more suitable for applications in biological systems (Malik *et al*., 2014).

The antibacterial properties of silver have been known since the late 1800's (Politano *et al*., 2013) and several studies have demonstrated the medicinal properties of silver nanoparticles (or colloidal silver) (Ge *et al*., 2014). A common chemical synthesis method for silver nanoparticles is the reduction of silver nitrate ($AgNO_3$) using tri-sodium citrate (Rashid *et al*., 2013). Several studies have shown that plant extracts can be used in biosynthesis methods to produce silver nanoparticles (Ahmed *et al*., 2016). These nanomaterials are often evaluated for their antibacterial activities. The synthesis of silver nanoparticles from plants with known antibacterial activities has the potential to produce antimicrobial agents that are exponentially more toxic to the microorganisms than the silver nanoparticles or plant extracts alone. Such nanoparticles could possibly provide us with novel antimicrobial agents that can address multidrug resistance.

2.4.2 Applications and promises of nanotechnology in medicine

Nanotechnology promises numerous developments in the health industry. There is increasing optimism that it will yield great advances in the early detection, diagnosis and treatment of diseases (Azzawi *et al*., 2016; De Jong and Borm, 2008). Because of their small size and increased surface area, nanoparticles can adsorb and carry other molecules such as proteins, drugs, probes and nucleic material effectively (Abhilash, 2010). Nanoparticles can be applied in site specific drug delivery and targeted therapy. Effective treatment to cancer or any disease requires the drug to get specifically to the target and exert its effect on it. This active targeting can be achieved by the functionalization of nanoparticles with ligands such as antibodies and peptides (Zhong *et al*., 2014; Gu *et al*., 2007). Site specific drug targeting will significantly reduce side effects as the drug will be deposited mostly on the diseased region. The drug will therefore be less toxic while maintaining its therapeutic effects (Ingale and Chaudhari, 2013).

Nanomaterial can also be applied in tissue engineering were it helps reproduce or repair

damaged tissues especially in organ transplants or artificial implants. In artificial bone-implants nanoparticles can reduce the chances of rejection and stimulate the production of osteoblasts (Singh, 2016). They can also be used to promote the process of wound healing in the different tissues. Nanotechnology can help in MRI contrast enhancement and also in the treatment of different diseases. They can be used in the treatment of neuro-degenerative disorders such as Parkinson's disease and Alzheimer's diseases as well as in treatment of some infections such as tuberculosis and HIV infections (Nikalje, 2015).

Nanotechnology can also be used for faster development of new and safe medicines. They can deliver new antimicrobial agents which can help overcome the issue of antimicrobial resistance. Banoe *et al* (2010) reported that zinc oxide nanoparticles can decrease the antibiotic resistance and enhance the antibacterial activity of ciprofloxacin against microorganisms. Silver has always been a good antimicrobial agent, production of silver nanoparticles will increase the efficacy of silver hence will provide an efficient and excellent antimicrobial agent (Pulit-Prociak and Banach, 2016).

2.4.3 Metallic nanoparticles

There are many types of nanoparticles which include liposomes, polymeric nanoparticles, nanocrystal, dendrimer, solid lipid nanoparticles and metallic nanoparticles (Desai, 2012). Metallic nanoparticles are one of the most common nanoparticles being used worldwide in different applications and research. These are nanoparticles produced from metals such as silver, aluminium, gold, zinc, carbon, titanium, palladium, iron and copper (Vadlapudi *et al*., 2013). The phenomenon of surface plasmon resonance (SPR) is used to confirm the presence of metal nanoparticles on the UV-vis machine. It occurs when an incident light beam strikes the surface of a metal resulting in a reduction in the intensity of the reflected light. The SPR makes it possible to measure the adsorption of molecules on the metal surfaces (Englebienne *et al*., 2003).

Metallic nanoparticles are useful in targeted therapy, drug delivery and in the diagnosis and treatment of various diseases. Iron nanoparticles are used in the treatment of cancer particularly Iron oxide nanoparticles which can be used for tumour treatment by magnetically induced hyperthermia (Lehner, 2013). Gold nanoparticles are also widely used in the treatment of cancer and in the delivery of molecules such as peptides, proteins and nucleic acids. When functionalised with quaternary ammonium groups, they can protect nucleic acids from enzymatic degradation (Suryaprakash, 2016). Metallic nanoparticles can also be used in diagnostics as probes to detect biomarkers in human blood. An example is gold

nanoparticles which can be used to detect carcinoembryonic antigen and alpha foetal protein, markers to breast and lung cancer (Islam and Uddin, 2017). Silver is one of the important metals in nanotechnology that have been routinely used for the synthesis of nanoparticles.

2.4.4 Silver nanoparticles

Silver has been used for a long time, since around the late 1800's (Luoma, 2013). Some of its numerous uses include the making and coating of coins and cutlery. This is because silver is a corrosion resistant metal. Its use is also popular in cosmetics and in environmental products such as antibacterial paints, disinfectants and pesticides (Chen *et al*., 2016). Because of its antibacterial properties, silver is widely used in the medical field. It is used as a coating for medical catheters (urinary, venous, drainage catheters) as well as surgical blades and needles to prevent contamination and infections. Silver have also been applied in the treatment of burn wounds (as silver sulfadiazine), diabetic skin ulcers and newborn eye infections (Konop *et al*., 2016). The synthesis of silver nanoparticles has become popular and their applications in different fields are increasing.

Silver nanoparticles are expected to enhance the effects of the silver metal. This is due to the small sizes of the nanoparticles which provide a larger surface area for the particles hence increasing their activity (Ahmed *et al*., 2016). The antimicrobial effects of silver nanoparticles have been determined by many researchers and they have been found to be effective against many Gram-positive and Gram-negative bacteria as well as fungi and viruses (Dakal *et al.*, 2016). The action of these nanoparticles against microorganisms involves a number of interlinked mechanisms. Silver nanoparticles adhere to the cell wall of the microorganism, penetrate it and cause structural changes in the cell membrane (Prabhu and Poulose, 2012). They can also penetrate the cell and cause damage to the intracellular structures such as ribosomes, mitochrondria, proteins and DNA. These nanoparticles also have the ability to induce oxidative stress by the generation of reactive oxygen species and free radicals in the cell. Some studies have also shown that silver nanoparticles are anti-inflammatory in nature and they can help in wound healing (Midha *et al*., 2015). Because of their satisfactory activity and the problem of antibiotic drug resistance, silver nanoparticles have become a promising alternate to antibiotic therapy.

2.4.5 Synthesis of nanoparticles

Two main processes are involved in the synthesis of nanoparticles regardless of the field or type of nanoparticle being synthesized. These include the bottom up approach which

involves the synthesis of nanostructures from smaller building blocks namely atoms and molecules (Grigore *et al*., 2016). In this approach, atoms are manipulated chemically or physically to self-assemble and form new nuclei which will in turn grow into a particle of nanoscale (nanoparticle) (Ochekpe *et al.,* 2009). The second process, the top down approach is the exact opposite of the bottom up approach. It involves the breakdown of bulk material into nanostructures with various lithographic techniques such as grinding and milling (Kavitha *et al.*, 2013).

2.4.5.1 Physical and chemical synthesis

Nanoparticles can be synthesized through physical, chemically and biological ways (Patra and Baek, 2014). Evaporation-condensation and laser ablation are the most important physical approaches. These techniques use a furnace tube under atmospheric pressure to produce nanoparticles (Iravani *et al*., 2014). The advantage of using this approach over chemical methods is that there is no solvent contamination in the prepared nanoparticles. However there are great disadvantages associated with this method and these include high energy consumption, large space occupied by the tube furnace and the long amount of time that is required to achieve thermal stability (Gudikandula and Maringanti, 2017).

Many chemical methods can also be used in the used in the synthesis of nanoparticles. The most popular chemical way of synthesizing nanoparticles is chemical reduction. It involves three main components namely the salt or acid specific to the type of nanoparticle being synthesized (for example silver nitrate is used for the synthesis of silver nanoparticles), reducing agents and capping agents (Rhashid *et al.*, 2013). Upon synthesis of nanoparticles, capping agents have to be added to stabilise the size and morphology of the nanoparticle as well as to protect the surface of the nanoparticle from aggregation (agglomeration). Polymers, surfactants and charged molecules are some of the capping agents that are used. These chemicals (reducing and capping agents) have high activity however they are toxic, hazardous and dangerous to the environment (Firdhouse and Lalitha, 2015). As a results, the nanoparticles produced using this method have limited applications in biomedicine because of their toxicity.

The main limitation with the chemical and physical methods of nanoparticle synthesis is that they are extremely costly and they involve the use of toxic chemicals and solvents which are unsafe for the environment. They are also difficult to upscale as this will be further increasing the cost and the environmental damage (Iravani *et al*., 2014). Therefore scientists are

exploring green methods of synthesizing nanoparticles as they eliminate most of the above mentioned limitations.

2.4.5.2 Biological (green) synthesis

Because of the limitations of chemical and physical methods for the synthesis of nanoparticles, biological or green processes were discovered. These are environmental friendly process of synthesizing nanoparticles mainly using plants and microorganisms (Shelar and Chavan, 2014). Much attention has been given to green nanotechnology because of its advantages which include, low-toxicity, easy and rapid synthesis, reproducibility and well defined morphology and controllable sizes of the synthesized nanoparticles (Shah *et al*., 2015). Many studies have shown that microorganisms such as actinomyces, fungi and yeast possess the ability to synthesize metallic nanoparticles. This is achieved by their ability to accumulate and detoxify heavy metals due to the presence of various reductase enzymes, which reduce metal salts to metal nanoparticles (Pantidos and Horsfall, 2014).

Plants however are more advantageous than microorganisms in the synthesis of metallic nanoparticles. Microorganisms can be toxic and can potentially cause infections (Rahman *et al*., 2011). Plants are non-pathogenic, easier to upscale, cheaper and more readily available than microorganisms. Furthermore nanoparticles synthesis with plants is faster than with microorganisms. This is because they do not need complex and time consuming procedures such as microbial sampling, isolation, culturing, and maintenance (Song and Kim, 2009). In the synthesis of nanoparticles, plants acts as both reducing and capping agents and uses water as a solvent thus greatly avoiding the use of toxic chemical reducing and capping agents. The specific components responsible for synthesis of nanoparticles in plants are unclear. However bioactive phytochemicals of the plants such as alkaloids, phenolic acids, polyphenols, proteins, sugars, and terpenoids are believed to have significant roles in the reduction of the metal salt and in the stabilization of synthesized nanoparticles (Singh *et al*., 2015). The bioactive phytochemicals in plant extracts can therefore be extracted and used directly in the synthesis of nanoparticles. This can also be done in microorganisms where specific compounds can be extracted for the synthesis of nanoparticles. Fig 2.6 shows the diagrammatic presentation of silver nanoparticles synthesis using plants. In this simple process, the plant extract containing the phytochemicals is mixed with the metal (silver) ions in solution and the silver nanoparticles are produced.

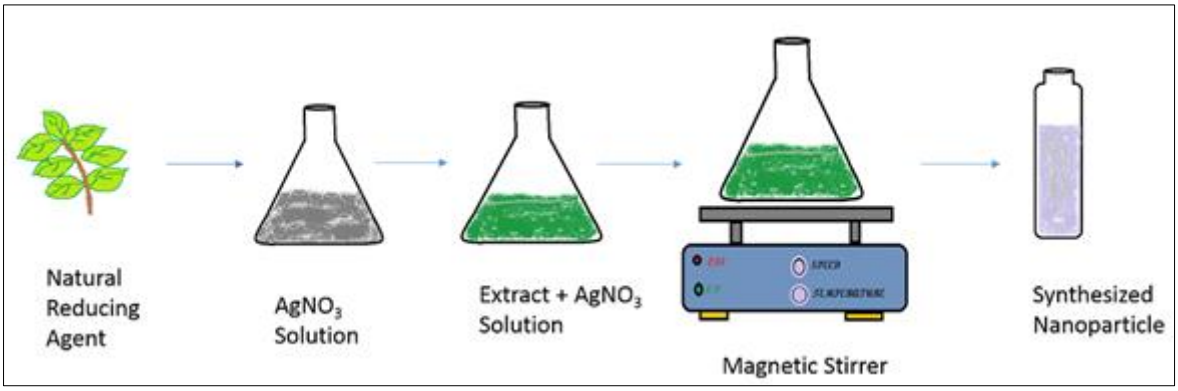

Figure 2.6: Biological synthesis of silver nanoparticles using plants

(Source: http://www.pharmatutor.org/articles/bionanoparticles-green-nanochemical-approach).

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CHAPTER THREE
SYNTHESIS AND CHARACTERISATION OF SILVER NANOPARTICLES FROM *COTYLEDON ORBICULATA*.

3.1 Abstract

The aim of this study was to determine if the aqueous extracts of *Cotyledon orbiculata* var *orbiculata* (*C. orbiculata*) are able to produce silver nanoparticles. Optimisation of silver nanoparticle synthesis using this plant was done at different conditions. These include; temperature (25 and 70°C), silver nitrate concentration (1 and 3mM), plant extract concentration (1.5, 3 and 6mg/ml) and time. Various techniques such as the UV-vis spectrophotometer, Dynamic Light Scattering (DLS) and High Resolution Transmission Electron Microscopy (HR-TEM) were used to characterize the nanoparticles. The results obtained shows that *C. orbiculata* is able to synthesize silver nanoparticles. The optimal conditions for the synthesis of silver nanoparticles using *C. orbiculata* were using 3mg/ml plant extract and 3mM silver nitrate concentration at 70°C while stirring for 2 hours. The UV-vis spectrum revealed a characteristic surface plasmon resonance peak at 420nm. Characterisation using HR-TEM showed the presence of spherical nanoparticles with sizes ranging from 20-40nm. These nanoparticles were proven to be silver nanoparticles by Energy Dispersive X-ray spectra (EDX) and their crystalline nature was shown by the Selected Area Diffraction (SAED) and the presence of lattice fringes.

Keywords

Cotyledon orbiculata, Silver nanoparticles, Characterisation, Synthesis and Spectrophotometer.

3.2 Introduction

Green nanotechnology is gaining much attention in the field of nanotechnology. This is because it is a safe and environmental friendly method to synthesize nanoparticles (Malik *et al*., 2014). Among metal nanoparticles (gold, copper, platinum and titanium), silver nanoparticles are greatly known for their excellent antibacterial activities (Konop *et al*., 2016). Many plant extracts have therefore been used in the synthesis of silver nanoparticles. Plants such as *Capparis spinosa, Datura stramonium, Ocimum tenuiflorum, Solanum tricobatum, Syzygium cumini, Centella asiatica, Citrus sinensis* and *Musa paradisiacal* have been used to synthesize silver nanoparticles (Gomathi *et al*., 2017; Benakashani *et al*., 2016; Logeswari *et al*., 2015; Ibrahim, 2015). Although many plants have been used in the synthesis of silver nanoparticles, the ability of some plant extracts to produce nanoparticles is still unknown. One of these plants is *C. orbiculata*, a succulent plant indigenous to South Africa. This plant is used traditionally as a source of medicine to treat skin diseases such as inflammation, boils and acne (Aremu *et al*., 2010). Silver nanoparticles synthesized using *C. orbiculata* plant extract can potentially have higher activity than the plant extract or the chemically produced silver nanoparticles. However, despite its popularity, its ability to synthesize nanoparticles is unknown. This study will therefore focus on the ability of *C. orbiculata* to synthesize silver nanoparticles.

The quality and quantity of synthesized nanoparticles is greatly affected by several synthesis parameters such as reaction time, reaction temperature, silver nitrate concentration and plant extract concentration (Patra and Baek, 2014). These parameters however can be regulated in different ways in-order to optimise the synthesis of nanoparticles. Due to the huge diversity of plants and their components it is important to optimise the synthesis of nanoparticles with respect to each specific plant and the type of nanoparticle being synthesized.

3.3 Methodology

3.3.1 Plant material

Fresh *C. orbiculata* plants were purchased from Van Der Berg Garden Village nursery in Stellenbosch, Cape Town. Before extraction, the plants were kept in their vases and maintained to keep them fresh.

3.3.2 Plant extracts preparation

Just before the process of extraction, the *C. orbiculata* leaves were cut from the plants. They were washed with tap water to remove insects and dirt; they were then rinsed with distilled

water and dried with paper towel. The fresh leaves (300g) were cut into small pieces and blended using a kitchen blender.

Extraction was done using the maceration method. The blended leaves were macerated in 600ml of distilled water overnight. There after the extracts were filtered using a Whatman filter paper no.1 followed by a 0.45µm syringe and then concentrated. Concentration was done by freeze drying the extracts. The dried extract powders were stored at 4°C until use.

3.3.3 Synthesis of silver nanoparticles

Aqua regia was used for cleaning purposes. It was made by adding nitric acid to hydrochloric acid in a volume ratio of 1:3. All glassware cleaned with aqua regia was rinsed thoroughly with distilled water and autoclaved before use. Silver nitrate was purchased from ACE chemicals. For the synthesis of silver nanoparticles, two concentrations of silver nitrate solution (1mM and 3mM) were prepared. Silver nitrate solution (5ml) was mixed with 1ml of the different concentrations of plant extract in glass tubes. The mixtures were incubated separately under stirring at 25°C (room temperature) or 70°C for different time intervals. The tubes were covered with foil to protect the silver nitrate from light. The synthesized nanoparticles were purified by centrifugation at 10 000rpm (Centrifuge 5417R (Eppendorf AG, Hamburg, Germany) for 10 minutes, discarding the supernatant and re-suspending the pellet in distilled water. This process was repeated three times. The reason for this is to remove excess plant material and phytochemicals.

3.3.4 Stability testing of silver nanoparticles

The stability of the synthesized silver nanoparticles in different biological media was evaluated. The media tested included Roswell Park Memorial Institute medium (RPMI) (cell culture medium), Muller-Hinton broth (MHB), Yeast Peptone broth (YPB) and LB broth (Luria-Bertani broth) (culture medium for microorganisms) and Phosphate-buffered saline (PBS). The assay was done immediately after the synthesised nanoparticles were purified. In glass test tubes 250µl of aqueous silver nanoparticles were mixed with the same volume of medium or buffer. The tubes were incubated at 25°C or 37°C for 0.5 to 3hours. The stability of these nanoparticles was evaluated by measuring the changes in the UV-Vis spectra.

3.3.5 Characterisation of silver nanoparticles

3.3.5.1 UV-Visible spectroscopy

The formation of silver nanoparticles was confirmed by the UV-Visible spectroscopy (POLARstar Omega microplate reader) at a wavelength range of 250nm – 800nm. This was done by aliquoting 300µl of the silver nanoparticles into the wells of a 96-well microtiter plate. The 96-well microtiter plate was carefully placed in a microplate reader and the surface plasmon resonance (SPR) for the silver nanoparticles was observed.

3.3.5.2 Dynamic Light Scattering

Dynamic Light Scattering (DLS) (Zetasizer malvern) was used to determine the particle size distribution, Poly Dispersity Index (PDI) and zeta potential of the nanoparticles. The synthesized and purified nanoparticles were placed in clean cuvettes. The cuvettes were wiped from outside and were inserted into the machine (Zetasizer malvern). The size and zeta potential readings of the nanoparticles were recorded. Polystyrene cuvettes were used for size measurement and the Disposable Capillary Cell (DTS1070) cuvettes were used for zeta potential measurement.

