Silver nanoparticles from biological extracts and their antimicrobial activities

by

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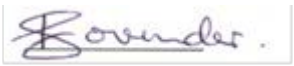
I, Dr Patrick Govender as supervisor of the MSc study hereby consent to the submission of this MSc Thesis.

Signed:  Date: 27/02/2017

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SUMMARY

Antimicrobial agents are an integral part of daily life and in most instances, misused by the consumer. This, combined with the inherent ability of microorganisms to rapidly develop resistance mechanisms, has led to the emergence and spread of multi-drug resistant pathogenic infections. For this reason, there is a great need for novel antimicrobial scaffolds to overcome the resistance posed by these microbes. The advent of nanotechnology provides an attractive avenue for the development of new or improved antimicrobial compounds. In this regard, metal nanoparticles such as silver, produced through nanotechnology have received increased attention as potential candidates for antimicrobial development. Consequently, concerted efforts have been extended to identify novel biological capping structures that could potentially increase the bioactivity of the produced particles. With this in mind, the current study was undertaken to explore the use of medicinal plant and bacterial extracts in silver nanoparticle production using a green synthetic method. With the use of sunlight, a recently reported primary energy source for nanoparticle synthesis, silver nanoparticles were prepared successfully from both extracts with attractive reaction times. Moreover, the derived nanoparticles were consistent in terms of their spherical shape and small size, both of which are advantageous physical attributes that contribute to enhanced bioactivity. Importantly, biomolecules present in the extracts displayed a dual functionality of reducing and stabilising produced nanoparticles. For the plant extracts, phytocompounds such as flavones; terpenoids and polysaccharides were detected in nanoparticle formation whilst in bacterial extracts protein functional groups were observed. Bioactivity screening revealed that silver nanoparticle preparations from both phylum extract type's exhibit promising antibacterial and antifungal activities. These results seemingly suggest that AgNPs produced in this study represent potential in the development of novel antimicrobial agents. Furthermore, the use of sunlight to drive nanoparticle formation is an attractive strategy that may be applied as an alternative energy source for future nanoparticle preparations.

This thesis is dedicated to Shalendra and my grandparents, Richard and Lilly Ramessur

BIOGRAPHICAL SKETCH

Jerushka S. Moodley was born on the 4th of June 1992 and grew up in the south coast of Durban, KwaZulu-Natal. She matriculated in 2009 from Strelitzia Secondary School. Her love for science led her to enroll for a Bachelor's Degree, at the University of KwaZulu-Natal, pursuing majors in Biochemistry and Microbiology. She completed her undergraduate degree in 2013 and decided to follow her interest in Biochemistry by pursuing a Bachelor of Science Honours degree in 2014, which she completed *cum laude*. Jerushka spends her spare time reading novels and nurturing her garden.

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PREFACE

This dissertation is presented as a compilation of five chapters.

Chapter 1	**General Introduction and Project Aims**
Chapter 2	**Literature Review** Biological synthesis of silver nanoparticles from plants and bacteria and their antimicrobial activities.
Chapter 3	**Research Results 1** Bioactivity of silver nanoparticles derived via green technology from *Moringa oleifera* leaf extracts.
Chapter 4	**Research Results 2** Antimicrobial activities of silver nanoparticles derived from bacterial extracts using green synthesis.
Chapter 5	**General Discussion and Conclusion**

CONTENTS

ABBREVIATIONS

°C	Degrees Celsius
Ag	Silver
Ag^+	Silver ions
$AgNO_3$	Silver nitrate
AgNP	Silver nanoparticle
ATCC	American Type Culture Collection
CFS	Cell free supernatant
F	Fresh
FD	Freeze-dried
h	Hours
l	Liter
ml	Milliliter
mm	Millimeter
min	Minutes
ppp	Parts per million
rpm	Revolutions per minute
UV	Ultraviolet
µg	Microgram
µl	Microliter

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Chapter 1

INTRODUCTION AND STUDY AIMS

1.1 INTRODUCTION

The interest in silver nanoparticles (AgNPs) as an alternative to current antibiotics has increased profoundly over the last few years. This is owed to the cumulative incidence of microbial drug-resistant infections and the lack of appropriate treatment thereof (Ventola, 2015). The World Health Organisation report of 2014, highlighted the probability of a post-antibiotic era in which common infections and minor injuries could potentially result in fatalities (World Health Organisation, 2014). Accordingly, concerted efforts have been extended by global pharmaceuticals to formulate new or improved antibiotics. However, despite high research cost-intensive investment in the last decade or so only two new classes of antibiotics have been introduced into the market (Projan, 2003, Walsh, 2003). The imperative need for the uncovering of novel antimicrobial scaffolds has led to the resurgence of silver, however, in its nano-particulate form (Rai, *et al.*, 2009).

The antimicrobial activities of AgNPs are well established and currently researchers are striving to develop greener synthetic strategies for their production (Ahmed, *et al.*, 2016, Kim, *et al.*, 2007). The use of nanotechnology for the synthesis of AgNPs from environmentally compatible bio-materials is evolving into an important branch of science and technology (Ponarulselvam, *et al.*, 2012). To this end, a variety biological extracts have been explored for the bottom-up synthesis of AgNPs (Velusamy, *et al.*, 2016). However, there is an ongoing search to identify novel capping structures for the production of AgNPs with increased bio-efficacies. In this context, this dissertation aims to derive potentially new promising AgNPs from medicinal plant- and bacterial-based extracts in a clean, green and cost-effective manner.

1.2 AIMS OF THE STUDY

The main aims of the MSc study described within this dissertation are as follows:

1. Green synthesis of AgNPs capped with phytocompounds extracted from leaves of the medicinal tree species *Moringa oleifera* and their bioactivity evaluation.
2. Green synthesis of AgNPs from the bacteriocin producer *Pediococcus acidilactici* and non-producer *Bacillus subtilis* and an evaluation of their antimicrobial activities.

This dissertation is divided into five chapters of which, the first is this introductory **Chapter 1**.

In **Chapter 2**, a detailed literature review is presented encompassing the biological synthesis of AgNPs from plants and bacteria and their antimicrobial activities.

In **Chapter 3**, aqueous leaf extract samples of the medicinal tree species *M. oleifera*, were employed in the sunlight driven synthesis of AgNPs. The produced AgNPs were characterised in terms of their yield, morphology and bioactivity. The novelty aspect of this research was the production of *M. oleifera* leaf derived AgNPs using sunlight irradiation as an alternative energy source and characterisation of their bioactivity in terms of their minimal inhibitory concentrations.

