An investigation into the antimicrobial and immune modulatory effects of *Sutherlandia frutescens* and *Salvia africana-lutea* extracts and their respective biogenic nanoparticles.

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By

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ABSTRACT

An investigation into the antimicrobial and immune modulatory effects of *Sutherlandia frutescens* and *Salvia africana-lutea* extracts and their respective biogenic nanoparticle*s*

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The prevalence of cancer and antibiotic-resistant microbes has led to the necessity of affordable, efficacious, non-toxic and easily accessible therapeutic approaches. There is numerous anecdotal evidence that African Traditional Medicines (ATM), in particular medicinal plants, have anticancer, antimicrobial and "immune booster" activities. The interaction of medicinal plants with the immune system could be a great asset in drug discovery. Two South African plants, *Sutherlandia frutescens* (cancer bush) and *Salvia africana-lutea* (brown dune sage) have been associated with a long history of traditional use in the treatment of a variety of ailments including cancer and infections. Additionally, the complexity of phytochemicals present in medicinal plants has led to their utilization in the synthesis of biogenic nanoparticles. Synthesis of colloidal biogenic nanoparticles using plant extracts could possibly enhance the bioactivity of the synthesising phytochemicals. The improved bioactivity of synthesised biogenic nanoparticles is probably due to the enhanced stability of synthesising phytochemicals within the nanoparticles, and increased surface area of nanoparticles giving rise to an improved delivery of beneficial phytochemicals to target cells.

The ability of *Salvia africana-lutea* (SAL) and *Sutherlandia frutescens* (SF) water extracts to reduce silver (Ag) and gold (Au) ions consequently producing biogenic nanoparticles was assessed. The optimum conditions for the synthesis of Ag and Au nanoparticles using SAL were 3.125 mg/ml plant extract reacting with 3 mM

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$AgNO_3$ and 1 mM $NaAuCl_4 \cdot 2H_2O$ respectively at 70 °C for 24 hours shaking at 65 rpm whilst those for the synthesis of Ag nanoparticles using SF (SF AgNP) were 6.25 mg/ml plant extract reacting with 3 mM $AgNO_3$ at 70 °C for 24 hours shaking at 65 rpm. The UV-vis spectrophotometry, Dynamic Light Scattering (DLS), High-Resolution Transmission Electron Microscopy (HR-TEM), and Fourier Transform Infrared (FT-IR) spectroscopy were further employed to confirm the successful synthesis of the Ag and Au nanoparticles.

The antimicrobial activity of the synthesised biogenic nanoparticles and various SAL and SF extracts (water, ethanol and acetone) against wound infecting microorganisms was also investigated. Ethanol and acetone extracts exhibited stronger antimicrobial activity in comparison to the water extracts of both plants. The high minimum inhibitory concentration (MIC) values reported did not categorize most of the extracts as noteworthy antimicrobial agents (MIC values > 1 mg/ml) except for ethanol and acetone SAL extracts (MIC = 0.78 mg/ml) against *S. pyogenes.* The SAL AgNPs and SF AgNPs displayed significant inhibitory activity (MIC values ≤ 1 mg/ml) against all selected microorganisms except for the activity of SF AgNPs against *C. albicans* (MIC = 2 mg/ml).

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The immunomodulatory effects of the various SAL and SF treatments were assessed. The cell proliferation reagent Water Soluble Tetrazolium-1 (WST-1) was employed in the selection of non-toxic extracts and biogenic nanoparticle concentrations to treat the monocyte-derived macrophage cell line THP1 and natural killer cell line NK-92. The selected non-toxic concentrations were then utilised to assess the effects of the extracts and nanoparticles on cytokine production and secretion by THP-1 macrophage and NK-92 using Enzyme-Linked Immunosorbent Assay (ELISA). Varying inflammatory activities were induced by the different treatments when their effects on the production and secretion of interleukin-6 (IL-6), tumour necrotic factor-α (TNF-α) and interleukin-1β (IL-1β) by THP-1 macrophage and interleukin-10 (IL-10) and interferon-γ (IFN-γ) by NK-92 were analysed. However, most of the treatments decreased the levels of pro-inflammatory

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cytokines, suggesting their anti-inflammatory activity. Subsequently, the co-culture of target cells (HaCaT, Hela and PC-3) with treated effector cells (NK-92) (treated with water, ethanol and acetone extracts of SAL and SF and synthesised biogenic nanoparticles) was performed. At an effector:target (E:T) ratio of 10:1, the viability of PC-3 and Hela cells was significantly decreased in the presence of SAL AgNPs, SAL AgNPs and SF AgNPs treated NK-92 cells. These affected target cells were then assessed for intracellular reactive oxygen species (ROS) production post co-culture. ROS production by the PC-3 and Hela cells was increased when compared to the negative control (untreated cells). This could mean the NK-92 cells were stimulated by the nanoparticles to release cytotoxic compounds, other than the cytokines assessed, which possibly stressed the target cells resulting in their decreased viability. The amplified bioactivity of the biogenic nanoparticles was observed when their activities were compared to those displayed by the synthesising plant extracts.

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KEYWORDS

Antimicrobial

Cancer

Immune modulation

Immune system

Macrophages

Microorganisms

Natural killer cells

Nanoparticles

Salvia africana-lutea

Sutherlandia frutescens

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DECLARATION

I declare that ***An investigation into the antimicrobial and immune modulatory effects of Sutherlandia frutescens and Salvia africana-lutea extracts and their respective biogenic nanoparticles*** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Phumuzile Dube Date: 28/02/2020

Signature:
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arrived at, are those of the author, and are not necessarily to be attributed to the National Research Foundation.

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DEDICATION

To my loving family. As you always said “**It is Our Doctorate**”. Here is to our wonderful achievement.

And

To my glorious Heavenly father. All Glory to you!!

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
Ag	Silver
AgNP	Silver nanoparticles
Au	Gold
AuNP	Gold nanoparticles
ATCC	American Type Culture Collection
DLS	Dynamic Light Scattering
EDX	Energy Dispersive X-ray spectra
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
HACAT	Human keratinocyte cell line
Hela	Human uterine cervical carcinoma
HIV	Human Immunodeficiency Virus
HR-TEM	High-Resolution Transmission Electron Microscopy
IL	Interleukin
IFN	Interferon
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MHA	Müller-Hinton Agar
MHB	Müller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NK	Natural Killer
NK-92	Human natural killer cell line
PBS	Phosphate-buffered Saline
PC-3	Human prostate cancer cell line
PDI	Poly Dispersity Index
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive Oxygen Species

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RPMI	Roswell Park Memorial Institute
SAED	Selected Area Electron Diffraction
SAL	*Salvia africana-lutea*
SAL AgNP	*Salvia africana-lutea* silver nanoparticle
SAL AuNP	*Salvia africana-lutea* gold nanoparticle
SF	*Sutherlandia frutescens*
SF AgNP	*Sutherlandia frutescens* silver nanoparticle
SPR	Surface plasmon resonance
THP-1	Tamm-Horsfall Protein 1
TNF	Tumour Necrosis Factor
WHO	World Health Organisation
WST-1	Water Soluble Tetrazolium-1
YPB	Yeast Peptone Broth

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PREFACE

This thesis is submitted in fulfilment of the requirements for the degree of Doctor Philosophiae in the Department of Biotechnology, University of the Western Cape. In chapter 1 a description of the research problem, brief background of the research project and the projected aims and objectives is provided. Chapter 2 provides a literature review that discusses basic concepts related to the project and supplies a rationale for the performance of the project. Three articles which will be submitted for publication are chapters 3, 4 and 5. The three chapters highlight the main aims of this project. They all have separate abstracts, introductions, methods and materials, results, discussions and conclusions. It should be noted that these three chapters contain some similar methods and materials. Chapter 6 is the general discussion summarising the integrated results of the entire thesis.

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CHAPTER ONE

INTRODUCTION

1.1. Statement of the research problem

The rise of cancer incidence and antimicrobial resistance in human pathogens poses a growing challenge to medicine and public health. Due to the high prevalence of HIV and AIDS in Africa, South Africa in particular, the immunocompromised population (who are more susceptible to infections and cancer development) is increasing daily, resulting in high morbidity and mortality. The microorganisms of choice are medically significant and have a high prevalence worldwide, especially in developing countries (Mendelson, 2014). These bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Staphylococcus epidermidis,* Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*) and fungus (*Candida albicans*) infect wounds thereby delaying the wound healing process. If left untreated, these infections may lead to the development of more serious complications including chronic wounds and septicaemia. There is no doubt that the modulation of the immune system can be used as an effective treatment of cancer and infections. Immune modulating drugs such as Ipilimumab (Yervoy; Bristol-Myers Squibb), Nivolumab (Opdivo; Bristol-Myers Squibb), and Pembrolizumab (Keytruda; Merck Sharp and Dohme Corp) have been approved by the Food and Drug Administration (FDA) in the treatment of cancer (West, 2015).

There are claims that several Complementary and Alternative Medicines (CAM) and African Traditional Medicines (ATM) have “immune booster” activity. In South Africa, the use of ATM prepared from plants such as *Sutherlandia frutescens* (cancer bush) and *Salvia africana-lutea* (brown dune sage) is very common amongst HIV positive patients. Several studies have reported that HIV positive patients use these herbal medicines in conjunction with anti-retroviral treatments as “immune boosters” (Huang *et al*., 2014, Lamorde *et al*., 2010). There are also anecdotal claims that these “immune booster”

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herbal medicines can be used to treat infections and cancer (Kumar *et al.*, 2012, Gebreyohannes and Gebreyohannes, 2013). However, there is very little scientific evidence to support these claims. Additionally, the synthesis of biogenic nanoparticles using medicinal plants has shown great promise in medical research. Their improved bioactivity when compared to that of the synthesising crude extracts has led to increased scientific interest (Philip *et al.*, 2011, Ahmed *et al.*, 2015). Henceforth, the bio-application of biogenic nanoparticles alongside their synthesising plant extracts is important in the pursuit of novel therapeutic approaches against cancer and infections.

Due to this lack of adequate research on the immunomodulatory and antimicrobial effects of medicinal plants in conjunction with their respective biogenic nanoparticles, the current study hence aims to investigate the effects of extracts and biogenic nanoparticles of *S. frutescens* and *S. africana-lutea* on natural killer (NK) cells and macrophages. In immune-competent individuals, a rapid activation of the innate (the first line of immune defence) immune system in response to foreign pathogens often results in a successful immune response. Stimulating the activity of immune cells in patients with suppressed immunity may be a beneficial therapeutic tool in the detection and destruction of infectious agents. The innate immune system comprises of a variety of cells including the natural killer cells (NK) and macrophages. NK cells and macrophages have been shown to possess the ability to kill infectious agents (viral and bacterial) and cancerous cells without prior sensitization. Previous studies have shown that NK cells and macrophages play an important role in the detection and eradication of bacterial, fungal and viral infections in the body (Gao and Williams, 2015). The cytotoxic activity of NK cells towards cancerous cells has been investigated as a target-specific treatment of cancer (Geller and Miller, 2011, Cheng *et al.*, 2013). Due to the potential of immunotherapy, several clinical methods have been used to activate NK cells and macrophages. Investigating the antimicrobial and immunomodulatory effects of extracts and biogenic nanoparticles prepared from *S. frutescens* and *S. africana-lutea* may substantiate the claims that these medicinal plants have both antimicrobial and “immune booster” activities.

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1.2. Background of the research problem

Wound infections are a frequent complication of patients with suppressed immune systems. The immunosuppressed state can be caused by different conditions including HIV/AIDS, cancer and surgical procedures. In South Africa, over 5.6 million people are infected with HIV/AIDS (UNAIDS, 2011). The rise in the immunocompromised population has increased the prevalence of wounds infected by antibiotic-resistant microorganisms such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Candida albicans*. The increased resistance of these microorganisms to existing conventional treatment, including ampicillin, fluconazole, and ciprofloxacin is exacerbating this problem, increasing morbidity, mortality and economic strains in health care systems globally. Resurgence of these resistant strains is the greatest threat to patient safety in public and private hospitals across South Africa (Mendelson, 2014). The serious problems associated with these infectious agents are necessitating the urgent need for alternative antimicrobial agents that are easily accessible, safe and efficacious.

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Medicinal plants have been used to treat human diseases for centuries. Anecdotal claims such as medicinal plants are "immune boosters", antimicrobial and anticancer agents have propagated their wide use. Their good therapeutic performance, affordability and low toxicity has also contributed to their popularity (Lin *et al*., 2008). It has been reported that 80% of the population in developing countries use medicinal plants in the treatment of diseases (Maroyi, 2013). Many of these medicinal plants have demonstrated significant potential in the treatment of diseases such as HIV/AIDS, cancer, malaria, diabetes, mental disorders, and microbial infections. The medicinal plants are the richest resource of biological material used in modern and traditional medicinal systems (Das *et al*., 2010). Bioactive phytochemicals such as saponins, tannins, flavonoids, alkaloids, and polyphenols usually demonstrate curative properties (Sofowora, 1993). In recent years, there has been a growing interest in naturally occurring compounds with anti-cancer activity. In the past 20 years, 25% of all medicinal drugs were directly obtained from plants while another 25% was derived from naturally occurring compounds (Gali-Muhtasib *et al*., 2006). There are many examples of plant-derived anti-cancer drugs such as taxol isolated

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from the bark of the western yew tree (*Taxus breviolia*), camptothecin isolated from the Chinese ornamental tree (*Camptothecaa cuminata*), vinblastine and vincristine isolated from the Madagascar periwinkle (*Catharanthus roseus*), and combretas statins isolated from the South African bush willow tree (*Combretum caffrum*) (Shah *et al*., 2013). Despite the recent interest in high throughput drug discovery methods such as molecular modelling, combinatorial chemistry and other synthetic chemistry techniques, natural products such as medicinal plants remain an important source of drug discovery and novel chemical entities (Karthikeyan *et al*., 2015).

Sutherlandia frutescens (cancer bush) and *Salvia africana-lutea* (brown dune sage) are two indigenous South African plants that have been used as traditional medicine for the treatment of various conditions such as fever, internal cancers, cough, diarrhoea, wounds, depression, and anxiety (Nielsen *et al*., 2012, Faleschini *et al*., 2013). The overall acceptance of medicinal plants has led to their increased inclusion in various experimental processes producing novel compounds with improved properties (Sapsford *et al*., 2013). Nanotechnology is a rapidly developing field that makes use of inert metals in the production of beneficial nanomaterials. The bio-reduction of metallic ions by the various metabolites within the plant extracts may form biogenic nanoparticles with high therapeutic potential (Elbagory *et al*., 2017). The improved bio-activity of the resulting biogenic nanoparticles compared to that of the synthesising plants has also elevated scientific interest.

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1.3. Research aim and objectives

The main aim of this research study is to investigate the antimicrobial and immune modulatory effects of two South African medicinal plants (*S. frutescens* and *S. africana-lutea*) and their respective nanoparticles. The objectives are as follows:

1. Investigate the ability of *S. frutescens* and *S. africana-lutea* water extracts in the synthesis silver (Ag) and gold (Au) nanoparticles.
2. Investigate the antibacterial activity of *S. frutescens* and *S. africana-lutea* extracts and respective Ag nanoparticles against medicinally important bacteria;

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Staphylococcus aureus, MRSA, *Streptococcus pyogenes, Staphylococcus epidermidis* and *Pseudomonas aeruginosa.*

3. Investigate the antifungal activity of *S. frutescens* and *S. africana-lutea* extracts and respective Ag nanoparticles against *Candida albicans.*
4. Investigate the effects of *S. frutescens* and *S. africana-lutea* extracts and respective nanoparticles on cytokine production of macrophages and natural killer (NK) cells.
5. Investigate the effects of treated NK-92 cells (treated using *S. frutescens* and *S. africana-lutea* extracts and respective nanoparticles) on cancerous (PC-3 and Hela) and non-cancerous (HaCaT) cells in the co-culture.
6. Investigate the effects of activated NK-92 cells towards ROS production of target cells in co-culture.

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CHAPTER TWO

LITERATURE REVIEW

2.1. Cancer

2.1.1. Cancer development

Cancer can be defined as the uncontrolled growth of abnormal cells due to mutations in genes controlling cell survival and growth (Figure 2.1). Hanahan and Weinberg defined cancerous cells as those able to promote angiogenesis, warrant continuous replication, evade growth suppressors, support proliferative signalling, initiate invasion and metastasis, and withstand cell death (Hanahan and Weinberg, 2011). Additional characteristics focusing mainly on the relationship of cancer cells with the immune system were also identified. These are their capacity to elude immune destruction and their chronic inflammation potential resulting in tumour growth promotion (Edge and Compton, 2010). Cancer development has been accepted as a multistep process that may occur in almost any tissue. The spreading (metastasis) of these fast-growing abnormal cells to vital organs can affect their function resulting in death (Azmi *et al.*, 2013).

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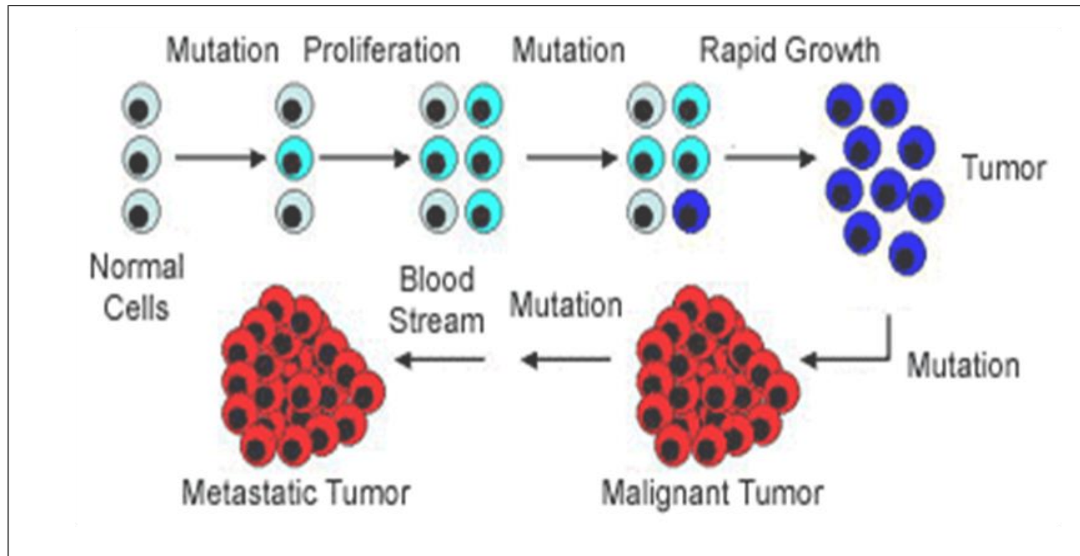

Figure 2.1: Model of cancer development and progression. Uncontrolled division of abnormal cells due to mutations in genes directing cell survival and growth. Adapted from Maggie, 2015.

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2.1.2. Prevalence and Health burden of cancer

The World Health Organization (WHO) reported non-communicable diseases including cancer as the most significant cause of mortality globally. Cancer affects millions of people all over the world and is, therefore, an important global health problem (Vineis and Wild, 2014). This disease accounts for more than 6 million deaths a year worldwide, with an estimated 10 million new cases diagnosed annually. It is predicted that cancer will become the main cause of morbidity and mortality in the coming decade (Bratu *et al.*, 2011). GLOBOCAN stated 14.1 million new cancer cases were reported whilst 8.2 million cancer caused deaths occurred in the year 2012 (Torre *et al.*, 2015). With the decrease in the incidence of heart disease and increased prevalence of confirmed cancer risk factors including tobacco smoking, obesity and sedentary lifestyles, cancer was reported in 2016 to be the leading cause of death in 21 countries. Developing countries, in which 82% of the world's population was reported to reside, accounted for 57% new cases and 65% cancer deaths (Siegel *et al.*, 2016).

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Epidemiologists were reported to speculate an escalation of cancer cases with 60% of new cases originating from Africa, Asia and South America (Sartorius *et al*., 2016). These assumptions were confirmed by the 2018 GLOBOCAN report which highlighted an increased global cancer burden of 18.1 million new cases and 9.6 million cancer caused deaths. This elevated cancer incidence is a result of the populations' exposure to cancer risk factors due to socio-economic development. GLOBOCAN further reported on the displacement in developing countries of poverty-associated and infection-associated cancers by those commonly observed in developed countries (Bray *et al*., 2018).

The World Cancer Research Fund ranked South Africa 50th on a list of countries with the highest cancer prevalence rates (Ferlay *et al*., 2015). WHO indicated that cancer is the 3rd most significant cause of death in South Africans less than 70 years of age as indicated in Figure 2.2. Data published by Statistics SA showed that 8% of all deaths in South Africa were caused by cancer (Jemal *et al*., 2011). Exacerbating the cancer mortality rate is the increased HIV/AIDS population of South Africa. Bohlius and colleagues reported that HIV positive children are at high risk of developing cancer with an 82 in every 100 000 people incident rate (Bohlius *et al*., 2016).

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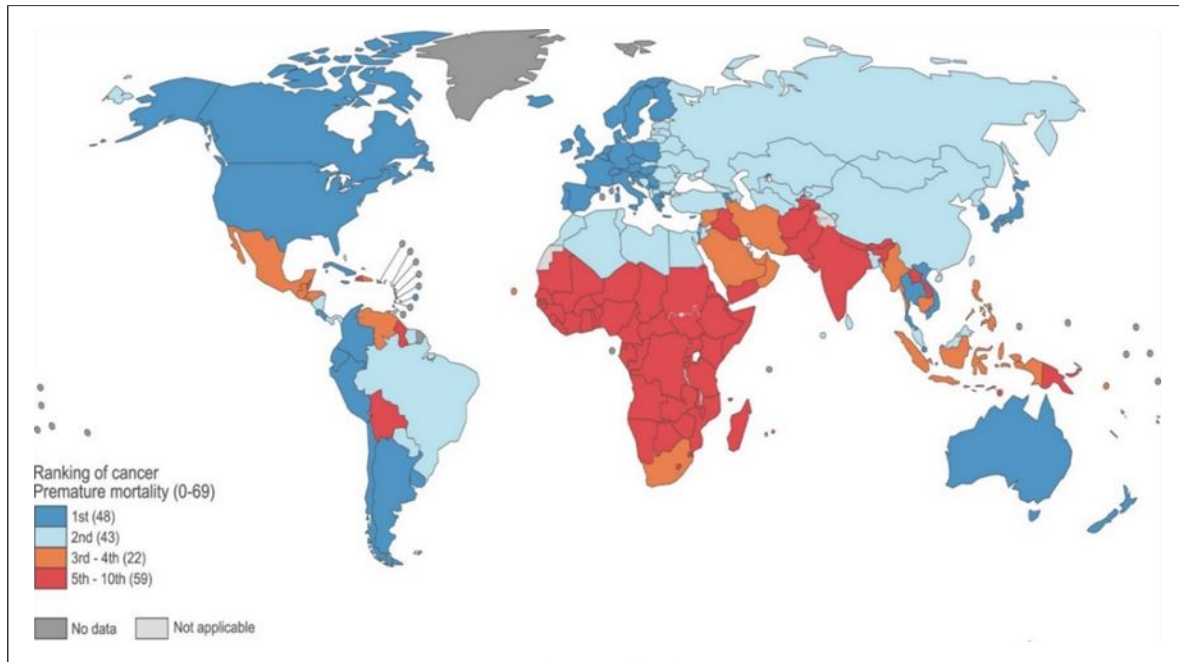

Figure 2.2: Global rankings of death caused by cancer in individuals less than 70 years of age in 2015. South Africa was ranked 3rd-4th. Adapted from Bray *et al*., 2018.

2.1.3. Economic burden of cancer

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Cancers detrimental effects are not confined to the diagnosed patients alone but equally affect the society and economy. In the United States of America (USA), 62% of all bankrupt individuals in 2015 were as a result of healthcare costs (Mahoney, 2015). In 2012, 11 cancer drugs approved by the Food and Drug Administration (FDA) in the USA were priced ZAR1.5 million per year per patient. Cancer care expenditure is expected to increase from $125 billion in 2010 to almost $173 billion in 2020 (Kelly *et al*., 2015). Dr Leonard Saltz, during the 2015 American Society of Clinical Oncology annual meeting, commented that the new-generation immunotherapy drug ipilimumab cost "4 000 times the cost of gold". This new-generation drug is reported to be sold at discounted prices in developing countries, however, these discounted prices are viewed exorbitant by most of the population. In South Africa, the cost of ipilimumab is ZAR1 million whilst that of trastuzumab is ZAR25 000 per treatment (Sartorius *et al*., 2016). This is mainly due to the high costs associated with drug development. The cost escalation coupled with the

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expected increase in cancer cases in South Africa has necessitated the development of more affordable and easily accessible treatment and management strategies for cancer.

2.1.4. Cancer risk factors

Though the general prevention and treatment of certain cancers have recently improved, the overall cancer burden is increased due to the ageing global population and varying risk factors. Advancement in technology coupled with socio-economic development have led to an increased adoption of the sedentary lifestyle by populations' in both developed and developing countries. Sedentary lifestyle as a behavioural trait increase the probable exposure of individuals to cancer risk factors throughout their lifespan resulting in the elevated prevalence of cancer. Certain factors have been reported to incline individuals toward the development of specific cancers (Azzani *et al.*, 2015, Parkin *et al.*, 2014). Cancer risk factors have been categorized as either intrinsic or extrinsic, with intrinsic or unavoidable cellular factors modestly contributing to cancer development. The intrinsic factors result in mutations in the DNA sequence following cellular replication. Extrinsic factors are environmental or behavioural influences promoting the occurrence of mutagenesis, a process by which a mutation occurs due to a change in an organism's genetic information. Of all cancer cases 70-90% are thought to be caused by extrinsic factors whilst the remaining 10-30% are linked to intrinsic factors. An estimated 50% of all reported cancer cases are caused by avoidable behaviours including smoking, alcohol consumption and food overindulgence (Wu *et al.*, 2016, O'Leary *et al.*, 2018).

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Tobacco smoke contains at least 60 carcinogenic chemicals which increase the developmental risk of 17 cancer types including bladder, kidney, liver, stomach, colon, rectum, cervix, throat, lung, larynx, oesophageal and mouth cancer (O'Leary *et al.*, 2018). The mechanism by which tobacco causes genomic damage resulting in mutations and consequently cancer development remain misunderstood. However, the main mutational signature observed in tissues directly exposed to tobacco carcinogens is the erroneous replication of DNA. Furthermore, DNA base substitution has been reported to occur more

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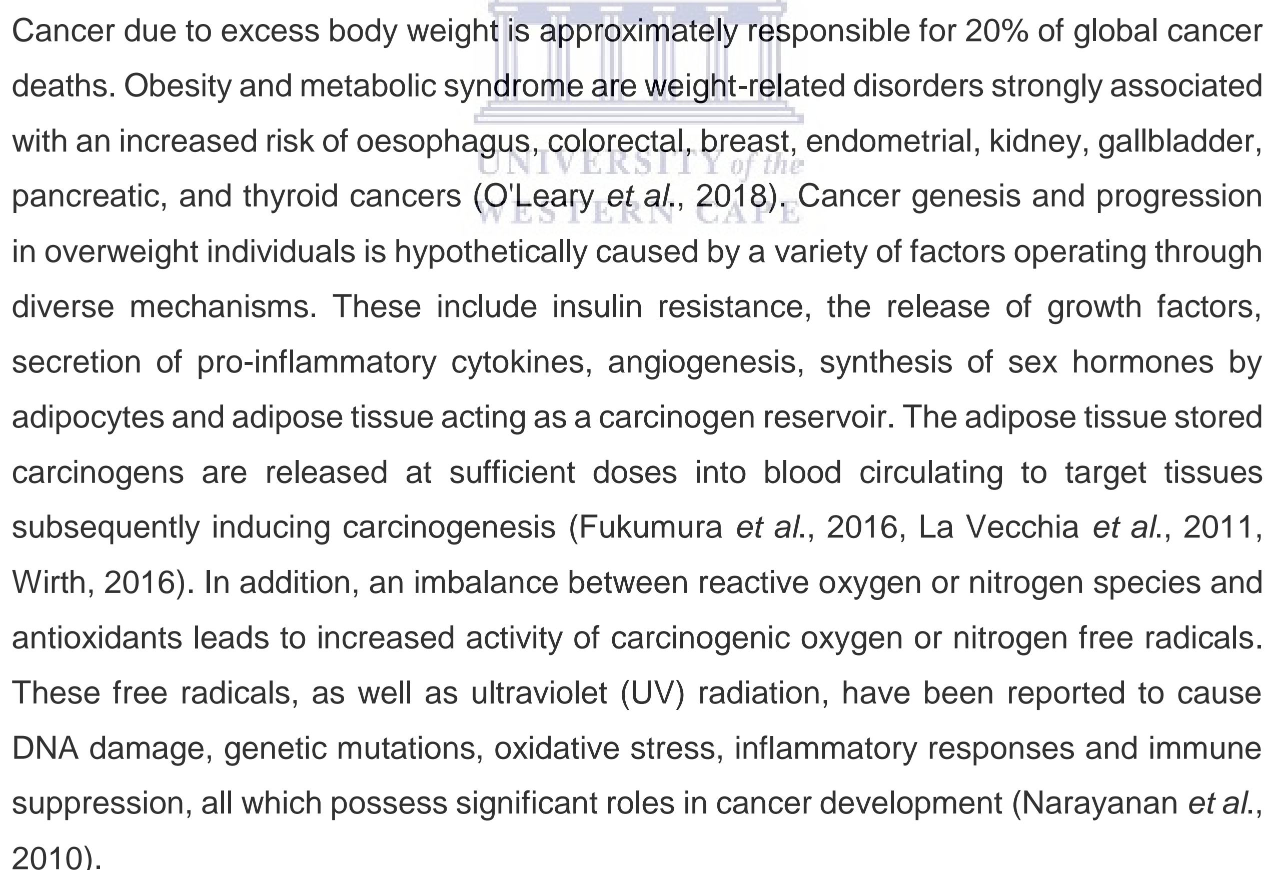
frequently in smokers (Alexandrov *et al.*, 2016). Alcohol use, on the other hand, has been directly associated with 7 cancers namely breast, mouth, oesophagus, liver, larynx, pharynx, and colorectal cancer. It is estimated that 5.8% of cancer mortality rates are related to alcohol consumption, globally. Majority of these findings have been associated with an alcohol intake of 3-3.5 glasses a day (Connor, 2017). The mechanisms by which alcohol consumption causes cancer is target dependent. Acetylaldehyde, the carcinogenic by-product of ethanol oxidation in the liver, causes DNA damage leading to the development of alcohol-related pharynx, larynx, mouth, liver and oesophagus cancer (Boffetta and Hashibe, 2006). With respect to alcohol-associated breast cancer, oestrogen metabolism interference is observed resulting in the hormones display of carcinogenic traits either directly or via oestrogen receptors (Scoccianti *et al.*, 2014, Seitz *et al.*, 2012).



Cancer due to excess body weight is approximately responsible for 20% of global cancer deaths. Obesity and metabolic syndrome are weight-related disorders strongly associated with an increased risk of oesophagus, colorectal, breast, endometrial, kidney, gallbladder, pancreatic, and thyroid cancers (O'Leary *et al.*, 2018). Cancer genesis and progression in overweight individuals is hypothetically caused by a variety of factors operating through diverse mechanisms. These include insulin resistance, the release of growth factors, secretion of pro-inflammatory cytokines, angiogenesis, synthesis of sex hormones by adipocytes and adipose tissue acting as a carcinogen reservoir. The adipose tissue stored carcinogens are released at sufficient doses into blood circulating to target tissues subsequently inducing carcinogenesis (Fukumura *et al.*, 2016, La Vecchia *et al.*, 2011, Wirth, 2016). In addition, an imbalance between reactive oxygen or nitrogen species and antioxidants leads to increased activity of carcinogenic oxygen or nitrogen free radicals. These free radicals, as well as ultraviolet (UV) radiation, have been reported to cause DNA damage, genetic mutations, oxidative stress, inflammatory responses and immune suppression, all which possess significant roles in cancer development (Narayanan *et al.*, 2010).

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2.1.5. Conventional cancer treatment

The main goal of cancer treatments is to preserve the short and long-term quality of life for diagnosed patients. Though cancer therapy is considered multimodal, the treatment strategy administered is dependent on numerous factors including malignancy type, cancer progression, genetic and histologic nature of a tumour, and previously administered therapies (Schnipper *et al*., 2015). Oncologists use the above criteria to enhance the prognosis of the diagnosed cancer, prescribing the ideal treatment plan for each patient to increase the probability of a positive outcome. The control of cancerous cells' survival and death is the main target of all existing cancer treatments. The major approaches to achieving tumour control include surgery, chemotherapy, and radiotherapy. However, due to increased recurrence and metastases cases of cancer after a series of therapy, there is a need for the development of drugs which can specifically target the cancerous cells without causing adverse effects. Furthermore, treatment-linked side effects which vary in nature and extent between individuals undergoing similar treatments increase the problems associated with existing cancer treatments (Frey *et al*., 2014). The side effects together with the general unacceptability of the clinical outcomes necessitate the identification of novel strategies for the treatment and management of the disease. Consequently, herbal drugs which include medicinal plants, herbal complexes and compounds, and immune therapy have been identified as an appropriate adjunct to standard cancer therapies.

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2.1.5.1. Immunomodulation as a treatment for cancer

The immune system is an extensive network of tissues and specialised cells intended to detect and eliminate foreign materials (e.g. bacteria and viruses) as well as damaged and harmful cells (e.g. cancer cells) present in the body. However, cancer cells have developed mechanisms to either suppress the body's immune system or hide from immune cells designed to detect and destroy them. It has been demonstrated that tumour derived factors alter the phenotype of modulated dendritic cells (DC) resulting in impaired DC with lower levels of CD40, CD80, DC86 expression, contributing to the suppression

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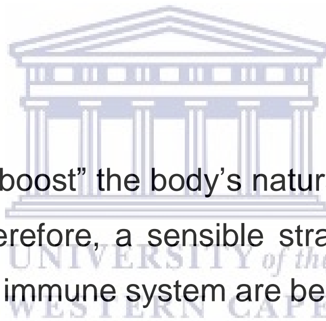
of host T cells (Munn *et al*., 2002). Immune suppression is modulated by $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (Treg) and other immune suppressor cells. Transformation of normal $CD4^+$ T cells into Treg is aided by the transforming growth factor beta (TGF-β) produced by tumour cells (Jacobs *et al*., 2012). It has been reported that tumour cells are able to shed off surface antigens or down-regulate the expression of potent surface molecules required for their recognition by immunity cells. Furthermore, tumour variants resistant to the immune system effectors are expressed during a process termed immuno-editing which results from constant tumour division due to genetic instability of cancer cells (Vinay *et al*., 2015). Henceforth tumour cells either become weak immune cell stimulators or target sites subsequently evading the hosts' immune system. Tumour cells also produce or release chemical factors that either induce apoptosis or modulate the function of the immune cells, therefore, directly interfering with the hosts' immune system (Whiteside, 2006, Finn, 2012).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Immunotherapy, which aims to "boost" the body's natural defence mechanisms to detect and destroy cancer cells is, therefore, a sensible strategy to combat cancer. Several strategies aimed at boosting the immune system are being investigated as treatments for cancer. A number of food and drug administration (FDA) approved immunotherapeutic anticancer drugs have recently come onto the market (Table 2.1).

Table 2.1: List of FDA approved immunotherapeutic anticancer drugs.

Drug	Trade name	Targeted cancer	Mechanism	FDA year of approval	Reference
Ipilimumab	Yervoy®	Melanoma	Targets a protein receptor (CTLA-4) that downregulates the immune system.	2011	Robert *et al*., 2011
Sipuleucel-T	Provenge®	prostate cancer	Autologous cell immunotherapy that stimulates the immune system	2010	Kantoff *et al*., 2010
Nivolumab	Opdivo®	renal cell carcinoma	Blocks signals that prevent T-cell activation.	2015	Kazandjian *et al*., 2016
Atezolizumab	Tecentriq®	bladder cancer	Blocks inhibitory signals related to T-cell activation.	2016	Fellner, 2016
Elotuzumab	Emplicit®	multiple myeloma	Directly activates natural killer (NK) cells.	2015	Fellner, 2016

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2.1.5.2. Natural killer (NK) cells as an antitumour defence mechanism

Natural killer (NK) cells are a distinct group of lymphocytes that are part of the innate immune system. Hellström and colleagues were the first researchers to report on the anti-tumour activities of NK cells in 1968 (Hellstrom *et al.*, 1968). Generally, the innate immune system directly or indirectly regulates cancer development. Directly, the immune cells either release free radicals that damage the DNA or regulate intracellular pathways of the cancerous cells. The indirect mechanisms include the production of growth factors, cytokines and chemokines that restrict tumour angiogenesis and upregulate the antitumour adaptive immune responses (de Visser *et al.*, 2006).

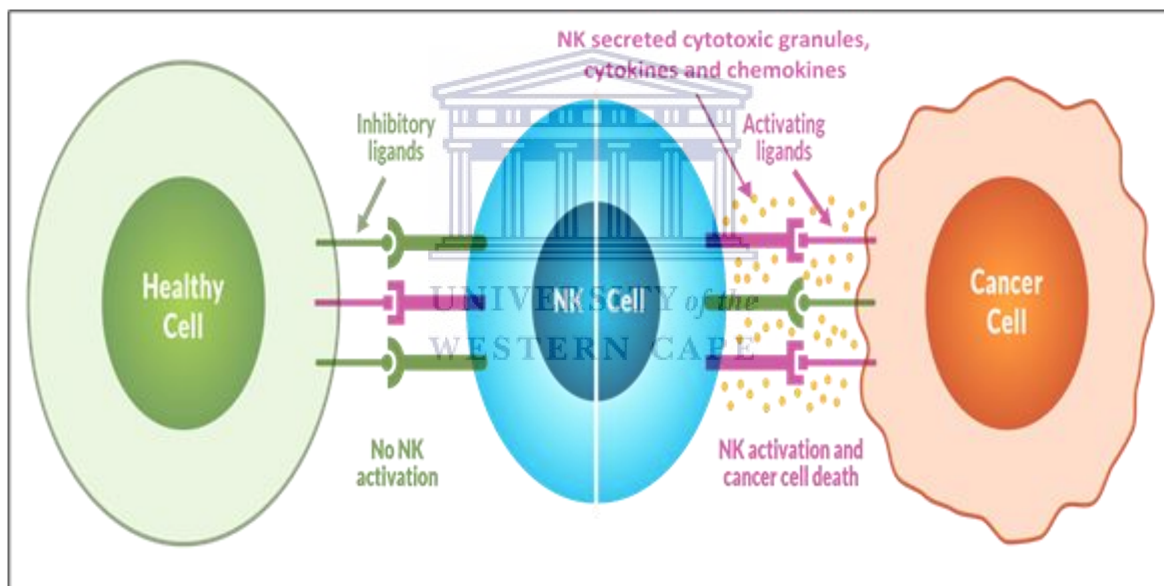

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Figure 2.3: Mechanism of NK cell inhibition and activation. Healthy cells express more inhibitory ligands, while cancer cells express more activating ligands that recognised by surface receptors on NK cells resulting in the activation of NK cells. Adapted from Campana, 2015.