3.3.5.3 High-resolution transmission electron microscopy

The morphology and the mean particle size were determined by High-resolution transmission electron microscopy (HRTEM) analysis (FEI Tecnai G2 20 field-emission gun). For the preparation of HR-TEM, aqueous silver nanoparticles were loaded onto a carbon coated copper grid, the grid was dried under a Xenon lamp for 10 min and was analysed under the microscope. The mean particle size was determined using the Image J software. Selected Area Diffraction (SEAD) and Energy dispersive X-ray spectra (EDX) were also analysed on the same samples using TEM. SAED is used to characterize the crystalline nature of particles and EDX gives the qualitative and quantitative status of the elements involved in formation of nanoparticles.

3.4 Results

3.4.1 Optimisation of silver nanoparticle synthesis

Different plants differ in their phytochemical composition and therefore their ability to synthesize nanoparticles also differs. It is therefore imperative to optimise the synthesis of nanoparticles pertaining to the specific plant extracts. The synthesis of silver nanoparticles from *C. orbiculata* water extract was optimised by varying different parameters such as reaction temperature, reaction time and concentration of both silver nitrate and plant extracts.

The synthesis of nanoparticles was first observed by the colour change of the solution from colourless to yellow then brown as shown in Fig 3.1. Nanoparticles were synthesized at two different temperatures (25°C and 70°C). No colour change was observed at 25°C for all samples, suggesting that no nanoparticles were synthesized at this temperature. Synthesis of silver nanoparticles was therefore done at 70°C.

For the optimisation of synthesis with regards to silver nitrate concentration, two concentrations of silver nitrate (1mM and 3mM) were used. Of these two concentrations, 3mM produced nanoparticles with a more intense brown colour and had smooth and higher absorbance curves as compared to 1mM. The UV-vis results are represented in Fig 3.2. Subsequent nanoparticle synthesis was therefore done using 3mM silver nitrate. The effect of the concentration of the plant extract was also evaluated; the concentrations of plant extract used were 48, 24, 12, 6, 3 and 1.5mg/ml. No colour change was observed in the 3 highest concentrations (48, 24, 12mg/ml). Colour change was first observed in 6mg/ml nanoparticles after 15mins of synthesis. The synthesis of nanoparticles by the 3 lowest concentrations (6, 3 and 1.5mg/ml) was confirmed by the UV-vis spectroscopy and the results are shown in Fig 3.3.

To optimise the reaction time, nanoparticles were synthesized until the there was no further colour change of the solution. Synthesis was also stopped when the UV-vis absorbance curves become rough and uneven as shown in Fig 3.3A and B at 3hours and in Fig 3.3C at 2 hours. This suggested complete reduction of the silver nitrate and complete synthesis of the silver nanoparticles. The silver nanoparticles were characterised by UV-vis at a wavelength range of 250-800nm. This instrument is commonly used to characterise the optical properties of metal nanoparticles. The presence of a UV-vis spectrum in the SPR range of 400-500nm confirms the presence of silver nanoparticles (Ashraf *et al.*, 2016).

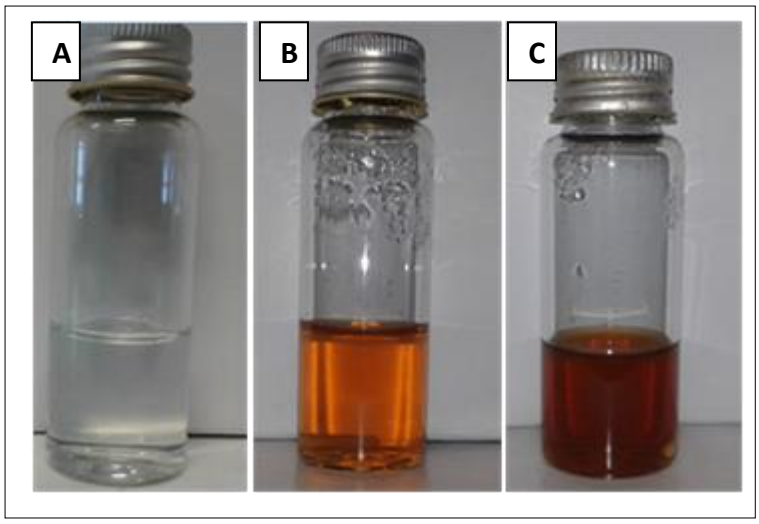

Figure 3.1: Change in colour intensity over time during silver nanoparticle synthesis

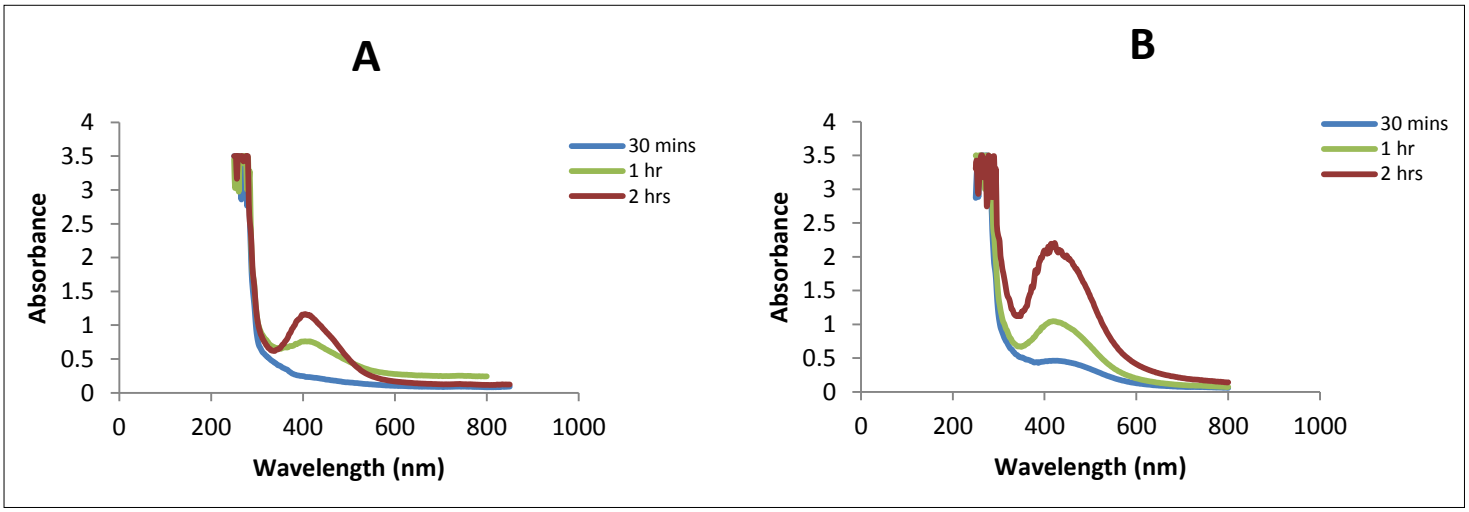

Figure 3.2: A comparison of the UV- vis absorption spectra of silver nanoparticles synthesised using two different $AgNO_3$ concentrations

A represents synthesis using 1mM $AgNO_3$ solution and **B** represents synthesis using 3mM $AgNO_3$ solution.

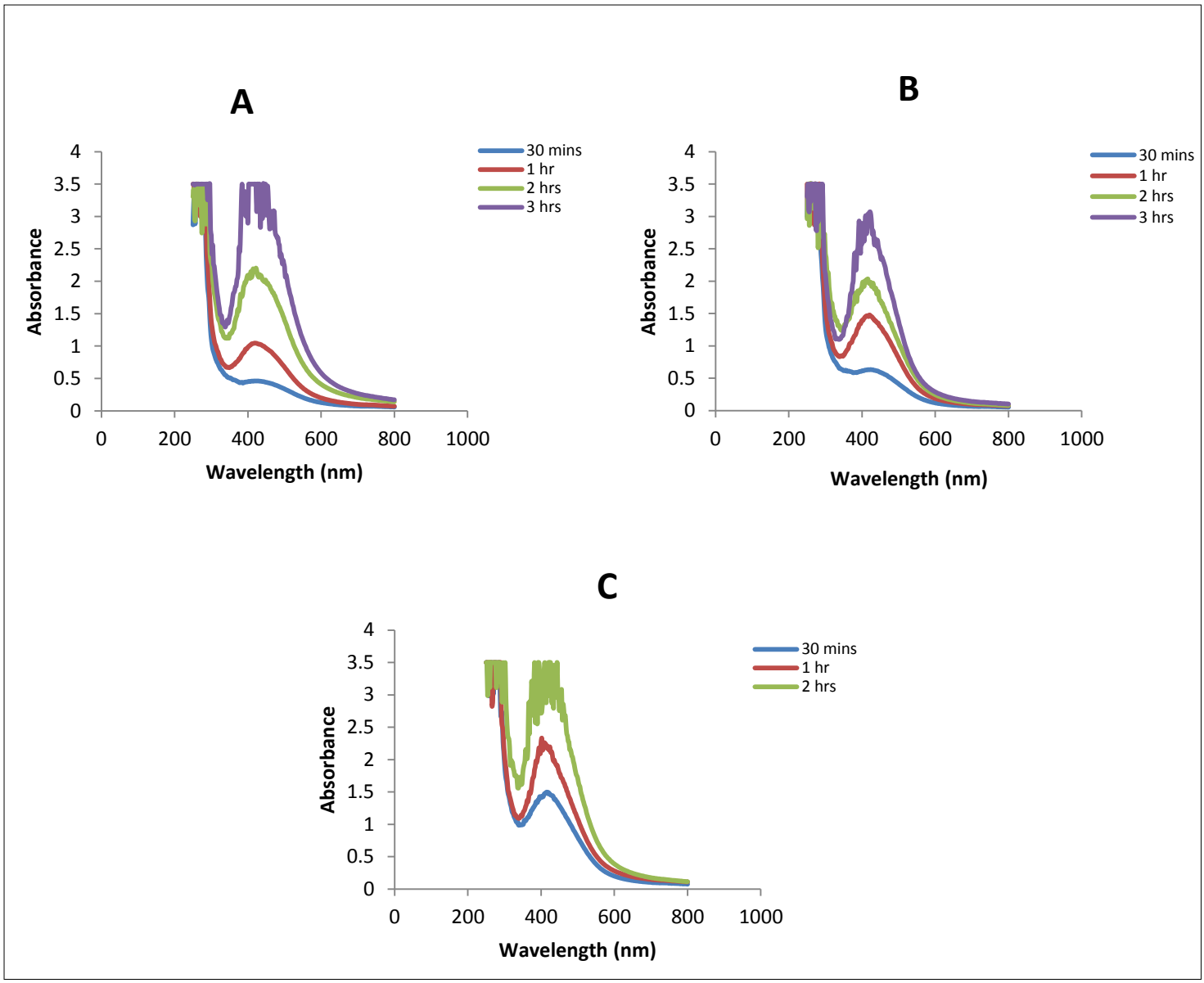

Figure 3.3: Time dependant changes in the UV- vis absorption spectra of silver nanoparticles overtime, synthesized at 3 different concentrations of *C. orbiculata* plant extract

A represents synthesis at 1.5mg/ml, **B** represents synthesis at 3mg/ml and **C** represents synthesis at 6mg/ml of the plant extract.

3.4.2 Dynamic Light Scattering

The DLS was used to measure three other important properties of the nanoparticles. These are size distribution, zeta potential and Poly Dispersity Index (PDI). Zeta potential measures the charge of the particles however it can also be used to determine if the nanoparticles are likely to be stable. The charge is a reflection of the repulsion forces between the particles, larger charges signify more stable particles (Chanda *et al*., 2010). PDI is another property that can be determined by the DLS. The PDI is a measure of the width of molecular weight distribution; it measures the distribution of particles with a value from 0 to 1. A sample with a PDI of less than 0.2 is considered to be monodispersed whereas PDI values of 0.7 suggests a broad size distribution of the particles (Mazzonello *et al*., 2017). Table 3.1 shows the zeta potential, PDI and size distribution of the synthesized silver nanoparticles. All the nanoparticles had PDIs of less than 0.2 and an average zeta potential charge of -19mV.

Table 3.1: Size, poly dispersity index (PDI) and zeta potential of the silver nanoparticles synthesized using different concentrations of *C. orbiculata* extract at 70°C for 2 hours using 3mM silver nitrate

Plant extract concentration	**6mg/ml**		**3mg/ml**		**1.5mg/ml**	
Average size	106nm		110nm		137nm	
Peak 1 (size and % Intensity)	201nm	17%	210nm	22.1%	230nm	27.7%
Peak 2 (size and % Intensity)	45nm	83%	57nm	77.9%	66nm	72.3%
PDI	0.07		0.15		0.1	
Zeta potential	-19.1		-19.5		-18.2	

3.4.3 Stability of the nanoparticles

Stability is a crucial aspect of nanoparticles. Nanoparticles need to remain stable in different environments in-order to maintain their properties and successfully execute their potential application. A loss in their stability can result in them aggregating which will consequently lead to the loss of their properties (Gambinossi *et al*., 2015). Aggregation of silver nanoparticles can also result in an increase of their cytotoxicity which can adversely affect their suitability for applications in therapeutics (Gilbert *et al*., 2009; Wick *et al*., 2007). Evaluating the stability of the nanoparticles in biological media before exposing them to cells and microorganisms or before in-vivo application is therefore of great importance as it

ensures accurate results and conclusions.

The stability of the *C. orbiculata* silver nanoparticles in biological media was determined using Roswell Park Memorial Institute medium (RPMI), Muller-Hinton broth (MHB), Yeast Peptone broth (YPB), Luria-Bertani (LB) and Phosphate-buffered saline (PBS). The stability of the silver nanoparticles in the respective media was evaluated by measuring the changes in UV-Vis spectra after 1, 3, 5, 20 and 24 hours. Of the three concentrations of *C. orbiculata* used for the synthesis of nanoparticles, 3mg/ml produced nanoparticles that were fairly stable in RPMI, MHB, YPB and PBS. The SPR characteristics of these nanoparticles did not change throughout the time of incubation in the biological media. The UV-vis spectra showing stability of the silver nanoparticles (synthesised using 3mg/ml *C. orbiculata* extract) at 25°C and 37°C is shown in Fig 3.4 and 3.5, respectively. Stability of the nanoparticles was evaluated at 25°C and 37°C because incubation in most biological assays is done at these temperatures. The nanoparticles also need to be stable at 37°C (body temperature) to effectively execute their use in therapeutics.

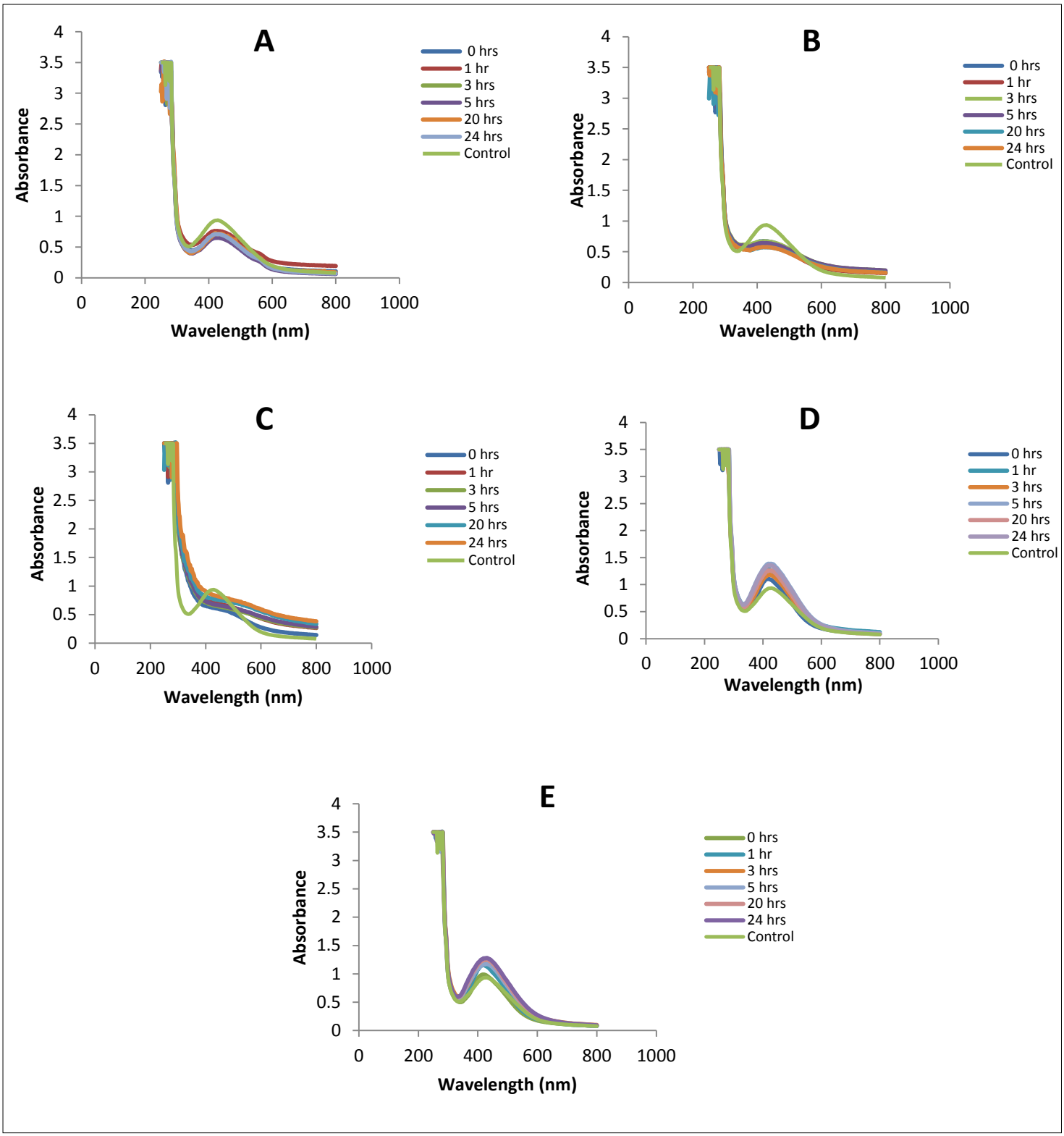

Figure 3.4: Changes in the UV-vis absorption spectra of silver nanoparticles at 25°C

Silver nanoparticles were synthesized using 3mg/ml plant extract. Stability was tested in (A) RPMI, (B) MHB, (C) YPB, (D) LB broth and (E) PBS over 24 hours. The control represents the nanoparticles in water.