The use of sunlight as a promising alternative to drive AgNP formation led to its application in AgNP synthesis from bacterial extracts in **Chapter 4**. Novel AgNPs were produced from the antimicrobial peptide producing bacterium *P. acidilactici* and a non-producing *B. subtilis* strain. In addition, the produced AgNPs were characterised in terms of their morphology and bioactivity.

Lastly, **Chapter 5** comprises of a general discussion and conclusion along with potential ideas for future research.

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Chapter 2

LITERATURE REVIEW

Biological synthesis of silver nanoparticles from plants and bacteria and their antimicrobial activities

Biological synthesis of silver nanoparticles from plants and bacteria and their antimicrobial activities

2 INTRODUCTION

The antimicrobial potential of silver (Ag) and Ag-based solutions has long been established, however, their application was considered obsolete upon the discovery of antibiotics (Alexander, 2009, Kim, *et al.*, 2007). In recent years, the developing crisis of multi-drug resistant pathogenic infections has led to the resurgence in this metal, however, with the use of nanotechnology to generate its nanoparticle form. For this reason, tremendous efforts have been extended in nanotechnology, particularly in the development of green synthetic strategies for silver nanoparticle (AgNP) production to facilitate their use in antimicrobial therapeutic applications.

2.1 CONVENTIONAL NANOPARTICLE SYNTHETIC STRATEGIES

Established technologies for AgNP synthesis and other metal preparations can be categorised distinctly into two approaches, namely: “top to bottom”, which is normally employed by physicists and “bottom to up”, a construction favourite of chemists (Biswas, *et al.*, 2012, Thakkar, *et al.*, 2010). Both approaches converge at the nanodimension, but vary drastically in the synthetic technology. “Top to bottom” approaches apply various physical methods such as grinding, milling, sputtering, evaporation-condensation and thermal/laser ablation to break down bulk solid materials to their nanoparticulate form. “Bottom to up” approaches entail various chemical and biological methods to synthesise nanoparticles by the self-assembly of atoms such as Ag^{+} into nuclei that further develop into nano-sized particles (Ahmed, *et al.*, 2016).

Important physical “top to bottom” methods for nanoparticle preparation include evaporation-condensation and laser ablation techniques (El-Nour, *et al.*, 2010). Evaporation-condensation applies a tube furnace at atmospheric temperature wherein primary material (metal Ag) contained in a boat; is centred in the furnace and vaporised into a carrier gas (Ahmed, *et al.*, 2016). A number of inadequacies have been identified with this technique, for example, the furnace occupies a large space, requires high energy input whilst raising the environmental temperature around the source material and

requires long durations to achieve thermal stability. Additionally, a major drawback to this type of synthesis is the resulting imperfections in the surface structure of the derived nanoparticles which can ultimately alter their physical properties (Ahmed, *et al.*, 2016, Iravani, *et al.*, 2014). In laser ablation, irradiation is used to remove material from a bulk metal in solution. The efficacy of this technique and characteristics of nascent particles is largely dependent on a number of parameters including the wavelength of the laser, duration of laser pulses, laser fluence, ablation duration and the effective liquid medium with or without surfactants (Chen and Yeh, 2002, Kim, *et al.*, 2005). An important advantage of laser ablation for AgNP preparation is the absence of chemicals in solution which could potentially contaminate the nanoparticle preparation (Tsuji, *et al.*, 2002).

Regarding "bottom to up" approaches, wet chemical reduction is the most frequently practiced method for nanoparticle preparation (Iravani, *et al.*, 2014) although, several other methods have been reported (Amin, *et al.*, 2009, Saion, *et al.*, 2013, Yang and Pan, 2012, Yin, *et al.*, 2003). As the name suggests, wet chemical reduction involves the reduction of a metal salt precursor in aqueous or organic solution. Various organic and inorganic compounds successfully utilised as reducing agents in the synthesis of AgNPs include: ascorbate; borohydride; citrate; elemental hydrogen; formaldehyde; N-N-dimethyl formamide (DMF); Tollen's reagent; and polyethylene glycol blocks (Chou and Ren, 2000, Iravani, *et al.*, 2014, Pillai and Kamat, 2004). In addition to reducing agents, protective stabilising agents are also included in the reaction solution to prevent agglomeration of nascent nanoparticles (Bai, *et al.*, 2007, Kapoor, *et al.*, 1994). With stability achieved, this method can be useful for the production of high nanoparticle yields with low preparation costs (Song, *et al.*, 2009). However, the efficacy of this method is challenged by the potential contamination of nascent nanoparticles by precursor chemicals, the use of toxic solvents and the generation of hazardous by-products (Iravani, 2011, Thakkar, *et al.*, 2010).

Evidently, the aforementioned physical and chemical methods have certain limitations that restrict their use in the preparation of nanoparticles for biological applications (Patra and Baek, 2014). In this regard, concerted efforts have been extended to develop nanoparticle synthetic strategies that are environmentally sound. Essentially, this would entail the use of benign, biotechnological tools and has given rise to the concept of green

technology. This technology can best be described as the use of biological routes such as plants and microorganisms or their byproducts in the synthesis of nanoparticles (Dauthal and Mukhopadhyay, 2016, Patra and Baek, 2014, Rajput, *et al.*, 2016). These bio-inspired methods **(Fig. 2.1)** are not only environmentally welcoming but are cost effective and can be easily up-scaled for large productions (Dhuper, *et al.*, 2012).