NK cells detect cancer cells through cell surface receptor recognition and can induce cell death through the release of cytotoxic granules containing perforin and various granzymes (Figure 2.3). The secreted compounds cause the perforation and subsequent apoptotic death of the targeted cancer cells via caspase-dependent and –independent

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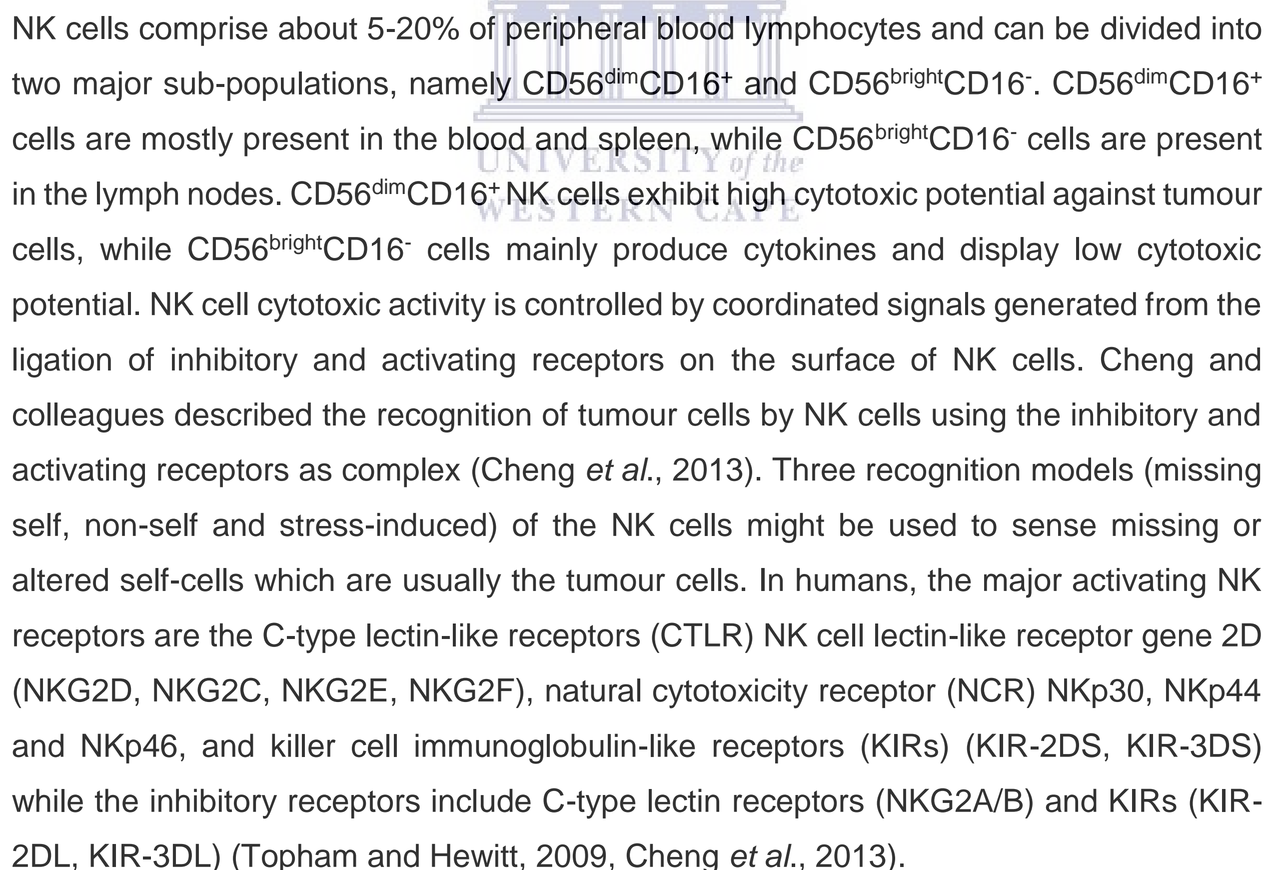
pathways (Kokura, 2016, Vivier *et al*., 2012). NK cells can also eliminate cancer cells through the expression of death receptor ligands such as Fas Ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), which causes apoptosis in the cancer cells. The expressed tumour necrosis factor (TNF) family members FasL and TRAIL induce tumour cell apoptosis by interacting with FasL and TRAIL receptors expressed on the tumour cells. NK cells are also able to secrete various effector molecules, which possess anti-tumour functions including stimulation of adaptive immunity and restriction of tumour angiogenesis (Screpanti *et al*., 2001, Sutlu and Alici, 2009, Cheng *et al*., 2013). An 11-year survey observing the association of cancer incidence and death with cytotoxicity of peripheral-blood lymphocytes was performed. It reported that a low NK cell cytotoxicity in the peripheral blood correlates with an increased risk for cancer (Levy *et al*., 2011). This further confirms the function of NK cells in eradicating cancerous cells in patients.



NK cells comprise about 5-20% of peripheral blood lymphocytes and can be divided into two major sub-populations, namely CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-}. CD56^{dim}CD16^{+} cells are mostly present in the blood and spleen, while CD56^{bright}CD16^{-} cells are present in the lymph nodes. CD56^{dim}CD16^{+} NK cells exhibit high cytotoxic potential against tumour cells, while CD56^{bright}CD16^{-} cells mainly produce cytokines and display low cytotoxic potential. NK cell cytotoxic activity is controlled by coordinated signals generated from the ligation of inhibitory and activating receptors on the surface of NK cells. Cheng and colleagues described the recognition of tumour cells by NK cells using the inhibitory and activating receptors as complex (Cheng *et al*., 2013). Three recognition models (missing self, non-self and stress-induced) of the NK cells might be used to sense missing or altered self-cells which are usually the tumour cells. In humans, the major activating NK receptors are the C-type lectin-like receptors (CTLR) NK cell lectin-like receptor gene 2D (NKG2D, NKG2C, NKG2E, NKG2F), natural cytotoxicity receptor (NCR) NKp30, NKp44 and NKp46, and killer cell immunoglobulin-like receptors (KIRs) (KIR-2DS, KIR-3DS) while the inhibitory receptors include C-type lectin receptors (NKG2A/B) and KIRs (KIR-2DL, KIR-3DL) (Topham and Hewitt, 2009, Cheng *et al*., 2013).

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NKG2D receptors play a key role in the recognition of cancer cells by NK cells, as they are rarely expressed on normal cells. Human cytomegalovirus (HCMV) UL16-binding proteins (ULBP16), MHC class I chain-related protein A (MICA) and B (MICB) are NKG2D ligands that are overexpressed on the surface of cancer cells. ULBP16 is expressed on primary leukaemia, glioma and melanoma tumour cells, whilst MICA by melanoma, some primary leukaemia and many isolates from lung, kidney, prostate, ovary, colon and breast carcinoma, and MICB by breast, prostate, pancreatic, gastric and ovarian cancers. The expression levels of these cell surface glycoproteins increase when cells are under stress, transformed or damaged. Increased expression of the NKG2D receptors results in cancer cells being more susceptible to NK killing as they act as “kill me” signals. However, it has been shown that cancer cells could escape NKG2D facilitated NK cell recognition by shedding cell surface NKG2D receptors, a process mediated by the action of endoplasmic reticulum protein 5 (ERp5). ERp5 induces a conformational change of MICA and MICB by binding to a six amino acid motif in the α3 domain of the NKG2D receptors (Aguera-Gonzalez *et al*., 2009). This action results in the decreased recognition of NKG2D receptors expressed on cancer cells. It has been reported that soluble MICA and MICB were present in the sera of patients with a variety of malignancies (Ghadially *et al*., 2017). Drugs that can increase the expression of NKG2D ligands in cancer cells are therefore a suitable strategy to treat cancer. Dacarbazine (DTIC) which has been used to treat patients with inoperable metastatic melanoma has been shown to increase the expression of NKG2D ligands in melanoma cells (Ugurel *et al*., 2013).

The ability of activated NK cells to detect and destroy cancer cells can be exploited for the treatment of cancer. Several attempts have been made to use NK cells in this way. Examples of this are autologous and allogeneic immunotherapy. Autologous NK cell infusion is when a patient’s own immune cells (NK cells have been used, but activated cytotoxic T lymphocytes or dendritic cells can also be used) are removed, activated and placed back into the patient. Allogenic NK immunotherapy, which is the most promising of the two therapies, involves the activation and transfusion of donor immune cells into the patient. This method, however, has increased risk of host versus graft disease

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(Klingemann *et al*., 2016). Autologous clinical trials have not been successful (Ljunggren and Malmberg, 2007) due to the compromised state of the NK cells. The underlying disease and its treatment results in the relative ineffectiveness of the autologous NK cells. The use of therapeutic drugs able to activate NK cells has proven more successful in the treatment of cancer. Lenalidomide, thalidomide, and pomalidomide are examples of FDA approved immunomodulatory drugs that are able to stimulate the cytotoxic functions of NK cells (Dahlberg *et al*., 2015). A study by Xu and colleagues showed that lenalidomide activates T cells to secrete IL-2, which in turn activates NK cell cytotoxicity (Xu *et al*., 2013).

2.2. Wound infections

2.2.1. Prevalence of wound infections

In recent years, the epidemiology of invasive infectious diseases has increased (Zarrin and Jundishapur, 2009). The resurgence of highly infectious microbes resistant to conventional treatment is an important cause of morbidity and mortality globally, mostly in the low-income countries (Coetzee *et al*., 2013, Mudau *et al*., 2013). Microbiologically, the main function of intact skin is to prevent surface microorganisms from invading and colonizing underlying tissue hence preventing or retarding infection development. A breach of the multi-functional skin may lead to the invasion of the body by pathogenic microorganisms resulting in the development of serious conditions including chronic wounds and septicaemia (Lai-Cheong and McGrath, 2017).

2.2.2. Wound healing and development of wound infections

Wound healing is described in four phases' namely hemostasis, inflammation, proliferation and maturation (Serra *et al*., 2015). The process involves the interaction between different cells namely endothelial, epithelial, keratinocytes, fibroblasts and langerhans (Figure 2.4). The increased proliferation of highly virulent microorganisms has been shown to affect different stages of wound healing. At the wound site bacteria tend

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to develop biofilms under which the microorganisms are protected from the host immune system. Microbial infections are known to be treated by antibiotics, however, this biofilm development has contributed significantly to the microorganisms' antibiotic resistance. In 2008 it was estimated that 99.9% of the global microbial biomass existed as biofilms (Bjarnsholt *et al*., 2008).

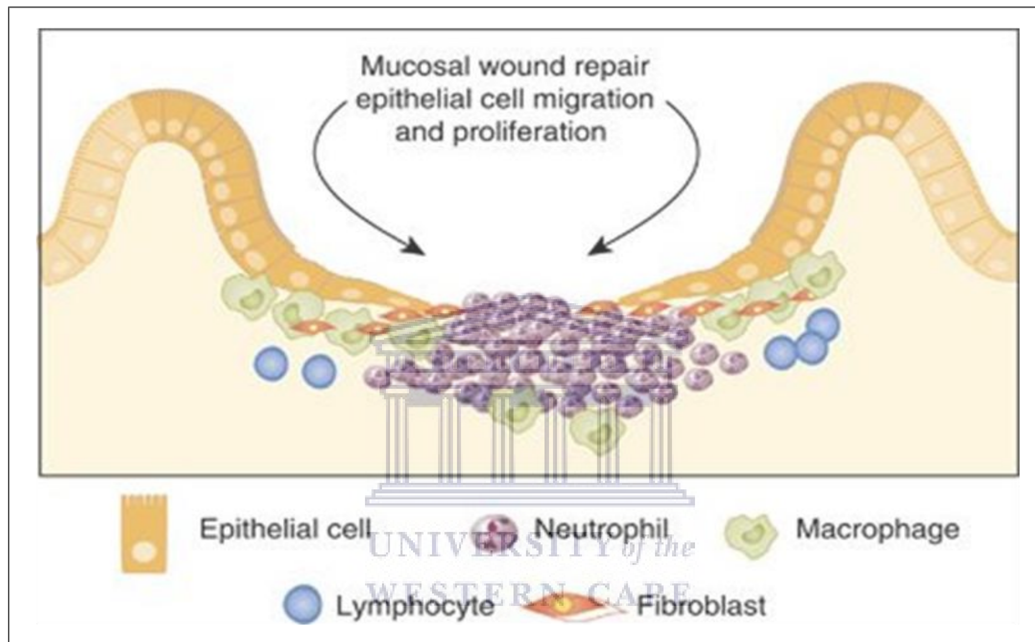

Figure 2.4: Role of the inflammatory components in wound healing. The major inflammatory components that are involved in the different phases of wound repair, include epithelial cells, keratinocytes, fibroblasts, neutrophils, macrophages and lymphocytes. Reprinted with permission from Springernature (Leoni *et al*., 2015).

2.2.3. Prevalent wound infecting microorganisms

To date, aerobic and facultative infectious microorganisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Streptococcus pyogenes* and *Candida albicans* are currently posing a great challenge to the medical sector, due to their widespread antibiotic resistance (Dumville *et al*., 2013). *Pseudomonas aeruginosa* and MRSA have been listed

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by the Infectious Diseases Society of America as part of the six most pathogenic microorganisms to which novel effective therapies are urgently required. These infectious microbes are also the primary causes of delayed wound healing. They currently represent an important cause of morbidity and mortality in developing countries, warranting the urgent need for alternative antimicrobial agents that are easily accessible, affordable, safe and efficacious.

2.2.3.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) of the *Pseudomonadaceae* family is an aerobic Gram-negative rod-shaped bacteria first isolated from skin wounds in 1882. It mainly inhabits water and soil, posing a serious problem in a variety of settings including hospitals. The single flagellum (Figure 2.5) allows for efficient surface attachment and motility of *P. aeruginosa*. Furthermore, the ability of the bacteria to form biofilms permits it to survive harsh environments (Peekate and Abu, 2017). Biofilm formation allows for the bacterium to localize within the deepest regions of the wound. Wounds infected by *P. aeruginosa* tend to occupy a greater area and portray delayed or inhibited healing processes (Gjodsbol *et al*., 2006, Gjodsbol *et al*., 2012). The numerous virulent factors including hydrogen cyanide, cytotoxins, exotoxins, blue phenazine pigment pyocyanin, enzymes (elastase, haemolysin-phospholipase C, proteases) and exoenzymes (ExoS, ExoT, ExoU, ExoY) elevate the harmful effects of *P. aeruginosa*.

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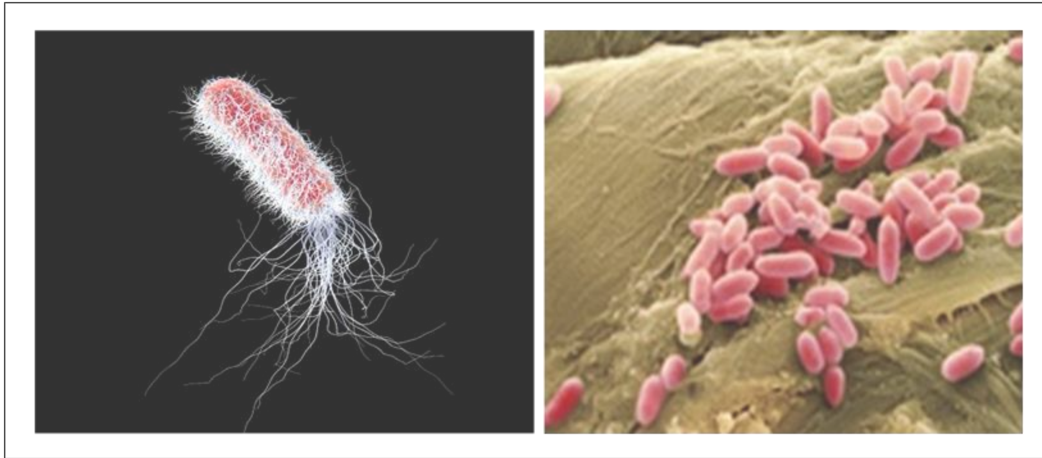

Figure 2.5: **Scanning electron micrographs and morphology of the *P. aeruginosa*.** Gram-negative rod-shaped bacteria with a single flagellum. Adapted from Gschmeissner, 2013, Kon, 2019.

2.2.3.1.1. Prevalence of *P.* aeruginosa infections

The emergence and spread of infections caused by the multi-drug resistant, *P. aeruginosa* is of great concern. This opportunistic pathogen is a significant cause of hospital-acquired (e.g. bloodstream infections, urinary tract infections, surgical wound infections, skin infections in burn injuries, and pneumonia) and community-acquired (e.g. skin and soft tissue infections and keratitis) infections. However, most *P. aeruginosa* infections are often hospital-acquired than community acquired (Percival *et al.*, 2012). Individuals at high risk of developing such infections include those with compromised immune systems, those admitted long term in hospital settings, those diagnosed with cystic fibrosis and those prescribed long term antimicrobial therapies. These infections are sometimes life-threatening when left untreated and occur in individuals with impaired immunity. Numerous studies have reported on the nosocomial nature of *P. aeruginosa* infections as well as its extensive involvement with skin infections (Percival *et al.*, 2012, Salisbury *et al.*, 2018, Weiner *et al.*, 2016). In the intensive care unit (ICU), *P. aeruginosa* has been associated with 13.2-22.6% of nosocomial infections and noted as the most common cause of nosocomial pneumonia (Driscoll *et al.*, 2007, Behnia *et al.*, 2014). It was reported that 80% of adults diagnosed with cystic fibrosis have chronic *P. aeruginosa* infection (Hoiby, 2011). Furthermore, 52.2% of chronic leg ulcer cases reported in 2010 were

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complicated by *P. aeruginosa* infections (Storm-Versloot *et al*., 2010). A 21-day retrospective study of patients diagnosed with *P. aeruginosa* bloodstream infections from two university hospitals in Italy reported a staggering 33.9% mortality rate (Tumbarello *et al*., 2011). Another retrospective study reported an 80% fatality rate in neutropenic patients infected with *P. aeruginosa* in the haematology ward of a South African tertiary academic hospital (Mudau *et al*., 2013). Recently, the Centre for Disease Control and Prevention reported that *P. aeruginosa* accounted for 8% of all healthcare-associated infections recorded in 2 039 hospitals (Weiner *et al*., 2016).

2.2.3.1.2. Resistance mechanisms of *P. aeruginosa*

Antimicrobial resistance is an increasing global health concern caused by a multiple of factors. *P. aeruginosa* is one such bacterium that has demonstrated resistance to most antibiotics. Compared to other microorganisms, *P. aeruginosa* is difficult to eradicate due to its intrinsic, and acquired resistance. The intrinsic mechanisms of *P. aeruginosa* include:

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- the low permeability of its outer membrane which acts as a barrier in the uptake of antimicrobial agents drastically reducing antibiotic uptake (Breidenstein *et al*., 2011)
- induced expression of various efflux pumps (Tumbarello *et al*., 2011).
- production of β-lactamase capable of enzymatically modifying β-lactam antibiotics (Alvarez-Ortega *et al*., 2010).

P. aeruginosa is able to acquire inheritable traits that further decrease its antibiotic susceptibility. These include antibiotic resistance genes attained either by conjugation, transduction or transformation harboured within deoxyribonucleic acid (DNA) and mutational resistance that results in the alteration of antibiotic target sites (Breidenstein *et al*., 2011).

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2.2.3.1.3. Conventional treatment of *P. aeruginosa* infections

Systemic administration of antimicrobials is ideal in the treatment of *P. aeruginosa* caused infections. Though early detection of *P. aeruginosa* infections can improve prognosis, it cannot guarantee the success of treatment. The most common drugs used to treat *P. aeruginosa* infections include ceftazidime, piperacillin/tazobactam, ciprofloxacin, meropenem, ertapenem and amikacin with piperacillin/tazobactam being reported as more efficacious (Serra *et al*., 2015). However, the prolonged use of these drugs has been associated with adverse side effects including tendinitis and drug fever. The overuse of the antibiotics has also been reported to result in *P. aeruginosa* developing resistance to prescribed antibiotics previously identified as effective e.g. third and fourth generation cephalosporins and aminoglycosides (gentamicin and amikacin). Henceforth, the need for less detrimental and less common antimicrobial agents like medicinal plants and nanoparticles in the treatment of *P. aeruginosa* infections (Breidenstein *et al*., 2011).

2.2.3.2. Methicillin sensitive and resistant *Staphylococcus aureus*

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Like *P. aeruginosa*, *Staphylococcus aureus* (*S. aureus*) is a common opportunistic pathogen. *S. aureus,* a Gram-positive coccus (Figure 2.6), is considered both a pathogen and commensal depending on its relationship with the host. *S. aureus* has a higher pneumonia-causing frequency in comparison to *P. aeruginosa*, making it a very dangerous pathogen since pneumonia is the chief cause of adolescent death worldwide (Rasigade and Vandenesch, 2014, Reed *et al*., 2012, Rasigade *et al*., 2014). As a commensal, it inhabits either the skin or mucous membranes of 30-50% of the worlds' population. The pathogenic characteristics of *S. aureus* are attributed to its complex virulent factors: DNAse, α-haemolysin, β-haemolysin, σ-haemolysin and staphylococcal enterotoxins (e.g. SER to SET, SEG to SEI, SEA to SEE) (Argudin *et al*., 2010, Stefani *et al*., 2012). The emergence of the methicillin-resistant *S. aureus* (MRSA) strain led to increased scientific interest of the microorganism. Though microscopically similar to the methicillin-sensitive *S. aureus* (MSSA), MRSA expresses the *mec*A gene which makes it resistant to the antibiotic methicillin. Furthermore, some virulent factors absent in MSSA

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are frequently expressed in MRSA such as the leukocytic toxin Panton-Valentine leukocidin responsible for severe cutaneous infections (Goss and Muhlebach, 2011). The resistant nature of MRSA has led to it being identified as a major cause of healthcare-associated and community-associated infections (Kock *et al*., 2010). The basic structure of MSSA and MRSA consists of a peptidoglycan cell wall, surface proteins, circular chromosome and microcapsule. The gold pigmentation of the *S. aureus* colonies on agar plates differentiate MSSA and MRSA from other staphylococcal species.

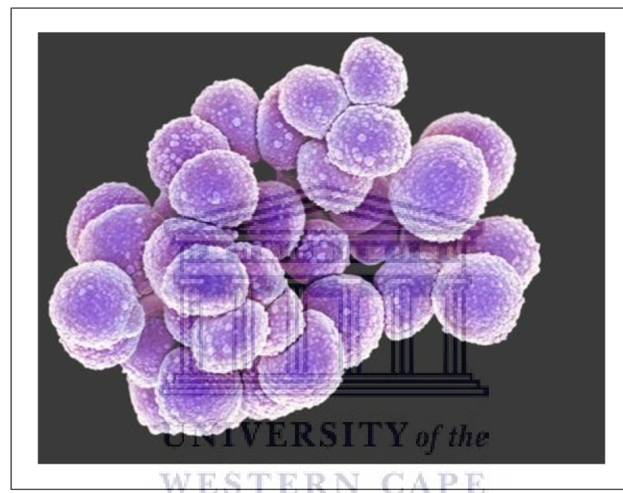

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Figure 2.6: Scanning electron micrograph and morphology of the *S. aureus*. Gram-positive spherical shaped in clusters. Adapted from SciencePhotoLibrary, 2018.

2.2.3.2.1. Prevalence of MSSA and MRSA infections

Respiratory ailments are the most important cause of mortality in developing countries (Madikizela *et al*., 2013). MSSA and MRSA are associated with the development of noteworthy respiratory infections. They are also important causative agents of a wide array of infections ranging from localised skin and soft tissue eruptions to more life-threatening conditions such as bone, joint and central nervous system infections, sepsis, bacteraemia and endocarditis (Tong *et al*., 2015, David and Daum, 2010). In patients between the ages of 9 and 11 years, MRSA was identified as the main bloodstream infecting pathogen (Tohidpour *et al*., 2010). MRSA was also reported the major bacterium responsible for community-acquired infections in young males (Choucair and Boustani,

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2018). The microbiological analysis of 438 pus samples revealed that 318 pus samples were culture positive for *S. aureus*, in India (Sangwan, 2016). Over a 5 year period (2007-2011), 26% of bacteraemia cases, 72% of all nosocomial infections and 8.8% of bacteraemia mortalities in a Cape Town children hospital (South Africa) were a result of *S. aureus* (Naidoo *et al*., 2013).

2.2.3.2.2. Resistance mechanisms of MSSA and MRSA

The emergence of virulent *S. aureus* strains resistant to methicillin, vancomycin, fluoroquinolone, tetracycline and macrolide-resistant is a heavy predicament worldwide. The existence of these resistant *S. aureus* strains is a result of the different resistance mechanisms possessed by the bacterium. These mechanisms include enzymatic inactivation of antibiotic, spontaneous gene mutation and decreased antibiotic affinity (Duran *et al*., 2012). Further intensifying its resistance is the microcapsule surrounding the bacterium made of the anti-phagocytic polysaccharide microcapsular polysaccharide serotype which protects *S. aureus* from the phagocytic pathways of the hosts' immune system (Pantosti and Venditti, 2009). In addition to these resistance mechanisms MRSA, the most common of the resistant *S. aureus* strains, has adopted unique resistance towards methicillin. Methicillin inhibits the growth of bacteria by blocking the penicillin-binding proteins (PBP) which function to maintain the bacterial cell wall. In MRSA the expressed mec A gene encodes for the modified penicillin-binding protein (PBP2a) which replaces the PBP decreasing the affinity of the bacterium for β-lactams such as penicillin, cephalosporin and carbapenems (Monecke *et al*., 2011, Stefani *et al*., 2012).

2.2.3.2.3. Conventional treatment of MSSA and MRSA infections

S. aureus infected patients require extended antibiotic therapy to prevent infection relapse (Tong *et al*., 2015). The sensitivity and resistance of *S. aureus* strains has been extensively studied. Though vancomycin has been the ideal antibiotic in the treatment of *S. aureus* infections, strains with decreased vancomycin sensitivity have been identified

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(van Hal and Fowler, 2013). A South African study, carried out in the Mafikeng area identified *S. aureus* strains resistant to methicillin, penicillin, ampicillin, erythromycin, streptomycin, and oxytetracycline (Ateba et al., 2010). Oxacillin, cefazolin, doxycycline, clindamycin and sulfamethoxazole have been identified as treatments for MSSA infections whilst linezolid, daptomycin, tigecycline and ceftaroline are utilised to address MRSA infections. Regardless of their significant antimicrobial potential, the routine prescription of these antibiotics is not yet established due to their high cost. Furthermore, prolonged intake of these antimicrobial agents has been linked to the development of different side effects including nephrotoxicity (Tong *et al*., 2015). These factors increase the global demand for more efficacious, affordable and less detrimental antimicrobial agents in the treatment of MSSA and MRSA infections.

2.2.3.3. *Streptococcus pyogenes*

Streptococcus pyogenes (*S. pyogenes*) is a Gram-positive coccus (Figure 2.7) responsible for infections ranging from acute to life-threatening. Usually referred to as group A streptococci (GAS) due to their expression of group A antigen, this human pathogen causes toxic shock syndromes, skin, soft tissue, and respiratory infections. *S. pyogenes* is able to adapt to the hosts harsh environment (Lamagni *et al*., 2009, Johansson *et al*., 2010). This adaptation is a result of the expression of complex virulence factors referred to as response regulators (RRs). The RRs serve as a mean of communication between the adaptive mechanisms of the bacterium and their surrounding environment (Johansson *et al*., 2010).

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Figure 2.7. **Scanning electron micrograph and morphology of the *S. pyogenes.*** Gram-positive spherical shaped bacteria. Adapted from Batra, 2018.

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2.2.3.3.1. Prevalence of *S. pyogenes* infections

The increasing prevalence of severe GAS infections such as streptococcal toxic shock syndrome and necrotizing fasciitis is a global concern. A survey conducted in 194 hospitals in France identified 664 cases of invasive GAS infections and reported a 3.1 per 100 000 people global annual incidence (Lepoutre *et al*., 2011). A more recent study isolated GAS from 1 069 throat swab samples collected from paediatric patients in China, confirming the vulnerability of children to GAS infections (Gao *et al*., 2017). GAS infections contribute significantly to the mortality and morbidity in developing countries, with a reported adolescent fatality rate of 15% (Engel *et al*., 2014).

2.2.3.3.2. Resistance mechanisms of *S. pyogenes*

More than 40 encoding genes within GAS are responsible for its virulence nature. The random expression of these genes results in the emergence of different GAS strains resistant to antibiotic therapies (Richter *et al*., 2008). The rise in the prevalence of macrolide-resistant GAS strains is posing a global clinical problem. 40 GAS strains were reported to exhibit resistance to the antibiotic tetracycline (Camara *et al*., 2013).

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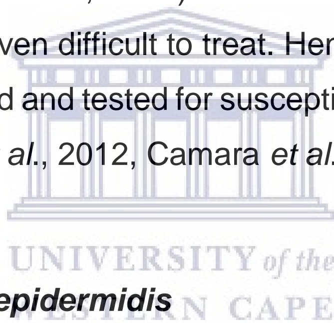
Prolonged and increased administration of antibiotics is the main contributory factor to the elevated resistance (Albrich *et al*., 2004). Target site modification and drug efflux mechanisms within the bacterium also contribute towards its antibiotic resistance (Richter *et al*., 2008). Henceforth, unconventional therapies need to be developed to efficiently combat the pathogen**.**

2.2.3.3.3. Conventional treatments of *S. pyogenes* infections

GAS is sensitive to penicillin and vancomycin and resistant to numerous antibiotics including erythromycin, clindamycin, tetracycline and rifampin (Rubio-Lopez *et al*., 2012). However, it has been reported that penicillin exhibits decreased activity against severe invasive GAS infections (Sawai *et al*., 2007). Due to the existence of different strains, GAS related infections have proven difficult to treat. Hence it is important for the isolated strain to be successfully identified and tested for susceptibility to specific antibiotics before their administration (Shulman *et al*., 2012, Camara *et al*., 2013).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2.2.3.4. *Staphylococcus epidermidis*

Staphylococcus epidermidis (*S. epidermidis*) is a clinically prominent Gram-positive coccus commonly encountered on human epithelial tissues as well as in hospital settings (Figure 2.8). *S. epidermidis* usually becomes pathogenic if the skin barrier is breached or when the hosts' immune system is compromised. The coagulase-negative staphylococcus has been identified as the main causative agent of bacteraemia. It is responsible for about 40-90% of all reported medical device linked infections (Otto, 2009, Byrd *et al*., 2017). Aggravating this problem is the increase in antibiotic resistance of *S. epidermidis*, making their eradication a difficult task. The bacterium has been observed to display pathogenic behaviour in hospital environments confirming its nosocomial nature. It accomplishes this through the secretion of toxins that are detrimental to the hosts' health such as delta-toxin, C3 enterotoxin and staphylococcal enterotoxin-like toxin (Namvar *et al*., 2014).

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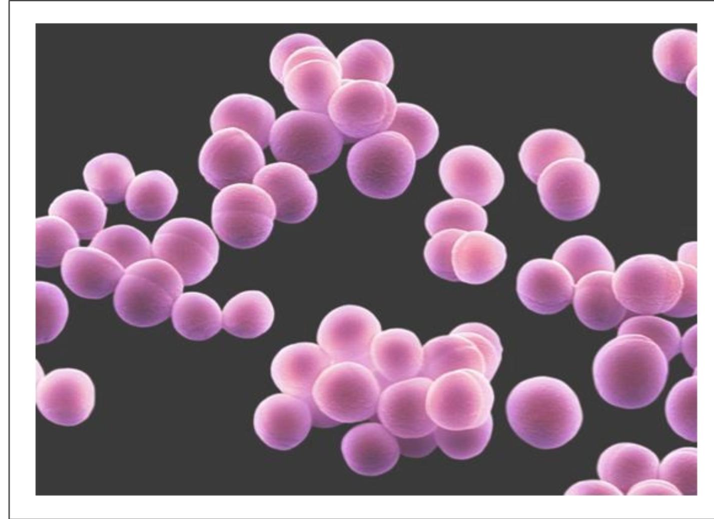

Figure 2.8. **Scanning electron micrograph and morphology of the *S. epidermidis.*** Gram-positive coccus. Adapted from Gschmeissner, 2016. .

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2.2.3.4.1. Prevalence of *S. epidermidis* infections

Most *S. epidermidis* caused infections are of a chronic nature and include nasal, mucosal and medical-device related infections. The infections associated with medical devices such as prosthetics are a serious concern in health centres globally. Methicillin resistant *S. epidermidis* was reported to colonize the nasal cavities of children in Japan (20%) and army draftees in the USA (7%) (Namvar *et al.*, 2014).

2.2.3.4.2. Resistance mechanisms of *S. epidermidis*

Due to the widespread use of antibiotics, *S. epidermidis* has recently developed notable antibiotic resistance. Global surveys have revealed that 60-85% of isolated *S. epidermidis* strains display methicillin resistance. This has been reported to be due to the expression of the *mec*A gene carried by the staphylococcal cassette chromosome (SCC); similar to MRSA (Kleinschmidt *et al.*, 2015, Leite *et al.*, 2011). Furthermore, biofilm formation is a key factor in the pathogenesis of *S. epidermidis* which allows the bacterium to be rooted in an extracellular matrix. The biofilm protects the cocci from the hosts' immune system resulting in persistent chronic infections. *S. epidermidis* produces exo-enzymes,

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protective surface polymers, cytolytic phenol-soluble modulin (PSM) that participate in biofilm development (Chong *et al*., 2016). The bacterium has the ability to inactivate β-lactam antibiotics through the action of β-lactamase which destroys their ability to bind to PBP's (Namvar *et al*., 2014).

2.2.3.4.3. Conventional treatment of *S. epidermidis* infections

Numerous studies have reported on the resistance of *S. epidermidis* to the anti-staphylococcal drug methicillin due to *mec*A gene expression (Leite *et al*., 2011, Qin *et al*., 2017). To date vancomycin, daptomycin, and dalbavancin are the most important antimicrobial therapies utilised in the treatment of *S. epidermidis* caused infections (McManus *et al*., 2015). However, these drugs have been shown to be less effective if the bacteria produce a biofilm (Morgenstern *et al*., 2016). Rifampicin is one drug associated with good therapeutic outcomes with respect to biofilm development. It is important to note that rifampicin has a high probability to lose its effectiveness due to the development of resistance (Wi *et al*., 2018). Antibiotics such as linezolid and tigecycline have been associated with low *S. epidermidis* resistance, intensifying their popularity (Namvar *et al*., 2014).

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2.2.3.5. *Candida albicans*

Due to their omnipresence, fungal infections are also significant contributors to morbidity and mortality. The *Candida* genus consists of 17 species of which *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* are the most invasive strains. *Candida albicans* (*C. albicans*) is present in the gastrointestinal tract, vaginal and oral mucosa of about 75% of the worlds' population. In immunocompetent individuals this colonization is manageable whilst in immunocompromised patients, this causes infections commonly referred to as candidiasis (Kim and Sudbery, 2011, Mayer *et al*., 2013, Rane *et al*., 2013). Its unique ability to grow and survive as yeast, pseudohyphae and hyphae has made it the subject of extensive research (Figure 2.9). This morphological triad is assumed to be an important virulent

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characteristic of the dimorphic fungi (Mayer *et al*., 2013). The fungus disease spectrum is increased by its ability to colonize almost every body part from superficial to deep tissue.

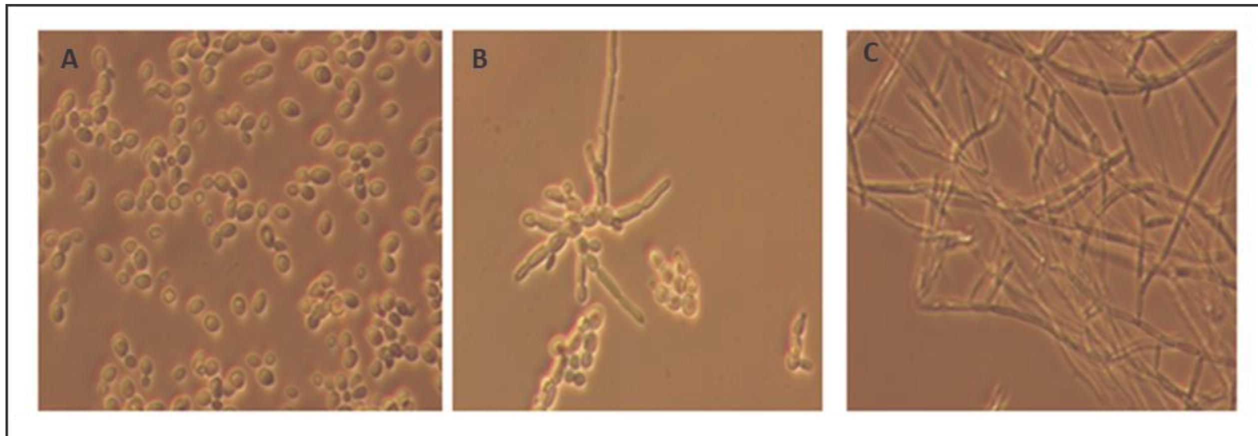

Figure 2.9. Microscopic image of the fungus *C. albicans*. Different morphological states of the fungus include yeast (A), pseudohyphae (B), hyphae (C). Adapted from Drummond, 2016.

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2.2.3.5.1. Prevalence of *C. albicans* infections

C. albicans is the most significant cause of nosocomial infections, accounting for 40% of bloodstream infections and 15% of sepsis cases (Nobile and Johnson, 2015). In the USA *C. albicans* was reported to be the cause of 6.8% of all recorded nosocomial infections (Hidron *et al*., 2008, Rane *et al*., 2013). The ability of *C. albicans* to:

- attach to host tissue surfaces,
- evade hosts' immune system through morphogenesis
- produce tissue-damaging hydrolytic enzymes e.g. haemolysin, phospholipases and proteases
- develop biofilms on both host tissue and medical devices

attribute significantly to its pathogenicity (Pfaller, 2012, Mayer *et al*., 2013, Tobudic *et al*., 2012). Candidiasis, the mucous membrane infections caused by *C. albicans,* often occurs in diabetic and immunocompromised patients. Candidiasis is associated with a mortality

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rate of 10-49% among the hospitalized and immunocompromised population (Masevhe *et al*., 2015, Pfaller and Diekema, 2007). The immunocompromised populace includes; patients with diabetes, cancer and HIV/AIDS, patients ingesting prescribed antibiotics and patients undergoing immunosuppressant therapy. With an HIV/AIDs incidence of about 5.6 million, candidiasis is considered a serious economic and health care burden in South Africa. About 90% of HIV/AIDS patients develop oropharyngeal candidiasis during various stages of AIDS progression (UNAIDS, 2011, Liu *et al*., 2011). The increased prevalence of these conditions that weaken the immune system has consequently led to the epidemiological escalation of *C. albicans* infections. A study documented that 79.1% of HIV infected children in Africa develop oral candidiasis, confirming the high incidence of oral candidiasis in HIV/AIDS infected children (Gaitan-Cepeda *et al*., 2015, Charone *et al*., 2013).

2.2.3.5.2. Resistance mechanisms of *C. albicans*

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The cost of care and length of hospital admission due to deep-seated invasive candidiasis remain significantly high. Furthermore, the emergence of drug-resistant *C. albicans* strains further intensifies the problems associated with treatment. Hence, the development of novel affordable antifungal drugs is a critical requirement. The ability of the fungus to exist in three forms at any given time and energy-requiring efflux pumps (BENr, CDR1 and CRR2) encoded by specific genes were identified as the main causes of *C. albicans* resistance (Rane *et al*., 2013). A study conducted to scientifically investigate the azole resistance of *C. albicans* reported that the inactivation of the ERG3 gene that encodes for sterol Δ 5.6-desaturase, an enzyme utilised in the synthesis of ergosterol is a noteworthy mechanism for the increased resistance (Vale-Silva *et al*., 2012). Additionally, the genetic transformation of the drugs target-site within the fungus renders the prescribed anti-fungal treatments non-functional (Pfaller, 2012).

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2.2.3.5.3. Conventional treatment of *C. albicans* infections

The first choice for treating *C. albicans* in HIV/AIDS are the azole antifungal agents, specifically fluconazole. However due to prolonged and frequent exposure of the fungal strains to these azoles, therapeutic failure and drug resistance has since emerged posing a challenge to clinicians (Liu *et al*., 2011). Regardless of this fact, in the case of severe fetal candida infection, fluconazole administration prevents fetal injury related to intra-amniotic infections (Maneenil *et al*., 2015).

2.2.4. Immunomodulation in the treatment of microbial infections

Throughout our lifetime the immune system is continuously interacting with commensal and pathogenic microorganisms. The pathogens, in most cases, are efficaciously targeted by the immune system and prevented from causing infections. Due to the effectiveness of immune defences, the ability to modulate the immune system, either by boosting or suppression, has been therapeutically useful in a variety of conditions. Currently, there is an elevated prevalence of antibiotic-resistant microbial infections as well as a significant decline in the discovery of novel antibiotics (Hofer, 2019). Henceforth, there is increased interest in the exploitation of the hosts' natural anti-infection mechanisms to enhance therapeutic benefits. The most promising approach involves the direct modulation of the hosts' immune system in an attempt to eradicate the infectious agents with limited inflammatory induced tissue injury (Figure 2.10). The possibility of infecting agents developing resistance is eliminated, as the therapeutic target is the host rather than the invading pathogen (Silva *et al*., 2016, Tse *et al*., 2017).