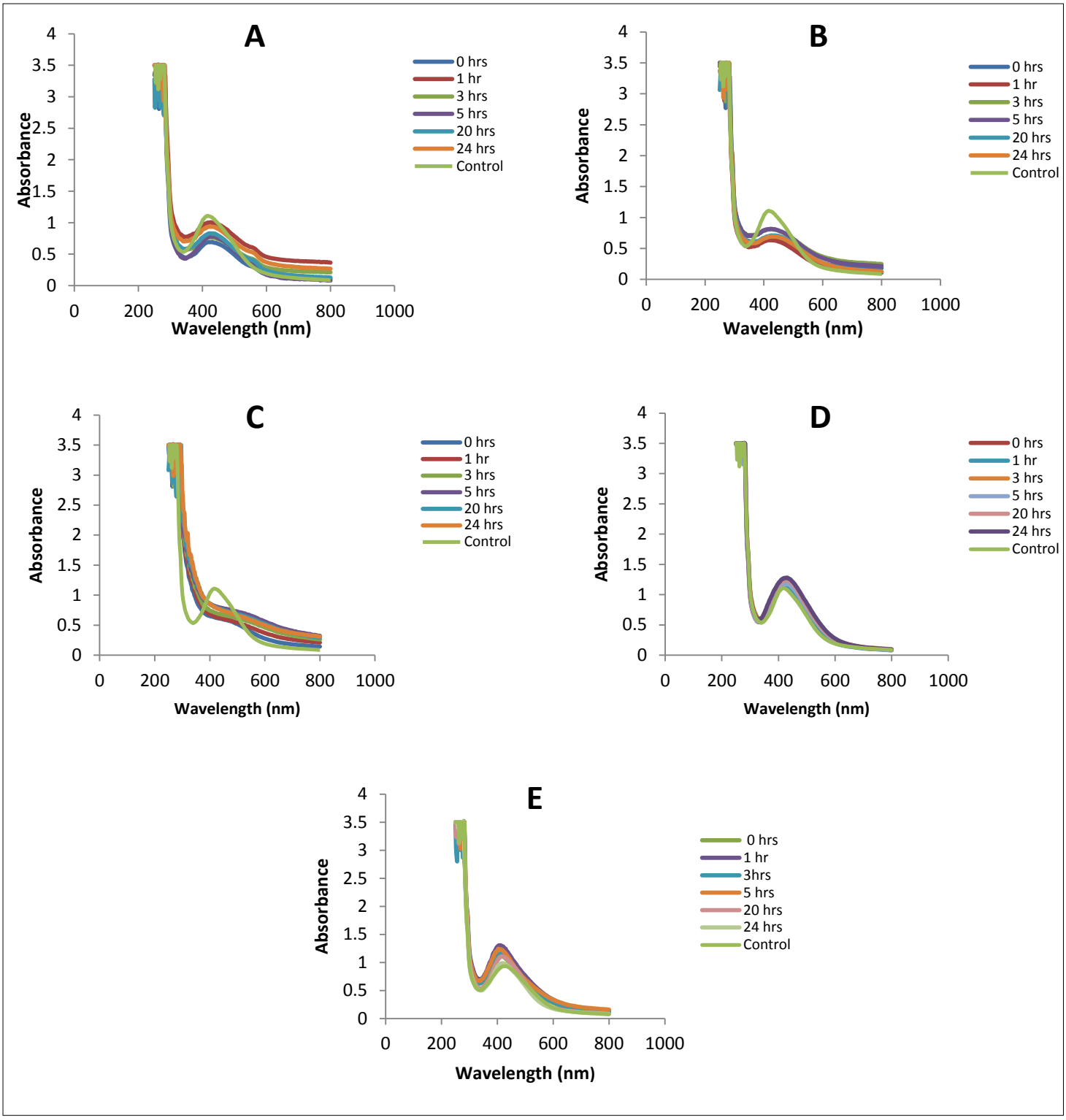

Figure 3.5: Changes in the UV-vis absorption spectra of silver nanoparticles at 37°C

Silver nanoparticles were synthesized using 3mg/ml plant extract. Stability was tested in (**A**) RPMI, (**B**) MHB, (**C**) YPB, (**D**) LB broth and (**E**) PBS over 24 hours. The control represents the nanoparticles in water.

3.4.4 Characterisation of silver nanoparticles by HR-TEM, SAED and EDX

Analysis with HR-TEM displayed the morphology of the silver nanoparticles as spherical. The size of these nanoparticles ranged from 20-40nm with a few larger particles of approximately 60nm (Fig 3.6). The crystalline nature of the nanoparticles was shown by the presence of lattice fringes on the particles (Fig 3.7A). The fringe spacing was measured and was found to be 0.229nm. This was confirmed by the selected electron diffraction (SAED) pattern (Fig 3.7B). The rings were indexed and were found to correspond to the (111), (200), (220) and (311) crystalline planes of the face centred cubic crystalline structure of metallic silver (Tippayawat *et al*., 2016). The elements, carbon, oxygen, copper, silicon, sulphur, chloride and silver were detected by the Energy dispersive X-ray spectra (EDX) as shown in Fig 3.8.

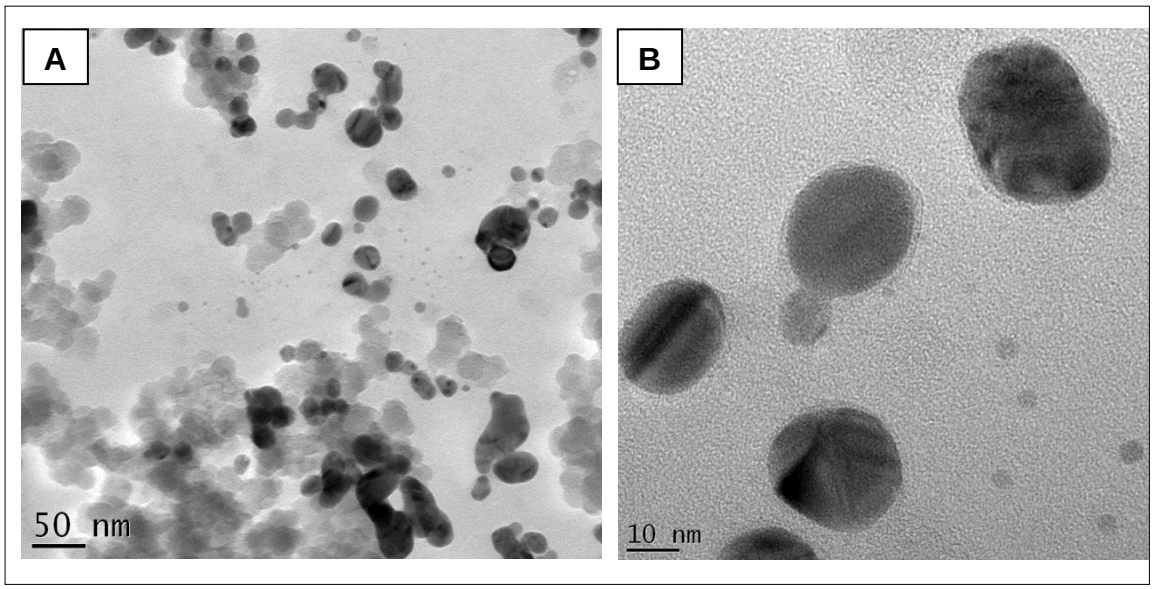

Figure 3.6: HR- TEM images of silver nanoparticles synthesized using *C. orbiculata* water extract

A shows the nanoparticles at a 50nm scale while **B** shows the nanoparticles at a 10nm scale

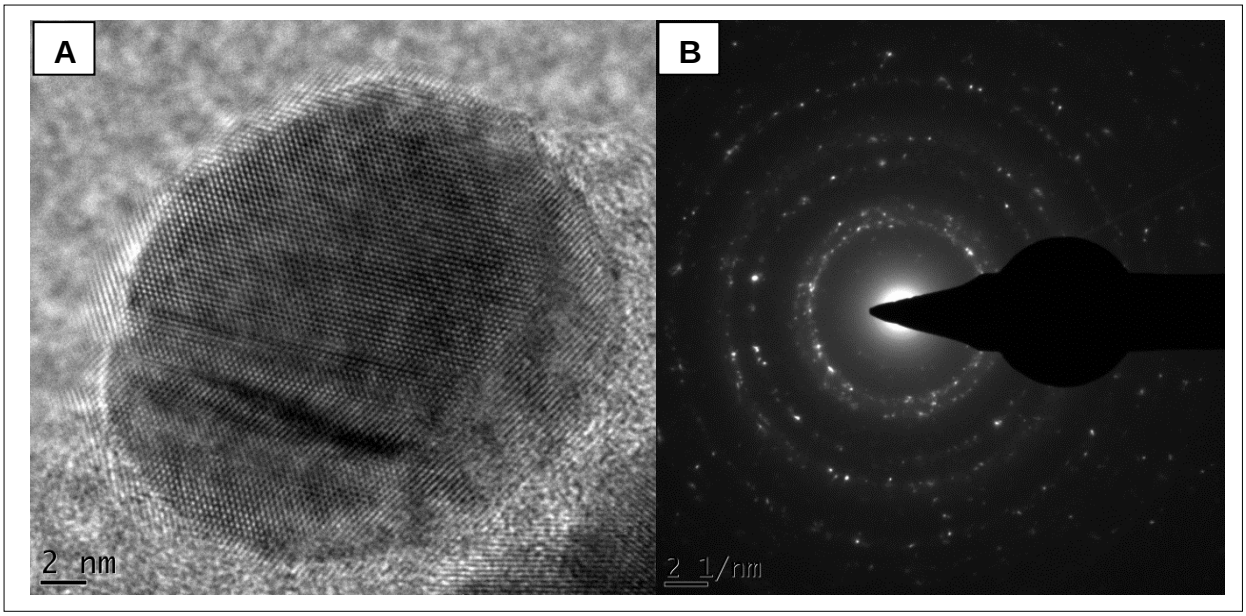

Figure 3.7: The crystalline nature of *C. orbiculata* synthesized silver nanoparticles

(A) shows the lattice fringes and **(B)** shows SAED.

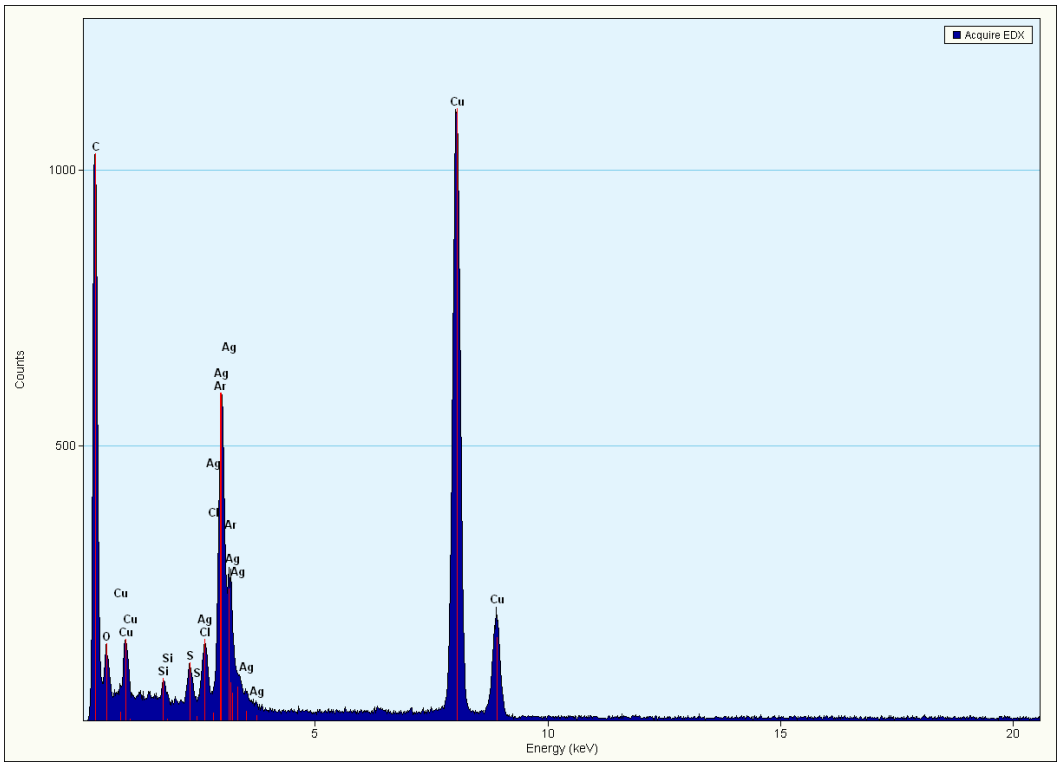

Figure 3.8: Energy dispersive X-ray spectra (EDX) of silver nanoparticles synthesized using *C. orbiculata*

3.5 Discussion

3.5.1 Confirmation of silver nanoparticle formation

The synthesis of silver nanoparticles was indicated by a colour change in the reaction mixture (silver nitrate and *C. orbiculata* extract solution). After the addition of the plant extract to the silver nitrate solution, the immediate colour of the mixture was at first colourless (Fig 3.1A). However with time the colour changed to yellow then brown as shown in Fig 3.1B and C, respectively. The change in colour of the solution is due to the formation of silver nanoparticles (Perveen and Al-Sulami, 2017). It is known that silver nanoparticles exhibit a yellowish brown colour due to the excitation of their surface plasmon (SP) vibrations (Veerasamy *et al*., 2011). Silver nanoparticles are formed by the reduction of the silver salt to silver ions (Ag^+ to Ag^0) (Iravani *et al*., 2014). The phytochemicals in the plant extracts act as reducing agents in the synthesis of nanoparticles. Phytochemicals such as alkaloids, terpernoids and flavonoids have reducing properties and may be responsible for the formation of nanoparticles (Bhumi *et al*., 2015).

The synthesis of silver nanoparticles was confirmed by measuring the UV-vis spectra at a wavelength range of 200-800nm. The maximum absorbance of the synthesized nanoparticles was around 420nm which is a characteristic of silver nanoparticles. Silver nanoparticles have an SPR at a range of around 400nm-500nm (Ashraf *et al.*, 2016). SPR of lower wavelengths suggest that smaller nanoparticles were formed and vice versa (Djuhana *et al*., 2016).

3.5.2 Effects of silver nitrate concentration on the synthesis reaction

Different parameters of synthesis were evaluated to determine the optimal conditions of silver nanoparticles synthesis with *C. orbiculata* plant extract. These include the concentration of the plant extract and silver nitrate solution, reaction temperature and reaction time. Two commonly used silver nitrate concentration namely 1mM and 3mM were used for the synthesis of silver nanoparticles. Previous studies have used of 1mM silver nitrate for the green synthesis of silver nanoparticles (Kumar *et al*., 2014; Logeswari *et al*., 2015). On the other hand, there are studies where 3mM silver nitrate was used as the best concentration for nanoparticle synthesis such as Kagithoju *et al* (2015). In a study by Singh *et al* (2015), 4 different concentrations (2, 3, 4, and 5mM) of silver nitrate were used in the synthesis of nanoparticles using 5% *Latana camara* leave extract and 3mM was found to be the best concentration. In this study the concentration of silver nitrate also had an effect on the synthesis of silver nanoparticles using *C. orbiculata* plant extract. It can be seen from Fig 3.2 that 3mM silver nitrate was better than 1mM in the synthesis of silver nanoparticles. In

Fig 3.2B, 3mM silver nitrate synthesized more nanoparticles than 1mM (Fig 3.2A) in the same time frame. This is evident from the height of the absorbance curves which gives an indication of the number of the nanoparticles produced as the absorbance is proportionate to the concentration of the silver nanoparticles in a solution (Elbagory *et al*., 2016).

3.5.3 Effects of *C. orbiculata* plant extract concentration on the synthesis reaction

Another parameter evaluated was the concentration of the plant extract. Of the different plant extract concentrations tested (48, 24, 12, 6, 3, and 1.5mg/ml) only 3 concentrations (6, 3, and 1.5 mg/ml) showed nanoparticle synthesis as determined by a change in colour of the respective solutions after addition of silver nitrate. At 48, 24 and 12mg/ml no colour change was observed suggesting no nanoparticles were synthesized. This further suggests that very high concentrations of plant extract concentrations hinder the formation nanoparticles. In a study done by Benakashani and colleagues (2016) which investigated the effects of varying concentrations of plant extract on nanoparticle synthesis, the results obtained indicated that an increase in the plant extract concentration lead to an increase in the formation of nanoparticles. This is similar to the findings in this study where an increase in *C. orbiculata* extract concentration enhanced the formation of silver nanoparticles. As it can be observed in Fig 3.3, the absorption spectrum of the nanoparticles became sharper with increase in plant extract concentration from A (1.5mg/ml) to C (6mg/ml). There was also a blue shift in the absorption spectrum indicated by the change of lambda λ_{max} from 420nm to 402nm for 1.5mg/ml and 6mg/ml respectively. The sharpness of the peak and the reduction of the λ_{max} both suggest the formation of smaller monodispersed nanoparticles with increasing concentrations of plant extract (Fatimah, 2016).

Increase in the extract concentration also increased the intensity of the colour and yield of the silver nanoparticles produced. This is because at high extract concentrations there are increased amounts of the phytochemicals therefore increased amounts of reducing agents leading to quicker reduction of silver nitrate compared to lower concentrations (Ibrahim, 2015). This can be explained by the different time periods observed to complete the reduction of silver ions. As reduction is faster at high extract concentrations, the 6mg/ml concentration took about 2 hours for the reduction of silver nitrate whereas for lower concentrations there was complete silver nitrate reduction after 3hours. Complete reduction was indicated by the formation of rough and irregular UV-vis absorbance curves as observed in Fig 3.3, as well as permanent colour of nanoparticles (that is there was no further colour change of the reaction mixture). Verma and Mehata (2016) had the same observation; their silver nanoparticles synthesized using *Azadirachta indica* water extracts also showed a

complete colour change which was permanent as an indicating complete reduction. In this study, a concentration 3mg/ml of the extract, 3mM silver nitrate and a synthesis time of 2 hours was considered the best conditions for silver nanoparticle synthesis using *C. orbiculata* plant extract.

3.5.4 Effects of temperature

Temperature is another important factor in the synthesis of nanoparticles. Lui *et al* (2011) suggested that high temperatures and pressures facilitate the synthesis of nanostructures. It has also been found that the synthesis temperature can affect the size, shape and yield of nanoparticles synthesized via plant extracts (Shah *et al*., 2015; Sathishkumar *et al*., 2010). A study based on *Citrus sinensis* extract showed a decrease in the size of nanoparticles with increasing temperature (Kaviya *et al*., 2011). At 25°C *Citrus sinensis* (sweet orange) peel extract produced particles with a size of 35nm but at 60°C the particle size decreased to 10 nm. In this study however, no nanoparticles were produced at 25°C for all plant extract concentrations and silver nitrate concentrations tested. Synthesis was only observed at 70°C. This result is similar to the study by Elbagory and colleagues (2016) were extracts of *Aspalathus hispida*, *Asparagus rubicundus*, and *Dicerothamnus rhinocertis*, did not produce any gold nanoparticles at 25°C. This might be because reducing phytochemicals in the plants require higher temperatures to initiate the reduction process.

3.5.5 Effects of reaction time

Reaction time is as important as other factors which affect the synthesis of silver nanoparticles. A colour change was observed within 15 minutes of nanoparticle synthesis. The colour gradually changed from colourless (Fig 3.1A) to yellow (Fig 3.1B) and finally brown (Fig 3.1C). This shows that the colour intensity of silver nanoparticles increased with time as the amount of silver nanoparticles in the solution increased (Ahmed *et al*., 2016). In Fig 3.3A the height of the absorbance curves increased with time indicating an increase in concentration the silver nanoparticles. At 30mins the absorbance reading was 0.46 but increased to 1.05 and 2.17 after 1 and 2 hours, respectively. This confirms the increase in nanoparticle concentration as there is a linear correlation of the absorbance and the concentration of nanoparticles in a solution (Elbagory *et al*., 2016). Based on these results the reaction time of 2 hours was selected as the best time for silver nanoparticle synthesis.