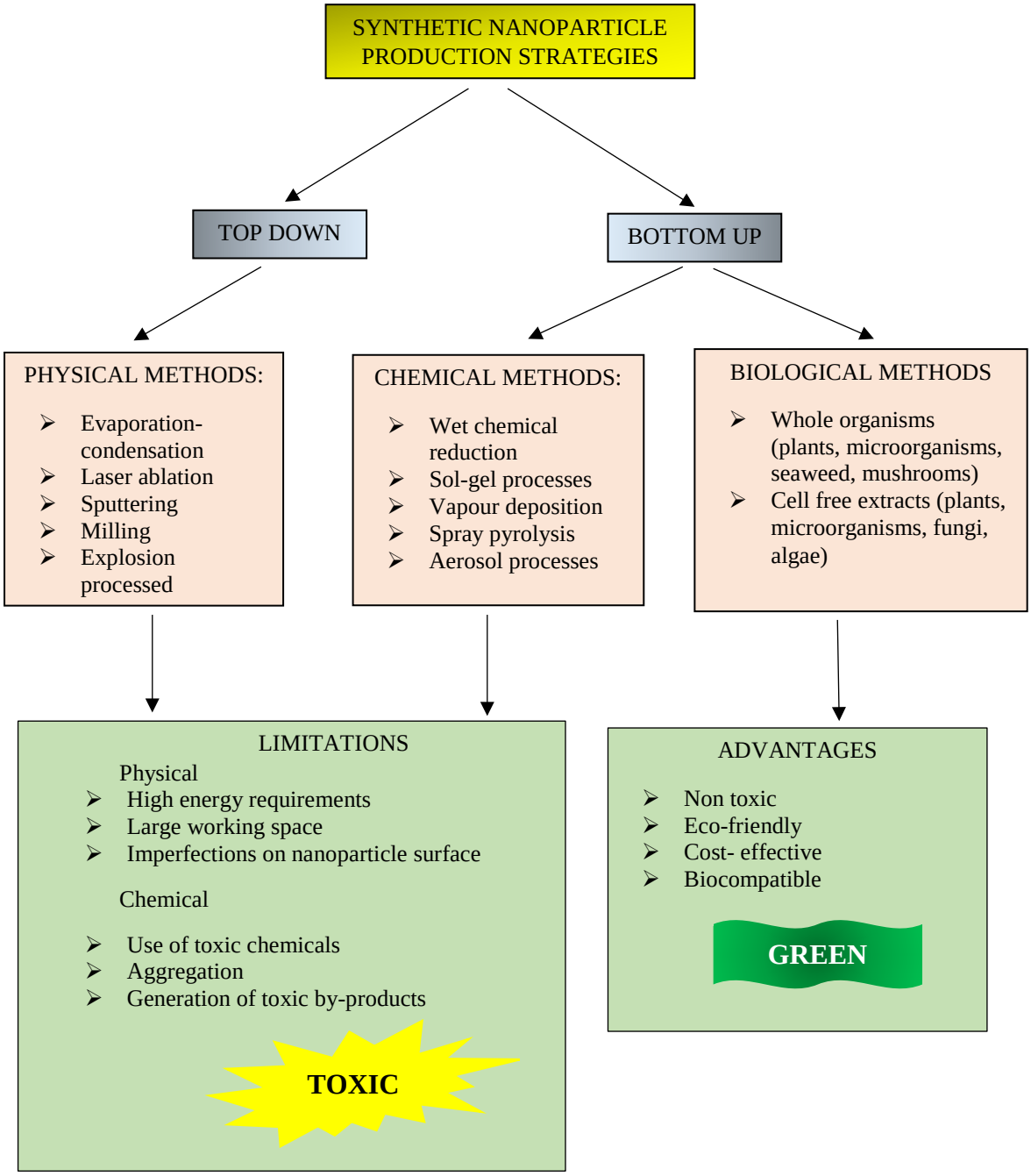

Figure 2.1 Different approaches for AgNP synthesis. Adapted from (Ahmed, *et al.*, 2016, Mittal, *et al.*, 2013).

2.2 BIOLOGICAL AGNP SYNTHETIC STRATEGIES

As previously eluded, biological approaches for AgNP synthesis employ the use of living organisms or their extracts as capping/reducing agents in a synthetic reaction .To date, a variety of biological entities have been explored for their Ag^+ reducing abilities and include viruses, bacteria, plants, algae, fungi, yeast and mammalian cells (Makarov, *et al.*, 2014, Mohanpuria, *et al.*, 2008, Pantidos and Horsfall, 2014, Thakkar, *et al.*, 2010, Velusamy, *et al.*, 2016). Biological synthesis can be divided into two strategies, specifically: bioreduction and biosorption. Bioreduction occurs when metal ions undergo chemical reduction into biologically stable complexes. Many organisms have displayed dissimilatory metal reduction involving the coupling of reduction with oxidation of an enzyme. The resulting stable, inert nanoparticles can then be safely extracted from the reaction mixture. Alternatively, biosorption involves the attachment of metal ions onto an organism itself, such as on the cell wall. Various bacteria, fungi and plants species express peptides or possess modified cell wall structures that are capable of binding metal ions, thereby forming stable complexes in the form of nanoparticles (Pantidos and Horsfall, 2014).

In this review, the use of plant and bacterial biological material for AgNP synthesis will be discussed. For a review on the use of alternative biological entities as AgNP factories, studies by the following authors are recommended (Pantidos and Horsfall, 2014, Singh, *et al.*, 2016, Velusamy, *et al.*, 2016).

2.2.1 AgNP synthesis from plants

Plants have shown the capacity to hyper-accumulate metals as a means to protect themselves from insects and herbivores. This observation has paved way for the technology known as phytoextraction, wherein plants are employed to extract minerals from various groundwater and soil sediments. Major applications of phytoextraction include the mining of precious metals from unfeasible ground sites (phytomining), stabilisation or recovery of non-naturally occurring contaminants (phytoremediation) and the addition of essential metals to growing crops. Interestingly, studies have unveiled that metals accumulated by the plant are usually deposited in the form of nanoparticles. This has stimulated interest for the use of plants as factories for nanoparticle synthesis (Makarov, *et al.*, 2014). Whole plants have been explored for the synthesis of nanoparticles when grown on the appropriate metal enriched substrates. Species such as

Brassica juncae (mustard greens) and *Medicago sativa* (alfalfa) have demonstrated the ability to accumulate AgNPs. For example, 50 nm sized AgNPs, at a high yield (13.6 % of total plant weight) were reported for *M. sativa* when grown on silver nitrate ($AgNO_3$) (Harris and Bali, 2008). Additionally, icosahedral gold nanoparticles of 4 nm size were observed in *M. sativa* and semi-spherical copper nanoparticles of 2 nm size were observed in *Iris pseudacorus* when the plants were grown on gold and copper salt enriched substrates, respectively (Gardea-Torresdey, *et al.*, 2002, Manceau, *et al.*, 2008).

Although whole plants can potentially serve as factories for nanoparticle synthesis, several disadvantages have been identified with this technology especially when up-scaling for industrial applications. For example, physical attributes of nanoparticles such as size and shape vary upon the localisation of the particles in the plant due to the differences in metal ion content in different plant tissues and the possibility of nanoparticle movement and penetration (Gardea-Torresdey, *et al.*, 2002). This heterogeneity of important bioactivity-determinants such as size and shape (Jeong, *et al.*, 2014, Pal, *et al.*, 2007) limit the use of these nanoparticles and especially in applications where mono-dispersed nanoparticle preparations are required. Furthermore, recovery of nanoparticles from living plants entails laborious extraction, isolation and purification procedures and may potentially result in low yields (Makarov, *et al.*, 2014).