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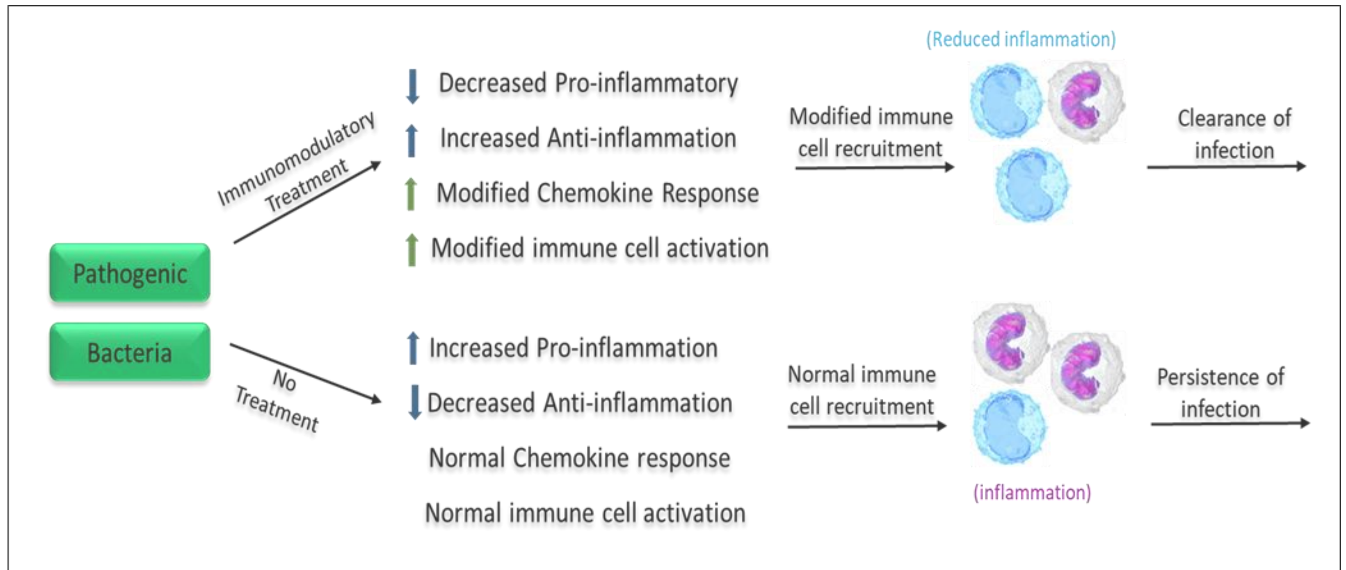

Figure 2.10. Direct modulation of the hosts' immune system in eradicating infectious agents with limited inflammatory induced tissue injury. Reduced inflammation results in accelerated infection clearance. Modified and reprinted with permission from Bentham Science Publishers Ltd (M Fura *et al*., 2017).

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2.2.5. Macrophages as an antimicrobial defence mechanism.

Macrophages have always been identified as the focal point of immune systems as they are the first and most evolved white blood cell observed categorizing them as helper cells for adaptive immunity (Van Belleghem and Bollyky, 2018). In wounds, macrophages play numerous roles including host defence due to their different phenotypic states i.e alternatively activated M2 (able to heal and/or repair) and classically activated M1 (able to inhibit and/or kill) as displayed in Figure 2.11.

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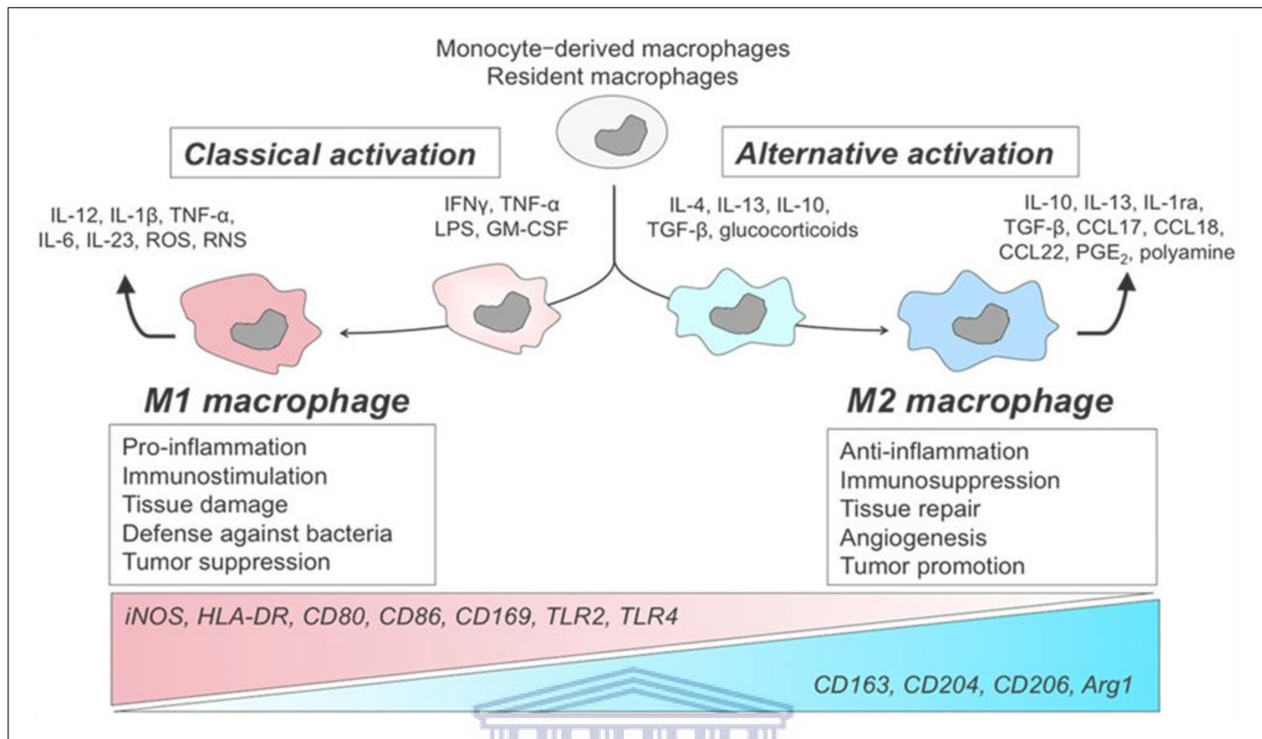

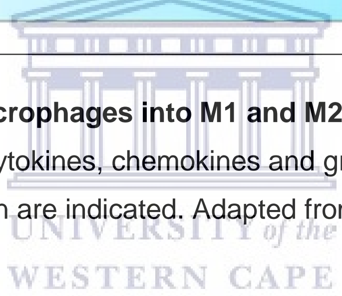

Figure 2.11`: Differentiation of macrophages into M1 and M2 subtypes in response to environmental stimuli. Activating cytokines, chemokines and growth factors as well as biochemicals secreted post activation are indicated. Adapted from Takeya and Komohara, 2016.

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M2-type macrophages maintain homeostasis by assisting with the repair and replacement of cells. It is also abundant during the wound healing process. However, in the presence of an infection or colonization by pathogens this type can modify itself into the inhibitory mode M1. The polarized macrophage subtype M1 and M2 responses play central roles in activating T cells to make Th1 and Th2-type responses respectively in the presence of pathogens (Mills *et al*., 2015). The activity of the M1-type macrophages, as well as nitric oxide (NO) production, has been associated with host protection. NO production has been documented to possess antifungal, antibacterial and antiviral effects on specific microorganisms (Mills *et al*., 2000, Cole *et al*., 2012). Innate activation involving the stimulation of the M1-type macrophage is dependent on pattern recognition of pathogens. Lipopolysaccharide (LPS), the most studied signal expressed by invading bacteria, is recognised by the Toll-like Receptor 4 (TLR-4) of the M1-type macrophage. The activation of TLR-4 induces Tirap (Toll-interleukin 1 receptor domain containing adaptor protein) and

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MyD88-dependent pathways that lead to the secretion of pro-inflammatory cytokines (e.g. IL-6, IL1-β, IFN-β, IL-12 and TNF), chemokines (e.g. CXCL11 and chemokine [C-C motif] ligand 2 CCL2) and antigen presenting molecules (e.g. antigen processing molecules and MHC) (Martinez and Gordon, 2014). Therefore, genetic mutations within the TRLs family has been linked to prolonged infections (Casanova *et al*., 2011).

2.3. African Traditional Medicines (ATMs)

The World Health Organization (WHO) defined ATMs as the "sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or societal imbalance, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing". Complementary and Alternative Medicines (CAM) which are traditional medicines adopted by cultures outside the indigenous populace have been widely used with 70% of the German and Canadian population being reported to have tried CAM at least once (Abdullahi, 2011). Medicinal plants, which are considered part of ATMs, contain substances or properties, in one or more organs, that can be used for therapeutic purposes (Sofowora *et al*., 2013). They are the richest resource of biological material used in modern and traditional medicine systems including the pharmaceutical and nutraceutical industries (Das, 2010). Different cultures have over many years acquired a wealth of indigenous knowledge on the use of plants to treat various ailments including wounds, coughs and colds, digestive problems, ulcers, as well as more serious conditions such as cancer, diabetes mellitus, hypertension and asthma (Ncube *et al*., 2013). *Aloe greatheadii, Centella asiatica, Dialium schlechteri* are just some of the indigenous South African medicinal plants traditionally used for the treatment of wounds (De Wet *et al*., 2013). The South African traditional medicine trade is believed to represent 5.6% of the annual National Health budget worth R2.9 billion, providing employment to at least 133 000 people (Mander *et al*., 2007).

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Prior to the realization that active phytochemicals within the plants are responsible for their medicinal efficacy, doctrine trends were utilised to identify medically useful plants. One example was the intake of red coloured herbs in the treatment of blood diseases. With technological advancement, isolation of the active compounds led to the development of commonly prescribed modern medicines. Morphine was the first pharmacologically recognized active compound isolated in pure form from a medicinal plant. With this, numerous alkaloids were then isolated from plants and used as beneficial drugs, revolutionising the pharmaceutical sector. These include caffeine (*Coffea arabia*), cocaine (*Erythroxylum coca*), morphine and codeine (*Papaver somniferum*), pilocarpine (*Pilicarpus jaborandi*), salicin (*Salix* species) and theophylline (*Camellia sinensis*) (Salim, 2008, Ahn, 2017). When the correlation between the traditional use of medicinal plants and the modern medicines discovered from those plants was studied, it was reported that 88 isolated compounds from 72 medicinal plants have been initiated into modern therapy. Majority of these compounds were observed to display identical or similar therapeutic function as those of the medicinal plants (Fabricant and Farnsworth, 2001). Currently, plants are still significant sources of medicines, particularly in developing countries that use plant-based traditional medicines for their healthcare. A more recent study reported that 25% of all medicines worldwide are derived from medicinal plants (van Wyk and Prinsloo, 2018). Regardless of medicinal plants being a fully entrenched practice in South Africa, they are not regulated. They are generally perceived as safe and more effective due to their natural constituents and their constant use for centuries. However, the effects of these medicinal plants are poorly documented. In the absence of scientific evidence to back up claims about the therapeutic efficacy of medicinal plants, these assertions will remain unsubstantiated and pose a potential public health problem.

2.3.1. Medicinal plants with immunomodulatory activity

There are claims that several CAMs and ATMs have “immune booster” activity. Medicinal plants with immune modulating phyto-compounds have shown great promise in the treatment of detrimental conditions. Medicinal plants that have been shown to possess this activity include *Hypoxis hemerocallidea* (African potato), *Allium sativum* (garlic),

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Zingiber officinale (ginger), *Pelargonium sidiodes* (black pelargonium), *Scilla natalensis* (wild squill) and *Eclipta postata* (false daisy) (Peltzer *et al*., 2011, Street, 2012, Khodadadi, 2016, Ngcobo and Gqaleni, 2016), presenting an alternative to chemotherapy and antibiotic therapy. In South Africa, the intake of teas prepared from common medicinal plants is very common amongst HIV positive patients (Owen-Smith et al., 2007, Kamatou, 2010). Several studies reported that HIV positive patients use these herbal medicines in conjunction with anti-retroviral treatments as “immune boosters” or “tonics” (Mahlangeni *et al*., 2018, Zou *et al*., 2016, Fomundam *et al*., 2015). There are also claims that these “immune booster” herbal medicines can be used to treat cancer and microbial infections (Nair, 2006). Although the immune modulating effects of herbal medicines prepared from plants such as *Echinacea purpurea, Panax ginseng* and *Astragalus membranaceus* have been studied, not much research has been done on the immune effects of *Sutherlandia frutescens* (cancer bush), and *Salvia africana-lutea* (brown sage).

2.3.2. *Sutherlandia frutescens* (cancer bush)

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Sutherlandia frutescens (L.) R.Br (Figure 2.12) , an endemic to Southern Africa, is known by over 25 names worldwide but is commonly referred to as the cancer bush (English) or kankerbos (Afrikaans) (Powrie, 2004). It has been recorded that this plant contains significant levels of free and protein-bound amino acids, together with non-protein amino acids. The documented high levels of the non-protein amino acid compound L-canavanine, a compound associated with anti-cancer and antimicrobial activity could explain the traditional use of the plant in cancer treatment (Ntuli *et al*., 2018). The complex interaction of different compounds within the plant such as Pinitol, γ-aminobutyric acid (GABA), flavonoids, triterpene glycosides and other compounds has further contributed significantly to the ethnopharmacological ability of *Sutherlandia frutescens* (*S. frutescens*) (van Wyk, 2008, Aboyade *et al*., 2014) . In South Africa, the drought-resistant *S. frutescens* has been used in a variety of traditional medicinal networks for the treatment of various ailments including stomach problems, internal cancers, diabetes and wounds, suggesting its curative activities (van Wyk, 2008).

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Figure 2.12. ***Sutherlandia frutescens*** **plant (DST, 2011)**.

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2.3.2.1. Bioactivities of *S. frutescens*

2.3.2.1.1. Antimicrobial activity of *S. frutescens*

The antimicrobial activity of *S. frutescens* has not been extensively documented. However, a hexane *S. frutescens* extract was shown to inhibit the growth of three bacteria; namely *S. aureus*, *E. faecalis* and *E. coli* with minimum inhibitory concentrations (MIC) of 0.31, 1.25 and 2.50 mg/ml respectively (Katerere and Eloff, 2005).

2.3.2.1.2. Anti-tumour activity of *S. frutescens*

The anticancer activity of *S. frutescens* has been extensively studied and recorded (Tai, 2004, Chinkwo, 2005, Steenkamp *et al*., 2006, Lin, 2016, Skerman *et al*., 2011, Aboyade *et al*., 2014, Stander *et al*., 2009, van der Walt, 2016). These studies reported that *S. frutescens* inhibit the growth of breast, ovarian and cervical cancer. *S. frutescens* was further associated with cytotoxic activity toward oesophageal cancer cells and prostate carcinoma and shown to induce apoptosis in malignant melanoma and leukemic cells.

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2.3.2.1.3. Immune modulatory effects of *S. frutescens*

It was reported that an ethanol extract of *S. frutescens* amplified the release of cytokines from a neutrophilic promyelocyte cell line HL-60 with the potential to activate other potent immunity cells (Faleschini, 2013). Lei and colleagues demonstrated that a decoction of *S. frutescens* exhibited potent immune stimulatory activity. The production of reactive oxygen species (ROS), tumour necrosis factor (TNF-α) and nitric oxide (NO) was increased when a murine macrophage cell line (RAW 264.7) was exposed to the plant material. Furthermore, the action of polysaccharides present within the decoction up-regulated TLR4 receptors and nuclear factor kappa-light-chain-enhancer of B cells (NF-KB) which are part of the immune signalling pathways (Lei *et al.*, 2015).

2.3.3. *Salvia africana-lutea* (brown dune sage)

Salvia africana-lutea (*S. africana-lutea*) (Figure 2.13), of the Lamiaceae family, is a well-known South African medicinal plants. Locally known in Afrikaans as geelblom-salie, the drought tolerant plant is mainly indigenous to the South African vegetation, specifically the Western Cape (van Jaarsveld, 2013). It has been utilised for decades in the treatment of a variety of human ailments including colds, flu, coughs and body sores with significant documentation (Etsassala *et al.*, 2019). This wide array of medicinal benefits has been the rationale of the plant's popularity. Rosmarinic and carnosic acid were shown to be the most abundant components of *S. africana-lutea*. These potent flavonoids are associated with significant anti-inflammatory, anti-microbial and anti-oxidant properties (van Wyk, 2004). It was also reported that the trimers and tetramers of the plant possess antioxidant, anti-cancer and antimicrobial activity (Kamatou *et al.*, 2010).

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Figure 2.13. *Salvia africana-lutea* plant (Kommetjie, 2014).

2.3.3.1. Bioactivities of *S. africana-lutea*

2.3.3.1.1. Antimicrobial activity of *S. africana-lutea*

The extracts made from the aerial portion of the plant displayed significant antimicrobial activity against *B. cereus* and *S. aureus* with MIC values of 0.75mg/ml, which categorizes them as potent antimicrobial agents (Kamatou *et al.*, 2007).

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2.3.3.1.2. Anti-tumour activities of *S. africana-lutea*

S. africana-lutea was shown to exert anticancer activity on breast adenocarcinoma (MCF-7) and the human colon cancer (HT-29) in a dose-dependent manner (Kamatou *et al.*, 2010). Further research to investigate the cytotoxic effects of *S. africana-lutea* on other cancer types is important for the full exploration of the plants anticancer potential.

2.3.3.1.3. Immune modulatory effects of *S. africana-lutea*

The scientific interest in the mechanisms by which *S. africana-lutea* exerts its medicinal effects is gaining momentum. It was reported that the plant exerts immune booster activity

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in patients infected with bacteria (Kamatou *et al*., 2007) but its immune stimulating potential with interest to cancer is poorly documented. Regardless of the strong beliefs in the immune-stimulating ability of the plant, the lack of scientific evidence to support this claim means this bioactivity remains speculative.

2.4. Nanotechnology

The multifunctional scientific field of nanotechnology is one of the most promising technologies of recent times. The main feature of nanotechnology is the synthesis of particles less than 100 nm in diameter that exhibit new and enhanced size-dependent properties compared to their raw material (Patil *et al*., 2019). The unique features of synthesised nanoparticles such as large surface area to volume ratio and small size allow them to bind and deliver small-sized beneficial compounds to target sites. Nanotechnology has been adopted into several industrial sectors including food packaging and safety, water treatment, electrokinetics, physics, chemistry, biotechnology, renewable energy technology (oil and gas), textiles, cosmetology and agriculture (Duncan, 2011, Baruah, 2016, Saxena, 2018, Qu *et al*., 2013). The greatest pinnacle associated with nanotechnology was the development of novel medicinal treatments (nanomedicine). Nanomedicine has been utilised in the diagnosis, treatment and prevention of varying ailments including cancer, pulmonary diseases and microbial infections, as well as aid surgery recovery (Digesu *et al*., 2016). To date, about 51 nanopharmaceuticals synthesised from liposomes, polymers, nanocrystals, proteins, micelles and inorganic reducing agents have been approved by the FDA and are available in clinical practices, whilst 77 are undergoing clinical trials (Singh, 2017).

2.4.1. Physical and chemical synthesis of nanoparticles

The transformation of materials into nanoparticles involves physical and chemical changes. Prior to the genesis of nanoparticle synthesis using biological entities, nanoparticle synthesis solely depended on a variety of chemical and physical methods such as photochemical, chemical and electrochemical reduction, lithography and laser

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ablation (Lukman *et al*., 2011, Tran *et al*., 2013, Yu, 2007). These methods allow the manipulation of the produced nanoparticles to exhibit desired characteristics. Though these methods are being utilised to-date, literature has associated them with deleterious implications. These include their expensive nature, their high labour intensity and their utilization of numerous toxic reducing inorganic and/or organic chemicals (e.g. hydrazine, salts of tartrate, sodium/potassium borohydrate, ascorbic acid, sodium citrate) that result in the production of environmentally detrimental by-products (Noruzi *et al*., 2011). These factors consequently affect their application in biological systems. With this in mind, alternatively safer and cost-effective nanoparticle synthesis methods were developed, such as biogenic nanoparticle synthesis.

2.4.2. Biogenic synthesis of nanoparticles

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The overall acceptance of medicinal plants has led to their increased inclusion in more eco-friendly and simple natured experimental processes such as nanoparticle synthesis, to produce novel compounds with improved properties (Sapsford *et al*., 2013). The biogenic synthesis of nanoparticles involves the production of nanoparticles using biological bodies such as bacteria, human cells, fungi or plant extracts, which contain various metabolites that are involved in the biogenic reduction of metal ions to form metallic nanoparticles (Elbagory *et al*., 2017). The use of plants is the most favoured approach as they are readily available, safer and contain a wide variety of reducing phytochemicals. Numerous studies have documented the successful synthesis of metallic nanoparticles using plants including *Pelargonium graveolens*, *Azadirachra indica*, *Capparis spinose*, and herbal teas (*Camellia sinensis* and rooibos) (Ahmed *et al*.,2016, Benakashani, 2016, Elbagory *et al*., 2016). Additionally, the enhanced activity of the biogenic nanoparticles when compared to that of the synthesising plants is well appreciated in phytomedicine. The enhanced activity is attributed to the expected increase in stability of bioactive phytochemicals within the nanoparticles and the large surface area of the synthesised nanoparticles. The reaction involves the reduction of metallic ions by phytochemicals including amino acids, polyphenols and saponins. The reduction is preceded by the nucleation steps which involves various capping agents that

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stabilize the synthesised nanoparticles. Examples of different plant phytochemicals involved in the various steps of biogenic nanoparticle synthesis are displayed in Figure 2.14.

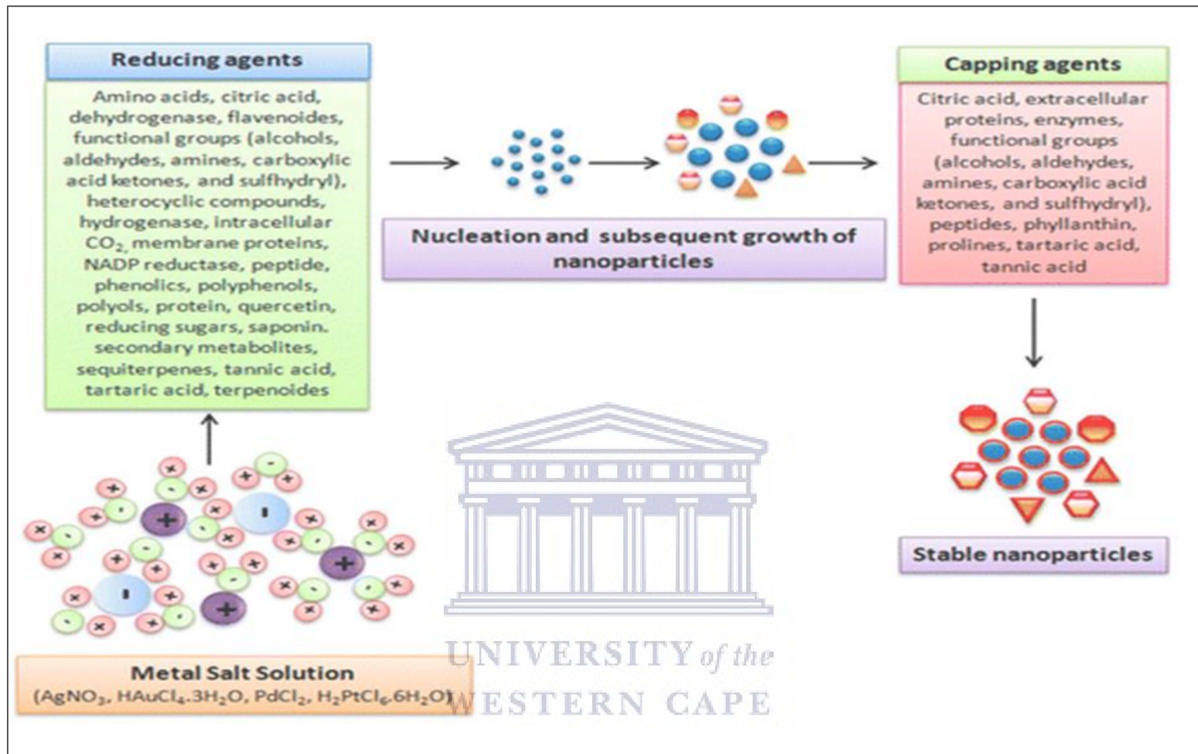

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Figure 2.14: Steps involved in the synthesis of biogenic nanoparticles using plant biomass and extracts. Reprinted with permission from Bentham Science Publishers Ltd (Akhtar *et al.*, 2013).

2.4.3. Metallic nanoparticles

Metal nanoparticles display unique magnetic, electronic, catalytic, and optical properties resulting in their use in a variety of applications. Among the metal nanoparticles used in biomedical applications, silver (Ag) and gold (Au) nanoparticles are appealing because of their unique biological properties and distinctive physicochemical properties (Tran *et al.*, 2013, Storm-Versloot *et al.*, 2010). These properties make them invaluable and extremely popular in a diverse range of consumer and pharmaceutical products. The popularity of Ag nanoparticles has been attributed to their bacteriostatic, bactericidal and fungicidal

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activity whilst that of Au nanoparticles is due to their cytotoxic, drug delivery and disease diagnostic potential (Elbagory *et al*., 2017, Digesu *et al*., 2016, Tran *et al*., 2013). The immunomodulatory effects of metallic nanoparticles on human immune cells has not been widely documented, further rationalizing the current study.

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CHAPTER THREE

BIOGENIC SYNTHESIS AND CHARACTERISATION OF NANOPARTICLES FROM *SALVIA AFRICANA-LUTEA* AND *SUTHERLANDIA FRUTESCENS.*

3.1. Abstract

The biogenic synthesis of nanoparticles from widely utilised medicinal plants is important due to the complexity of the phytochemical blueprint of the plants. The aim of this study was to determine if water extracts of *Salvia africana-lutea* (SAL) and *Sutherlandia frutescens* (SF) reduce silver (Ag) and gold (Au) ions consequently producing biogenic nanoparticles. The reactions were optimised by varying the synthesis conditions which includes time, plant extract concentration, silver nitrate ($AgNO_3$) and sodium tetrachloroaurate (III) dehydrate ($NaAuCl_4{\cdot}2H_2O$) concentration (1 mM and 3 mM), and temperature (25 ^oC and 70 ^oC). The observed colour change suggested nanoparticle synthesis, whereas confirmation and characterisation of the nanoparticles was achieved using UV-vis spectrophotometry, Dynamic Light Scattering (DLS), High-Resolution Transmission Electron Microscopy (HR-TEM), and Fourier Transform Infrared (FT-IR) spectroscopy. SAL was able to synthesise both Ag (SAL AgNPs) and Au (SAL AuNPs) nanoparticles, whilst SF was able to synthesise Ag (SF AgNPs) nanoparticles only. The optimum conditions for the synthesis of SAL AgNP and SAL AuNP were 3.125 mg/ml plant extract with 3 mM $AgNO_3$ and 1 mM $NaAuCl_4{\cdot}2H_2O$ respectively at 70 ^oC for 24 hours shaking at 65 rpm whilst those for the synthesis of SF AgNP were 6.25 mg/ml plant extracts with 3 mM $AgNO_3$ at 70 ^oC for 24 hours shaking at 65 rpm. The absorbance spectra revealed the characteristic surface plasmon resonance (SPR) peak between 400-500 nm and 500-600 nm corresponding to Ag and Au nanoparticles respectively. The HR-TEM images displayed the presence of spherical and polygon shaped nanoparticles with varying sizes, majority ranging between 6-8 nm and 10-15 nm for SAL AgNPs and SAL AuNPs, respectively, and 15-20 nm for SF AgNPs. The Energy Dispersive X-ray spectra

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(EDX) and Selected Area Electron Diffraction pattern (SAED) further confirmed the successful synthesis of the Ag and Au nanoparticles by displaying characteristic optical adsorption peaks and lattice fringes. The FT-IR was further employed to possibly identify the functional groups involved in the synthesis of the Ag and Au biogenic nanoparticles.

Keywords: Characterisation, gold nanoparticles, silver nanoparticles, *Salvia africana-lutea, Sutherlandia frutescens*, synthesis.

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3.2. Introduction

The scientific interest in the biogenic synthesis of colloidal/metallic nanoparticles using biological molecules has recently gained momentum. This is due to the general perception that biogenic nanoparticles are safe and environmentally benign in comparison to metallic nanoparticles synthesised using physical and chemical methods which have been reported to be toxic, labour intensive and expensive. Additionally, the use of harmful chemicals in the synthesis has been shown to limit the biological use of the nanoparticles (Shankar *et al.*, 2004).

Of the currently utilised biological systems, plants are most favoured as they are less toxic, readily available and contain a wide variety of phytochemicals that can potentially reduce metal ions. Silver, gold, copper, platinum and titanium metallic nanoparticles are widely used, with silver (Ag) and gold (Au) being the most popular due to their unique biological and optical properties. Numerous plants such as *Medicago sativa*, *Azadirachta indica*, *Aspalathus hispida, Asparagus ribicundus*, and *Dicerothamnus rhinocer* (Elbagory *et al.*, 2016, Lukman *et al.*, 2011, Ahmed *et al.*, 2016) have been used in the synthesis of Ag and Au nanoparticles. *Salvia africana-lutea* (SAL) and *Sutherlandia frutescens* (SF) are two indigenous South African plants with a long history of use in traditional medicine. SAL has been used in the treatment of skin and gastric disorders, whilst SF is traditionally known for its anticancer activity (Kamatou *et al.*, 2007, Kamatou *et al.*, 2010, Katerere and Eloff, 2005). Nanoparticles synthesised from medicinal plants can potentially have higher bioactivity in comparison to the crude extracts.

The bio-reduction reaction and characteristics of the nanoparticles are both affected by different factors including the concentrations of the reactants, temperature and time. It is therefore important to optimise these parameters in the nanoparticle synthesis reaction. Additionally, the differences in the chemical composition of medicinal plants may also affect the properties and yield of the synthesised nanoparticles, further justifying the need for the optimisation of the synthesis procedure.

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This study aimed to determine if water extracts of SAL and SF reduce silver (Ag) and gold (Au) ions consequently producing biogenic nanoparticles. The effect of time, plant extract concentration, silver nitrate ($AgNO_3$) and sodium tetrachloroaurate (III) dehydrate ($NaAuCl_4 \cdot 2H_2O$) concentration (1 mM and 3 mM), and temperature (25 ^oC and 70 ^oC) was also evaluated to optimise the synthesis of Ag and Au nanoparticles.

3.3. Methodology

3.3.1. Chemicals and apparatus

Mueller hinton broth (MHB), sodium tetrachloroaurate (III) dehydrate ($NaAuCl_4 \cdot 2H_2O$), silver nitrate ($AgNO_3$), Yeast Peptone broth (YPB) and Greiner bio-one 96 well flat bottom polystyrene microplates were acquired from Sigma-Aldrich (St. Louis, USA). Bovine Serum Albumin (BSA) was purchased from Miles Laboratories (Pittsburgh, PA, USA). Dulbecco's Phosphate Buffered Saline (DPBS), Minimum Essential Medium Eagle-Alpha Modification (MEM-α) and Roswell Park Memorial Institute medium (RPMI) was acquired from Thermofischer scientific (Waltham, Massachusetts, USA).

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3.3.2. Plant material

Fresh whole plants of *Sutherlandia frutescens* (SF) and *Salvia africana-lutea* (SAL) were supplied by Harry Goeman nursery (Kommetjie Road, Sunnydale, Cape Town). The plants were grown from a reliable source of seed. They were harvested during autumn and were not flowering during harvest time.

3.3.3. Plant extraction

The leaves and stem of the plants were harvested, washed with distilled water and air dried in the shade for 2 weeks. After drying, the plant material was finely ground and extracted. Water extracts were prepared by adding 50 ml of boiling distilled water to 5 g

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of plant material. The concoction was left stirring for 24 hours at 25 ºC, after which it was filtered through flex wool to entrap residual plant material. The extract was further filtered using Whatman no.4 mm paper followed by micro-filtering using 0.45 µm filter, and subsequently freeze dried (VirTis Genesis 25 ES Freeze drier, SP Scientific, Warminster, USA). The glassware was sterilized prior to the extraction process to limit contamination. The dried extract was weighed and stored at 4 ºC in the dark for future use.

3.3.4. Optimisation of conditions for the synthesis and screening of Ag and Au nanoparticles

This was performed in a griener flat-bottom 96 well plate as described by Elbagory *et al* (2016).

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3.3.4.1. Optimisation of concentration

The water extract (40 µl) of SAL and SF was added at decreasing concentrations (50 to 1.5625 mg/ml) to each well. To this 200 µl of $AgNO_3$ or $NaAuCl_4{\cdot}2H_2O$ was added for the synthesis of silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) respectively, thus making a reaction mixture of 1:5 (Plant extract: $AgNO_3$ or $NaAuCl_4{\cdot}2H_2O$). The AgNPs and AuNPs were synthesised using 1mM and 3mM of $AgNO_3$ and $NaAuCl_4{\cdot}2H_2O$ respectively.

3.3.4.2. Optimisation of temperature

Synthesis was performed at two temperatures, 25 and 70 ºC. The $AgNO_3$ and $NaAuCl_4{\cdot}2H_2O$ were all preheated at the specific synthesis temperature prior to synthesis. The AgNPs were synthesised in the dark to prevent the photoactivation of $AgNO_3$. The plates were covered to minimise the effects of evaporation.

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3.3.4.3. Optimisation of reaction time

The synthesis took place over a 24 hour period and the UV-vis (ultraviolet-visible) spectra of each sample was measured after 1, 3, 6 and 24 hours using a POLARstar Omega spectrophotometer (BMG labtech, Germany). The synthesis of AgNPs and AuNPs was then up-scaled to a final volume of 100 ml for further characterisation, stability assays and application using the determined optimum synthesis conditions.

3.3.5. Characterisation of synthesised Ag and Au nanoparticles.

3.3.5.1. UV-Vis spectroscopy

The surface plasmon resonance (SPR) of the synthesised nanoparticles was measured by recording the UV-vis spectra (POLARstar Omega spectrophotometer, BMG labtech, Germany) ranging from 300 nm to 700 nm for AgNPs and 400 nm to 700 nm for AuNPs.

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3.3.5.2. Dynamic Light Scattering (DLS)

Following up-scaled synthesis, the synthesised AgNPs and AuNPs were centrifuged at 13 000 rotations per minute (rpm) using the Eppendorf AG centrifuge 5417R with a standard rotor (F-45-30-11) for 15 minutes. The pellets washed three times as described by Elbagory *et al* (2016) with autoclaved distilled water. This was performed to remove any residual phytochemicals and $NaAuCl_4 \cdot 2H_2O$ or $AgNO_3$ not utilised in the nanoparticle synthesis reaction. After each wash, the nanoparticles were re-suspended in volumes of water that was equal to total reaction volume. This was done to maintain the same nanoparticle concentration after washing the nanoparticles. The hydrodynamic size, Poly Dispersity Index (PDI) and zeta potential of the synthesised nanoparticles were determined using Dynamic Light Scattering (DLS) (Zetasizer Nano ZS90, Malvern Instruments Ltd, UK). The washed nanoparticles were placed into either the polystyrene cuvette for hydrodynamic size and PDI determination, or the Disposable Capillary Cell (DTS1070) cuvettes for zeta potential analysis.

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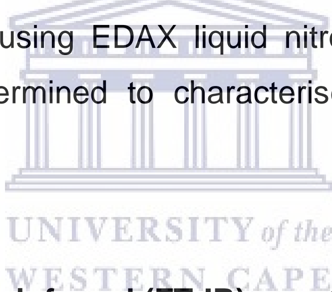
3.3.5.3. High-resolution Transmission Electron Microscopy (HR-TEM), Energy Dispersive X-ray spectra (EDX) and Selected Area Electron Diffraction (SAED) pattern

In the determination of size and morphology of the synthesised Ag and AuNPs, a drop of the samples solution was placed onto a carbon-coated copper grid. These were allowed to dry for 10 minutes under a Xenon lamp, after which the grids were analysed under the transmission electron microscope (Field Emission Transmission Electron Microscope, Tecnai F20, FEI Company, Oregon USA) as described by Elbagory *et al* (2016). The microscope was operated at an acceleration voltage of 200 kV in a bright field mode. Concurrently, the Energy Dispersive X-ray spectra (EDX) were collected using a lithium doped silicon detector cooled using EDAX liquid nitrogen. Selected Area Diffraction pattern (SAED) was also determined to characterise the crystalline nature of the synthesised nanoparticles.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3.3.5.4. Fourier transform infrared (FT-IR) spectroscopy

FT-IR analysis was performed according to a previously reported method using the PerkinElmer spectrum one FT-IR spectrophotometer (Waltham, MA, USA) as described by Khan *et al* (2013). In eppendorf tubes, 1 ml of synthesised Ag and Au nanoparticles were centrifuged and the pellets air dried at 25 ^{o}C. The dried pellets were weighed and the quantity of synthesised nanoparticles was determined as the mass of dried pellet in 1 ml of synthesised nanoparticles. The purified dried nanoparticles were mixed with potassium bromide (KBr) and pressed into a pellet for analysis. Pressed pure KBr was used for background correction.

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3.3.6. Stability analysis of synthesised Ag and Au nanoparticles.

In a 96 well plate, the washed nanoparticles (100 µl) were incubated with an equal volume of the biological media as described by Elbagory *et al* (2017). The *in vitro* stability of synthesised Ag and AuNPs was tested by incubating the nanoparticles with biological media (Minimum Essential Medium Eagle-Alpha Modification (MEM-α), Roswell Park Memorial Institute medium (RPMI)), Bovine Serum Albumin (BSA), and Dulbecco's Phosphate Buffered Saline (DPBS). Synthesised AgNPs were also incubated with Yeast Peptone Broth (YPB) and Mueller Hinton Broth (MHB). The stability of the synthesised nanoparticles was determined by observing the changes in the UV-Vis spectra (POLARstar Omega spectrophotometer, BMG labtech, Germany) after 0, 1, 12 and 24 hours.

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3.4. Results

3.4.1. Establishing the optimum conditions for the synthesis of Ag and Au nanoparticles

The synthesis of Ag and AuNPs from both *S. frutescens* (SF) and *S. africana-lutea* (SAL) was optimised by varying reaction temperature, reaction time, plant extract concentration and $NaAuCl_4{\cdot}2H_2O$ or $AgNO_3$ concentration. The first indication of successful biosynthesis of nanoparticles was a uniform colour change as illustrated in Figure 3.1. AgNP synthesis produced a brown solution for both SF and SAL. AuNP synthesis with SAL produced a red-violet solution, while AuNP synthesis with SF produced a clear solution with visible particulates.

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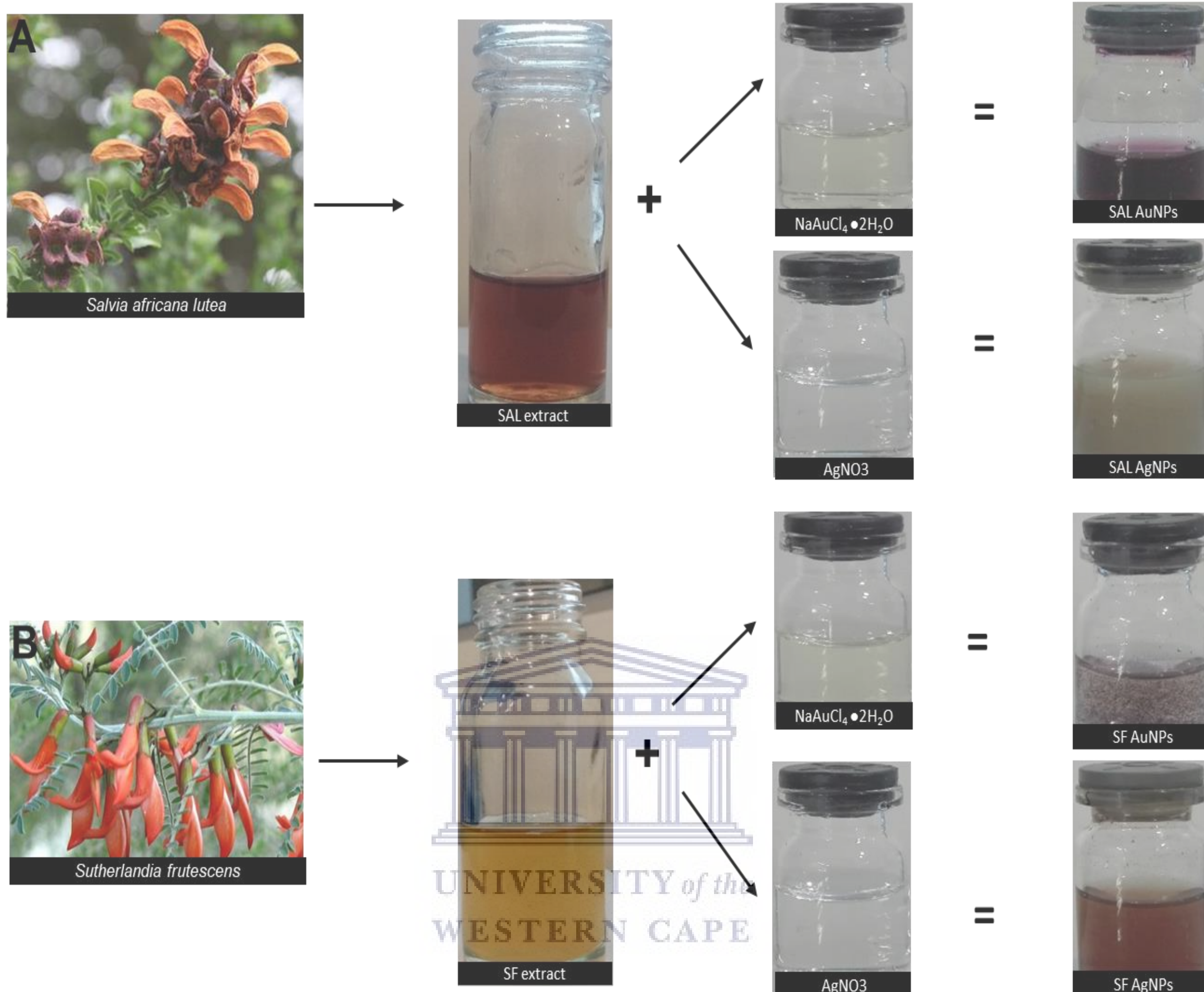

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Figure 3.1: Illustration of synthesis of silver and gold nanoparticles from *S. africana-lutea,* and *S. frutescens.*

A represents biogenic nanoparticle synthesis using *Salvia africana-lutea* extracts and **B** using *Sutherlandia frutescens* extracts.