3.5.6 Zeta potential, size and PDI

The size distribution, zeta potential and PDI of the silver nanoparticle was determined by DLS. The results were as shown in table 3.1. The average particle sizes were 66, 57 and 45nm for nanoparticles synthesized using 1.5, 3 and 6mg/ml *C. orbiculata* extract, respectively. As it can be seen that the average size of the particles decreased as the concentration of the *C. orbiculata* extract increased. This corresponds to the findings in Fig 3.3 where the spectrum of the nanoparticles became sharper as the plant extract concentration increased. Sharp absorption spectrum of nanoparticles suggests smaller nanoparticles compared to broader ones. This data is in agreement with previous studies done using banana peel extract (Ibrahim, 2015) and *Capparis spinosa* extract (Benakashani *et al*., 2016). However Verma and Mehata's findings were contradictory since the nanoparticle size in their study increased with increasing extract concentration (Verma and Mehata, 2016).

The nanoparticles had an average zeta potential of -19mV. This high negative value connote that the nanoparticles have long term stability and are highly dispersed due to negative-negative repulsion forces (Mukherjee *et al*., 2014). Metal nanoparticles with a large negative zeta potential tend to repel each and therefore do not aggregate. However those with low zeta potential values easily aggregate due to the absence of repulsive forces (Saeb *et al*., 2014). According to Ardani *et al* (2017), nanoparticles with zeta potential values in the range of ±0-10 mV, ±10 – 20 mV, ±20 - 30 mV and > ± 30 mV are considered to be highly unstable, relatively stable, moderately stable and highly stable in that respective order. Based on this, it can be concluded that the nanoparticles shown in table 3.1 are therefore all relatively stable. Nanoparticle size distribution can be expressed through the PDI value. A PDI value of 0.1-0.2 suggests that the nanoparticles are monodispersed. This means that most of the particles in the sample are of the same shape and size. Increase in the PDI value indicates increase in the broadness of the particle size distribution (Mazzonello *et al*., 2017). The nanoparticles synthesized in this study have PDI values of 0.07, 1.5 and 1.0 meaning they are moderately dispersed (Bhattacharjee, 2016).

3.5.7 Stability of the silver nanoparticles in biological media

Nanoparticles are used for various applications both in-vivo and in-vitro. It is therefore crucial to confirm their stability before using them as unstable nanoparticles lose their properties. Fig 3.4 and 3.5 show the stability of silver nanoparticles produced from 3mg/ml *C. orbiculata* extracts at 25°C and 37°C, respectively. The stability of the nanoparticles was evaluated in 5 different biological media over a 24 hour period. The addition of the nanoparticles to solution

of RPMI, LB broth and PBS did not change the absorption spectra of these nanoparticles. This shows that the SPR characteristics of the nanoparticles did not change throughout the incubation time implying that the nanoparticles are stable in the mentioned media (Elbagory *et al*., 2016).

The nanoparticles did not show much stability in MHB and YPB at both temperatures. The addition of the nanoparticles to MHB resulted in flattening (especially at 25°C) of the absorption spectra which might be because some of the nanoparticles disintegrated. This flattening was observed from time 0, which suggests that, the effects of MHB and YPB occurs as soon as the nanoparticles are added to the media. Although MHB only moderately affected the absorption spectra of the nanoparticles, YPB resulted in a complete flattening of the absorption spectra. *C. orbiculata* silver nanoparticles were more stable in LB broth than MHB and YPB. LB broth is therefore a more suitable bacterial growth medium to test the antimicrobial activity of the silver nanoparticles.

3.5.8 HR-TEM, SAED and EDX

The morphology and size of the silver nanoparticles was analysed by HR-TEM. HR-TEM analysis showed that the *C. orbiculata* silver nanoparticles were spherical of different sizes as shown in Fig 3.6. The diameter sizes of the nanoparticles ranged from 20–40nm with a few larger particles of approximately 60nm. This agrees with the results obtained by DLS in Table 3.1 (peak 2). The average particle sizes recorded in Table 3.1 are however much larger than the actual nanoparticles. This might be due to the presence of some aggregates giving a large average size. Similar results were also obtained in a study by Kittler *et al* (2010) were the DLS measured higher average diameter of the PVP coated silver nanoparticles compared to SEM. According to them, this might be because the DLS tends to overestimate the fraction of large particles due to their much higher scattering efficiencies.

The crystalline nature of the silver nanoparticles was shown by the lattice fringes on the particles as it can be seen in Fig 3.7A. This was confirmed by the SAED pattern shown in Fig 3.7B. The EDX analysis which gives the status of the elements involved in the nanoparticle formation confirmed that the nanoparticles contained silver. An optical adsorption peak was observed at 3keV due to the presence of silver. This is consistent with previous studies where similar results were obtained (Paulkumar *et al*., 2017; Bashir and Qureshi, 2015). According to Magudapatty *et al* (2001), silver nanocrystals typically have an optical absorption peak approximately at 3keV due to their surface plasmon resonance. Adsorption peaks were also observed for other elements which include carbon, oxygen, silicon, sulphur,

chloride and copper. The presence of carbon, oxygen, chloride and sulphur is likely to be from the phytochemicals involved in the reduction and the capping processes during nanoparticle synthesis. The presence of copper and silicon is however due to the TEM grid onto which the sample was placed (Rodríguez-León *et al*., 2013).

The silver nanoparticles produced in this study have properties similar to those silver nanoparticles reported in several other studies using extracts of *Crocus sativus* L (Bagherzade *et al*., 2017), *Datura stramonium* (Gomathi *et al*., 2017)*, Urtica dioica* (Jyoti *et al*., 2016), *Azadirachta indica* (Verma and Mehata, 2016)*,* Olive oil (Khalil *et al*., 2014) and tea leaves (Sun *et al*., 2014).

3.6 Conclusion

The water extract of *C. orbiculata* was successfully used to produce silver nanoparticles. Different parameters were evaluated for the optimisation of nanoparticle synthesis. These parameters include reaction temperature, reaction time, silver nitrate concentration and plant extract concentration. The optimal conditions for the synthesis of the nanoparticles were 3mg/ml of the *C. orbiculata* extract, 3mM silver nitrate at 70°C for 2hours. HR-TEM showed that the synthesized nanoparticles were spherical in shape, crystalline in nature with sizes ranging from 20-40nm. The morphology and nature of these nanoparticles correlate with most silver nanoparticles produced using other plant extracts in previous studies.

3.7 References

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CHAPTER FOUR
ANTIMICROBIAL EFFECTS OF *COTYLEDON ORBICULATA* EXTRACTS ON SKIN INFECTION CAUSING MICROORGANISMS.

4.1 Abstract

Plants have recently received a lot of attention in the scientific research field because of their different medicinal properties, such as antimicrobial, anti-inflammatory and anticancer properties. Attention to the antimicrobial properties of medicinal plants is mainly attributed to the growing challenge of antimicrobial resistance. Antimicrobial resistance (AMR) is defined as the ability of microorganisms such as bacteria, viruses and fungi to grow in the presence of drugs that would normally kill or limit their growth (Crouch *et al*., 2015). As a result of this resistance, standard treatments become ineffective and infections with the resistant organisms become very difficult to treat (Berdy, 2012). Medicinal plants have been used traditionally to treat many human ailments. The popularity of medicinal plants is wide spread particularly in developing countries. South Africa is one such county with a rich biodiversity of around 30 000 different plant species (Street and Prinsoloo, 2013). *Cotyledon orbiculata* is one of the indigenous plants of South Africa that is traditionally used in the treatment of acne, boils, earache and toothache. These traditional uses suggest that the plant may possess some antimicrobial properties.

In this study, the antimicrobial properties of *Cotyledon orbiculata* were determined. *C. orbiculata* was obtained from the Van Der Burg Garden village nursery in Stellenbosch, Cape Town. Aqueous, methanol and chloroform extracts of the fresh leaves were evaluated for antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, Methicillin resistant *Staphylococcus aureus* (MR*SA*), *Pseudomonas aeruginosa* and *Candida albicans*. The microdilution assay was used to determine the minimum inhibitory concentration (MIC) of the extracts. Results obtained revealed that all extracts of *C. orbiculata* have antimicrobial properties against all the microorganisms tested. The MICs of the extracts ranged from 3.13 to 50mg/ml and the MBCs from 6.25 to >100mg/ml. The methanol extract exhibited better antimicrobial activity in comparison to the others extracts. The water extract had better antifungal properties, whereas the chloroform extract showed the least activity in both antibacterial and antifungal studies. Silver nanoparticles produced from *C. orbiculata* also had antimicrobial activity against all the microorganisms that were tested. The MIC of the nanoparticles against these microorganisms ranged from 5-80µg/ml and the MBC from 20-160µg/ml. The silver nanoparticles were found to have more antimicrobial activity than the water extract (from which they were produced).

Keywords

Cotyledon orbiculata, Antimicrobial Resistance, Minimum inhibitory concentration, Medicinal Plants and Microorganisms.

4.2 Introduction

The development of antimicrobial resistance to present day drugs has posed great challenges to global health care and has been considered a universal concern and major public health problem by the World Health Organisation (WHO) in 2014 (WHO, 2014). The eradication of some common microorganisms has become almost impossible, due of their ability to gain resistance to most proposed drugs. Common microorganisms such as *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *C. albicans* have become increasingly resistant to many antibiotics. Antimicrobial resistance is the ability of microorganisms to grow in the presence of drugs or antimicrobial agents that would normally limit their growth (Crouch *et al*., 2015). Acquisition of the *mecA* gene renders *S. aureus* even more resistant; with this gene *S. aureus* becomes resistant to methicillin and is named methicillin-resistant *S. aureus* (MR*SA*). MR*SA* is resistant to all penicillins, cephalosporins and carbapenems (Karmakar *et al*., 2016). *S. epidermidis* shows resistance to many different classes of antibiotics including β-lactams, macrolides and aminoglycosides (Vandecandelaere *et al*., 2017). This is mainly due their possession of a biofilm. *P. aeruginosa* can also form biofilms which allows it to survive harsh conditions such as very high temperatures and different chemicals. This microorganism is highly resistant to many antibiotics due to the barrier provided by its impermeable outer membrane (Ochoa *et al*., 2013). Due to its ability to acquire mechanisms of resistance to multiple groups of antimicrobial agents, therapeutic options are greatly limited for *P. aeruginosa* (Yayan *et al.*, 2015). *C. albicans* which is the main cause of invasive candidiasis have also developed resistance to common antifungal drugs such as azoles. This is due to the prolonged and frequent use of these drugs (Vila *et al*., 2017). These microorganisms are the main causes of community and hospital acquired infections, necessitating the search for alternate effective antibacterial agents (Rahman *et al*., 2011).

Plants have been used traditionally as a source of medicine for centuries. Medicinal plants were found to possess beneficial biological activities such as antimicrobial, anticancer, antioxidant, antihelminth, analgesic and wound healing activity (Sasidharan *et al*., 2011). This is due to their metabolites which include alkaloids, glycosides, terpenoids, saponins, steroids, flavonoids, tannins, quinones and coumarins (Das *et al*., 2010). In developing countries, it has been reported that 80% of the population still use plants to treat diseases and many other ailments (Maroyi, 2013).

Use of medicinal plants is preferred over conventional medicines because plants are safer, cheaper, environmental friendly and more acceptable as they are part of peoples' own culture (Mahima *et al*., 2012). Many plants have also been used in the field of nanotechnology to synthesize nanoparticles. Nanotechnology is a field of science which deals with the synthesis, development and use of materials ranging in nanometers (Kaviya *et al.*, 2011). The synthesis of nanomaterial using plants is called as green nanotechnology. This field aims to synthesize nanomaterials that are more bio-friendly using safe and environmental friendly methods (Malik *et al*., 2014). Silver nanoparticles are greatly known for their excellent antibacterial activities. It is therefore important to evaluate the antimicrobial activity of the silver nanoparticles synthesized using different plant extracts (Dakal *et al.*, 2016).

South Africa has a large diversity of plant species, many of which have medicinal properties (Elisha *et al*., 2017). *Cotyledon orbiculata* is one of the indigenous plants of South Africa. It is a small shrub with fleshy leaves and is often referred to as "pig's ears" because of its oval shaped grey-green leaves, which are very variable with red or pale margins (Rowley, 2007). *C. orbiculata* is popular for its traditional use in the treatment of ailments such as acne, boils, earache and toothache (Aremu *et al*., 2010). Its traditional use suggests that it may possess some antimicrobial properties, however, there is very little scientific evidence to support this; this study will therefore evaluate the antimicrobial effects of different solvent extracts and silver nanoparticles of *C. orbiculata*.

4.3 Methodology

4.3.1 Plant material

Fresh *C. orbiculata* plants were obtained from Van Der Burg Garden Village nursery in Stellenbosch, Cape Town. The plants were kept in their vases before extraction to keep them fresh.

4.3.2 Preparation of plant extracts

Just before the process of extraction, the *C. orbiculata* leaves were cut from the plants. They were washed with tap water to remove insects and dirt, and then they were rinsed with distilled water and dried with paper towel. The fresh leaves (300g) were cut into small pieces and blended using a kitchen blender.

Extraction was done using the maceration method. Three solvents of different polarity (water, methanol and chloroform) were used for the extraction. The blended leaves were macerated in 600ml of the different solvents overnight. There after they were filtered using a Whatman filter paper no.1 followed by a 0.45µm syringe and then concentrated. The concentration of the methanol and chloroform extracts involved the evaporation of the solvents using a rotary evaporator (Buchi Rotavapor 11). The extracts were then dried under the fume hood in the dark until all excess solvent had evaporated. The freeze drying method was used for the concentration of the water extract. Freeze drying is a water removal process that is based on the principle of sublimation. Sublimation is when a solid is changed into a gas phase without entering a liquid phase (Azwanida, 2015). Freeze drying involves three main stages namely the freezing phase, primary drying (sublimation) and secondary drying (adsorption) phase. In this method samples are frozen at –80°C and are dried under high vacuum speeds (Liu, 2006). The dried extract powders were stored at 4°C until use.

4.3.3 Microorganisms

Microorganisms used for antimicrobial evaluation in this study include the Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), Methicillin resistant *Staphylococcus aureus* (33591) and *Staphylococcus epidermidis* (ATCC 12228), the Gram-negative bacterium *Pseudomonas aeruginosa* (ATCC 27853) and the fungus *Candida albicans* (ATCC 10231). The selected pathogens represent pathogenic species commonly associated with nosocomial and severe skin infections. They are amongst the most common pathogens isolated from patients in South Africa. These pathogens are also a great cause of concern with regards to conventional antibiotic therapy because of their ability to acquire antimicrobial resistance rendering many antimicrobials ineffective (Sydnor and Perl, 2011).

4.3.4 Culture of microorganisms

All culture media was prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the media was cooled to around 40-45°C, poured into sterile petri dishes in a laminar flow and left to set. All media was stored at 4°C.

The microorganisms were obtained from Thermo Fisher Scientific. The microorganisms were first cultured in nutrient broth at 37°C for 24hours, after which they were sub-cultured on Müller Hinton agar (MHA) and incubated under the same conditions. *Candida albicans* was

sub-cultured on Sabouraud dextrose agar (SDA) plates for 48 hours. From the cultures, Gram stains were done as a simple confirmation test for the microorganisms (Opota *et al*., 2015). The microorganisms were maintained by constant sub-culturing.

4.3.5 Antimicrobial Inhibition

4.3.5.1. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations (MIC) of the extracts against the different organisms were determined. MIC is the lowest concentration of an antimicrobial that inhibits visible growth of microorganisms after incubation (Mostafa *et al*., 2017). This assay is simple, easy to perform and has high reproducibility of results (Levison and Levison, 2009).

Plant extracts were weighed and dissolved in appropriate solvents, to give a final concentration of 100mg/ml. The aqueous extract was dissolved in autoclaved distilled water, whereas organic extracts were dissolved in 10% dimethyl sulfoxide (DMSO) (Langfield *et al*., 2004). The extracts were mixed with a vortex for faster complete dissolution. Ampicillin (2.5mg/ml) was used as the positive control for bacteria, because it has been shown to have anti-bactericidal activity both gram positive and negative microorganisms (Sharma *et al*., 2013). Fluconazole (10mg/ml) was used as the positive control for the fungus, as it is the most common drug used in the treatment of *C. albicans* infections (Gulati and Nobile, 2016). Extraction solvents were used as the negative controls.

The 96-well plates were prepared by dispensing 50µl of Mueller Hinton broth (MHB) for bacteria and yeast peptone broth (YPB) for *C. albicans* into each well. 100µl of the extracts were added into the first row of the plate in triplicate. These were serially diluted by column, using a multichannel pipette set at 50µl. 50µl of mixture was discarded from the last well, leaving each well with a volume of 50µl of medium. Microbial suspensions of the bacteria and fungus were made in MHB and YPB respectively. They were diluted until they reached a 0.5 McFarland standard (standard is approximately 1×10^8 CFU/ml) (Baris *et al*., 2006). A volume of 50µl of the microbial suspensions was added to all the repective wells. The plate was then sealed and incubated for 18 - 24 hours at 37°C. In the case of silver nanoparticles, the MIC assay was conducted in the same way as described above however the Luria Bertani broth was used as the microbial medium for both the bacteria and the fungi.

Alamar Blue was used as an indicator of inhibition. After incubation, 10µl of Alamar Blue was added to each well and the plate was incubated for 1- 3 hours away from light. In the presence of active bacteria, the blue dye, Alamar Blue, is reduced to a pink fluorescent dye (Rampersad, 2012). Therefore, inhibition of bacterial growth by the extract would be determined by the wells that remained blue. The MIC values were recorded as the concentrations in the last wells with the blue colour. The MIC was also recorded with a spectrophotometer at fluorescence intensity 600nm and was considered as the last concentration before a sharp increase in fluorescence intensity. All the screening was done in triplicates.

4.3.6 Minimum Bactericidal and Fungicidal Concentrations

The minimum bactericidal (MBC) and fungicidal (MFC) concentrations were determined by sub-culturing a loopful of media from the wells that showed no growth of microorganisms during MIC. The culture plates were incubated at 37°C for 18-24hours.The MBC/MFC was recorded as the lowest concentration at which no growth was observed (Pandian *et al*., 2016).

4.3.7 Statistical analysis

Statistical analysis of the data was done using the Graphpad prism5 software. The results were expressed as mean $\pm$ standard error of the mean (SEM). The significance of the antimicrobial activity of *C. orbiculata* extracts was determined using the Two-way analysis of variance (ANOVA). The Bonferroni analysis was used to compare the activity of the different plant extracts against the test microorganisms. $P< 0.05$ values were considered to be statistically significance.

4.4 Results

4.4.1 Plant extracts

After extraction, the mass of the dry extracts were weighed and recorded as shown in Table 4.1. Although the same weight of fresh extracts was used for extraction with all solvents, methanol produced more extract (in weight) in comparison to the other extracts whereas chloroform produced the least amount.

Table 4.1: Weight of dry plant extracts produced after extraction

Extracts	Weight of fresh leaves (g)	Weight of dry extracts (g)
Aqueous	600	2.70
Methanol	600	4.27
Chloroform	600	2.33

4.4.2. Identification of Microorganisms

After culturing of microorganisms, their growth was observed. Gram staining was done as a quick test to confirm the organisms from culture. Microscopy of *S. aureus, S. epidermidis* and MRSA showed the presence of Gram-positive cocci in clusters. Evaluation of microscopy of *C. albicans* showed Gram-positive budding yeast cells which were oval in shape. *P. aeruginosa* microscopy presented with pink rods (bacilli), this confirms the presence of Gram-negative bacillus. The Gram stain images are shown in figure 4.1.