The use of plant broths/extracts in nanoparticle synthesis was introduced by Shanker *et al*., (2003). In their study, compounds responsible for the reduction of metal ions were extracted and used in as reducing agents in a synthetic reaction mixture, resulting in the extracellular production of nanoparticles (Shankar, *et al.*, 2003). This strategy tentatively offers several advantages compared to the use of whole plants. For example, nanoparticle formation occurs considerably faster as opposed to whole plants which require diffusion of metal ions throughout the plant body. Additionally, the use of extracts would be more economical due to the ease of purification (Makarov, *et al.*, 2014).

This *in vitro* approach has been actively developed and applied to variety of plant flora for the synthesis of AgNPs (Iravani, 2011). Various organ extracts: stem, root, leaf, bark, fruit and fruit peel have demonstrated the ability to reduce Ag^+. Particularly, biomolecules **(Fig. 2.2)** such as proteins, amino acids, enzymes, polysaccharides, alkaloids, tannins, phenolics, saponins, terpenoids and vitamins present in the extracts act as both reducing agent and stabilising agents (Ahmed, *et al.*, 2016).

2.2.1.1 Plant metabolites involved in nanoparticle synthesis

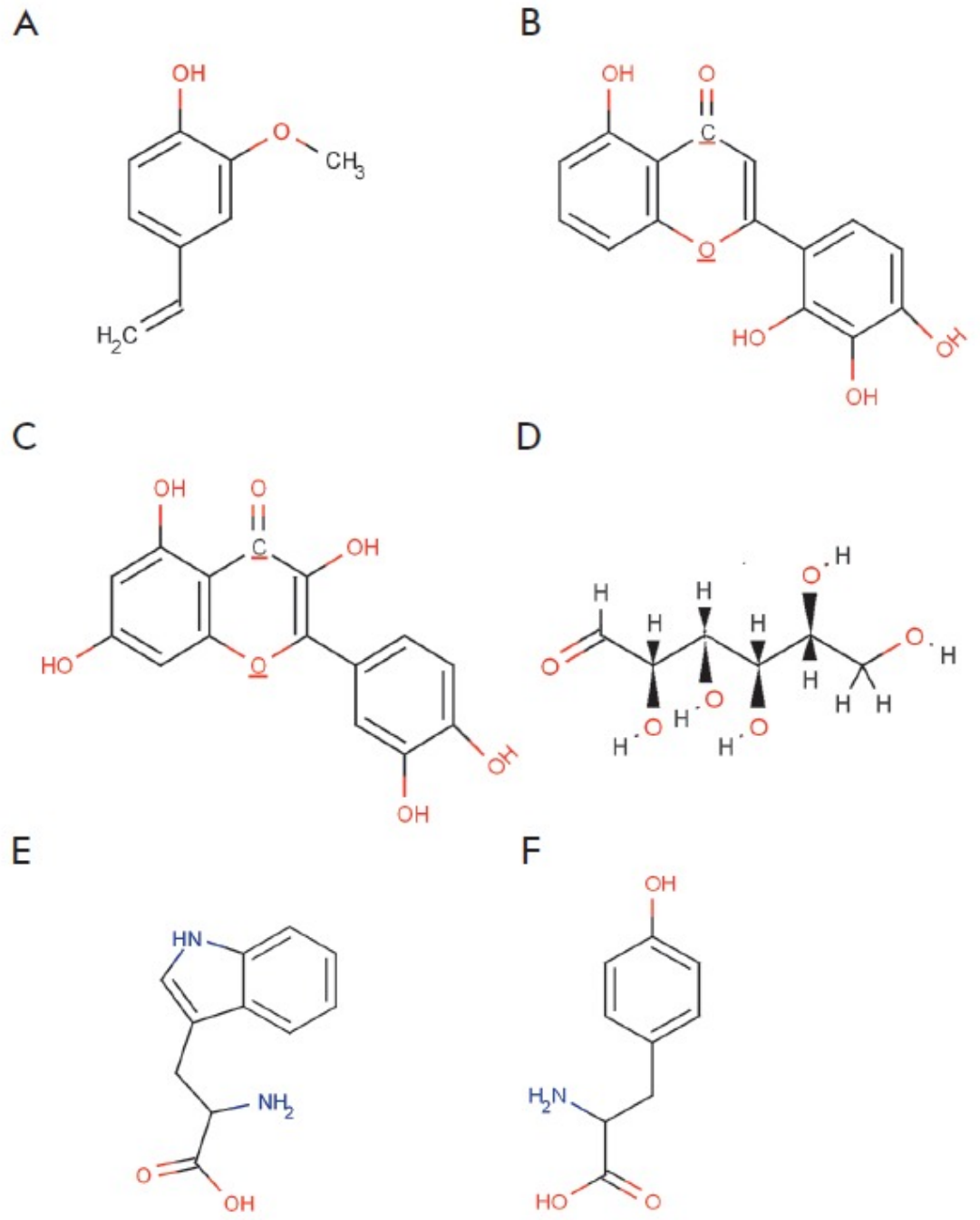

Figure 2.2 Major plant metabolites involved in the synthesis of metal nanoparticles: (A)-terpenoids (eugenol); (B & C)-flavonoids (luteolin, quercetin); (D)-a reducing hexose with the open chain form; (E & F)-amino acids (tryptophan, tyrosine) (Makarov, *et al.*, 2014).

Terpenoids are a class of diverse organic polymers manufactured in plants from five-carbon isoprene units and display strong antioxidant activities. In a previous study by Shankar *et al*., (2003) involving gold nanoparticle synthesis from geranium leaf extracts, it was suggested that these polymers were actively involved in the reduction of gold ions into stable nanoparticles (Shankar, *et al.*, 2003). Later Singh *et al*., (2010) reported that eugenol, the main terpenoid found in *Szyygium aromaticum* (clove), played an important role in the reducing $AgNO_3$ and $HAuCL_4$. The Fourier transform infrared (FTIR) spectroscopy analysis of their study suggests that the dissociation of the proton from the OH group in eugenol leads to the formation of intermediate resonance structures which are capable of undergoing further oxidation. This latter reaction may be coupled to the reduction of Ag^+ and subsequent formation of stable AgNPs (Singh, *et al.*, 2010).