Abbreviations: $AgNO_3$: silver nitrate; AgNP: silver nanoparticle; AuNP: gold nanoparticle; mM: millimolar; $NaAuCl_4 \cdot 2H_2O$: sodium tetrachloroaurate (III) dehydrate; SAL AgNPs: *Salvia africana-lutea* silver nanoparticles; SAL AuNPs: *Salvia africana-lutea* gold nanoparticles: SF AgNPs: *Sutherlandia frutescens* silver nanoparticles; SF AuNPs: *Sutherlandia frutescens* gold nanoparticles.

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3.4.1.1. Determining the optimum concentration (OC) of reactants in synthesising and screening Au and Ag nanoparticles

It has been documented that both 1 mM and 3 mM of $AgNO_3$ and $NaAuCl_4 \cdot 2H_2O$ are commonly used for Ag and AuNP synthesis respectively (Obaid *et al.*, 2017, Akhtar *et al.*, 2013, Benakashani, 2016). Similar concentrations of $AgNO_3$ and $NaAuCl_4 \cdot 2H_2O$ were thus employed in this study. It was observed that the colour changes were more rapid and homogenous for 1 mM $NaAuCl_4 \cdot 2H_2O$ reacting with SAL plant extracts as well as 3 mM $AgNO_3$ reacting with SAL and SF plant extracts. Hence, 1 mM $NaAuCl_4 \cdot 2H_2O$ was selected for the synthesis of *Salvia africana-lutea* gold nanoparticles (SAL AuNPs) and 3 mM $AgNO_3$ as the optimum concentration for *Salvia africana-lutea* silver nanoparticles (SAL AgNPs) and *Sutherlandia frutescens* silver nanoparticles (SF AgNPs) synthesis. With respect to plant extract concentrations, the plant extract concentrations used were 50, 25, 12.5, 6.25, 3.125 and 1.5625 mg/ml. The reactions were monitored for colour change at different time intervals for 24 hours. SF extracts were observed to produce a brown solution at 6.25 and 3.125 mg/ml with $AgNO_3$, while SAL extracts produced a brown solution with $AgNO_3$ and violet-red with $NaAuCl_4 \cdot 2H_2O$ at concentrations of 3.125 mg/ml and 1.5625 mg/ml. The UV-vis spectrum was recorded at specific time points as shown in Figure 3.2. The screen for AgNP synthesis showed absorbance peaks for SAL and SF with 3 mM $AgNO_3$ while the screen for AuNP synthesis showed absorbance peaks for SAL with 1 mM and 3 mM $NaAuCl_4 \cdot 2H_2O$ but not SF. For both Ag and Au nanoparticles, increased plant extract concentration (12.5, 25 and 50 mg/ml) resulted in increased absorbance intensity. Hence 6.25 mg/ml of SF plant extract was selected for SF AgNP synthesis while 3.125 mg/ml SAL plant extract was selected for the optimum synthesis of both SAL Ag and AuNPs.

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3.4.1.2. Determining the optimum reaction temperature for Au and Ag nanoparticle synthesis

The Ag and Au nanoparticles were synthesised over a 24 hour period at both 25 and 70 ^{o}C. However, no colour change was observed for all reactions at 25 ^{o}C. Furthermore, absence of absorbance peaks on the UV-vis spectra, similar to those observed at 70 ^{o}C for SF AuNPs shown in Figure 3.2, was observed for all nanoparticle synthesis at 25 ^{o}C. Hence, 70 ^{o}C was selected as the optimum temperature for SAL AgNP, SAL AuNP and SF AgNP synthesis due to the presence of absorbance peaks associated with AgNP and AuNP synthesis.

3.4.1.3. Determining the optimum reaction time for Au and Ag nanoparticle synthesis

The UV-vis absorbance spectra was measured after 1, 3, 6 and 24 hours. As the synthesis progressed the intensity of the absorbance peaks increased. A maxima absorbance between 400 and 500 nm for SAL AgNPs and SF AgNPs, and between 500 and 600 nm for SAL AuNPs was observed (Figure 3.2). The optimum synthesis conditions are summarised in Table 3.1.

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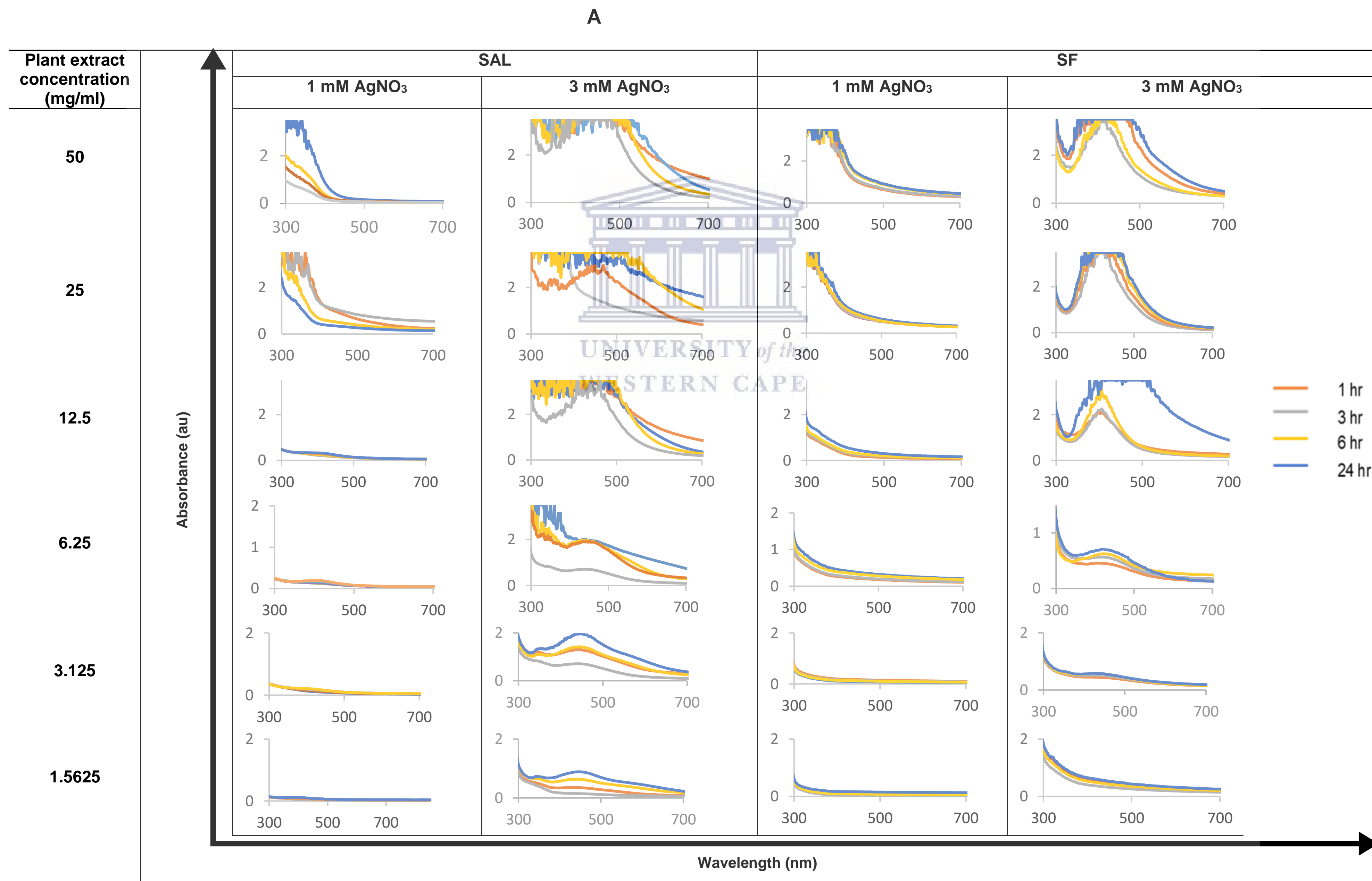

Figure 3.2: Effects of reaction time, plant extract concentration and $AgNO_3$ or $NaAuCl_4{\cdot}2H_2O$ concentration on UV-vis spectra of synthesised nanoparticles.
A represent synthesis of Ag nanoparticles using SAL and SF with $AgNO_3$ and **B** represents Au nanoparticles using SAL and SF with $NaAuCl_4{\cdot}2H_2O$

Abbreviations: $AgNO_3$: silver nitrate; au: arbitrary units; hr: hour; mg/ml: milligram per millilitre; mM: millimolar; nm: nanometre; $NaAuCl_4{\cdot}2H_2O$: sodium tetrachloroaurate (III) dehydrate; SAL: *Salvia africana-lutea*; SF: *Sutherlandia frutescens*.

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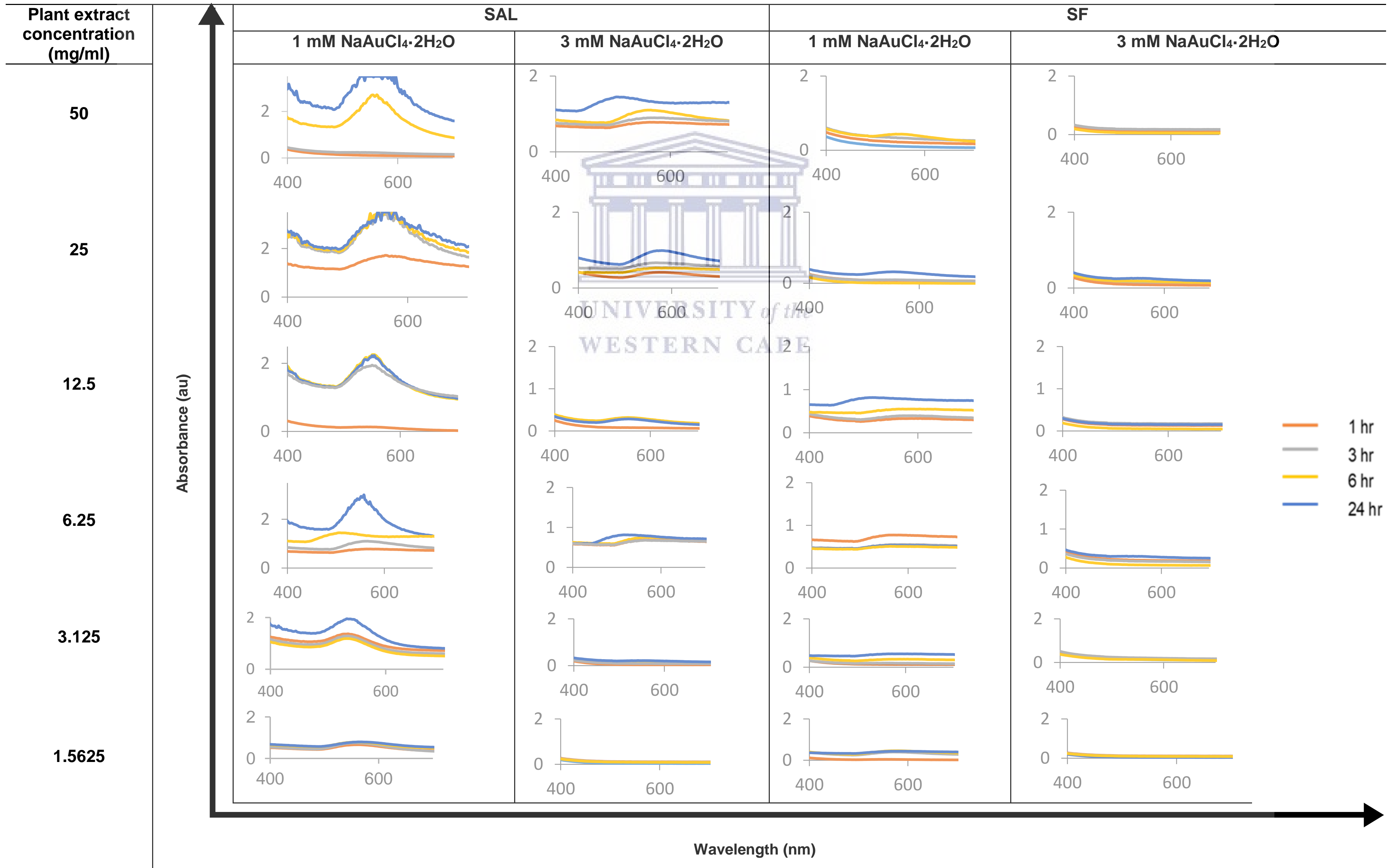

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Table 3.1: Summary of optimum conditions of AgNP and AuNP synthesis using SAL and SF plant extract.

Plant name	Metal ion	Nanoparticle abbreviation	Optimum plant extract concentration (mg/ml)	Optimum $NaAuCl_4 \cdot 2H_2O$ / $AgNO_3$ concentration (mM)	Optimum synthesising temperature (°C)	Optimum synthesising time (hrs)
Salvia africana-lutea	Silver	SAL AgNP	3.125	3	70	24
Salvia africana-lutea	Gold	SAL AuNP	3.125	1	70	24
Sutherlandia frutescens	Silver	SF AgNP	6.25	3	70	24

Abbreviations: $AgNO_3$: silver nitrate; hrs: hours; mg/ml: milligram per millilitre; mM: millimolar; $NaAuCl_4 \cdot 2H_2O$: sodium tetrachloroaurate (III) dehydrate; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SF AgNP: *Sutherlandia frutescens* silver nanoparticles; °C: degrees Celsius.

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3.4.2. Characterisation of the synthesised Au and Ag nanoparticles by Dynamic Light Scattering

Dynamic Light Scattering (DLS), is a technique commonly used in the determination of particle size in colloidal suspensions (Hoo *et al.*, 2008). It was used to determine three important characteristics of synthesised Ag and Au nanoparticles. These are hydrodynamic size, Poly Dispersity Index (PDI) and zeta potential which can be used to predict the behaviour of nanoparticles in biological media. The surface charge of colloidal nanoparticles was also determined using the zeta potential technique. Table 3.2 shows the charge, size and distribution of the synthesised nanoparticles. All the synthesised nanoparticles displayed a PDI greater than 0.2 and zeta potential less than -30 mV.

Table 3.2: Average hydrodynamic size, PDI and ZP of the AgNPs and AuNPs synthesised using the optimum synthesising conditions.

Nanoparticle	Hydrodynamic size (nm±SD)	PDI±SD	ZP (mV±SD)
SAL AgNP	34.63±0.53	0.63±0.03	-41.1±2.00
SAL AuNP	63.27±0.94	0.51±0.03	-34.7±1.39
SF AgNP	261.2±8.40	0.612±0.02	-35.7±1.53

Abbreviations: Ag: silver; Au: gold; mg/ml: milligram per millilitre; mV: millivolt; nm: nanometre; PDI: poly dispersity index; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SD: standard deviation; SF AgNP: *Sutherlandia frutescens* silver nanoparticles; ZP: zeta potential.

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3.4.3. Characterisation of the synthesised Au and Ag nanoparticles using HR-TEM, EDX and SAED

HR-TEM was employed to analyse the morphology and size of the synthesised nanoparticles. The synthesised Ag and Au nanostructures were polymorphic with varying sizes. This size variation was more pronounced with SF AgNPs, as seen in Figure 3.3. Although the predominating shape was spherical, SAL was also observed to produce polygon shaped Ag and Au nanoparticles.

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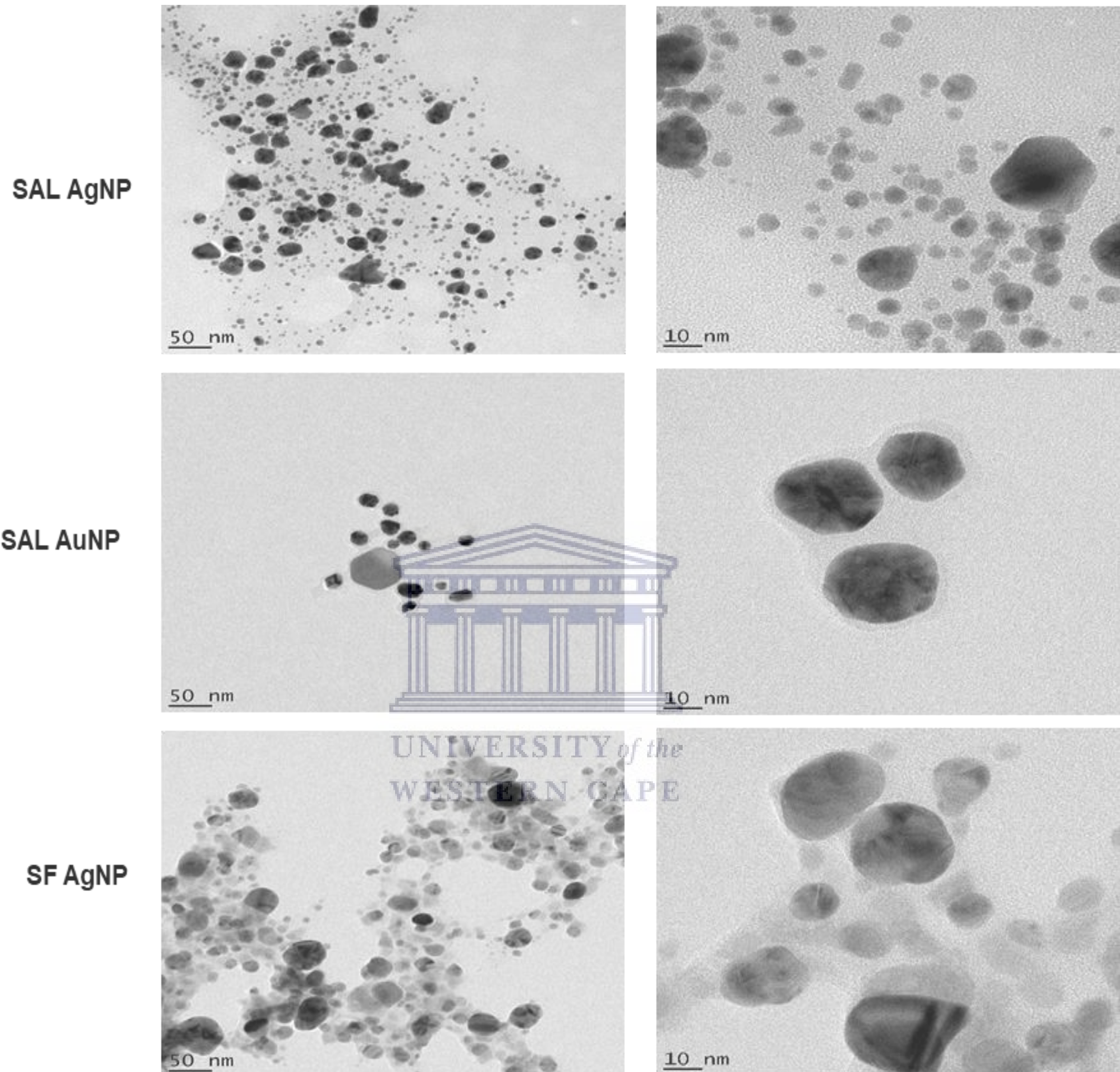

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Figure 3.3: High Resolution Transmission Electron Microscopy (HR-TEM) images of the nanoparticles.

Abbreviations: nm: nanometre; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SF AgNP: *Sutherlandia frutescens* silver nanoparticles.

Size distribution curves for the synthesised nanoparticles measured from the HR-TEM images using the imageJ software are indicated in Figure 3.4. The highest frequency of SAL AgNPs and SAL AuNPs were ranging from 6-8 nm and 10-15 nm respectively whilst

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the highest frequency of SF AgNPs was between 15-20 nm. The nanoparticle diameter as detailed by HR-TEM is summarised in Table 3.3.

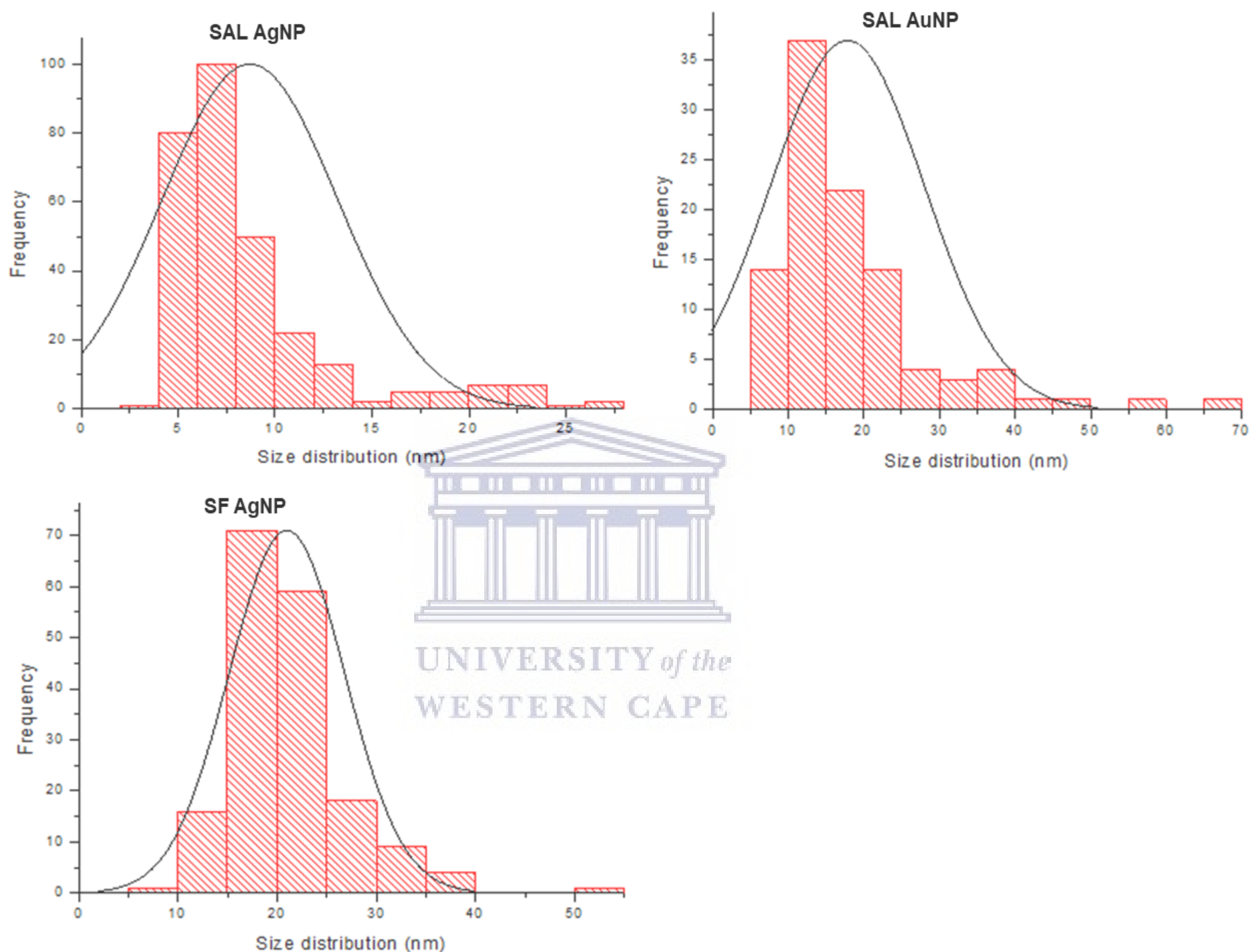

Figure 3.4: Size distribution curves for synthesised Ag and Au nanoparticles as detailed by HR-TEM.

Abbreviations: nm: nanometre; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SF AgNP: *Sutherlandia frutescens* silver nanoparticles.

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Table 3.3: Average particle diameter (PD) of synthesised nanoparticles

Nanoparticle	Average PD(nm±SD)
SAL AgNP	8.71±5.13
SAL AuNP	17.84±10.17
SF AgNP	21±5.82

Abbreviations: nm: nanometre; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SD: standard deviation; SF AgNP: *Sutherlandia frutescens* silver nanoparticles; PD: particle diameter.

The gold and silver ions were detected using EDX analysis as shown in Figure 3.5. The position of strong optical adsorption peaks for Au ions was observed at 2.3, 9.7 and 11.3 keV and silver ions between 2.5 and 4 keV. The presence of nickel, copper, carbon, argon, oxygen and chlorine peaks were also found and is discussed in section 3.5.3.2.

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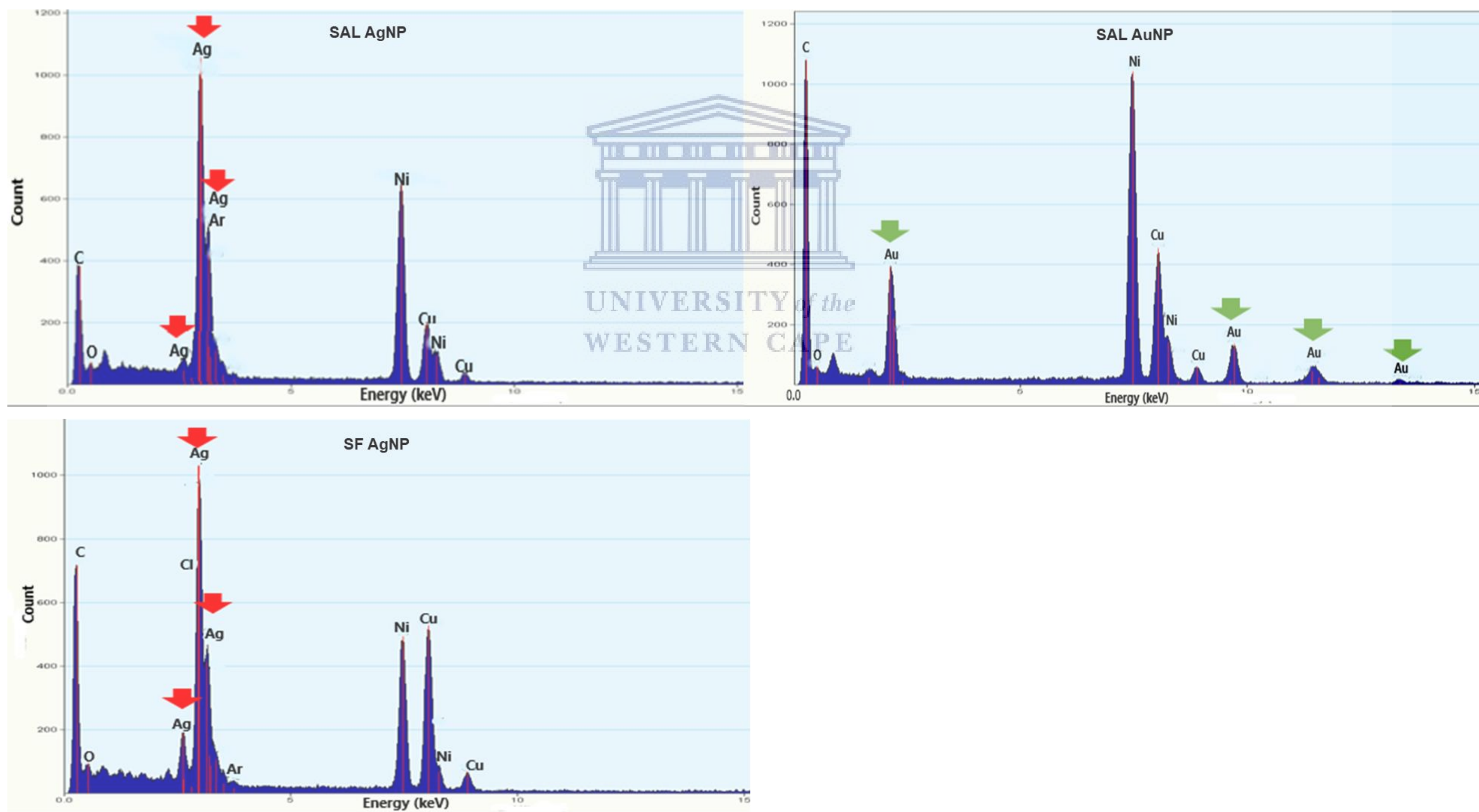

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Figure 3.5: Energy Dispersive X-ray Spectroscopy (EDX) spectra of SAL AgNPs, SAL AuNPs and SF AgNPs. The red arrows show Ag and green arrows Au peaks.

Abbreviations: keV: kilo-electronvolt; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SF AgNP: *Sutherlandia frutescens* silver nanoparticle.

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The crystalline nature of the synthesised Au and Ag nanoparticles was observed as the lattice fringes with specific spacing, as illustrated in Figure 3.6. The SAL AgNPs and SF AgNPs had a fringe spacing of 0.228 and 0.226 nm respectively, whilst the SAL AuNPs had 0.233 nm fringe spacing. The SAED patterns which shows the crystalline nature of the synthesised Ag and Au nanoparticles are also displayed. After indexing, the rings for SAL AuNPs were found to correspond with the (111), (200), (220) and (311) face-centered cubic (fcc) of gold, whilst those for SAL AgNPs and SF AgNPs were shown to concur with (111), (200), (220), (222) and (311) fcc of silver (Elbagory *et al.,* 2016).

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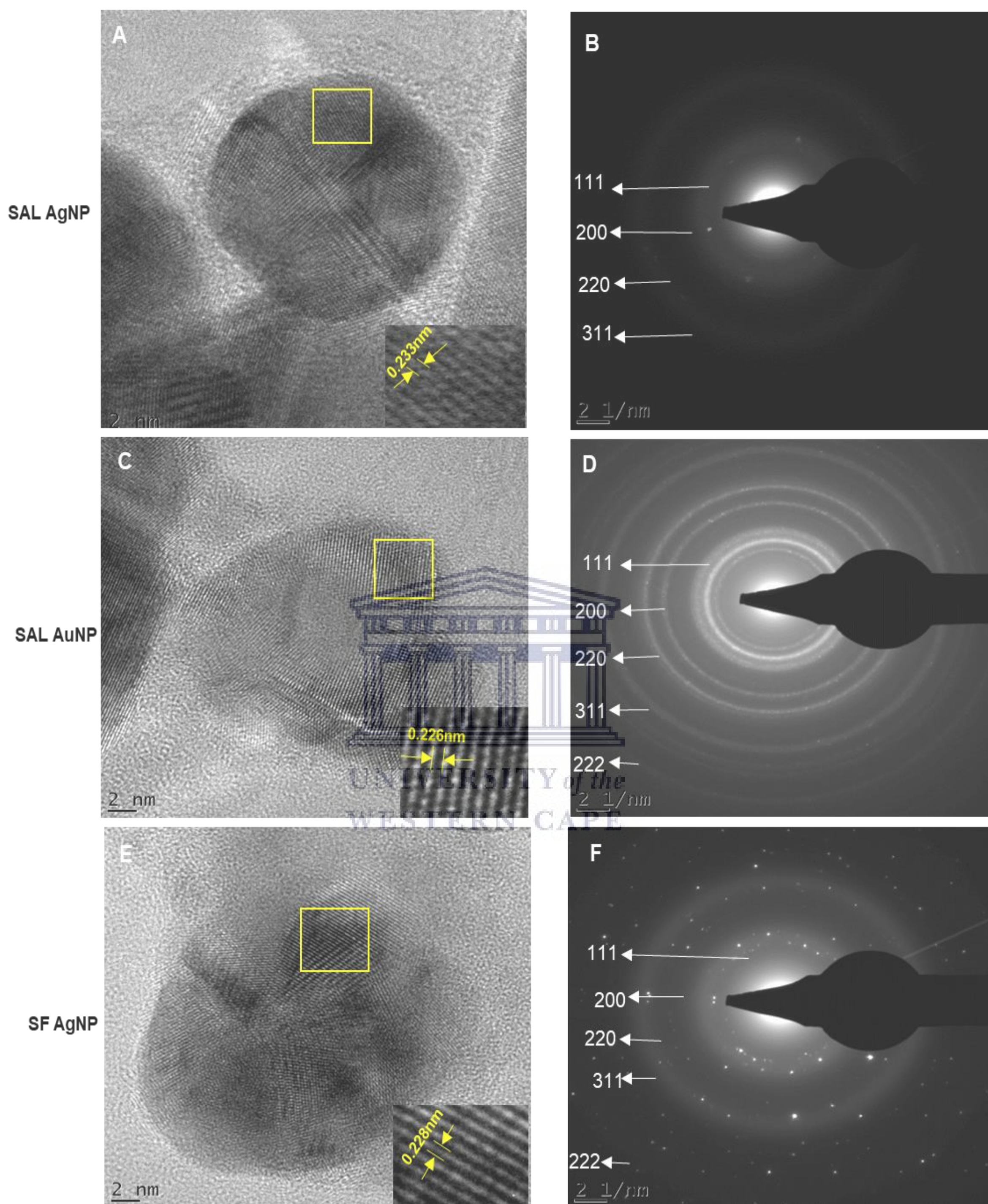

Figure 3.6: HR-TEM images and SAED analysis of the nanoparticles. **A**, **C** and **E** show HR-TEM and lattice fringes. The inserts on **A**, **C** and **E** shows the measurement of the lattice fringes for the respective nanoparticle. **B**, **D** and **F** show the SAED.

Abbreviations: nm: nanometre; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SF AgNP: *Sutherlandia frutescens* silver nanoparticle.

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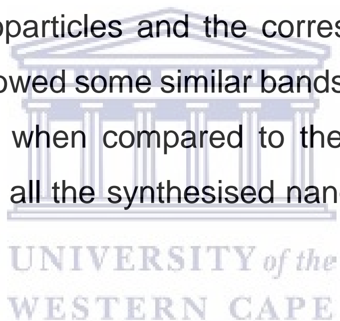
3.4.4. Characterisation of the synthesised Au and Ag nanoparticles by Fourier transform infrared (FT-IR) spectroscopy

FT-IR measurements were done to identify the functional groups in *S. africana-lutea* and *S. frutescens* responsible for the stabilization of the synthesised Au and Ag nanoparticles through capping. The Infrared (IR) spectrum of SAL AgNPs displayed intense bands at 3929.12, 3472.9, 3414.29, 3241.07, 2920.96, 2049.04, 1625.92, 1237.48, 618.97 and 480.39 cm^{-1}, whereas intense bands for SAL AuNPs were observed at 3928.09, 3706, 3563.05, 3480.81, 3405.97, 3236.06, 2907.03, 2045.03, 1618.43, 1513.16, 1257.18, 855.96, 620.74, 482.42 and 424.19 cm^{-1}. In the IR spectrum of SF AgNPs, prominent absorption bands were located at 3551.94, 3474.66, 3411.17, 3238.56, 2911.52, 2053.49, 1781.7, 1625.97, 1259.13, 1041.76, 620.31, 481.75 and 420.26 cm^{-1} (Figure 3.7). The biosynthesised nanoparticles and the corresponding plant extracts used to synthesise the nanoparticles showed some similar bands, whilst some of the bands of the nanoparticles appeared shifted when compared to the FT-IR spectra of the extracts. Similar bands were observed in all the synthesised nanoparticles. The band shifts were summarised in Table 3.4.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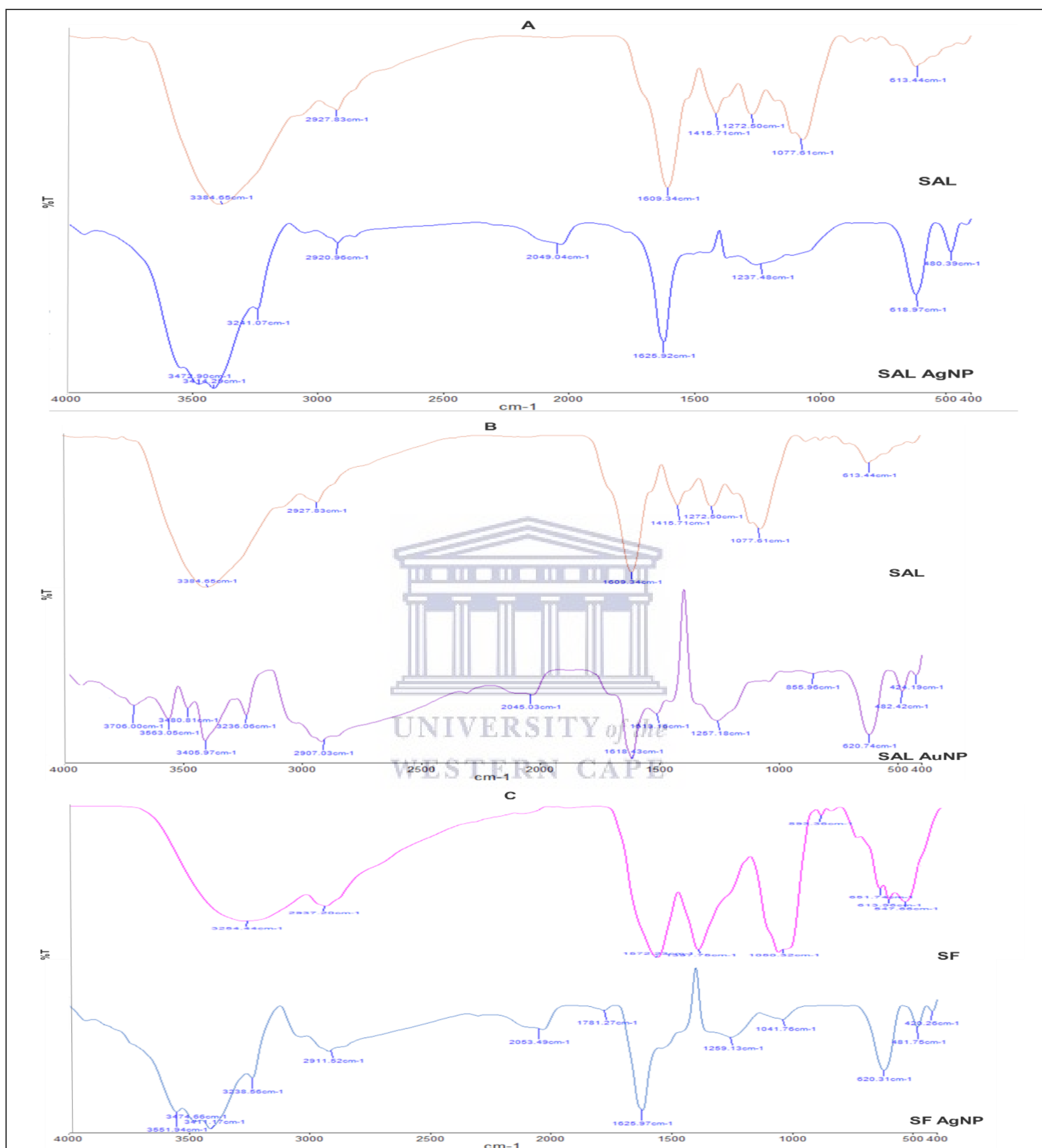

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Figure 3.7: FT-IR spectra of plant extract and synthesised nanoparticles. A shows the FT-IR spectra of SAL and SAL AgNP, **B** shows the FT-IR spectra of SAL and SAL AuNP and **C** shows the FT-IR of SF and SF AgNP

Abbreviations: cm^{-1}: per centimetre; SAL: *Salvia africana-lutea*; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SF: *Sutherlandia frutescens*; SF AgNP: *Sutherlandia frutescens* silver nanoparticle; %T: percentage transmission.

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Table 3.4: Comparison of FT-IR spectra peaks of SAL and SF aqueous extracts and their respective synthesised nanoparticles

	FT-IR peaks in water extracts (cm^{-1})	FT-IR peaks in NPs (cm^{-1})	Shift value*(cm^{-1})	Possible functional groups
SAL AgNP	3384.65	3414.29	-29.64	O-H (Alcohols)
	2927.83	2920.96	6.87	C-H (Alkanes)
	1609.34	1625.92	-16.58	C=C (Aromatics)
	1272.5	1237.48	35.02	C-O (Aromatic esters, Ethers, Carboxylic acids)
SAL AuNP	3384.65	3405.97	21.32	O-H (Alcohols)
	2927.83	2907.03	20.8	C-H (Alkanes)
	1609.34	1618.43	-9.09	C=C (Aromatics)
	1272.5	1257.18	15.32	C-O (Aromatic esters, Ethers, Carboxylic acids)
SF AgNP	3254.44	3238.56	15.88	N-H (Amine)
	2937.2	2911.52	25.68	C-H (Alkanes)
	1050.32	1041.76	8.56	C-O-C

*The shift values were calculated by subtracting the transmittance peak of synthesised NPs from the transmittance peak of the aqueous extracts.