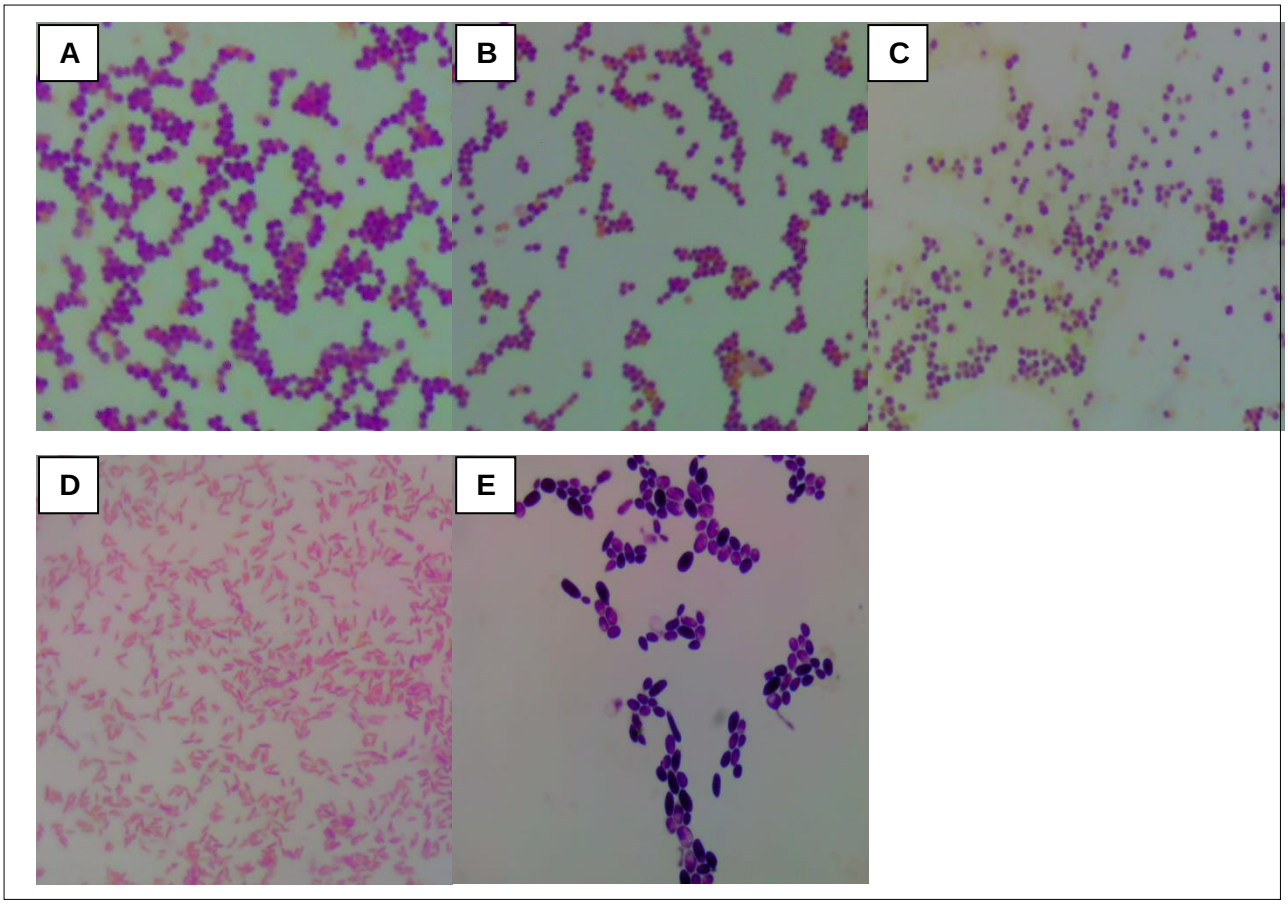

Figure 4.1: Gram stain images of microorganisms

(A) *S. aureus*, **(B)** MR*SA*, **(C)** *S. epidermidis*, **(D)** *P. aeruginosa* and **(E)** *C. albicans.* The images were taken using a light microscope at the X100 magnification.

4.4.3 Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of the *C. orbiculata* plant extracts was evaluated by determining the MIC of these extracts using the broth microdilution assay (microtitre plate method) (Eloff, 1998). This method was chosen over the broth macrodilution method because it has less room for errors and is more economical in the use of both the media and plant extracts (Balouiri *et al*., 2016; Jorgensen and Ferraro, 2009). The MIC is defined as the lowest concentration of an antimicrobial agent that inhibits visible microbial growth of microorganisms after incubation of 18-24hours (Mostafa *et al*., 2017; Das *et al*., 2010). The microdilution method can be enhanced by the addition of resazurin dyes. These dyes aid the visual determination of an accurate MIC (Elshikh *et* al., 2016). In this study, the dye, Alamar Blue was used; it is a blue, non-fluorescent dye that is reduced to a pink fluorescent dye by cellular activity (O'Brien, *et al*., 2000). Visually, the MIC was defined as the concentrations in the last wells with the blue colour, and on a spectrophotometer the MIC was defined as the last concentration before a sharp increase in fluorescence values after incubation with Alamar Blue.

The MIC of the plant extracts against the different microorganisms is shown in Table 4.2. Water, methanol and chloroform extracts exhibited some degree of activity against the test microorganisms. Methanol extract was the most active with MIC values of 6.25, 6.25, 6.25, 3.13 and 12,5mg/ml against *S. aureus*, *S. epidermidis*, MRSA, *P. aeruginosa* and *C. albicans* respectively. The least active extract was chloroform with MIC values of 25, 25, 25, 50 and 12.5mg/ml against *S. aureus*, *S. epidermidis*, MRSA, *P. aeruginosa* and *C. albicans* respectively. Interestingly, each extract showed similar activity against all Gram-positive bacteria. For example, the MIC for water, methanol and chloroform extracts against all the Gram-positive bacteria was 12.5, 6.25 and 25mg/ml respectively. To rule out the possibility of contamination among the Gram-positive bacteria, a coagulase test was done. *S. aureus* and MRSA were both coagulase positive whereas *S. epidermidis* was coagulase negative (data not shown). Also, their susceptibility to the positive control (ampicillin) was different, confirming that they were different organisms. *P. aeruginosa* was the least susceptible microorganism to the chloroform and water extracts, these extracts had MIC values of 50mg/ml against this microorganism. On the other hand *P. aeruginosa* was the most susceptible microorganisms to the methanol extract (MIC of 3.13mg/ml).

The extracts also exhibited some antifungal properties. Water, methanol and chloroform extracts had MIC values of 6.25, 12.5 and 12.5mg/ml against *C. albicans*. From this data it can be noted that the water extract had the most activity against *C. albicans* as compared to other extracts. The microorganisms showed different susceptibility to the positive controls (ampicillin for bacteria and fluconazole for *C. albicans*). The most susceptible bacteria was *S. epidermidis* followed by *S. aureus* then MRSA and lastly *P. aeruginosa* as shown in Table 4.2. Graphical comparison of the antimicrobial activity of the plant extracts is shown in Fig. 4.2, whereas Fig. 4.3 is more detailed and shows the degree of susceptibility of each microorganism to the extracts.

Table 4.2: MIC values of different extracts of *C. orbiculata*

	MIC (mg/ml)				
Microorganisms	**Water[a] Extract**	**Methanol[b] Extract**	**Chloroform[c] Extract**	**Ampicillin**	**Statistical significance (*P*)**
S. aureus	12.5	6.25	25	0.02	ab* ac*** bc***
S. epidermidis	12.5	6.25	25	0.04	ab** ac*** bc***
MRSA	12.5	6.25	25	0.31	ab* ac** bc***
P. aeruginosa	50	3.13	50	1.25	ab^{ns} ac^{ns} bc**
C. albicans	6.25	12.5	12.5		ab*** ac*** bc^{ns}

Abbreviations: ^{NS}P>0.05 * *P*<0.05; ***P*<0.01; ****P*<0.001.
Fluconazole MIC for *C. albicans* was 0.06mg/ml

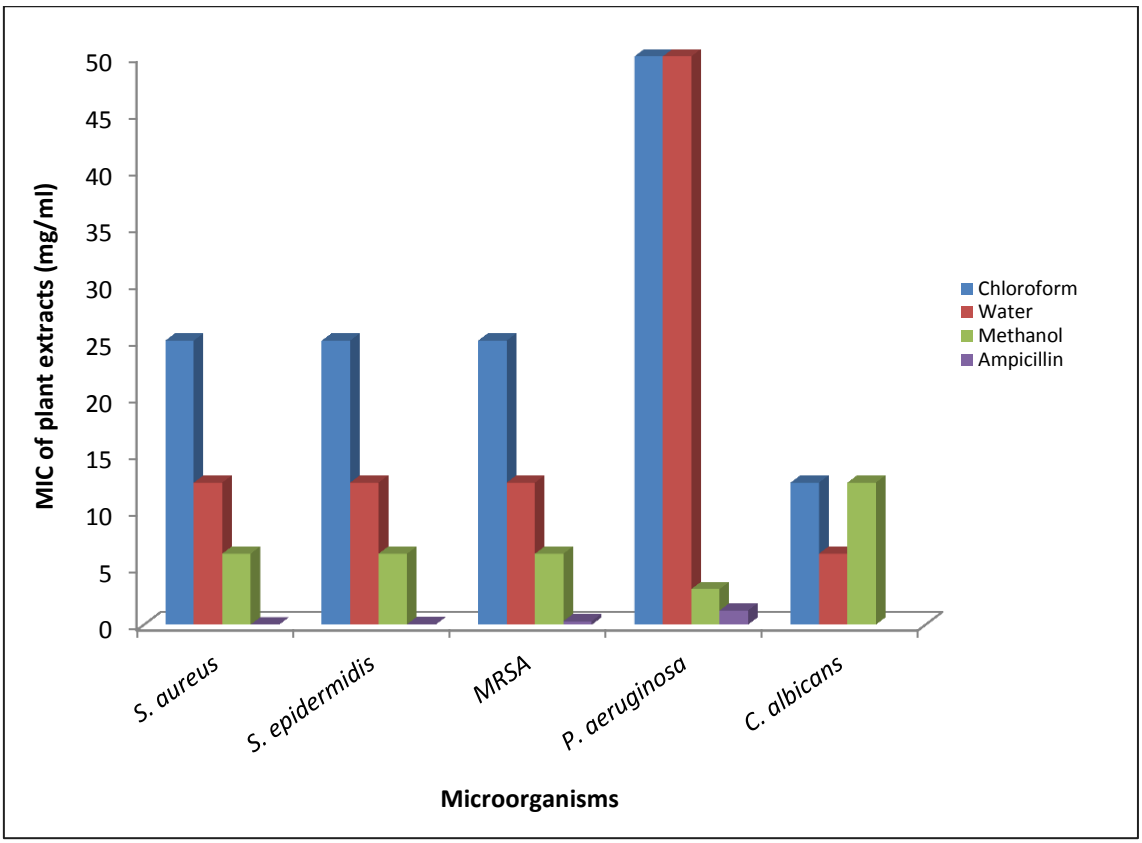

Figure 4.2: Comparative analysis of the MICs of *C. orbiculata* extracts

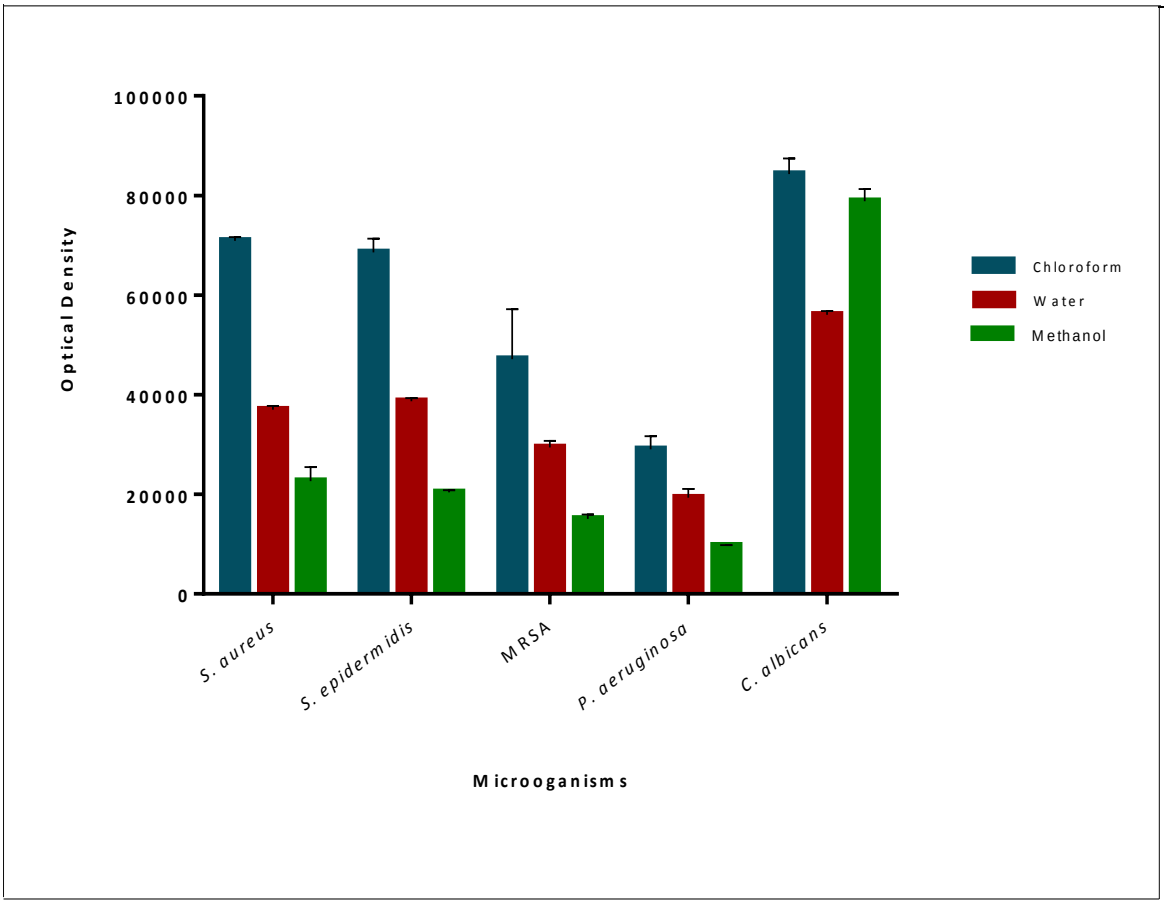

Figure 4.3: Levels of susceptibility of the microorganism to the *C. orbiculata* extracts at their MICs

4.4.4 Minimum Bactericidal and Fungicidal Concentrations

The bactericidal and fungicidal properties of the plant extracts were determined as minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) respectively. MBC and MFC are defined as the lowest concentration of an antimicrobial agent that kills 99.9% of a microorganism and completely stops its growth (Hafidh *et al*., 2011). Table 4.3 shows the MBC values of the respective plant extracts. The MBC of the extracts ranged from 6.25 to >100mg/ml, this range is higher than that of the MICs of these extracts. This indicates that higher extract concentrations are needed to completely kill the microorganisms, than to inhibit their growth. However, for the methanol extract against *S. epidermidis* and MRSA the MIC and MBC were at the same concentration. The chloroform extract had the least bactericidal activity as it showed MBC values of 100 and >100 mg/ml for all microorganisms. The plant extracts all exhibited MFCs of >100mg/ml against *C. albicans*. This shows that these extracts are poor fungicidal agents as they need very high concentrations to completely kill *C. albicans*. The MFC of fluconazole against *C. albicans* was 0.1mg/ml. Comparison of the MBCs and MFCs of the extracts is shown in Fig 4.4.

Table 4.3: MBC and MFC values of *C. orbiculata* extracts

MBC/MFC(mg/ml)				
Microorganisms	**Water extract**	**Methanol Extract**	**Chloroform extract**	**Ampicillin**
S. aureus	25	12.5	100	0.04
S. epidermidis	25	6.25	100	0.04
MRSA	50	6.25	>100	0.63
P. aeruginosa	100	25	100	>10
C. albicans	>100	>100	>100	

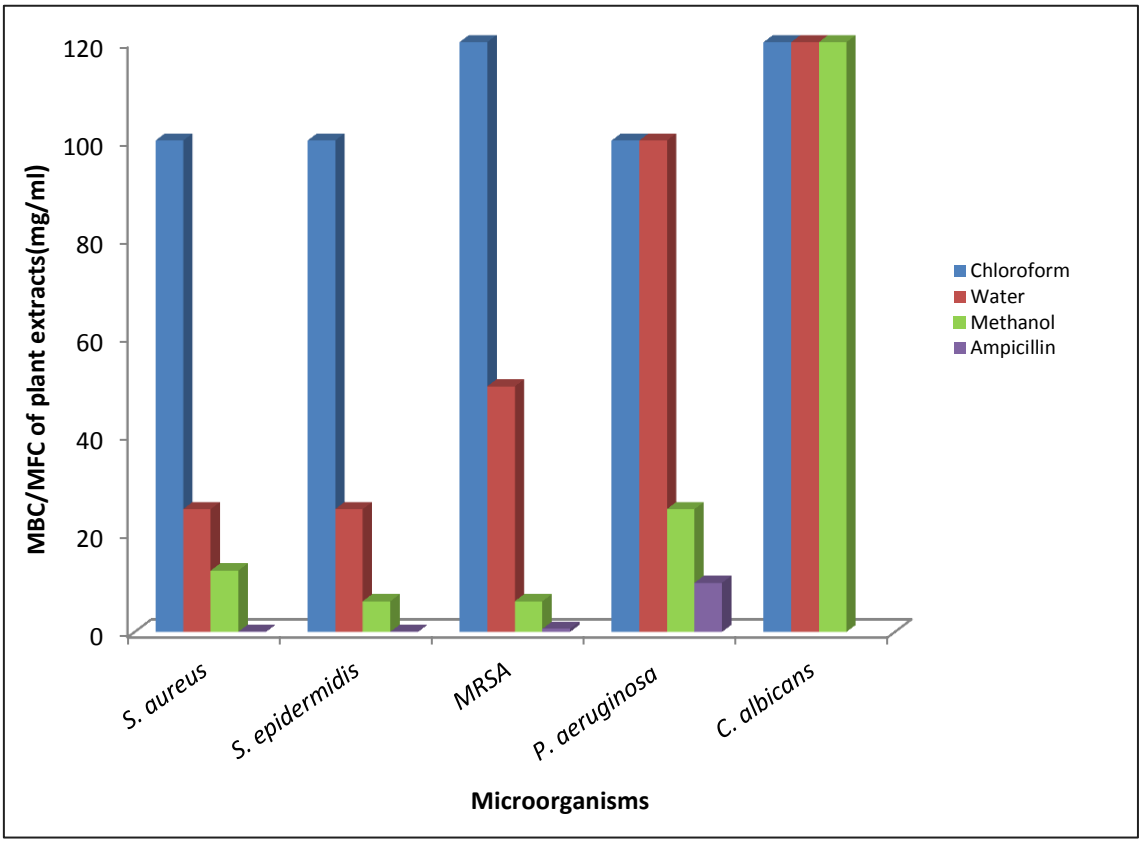

Figure 4.4: Comparative analysis of the MBC and MFC of *C. orbiculata* extracts

4.4.5 Antimicrobial activity of silver nanoparticles synthesized using *C. orbiculata*

C. orbiculata silver nanoparticles also exhibited some antimicrobial activity. The antibacterial and antifungal effects of *C. orbiculata* silver nanoparticles are shown in Table 4.4. The nanoparticles were most active against *P. aeruginosa* with MIC and MBC values of 5 and 20µg/ml respectively. The lowest activity was found against *C. albicans* with MIC and MFC values 80 and 160µg/ml respectively. The silver nanoparticles exhibited the same inhibitory activity against *S. aureus* and *S. epidermidis* with the MIC value of 20µg/ml. However their bactericidal effects differed as shown by the different MBC values against these microorganisms (MBC against *S. aureus* and *S. epidermidis* was 40 and 20µg/ml, respectively). MRSA was the most resistant bacteria with MIC and MBC values of 80 and 160µg/ml respectively.