Flavonoids are made up of a large group of polyphenolic compounds containing various classes such as anthocyanins, isoflavonoids, flavonols, chalcones, flavones and flavanones. There are several functional groups present on flavonoid compounds that are able to participate in nanoparticle formation. It has been hypothesised that the tautomerization of flavonoids from the enol to keto form releases a reactive hydrogen atom that can participate in the reduction of metal ions. For example, studies involving AgNP synthesis from *Ocimum sanctum* extracts indicate that synthesis is likely to be the result of tautomerization of the flavonoids luteolin and rosmarinic acid (Ahmad, *et al.*, 2010). Additionally, some flavonoids can chelate metal ions with their carbonyl groups or π-electron. Quercetin is an example of a flavonoid with strong chelating activity (Makarov, *et al.*, 2014). These mechanisms may explain the prevalence of flavonoid groups adsorbed on to the surface of AgNPs derived in previous studies (Gannimani, *et al.*, 2014, Veerasamy, *et al.*, 2011). Further indication of flavonoid involvement in nanoparticle synthesis is provided by a study using *Lawsonia inermis*, in which the flavonoid apiin was extracted and successfully employed in the synthesis of gold and Ag nanoparticles (Kasthuri, *et al.*, 2009).

Sugars contained in plant extracts are also capable of inducing nanoparticle formation. It is known that monosaccharides in the linear form containing an aldehyde (eg. glucose), are capable reducing agents (Makarov, *et al.*, 2014). Monosaccharides harbouring a keto-group may act as antioxidants upon tautomeric transformation from a ketone to an aldehyde (eg. fructose). In this regard, glucose is reportedly more efficient at metal ion

reduction than fructose due to the kinetics of tautomerism from a ketone to an aldehyde which limits the reducing potential of fructose. Disaccharides and polysaccharides may also participate in the reduction of metal ions however, this is largely dependent on the ability of their monosaccharide components to take on an open chain configuration within an oligomer. Examples include lactose and maltose. In contrast, sucrose is unable to participate in metal ion reduction because the linkage of its glucose and fructose monomers restrict the formation of open chains. However, when sucrose was placed in tetrachloroauric and tetrachloroplatinic acids, nanoparticle formation proceeded (Panigrahi, *et al.*, 2004). This may be due to the acidic hydrolysis of sucrose yielding glucose and fructose. In general, it is suggested that nanoparticle formation by sugars is occurs by the oxidation of an aldehyde group into a carbonyl group which subsequently leads to the reduction of metal ions and nanoparticle formation (Shankar, *et al.*, 2003).

FTIR analysis of plant derived metal nanoparticles have revealed the presence of proteins on their surface, suggesting that proteins may also possess metal ion reducing ability. However, amino acids have displayed differences in their potential for metal ion reducing and binding efficiencies. For example, lysine, cysteine, arginine and methionine have been shown to bind Ag^{+}. In a separate study, aspartate was used to reduce tetrachloroauric acid forming nanoparticles, whilst valine and lysine did not possess this ability. Amino acids capable of binding metal ions are thought to do so through their amino or carboxyl groups or through side chain groups: carboxyl groups of aspartic and glutamic acid, imidazole ring of histidine, thiol of cysteine, thioether of methionine, hydroxyl group of serine; threonine and tyrosine, carbonyl groups of asparagine and glutamine (Makarov, *et al.*, 2014).

Linkage of amino acids in a peptide chain may also affect the ability of individual amino acids to bind and reduce metal ions. For example, the R-carbon of amines and carboxylic acids in a peptide bond are inaccessible for association with metal ions. However, the free side chains of individual amino acids can still participate in binding and reduction of metal ions although, this is largely dependent on the amino acid sequence. Tan *et al.*, (2010) demonstrated that synthesised peptides derived from amino acids with strong binding abilities and high reducing activities displayed lower reduction than expected (Tan, *et al.*, 2010). A previous study suggested that protein molecules capable of nanoparticle formation display a strong attraction of metal ions to the regions on the

molecule responsible for reduction however, their chelating activity is limited (Glusker, *et al.*, 1999). It was also suggested that the amino acid sequence of a protein can influence the size, shape and yield of derived nanoparticles. For example, the synthetic peptide GASLWWSEKL was found to rapidly reduce metal ions forming a large number of small nanoparticles (<10 nm), however, replacement of the N- and C- terminal residues forming the peptide SEKLWWGASL led to slower reduction and formation of larger nanospheres and nanotriangles (40 nm). These findings seemingly suggest that peptides and proteins present in plant extracts probably play a vital role in determining nanoparticle size and shape and potentially affect the overall yield of the nanoparticles (Tan, *et al.*, 2010).

2.2.2 AgNP synthesis from bacteria

There exists a vast array of literature pertaining to the use of bacteria as factories for nanoparticle synthesis (Iravani, 2014, Singh, *et al.*, 2015). Bacteria have a marked advantage over other microbial systems such as fungi due to their abundance, rapid growth rate, cheap cultivation and the relative ease of their manipulation (Pantidos and Horsfall, 2014). Their ubiquitous nature has led to their exposure and proliferation in many environmental extremes and ultimately depends on the natural defence mechanisms of these microorganisms to resist the effects posed by environmental stresses (Shah, *et al.*, 2015). Bacteria have demonstrated these defence mechanisms in a number of non-optimal growth conditions including environments contaminated with metal ions.

AgNP synthesis by bacteria can occur intracellularly or by the use of their extracts (Singh, *et al.*, 2015). Several studies have reported intracellular synthesis by a variety of bacterial species and as similarly reported for the use of whole plants, this technology is associated with long duration periods for nanoparticle synthesis. For example Pugazhenthiran *et al.*, (2009) reported an incubation time of 7 days for AgNP synthesis from *Bacillus* sp. (Pugazhenthiran, *et al.*, 2009). Kalimuthu *et al.*, (2008) reported a reaction time of 24 hours for AgNP synthesis by *Bacillus licheniformis* (Kalimuthu, *et al.*, 2008). Although this reaction time was more industrially significant, the authors reported an additional extraction to acquire the derived nanoparticles. Synthesis of AgNPs by the use of bacterial cell free supernatant (CFS) extracts was reported by Shahverdi *et al.*, (2007). Interestingly, nanoparticle synthesis occurred within five minutes of Ag^{+} coming into contact with the CFS (Shahverdi, *et al.*, 2007). Thus, this method presents the greatest

potential for industrial production of AgNPs from bacteria. Several other studies have reported on the production of AgNPs from bacterial CFS extracts but not at the previously stated formation rate (Gurunathan, *et al.*, 2009, Saifuddin, *et al.*, 2009). This seemingly suggests that bacterial extracts differ in their metal ion reducing abilities and may require an external energy source to accelerate nanoparticle formation.