Abbreviations: cm^{-1}: per centimetre; NPs: nanoparticles; SAL: *Salvia africana-lutea*; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SF: *Sutherlandia frutescens*; SF AgNP: *Sutherlandia frutescens* silver nanoparticle.

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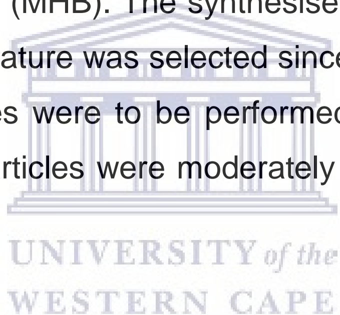
3.4.5. Stability of synthesised Ag and Au nanoparticles in biological media

The stability of synthesised nanoparticles in solvents is an important parameter for their application. Stable nanoparticles are notably evenly distributed and do not agglomerate/aggregate when placed in a solvent. Changes in the UV-vis spectra of nanoparticles over time in the presence of a solvent can be used as an indication if the nanoparticles are stable. In this study, the stability of the synthesised nanoparticles was determined by evaluating changes in the UV-vis spectra over a period of 24 hours as shown in Figure 3.8. The UV-vis changes were observed after 0, 1, 12 and 24 hours incubation of the synthesised nanoparticles in Minimum Essential Medium Eagle-Alpha Modification (MEM-α), Roswell Park Memorial Institute medium (RPMI), Bovine Serum Albumin (BSA), Dulbecco's Phosphate Buffered Saline (DPBS), Yeast Peptone Broth (YPB) and Mueller Hinton Broth (MHB). The synthesised nanoparticles and media were incubated at 37 ^{o}C. This temperature was selected since most of the intended biological applications of the nanoparticles were to be performed at 37 ^{o}C in these media. The synthesised Ag and Au nanoparticles were moderately stable, depicted by the minimal changes in the UV-vis spectra.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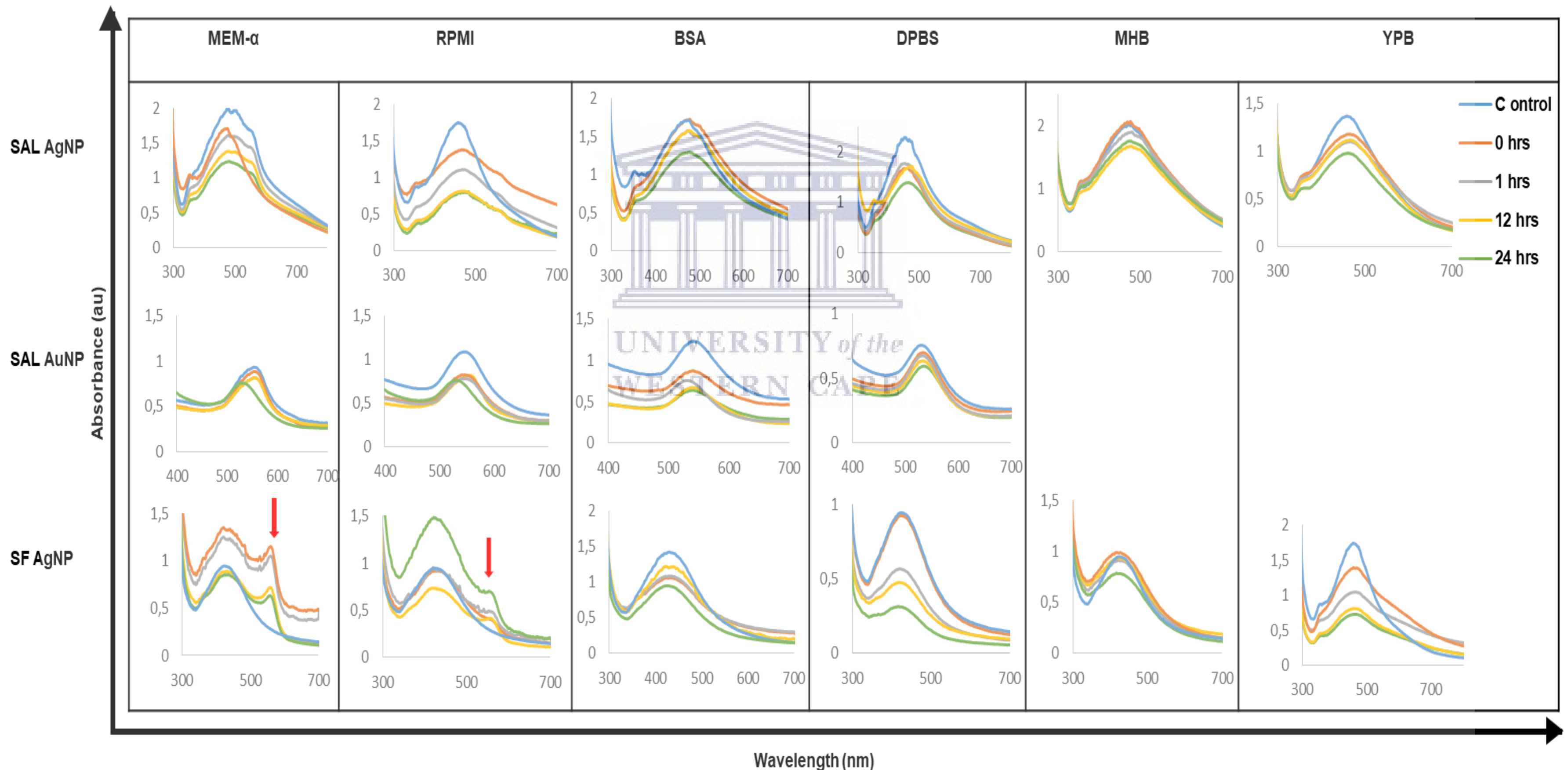

Figure 3.8: Stability UV-Vis graphs of synthesised nanoparticles incubated in biological media at 37 ºC. Arrows show the peaks due to the phenol red content in the respective media.

Abbreviations: au: arbitrary units; BSA: Bovine Serum Albumin; DPBS: Dulbecco's Phosphate Buffered Saline; hrs: hour; MEM-α: Minimum Essential Medium Eagle-Alpha Modification; MHB: Mueller Hinton Broth; nm: nanometre; RPMI: Roswell Park Memorial Institute medium; SAL AgNP: *Salvia africana-lutea* silver nanoparticle; SAL AuNP: *Salvia africana-lutea* gold nanoparticle; SF AgNP: *Sutherlandia frutescens* silver nanoparticles, YPB: Yeast Peptone Broth.

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3.5. Discussion

3.5.1. Synthesis of Ag and Au nanoparticles

The synthesis of Ag and Au nanoparticles was suggested by the formation of a colour change after $AgNO_3$ or $NaAuCl_4 \cdot 2H_2O$ was reacted with the optimum plant extract concentration and incubated. The synthesised Ag nanoparticles exhibited the characteristic brown colour whilst the Au nanoparticle solution appeared red-violet (Figure 3.1). Due to the absence of a uniform colour change after the commencement of reaction, *S. frutescens* (SF) was deemed to require other synthesis conditions besides those assessed in the study to enable the SF extracts to reduce the $NaAuCl_4 \cdot 2H_2O$. The uniform colour change is a result of surface plasmon resonance (SPR) of the synthesised nanoparticles (Ahmed *et al*., 2016). For optimising nanoparticle synthesis, the reactions were considered complete when no further colour change was observed. The UV-vis spectra confirmed the synthesis of nanoparticles by displaying peaks characteristic of Ag and Au nanoparticles. The intensity of the absorbance peak is reflective of the concentration of synthesised nanoparticles. The SPR for Ag nanoparticles produce λ-max absorbance peaks between 400 and 500 nm, whilst the λ-max for Au nanoparticles is between 500 and 600 nm. These optical characteristics are a result of the excitation of the longitudinal plasmon vibration (Kasthuri *et al*., 2009, MubarakAli *et al*., 2011). The absence of a homogenous colour change and characteristic absorbance peak has been associated with a failure of nanoparticle synthesis (Philip *et al*., 2011) as observed when SF is reacted with $NaAuCl_4 \cdot 2H_2O$. The λ-max absorbance peaks of *S. africana-lutea* Ag and Au nanoparticles were around 472 and 532 nm respectively whereas for *S. frutescens* Ag nanoparticles was around 432 nm indicating corresponding nanoparticle synthesis.

3.5.2. Optimisation of different synthesising parameters

The analysis of the morphology and size of synthesised nanoparticles is of great importance because the size and shape of biogenic nanoparticles vary affecting their

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biological application. This variation is a result of the uniqueness of the phytochemical blueprint of the plants. Additionally, the synthesising conditions i.e. time, temperature, and reactants concentration affect the quality and quantity of the nanoparticles (Elbagory *et al.*, 2016). Numerous studies have used varying concentrations of $AgNO_3$ and $NaAuCl_4{\cdot}2H_2O$ in nanoparticle synthesis (Kumar *et al.*, 2014, Arunachalam *et al.*, 2013, Logeswari *et al.*, 2015, Elbagory *et al.*, 2016). However, 3 mM $AgNO_3$ and 1 mM of $NaAuCl_4{\cdot}2H_2O$ have consistently shown to be suitable concentrations for biogenic nanoparticle synthesis. This was confirmed by the observations of this study, which reported a more defined colour change when SAL AgNPs, SAL AuNPs and SF AgNPs were synthesised.

The effects of plant extract concentration were also evaluated. Different concentrations of SAL and SF extracts (50, 25, 12.5, 6.25, 3.125 and 1.5625 mg/ml) were reacted with $AgNO_3$ and $NaAuCl_4{\cdot}2H_2O$ (1 mM and 3 mM) to produce Ag and Au nanoparticles respectively. All the higher extract concentrations assessed (50, 25 and 12.5 mg/ml) showed colour change suggesting successful nanoparticle synthesis. In contrast no colour changes were observed for SF extracts reacted with $NaAuCl_4{\cdot}2H_2O$, suggesting the unsuccessful synthesis of SF AuNPs. However, the absorbance peaks produced at higher plant concentrations were noisy suggesting that high plant extract concentrations result in a higher yield of nanoparticles. Furthermore, at lower plant extract concentrations, 6.25 mg/ml for *S. frutescens* extract with $AgNO_3$ and 3.125 mg/ml for *S. africana-lutea* extract with $AgNO_3$ and $NaAuCl_4{\cdot}2H_2O$ displayed more defined absorbance peaks. Broader peaks were observed when 1.5625 mg/ml SAL extract synthesised SAL AgNPs, 3.125 mg/ml SF synthesised SF AgNPs and 1.5625 mg/ml SAL synthesised SAL AuNPs. This could be due to the insufficiency of biomolecules required for capping and stabilization of the synthesised nanoparticles (Philip *et al.*, 2011). The variations in the values of absorbance signifies changes in the nanoparticle size and concentration (Tripathy *et al.*, 2010). Sharper absorbance peaks observed for SAL AgNPs and SAL AuNPs corresponded with the generally smaller sized nanoparticles while broader

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absorbance peaks were observed for larger sized SF AgNPs. This observation was in agreement with observations reported by Ibrahim (2015).

The reaction time was optimised by monitoring the reaction over different time intervals. Interestingly, for all nanoparticles the intensity of the λ-max absorbance peaks increased with time, indicating increasing quantity of nanoparticles produced. Elbagory and colleagues reported on the correlation of the absorbance and number of nanoparticles in solution (Elbagory *et al.*, 2016). As the reaction temperature was increased, the synthesis rate of both Ag and Au nanoparticles also increased. Hence, the synthesis temperature of 70 ^{o}C allowed for the successful synthesis of SAL AgNPs, SAL AuNPs and SF AgNPs. Increase in synthesis temperature was previously shown to increase both the synthesis rate and final conversion of metallic ions to nanoparticles (Song and Kim, 2009).

3.5.3. Characterisation of synthesised nanoparticles

3.5.3.1. The characterisation of synthesised nanoparticles using DLS

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The average hydrodynamic size, zeta potential and PDI of the synthesised nanoparticles were determined using DLS and are shown in Table 3.2. Hydrodynamic size is depended on the interaction of nanoparticles with the solvent the nanoparticles are suspended in. The average hydrodynamic size reported was 34.63, 63.27 and 261.20 nm for SAL AgNPs, SAL AuNPs and SF AgNPs respectively. All synthesised nanoparticles had average zeta potentials of less than -30 mV (Table 3.2). Such high negative zeta potential values suggest that synthesised nanoparticles are considered stable (Ardani *et al.*, 2017). This stability is a result of the strong repulsion forces existing between the negatively charged nanoparticles, preventing their aggregation. To express the distribution of synthesised nanoparticles in colloid, the PDI was measured. PDI measures the distribution of synthesised nanoparticles with values between 0 and 1. A PDI value between 0.1 and 0.2 suggests that synthesised nanoparticles are monodispersed i.e. they are of similar size and shape (Clogston and Patri, 2011). However, the PDI values in this work were gretaer than 0.2, suggesting pnanoparticle formation of different size and

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shape i.e polydisperse. These results augur well with the HR-TEM results, as will be seen later.

3.5.3.2. The characterisation of synthesised Au and Ag nanoparticles using HR-TEM, EDX and SAED

The HR-TEM was performed to observe the morphology and to determine the core size of the synthesised nanoparticles. The HR-TEM revealed the presence of nanoparticles with different sizes and shapes as seen in Figure 3.3. This observation was in line with the high PDI values reported in Table 3.1. The hydrodynamic size of the synthesised nanoparticles was 34.63, 63.27 and 261.20 nm whilst the HR-TEM core size was 8.71, 17.84 and 21 nm for SAL AgNPs, SAL AuNPs and SF AgNPs respectively. Such apparent discrepancies between size determination done by HR-TEM and DLS has been reported previously (Elbagory *et al*., 2016). The hydrodynamic size is typically bigger than the HR-TEM derived size as it analyses interaction of the nanoparticles surface with the solvent (Khan *et al*., 2013) whilst HR-TEM measures the core size of the nanoparticles. Hydrodynamic size is important in understandind and otpimising the nanoparticles perfomance in biological assays (Clogston and Patri, 2011). Based on these results, our observations concurred with previously reported studies. This geometrical and size variation has been widely reported for biogenic nanoparticles and has been associated with the phytochemical profile of the selected plants (Elbagory *et al*., 2016, Kumar *et al*., 2017, Dipankar and Murugan, 2012). The common phytochemical groups responsible for the reduction of Ag and Au ions in the synthesis of nanoparticles have been identified as flavonoids, alkaloids, flavones, amino acids, steroids, polyphenols and proteins (MubarakAli *et al*., 2011).

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The elemental profile of the nanoparticles is shown on the EDX spectra (Figure 3.5). The presence of strong optical adsorption peaks corresponding to Ag (2.5-4 keV) on the spectra for SAL AgNPs and SF AgNPs, and Au (2.3, 9.7 and 11.3 keV) for SAL AuNPs further confirms the successful synthesis of the Ag and Au nanoparticles. Additionally,

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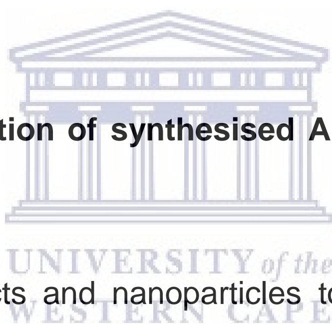
adsorption peaks corresponding to nickel, copper, carbon, argon, oxygen and chlorine elements were also observed. These elements could be from the grid utilised during HR-TEM as well as plant extracts and $AgNO_3$ or $NaAuCl_4{\cdot}2H_2O$ involved in the nanoparticle synthesis. The lattice fringes present on the synthesised nanoparticles confirms the crystalline nature of the nanoparticles (Figure 3.6). This was further confirmed by the SAED pattern observed. The lattice fringe spacing was similar to that previously reported for Ag or Au nanoparticles synthesised from *Murraya koenigii* (Philip *et al*, 2011), *Iresine herbstii* (Dipankar and Murugan, 2012), *Pulicaria glutinosa* (Khan *et al*, 2013) and *Medicago sativa* (Lukman *et al*, 2011). Ring positioning for the synthesised AuNPs (111, 200, 220, 311 and 222 fcc) and AgNPs (111, 200, 220 and 311 fcc) on the SAED were in line with numerous published studies (Philip and Unni, 2011, Yin *et al.*, 2010, Elbagory *et al.*, 2016, Kumar *et al.*, 2017).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3.5.3.3. The characterisation of synthesised Au and Ag nanoparticles using FT-IR spectroscopy

FT-IR was done on the extracts and nanoparticles to identify the possible common functional groups between the extract and the nanoparticles. The Infrared (IR) spectroscopy shows the vibrations of molecular bonds and hence provides information on the nature of the bonds and functional groups in the molecules. The generated information can be utilised to identify functional groups from phytochemicals that are involved in the bioreduction of $AgNO_3$ and $NaAuCl_4{\cdot}2H_2O$. Numerous studies suggest that different phytochemicals play a role in the synthesis of biogenic AgNP and AuNP (Elbagory *et al.*, 2017, MubarakAli *et al.*, 2011, Philip *et al.*, 2011). Figure 3.7 shows the presence of several functional groups that are present in both the extract and nanoparticles. The FTIR spectra of nanoparticles capped with SAL and SF are observed to have shifted to either high or low wavenumber as compared to that of the pure extracts. The shifts are summarised in Table 3.4 and suggest the involvement of the corresponding functional groups in the synthesis of the nanoparticles. Interestingly, similar peaks were observed in the FT-IR spectra of synthesised nanoparticles from both plants, suggesting

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similar functional groups and therefore similar phytochemicals may be involved in the synthesis of the nanoparticles. For instance, visible bands at 2920.96, 2907.03 and 2911.52 cm^{-1} may be due to the C-H stretch alkanes whilst the defined bands at 3414.29, 3405.97 and 3411.17 cm^{-1} for SAL AgNPs, SAL AuNPs and SF AgNPs respectively, correspond with the O-H group (Figure 3.7). Additionally, SAL AgNPs and SAL AuNPs also displayed respective visible bands at 1237.48 and 1257.18 cm^{-1} suggesting the stretching vibration of the C-O functional group. The weak but notable band at 1041.76 cm^{-1} for the SF AgNPs could be assigned as absorption bands for –C-O-C- whilst the 3238,56 cm^{-1} band correspond to the N-H functional group. The bands corresponding to the C-O and O-H bonds on the FT-IR spectra indicate the possible involvement of phenolic acids, carbohydrates, flavonoids and terpenoids in the capping and stabilization of the synthesised nanoparticles (Philip and Unni, 2011, Philip *et al.*, 2011, Kumar *et al.*, 2017). The HPLC phytochemical profile of *S. africana-lutea* has revealed that the plants' are high in flavonoid content especially rosmarinic and carnosic acid (Kamatou *et al.*, 2010). The involvement of compounds that contain hydroxyl and carbonyl groups in nanoparticle synthesis has been reported in numerous studies (Philip and Unni, 2011, Philip *et al.*, 2011, Yin *et al.*, 2010). A study by Aboyade and colleagues reported that *S. frutescens* contained significant levels of free and protein-bound amino acids (Aboyade *et al.*, 2014). Balashanmugam and colleagues suggested that amino acids and proteins may act as stabilizers of nanoparticles after the bioreduction process (Balashanmugam *et al.*, 2016). The shifted band at 3238.56 cm^{-1} in the FT-IR spectrum of SF AgNPs, is probably attributed to the N-H group of amines, affirming the above suggestion. The benign nature of the identified capping and stabilizing agents may allow the use of the synthesised nanoparticles in the food, medicinal and cosmetic industries. However, further analysis of these synthesised nanoparticles is required for the precise identification of the actual molecules responsible for the synthesis of nanoparticles.

3.5.3.4. Stability of nanoparticles in biological media

Any biomedical application of the nanoparticles would first require *in vitro* and *in vivo* testing. It is therefore important to confirm the stability of these nanoparticles in biological

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environments. Biologically stable nanoparticles do not aggregate when exposed to biological media over an extended period of time (Khan *et al*., 2013). The stability of the synthesised nanoparticles using *S. africana-lutea* and *S. frutescens* extracts is reported in Figure 3.8. Uv-vis analysis was used to determine changes in the stability of the nanoparticles. The stability of the nanoparticles was evaluated at 37 ^{o}C since most *in vitro* and *in vivo* applications are performed at this temperature. When the SAL AgNPs and SF AgNPs were placed to MHB, the absorption peaks did not change, implying that the nanoparticles are stable in the respective media. The absorption peaks of the SAL AuNPs flattened after being incubated in MHB and YPB, suggesting their unstable nature in the microbial media. Even though the absorption spectra of the synthesised nanoparticles in the other biological media (MEM-α, RPMI, BSA, DPBS and YPB) broadened slightly with time, the resulting λ-max corresponding to the SPR of AgNPs and AuNPs did not change, meaning synthesised SAL AgNPs, SAL AuNPs and SF AgNPs were still present in solution. The broadening and slight flattening might be as a result of some of the synthesised nanoparticles disintegrating or agglomerating within the selected media (Ahmed *et al*., 2016). Agglomeration has been reported to result in changes in the surface morphology of the synthesised nanoparticles (Zimbone *et al*., 2014). A second red–shifted peak was observed for SF AgNPs in MEM-α and RPMI and shown by the red arrows in Figure 3.8, which could be associated with the phenol content of the media. However, additional assays will be required for the confirmation of this assumption.

3.6. Conclusion

S. africana-lutea and *S. frutescens* water extracts were used to successfully produce Ag nanoparticles (SAL AgNPs and SF AgNPs). Furthermore, the water extract of *S. africana-lutea* was also used to produce SAL AuNPs. The successful synthesis of the biogenic nanoparticles was confirmed and the nanoparticles were characterised using different characterisation tools, namely UV-vis spectroscopy, DLS, HR-TEM and FT-IR. Various parameters that could affect nanoparticle synthesis were assessed for the optimum nanoparticle synthesis. These included reaction time, reaction temperature, plant extract concentration and $NaAuCl_4{\cdot}2H_2O$ or $AgNO_3$ concentration. The optimum conditions for

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the synthesis of SAL AgNPs and SAL AuNPs were 3.125 mg/ml plant extract with 3 mM $AgNO_3$ and 1 mM $NaAuCl_4{\cdot}2H_2O$ respectively at 70 ^oC for 24 hours shaking at 65 rpm, whilst those for the synthesis of SF AgNPs were 6.25 mg/ml plant extract with 3 mM $AgNO_3$ at 70 ^oC for 24 hours shaking at 65 rpm. The HR-TEM displayed the presence of spherical and polygon shaped biogenic nanoparticles of varying sizes. This observation was in line with most Ag and Au nanoparticles synthesised using plant extracts. Thus, *S. africana-lutea* and *S. frutescens* could be used for the efficient and simple synthesis of biogenic nanoparticles.

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3.7. References

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CHAPTER FOUR

ANTIMICROBIAL ACTIVITY OF *SALVIA AFRICANA-LUTEA* AND *SUTHERLANDIA FRUTESCENS* PLANT EXTRACTS AND THEIR RESPECTIVE SILVER NANOPARTICLES

4.1. Abstract

The increase in the use of medicinal plants such as *Salvia africana-lutea* (*S. africana-lutea*) and *Sutherlandia frutescens* (*S. frutescens*) is attributed to experimental studies done on their bioactivities. This is due to their rich source of biological material used in both modern and traditional medicinal systems. Their use in the treatment of infected wounds has always been accepted. However, elevation of microbial resistance has been reported. This has led to increased interest in enhancing the antimicrobial activity of these plant therapeutics. Nanoparticle synthesis using medicinal plants is one such approach that has recently gained popularity in various scientific fields. Nanoparticles synthesised from medicinal plants can potentially possess higher bioactivity in comparison to the crude extracts. The possible elevated stability and concentration of the beneficial phytochemicals within the synthesised colloidal biogenic nanoparticles may improve the antimicrobial activity of the produced nanoparticles.

Although the production of SAL AgNPs and SF AgNPs has been updated in the literature, their antimicrobial effects are not documented. Henceforth, the antimicrobial activity of water, ethanol and acetone extracts of *S. africana-lutea* and *S. frutescens* and the respective Ag nanoparticles from these extracts were tested against pathogens (*S. pyogenes*, *S. epidermidis*, *S. aureus, P. aeruginosa,* MRSA*, C. albicans*) that are prevalent in wound infections.

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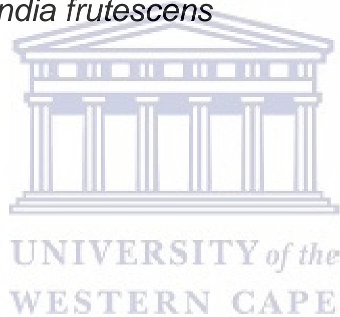
Ethanol and acetone extracts exhibited stronger antimicrobial activity in comparison to the water extracts of both plants. However, the high MIC values reported did not categorize the extracts as noteworthy antimicrobial agents (MIC values >1 mg/ml) except for the ethanol and acetone extracts of *S. africana-lutea* against *S. pyogenes.* These two extracts had an MIC value of 0.78 mg/ml against *S. pyogenes*. On the other hand, SAL AgNPs and SF AgNPs displayed significant inhibitory activity (MIC values ≤ 1 mg/ml) against all selected microorganisms except for SF AgNPs against *C. albicans* for which the MIC was 1.5 mg/ml. This study showed that Ag nanoparticles have higher antimicrobial activity than the crude extracts.

Keywords: Antimicrobial, microorganisms, *Salvia africana-lutea,* SAL AgNPs, SF AgNPs, nanoparticles, *Sutherlandia frutescens*



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4.2. Introduction

With the increase in the incidence of antibiotic-resistant microbes, it is of utmost importance that alternative treatment approaches be developed. Western medicines have been used across the globe in the treatment of different symptoms and manifestations caused by pathogenic microorganisms. However, research has shown that these treatment modalities with prolonged use could cause more harm than good. They have been associated with exacerbated antibiotic resistance (Wang *et al.*, 2017, Spellberg *et al.*, 2016) necessitating the need for new therapeutic approaches. Since 88 isolated compounds from 72 medicinal plants have been initiated into modern therapy, the scientific interest of complimentary medicines which includes traditional medicines in the treatment of antibiotic resistant infections has increased. The World Health Organisation (WHO) defined traditional medicines as 'the sum of the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness' (Abdullahi, 2011). Of the known traditional medicines, medicinal plants are the most commonly used in South Africa and Africa at large. This is due to the general social acceptance, availability, perceived low detrimental side effects and low cost of medicinal plants (De Wet *et al.*, 2013). Through mostly anecdotal evidence the use of medicinal plants have been associated with good prognosis in patients with cancer, diabetes, cardiac conditions, skin ailments, infections, gastrointestinal disorders and wound infections (Street and Prinsloo, 2012, Khodadadi, 2016, Ncube *et al.*, 2013).

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Wounds are a result of physical or thermal damage disrupting the continuity of the epithelial lining of the skin (Dhivya *et al.*, 2015). The wound healing process is described in three phases' namely inflammatory, proliferation and maturation. Proliferation of highly virulent microorganisms in wounds has been shown to affect the different stages of wound healing slowing the healing process (Serra *et al.*, 2015). If infected wounds are left untreated, they potentially lead to more life-threatening conditions such as septicaemia.

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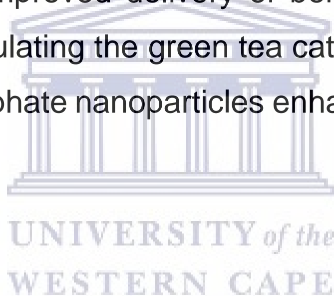
Medicinal plants can either directly promote wound healing by propagating any of the wound healing phases or kill infectious microbes that can potentially infect the wound.

Numerous plants which includes the indigenous *Salvia africana-lutea* (SAL) and *Sutherlandia frutescens* (SF) have a long history of use in the treatment of skin infections and wounds possibly due to their suspected antimicrobial activity (Kamatou *et al*., 2007, Katerere and Eloff, 2005). The synthesis of colloidal biogenic nanoparticles using plant extracts as reducing agents and stabilizers could possibly improve the bioactivity of the synthesising extracts. The resultant nanoparticles could have enhanced bioactivities compared to the bulk plant extracts. The expected increase in stability of bioactive phytochemicals within the nanoparticles and the large surface area of synthesised nanoparticles give rise to an improved delivery of beneficial phytochemicals to target cells. It was shown that encapsulating the green tea catechin (-)-epigallocatechin gallate (EGCG) in chitosan-tripolyphosphate nanoparticles enhanced the absorption and stability of EGCG (Dube *et al*., 2011).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The antimicrobial activity of water, ethanol and acetone extracts of SAL and SF and their respective silver nanoparticles was assessed in the study. Six of the most prevalent wound-infecting and reported antibiotic resistant microorganisms (*Pseudomonas aeruginosa*, *Staphylococcus aureus,* MRSA, *Staphylococcus epidermis*, *Streptococcus pyogenes* and *Candida albicans*) were used in the current study.

4.3. Methodology

4.3.1. Chemicals and apparatus

Acetone, ampicillin, blood agar, dimethyl sulfoxide (DMSO), ethanol, fluconazole, Mueller Hinton agar, Mueller Hinton broth, Sabouraud Dextrose 4% agar and Yeast Peptone broth were all acquired from Merck (Darmstadt, Germany). All solvents used throughout the

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study were of analytical reagent grade. Sterilized distilled water was used throughout the study. Greiner crystal clear 96-well flat bottom microplates were from Sigma-Aldrich (St. Louis, USA).

4.3.2. Plant material

Refer to section 3.3.2.

4.3.3. Plant extraction

Leaves and stems were collected from the plants, washed with distilled water and air dried in the shade for 2 weeks. After drying, the plant material was finely ground using a blender and extracted. Water extracts were prepared by adding 50 ml of boiling distilled water to 5 g of plant material, whilst the ethanol and acetone extracts were prepared by adding 50 ml of the respective solvent to 5 g of plant material. The mixture was left stirring for 24 hours at 25 ^{o}C after which it was filtered through flex wool to entrap residual plant material. The extracts were then filtered using Whatman no.4 mm paper. Ethanol and acetone filtrates were concentrated using the rotary evaporator to a fifth of their initial volume and finally air dried under a laminar fume hood yielding crude extracts. The water extracts were dried using the freeze drier. All extracts were weighed and stored at 4 ^{o}C in dark sealed containers for future experimental use.

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4.3.4. Synthesis of Ag nanoparticles

Refer to section 3.3.4. The unstable nature of SAL AuNPs in microbial media (MHB and YPB), resulted in their exclusion in the antimicrobial study.

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4.3.5. Microorganisms

The microorganisms chosen were the Gram-positive bacteria *Staphylococcus aureus* (*S. aureus,* ATCC 25923), methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591)*, Staphylococcus epidermidis* (*S. epidermidis,* ATCC 12228) and *Streptococcus pyogenes* (*S. pyogenes,* ATCC 19615), the Gram-negative bacterium *Pseudomonas aeruginosa* (*P. aeruginosa,* ATCC 27853) and the opportunistic fungus *Candida albicans* (*C. albicans,* ATCC 10231), representing the three main groups of microorganisms. These microorganisms are associated with severe infections affecting the skin and wound healing. *S. aureus*, *S. epidermidis*, *P. aeruginosa,* MRSA were cultured on blood agar, *S. pyogenes* on cooked blood agar whilst *C. albicans* on sabouraud dextrose agar (SDA). Microorganisms were kept viable by routine sub-culture and stocks stored at -80 ^{o}C in 25% glycerol.

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4.3.6. Agar well diffusion assay

The agar well diffusion assay was performed as described by Balouiri *et al* (2016) with some modifications. Single microbial colonies were cultured in Mueller Hinton broth (MHB) (*S. aureus*, *S. epidermidis*, *P. aeruginosa,* MRSA, *S. pyogenes*) and Yeast Peptone broth (YPB) (*C. albicans*) for 4 hours at 37 ^{o}C in a horizontal type-shaking incubator (LM-530D, Taiwan). A 0.5 McFarland standard equivalent to 1 × 10^8 CFU/ml microorganisms was prepared from the microbial suspensions. The surface of Mueller Hinton agar (MHA) (bacteria) and SDA (yeast) plates were inoculated with the respective microbe by spreading the microorganisms over the agar surface using sterile swabs. The stock solutions of ethanol and acetone extracts were prepared in 10% dimethylsulfoxide (DMSO) and diluted to the desired concentrations. The water extracts were dissolved in sterilized water. Holes (6 mm in diameter) were punched using a sterile cork borer and 50 µl of the extract solution or nanoparticles (1.5 mg/ml) was introduced into the holes. Two plant extract concentrations were selected; 50 mg/ml and one corresponding to the plant extract concentration utilised in the synthesis of *S. africana-lutea* Ag nanoparticles (SAL AgNPs) and *S. frutescens* Ag nanoparticles (SF AgNPs) (3.125 mg/ml for SAL and

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6.25 mg/ml for SF). The positive control used for all bacteria (*S. aureus*, *S. epidermidis*, *P. aeruginosa,* MRSA, *S. pyogenes*) was the broad spectrum antibiotic ampicillin (25 µg/ml), whilst for *C. albicans* fluconazole (25 µg/ml) was used. The respective extraction solvents served as the negative controls. The agar plates were incubated at 37 ^{o}C for 24 hours. As antimicrobial active test agents diffused in the agar, they inhibited the growth of the microbial strains forming clear zones of inhibition. The inhibition zones were measured in millimeters (mm) from the circumference of the well to that of the growth-free zones and recorded. All samples were tested in triplicate (*n*=3).

4.3.7. Determining the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) for each sample was determined using the microtitre plate method (Balouiri *et al.*, 2016). Microbial suspensions in Mueller-Hinton (for bacteria) and Yeast Peptone (for fungi) broths were prepared by inoculating respective broth with single colonies and incubating the cultures for 4 hours at 37 ^{o}C in a horizontal type-shaking incubator (LM-530D, Taiwan) from which a McFarland No. 0.5 standard at a spectrophotometric wavelength of 625 nm was prepared. The positive control (to confirm microbial susceptibility) for *S. pyogenes*, *S. epidermidis*, *S. aureus, P. aeruginosa,* and MRSA was ampicillin (25 µg/ml), and that for *C. albicans* was fluconazole (25 µg/ml). The respective extraction solvents and 1% DMSO served as the negative controls. The test samples assessed for MIC were those that displayed zones of inhibition during the agar disk diffusion assay. Under a laminar air flow, 96-well microtitre plates were aseptically prepared. The extract (200 µl of 50 mg/ml solution prepared in 1% DMSO) or nanoparticles (200 µl of 1.5 mg/ml solution) was placed in one of the wells. Broth (100 µl) was paced in the adjacent wells. Serial dilutions of the extracts and SAL AgNPs or SF AgNPs were prepared by removing 100 µl of the 50 mg/ml plant extract or 1.5 mg/ml nanoparticles and adding it to one of the wells containing the 100 µl broth to produce a mixture containing 25 mg/ml extract or 0.75 mg/ml nanoparticles. The dilution of the extracts and nanoparticles were sequentially repeated until a concentration range of 0.39 mg/ml for plant extracts and 0.012 mg/ml for nanoparticles were generated. This was followed by the addition of the specific microbes (100 µl) in all wells except the

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hindrance wells. The hindrance well served as a comparative well in order to visually identify wells without microbial growth due to inhibition. The plates were covered to minimize evaporation and incubated for 24 hours at 37 ^{o}C. After incubation, the absorbance reading at 625 nm was recorded for each plate. The MIC, determined by the spectrophotometric method was defined as the concentration at which there was a sharp decline in the absorbance value after incubation (Devienne and Raddi, 2002). This was confirmed by visually comparing each well to the corresponding hindrance well. The MIC was the lowest concentration at which no visible growth was observed. The experiment to determine MIC was done in triplicate.

4.3.8. Determining the Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentrations (MFC)

The lowest concentrations at which the test samples eradicated the respective bacteria (minimum bactericidal concentration (MBC)) and fungi (minimum fungicidal concentration (MFC)) were determined by sub-culturing a single loopful of the sample from all wells that did not show visible growth after MIC determination as described by Owuama (2017). Bacterial samples were sub-cultured on MHA whilst the fungus was sub-cultured on SDA. These were incubated for 18 (bacteria) and 48 (fungus) hours at 37 ^{o}C. The lowest concentration at which no growth was observed was recorded as either the MBC or MFC.

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4.3.9. Statistical analysis

The antimicrobial data are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the antimicrobial activity of the different plant extracts and Ag nanoparticles was achieved using the Bonferroni Multiple comparison analysis. Comparisons were considered significant at $P < 0.05$. GraphPad^{TM} PRISM6 software package was used for all statistical evaluations and graphical representations. All experiments were done in triplicates (n=3).

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4.4. Results

4.4.1. The antimicrobial activity of *Salvia africana-lutea extracts* and Ag nanoparticles against selected microorganisms.

4.4.1.1. Assessment of antimicrobial activity by agar well diffusion assay

Ampicillin (25 µg/ml) served as the anti-bacterial positive control and inhibited the growth of Gram-positive *S. aureus,* MRSA, *S. epidermidis, S. pyogenes,* and Gram-negative bacterium *P. aeruginosa* with zones of 14 mm, 13 mm, 15 mm, 16 mm and 10 mm, respectively. The antifungal agent Fluconazole (25 µg/ml) served as a positive control for *C. albicans* with an inhibition zone of 8 mm. The negative controls (extraction solvents) did not have any effect on the respective microorganisms and thus no zones of inhibition were observed around these wells. Both the polar (water, ethanol) and non-polar (acetone) extracts exhibited some degree of antimicrobial activity against the selected Gram-positive bacterial strains and fungus (Table 4.1). Of the extracts tested, ethanol and acetone proved most potent with acetone being most active. The activity of acetone extract against all tested microbial strains except *S. epidermidis* was not significantly different to that displayed by SAL AgNPs. Low extract concentrations (3.125 mg/ml) did not display visible antimicrobial activity whilst the higher extract concentrations (50 mg/ml) reported in Table 4.1 showed observable antimicrobial activity. The difficulty in dissolving higher concentrations of plant extracts did not allow for higher extract concentrations to be tested. When comparing the activity of the water extracts to that of the SAL AgNPs (1.5 mg/ml), the SAL AgNPs were more potent. The inhibitory activities of water extracts compared to that of the ethanol extracts, acetone extracts and SAL AgNPs were significantly ($P < 0.05$) different against *S. aureus,* MRSA, *S. pyogenes*, *S. epidermidis* and *P. aeruginosa.* The antimicrobial activities of ethanol extract compared to the inhibitory activities of the acetone extract and SAL AgNPs against MRSA were also significantly different ($P < 0.05$). Interestingly, the activity of the different plant extracts and SAL AgNPs against *C. albicans* were not significantly different ($P > 0.05$). Amongst the bacterial strains, *S. epidermidis* was the most sensitive whilst *P. aeruginosa* and *C. albicans* displayed the least sensitivity towards the test agents.

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Table 4.1: Antimicrobial inhibition using SAL by agar well diffusion.

Microorganisms	Test samples									
	Water[a]	Ethanol[b]	Acetone[c]	SAL AgNP[d]	Statistical significance					
	Average zones of inhibition (mm±SEM)				ab	ac	ad	bc	bd	cd
Staphylococcus aureus	2±1	8.5±0.5	9.5±0.5	9.5±0.5	****	****	****	ns	ns	ns
MRSA	4.7±0.7	6±0.6	8±1	9±0	*	****	****	**	***	ns
Streptococcus pyogenes	4.5±0.5	7.5±0.5	9±0.6	9.5±0.5	**	****	****	ns	*	ns
Staphylococcus epidermidis	4±0.6	9±0.6	9±0.6	11.5±0.5	****	****	****	ns	*	*
Pseudomonas aeruginosa	0	5±0.6	6±1	5.7±0.3	****	****	****	ns	ns	ns
Candida albicans	2.3±0.3	2.7±0.3	3±0.3	3.7±0.7	ns	ns	ns	ns	ns	ns

The inhibition of the microorganisms was performed using water (a), ethanol (b) and acetone (c) extracts at 50 mg/ml and SAL AgNPs (d) at 1.5 mg/ml. Ampicillin inhibited the growth of *S. aureus*, MRSA, *S. epidermidis*, *S. pyogenes*, and *P. aeruginosa* with zones of 14 mm, 13 mm, 15 mm, 16 mm and 10 mm, respectively. Fluconazole inhibited the growth of *C. albicans* with an inhibition zone of 8 mm. ***Abbreviations:*** AgNPs: silver nanoparticles; mm: millimeter; ns: not significant; SEM: standard error of the mean (for n=3). *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$; SAL: *Salvia africana lutea*.