Table 4.4: Antimicrobial properties of silver nanoparticles synthesized using *C. orbiculata* water extract

	Silver nanoparticles	
Microorganisms	**MIC (µg/ml)**	**MBC/MFC (µg/ml)**
S. aureus	20	40
S. epidermidis	20	20
MRSA	40	80
P. aeruginosa	5	20
C. albicans	80	160

4.5 Discussion

C. orbiculata is used traditionally as an antimicrobial agent to treat skin-related infections such as acne and boils. It is also used in the treatment of earache and toothache (Kumari *et al*., 2016). However there is little evidence of the antimicrobial properties of this plant in the literature. This study therefore aimed to validate the antibacterial and antifungal properties of *C. orbiculata* against some of the most common skin pathogens. The minimum inhibitory concentrations of *C. orbiculata* extracts and silver nanoparticles against the microorganisms; *S. aureus, S. epidermidis,* MRSA*, P. aeruginosa* and *C. albicans* were therefore determined.

In classifying antibacterial activity against Gram-positive and Gram-negative microorganisms, Gram-positive microorganisms would be more susceptible to the antimicrobial agent than the Gram-negative (Rahman *et al.*, 2011). This is because unlike Gram-positive bacteria, Gram-negative bacteria possess an outer membrane containing lipopolysaccharide. This membrane is difficult to penetrate and acts as a barrier for most antibacterial agents (Fair and Tor, 2014; Silhavy *et al*., 2010; Delcour, 2010). Findings from this study support this view, as the Gram-negative *P. aeruginosa* seemed more resistant than the Gram positive bacteria to the water and chloroform extracts which had the highest MIC values of 50mg/ml for this organism (Table 4.2). *P. aeruginosa* is a highly resistant microorganism associated with community and hospital-acquired infections in immunocompromised individuals (Ulloa-Urizar *et al*., 2015). Its resistance can be attributed to the lipopolysaccharides present on its outer membrane which renders it impermeable to most antibacterial compounds (Madikizela *et al*., 2013).

Results obtained from the methanol extract against *P. aeruginosa* were rather promising. The methanol extract had an MIC of 3.13 mg/ml against *P. aeruginosa*; this was the lowest MIC recorded in this study, implying that the methanol extract could inhibit the growth of *P. aeruginosa* at a concentration of 3mg/ml. This result is consistent with other studies where *P. aeruginosa* was more susceptible to inhibition by plant extracts as compared to Gram-positive microorganisms (Elisha *et al*., 2017; Makhafola and Eloff, 2012). This suggests the presence of a polar phytochemical in this extract that has high inhibiting effects on the growth of *P. aeruginosa*.

The Gram-positive bacteria used in this study included *S. aureus*, MRSA and *S. epidermidis.* These microorganisms cause a wide variety of infections. *S. aureus* and MRSA are the primary causes of skin and soft tissue infections, and they can also cause deep-seated infections such as endocarditis and bacteraemia (Taddesse *et al*., 2014). These microorganisms are also the main causes of nosocomial infections, which usually result in life threatening conditions (Grema *et al*., 2015). *S. epidermidis* causes infections in patients with indwelling foreign bodies such as heart valves and intravenous catheters (Buttner *et al.*, 2015). Table 4.2 displayed intriguing results with regards to Gram-positive microorganisms. Each plant extract had the same MIC value for all the Gram-positive microorganisms, meaning the organisms were susceptible to the extracts at the same concentrations. This might be due to the fact that these microorganisms are in the same genus; they are all Gram-positive cocci (staphylococci). The antibacterial agent in the plant extract might therefore be targeting a common structure or section in the organisms, hence using almost the same

concentration to inhibit their growth. Fig 4.3 shows the degree of susceptibility of the microorganisms to the plant extracts. Even though the microorganisms had the same MIC, MRSA was more susceptible to all the extracts than the other Gram-positive microorganisms. This finding was statistically significant ($P<0.05$) as shown in both Table 4.2 and Fig 4.3. Table 4.4 shows the different MBCs exhibited by the extracts against the microorganisms. Methanol extract showed the best bactericidal activities against both the Gram-positive and negative microorganisms whereas chloroform had the least bactericidal activity with MBCs starting from 100mg/ml against all the microorganisms.

The antifungal properties of this plant were evaluated against *C. albicans*. *C. albicans* is one of the most important opportunistic fungal pathogens causing oropharyngeal candidiasis in HIV patients, and in infants of less than a month, as well as in premature and HIV-infected babies. This plant showed some antifungal activity against *C. albicans*, with the MICs of 6.5, 12.5 and 12.5mg/ml for the water, methanol and chloroform extracts respectively. It can be observed from Table 4.2 that the water extract had the best activity against *C. albicans*. This finding is very encouraging as many reports often state that water extracts lack bioactivity (Madikizela *et al*., 2013; Mulaudzi *et al*., 2009). This is also a good result as it clearly validates the antifungal nature of the plant, since the water extracts mimic the way the plant is used traditionally. However, the plant extracts showed poor fungicidal activity, as they are only fungicidal at very high concentrations; the MFC of all extracts was beyond the highest concentration (100mg/ml) tested in the study.

4.5.1 Comparative analysis of the antimicrobial efficacies of different plant extracts

When comparing the different plant extracts of *C. orbiculata*, it can be observed in Fig 4.2 and 4.3 that the methanol extract had the best antibacterial activities. It exhibited the lowest MICs and MBCs compared to other extracts even with the highly resistant microorganisms, MRSA and *P. aeruginosa*. This might be because the solvent methanol extracts more compounds from plants than other solvents (Table 4.1). Methanol extracts were found to extract saponins which have antimicrobial activity (Ncube *et al*., 2008; Masoko and Eloff, 2006). The water extract proved to be the second best antibacterial agent in this study; however it showed better antifungal properties than other extracts in inhibiting the growth of *C. albicans*. Unfortunately, all the extracts exhibited poor fungicidal activities against this particular fungus. The water extract in this study displayed better antimicrobial activity than most plants; this might be because fresh plants were used in this study. A study done by Juniad *et al* (2006) proved that fresh plant water extracts exhibit better antimicrobial properties than the dried plant extracts. In their study, phytochemical analysis showed the

presence of phytochemicals in fresh plant extracts in appreciable quantities, whereas only traces of these phytochemicals were seen in the dry extracts of the same plant. Chloroform showed the least activity compared to the other extracts as it had the highest MICs and MBCs.

The difference in the activity of these extracts is because of the different extraction solvents used. Methanol is a polar solvent used for the extraction of polar chemical compounds; chloroform is non-polar and is used for the extraction of non-polar compounds (Ncube *et al*., 2008). Water is also a polar solvent, however most antimicrobial active components that have been identified are reported to be insoluble in water (Yamaji *et al*., 2005; Nang *et al*., 2007; Cowan, 1999). This explains why methanol extracts have better antimicrobial activity than water extracts.

A noteworthy antimicrobial agent is defined as an agent with an MIC reading below 1mg/ml (Ncube *et al*., 2008; Van Vuuren, 2008; Rìos and Recio, 2005). However all the extracts in this study exhibited MIC values of more than 1mg/ml. Contrary to this study, a study done by Mabona (2013) to show the antimicrobial effects of *C. orbiculata* displayed this plant as a noteworthy antimicrobial agent with the solvent mixture of dichlromethane and methanol at a ratio of 1:1. This solvent might therefore the best solvent for the extraction of antimicrobial compounds of this plant. This study however, showed better activity of the water extract as compared to the study by Mabona (2013).

4.5.2 Antimicrobial activity of silver nanoparticles synthesized using *C. orbiculata*

The results obtained in Table 4.4 shows that silver nanoparticles synthesized by *C. orbiculata* have antibacterial activity. This is supported by many studies which stated that silver nanoparticles are great antibacterial and bactericidal agents (Benakashani *et al*., 2016; Rai *et al*., 2012; Lara *et al*., 2011). The high antibacterial activity of silver nanoparticles is attributed to their large surface area, which provides better contact of the nanoparticles with the cell wall of the microorganisms (Ahmed *et al*., 2016). The silver nanoparticles were more active against the Gram-negative bacteria (*P. aeruginosa*) as compared to the Gram-positive bacteria (*S. epidermidis, S. aureus* and MRSA). This is likely due to the different membrane structures and compositions in the cell wall of the microorganisms (Gomathi *et al*., 2017). Gram-positive bacteria have a very thick layer of peptidoglycan in their cell wall whereas in Gram-negative bacteria, the cell wall contains a thin peptidoglycan layer (Snega *et al*., 2015). These results are in agreement with other findings in literature where Gram-negative bacteria were more susceptible to silver nanoparticles than Gram-positive bacteria (Gomathi *et al*.,

2017; Benakashani *et al*., 2016; Ibrahim, 2015).

The silver nanoparticles also showed some antifungal activity against *C. albicans* however the antifungal activity was lower than the antibacterial activity (Table 4.4). These results correspond to findings by Dobrucka and Dlugaszewska (2015) and Ibrahim (2015). In their studies the silver nanoparticles synthesized from *Arnicea anthodium* and Banana peel extract showed more activity against bacteria as compared to the fungus *C. albicans*.

4.5.3 Comparative analysis of the antimicrobial activity of the silver nanoparticles and the water extracts

Silver nanoparticles were synthesized from the water extract of *C. orbiculata*. The silver nanoparticles exhibited better antimicrobial activity than the water extract. The MICs of the nanoparticles against the microorganisms were much lower (µg/ml) than those of the water extracts (mg/ml). Low MICs represent high antimicrobial activity and vice versa (Chitemere and Mukanganyama, 2011). The excellent activity of the silver nanoparticles is attributed to their large surface area which allows them to be in better contact with the microorganisms. It also allows better contact of the phytochemicals and the microorganisms. This increases their toxicity to the microorganisms hence increasing their effects (Ahmed *et al*., 2016).

In the synthesis of silver nanoparticles, phytochemicals act as reducing agents, capping agents and stabilisers of the nanoparticles. In this process, the phytochemicals are also stabilised and are isolated or purified from the extract (Chuan *et al*., 2015). The phytochemicals can therefore exert their activities effectively without hindrance from other compounds in the extract. In the water extract there might be impurities or other compounds that are incompatible with the biologically active phytochemicals thus reducing their activity (Bonifácio *et al*., 2014; Saraf, 2010).

4.6 Conclusion

From the results of this study, it can be concluded that *C. orbiculata* possesses both antibacterial and antifungal properties, although it is not a very strong fungicidal agent. The methanol extract proved to be best antibacterial agent among the extracts used in this study. The water extract, however, was the best antifungal agent. All the plant extracts except for the methanol extract, had more activity against the Gram-positive bacteria than the Gram-negative bacteria. This was exhibited in the both MICs and the MBCs of these plant extracts. It has also been shown that although an extract may have the same MIC values for different organisms, the level of susceptibility of the organisms to that extracts may still differ.

Silver nanoparticles produced by *C. orbiculata* extract showed good antibacterial and antifungal properties. The nanoparticles exhibited more antibacterial activity against the Gram-negative bacteria than Gram-positive bacteria. This is because of their different cell wall membrane compositions. Silver nanoparticles had more antimicrobial activity than the water extract, probably because of the phytochemicals present on the nanoparticles. Further studies on these phytochemicals are important as they could potentially lead to the discovery of new chemical entities that may be used in the development of effective antimicrobial agents.

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CHAPTER FIVE
THE IMMUNOMODULATORY PROPERTIES OF EXTRACTS AND SILVER NANOPARTICLES PRODUCED *FROM COTYLEDON ORBICULATA*.

5.1 Abstract

Cotyledon orbiculata is a well-known South African plant that is used in the treatment of antimicrobial infections and inflammations of the skin. The immunomodulatory properties of this plant are still largely unknown. The focus of this study was therefore to determine the immunomodulatory effects of *C. orbiculata* extracts (water, chloroform and methanol) and silver nanoparticles produced from the water extract of this plant. THP-1 cells were treated with lipopolysaccharide (LPS) for 6 hours to stimulate cytokine production. The pre-treated cells were incubated with the plant extracts (60mg/ml) and silver nanoparticles (1.3µg/ml). After 24hours the extracellular supernatants of the cells were harvested and evaluated for cytokines production using the enzyme-linked immunosorbent assay (ELISA). All treatments with the extracts and silver nanoparticles significantly inhibited the secretion of the cytokines TNF-alpha, IL-1 beta and IL-6 in LPS stimulated THP-1 cells. These findings suggest that *C. orbiculata* has anti-inflammatory properties.

Keywords

Cotyledon orbiculata, Silver nanoparticles, Lipopolysaccharide, Cytokines and Immunomodulation.

5.2 Introduction

The immune system plays a major role in protecting the body against infections by pathogenic microorganisms. Macrophages, through their role in antigen presentation and cytokine secretion play a major role in fighting infections (Oishi and Manabe, 2016). During a bacterial infection, macrophages are activated to secrete pro-inflammatory cytokines which will act against the infection by causing an inflammation (Cekici *et al*., 2015). The most common pro-inflammatory cytokines include TNF alpha, IL-1 beta and IL-6 (Rider *et al*., 2016). Generally these cytokines leads to the onset of fever which attempts to eliminate pathogens, however higher concentrations of these cytokines for prolonged periods can lead to tissue damage and complications such as rheumatoid arthritis (Gulati *et al*., 2016). Therefore antimicrobial treatments with the ability to modulate cytokine production might be promising in effectively treating infections.

A large number of plants have been shown to modulate the immune system in different ways. Medicinal plants have been reported to possess anti-inflammatory, anti-stress and anti-cancer activity through their ability to modulate the immune system. Immunomodulation is when an immune response is altered to a desired level (Mukherjee *et al*., 2014). The alteration of an immune response may result in an increase (immunostimulation) or decrease (immunosupression) in the responsiveness of the immune system (Vaseeharan and Thaya, 2014).

Although the immunomodulatory effects of many plant extracts have been reported, very little is known about the immunomodulatory effects of *C. orbiculata*. The study done by Amabeoku and Kabatende (2012) is the only study that evaluated the immunomodulatory effects of *C. orbiculata* methanol extract. This study was done using the rat paw carrageenan-induced oedema test; *C. orbiculata* methanol extract was found to have anti-inflammatory properties. However in this study the immunomodulatory effects of the plant extracts and silver nanoparticles produced from the water extract of this plant were evaluated using the THP-1 monocytic cell line. These cells greatly resemble primary monocytes and macrophages in morphology, differentiation properties and function. Hence they have been used extensively in the in-vitro study of macrophages (Yang *et al*., 2016).

5.3 Methodology

5.3.1 Plant material

Refer to section **4.3.1**

5.3.2. Plant extracts preparation

Refer to section **4.3.2**

5.3.3 Synthesis of silver nanoparticles

Refer to section **3.3.3**

5.3.4 Cell culture

The human monocytic leukaemia cell line THP-1 was obtained from Dr Admire Dube (School of Pharmacy, University of Western Cape). THP-1 cells were cultured in cell culture flasks in Roswell Park Memorial Institute (RPMI) medium 1640 containing 10% fetal bovine serum (FBS) and 1% Pen strep (penicillin and streptomycin). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in a SL SHEL LAB incubator.

5.3.5 Differentiation of THP-1 cells

THP-1 monocytes were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) obtained from Sigma Aldrich. Cells were seeded in 24 well plates at a density of $2x10^5$ cells/ml and were treated with PMA at different concentrations of 50, 100, 200nM. The cells were incubated for 3 days at 37°C and 5% CO_2 in a humidified atmosphere of 5% CO_2 in a SL SHEL LAB incubator. The culture media was replaced with PMA free media (resting phase) for 24hours (Richter *et al*., 2016). The morphological changes were assessed and the best PMA concentration to use was determined.

5.3.6 Cell viability

Cell viability was evaluated using the WST-1 assay (obtained from Sigma Aldrich). This assay was done to determine the toxicity of the *C. orbiculata* plant extracts and silver nanoparticles towards THP-1 cells. The WST-1 assay was performed in 96 well cell culture plates. Cells were differentiated with 100nM PMA at a density of $2x10^5$ cells/ml. Differentiated cells were treated with different concentrations of *C. orbiculata* plant extracts and silver nanoparticles. The cells were incubated for 24hours at 37°C in a humidified atmosphere of 5% CO_2. After treatment the media was removed from the wells and was replaced with media containing 10% WST-1 reagent. The cells were incubated for 3hrs. The absorbance of the 96 well plate was measured at 440nm (reference 630nm) using the POLARstar Omega plate reader. Cell viability was expressed as a percentage of the absorbance of treated cells

to control (untreated) cells.

5.3.7 Determination of cytokine responses

Stimulation of cells for cytokine determination was done using lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (LPS was obtained from Sigma Aldrich). PMA differentiated THP-1 cells were stimulated with 1µg/ml LPS (Sánchez-Quesada *et al*., 2015) for 6hrs in 24 well plates. Stimulated cells were incubated at 37°C for 24hours with the plant extracts or silver nanoparticles. The LPS control was stimulated with LPS but not treated with extracts or the nanoparticles and was also incubated for 24hrs. The cell supernatants were transferred from the 24 well plates into Eppendorf tubes. The tubes were centrifuged at 1500rpm (Centrifuge 5417R (Eppendorf AG, Hamburg, Germany)) for 10minutes; supernatants were collected and stored at -80°C until measurement of cytokines. The production levels of the cytokines IL-1β, IL-6 and TNF-α were determined using ELISA kits (Bioo Scientific, USA). This assay was performed according to the manufacturers' instructions.

5.3.8 Statistical analysis

Statistical analysis of the data was done using the Graphpad prism6 software. The results were expressed as mean ± standard error of the mean (SEM). The significance of the cell viability and immunomodulatory effects of *C. orbiculata* extracts and silver nanoparticles were determined using the Two-way analysis of variance (ANOVA). The Dunnett's and Turkey's multiple comparison analysis were used for comparisons between the different extracts and the controls. $P < 0.05$ values were considered to be statistically significance.

5.4 Results

5.4.1 Differentiation of THP-1 monocytes into macrophages

The differentiation of THP-1 monocytes into macrophages using PMA has been reported in many studies (Vasandan *et al*., 2016; Hayman *et al*., 2017). However different concentrations of PMA have been used for this purpose. In this study THP-1 cell differentiation was compared at three different PMA concentrations (50,100 and 200nM). After differentiation all the cells had adhered to the bottom of the wells however the levels of differentiation differed. This was reflected by the morphological changes of the cells as shown in Fig 5.1. The cells showed better differentiation with 100nM PMA than at the other two concentrations. At this concentration the surface area of the cells had greatly increased and exhibited clear expansion of the cell organelles. At 200nM PMA the cells showed great differentiation features however there were signs of cell disintegration. The concentration of PMA chosen was therefore 100nM and was used throughout the study.