2.2.2.1 Bacterial metabolites involved in nanoparticle synthesis

As previously stated, metal nanoparticle synthesis in bacteria may potentially occur through resistance mechanisms attained by these organisms to overcome the toxic effects of metals. These strategies include: redox state changes, efflux systems, intracellular precipitation, metal accumulation and extracellular formation of complexes **(Fig. 2.3)** (Shah, *et al.*, 2015). In an early study, Slawson *et al*., (1992) observed that the Ag resistant strain *Pseudomonas stutzeri* AG259, was capable of accumulating AgNPs (35-46 nm) within its periplasmic space. The formation of these nanoparticles was thought to have occurred by a mechanism involving the NADH-dependent reductase enzyme which undergoes oxidation to form NAD^+. The lost free electron may potentially reduce Ag^+ to AgNPs (Slawson, *et al.*, 1992). Later, He *et al*., (2007) reported that the NADH-dependent reductase enzyme may similarly participate in the extracellular formation of gold nanoparticles by the bacterium *Rhodopseudomonas capsulata* (He, *et al.*, 2007). Other studies have reported nanoparticle formation without the use of biological enzymes. Non-enzymatic nanoparticle synthesis by a *Corynebacterium* sp. was reported by Sneha *et al*., (2010). Organic functional groups present at the cell wall were thought to induce metal ion reduction (Sneha, *et al.*, 2010). Sintubin *et al.*, (2009) proposed a two-step mechanism for AgNP formation by several lactic acid bacteria, involving biosorption of Ag^+ on the cell wall which is coupled to the subsequent reduction of these ions to form the nanoparticles (Sintubin, *et al.*, 2009). Parikh *et al*., (2008) identified a gene homologue in a Ag-resistant *Morganella* strain with a 99% nucleotide sequence similarity to a periplasmic Ag-binding protein-encoding gene (Parikh, *et al.*, 2008). Johnston *et al*., (2013) further reported the production of a small non-ribosomal peptide, delftibactin by *Delftia acidovorans* which they believed to be associated with a resistance mechanism. By producing inert gold nanoparticles bound to delftibactin, gold ions no longer caused toxicity to the cells (Johnston, *et al.*, 2013).

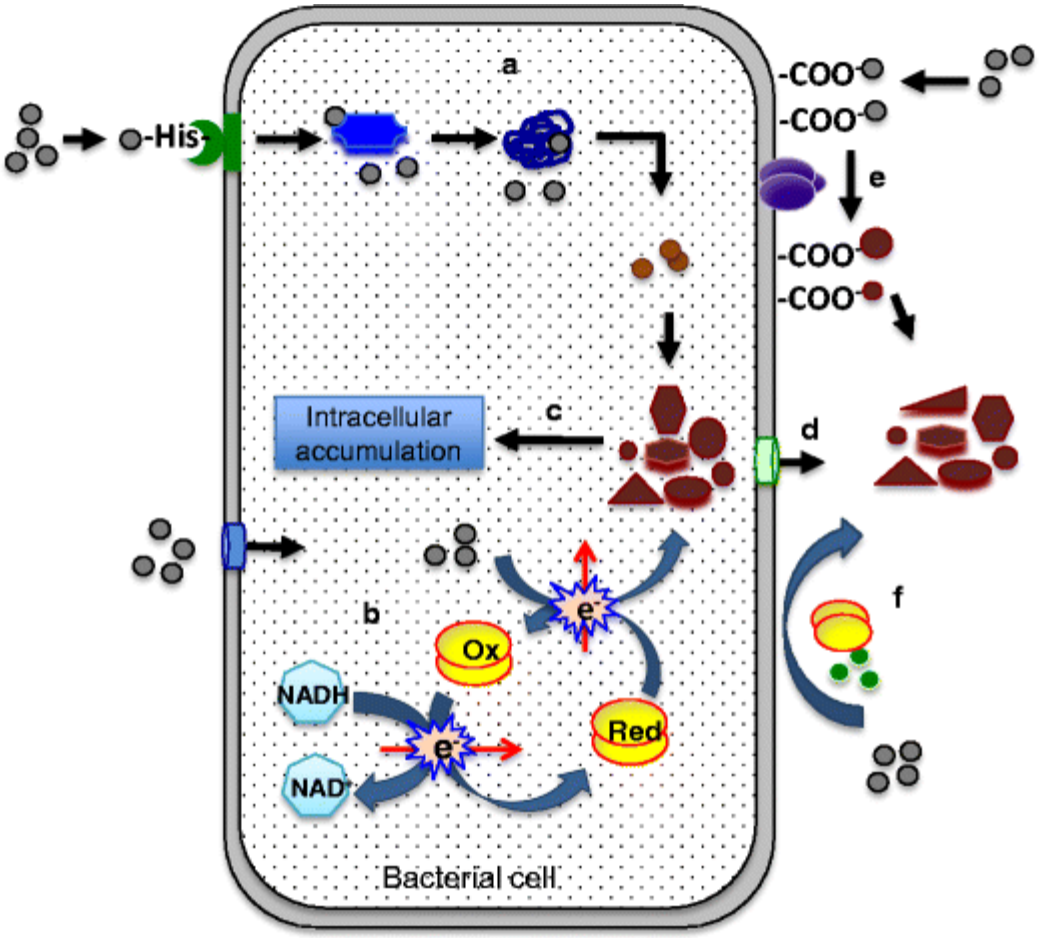

Figure 2.3 Metabolites and mechanisms involved in AgNP synthesis in bacteria: (a)-uptake of Ag^+ and activation of reduction machinery; (b)-electron shuttle system involving various cofactors and enzymes; (c & d)- intra or extracellular localisation of AgNPs; (e)-electrostatic interaction between Ag^+ and cell wall peptides/proteins & (f)-extracellular reduction by enzymes or other metabolites released in solution (Singh, *et al.*, 2015).