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4.4.1.2. Assessment of antimicrobial activity by Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration or Minimum Fungicidal Concentration (MBC or MFC) assays

The ethanol and acetone extracts displayed similar MIC values of 6.25, 0.78, 6.25 and 25 mg/ml against *S. aureus, S. pyogenes, S. epidermidis,* and *P. aeruginosa* respectively. Their MBC values were also similar against the above-stated microorganisms (*S. pyogenes* - 3.125 mg/ml, *S. epidermidis* - 25 mg/ml, and *P. aeruginosa* - 50 mg/ml) except for *S. aureus* and MRSA. The water extract was the least active against the selected microorganisms with MIC values of 50 mg/ml and above. The SAL AgNPs exhibited the most antimicrobial activity against *S. aureus,* MRSA, *S. pyogenes*, *S. epidermidis*, *P. aeruginosa* and *C. albicans* with MIC values of 0.1875, 0.375, 0.09375, 0.1875, 0.375 and 0.75 mg/ml respectively (Table 4.2). The MIC values of SAL AgNPs were 33, 8, 33,

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66 and more than 66 folds greater than those of ethanol and acetone against *S. aureus, S. pyogenes, S. epidermidis, P. aeruginosa* and *C. albicans* respectively. The MIC values of SAL AgNPs against MRSA were 66 and 33 folds greater than those of ethanol and acetone extracts. This significant antimicrobial activity of SAL AgNPs was confirmed by the MBC values less than 1 mg/ml and MFC value of 2 mg/ml.

Table 4.2: MIC (mg/ml) and MBC or MFC (mg/ml) of *S. africana lutea* extracts and Ag nanoparticles against various microorganisms.

Microorganisms	Test Samples							
	Water		Ethanol		Acetone		SAL AgNP	
	MIC (mg/ml)	MBC/MFC (mg/ml)	MIC (mg/ml)	MBC/MFC (mg/ml)	MIC (mg/ml)	MBC/MFC (mg/ml)	MIC (mg/ml)	MBC/MFC (mg/ml)
Staphylococcus aureus	>50	-	6.25	12.5	6.25	25	**0.1875**	0.75
MRSA	>50	-	25	50	12.5	25	**0.375**	0.75
Streptococcus pyogenes	50		**0.78**	3.125	**0.78**	3.125	**0.09375**	0.1875
Staphylococcus epidermidis	>50	-	6.25	25	6.25	25	**0.1875**	0.375
Pseudomonas aeruginosa	>50	-	25	50	25	50	**0.375**	0.75
Candida albicans	>50	-	>50	-	>50	-	**0.75**	2

Abbreviations: AgNPs: silver nanoparticles; MBC: Minimum Bactericidal Concentration; MFC: Minimum Fungicidal Concentration; mg/ml: milligram per millilitre; SAL: *Salvia africana lutea*. **MIC < 1 mg/ml are in bold.**

4.4.2. The antimicrobial activity of *Sutherlandia frutescens extracts* and Ag nanoparticles against selected microorganisms.

4.4.2.1. Assessment of antimicrobial activity by agar well diffusion assay

The ethanol and acetone extracts of *S. frutescens* (SF) and SF AgNPs exhibited some antimicrobial activity against *S. aureus*, MRSA, *S. pyogenes*, *S. epidermidis,* and *P.*

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aeruginosa as shown in Table 4.3. Of all microorganisms selected, *S. pyogenes* displayed some sensitivity towards the water extract whilst *C. albicans* only displayed sensitivity to the SF AgNPs. Generally, the water extract was less active whilst the activity of SF AgNPs was the most potent against selected microorganisms. Ethanol and acetone extracts displayed similar inhibitory potential as their activity was not significantly different against selected microorganisms. The difficulty in suspending higher masses of the dried extracts limited the testing of higher extract concentrations (>50 mg/ml).

Table 4.3: Antimicrobial inhibition using SF by agar well diffusion

Microorganisms	**Test samples**									
	Water[a]	**Ethanol**[b]	**Acetone**[c]	**SF AgNP**[d]	**Statistical significance (*P*)**					
	Average zones of inhibition (mm±SEM)				ab	ac	ad	bc	bd	cd
Staphylococcus aureus	0	2±0.3	2±0	5±0.7	**	**	***	ns	***	***
MRSA	0	1.3±0.3	3±0.3	3±0.3	*	***	***	*	**	ns
Streptococcus pyogenes	1±0.3	3±0.7	3±0.9	4±0.3	**	**	***	ns	ns	ns
Staphylococcus epidermidis	0	1±0.3	3±0.7	3±0.3	*	***	***	**	**	ns
Pseudomonas aeruginosa	0	1±0	2±0.3	3±0	*	**	***	*	**	*
Candida albicans	0	0	0	2.7±0.3	ns	ns	**	ns	**	**

The inhibition of the microorganisms was performed using water (a), ethanol (b) and acetone (c) extracts at 50 mg/ml and SF AgNPs (d) at 1.5 mg/ml. Ampicillin inhibited the growth of *S. aureus*, MRSA, *S. epidermidis*, *S. pyogenes*, and *P. aeruginosa* with zones of 14 mm, 13 mm, 15 mm, 16 mm and 10 mm, respectively. Fluconazole inhibited the growth of *C. albicans* with an inhibition zone of 8 mm.
Abbreviations: AgNPs: silver nanoparticles; mm: millimetre; ns: not significant; SEM: standard error of the mean (for n=3), SF: *Sutherlandia frutescens*. *P<0.05; **P<0.01; ***P<0.001.

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4.4.2.2. Assessment of antimicrobial activity by Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration/Minimum Fungicidal Concentration (MBC/MFC) assays

The SF AgNPs were the most active against *S. aureus,* MRSA, *S. pyogenes*, *S. epidermidis*, *P. aeruginosa* and *C. albicans* with MIC values of 0.375, 0.75, 0.75, 0.75, 0.75 and 1.5 mg/ml, and MBC or MFC values of 0.75, 3, 1.5, 1.5, 0.75 and 3 mg/ml respectively (Table 4.4). The least active of all the test samples was the water extract which exhibited MIC values of 50 mg/ml and above against all the selected microorganisms. Ethanol and acetone extracts displayed similar activity against *S. pyogenes* with MIC of 25 mg/ml.

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Table 4.4: MIC (mg/ml) and MBC or MFC (mg/ml) of *S. frutescens* extracts and Ag nanoparticles against various microorganisms.

Microorganisms	Water		Ethanol		Acetone		SF AgNP	
	MIC (mg/ml)	MBC/MFC (mg/ml)	MIC (mg/ml)	MBC/MFC (mg/ml)	MIC (mg/ml)	MBC/MFC (mg/ml)	MIC (mg/ml)	MBC/MFC (mg/ml)
Staphylococcus aureus	>50	-	50	50	25	50	**0.375**	0.75
MRSA	>50	-	50	-	25	25	**0.75**	3
Streptococcus pyogenes	50	-	25	25	25	50	**0.75**	1.5
Staphylococcus epidermidis	>50	-	>50	-	25	25	**0.75**	1.5
Pseudomonas aeruginosa	>50	-	>50	-	50	50	**0.75**	0.75
Candida albicans	>50	-	>50	-	>50	-	1.5	3

Abbreviations: AgNPs: silver nanoparticles; MBC: Minimum bactericidal Concentration; MFC: Minimum Fungicidal Concentration; mg/ml: milligram per millilitre, SF: *Sutherlandia frutescens*. **MIC < 1 mg/ml are in bold.**

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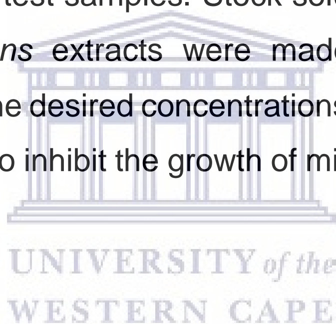
4.5. Discussion

The phytochemical uniqueness of *S. africana-lutea* and *S. frutescens* has significantly attributed to their long history of use. However, the antimicrobial activity of both these plants has not been widely documented in comparison to other bioactivities including anti-inflammatory and anticancer activity (Kamatou *et al*., 2008, Nielsen *et al*., 2012, Mabusa *et al*., 2017). The antibacterial and antifungal activity of water, ethanol, and acetone extracts of *S. africana-lutea* and *S. frutescens* and their respective Ag nanoparticles (SAL AgNPs and SF AgNPs) were evaluated using the agar well diffusion assay, minimum inhibitory concentration assay, and minimum bactericidal or fungicidal concentration assay. In general, these assays allowed for the qualitative (agar well diffusion) and quantitative (MIC, MBC or MFC) analysis of either the sensitivity or resistance of the selected microorganisms to the test samples. Stock solutions of ethanol and acetone *S. africana-lutea* and *S. frutescens* extracts were made using 10% dimethylsulfoxide (DMSO) and dilutions made to the desired concentrations which had less than 1% DMSO. At 1% DMSO was reported not to inhibit the growth of microorganisms (Baris *et al*., 2006, Langfield *et al*., 2004).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S. epidermidis is the causative agent of a variety of infections and in recent years has become an important cause of nosocomial infections. The bacterium forms biofilms that protect it from eradication by the immune system and antibiotic treatment (Vuong, 2002, Otto, 2009). Hence the need for novel therapeutic approaches for the treatment of such infections. Amongst the bacterial strains, *S. epidermidis* was more sensitive to the *S. africana-lutea* extracts and SAL AgNPs as seen in Table 4.1. *S. frutescens* extracts and SF AgNPs displayed most activity against *S. pyogenes*, as shown in Table 4.3. *S. pyogenes* is known to cause skin and soft tissue infections. *P. aeruginosa* and the fungus *C. albicans* were the most resistant strains. Gram-negative bacteria including *P. aeruginosa* possess an outer membrane that decreases the permeability of the cell wall to numerous agents (Winstanley *et al*., 2016). Henceforth, *P. aeruginosa* is expected to display certain levels of resistance towards different antimicrobial agents. *C. albicans* on the other hand has been reported to be able to grow and survive in yeast, pseudohyphae

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and hyphae forms enhancing their resistance to eradication (Rane *et al*., 2013, Whaley *et al*., 2016). The test samples were more potent against the bacterial strains in comparison to *C. albicans*. Though the activity of the positive controls was stronger than those displayed by the test samples, the inhibitory activity of the extracts, SAL AgNPs and SF AgNPs against *S. aureus,* MRSA, *S. pyogenes*, *S. epidermidis*, *P. aeruginosa* and *C. albicans* was noteworthy.

The unique features and characteristics of the plant, extracting solvent and selected microbial strains result in the selective sensitivity of the specific microorganisms to the various extracts of *S. africana-lutea* and *S. frutescens*. Tian and colleagues suggested that the more polar the extracting solvent the lower the inhibitory activity of the extract (Tian *et al*., 2009). This was supported by the weak inhibitory effect of the water (highly polar solvent) extracts against all the selected microorganisms. Ethanol, which is the second most commonly utilised solvent after methanol for the preliminary investigation of antimicrobial activity of plants, is known for its ability to extract polyphenols which have been shown to exhibit antimicrobial activity (Daglia, 2012, Valle Jr *et al*., 2015). Acetone, which has both polar and nonpolar characteristics, is able to extract polar as well as a non-polar phytochemicals. In addition to the polyphenols, it is able to extract lipophilic bioactive compounds. This results in an amplified activity of the acetone plant extracts (Ginovyan, 2017, Arunkumar and Muthuselvam, 2009). This was made evident by the significant inhibition of the study microorganisms by both the ethanol and acetone extracts with acetone extracts generally exhibiting stronger inhibitory potential.

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Biogenic silver nanoparticles have been widely shown to possess antimicrobial activity This study is no different, as the zones of inhibition caused by 1.5 mg/ml of SAL AgNPs and SF AgNPs was either significantly similar or greater compared to those resulting from 50 mg/ml of water, ethanol and acetone extracts. The current study took a step further and compared the activity of plant extracts at similar concentrations as the optimum concentration for Ag nanoparticle synthesis (3.125 mg/ml for *S. africana-lutea* and 6.25mg/ml for *S. frutescens*). All the extracts at these low concentrations did not show

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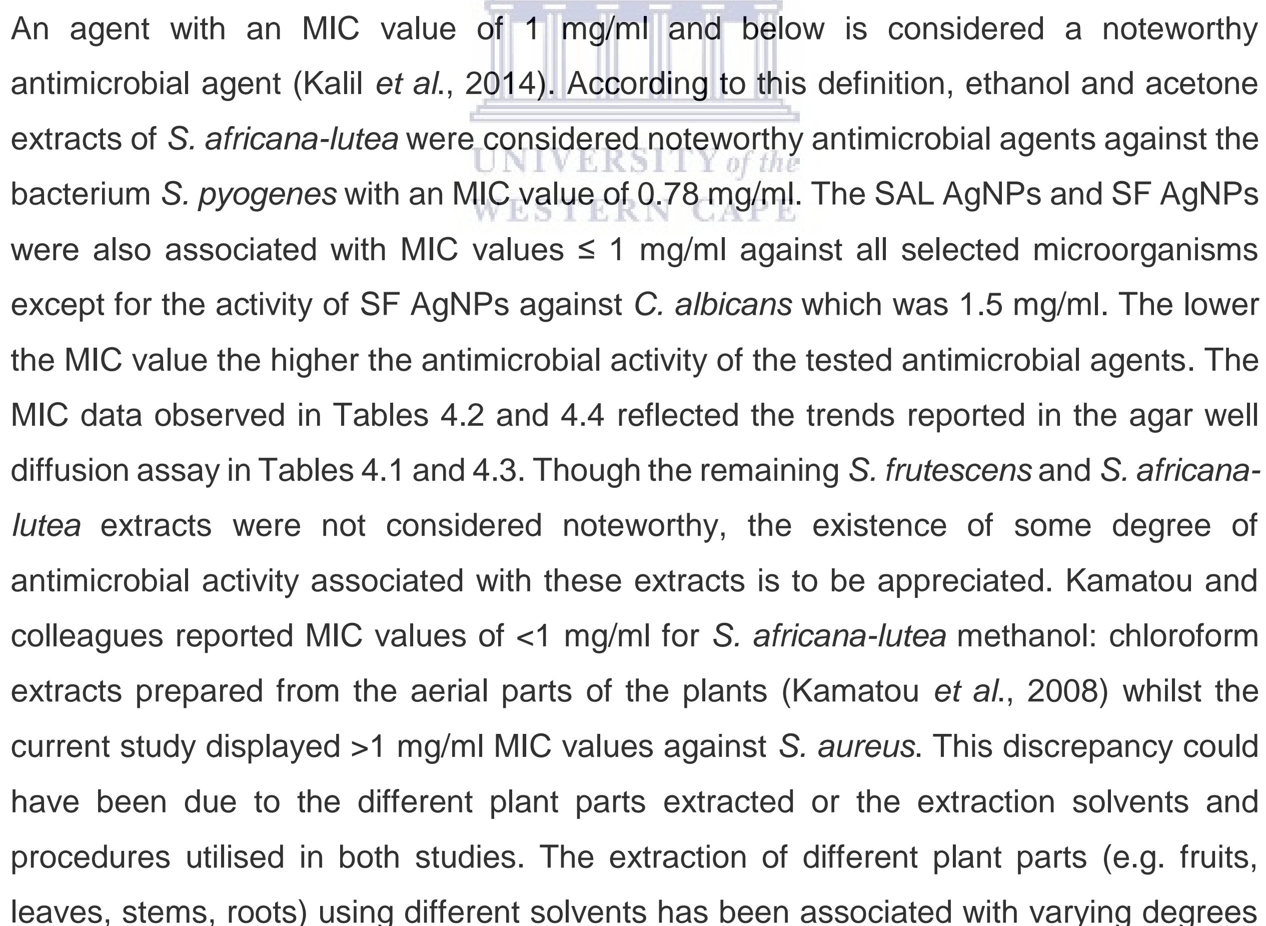
zones of inhibition including the water extracts from which the nanoparticles were synthesised. This probably suggests that the reducing, capping and stabilizing phytochemicals of the synthesised nanoparticles display a certain degree of antimicrobial activity. During nanoparticle synthesis, these bioactive phytochemicals are possibly incorporated into the nanoparticles. Within the synthesised nanoparticles the phytochemicals are probably concentrated and stabilized increasing the antimicrobial activity of the Ag nanoparticles compared to that of the crude water extracts. Additionally, it is possible that during nanoparticle production phytochemicals are altered resulting in stronger antimicrobial activity. These hypotheses probably explain the enhanced antimicrobial activity observed for the biogenic silver nanoparticles (SAL AgNPs and SF AgNPs) when compared to that displayed by the water extracts used in the nanoparticle synthesis reaction. However, additional assays are needed to confirm these assertions.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An agent with an MIC value of 1 mg/ml and below is considered a noteworthy antimicrobial agent (Kalil *et al*., 2014). According to this definition, ethanol and acetone extracts of *S. africana-lutea* were considered noteworthy antimicrobial agents against the bacterium *S. pyogenes* with an MIC value of 0.78 mg/ml. The SAL AgNPs and SF AgNPs were also associated with MIC values ≤ 1 mg/ml against all selected microorganisms except for the activity of SF AgNPs against *C. albicans* which was 1.5 mg/ml. The lower the MIC value the higher the antimicrobial activity of the tested antimicrobial agents. The MIC data observed in Tables 4.2 and 4.4 reflected the trends reported in the agar well diffusion assay in Tables 4.1 and 4.3. Though the remaining *S. frutescens* and *S. africana-lutea* extracts were not considered noteworthy, the existence of some degree of antimicrobial activity associated with these extracts is to be appreciated. Kamatou and colleagues reported MIC values of <1 mg/ml for *S. africana-lutea* methanol: chloroform extracts prepared from the aerial parts of the plants (Kamatou *et al*., 2008) whilst the current study displayed >1 mg/ml MIC values against *S. aureus*. This discrepancy could have been due to the different plant parts extracted or the extraction solvents and procedures utilised in both studies. The extraction of different plant parts (e.g. fruits, leaves, stems, roots) using different solvents has been associated with varying degrees

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of bioactivity (Azmir *et al.*, 2013). Additionally, the seasonal variation of harvested plants may have affected the phytochemical composition of the plants (Arraiza *et al.*, 2012). The different MBC and MFC values exhibited by the extracts and SAL AgNPs or SF AgNPs against the microorganisms were shown in Tables 4.2 and 4.4. Water extracts did not display any microbicidal effects at a concentration of 50 mg/ml. The MBC and MFC values for the *S. africana-lutea* and *S. frutescens* extracts and SAL AgNPs or SF AgNPs were either equal to or higher than the MIC values. Equal MIC and MBC or MFC meant the lowest concentrations inhibiting the visual growth of the microbes also eradicated the microbes (Krishnan *et al.*, 2015).

4.6. Conclusion

The result of this study indicates that *S. frutescens* and *S. africana-lutea* may be beneficial in reducing the risk of infectious diseases in immunocompromised patients. However, the biogenic silver nanoparticles synthesised using *S. frutescens* and *S. africana-lutea* as reducing agents exhibited amplified antimicrobial activity in comparison to the water extracts. The enhanced activity of the biogenic silver nanoparticles when compared to the antimicrobial activity of the synthesising plant extracts was in agreement with previously reported studies (Kathiravan *et al.*, 2015, Dar *et al.*, 2013, Ahmed *et al.*, 2016). According to our findings, we conclude the extracts and biogenic silver nanoparticles of *S. frutescens* and *S. africana-lutea* as effective health promoting agents. These agents can eventually be incorporated into different products such as bandages and topical ointments for the treatment of open wounds prone to infections. It is therefore recommended that further investigations for clinical applications of these agents be assessed.

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CHAPTER FIVE

IMMUNOMODULATORY EFFECTS OF *SALVIA AFRICANA-LUTEA* AND *SUTHERLANDIA FRUTESCENS* PLANT EXTRACTS AND THEIR RESPECTIVE NANOPARTICLES

5.1. Abstract

The immunomodulatory effects of *S. africana-lutea* and *S. frutescens* water, ethanol and acetone extracts, as well as the synthesised nanoparticles (SAL AgNPs, SAL AuNPs, SF AgNPs), were assessed using *in vitro* bioassays. The non-adherent monocytic cell line, THP-1, was differentiated to mimic macrophages using phorbol 12-myristate 13-acetate (PMA). The cell proliferation reagent WST-1 was used to identify the highest concentrations of extracts and respective nanoparticles that are nontoxic to THP-1 macrophages and natural killer cell line NK-92. The treatments displayed varying levels of anti-inflammatory activity when their effects on the production and secretion of interleukin-6 (IL-6), tumour necrotic factor-α (TNF-α) and interleukin-1β (IL-1β) by the THP-1 macrophage and interleukin-10 (IL-10) and interferon-γ (IFN-γ) by the natural killer cell line NK-92 were analysed. Of all the extracts analysed, the ethanol extracts of *S. frutescens* and *S. africana-lutea* generally exhibited the greatest inhibition of the cytokines. Though most of the treatments decreased the production of pro-inflammatory cytokines by THP-1 macrophage and NK-92 cell line, the water extract of *S. frutescens* increased the production of the pro-inflammatory cytokine IFN-γ whilst SAL AuNPs increased the productions of the pro-inflammatory cytokines IL-1β and IFN-γ. When target cells (HaCaT, Hela and PC-3) were co-cultured with the treated effector cell line NK-92 (treated with water, ethanol and acetone extracts of *S. africana-lutea* and *S. frutescens* and their respective nanoparticles) at an effector:target ratio of 10:1, the viability of PC-3 and Hela cells was significantly decreased in the presence of nanoparticle-treated NK-92 cells. The target cells susceptible (PC-3 and Hela) to the nanoparticle-treated NK-92 cells were then assessed for intracellular ROS production post co-culture. The

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intracellular ROS production was increased in all the target cells when compared to the negative control (untreated cells). This could mean the NK-92 cells are activated by the nanoparticles to release cytotoxic compounds that eventually stress the target cells resulting in their increased production of intracellular ROS and decreased viability.

Keywords: cytokines, Interferon-γ, Interleukin-1β, Interleukin-6, Interleukin-10, nanoparticles, Salvia *africana-lutea, Sutherlandia frutescens*, Tumour necrotic factor-α.

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5.2. Introduction

The immune system constantly provides the body with protection against an array of pathogenic microbes as well as damaged and harmful cells. This complex system consists of an extensive network of tissues and specialized cells. Macrophages and natural killer (NK) cells play a crucial role in the detection and elimination of harmful foreign material which include infectious microbes as well as tumour cells present in the body (Iwasaki and Medzhitov, 2010). These immune cells are a major part of the innate immune system. NK cells are capable of recognizing cancer cells through cell surface receptor recognition and induce cell death through the release of cytotoxic granules and cytokines (Kokura, 2016, Vivier *et al*., 2012). Macrophages, on the other hand, depend on pattern recognition to detect infectious microbes (Martinez and Gordon, 2014). In the presence of cancerous cells, the NK cells are activated and are able to eliminate these abnormal cells through the expression of death receptor ligands and secretion of cytokines including the pro-inflammatory IFN-γ and anti-inflammatory IL-10 (Biron *et al*., 1999, Vivier *et al*., 2012, Levy *et al*., 2011). During bacterial infections, the activated macrophages release pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α that work against the infection through the induction of an inflammatory response. Though generally beneficial, a prolonged release of these cytokines for extended periods of time can lead to severe tissue damage as well as complications including rheumatoid arthritis, psoriasis, eczema and multiple sclerosis. Immune responses can either be suppressed or activated by a cascade of regulated pathways. An interference in this balance can result in increased susceptibility to autoimmune diseases or infections depending on whether the immune system is excessively activated or suppressed (Haase *et al*., 2012). Immunotherapies that can stimulate or suppress immune responses can be used for the treatment of different ailments including cancer, psoriasis, eczema, Crohns disease and rheumatoid arthritis (Coutinho and Chapman, 2011). Henceforth, the use of therapeutics that modulate cytokine secretion by NK cells and macrophages may be a promising approach in the efficient treatment of cancer as well as infections (Gordon and Martinez, 2010, Cavaillon, 1994).

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The biogenic synthesis of nanoparticles using plants is gaining interest as they are perceived safe and environmentally benign (Souri *et al*., 2018). It is likely that the biogenic nanoparticles could have similar or enhanced bioactivities to the synthesising crude extracts depending on whether the bioactive phytochemicals are involved in the synthesis of the nanoparticles (Rao *et al*., 2016, Lee *et al*., 2014).

The use of medicinal plants to "boost" the immune system is a very old practice. Medicinal plants may be used to alter the immune system. Several studies have reported on the anti-inflammatory and immune modulating potential of plants including *Aloe vera*, *Carica papaya*, *Centella asiatica*, *Thymus vulgaris* and *Zataria multiflora* (Amirghofran *et al*., 2011, Otsuki *et al*., 2010, Mukherjee *et al*., 2014, Alamgir and Uddin, 2010). *Sutherlandia frutescens* (*S. frutescens*) and *Salvia africana-lutea* (*S. africana-lutea*) are taken as "tonics" by HIV positive patients presumably to activate or "boost" the immune system. However, very little is known about their exact interaction with the hosts' immune system. Though few studies have shown the anti-inflammatory activity of *S. frutescens* (Lei *et al*., 2015, Faleschini *et al*., 2013), the immune modulatory effects of *S. africana-lutea* extracts as well as nanoparticles synthesised from both plants is not known. This study aims at addressing this problem by investigating the immunomodulatory effects of *S. africana-lutea* and *S. frutescens* extracts and their respective nanoparticles.

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5.3. Methodology

5.3.1. Chemicals and apparatus

The 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), Corning® HTS Transwell®-24 well polystyrene permeable support, Lipopolysaccharide (LPS), Pen-strep (penicillin and streptomycin), phorbol 12-myristate 13-acetate (PMA), sodium bicarbonate and cell proliferative reagent WST-1 were acquired from Sigma Aldrich (St. Louis, USA). Dulbecco's Phosphate Buffered Saline (DPBS), Dulbecco's Modified Eagle Media (DMEM), fetal bovine serum (FBS), horse

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serum, interleukin 2 (IL-2), L-glutamine, Minimum Essential Medium Eagle-alpha Modification (MEM-α), Roswell Park Memorial Institute medium (RPMI) were supplied by Thermofischer scientific (Waltham, Massachusetts, USA). All ELISA kits were produced by Bioo Scientific, PerkinElmer (Austin, Texas, USA).

5.3.2. Plant material

Refer to section 3.3.2.

5.3.3. Plant extraction

Refer to section 4.3.3.

5.3.4. Nanoparticle synthesis

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Refer to section 3.3.4.

5.3.5. Cell culture

The cell lines utilised in this study were obtained from the American Type Culture Collection (ATCC) (Table 5.1). NK-92 cells were cultured in Minimum Essential Medium Eagle-alpha Modification (MEM-α) containing 2 mM L-glutamine, 12.5% horse serum, 100 U/ml interleukin 2 (IL-2), 1% Pen-strep (penicillin and streptomycin) and 1.5 g/L sodium bicarbonate. The THP-1 cell line was cultured in Roswell Park Memorial Institute medium (RPMI) containing 20% fetal bovine serum (FBS). PC-3 cells were cultured in RPMI whilst HaCaT and Hela cell lines were grown in Dulbecco's Modified Eagle Media (DMEM) all supplemented with 10% FBS and 1% pen-strep. All supplemented media will henceforth be referred to as complete media. The cells were cultured at 37 ^{o}C in a 5% CO_2 humidified SL SHEL LAB incubator.

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Table 5.1: Cell lines used in this study.

Cell line	Acronym	Disease	Species
Natural killer cells	NK-92	Normal	Homo sapiens
Macrophages	THP-1	Normal	Homo sapiens
Prostate cancer	PC-3	Adenocarcinoma	Homo sapiens
Cervical cancer	Hela	Adenocarcinoma	Homo sapiens
Human melanocytes	HaCaT	Normal	Homo sapiens

5.3.6. Differentiation of THP-1 cells

THP-1 cells were differentiated into monocyte-derived macrophages using phorbol 12-myristate 13-acetate (PMA) according to a previously described method (Daigneault *et al.*, 2010) with some modifications. The cells (at a density of $2x10^5$ cells/ml) were seeded in 24 well plates and treated with 25 nM of PMA. The cells were incubated in a 5% CO_2 humidified SL SHEL LAB incubator at 37 ^oC for 3 days. The PMA containing media was replaced with PMA free complete RPMI media and cells were allowed to rest for 24 hours. The morphological changes of the cells were monitored daily to ensure efficient differentiation.

5.3.7. Cell viability assay

The colourimetric assay, WST-1 was used as described by the manufacturer to determine the toxicity of *S. africana-lutea* and *S. frutescens* extracts and their respective nanoparticles towards NK-92, THP-1, PC-3, Hela and HaCaT cell lines. The assay was also employed to determine the toxicity of pre-treated (i.e. activated) NK-92 cell lines towards HaCaT, PC-3 and Hela in a co-culture experimental set-up (section 5.3.9). The cells were seeded at a density of $2x10^5$ cells/ml in 96 well plates and treated with decreasing concentrations of *S. africana-lutea* and *S. frutescens* extracts and respective nanoparticles for 24 hours. For THP-1 cells, prior to treatment, the cells were differentiated as described in section 5.3.6. For all cell lines, following the treatment with

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water, ethanol and acetone extracts of *S. africana-lutea* and *S. frutescens* and their respective nanoparticles the cells were washed with DPBS. Complete media containing 10% WST-1 was added to each well. Untreated cells served as the negative controls and cells treated with 10% DMSO served as the positive controls. After 4 hours incubation at 37 ^{o}C, the absorbance of the plates was measured using the POLARstar Omega spectrophotometer (BMG labtech, Germany) at 440 nm using a reference wavelength of 630 nm. Cell viability was calculated using the formula below:

$$\textbf{\% Cell viability} = \frac{\text{Absorbance of treated cells - Absorbance of Blank}}{\text{Absorbance of negative control - Absorbance of Blank}} \times 100$$

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5.3.8. Assessing the cytokine profile of treated THP-1 and NK-92 cells

Lipopolysaccharide (LPS) derived from *Escherichia coli* 0111: B4 was used to stimulate the THP-1 macrophages as described by Sánchez-Quesada and colleagues (2015). THP-1 macrophages (in 24 well plates) were stimulated by adding 1 μg/ml LPS to the cells and incubating the cells for 6 hours at 37 ^{o}C. LPS supplemented media was replaced with complete RPMI containing the plant extract or nanoparticles at the specified non-toxic concentrations as determined in section 5.3.7. The THP-1 macrophages were incubated for a further 18 hours. THP-1 macrophages treated with LPS alone represented the positive control. The non-adherent NK-92 cell lines were not treated with LPS. These cells were seeded in 24 well plates at a density of $2x10^5$ cells/ml with the respective treatment (plant extracts or nanoparticles) in completed MEM-α media. For both THP-1 macrophages and NK-92 cells, the negative control cells were not subjected to any treatment. The supernatants from both the THP-1 macrophages and NK-92 cells were collected into labelled 1.5 ml Eppendorf tubes after 24 hours. The tubes were centrifuged at 13,000 rotation per minutes (rpm) using the Eppendorf AG centrifuge 5417R with a standard rotor (F-45-30-11) for 5 minutes. Centrifugation allows for the settling of residual nanoparticles and cells into a pellet whilst conserving the cytokines in supernatant. The

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supernatants were transferred into clean labelled 1.5 ml Eppendorf tubes and stored at -80 ^{o}C until cytokine profiling was performed. THP-1 macrophage supernatants were used to assess IL-1β, IL-6 and TNF-α, while NK-92 supernatants were used to assess IL-10 and IFN-γ using enzyme-linked immunosorbent assay (ELISA). The assays were performed as described by the manufacturer.

5.3.9. Assessing the effect of treated NK-92 cells on cancer cells in co-culture

Prior to the commencement of the co-culture assay, the cell viability of all target cell lines (HaCaT, PC-3, and Hela) in MEM-α with specific extracts or nanoparticles at concentrations previously determined as non-toxic to the NK-92 cells was assessed. NK-92 cells were washed in DPBS, re-suspended in MEM-α culture medium lacking IL-2, and allowed to continue growing in culture. The target cells were seeded into the lower compartment (Figure 5.1) of the Corning® HTS Transwell®-24 well polystyrene permeable support and incubated for 24 hours to allow cell adherence. Spent media was replaced with 600 µl of extract or nanoparticles containing complete MEM-α. The effector cells (NK-92) suspended in test samples with complete MEM-α media was placed into the upper compartment at 10:1 effector:target (E:T) cell ratio. The lower and upper compartments are separated by a permeable polystyrene membrane with 0.4 µm wide pores (pore size allows media exchange between the two compartments whilst preventing cellular migration). Negative control wells were plated with untreated target and effector cells. These plates were incubated at 37 ^{o}C for 24 hours, after which the upper compartments were discarded and the cell viability assay was performed on the target cells. The cell viability was calculated as stated in section 5.3.7.

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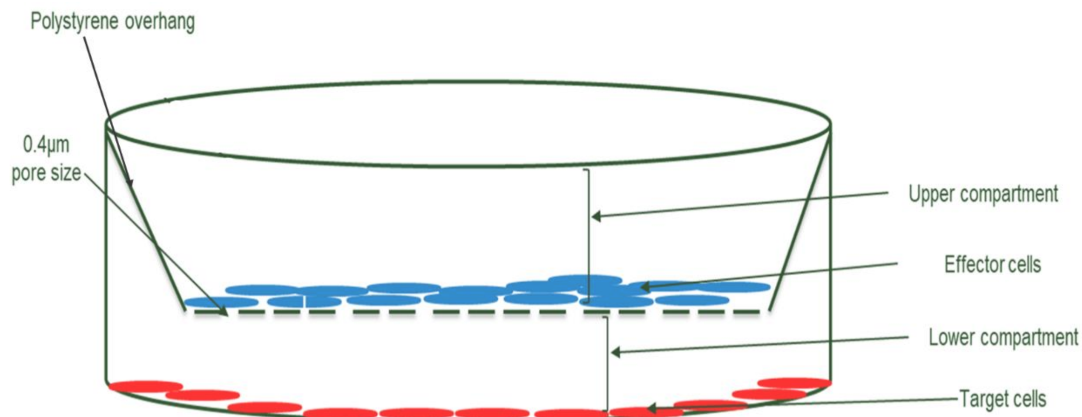

Figure 5.1: The co-culture experimental set-up for a single well of the Corning® HTS Transwell®-24 well polystyrene permeable support.

5.3.10. Assessment of oxidative stress

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The co-culture assay was set up as described in section 5.3.9. After discarding the upper compartment, the reactive oxygen species (ROS) assay was carried out on the target cells on the lower compartment. The ROS assay was done using the 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) molecular probe that allows for the quantification of intracellular ROS. The intracellular ROS oxidizes the probe, converting it to a highly fluorescent CM-DCF which is detectable using flow cytometry. Hydrogen peroxide treated target cells were used as a positive control and the untreated target cells as the negative control. The cells were stained using the CM-H_2DCFDA dye for 30 minutes at 37 ^{o}C. Thereafter, the dye was removed and the cells washed with PBS. The adherent cells were trypsinized, collected, centrifuged at 3,000 rpm for 3 minutes and the pellet re-suspended in PBS. The quantification of intracellular ROS was done at 488 nm using a BD AccuriTM C6 flow cytometer.

5.3.11. Statistical analysis

The immunomodulatory activity of *S. africana-lutea* and *S. frutescens* extracts and their respective nanoparticles was statistically analysed using the Bonferroni Multiple

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comparison analysis. Comparisons were considered significant at $P < 0.05$. GraphPad™ PRISM6 software package was used for all statistical evaluations and graphical representations. All experiments were done in triplicates (n=3).

5.4. Results

5.4.1. Differentiation of non-adherent THP-1 monocytes into macrophages

The differentiation of the non-adherent THP-1 monocytes into adherent macrophages has been reported in numerous studies (Daigneault *et al.*, 2010, Bener *et al.*, 2016). PMA treatment has been shown to be more effective in inducing differentiation in comparison to other THP-1 differentiation inducers like 1,25-dihydroxy vitamin D3 (VD3) (Schwende *et al.*, 1996). Differentiation using PMA causes the cells to become adherent and morphological changes as shown in Figure 5.2 is induced. The daily observation of the cells undergoing differentiation revealed a consistent morphological change seen as pseudopodia development and size increment of maturing cells. These cells were now recognized as fully differentiated macrophages, henceforth THP-1 cells will also be referred to as macrophages interchangeably.

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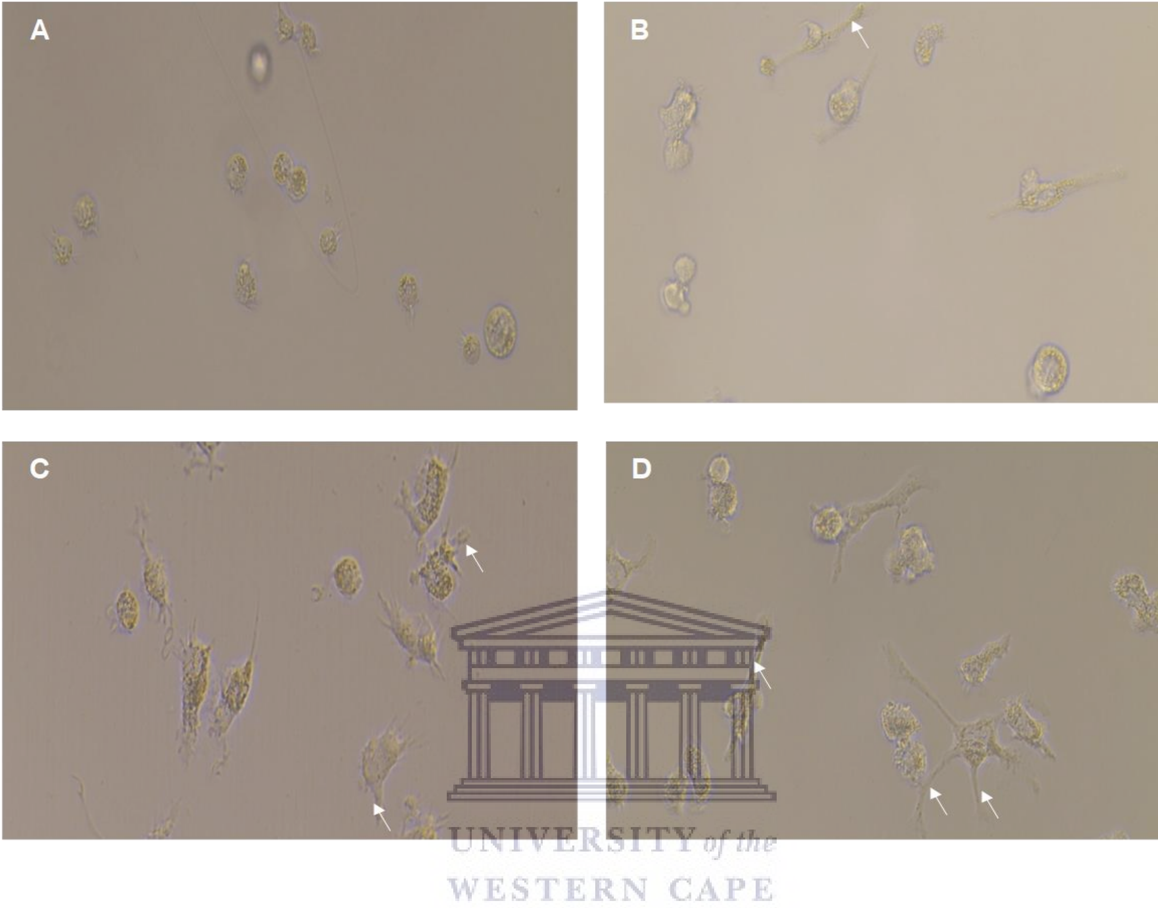

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Figure 5.2: Images of the morphological changes of PMA differentiated THP-1 cell line over time.

THP-1 morphological changes were observed over a period of 3 days. **(A)** represents Day 0, **(B)** represents Day 1, **(C)** represents Day 2 and **(D)** represents Day 3 of THP-1 cells exposure to PMA. The arrows point to pseudopodia developed as cells mature.