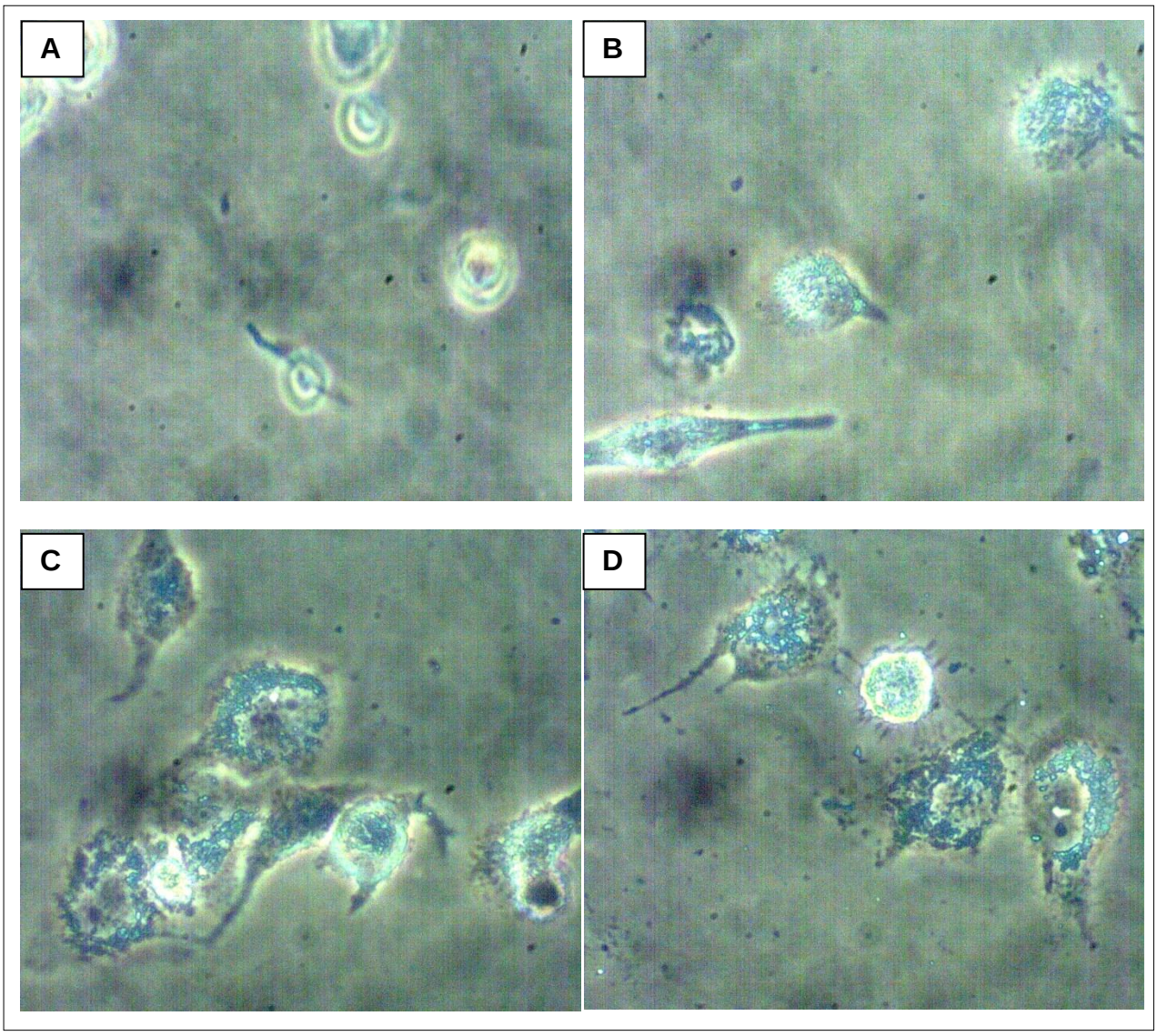

Figure 5.1: Images of PMA differentiated THP-1 macrophages

Differentiation was done using **(A)** 0nm PMA (undifferentiated), **(B)** 50nm PMA, **(C)** 100nm PMA and **(D)** 200nm PMA. The images were taken using a light microscope at a magnification of X50.

5.4.2 Cell viability

The toxicity of the water, methanol and chloroform extracts as well as silver nanoparticles of *C. orbiculata* to THP-1 cells was determined using the WST-1 assay (Fig 5.2 and 5.3). The extracts were found to be more toxic to the cells at higher concentrations (240µg/ml and above). Similarly the silver nanoparticles were only toxic at concentrations of 10µg/ml and above. The chloroform extract was the most toxic plant extract and the only extract that was able to significantly (P<0.05) reduce the viability (72%) of the cells at 240µg/ml. At a dose of 480µg/ml the viability of the cells treated with the chloroform extract was only 49% (P<0.0001). The methanol extract did not significantly affect the cell viability at 240µg/ml, but required a higher dose (480µg/ml) to significantly (P<0.01) reduce cell viability (63%). The water extract was the least toxic and did not induce significant toxicity in the cells at any of the concentrations that were tested.

Silver nanoparticles exhibited significant toxicity to the THP-1 cells at doses of 10 and 20µg/ml with percentage viabilities of 52 and 2% respectively. The silver nanoparticles are therefore more toxic than the plant extracts. Plant extract concentrations less than 120µg/ml and silver nanoparticle concentrations less than 5µg/ml did not exhibit any significant toxicity to the cells and were considered safe concentrations to use for the assessment of cytokine response in THP-1 cells. The doses 60mg/ml and 1.3µg/ml for the extracts and nanoparticles respectively, were therefore used in treating the THP-1 cells for cytokine determination.

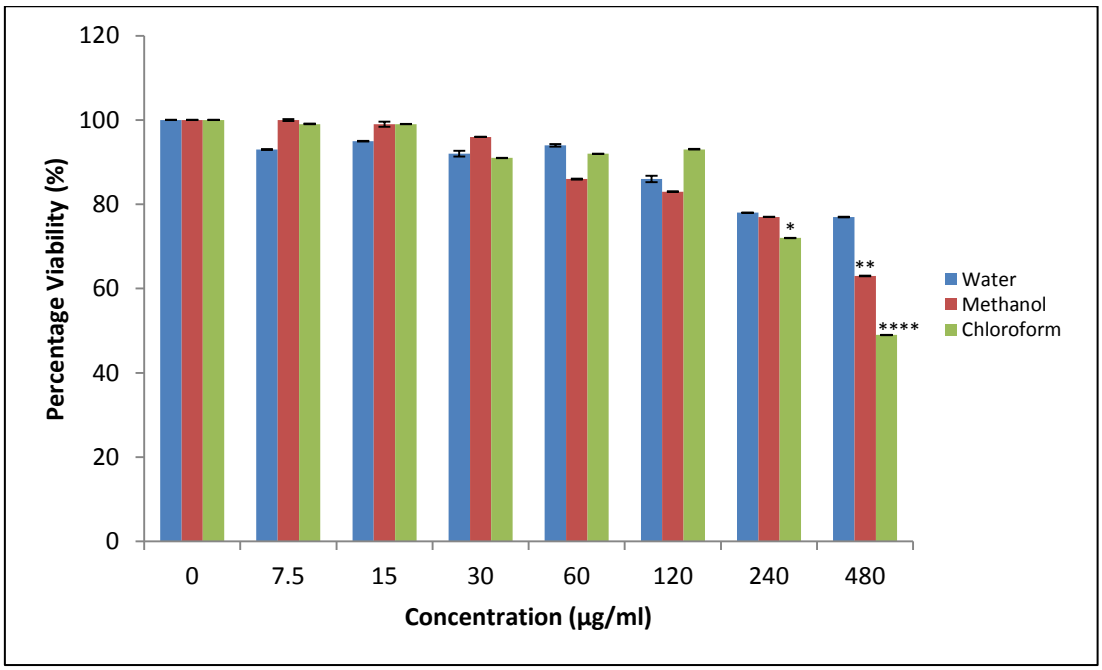

Figure 5.2: Cell viability of THP-1 cells treated with *C. orbiculata* extracts

THP-1 cell viability was assessed using the WST-1 assay. Each value represents mean ± SEM, statistical significance is indicated with * for *P*<0.05, ** for *P*<0.01 or **** for *P*<0.0001.

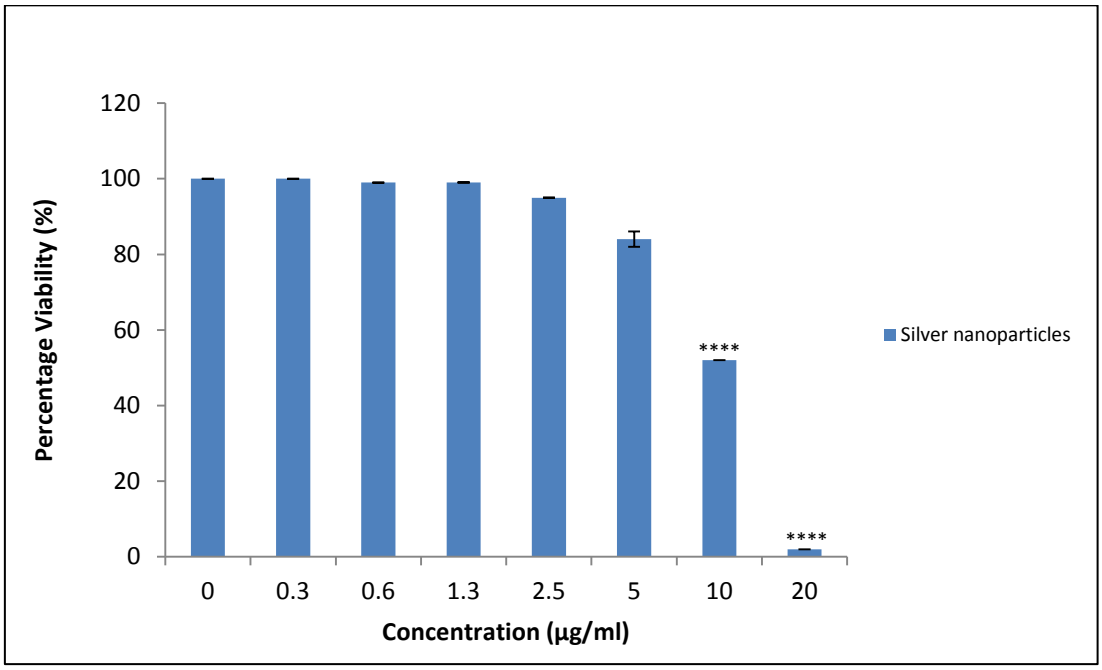

Figure 5.3: Cell viability of THP-1 cells treated with *C. orbiculata* silver nanoparticles

THP-1 cell viability was assessed using the WST-1 assay. Each value represents mean ± SEM, statistical significance is indicated with **** for *P*<0.0001.

5.4.3 Cytokine Determination

The ELISA assay was used to determine the effects of *C. orbiculata* plant extracts and silver nanoparticles on cytokine production in THP-1 cells. After differentiation with PMA, the cells were stimulated with LPS before exposure to the extracts. Lipopolysaccharides are biologically active substances found in the outer membrane of Gram-negative bacteria (Ramachandran, 2014). Exposure of THP-1 cells to LPS stimulates the production of inflammatory mediators such as cytokines which are essential for antibacterial defence. The levels of cytokine production in the presence of the extracts and silver nanoparticles are shown in Fig 5.4, 5.5 and 5.6.

Stimulation of the THP-1 macrophages with LPS resulted in an increase in the secretion of the cytokines TNF-alpha, IL-1 beta and IL-6. Treatment of the cells with the plant extracts and silver nanoparticles lowered the concentrations of the cytokines. The levels of TNF-alpha significantly decreased by approximately 2.5, 2, 1.5 and 3.5 fold in water, chloroform methanol extracts and silver nanoparticles treated cells, respectively (Fig 5.4). A comparison of the inhibitory activity of the extracts shows that there are significant differences (P<0.05) between the extracts. Cells treated with the water extract exhibited the greatest decrease in the TNF-alpha levels when compared to cells treated with the other extracts, whereas the methanol extract treated cells exhibited the lowest decrease. There was no statistical significance in the inhibitory between the water extracts and the silver nanoparticles. However it should be noted that the silver nanoparticle concentration was significantly lower than the concentration of the water extract.

A similar pattern of activity was observed with IL-1 beta. The levels of this cytokine decreased by about 3, 2, 1.5 and 10.5 fold in water, chloroform, methanol extracts and silver nanoparticles treated cells, respectively (Fig 5.5). A comparison of the inhibitory activity of the extracts to each other shows that the inhibitory activities of the extracts are significantly different. The water and methanol extracts exhibited the strongest and weakest inhibitory activity, respectively. However there was no significant difference between inhibitory effects of the water and chloroform extracts. The inhibitory effects of the silver nanoparticles to the secretion of IL-1 beta were much higher (P<0.05) compared to that of the water extracts.

The chloroform, water, methanol extracts and silver nanoparticles treated cells inhibited the secretion of IL-6 by approximately 20, 17.5, 6 and 7 fold, respectively (Fig 5.6). The pattern of the inhibitory activity on IL-6 was significantly different from the pattern observed for TNF-alpha and IL-1 beta. In this case, the highest and lowest inhibitory activity was seen with the chloroform and methanol extract, respectively (P<0.05). The inhibitory activity between the water extract and the silver nanoparticles showed no statistical significance.

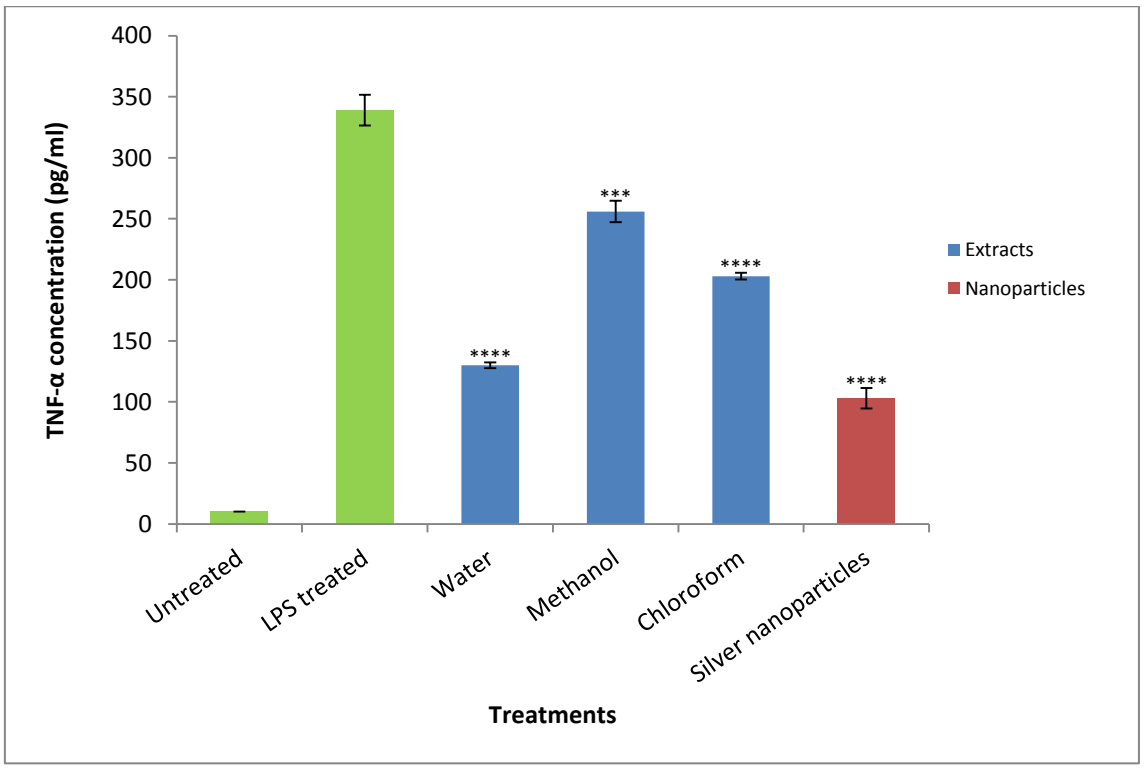

Figure 5.4: Effects of *C. orbiculata* extracts and silver nanoparticles on TNF-alpha secretion in LPS treated THP-1 cells

The concentrations used were 60mg/ml and 1.3µg/ml for the extracts and silver nanoparticles, respectively. Each value represents mean ± SEM, statistical significance of the extracts and silver nanoparticles treated cells to the LPS treated cells is indicated with *** for *P*<0.001 or **** for *P*<0.0001.

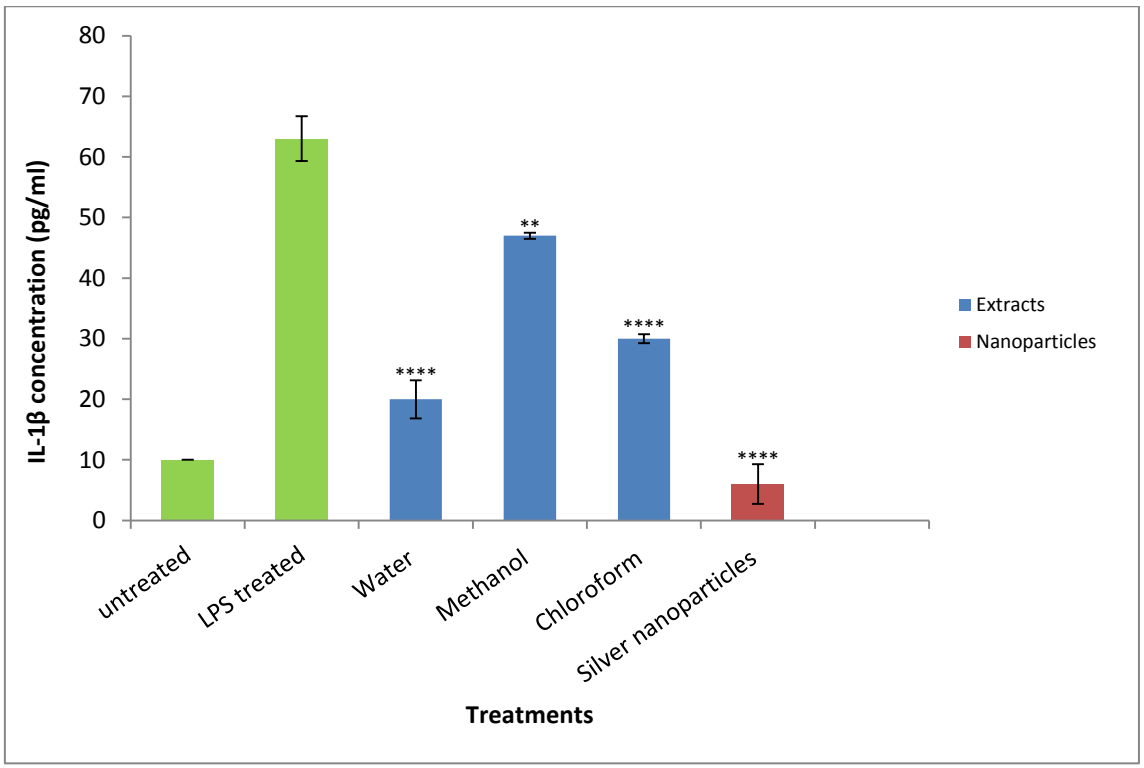

Figure 5. 5: Effects of *C. orbiculata* extracts and silver nanoparticles on IL-1 beta secretion in LPS treated THP-1 cells

The concentrations used were 60mg/ml and 1.3µg/ml for the extracts and silver nanoparticles, respectively. Each value represents mean ± SEM, statistical significance of the extracts and silver nanoparticles treated cells to the LPS treated cells is indicated with ** for *P*<0.01 or **** for *P*<0.0001.