2.2.3 Mechanism of nanoparticle synthesis

There are three main phases in the synthesis of metal nanoparticles from plants and plant extracts. Initially, an activation phase takes place during which metal ions are reduced from mono or divalent oxidation states to zero-valent states, followed by nucleation of the reduced atoms. This step is immediately followed by a growth phase where small neighbouring nanoparticles coalesce into larger particles with greater thermodynamic stability while further biological reduction occurs. As growth proceeds nanoparticles aggregate to form various shapes such as: cubes, spheres, triangles, hexagons, pentagons, rods and wires (Kim, *et al.*, 2010). Lastly, a termination phase follows in which

nanoparticles acquire the most energetically favourable conformation, which ultimately determines the final shape of the particles **(Fig. 2.4)** (Si and Mandal, 2007). This step is largely influenced by the ability of the plant extract to stabilise the resulting nanoparticles. For example, the high surface energy of nanotriangles results in their decreased stability. Such nanoparticles would then acquire a more stable morphology such as a truncated triangle to minimize Gibbs free energy, unless the stability is supported by the given extracts. It can be tentatively suggested that a similar mechanism occurs by the use of bacterial extracts since proteins and metabolites may also participate Ag^{+} reaction as previously stated.

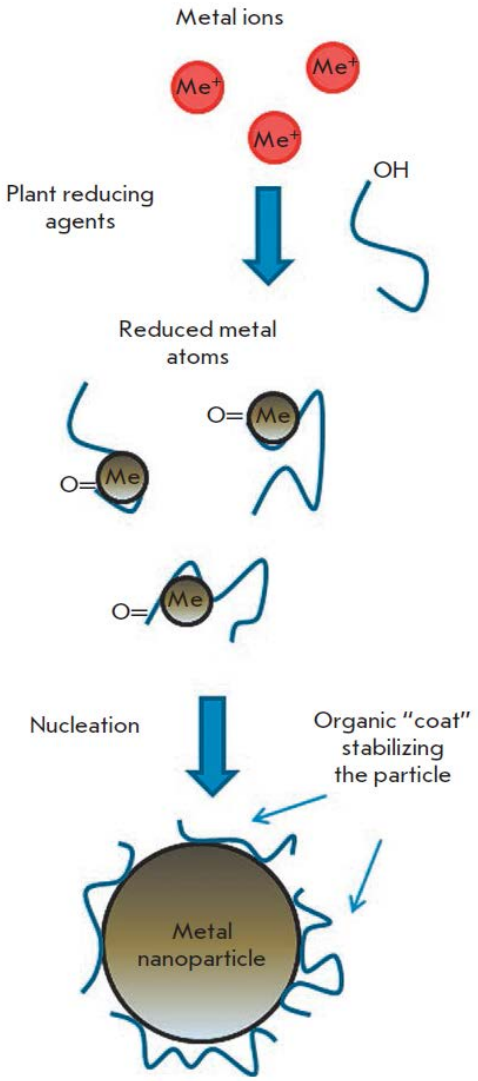

Figure 2.4 Schematic representation of nanoparticle synthesis using a plant extract (Makarov, *et al.*, 2014).

2.3 FACTORS AFFECTING BIOLOGICAL NANOPARTICLES

A number of controlling factors affect the synthesis and morphology of derived nanoparticles. Several researchers have associated these variations with the choice of adsorbate and catalyst used in the synthetic process (Ajayan, 2004, Patra and Baek, 2014). However, reaction parameters have also been shown to strongly affect the synthesis of nanoparticles from biological extracts.

2.3.1 pH

Studies have revealed that the pH of a reaction solution strongly influences the formation of the produced nanoparticles. Variances in reaction pH tend to induce variability in the shape and size of the produced nanoparticles. Lower acidic pH values tend to produce larger particles when compared to higher pH values. In a study employing *Avena sativa* (oat) biomass for the production of gold nanoparticles, larger particles (25-85 nm) where formed at pH 2 whilst smaller particles (5-20 nm) were formed at pH 3 and 4 (Armendariz, *et al.*, 2004). The researchers suggested that at pH 2, fewer functional groups were available for particle nucleation resulting in aggregation of the particles. A similar finding was observed in the synthesis of gold nanoparticles from the bacterium *Rhodopseudomonas capsulate.* At an increased pH of 7, spherical particles in the range of 10-20 nm in size were observed. In contrast, lowering the reaction pH to 4 resulted in the formation of nanoplates (He, *et al.*, 2007).

2.3.2 Temperature

Temperature is an important factor in any synthesis. With respect to nanoparticle formulation with the use of biological entities, temperature elevation has demonstrated catalytic behaviour by increasing the reaction rate and efficiency of nanoparticle formation. For example, a study on the influence of reaction temperature in the synthesis of AgNPs from neem leaf extracts suggested that temperature elevation (10-50°C) was correlated with enhanced reduction of Ag^{+} (Verma and Mehata, 2016). It was also noted that smaller sized AgNPs were produced at 50°C, similar to the finding of Kaviya *et al.*, (2011) in the production of AgNPs from *Citrus sinensis* peel extracts using varying temperatures (Kaviya, *et al.*, 2011). Similarly, this trend was observed in the production of AgNPs from the spent culture supernatants of *Escherichia coli* (Gurunathan, *et al.*, 2009). The authors tentatively suggested that the increased reaction rate might be due to

the effect of temperature on a key enzyme participating in nanoparticle responsible synthesis. However, the study importantly revealed that temperature elevation above 60˚C contrastingly favoured the production of larger sized particles. The reason for this observation was reported as follows: at high temperatures, kinetic energy of the molecules increase resulting in rapid reduction of Ag^+ (facilitating reduction and nucleation), to the detriment of secondary reduction on the surface of nascent particles in the growth phase. However, higher temperatures beyond the optimum are thought to increase the growth of the crystal around the nucleus, resulting in the production of larger particles (Gurunathan, *et al.*, 2009, Veerasamy, *et al.*, 2011).

Temperature has also been demonstrated to affect the structural form of nanoparticles. For example, AgNP synthesis using *Cassia fistula* extracts resulted in the formation of Ag nanoribbons at room temperature whilst spherical AgNPs were formed at temperatures above 60˚C (Lin, *et al.*, 2010). High temperatures in the study were thought to alter the interaction of plant biomolecules with the faces of Ag, inhibiting the coalescence of adjacent nanoparticles.

2.3.3 Sunlight irradiation

Sunlight irradiation, a recently reported primary energy source for nanoparticle formation, has been observed to derive AgNPs with desired physical attributes. Recent studies on sunlight driven AgNP synthesis using *Allium sativum* (garlic extract) and *Andrachnea chordifolia* ethanol leaf extract revealed that sunlight rapidly enhanced nanoparticle formation to produce spherical AgNPs with average diameters of 7.3 nm and 3.4 nm, respectively (Karimi Zarchi, *et al.*, 2011, Rastogi and Arunachalam, 2011). In addition, this use of sunlight has also been used in AgNP synthesis from *Bacillus amyloliquefaciens* CFS to produce circular and triangular crystalline AgNPs with an average diameter of 14.6 nm (Wei, *et al.*, 2012).