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Immunomodulatory effects of *S. africana-lutea* and *S. frutescens* extracts and synthesised nanoparticles on THP-1 macrophages

5.4.2. Determining the highest concentration of plant extracts and nanoparticles that are non-toxic to THP-1 cells

The cytotoxic effects of *S. africana-lutea* and *S. frutescens* extracts and synthesised *S. africana-lutea* gold nanoparticles (SAL AuNPs), *S. africana-lutea* silver nanoparticles (SAL AgNPs) and *S. frutescens* silver nanoparticles (SF AgNPs) were determined and reported (Figure 5.3 and 5.4). The effect was observed as directly proportional to the treatment concentration. The higher the treatment concentration the greater the toxicity effects towards the cells. Of the plant extracts assessed, the water extracts were observed as the least toxic. The water extracts (0.625 mg/ml) reduced the viability of THP-1 to 67% (*S. frutescens)* and 53% (*S. africana-lutea*) of cells. The acetone extracts were most toxic as they decreased the viability of THP-1 cells to 11% and 32% at a concentration of 0.625 mg/ml *S. frutescens* and *S. africana-lutea* respectively.

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The synthesised nanoparticles displayed a similar effect as the extracts. As the nanoparticle concentration increased, the viability of the treated THP-1 cells decreased. SF AgNPs reduced the viability of THP-1 to 2% at a concentration 0.5 mg/ml. The SAL AgNPs and SAL AuNPs reduced the viability of THP-1 to 44% and 45% respectively at a concentration of 0.5 mg/ml. At this concentration (0.5 mg/ml), SAL AgNPs and SAL AuNPs significantly decreased the viability of the THP-1 cells in comparison to the untreated cells. The cell viability of macrophages treated with SF AgNPs at concentrations of 0.031 mg/ml and higher was also observed as significantly different to that of the untreated cells. The untreated cells served as the negative control. *S. frutescens* extracts and SF AgNPs generally displayed more cytotoxic effects compared to *S. africana-lutea* extracts, SAL AgNPs and SAL AuNPs. The extract and nanoparticle concentrations that reduced the macrophages viability by 20% or less were considered

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non-toxic and hence used in the treatment of the THP-1 for cytokine determination (Table 5.2).

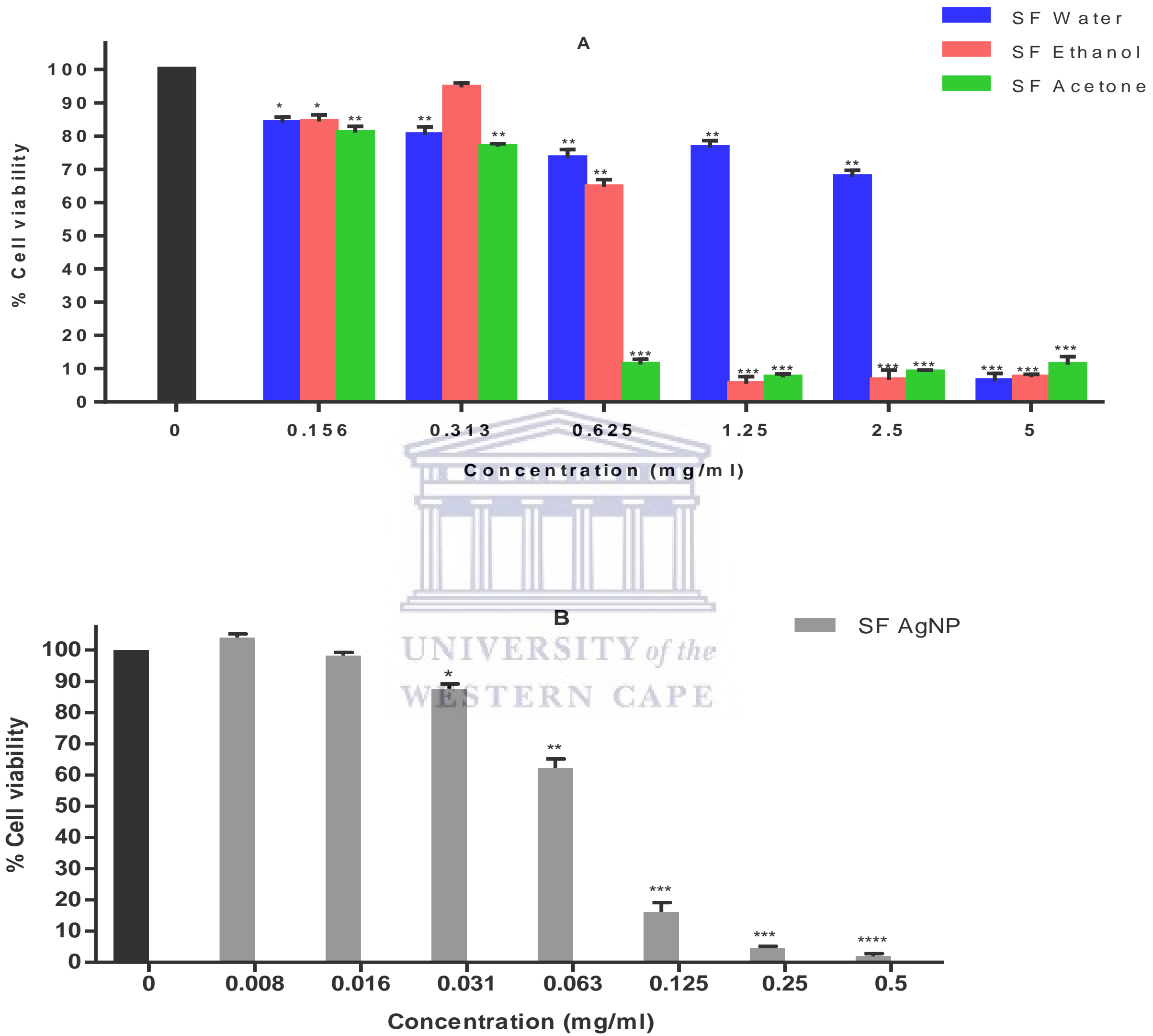

Figure 5.3: Cell viability of THP-1 cells treated with (A) water, ethanol, and acetone extracts of *S. frutescens* and (B) SF AgNP compared with untreated cells.

Abbreviations: mg/ml: milligram per millilitre; SF: *Sutherlandia frutescens*; SF AgNP: *Sutherlandia frutescens*; *P<0.05; **P<0.01; ***P<0.001, ****P<0.0001.

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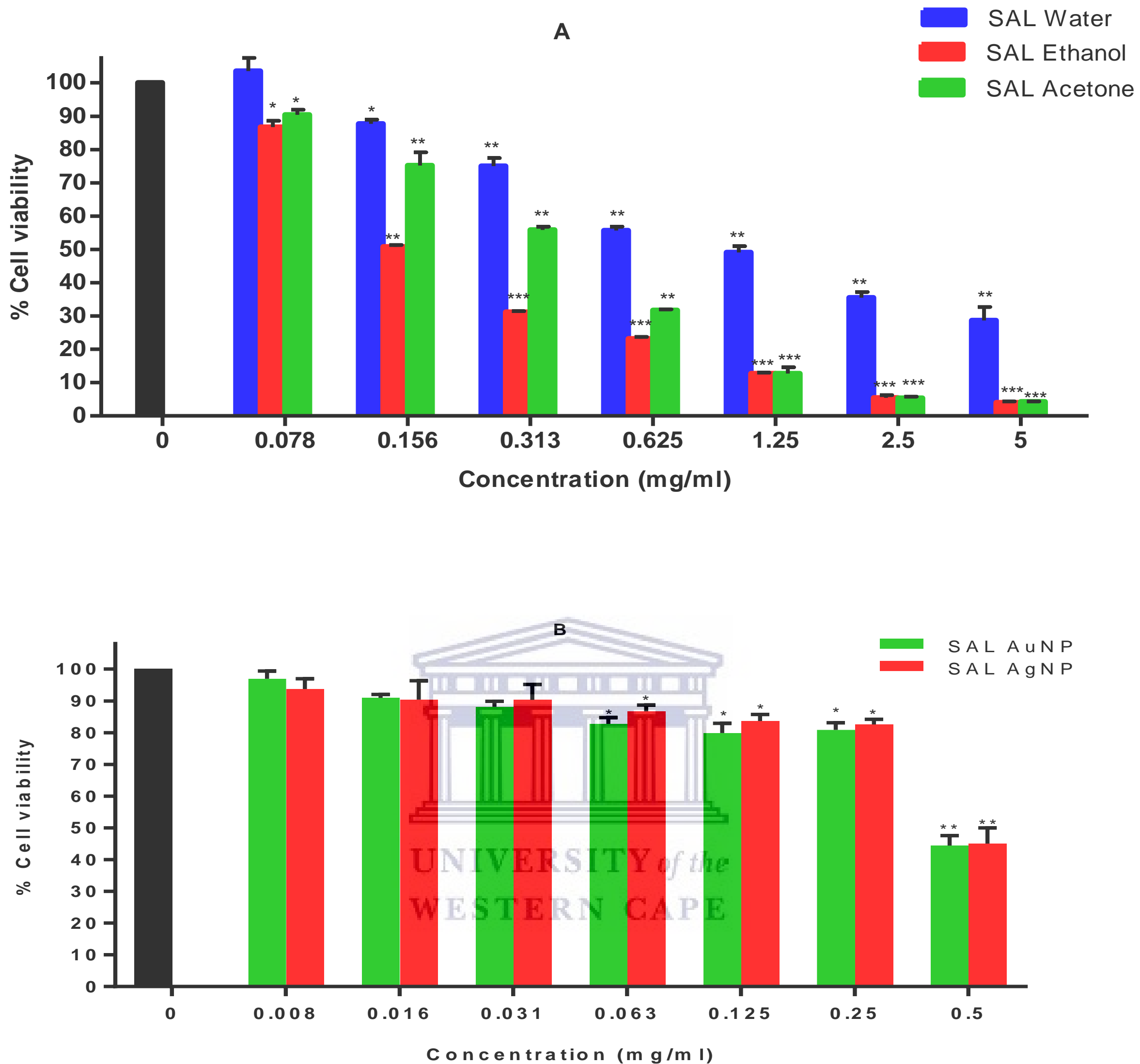

Figure 5.4: Cell viability of THP-1 cells treated with (A) water, ethanol, and acetone extracts of *S. africana-lutea* and (B) SAL AgNP and SAL AuNP compared with untreated cells.

Abbreviations: mg/ml: milligram per millilitre; SAL: *Salvia africana-lutea*; SAL AgNP: *Salvia africana-lutea* silver nanoparticle: SAL AuNP: *Salvia africana-lutea* gold nanoparticle; *P<0.05; **P<0.01; ***P<0.001.

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Table 5.2: Treatment concentrations selected to treat THP-1 cells for cytokine profiling according to % cell viability.

Treatment	Concentration (mg/ml)	Average % Cell viability
SF water extract	0.156	84
SF ethanol extract	0.313	95
SF acetone extract	0.156	81
SAL water extract	0.156	88
SAL ethanol extract	0.078	87
SAL acetone extract	0.078	90
SAL AgNP	0.25	89
SAL AuNP	0.25	84
SF AgNP	0.031	88

***Abbreviations*:** AgNP: silver nanoparticle; AuNP: gold nanoparticle; mg/ml: milligram per millilitre; SAL: *Salvia africana-lutea*; SF: *Sutherlandia frutescens*.

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5.4.3. Determining the effects of extracts and nanoparticles on cytokine secretion in THP-1 cells

The ELISA kit was used to determine the effects of *S. africana-lutea* and *S. frutescens* extracts and their respective nanoparticles on cytokine production in THP-1 cells. Lipopolysaccharide (LPS) was used to activate the differentiated THP-1 cell lines. LPS treatment leads to the stimulation of the macrophages to produce and secrete pro-inflammatory cytokines. Stimulation of differentiated THP-1 macrophages with LPS resulted in an increased secretion of IL-6, IL-1β, and TNF-α (Figure 5.5). However, treatment of the LPS-pre-treated THP-1 cells with *S. africana-lutea* and *S. frutescens* extracts and their respective nanoparticles lowered the concentrations of the analysed cytokines. The *S. frutescens* ethanol extract and *S. africana-lutea* water extract exhibited the greatest suppression of IL-6 production shown by the low concentrations of extracellular IL-6 (Figure 5.5A). The *S. africana-lutea* acetone extract induced the lowest decrease in IL-6 levels compared to all other treatments. The effects of the nanoparticles

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on the concentration of IL-6 were similar to those displayed by the plant extracts with the lowest decrease of IL-6 levels displayed by SAL AuNPs.

A similar observation was noted with TNF-α (Figure 5.5B). All the treatments resulted in a decrease in the extracellular levels of TNF-α. Though all the extracts reduced the TNF-α concentrations, the ethanol and acetone extracts of both plants significantly decreased the levels of TNF-α when compared to LPS treated THP-1 macrophages. It should be noted that the ethanol and acetone extracts of *S. africana-lutea* displayed the greatest decrease of TNF-α levels. Intriguingly, both the ethanol extract and SAL AuNPs displayed similar effects by reducing the levels of TNF-α by over 30 folds when compared to the levels of TNF-α induced by LPS treatment alone. When comparing the activity of the nanoparticles, the SF AgNPs reduced the TNF-α levels the least, with no significant difference to the TNF-α concentrations secreted by the LPS treated THP-1 macrophages.

The effects of the *S. frutescens* and *S. africana-lutea* water, ethanol, and acetone extracts and their respective nanoparticles on the secretion of IL-1β were similar to those displayed for IL-6 and TNF-α with a few exceptions. The SAL AuNPs were the only treatment that significantly increased the levels of the extracellular IL-1β (Figure 5.5C). The SAL AgNPs was also noted to slightly increase the extracellular level of IL-1β. On the other hand, the ethanol extract of *S. frutescens* significantly decreased the levels of IL-1β by 2 folds in comparison to the extracellular IL-1β concentrations of LPS treated macrophages.

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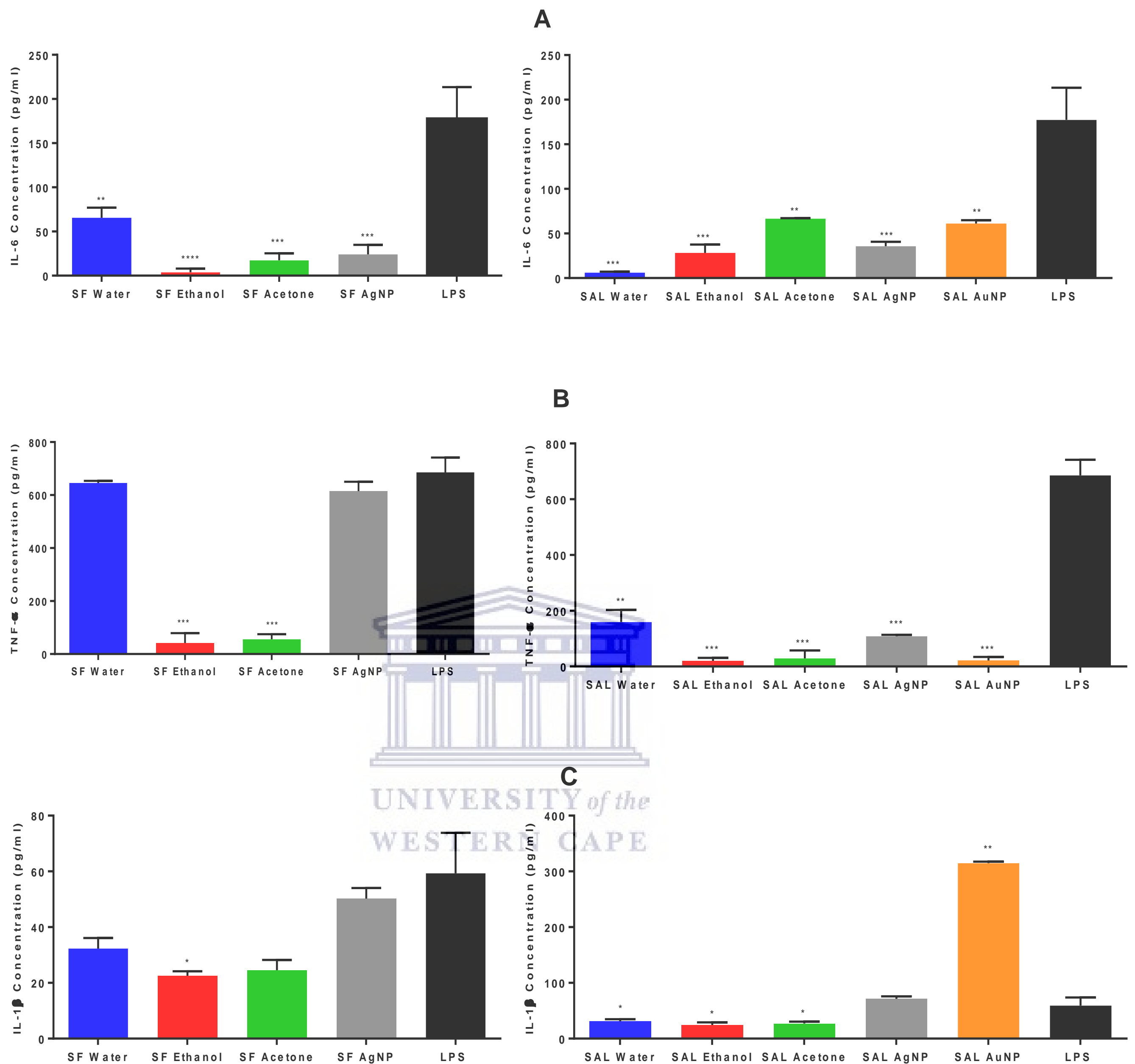

Figure 5.5: The effects of *S. frutescens* and *S. africana-lutea* extracts and respective nanoparticles on (A) IL-6, (B) TNF-α and (C) IL-1β secretion in LPS pre-treated THP-1 macrophage.

***Abbreviations*:** AgNP: silver nanoparticle; AuNP: gold nanoparticle; IL-1β: interleukin 1 beta; IL-6: interleukin 6; LPS: lipopolysaccharide; pg/ml: picogram per milliliter; SAL: *Salvia africana-lutea*; SF: *Sutherlandia frutescens*; TNF-α: Tumour necrotic factor alpha.

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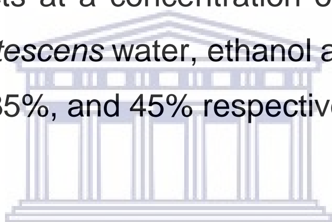
Immunomodulatory effects of *S. africana-lutea* and *S. frutescens* extracts and synthesised nanoparticles on NK-92 cells.

5.4.4. Determining the highest concentration of plant extracts and nanoparticles that are non-toxic to NK-92 cells

The toxicity effects of the *S. frutescens* and *S. africana-lutea* water, ethanol, and acetone extracts and their respective nanoparticles on NK-92 cells were analysed and reported (Figure 5.6 and 5.7). All extract concentrations significantly reduced the viability of the NK-92 cell lines when compared to the untreated cell with a few exceptions of water and ethanol *S. africana-lutea* extracts at a concentration of 0.078 mg/ml (Figure 5.6A and 5.7A). At 0.625 mg/ml the *S. frutescens* water, ethanol and acetone extracts reduced the viability of NK-92 cells to 35%, 35%, and 45% respectively.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The viability of NK-92 cells was 29%, 36% and 47% in water, ethanol and acetone *S. africana-lutea* extract concentrations of 0.625 mg/ml, 0.313 mg/ml and 0.625 mg/ml respectively. The SF AgNPs, SAL AgNPs and SAL AuNPs reduced the viability of NK-92 cells to 37%, 37% and 40% at 0.125 mg/ml, 0.25 mg/ml and 0.25 mg/ml nanoparticle concentration (Figure 5.6B and 5.7B). In general, the synthesised nanoparticles exhibited higher levels of toxicity towards the NK-92 cells compared to the plants' crude extracts. The treatment concentrations that resulted in cell viabilities of ≥ 80% were selected to treat NK-92 cells for cytokine profiling (Table 5.3).

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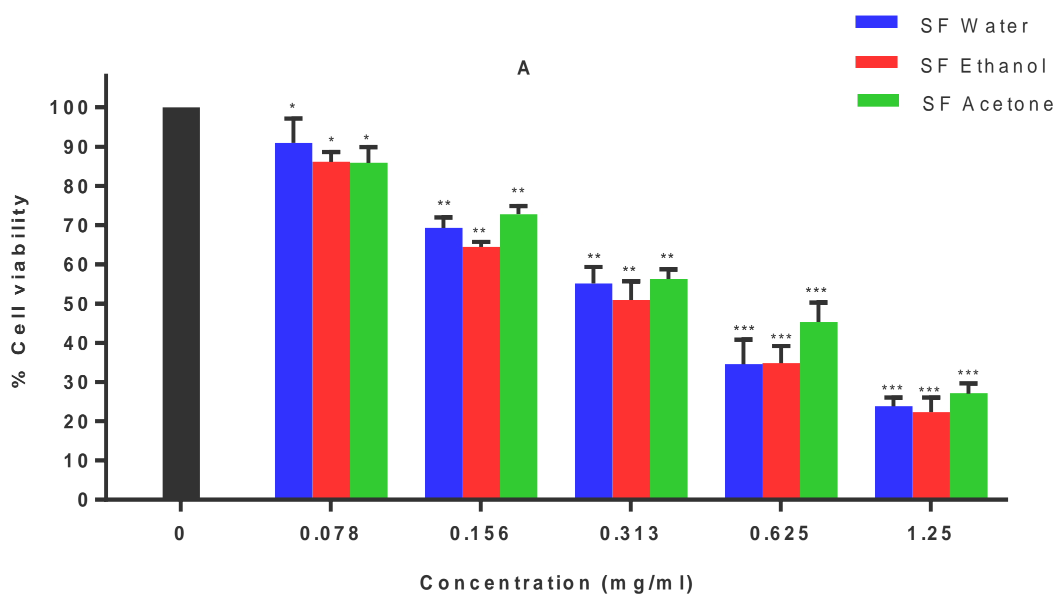

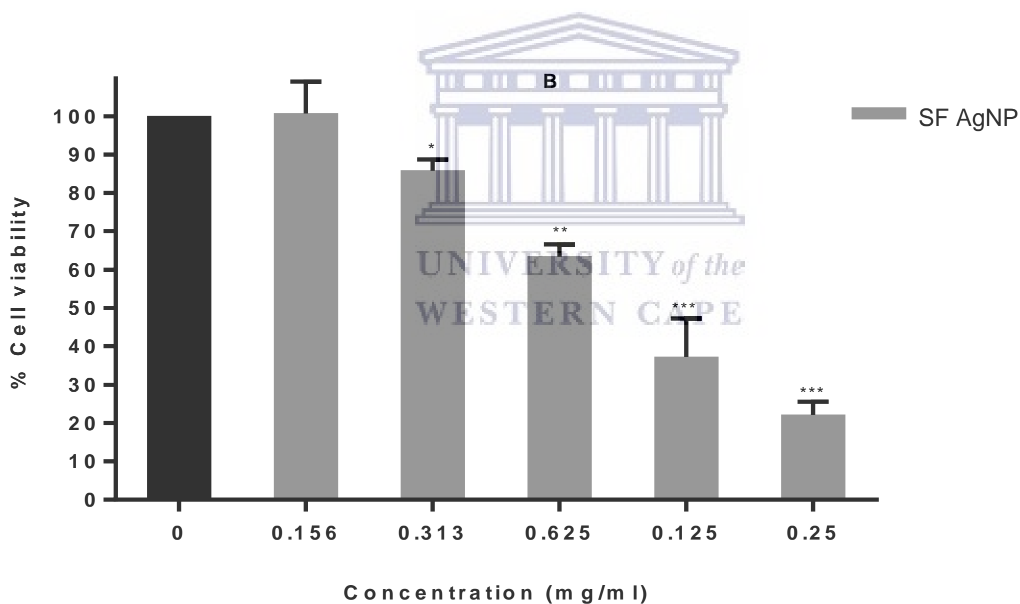

Figure 5.6: Cell viability of NK-92 cells treated with (A) water, ethanol, and acetone extracts of *S. frutescens* and (B) SF AgNP compared with untreated cells.

Abbreviations: mg/ml: milligram per millilitre; SF: *Sutherlandia frutescens*; SF AgNP: *Sutherlandia frutescens*; **P*<0.05; ***P*<0.01; ****P*<0.001.

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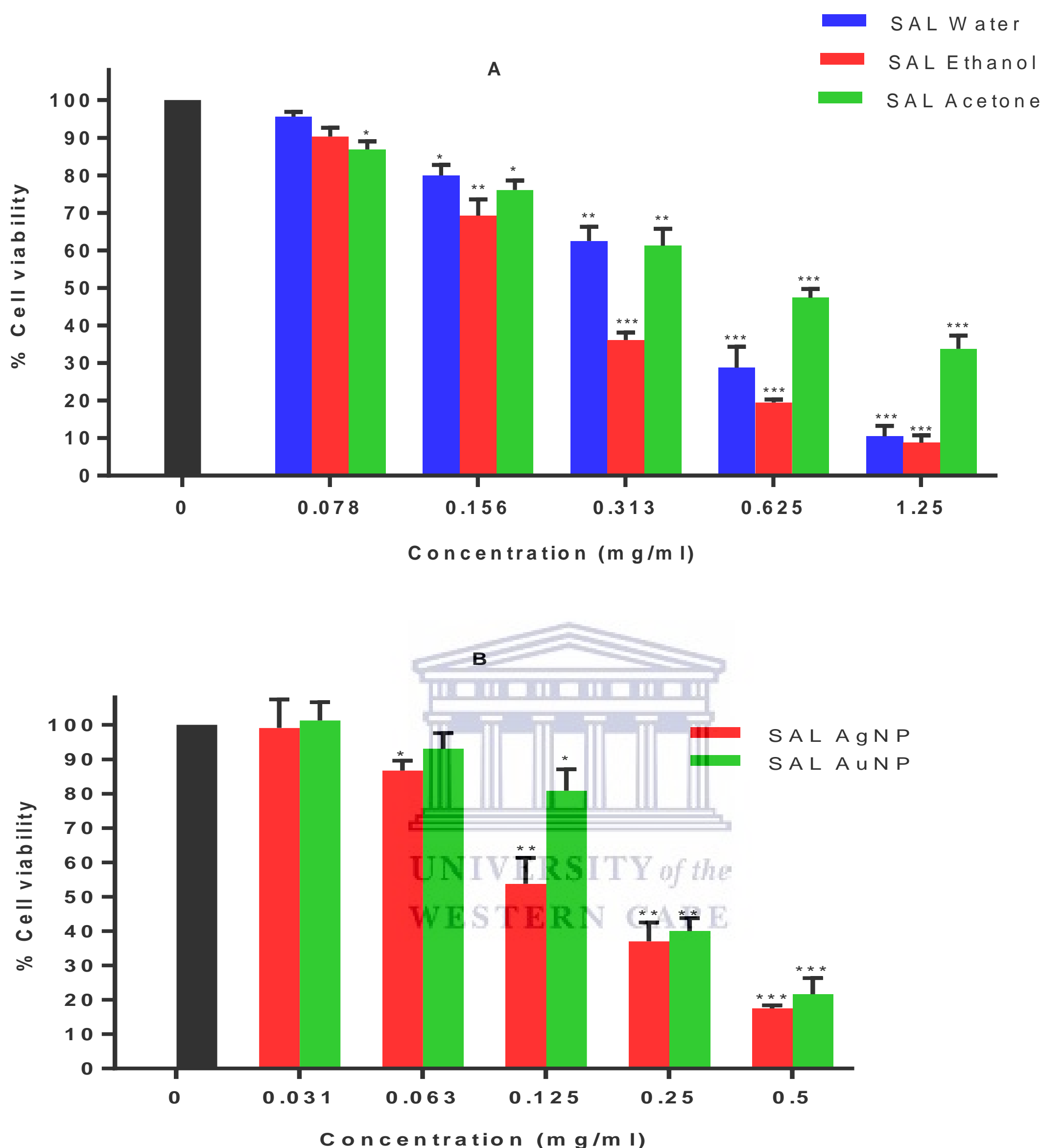

Figure 5.7: Cell viability of NK-92 cells treated with (A) water, ethanol, and acetone extracts of *S. africana-lutea* and (B) SAL AgNP and SAL AuNP compared with untreated cells.

Abbreviations: mg/ml: milligram per millilitre; SAL: *Salvia africana-lutea*; SAL AgNP: *Salvia africana-lutea* silver nanoparticle: SAL AuNP: *Salvia africana-lutea* gold nanoparticle; *P<0.05; **P<0.01; ***P<0.001.

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Table 5.3: Treatment concentrations selected for treating NK-92 for cytokine profiling according to % cell viability.

Treatment	Concentration (mg/ml)	Average % Cell viability
SF water extract	0.078	91
SF ethanol extract	0.078	86
SF acetone extract	0.078	86
SAL water extract	0.156	80
SAL ethanol extract	0.078	90
SAL acetone extract	0.078	87
SAL AgNP	0.063	81
SAL AuNP	0.125	87
SF AgNP	0.313	86

***Abbreviations*:** AgNP: silver nanoparticle; AuNP: gold nanoparticle; mg/ml: milligram per millilitre; SAL: *Salvia africana-lutea*; SF: *Sutherlandia frutescens*.

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5.4.5. Determining the effects of extracts and nanoparticles on cytokine secretion in NK-92 cells

The secretion of the pro-inflammatory cytokine IFN-γ was significantly increased by the treatment of NK-92 cells with *S. frutescens* water extract as well as SAL AuNPs. The acetone and ethanol *S. frutescens* extracts as well as the SF AgNPs significantly ($P<0.05$) decreased the levels of produced IFN-γ by the NK-92 as seen in Figure 5.8A. Ethanol and acetone extracts of *S. africana-lutea* induced significant ($P<0.05$) changes in the levels of IFN γ when compared to untreated NK-92 cells. Though the secretion of IL-10 by NK-92 cell line was increased as seen in Fig. 5.8B following treatment, SAL AgNPs and SF AgNPs were the only treatments shown to significantly increase the extracellular levels of IL-10.

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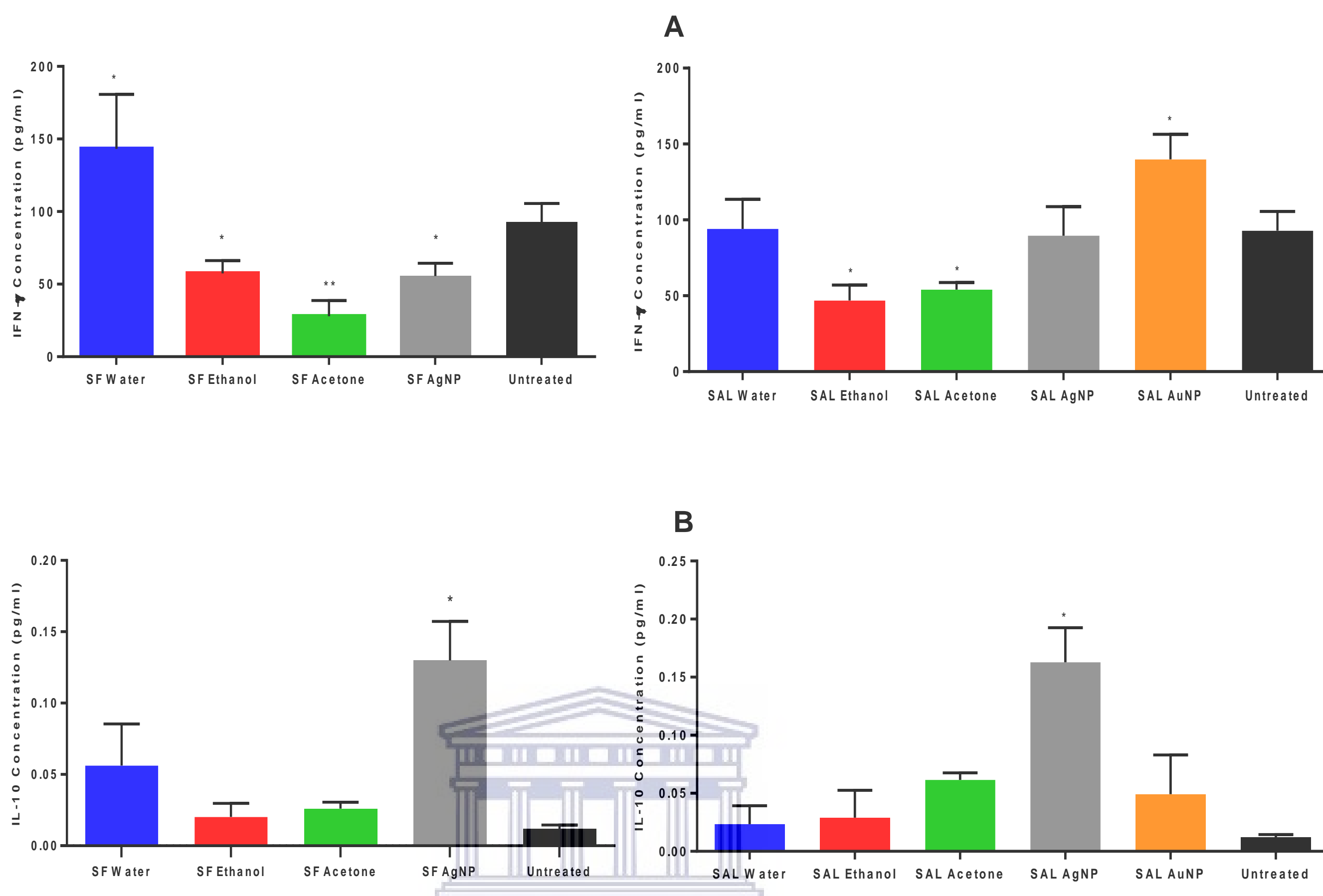

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Figure 5.8: The effects of *S. frutescens* and *S. africana-lutea* extracts and respective nanoparticles on (A) IFN-γ and (B) IL-10 and secretion in NK-92 cells.

***Abbreviations*:** AgNP: silver nanoparticle; AuNP: gold nanoparticle; IFN-γ: interferon gamma; IL-10: interleukin 10; pg/ml: picogram per milliliter; SAL: *Salvia africana-lutea*; SF: *Sutherlandia frutescens*; **P*<0.05: ***P*<0.01.

5.4.6. Co-culture assay

The co-culture assay was performed to analyse whether *S. africana-lutea* and *S. frutescens* extracts and their respective nanoparticles activate the effector (NK-92) cells to produce and release compounds that have cytotoxic effects towards the target cells (HaCaT, PC-3, and Hela). Prior to the commencement of the co-culture assay, two cell viability assessments were performed on these three cell lines. These were the viability of the target cells in the media that supports the optimum growth of the effector NK-92 cell line (MEM-α), and their viability after treatment with *S. africana-lutea* and *S. frutescens* extracts and their nanoparticles at concentrations previously shown to be non-

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toxic to the effector cell line NK-92. After culturing the target cells in MEM-α, the cells were shown to be viable in the MEM-α media with cell viabilities above 80%. These cell viabilities were not significantly different from the viability of the same cell lines cultured in their recommended culture media as shown in Figure 5.9.

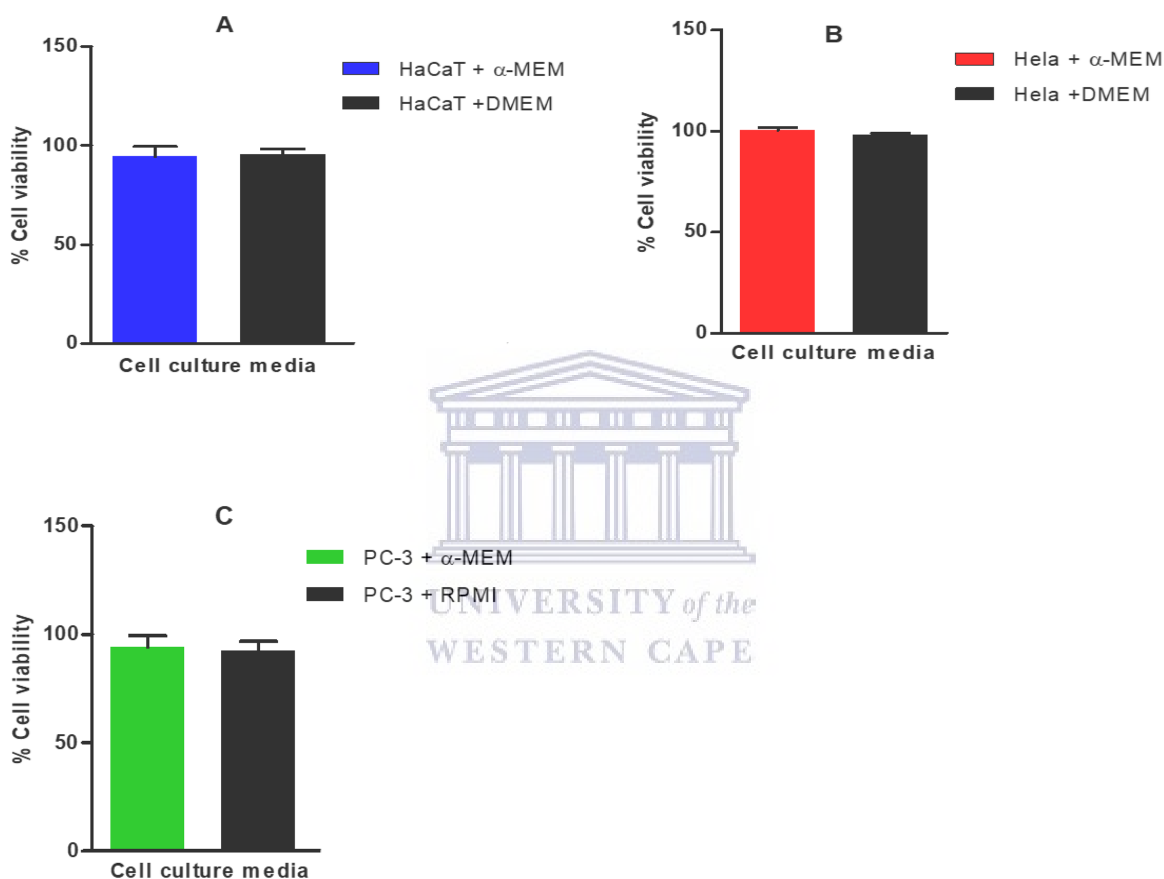

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Figure 5.9: Comparison of the Cell viability of target cells (HaCaT, Hela, and PC-3) in MEM-α to their viability in their recommended growth media.

The WST-1 assay was performed on **(A)** HaCaT, **(B)** Hela and **(C)** PC-3 cells to test their viability after incubating the cells in MEM-α for 24 hours in comparison to culturing the cells in their supporting media (HaCaT and Hela in DMEM and PC-3 in RPMI).

Furthermore, the selected extract and nanoparticle treatments did not exhibit any inhibitory activity towards the target cells as illustrated in Figure 5.10. These observations allowed for the commencement of the co-culture assay.

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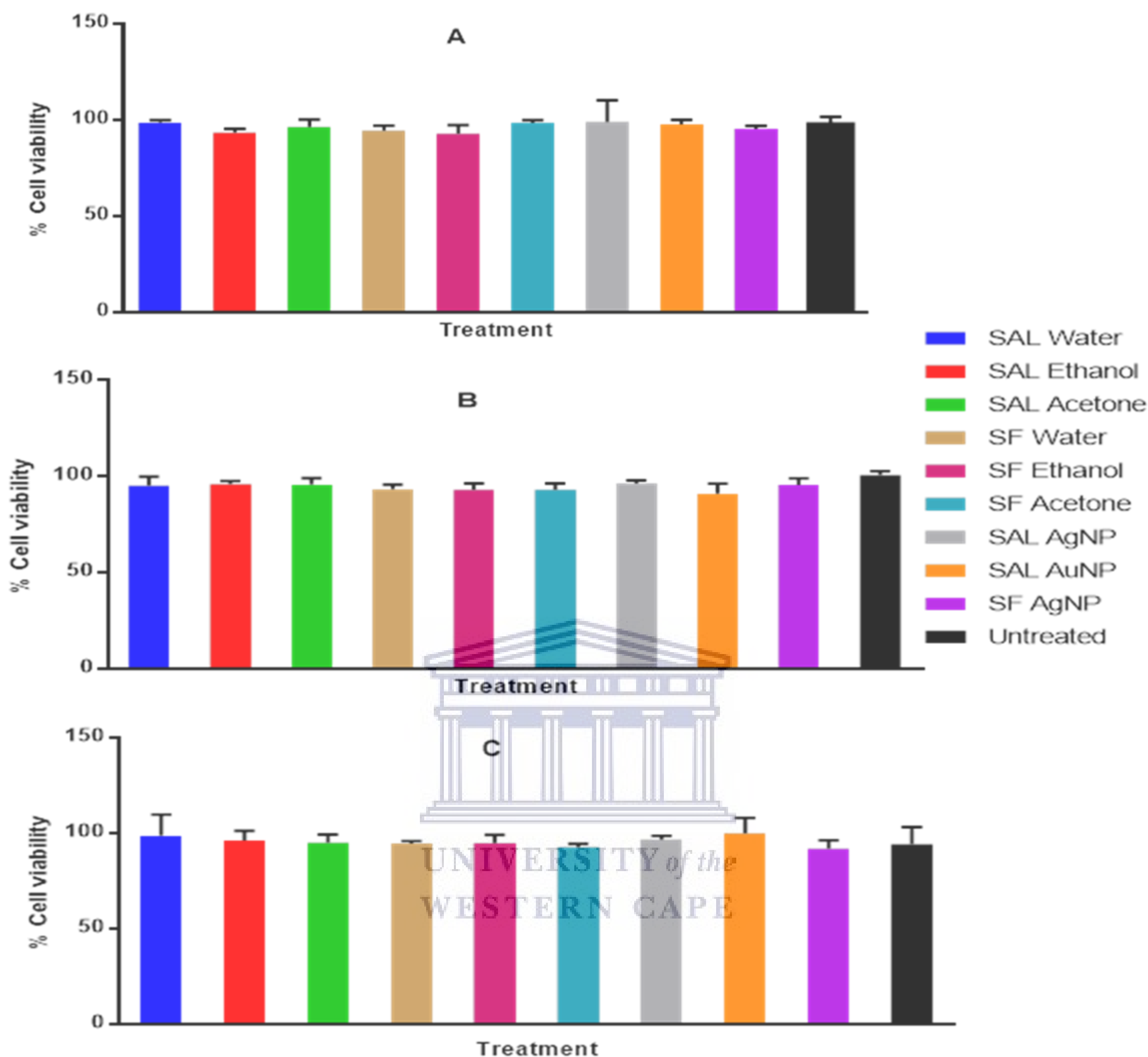

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Figure 5.10: Cell viability of target cells treated with water, ethanol, and acetone *S. africana-lutea* and *S. frutescens* extracts and respective nanoparticles.