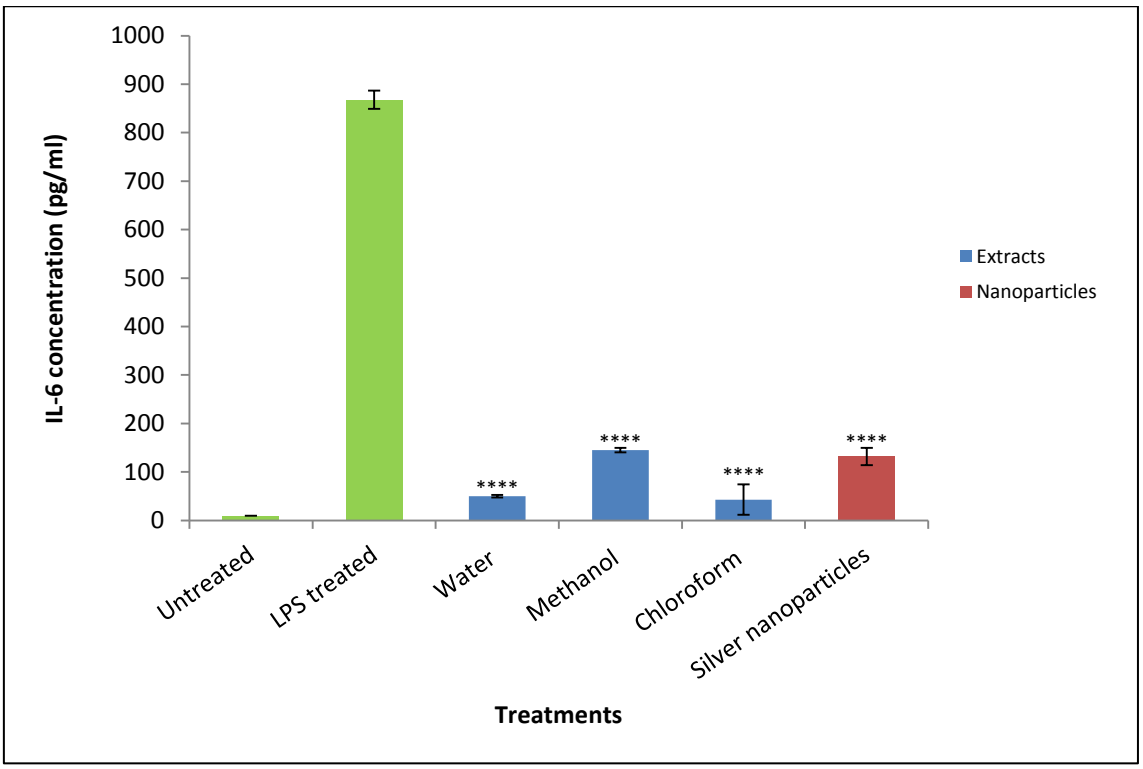

Figure 5. 6: Effects of *C. orbiculata* extracts and silver nanoparticles on IL-6 secretion in LPS treated THP-1 cells

The concentrations used were 60mg/ml and 1.3µg/ml for the extracts and silver nanoparticles, respectively. Each value represents mean ± SEM, statistical significance of the extracts and silver nanoparticles treated cells to the LPS treated cells is indicated with **** for *P*<0.0001.

5.5 Discussion

5.5.1 Differentiation of THP-1 cells

PMA a cognate of diacylglycerol is an activator of protein kinase C (PKC) (Dzietko *et al*., 2015; Goel *et al*., 2007). Activation of THP-1 monocytes by PMA causes differentiation of these cells into macrophages. Upon differentiation, morphological changes of the cells can be observed (Fig 5.1). Differentiated THP-1 cells increase in surface area and become larger; their adherence to glass and plastics is enhanced and they show expansion of the cytoplasm and of cytoplasmic organelles such as mitochondria and lysosomes (Smiderle *et al*., 2011). Other differentiation stimuli such as 1.25-dihydroxyvitamin D3 has been used, however differentiation of monocytes with PMA was shown to resemble the phenotype of human tissue macrophages more closely (Daigneault *et al*., 2010).

Although differentiation of THP-1 cells by PMA has been reported in numerous studies, multiple protocols have been used. Different concentrations of PMA have been used for THP-1 monocyte differentiation. In this study three concentrations of PMA were evaluated and 100nM which appears to be the most commonly used concentration was used for THP-1 cell differentiation. This is consistent with other studies (Malheiro *et al*., 2017; Richter *et al*., 2016; De Bruin *et al*., 2016) where 100nM PMA was used. However Vasandan *et al* (2016) used 20nM, Hayman *et al* (2017) and Sanchez-Queseda *et al* (2015) used 50nM, Genin *et al* (2015) used 150nM and Daigneault *et al* (2010) used 200nM.

5.5.2 The viability of THP-1 cells treated with *C. orbiculata* extracts and silver nanoparticles

In order to study the immunomodulatory effects of *C. orbiculata*, PMA activated THP-1 cells were treated with extracts of *C. orbiculata* and silver nanoparticles produced from the extracts. It was therefore important to determine the highest concentration of *C. orbiculata* extracts and silver nanoparticles that will not be toxic to the THP-1 cells. Cell viability assays are crucial as they allow for the determination of the cytotoxic effects of compounds to different cell lines in-vitro. A variety of methods such as the MTT, WST-1 and ATP assays can be used (Ngamwongsatit *et al*., 2008). In this study the WST-1 assay which is a simple, rapid method was used. This assay is based on the conversion of a water soluble tetrazolium salt into formazan by mitochondrial dehydrogenases, this occurs in viable cells but not in dead cells. The amount of viable cells present will determine the amount of the formazan produced. Therefore the number of cells can be quantified by the detection of the formazan product level (Heimer *et al*., 2016).

The cytotoxicity of *C. orbiculata* plant extracts and silver nanoparticles to THP-1 cells was determined and the results are shown in Fig 5.2 and 5.3 respectively. Extracts of *C. orbiculata* exhibited a dose dependent cytotoxicity towards THP-1 cells. These extracts were more toxic at higher concentrations. The plant extracts, particularly chloroform and methanol showed significant toxicity at concentrations of 240µg/ml and above. To the best of our knowledge this study is the first to evaluate the toxicity of *C. orbiculata* extracts towards THP-1 cells. The toxicity of some other plants such as *Casearia sylvestris*, *Thymus vulgaris* and *Caryocar brasiliense* to THP-1 cells have been done and the IC_{50} values of 124, 160 and 224µg/ml, respectively have been reported (Gusman *et al*., 2015; Ayesh *et al*., 2014).

At concentrations below 5µg/ml the *C. orbiculata* silver nanoparticles did not exhibit significant toxicity to the THP-1 cells. Concentrations of 10µg/ml and above were highly toxic to these cells (Fig 5.3). This finding is in agreement with other studies which reported 5µg/ml silver nanoparticles as the highest concentration that did not affect cell viability in different cell lines. In a study by Martinez-Gutierrez *et al* (2010) chemically synthesized silver nanoparticles were found to be cytotoxic to THP-1 cells at concentrations greater than 5µg/ml. Guranathan *et al* (2013) reported that biogenic silver nanoparticles (synthesized from *Bacillus funiculus*) were cytotoxic to breast cancer cells (MDA-MB 231) at concentrations of 10µg/ml and above.

5.5.3 Cytokine secretion

The immunomodulatory effects of *C. orbiculata* plants extracts and silver nanoparticles were evaluated using the THP-1 cell line. The cells were first differentiated using PMA and then treated with LPS before treatment with the extracts and silver nanoparticles. LPS treatment of the cells resulted in the production of pro-inflammatory cytokines (TNF-alpha, IL-1 beta and IL-6) however the addition of the extracts or the nanoparticles to the cells inhibited the production of these cytokines. These results cannot be attributed to cell cytotoxicity as the dosages used were shown not to affect cell viability (Fig 5.2 and 5.3). Treatments with the three extracts and the silver nanoparticles inhibited the production of TNF-alpha, IL-1 beta and IL-6. Amongst the extracts the inhibitory activity of the water extracts on TNF-alpha and IL-1 beta production was significantly higher than the methanol and chloroform extracts. However the inhibitory effects of the chloroform extract on IL-6 was much higher than the other extracts. The differences in the activity of the extracts can be ascribed to the difference in the phytochemical composition of the extracts. The three solvents (water, methanol and chloroform) have different polarities. Methanol and water are polar solvents; however water is more polar than the methanol solvent whereas chloroform is a non-polar solvent. Polar

solvents extracts polar chemical compounds and vice versa (Ncube *et al*., 2008).

Although silver nanoparticles were produced from the water extract, they exhibited higher inhibitory effect than the water extract. Based on the fact that the concentration of the silver nanoparticles was significantly lower compared to the extracts, it can also be concluded that the silver nanoparticles have a greater inhibitory effect on TNF-alpha, IL-1 beta and IL-6 secretion. This may suggest that the nanoparticles have a higher anti-inflammatory effect. The high activity of the nanoparticles might be due presence of active phytochemicals that have been used in the synthesis of these nanoparticles. Nanoparticles tend to increase the stability of phytochemicals (Chuan *et al*., 2015); they also act as carriers and deliver these active compounds at their desired targets inside the cells (Bonifácio *et al*., 2014). Most of the bioactive phytochemicals of extracts are poorly absorbed by cells because they are unable to cross the lipid membranes or have high molecular size which results in the loss of bioactivity and reduced efficacy (Saraf, 2010). Therefore phytochemicals loaded on nanoparticles will be more stable, highly absorptive and more active than those in the plant extracts (Chuan *et al*., 2015).

The results obtained in this study showed that *C. orbiculata* extracts and silver nanoparticles have anti-inflammatory properties as they decreased the production levels of the pro-inflammatory cytokines in LPS stimulated THP-1 cells (Fig 5.4, Fig 5.5 and Fig 5.6). This finding is consistent with the results by Amabeoku and Kabatende (2012) who reported that methanol extracts of *C. orbiculata* have anti-inflammatory activities. In their study, *C. orbiculata* attenuated carrageenan-induced rat right hind paw oedema which suggested that the plant species have anti-inflammatory effect. In this study however the anti-inflammatory properties of *C. orbiculata* were evaluated by determining the levels of pro-inflammatory cytokines in THP-1 cells. This study is the first to determine the anti-inflammatory properties of the water and chloroform extracts as well as silver nanoparticles produced from *C. orbiculata* extract. One of the traditional uses of *C. orbiculata* is to treat skin inflammations (Kumari *et al*., 2016). This study shows that the water extracts of this plant have anti-inflammatory properties and therefore provides scientific evidence for the traditional use of this plant. Other plants have also been shown to possess the same anti-inflammatory effects when tested using THP-1 cells. These plants include *Cinnamomum osmophloeum* (Chao *et al*., 2005), *Hypericum triquetrifolium* (Saad *et al*., 2016)*, Perganum harmala* (Hadieh *et al*., 2010)*, Thymus vulgaris* (Ocana and Reglero, 2012*), Casearia sylvestris* (Gusman *et al*., 2015) and *Helichrysum stoechas* (Bremner *et al*., 2009).

5.6 Conclusion

From the present study it can be concluded that water, methanol and chloroform extracts of *C. orbiculata* together with silver nanoparticles produced from this plant have anti-inflammatory activities. This confirms that *C. orbiculata* has immunomodulatory properties. This study therefore provides scientific evidence for the traditional use of this plant in inflammatory conditions of the skin. The pro-inflammatory cytokines TNF alpha, IL-1 beta and IL-6 induce inflammation, fever and tissue damage especially when secreted in high concentrations. Agents that reduce the secretion of these cytokines could be really helpful in relieving symptoms of inflammatory processes such as in skin infections, rheumatoid arthritis, inflammatory bowel disease and other autoimmune diseases. It has also been established that although low concentrations of *C. orbiculata* are safe to use, high levels of the plants extracts may become toxic for the cells.

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CHAPTER SIX
GENERAL DISCUSSION AND CONCLUSION

6.1 General discussion and conclusion

The focus of this study was to evaluate the antimicrobial and immunomodulatory properties of *C. orbiculata*. *C. orbiculata* is a South African plant that is commonly found in the Western Cape. It is used to treat many human ailments including skin infections, inflammation, earache and toothache (Amabeoku and Kabatende, 2012). One of the objectives in this study was to determine if the water extract of *C. orbiculata* can be used to synthesize silver nanoparticles. This was achieved by evaluating the different parameters that can affect the synthesis of nanoparticles. The parameters tested include reaction temperature (25 and 70°C), silver nitrate concentration (1 and 3mM), plant extract concentration (1.5, 3 and 6mg/ml) and reaction time. It was shown that the best conditions for the synthesis of silver nanoparticles using *C. orbiculata* water extract is a plant extract concentration of 3mg/ml, a silver nitrate concentration of 3mM and a reaction temperature of 70°C for 2 hours. Using these conditions spherical silver nanoparticles with average sizes of 20-40nm were produced. The synthesized nanoparticles correspond in morphology and nature to most silver nanoparticles produced using other plant extracts in previous studies (Bagherzade *et al*., 2017; Khalil *et al*., 2014).

The antimicrobial properties of the *C. orbiculata* extracts and silver nanoparticles and of were evaluated. The antimicrobial activity was tested against *S. epidermidis, S. aureus*, MRSA, *P. aeruginosa* and *C. albicans*. These pathogenic microorganisms are commonly associated with nosocomial and severe skin infections in South Africa (Mohammed *et al*., 2014). They are also a great cause of concern in conventional antibiotic therapy because of their ability to acquire antimicrobial resistance (Sydnor and Perl, 2011). It is therefore important to search for new antimicrobial agents that can inhibit the growth of these dangerous pathogens.

In this study, all *C. orbiculata* plant extracts exhibited antimicrobial activity at different degrees. The methanol extract had the most antibacterial activity followed by the water extract then lastly the chloroform extract. The high antimicrobial activity of methanol compared to other extracts is because the methanol solvent extracts more compounds from plants than other solvents (water and chloroform). One of these compounds is a saponin which is known to possess high antimicrobial activity (Ncube *et al*., 2008). The water extract also exhibited good antibacterial activity; however it showed better antifungal properties than

all the other extracts (*C. albicans* was the test microorganism). This finding clearly validates the antifungal properties of the plant since the water extract mimics the way the plant is used traditionally. *C. orbiculata* water extracts displayed better antimicrobial activity than most plants recorded in literature as many reports often state that water extracts lack bioactivity (Madikizela *et al*., 2013). This is probably because of the unique phytochemical composition of the plant. Although *C. orbiculata* proved to have antimicrobial activity, its activity was very low because it gave MIC values greater than 1mg/ml. Ncube *et al* (2008) and Van Vuuren (2008), defined a noteworthy antimicrobial agent as that with a MIC reading below 1mg/ml. However the antimicrobial properties of *C. orbiculata* should be acknowledged.

Silver nanoparticles synthesized using *C. orbiculata* extracts also exhibited great antimicrobial activity, because of their large surface area which allows better contact with the cell wall of the microorganisms (Ahmed *et al*., 2016). These nanoparticles were more active against bacteria than the fungus, *C. albicans.* It was seen that *P. aeruginosa* was more susceptible to the nanoparticles than the Gram-positive organisms. The antibacterial and antifungal properties observed for *C. orbiculata* silver nanoparticles in this study correlate with findings by previous studies in the literature (Gomathi *et al*., 2017; Benakashani *et al*., 2016; Ibrahim, 2015; Dobrucka and Dlugaszewska, 2015). The silver nanoparticles exhibited very high antimicrobial activity when compared to the water extract. This activity is attributable to the phytochemicals that are found on the nanoparticles. These are also possibly the phytochemicals responsible for the reduction, capping and stability of the silver nanoparticles.

Another part of this study was to determine the immunomodulatory effects of *C. orbiculata* plant extracts and silver nanoparticles. This was evaluated using the THP-1 macrophage cell line. This cell line resembles primary monocytes and macrophages in morphology and function hence they are used in the study of macrophages in-vitro (Smiderle *et al*., 2011). The cytotoxicity of the extracts and nanoparticles to the THP-1 cells was tested. The extracts exhibited dose dependent toxicity with increased toxicity at higher concentrations. Notable cytotoxicity of more than 20% was observed at 240µg/ml for all extracts. The silver nanoparticles also exhibited toxicity to the THP-1 cells. They showed very high levels of significant toxicity at 10µg/ml. This finding is consistent with other studies in literature which reported 5µg/ml silver nanoparticles as the highest concentration that does not affect the cell viability in different cell lines (Guranathan *et al*., 2013; Martinez-Gutierrez *et al*., 2010).

The immunomodulatory activities of *C. orbiculata* were evaluated by determining cytokine production (TNF alpha, IL 1 beta and IL 6) in THP-1 cells using the ELISA assay. The results obtained in this study showed that *C. orbiculata* extracts and silver nanoparticles have anti-inflammatory properties. The extracts showed different degrees of activity because of the differences in the polarity of the extraction solvents. Overall, the water extract had the most anti-inflammatory activity compared to the other extracts in inhibiting the secretion of the cytokines. The silver nanoparticles however showed more anti-inflammatory activity than the water extract. This activity might be because of the phytochemicals loaded on the nanoparticles. The nanoparticles are likely to increase the penetration of the phytochemicals in the cells thereby increasing their activity (Bonifácio *et al*., 2014). This may increase the effectiveness of silver nanoparticles when used in antimicrobial creams, ointments and wound dressings.

In conclusion, this study confirmed that *C. orbiculata* water extract can be used in the synthesis of silver nanoparticles. These nanoparticles and plant extracts (water, methanol and chloroform) of *C. orbiculata* have antimicrobial and immunomodulatory properties. The water extract gave promising results as it had good anti-inflammatory and antifungal properties compared to the other extracts. Future research on the phytochemicals presents in this extract might lead to the discovery of a new antimicrobial and anti-inflammatory compound. It was also seen that silver nanoparticles increased the effectiveness of the active compounds in water extracts. Therefore further studies can be done on the identification, isolation and purification of these compounds.

These findings greatly validate the use of this plant traditionally as a treatment of skin infections and inflammatory conditions. The knowledge acquired from this research can be used as leads in the production of new effective antimicrobial and anti-inflammatory drugs. Further studies on these extracts and nanoparticles are recommended as they can determine other medicinal properties that this plant may possess such as anticancer and antiviral properties. Comparisons of the activities of *C. orbiculata* produced silver nanoparticles and chemically synthesized silver nanoparticles are also recommended as it will show the extent of activity that is being provided by the phytochemical alone. The cytotoxicity of *C. orbiculata* has to be extensively researched on and the information should be passed on to the community and traditional healers of the Western Cape as they commonly use this plant as a source of medicine.

6.2 References

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