WST-1 assay was performed on **(A)** HaCaT, **(B)** Hela and **(C)** PC-3 cells to test their viability after treatment at concentrations previously shown as non-toxic towards NK-92 cell lines in MEM-α.

***Abbreviations*:** AgNP: silver nanoparticle; AuNP: gold nanoparticle; SAL: *S. africana-lutea*; SF: *S. frutescens.*

When HaCaT cells were co-cultured with treated NK-92 cells, the HaCaT cells remained viable. Additionally, their growth was not significantly decreased when compared to that of the treated HaCaT cells cultured in MEM-α as seen in Figure 5.11A. The Hela (Figure

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5.11B) and PC-3 (Figure 5.11C) cells displayed similar results as HaCaT after their co-culture with NK-92 cells treated with water, ethanol and acetone extracts of *S. frutescens* and *S. africana-lutea*. It was observed that co-culturing PC-3 and Hela cells with NK-92 cells treated with SAL AgNPs, SAL AuNPs and SF AgNPs significantly decreased the viability of PC-3 and Hela cells.

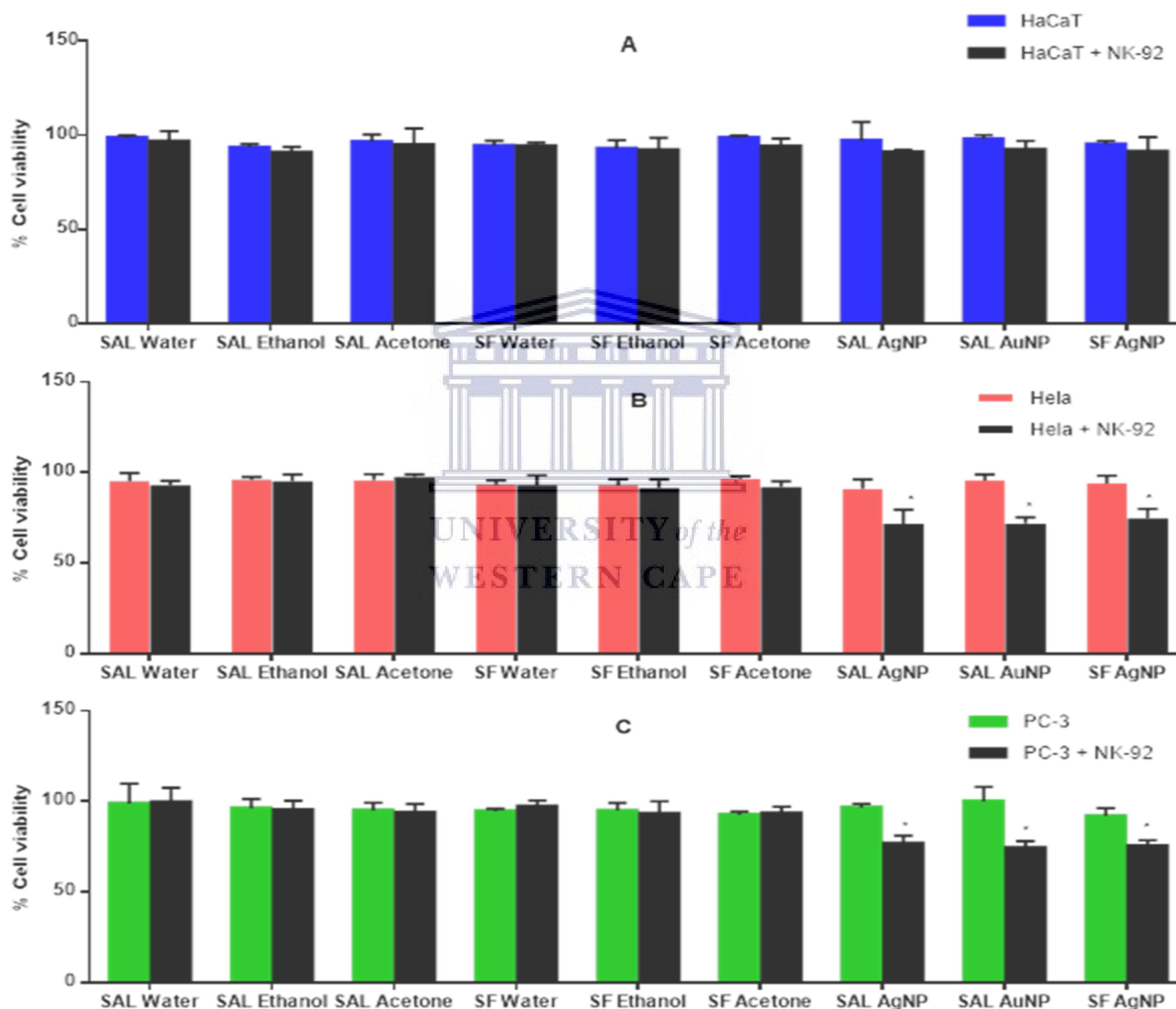

Figure 5.11: Cell viability of (A) HaCaT, (B) Hela and (C) PC-3 cells co-cultured with treated NK-92 cells.

The WST-1 assay was performed on treated target cells (HaCaT, Hela and PC-3) and the target cells following co-culture with treated effector cells (NK-92). The cell viability of the treated target cells was compared to that of the target cells co-cultured with the treated effector cell.

Abbreviations**:** AgNP: silver nanoparticle; AuNP: gold nanoparticle; SAL: *S. africana-lutea*; SF: *S. frutescens.*

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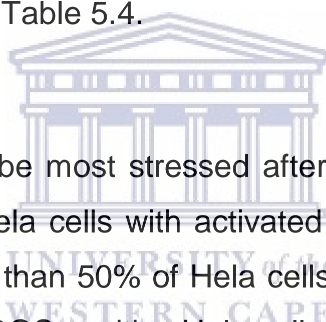
5.4.7. Evaluating the production of Intracellular Reactive Oxygen Species

The levels of intracellular reactive oxygen species (ROS) was measured using the CM-H_2DCFDA dye. The cell viability of two of the target cells (Hela and PC-3) significantly decreased after co-culture with activated NK-92 cells (activated by SAL AuNPs, SAL AgNPs and SF AgNPs). The production of ROS is an indication of oxidative stress. ROS production was therefore assessed in Hela and PC-3 cells that were co-cultured with activated NK-92 cells. Figure 5.12 displays an example of flow cytometry data in the form of a histogram comparing the untreated (ROS-) and hydrogen peroxide treated (ROS+) PC-3 and Hela cells. The ROS intensities are proportional to the height of the peaks observed. The comparison between the percentage of Hela or PC-3 cells positive for ROS following co-culture and the population of untreated Hela or PC-3 (negative control) cells producing ROS was reported in Table 5.4.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The Hela cells were shown to be most stressed after co-culture with activated NK-92 cells. Following co-culture of Hela cells with activated NK-92 cells (using SAL AgNPs, SAL AuNPs, SF AgNPs), more than 50% of Hela cells had increased ROS production, with the highest percentage of ROS positive Hela cells (63.58%) reported when the NK-92 cells were activated by SAL AuNPs. Though the percentage of PC-3 cells with increased ROS production was also increased following co-culture, the population of ROS positive PC-3 cells was lower than that of ROS positive Hela cells. The percentage of PC-3 cells with increased ROS production following co-culture with SF AgNPs activated NK-92 cells was not significantly ($P > 0.05$) different from the percentage of ROS positive untreated PC-3 cells.

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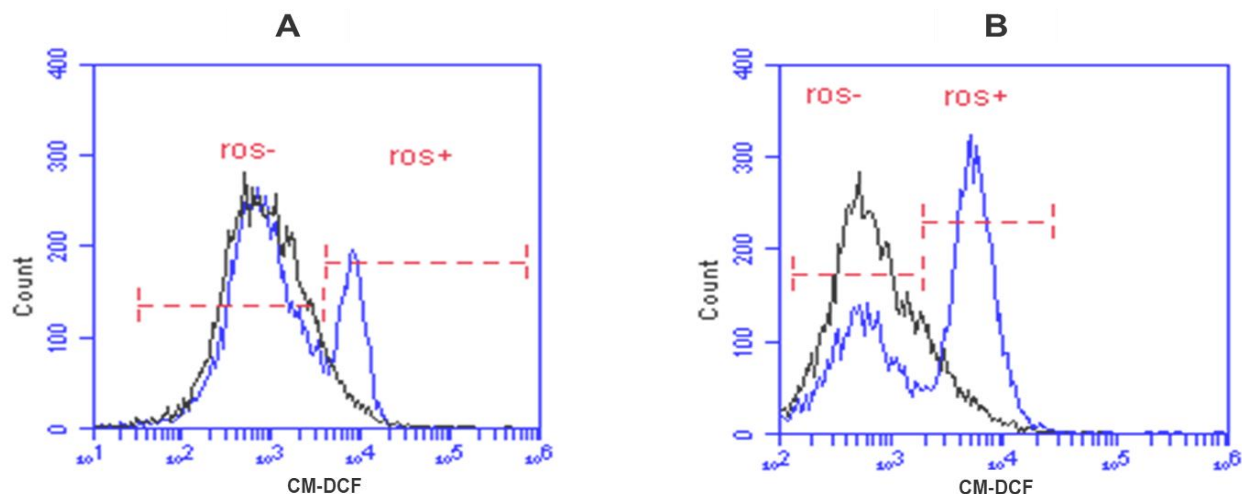

Figure 5.12: Histograms that represent (A) PC-3 and (B) Hela cells positive (hydrogen peroxide treated) and negative (untreated) for ROS.

The marked regions represent the cell populations' negative (ros-) and positive (ros+) for ROS. Black represents the negative control and blue represents the positive control.

Abbreviations**:** CM-DCF: 5-chloromethyl-2',7'-dichlorofluorescein; ROS: reactive oxygen species.

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Table 5.4: The comparison of percentage PC-3 and Hela cells with increased ROS production following co-culture with activated NK-92 cells.

Treatment	% ROS positive	
	PC-3	Hela
SAL AgNP	26,95±0,5*	52.23±0,6**
SAL AuNP	20,75±1,7*	63,58±0,8**
SF AgNP	13,65±2	56,25±1,4**
H_2O_2 treated	37.02±1.5**	79.57±2***
Untreated	10.93±0.2	21.38±0.9

Abbreviations**:** AgNP: silver nanoparticle; AuNP: gold nanoparticle; H_2O_2: hygrogen peroxide ROS: reactive oxygen species; SAL: *Salvia africana-lutea*; SF: *Sutherlandia frutescens*; **P*<0.05; ***P*<0.01; ****P*<0.001.

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5.5. Discussion

5.5.1. The differentiation of monocytic cell line THP-1 into mature macrophages

Monocytes obtained from the blood give rise to differentiated macrophages. The resulting macrophages develop specialized functions and become long-lived cells. PMA is a stimulus used to induce the *in vitro* differentiation of monocytic cell line THP-1 into macrophages (Lund *et al*., 2016). The rest phase in which the cells are cultured in media without PMA was reported to increase mitochondrial and lysosomal numbers, increase the cytoplasm to nuclear ratio and alter cell surface markers to patterns similar to those expressed on the surface of monocyte-derived macrophages (Daigneault *et al*., 2010). The use of PMA to differentiate monocytic THP-1 was shown to produce macrophages displaying phenotypic traits including pseudopodia similar to those of human macrophages (Figure 5.2).

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5.5.2. Effects of *S. africana-lutea* and *S. frutescens* extracts and their respective nanoparticles on cell viability

To study the immunomodulatory effects of the *S. africana-lutea* and *S. frutescens* extracts and their respective nanoparticles, it was important to evaluate the toxicity of the extracts and nanoparticles to THP-1 macrophages and NK-92 cells. The *in vitro* cytotoxicity effects of the treatments was assessed using the WST-1 (2-(-4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay. Though MTT (3-(4-5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is the most common colourimetric assay in the determination of cellular metabolism, it is time-consuming taking up to 47 hours to complete. WST-1 on the other hand, is less time consuming, taking up to 4 hours to complete (Yin *et al*., 2013). The WST-1 is a sulfonated tetrazolium salt able to be reduced to a water-soluble formazan by mitochondrial dehydrogenase (Tan and Berridge, 2000). Therefore, WST-1 was employed in our study to assess the number of healthy cells post-treatment.

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In the current study, a dose-dependent growth inhibition of THP-1 macrophages and NK-92 cells was observed when the cells were treated with the extracts and nanoparticles. The water extracts were the least toxic of all the extracts assessed. This was shown by the higher water extract concentrations required to reduce the cell viability of THP-1 (*S. frutescens* - 5 mg/ml; *S. africana-lutea* - 1.25 mg/ml) and NK-92 (*S. frutescens* - 0.625 mg/ml; *S. africana-lutea* - 0.625 mg/ml) to less than 50%. Extracts of these plants are used in traditional medicine and these low toxicities could mean that the extracts are relatively safe to consume. Of all the nanoparticles assessed for cytotoxic effects towards the THP-1 and NK-92 cells, SAL AgNPs and SAL AuNPs were reported as least toxic. This was decided upon by the nanoparticles concentrations required to reduce the viability of THP-1 (SAL AuNP – 0.5 mg/ml; SAL AgNP – 0.5 mg/ml; SF AgNP – 0.125 mg/ml) and NK-92 (SAL AuNP – 0.25 mg/ml; SAL AgNP – 0.25 mg/ml; SF AgNP – 0.125 mg/ml) cells to lower than 50%. GeneraThe higher the treatment concentration required to reduce the population of viable cells, the lower the cytotoxicity effects of the treatment.

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5.5.3. The immunomodulatory effect of *S. africana-lutea* and *S. frutescens* extracts and respective nanoparticles on THP-1 cells

The effects of the extracts and respective nanoparticles on THP-1 macrophages production of IL-6, TNF-α and IL-1β was also assessed. After differentiation of THP-1 monocytes using PMA, the cells were activated using LPS. The activation of the macrophages by invading microorganisms is an important first line cellular defence against infection. This LPS activation was done to mimic the activation of macrophages in the progression of an infection (Palsson-McDermott *et al*., 2015). LPS expressed on the surface of microorganisms can activate the secretion of pro-inflammatory cytokines, which includes IL-6, TNF-α, IFN-γ and IL-1β (Baek *et al*., 2018). LPS was observed to increase the production of the pro-inflammatory cytokines IL-6, TNF-α and IL-1β in THP-1 macrophages (Figure 5.5). However, the extracts and nanoparticles were seen to inhibit the production of these cytokines by THP-1 macrophages with a few exceptions. This observation could not be linked to cellular death as the treatment concentrations chosen

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did not significantly affect the viability of THP-1 macrophages (Table 5.2). The greatest inhibition of IL-6 (Figure 5.5A) was caused by the ethanol extract of *S. frutescens* and water extract of *S. africana-lutea*, whilst the ethanol and acetone extracts of *S. frutescens* and ethanol extract of *S. africana-lutea* greatly inhibited the production of TNF-α (Figure 5.5.B). The ethanol extract of *S. frutescens*, and the ethanol and acetone extracts of *S. africana-lutea* exhibited the highest inhibition of IL-1β (Figure 5.5.C). These contrasting inhibitory activities could have been due to the differences in the phytochemical profile of each extract. This could result from the differences in the properties of each plant as well as the extracting solvents (Dube *et al.*, 2017).

The activity of the nanoparticles was noted as different to that exhibited by the water extract, though the nanoparticles are synthesised from the water extracts. This observation was in agreement with numerous studies that reported on the differences between the bioactivities of synthesised nanoparticles and the crude extracts the nanoparticles were synthesised from (Ahmed *et al.*, 2015, Huang and Chang, 2009). The activity of the nanoparticles was enhanced as expected when compared to the activity of the synthesising extracts. The SF AgNPs was observed to inhibit the production of IL-6 more than the water extract of *S. frutescens* suggesting the significant anti-inflammatory activity of the nanoparticle. Though SAL AgNPs and SAL AuNPs decreased the production of TNF-α and IL-6 by THP-1 macrophages compare to the LPS treated THP-1 macrophages, these nanoparticles increased the production of the pro-inflammatory cytokine IL-1β. These observations suggest that SAL AgNPs and SAL AuNPs may exert both anti-inflammatory and pro-inflammatory effects towards macrophages. However, confirmatory assays such as gene expression are needed to confirm the observation.

5.5.4. The immunomodulation effect of *S. africana-lutea* and *S. frutescens* extracts and respective nanoparticles on NK-92 cells

IL-10 has been reported to suppress the production and secretion of IFN-γ (Sheridan *et al.*, 2017). This is accomplished by the blocking activity of IL-10 towards the IFN-γ inducer

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IL-12 (D'Andrea *et al.*, 1993). This current study concurred with this observation, displayed by the increased levels of IL-10 (Figure 5.8B) coupled with decreased levels of IFN-γ (Figure 5.8A) secreted by NK-92 cells post treatment with a few exceptions when compared to the untreated NK-92 cells. The generally increased production of the anti-inflammatory IL-10 and decreased pro-inflammatory IFN-γ suggested the anti-inflammatory activity of the treatments. The water extract of *S. frutescens* and SAL AuNPs are the only two treatments that increased the production of both the pro-inflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-10 by NK-92 cells suggesting their anti- and pro-inflammatory effect towards NK cells. Further investigation including gene expression is required to elucidate these observations.

5.5.5. The effects of extract and nanoparticle treated NK-92 cells towards target cells in co-culture

The co-culture of nanoparticle treated NK-92 cells with the target cells PC-3 and Hela resulted in decreased cell viability of the target cells. The decreased cell viability could not be due to the direct inhibitory effects of the treatments as the cells were shown viable in the treatment concentrations selected (Figure 5.10). The general anti-inflammatory effects of these nanoparticles suggest that they inhibit the production and secretion of pro-inflammatory cytokines by NK-92 cells. Hence, the decrease in viability of the target cells may be a result of the nanoparticles activating the natural killer cells to produce and release other cytotoxic compounds which may include granzymes and perforin that have inhibitory effects towards these two target cells (Reefman *et al.*, 2010).

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5.5.6. The effects of activated NK-92 cells towards ROS production of target cells in co-culture

Though increased levels of reactive oxygen species (ROS) have been detected in almost all cancer types including prostate and cervical cancers, a balance between antioxidants and ROS is required for tumour progression. Uncontrolled elevation of intracellular ROS

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is important in arresting the proliferation of cancerous cells. Several anti-carcinogenic agents inhibit tumour progression by promoting excessive oxidative DNA damage of cancerous cells (Liou and Storz, 2010, Prasad *et al*., 2017, Tong *et al*., 2015). Therefore, prolonged cellular distress caused by excessive ROS production may potentially result in the activation of pathways which lead to cell death such as apoptosis. The co-culture of treated effector cells NK-92 (treated with SAL AgNPs, SAL AuNPs and SF AgNPs) and target cells (PC-3 and Hela) promoted intracellular ROS production in the target cells, as reported in Table 5.4. Henceforth the nanoparticles could possibly promote the production of cytotoxic compounds by NK cells, which may lead to the eventual inhibition of cancerous cells.

5.6. Conclusion

This study displayed that the water, ethanol and acetone extracts of *S. africana-lutea* and *S. frutescens* and their respective nanoparticles may be beneficial anti-inflammatory agents. The extracts and nanoparticles could be ideal therapeutic approaches in the treatment of inflammatory conditions such as rheumatoid arthritis, dermatitis and psoriasis. The synthesised nanoparticles further possessed great potential in the treatment of cancer as evident by the increased ROS production of the cancerous cell lines following their co-culture with treated NK-92. Further assays are recommended for the identification of the cytotoxic compounds released by the natural killer cells upon treatment with the synthesised nanoparticles resulting in the subsequent inhibition of cancerous cells in the co-culture set-up.

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CHAPTER SIX

6.1. General Discussion

The main aim of this research study was to investigate the antimicrobial and immune modulatory effects of two South African medicinal plants (*Salvia africana-lutea* (*S. africana-lutea*) and *Sutherlandia frutescens* (*S. frutescens*)) and their respective nanoparticles. The scientific interest in African Traditional Medicines (ATM) has gained steady momentum in the past decades. This is mainly due to their socio-economic and socio-cultural status in the African community. Of the ATMs used, medicinal plants are the most popular. Their use in the treatment of a variety of ailments is well documented (Taye *et al*., 2011, Nagori and Solanki, 2011, Delfan *et al*., 2014, De Wet *et al*., 2013, Koduru *et al*., 2007, Ashidi *et al*., 2010, Engel *et al*., 2011). The two selected medicinal plants have shown great medicinal potential as evidenced by their long history of traditional use in the treatment of different conditions including infections and cancer (Katerere and Eloff, 2005, Tai, 2004, Chinkwo, 2005, Steenkamp *et al*., 2006, Lin *et al*., 2016, Skerman *et al*., 2011, Aboyade *et al*., 2014, Stander *et al*., 2009, van der Walt, 2016, Kamatou *et al*., 2010, Kamatou *et al*., 2007). Synthesis of biogenic nanoparticles using medicinal plants has also shown great potential. The perception that biogenic nanoparticles are safe, environmentally benign and demonstrate enhanced activity when compared to the synthesising crude extracts has increased scientific interest (Elbagory *et al*., 2017, Ahmed *et al*., 2015). The improved bioactivity of biogenic nanoparticles could be due to the increased surface area of synthesised nanoparticles and stability of synthesising phytochemicals within the nanoparticles. Hence the investigation of both the crude extracts and respective biogenic nanoparticles of *S. africana-lutea* and *S. frutescens* in the study.

The ability to synthesise nanoparticles using water extracts of *S. africana-lutea* and *S. frutescens* was analysed. Different parameters were optimised in the synthesis of the biogenic nanoparticles including reaction time, temperature, plant extract and $AgNO_3$ or

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$NaAuCl_4·2H_2O$ concentration. The first indication of nanoparticle synthesis was the characteristic colour change. The UV-vis spectra confirmed the synthesis of nanoparticles by displaying peaks characteristic of silver (Ag) and gold (Au) nanoparticles. The synthesised Ag nanoparticle solution exhibited the characteristic brown colour whilst the Au nanoparticle solution appeared red-violet. *S. africana-lutea* was able to reduce $AgNO_3$ and $NaAuCl_4·2H_2O$ to successfully produce Ag and Au nanoparticles at a concentration of 3.125 mg/ml of plant extract using 3 mM $AgNO_3$ and 1 mM $NaAuCl_4·2H_2O$ respectively at 70 ^oC for 24 hours. The synthesised SAL AgNPs and SAL AuNPs had an average diameter of 8.71 and 17.84 nm respectively. The *S. frutescens*, on the other hand, reduced 3 mM $AgNO_3$ at an extract concentration of 6.25 mg/ml at 70 ^oC for 24 hours to produce Ag nanoparticles (SF AgNPs) with an average diameter of 21 nm. The absence of a homogenous colour change after *S. frutescens* reacted with $NaAuCl_4·2H_2O$ suggested a failure of *S. frutescens* Au nanoparticle (SF AuNPs) synthesis (Figure 3.1). The SPR for Ag nanoparticles produced λ-max absorbance peaks between 400 and 500 nm whilst the λ-max for Au nanoparticles were between 500 and 600 nm. The maximum absorbance of SAL AgNPs and SAL AuNPs were around 472 and 532 nm respectively, whereas for SF AgNPs was around 432 nm. These λ-max absorbance peaks therefore correspond to the expected λ-max values for Au and Ag nanoparticles (Kasthuri *et al.*, 2009). The absence of the characteristic λ-max absorbance peak after *S. frutescens* was reacted with $NaAuCl_4·2H_2O$ confirmed the failure of SF AuNP synthesis (Philip *et al.*, 2011). Characterisation of SAL AgNPs, SAL AuNPs and SF AgNPs using DLS, HR-TEM and FT-IR showed that the synthesised nanoparticles displayed similar characteristics to previously reported Ag and Au nanoparticles synthesised using plant extracts (Kamatou *et al.*, 2010, Philip *et al.*, 2011, Philip and Unni, 2011).

The antimicrobial effects of the synthesised Ag nanoparticles (SAL AgNPs and SF AgNPs), as well as that of the water, ethanol and acetone extracts of *S. africana-lutea* and *S. frutescens*, were evaluated using the agar well diffusion assay, minimum inhibitory concentration assay and minimum bactericidal or fungicidal concentration assay. The inhibitory activity was tested against prevalent wound infecting pathogens (*S. pyogenes*,

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S. epidermidis, *S. aureus, P. aeruginosa,* MRSA*, C. albicans*). Of all the microorganisms assessed, *P. aeruginosa* and the fungus *C. albicans* were observed to be the most resistant strains. *P. aeruginosa* possesses an outer membrane that decreases the permeability of the cell wall to numerous agents whilst *C. albicans* is able to exist in three forms (yeast, pseudohyphae and hyphae) that possess different characteristics and display varying levels of resistance to treatments (Winstanley *et al*., 2016, Mayer *et al*., 2013). Though considered not noteworthy antimicrobial agents due to their high MIC values (>1 mg/ml), the water extracts of both plants displayed some level of inhibitory activity, validating to some extent the claimed antimicrobial activity of the plants. The antimicrobial activities of the water, ethanol and acetone extracts of both plants were comparable. *S. africana-lutea* generally displayed higher levels of inhibition when compared to *S. frutescens* (Tables 4.1 and 4.3). The ethanol and acetone extract of *S. africana-lutea* and *S. frutescens* exhibited greater inhibitory activity compared to the water extracts against all selected bacterial and fungal strains, with acetone extracts of both plants generally displaying higher activity than the ethanol extracts. This could be due to acetone's ability to extract both polar and nonpolar phytochemicals with possible antimicrobial activity exacerbating the extent of inhibition displayed. Previously, Tian and colleagues associated *Galla chinensis* extracts of weaker polarity with elevated antimicrobial activity (Tian *et al*., 2009). This is in agreement with the findings of this study since water is the most polar of the solvents assessed exhibiting weaker activity, and acetone the least polar of the extracting solvents displaying the greatest inhibition.

The zones of inhibition recorded for the SAL AgNPs, SF AgNPs and acetone extracts were similar ($P > 0.05$) with a few exceptions (Table 4.1 and Table 4.3). This observation does not mean the activity of acetone extracts is similar to that of the Ag nanoparticles since the concentration of the Ag nanoparticles (1.5 mg/ml) is significantly lower than that of the acetone extracts (50 mg/ml). This shows that the activity of the Ag nanoparticles is greater in comparison to that displayed by the crude plant extracts from which the Ag nanoparticles were synthesised. This observation was confirmed by the low MIC values

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recorded for the Ag nanoparticles against the selected bacterial and fungal strains (Table 4.2 and 4.4).

To confirm whether the water, ethanol, and acetone extracts of *S. africana-lutea* and *S. frutescens* and their respective nanoparticles (SAL AgNPs, SAL AuNPs, SF AgNPs) displayed immune modulatory activity, their effects on macrophage and natural killer cells were assessed. The cell lines utilised for the immunomodulatory assays were the monocyte cell line THP-1 which mimics circulating macrophages after differentiation using PMA and the natural killer (NK) cell line NK-92. Generally, the extracts and nanoparticles displayed an anti-inflammatory activity. LPS found on the surface of invading infectious microorganisms stimulates the activity of macrophages and can activate the secretion of pro-inflammatory cytokines which includes interleukin 6 (IL-6), interferon gamma (IFN-γ), tumour necrotic factor alpha (TNF-α) and interleukin 1 beta (IL-1β) (Baek *et al.*, 2018). IL-6 regulates the immune system by exerting either pro- or anti- inflammatory actions depending on which of its two signalling pathway is activated (Scheller *et al.*, 2011). TNF-α is an important early cytokine that regulates other pro-inflammatory cytokines (Parameswaran and Patial, 2010). IL-1β activates the antigen-presenting cells, which in turn leads to the activation of adaptive immune cells against infections (Loiarro *et al.*, 2010). The treatment of THP-1 macrophages with LPS resulted in increased secretion of IL-6, TNF-α and IL-1β (Figure 5.5). The cytokine profile of LPS activated THP-1 macrophages and the extract or nanoparticle treated THP-1 macrophages (pre-treated with LPS) were compared. When the LPS pre-treated THP-1 macrophages were exposed to the *S. africana-lutea* and *S. frutescens* plant extracts, the levels of IL-6, TNF-α and IL1-β were reduced compared to the levels produced by LPS activated THP-1 macrophages. The ethanol extract of *S. frutescens* and water extract of *S. africana-lutea* showed the greatest inhibition of IL-6, whilst the ethanol and acetone extracts of *S. africana-lutea* and ethanol extract of *S. frutescens* possessed the highest inhibition towards TNF-α production. The ethanol extracts of *S. africana-lutea* and *S. frutescens* displayed greater inhibition of IL-1β. Generally, the ethanol extract of *S. frutescens* was deemed to have more anti-inflammatory activity when compared to water and acetone extracts of *S.*

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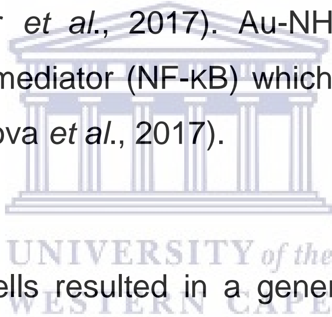
frutescens. The ethanol and acetone extracts of *S. africana-lutea* generally displayed stronger inhibition of cytokine production when compared to the activity of the *S. africana-lutea* water extract. The phytochemical composition of the extracts as determined by the nature of the plant and extracting solvent may explain the differences in activities of the extracts (Dube *et al*., 2017). The synthesised nanoparticles (SAL AuNPs, SAL AgNPs and SF AgNPs) reduced the levels of IL-6, TNF-α and IL-1β with a few exceptions which suggests that these nanoparticles exert an anti-inflammatory response in THP-1 macrophages. However, SAL AuNPs increased the levels of IL-1β when compared to the levels of IL-1β secreted by LPS activated THP-1 macrophages suggesting the pro-inflammatory activity of SAL AuNPs. Previous studies have reported on the pro-inflammatory activity of Au nanoparticles. Sodium polyacrylate stabilized citrate-Au nanoparticles were reported to possess pro-inflammatory activities and induced an *in vivo* influx of neutrophils (Durocher *et al*., 2017). Au-NH_2 nanoparticle induced the up-regulation of the inflammatory mediator (NF-κB) which is important in the activation of inflammatory responses (Tarasova *et al*., 2017).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The treatment of the NK-92 cells resulted in a generally observed anti-inflammatory response. This was shown by the increased production of the anti-inflammatory cytokine IL-10 and decreased secretion of the pro-inflammatory cytokine IFN-γ by the treated NK-92 cells with a few exceptions. Although the process is not well understood, IL-10 has been reported to suppress the production of IFN-γ by blocking the IFN-γ inducer IL-12 (D'Andrea *et al*., 1993, Sheridan *et al*., 2017). Only when IL-10 is combined with IL-18 can it enhance NK cell proliferation, cytotoxicity and IFN-γ production (Cai *et al*., 1999). Accelerated wound healing has been strongly associated with increased IL-10 (Kant *et al*., 2014, Leung *et al*., 2012). The extracts and nanoparticles increased the NK-92 production of IL-10 which suggests their beneficial potential in wound healing. The immune-defensive function of the IFN-γ is to activate the inflammatory response in the presence of abnormal cells recruiting other immune-potent cells including T cells and B cells (Wilke *et al*., 2011). The water extract of *S. frutescens* increased the production of IFN-γ. This pro-inflammatory activity could justify the use of *S. frutescens* in traditional

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medicines for the treatment of various ailments including internal cancers (van Wyk and Prinsloo, 2008). SAL AuNPs also increased the levels of IFN-γ further demonstrating the pro-inflammatory activity of SAL AuNPs. However, molecular studies which include gene expression are recommended to further study the effects of the *S. frutescens* water extract and SAL AuNPs on macrophages and NK cells as these treatments displayed both pro- and anti-inflammatory activity.

Inflammation is an important mechanism of the innate immune system to control infections (Ohkusa *et al*., 2004). During infections, the increased production of pro-inflammatory cytokines triggers the infiltration of infected tissues by innate immune cells However, the overproduction of the pro-inflammatory cytokines in response to infections may cause damage to the inflamed tissues due to oxidative stress or enzyme activity (Sfanos *et al*., 2013). Elevation of the pro-inflammatory cytokines IFN-γ, IL-1β and TNF-α is associated with inflammatory conditions which include Crohn's disease, eczema, dermatitis, psoriasis and rheumatoid arthritis (Akama *et al*., 2009, Haase *et al*., 2012). Lowering the production of the pro-inflammatory cytokines can be an ideal therapeutic approach in the treatment of inflammatory conditions (Feldmann and Maini, 2003). Henceforth, inflammatory disorders can benefit from anti-inflammatory therapies such as the *S. africana-lutea* and *S. frutescens* extracts and their respective nanoparticles.

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The co-culture assay was performed to analyse the effects of treated effector cells (NK-92) (treated using water, ethanol, acetone extracts of *S. africana-lutea* and *S. frutescens* and nanoparticles SAL AgNPs, SAL AuNPs and SF AgNPs) on target cells (HaCaT, PC-3, and Hela). Natural killer cells are known for their ability to kill abnormal cells especially cancerous cells, by producing and secreting pro-inflammatory cytokines including IFN-γ and cytotoxic granules containing perforin and granzymes once activated (Levy *et al*., 2011). Upon co-culturing treated NK-92 cells with the target cells, only the NK-92 cells treated with the synthesised nanoparticles exhibited significant levels of cytotoxicity towards the cancerous cell lines Hela and PC-3. This decrease in viability is not due to the direct cytotoxic effects of the treatments, as these were shown to be non-toxic towards

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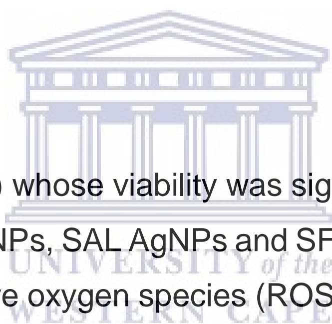
all the target cells (Figure 5.10). Since the release of the cytotoxic IFN-γ was decreased after treatment of NK-92 cells with the nanoparticles except for SAL AuNPs (Figure 5.8), the decrease in cell viability could be a result of the NK-92 cells releasing cytotoxic compounds other than the cytokines assessed. The NK-92 cells that behave in a similar way as the human NK cells, could probably have released cytotoxic granules upon treatment with the nanoparticles resulting in the decreased viability of the PC-3 and Hela cells. However, confirmatory assays to assess whether NK-92 cells activated with SAL AuNPs, SAL AgNPs and SF AgNPs produce perforin and granzymes should be conducted. The absence of inhibition of the noncancerous target cell line HaCaT by the treated NK-92 cells may suggest the general safety of these treatments and cancer specific toxicity upon application. The extract treated NK-92 cells did not exhibit any significant inhibition towards the target cells, further confirming the enhanced activity of the synthesised nanoparticles.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The target cells (PC-3 and Hela) whose viability was significantly decreased after the co-culture experiment with SAL AuNPs, SAL AgNPs and SF AgNPs treated NK-92 cells were assessed for intracellular reactive oxygen species (ROS) production following co-culture. This was performed to evaluate the effects of nanoparticle treated NK-92 cells on ROS production by target cells in co-culture. ROS production in PC-3 and Hela cells was increased following the co-culture experiment. Increased ROS production is associated with elevated levels of cellular stress that may potentially result in cell death (Liou and Storz, 2010). Though ROS are considered essential for the regulation of normal physiological function, excess cellular ROS production causes damage to important cellular components including proteins, nucleic acids and organelles. This can lead to the activation of cell death processes which include apoptosis (Redza-Dutordoir and Averill-Bates, 2016). However, an experiment set-up to assess ROS production of the target cells cultured in MEM-α should be done since MEM-α is not the recommended culture media to support both PC-3 and Hela cells. This would be an ideal approach in assessing any form of cellular stress due to PC-3 and Hela cells being cultured in MEM-α. Since the ROS production of PC-3 and Hela cells co-cultured with untreated NK-92 cells (denoted

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as untreated cells in Table 5.4) were lower than those detected in PC-3 and Hela cells co-cultured with activated NK-92, the increase in ROS production could be due to the activation of the NK-92 effector cells by the nanoparticles. Uncontrolled elevation of intracellular ROS is important in arresting the proliferation of cancerous cells. Several anti-carcinogenic agents inhibit tumour progression by promoting excessive oxidative DNA damage of cancerous cells (Prasad *et al*., 2017, Tong *et al*., 2015). Therefore, the nanoparticles could possibly promote the production of cytotoxic compounds by NK cells, which may lead to the eventual inhibition of cancerous cells. This suggests that the nanoparticles may play a beneficial role in the treatment of cancer.

The general inhibition of microbial growth, IFN-γ, IL-6, TNF-α, IL-1β production and promotion of IL-10 secretion by the SAL AgNPs, SAL AuNPs and SF AgNPs was amplified when compared to that of the synthesising water extracts. This could be due to nanoparticles acting as carriers that deliver the stabilized bioactive phytochemicals to the target sites. Their small size allows them to easily penetrate through the lipid membranes (Chuan *et al*., 2015, Bonifácio *et al*., 2014). Additionally, the beneficial phytochemicals are probably concentrated and stabilized within the synthesised nanoparticles increasing the activity of the nanoparticles. Furthermore, it is possible that during nanoparticle production phytochemicals are altered resulting in them displaying stronger activities. The enhanced biological activities of the biogenic nanoparticles concurred with previously reported studies (Ahmed *et al*., 2016, Benakashani, 2016, Elbagory *et al*., 2017).

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6.2. Conclusion and recommendations

In conclusion, the results obtained in this study suggest that constant intake of the aqueous extracts of *S. frutescens* and *S. africana-lutea* may prove beneficial to the human health, generally serving as natural therapeutics. The study confirms that these extracts have antimicrobial and immunomodulatory effects. However, the ethanol and acetone extracts generally exhibited higher levels of antimicrobial and immune modulating activity suggesting their great potential as health-promoting agents.

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Henceforth the identification and isolation of the bioactive compounds from these extracts is imperative using different experimental approaches which include HPLC and NMR. The enhanced bio-effect of all the synthesised nanoparticles (*S. africana-lutea* silver nanoparticles (SAL AgNPs), *S. africana-lutea* gold nanoparticle (SAL AuNPs) and *S. frutescens* silver nanoparticle (SF AgNPs)) was observed. Considering that this study shows that the plant extracts and respective nanoparticles have antimicrobial and anti-inflammatory properties, these treatments can potentially be used in the development of topical treatments for the autoimmune skin conditions such as psoriasis and eczema as well as microbial infections. To fully understand the activities of *S. africana-lutea* and *S. frutescens* extracts and respective nanoparticles on the immune system, it is recommended to evaluate their immunomodulatory effects on immune cells isolated from blood and in animal models. Further studies on these plants and synthesised nanoparticles are recommended to determine other medicinal properties possessed by these plants. This could eventually provide new leads in the on-going pursuit for novel antimicrobial, anti-inflammatory, and anti-cancer drugs. Additionally, the bioactive comparison of these biogenic nanoparticles and chemically synthesised nanoparticles is recommended, to show the extent of activity possessed by the phytochemicals involved in the nanoparticle synthesis.

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