2.4 EFFECT OF NANOPARTICLE MORPHOLOGY ON BIOACTIVITY

A variety of literature reports on the synthesis of AgNPs with differing morphologies. Understanding the effects of these morphological characteristics on bioactivity is therefore an important consideration when deriving nanoparticles for therapeutic purposes.

2.4.1 Size

Characteristically, AgNPs are small in size (1-100 nm) and therefore possess a large surface area that facilitates their interaction with bacterial cell membranes (Christian, *et al.*, 2008, Pal, *et al.*, 2007). However, it has been suggested that within this confined size range, AgNPs present a size-dependent inhibition spectrum. Martinez-Castanon *et al*., (2008) reported that AgNPs of 7 nm in size had minimum inhibitory concentration (MIC) values of 6.25 µg ml^{-1} and 7.5 µg ml^{-1} for *E. coli* and *Staphylococcus aureus*, respectively. In contrast, larger nanoparticles (29 nm) capped with the same reducing agent displayed higher MIC values for the respective strains (Martinez-Castanon, *et al.*, 2008). These results are in accordance with other studies that report nanoparticles of < 10 nm in size display improved bactericidal activities (Jeong, *et al.*, 2014, Morones, *et al.*, 2005).

2.4.2 Shape

The interaction of AgNPs of varying shapes with *E. coli* cells has unveiled that shape plays an important factor in bioactivity. Pal *et al*., (2007), reported that at a low Ag content of 1 µg, truncated triangular nanoparticles showed nearly complete inhibition of *E. coli* cells, whilst spherical nanoparticles with a total silver content above 12.5 µg displayed a reduction in colony forming units. Rod-shaped particles and $AgNO_3$ presented inferior activities when compared to truncated triangular and spherically shaped AgNPs (Pal, *et al.*, 2007).

Considering these factors and the aforementioned factors affecting synthesis of nanoparticles, it can tentatively be suggested that the fine tuning of reaction parameters such as pH or temperature may be applied in producing AgNPs with these desired physical attributes. However, the use of sunlight irradiation provides a promising alternative in this regard.

2.5 POTENTIAL APPLICATIONS OF BIOLOGICALLY DERIVED NANOPARTICLES

The physiochemical characteristics of metal nanoparticles render them applicable across a genre of multi-disciplinary fields for a variety of uses including: catalysis (Crooks, *et al.*, 2001); micro-electronics (Gittins, *et al.*, 2000); solar energy conversion (Kamat,

2007) amongst many others (Fedlheim and Foss, 2001). They have also been recognised for their potential in number of medical applications (Rai, *et al.*, 2016). However, the use of nanoparticles derived from physical and chemical synthetic routes raises health and toxicity concerns due to the nature of the reaction conditions which may ultimately affect the properties of the derived particles (Baker, *et al.*, 2013).

Biologically derived nanoparticles provide a greener alternative to nanoparticles derived from the aforementioned routes since, the synthesis methods used to derive these particles are clean and non-toxic (Ahmed, *et al.*, 2016). As a result, they are suitable for a number of biomedical applications including: cancer therapy; drug delivery; tumour detection; genetic disorder diagnosis; tissue repair; cell labelling; antimicrobial development; targeting and immunoassays and yet to be discovered applications (Ahamed, *et al.*, 2010, Bhadra, *et al.*, 2014, Huang, *et al.*, 2007, Khlebtsov and Dykman, 2011, Rai, *et al.*, 2016, Singh, *et al.*, 2016).

With respect to biologically derived AgNPs, their major exploitation exists in the development of antimicrobial agents due to their renowned microbial inhibitory activities and with the current status on antimicrobial drug resistance, these particles are being extensively sought after as possible alternatives to antibiotics (Kim, *et al.*, 2007, Rai, *et al.*, 2009). Given the nature of this study, the antimicrobial effects of AgNPs will be discussed so as to establish their potential in antimicrobial drug development.

2.6 SCREENING OF AGNP ANTIMICROBIAL ACTIVITIES

There exists an abundance of literature reporting on antimicrobial activities of biologically derived AgNPs (Awwad, *et al.*, 2013, Logeswari, *et al.*, 2012, Prasad and Elumalai, 2011, Sharma, *et al.*, 2009). Most of these studies utilise the disc diffusion assay (Biemer, 1973) or agar well diffusion assay (Perez, *et al.*, 1990) to establish inhibitory effects. Positive indication of inhibitory activities are visualised by zones of inhibition on a microbial lawn. Veersamy *et al.*, (2011) reported zones of inhibition of *S. aureus* and *E. coli* to be 15 mm and 20 mm respectively for AgNPs (20 µg ml^{-1}) derived from

mangosteen leaf extracts (Veerasamy, *et al.*, 2011). Similarly, Logeswari *et al.*, (2012) reported zones of inhibition of AgNPs synthesised from various plant extracts against several bacterial strains (Logeswari, *et al.*, 2012). Although diffusion techniques are preferred amongst researchers, they seem to be labour-intensive. In addition, many researchers do not establish the initial concentration of AgNP solution prior to antimicrobial evaluation (Jagtap and Bapat, 2013, Prasad and Elumalai, 2011). Such disparities make comparison between published data inapplicable (Allahverdiyev, *et al.*, 2011).

Determination of minimum inhibitory concentration (MIC) by the broth microdilution or macrodilution method (Eloff, 1998, Schwalbe, *et al.*, 2007) is easy to access and provides accurate information with respect to microbial susceptibility. Moreover MIC values are reported in various concentration units such as $\mu g\ ml^{-1}$, $\mu g\ l^{-1}$ or ppm thereby facilitating comparison between publications (Singh, *et al.*, 2015). These methods are therefore attractive for AgNP bioactivity analysis. Furthermore, determination of MICs is an important consideration for any therapeutic agent in development to assess their toxicity at the specified concentration range.

2.7 MECHANISMS OF AGNP MEDIATED ANTIMICROBIAL ACTIVITY

2.7.1 Bactericidal mechanisms

As previously mentioned, the antimicrobial effects of AgNPs are well established. However, a relatively confined amount of studies have been conducted to elucidate their mechanisms of antimicrobial action. These mechanisms are poorly understood and have failed to achieve consensus amongst researchers. Despite this, three common mechanisms of bactericidal activity have been proposed by various studies. These include the uptake of Ag^{+} (1), generation of reactive oxygen species (ROS) (2) and cell membrane disruption (3) **(Fig. 2.5)** (Marambio-Jones and Hoek, 2010).

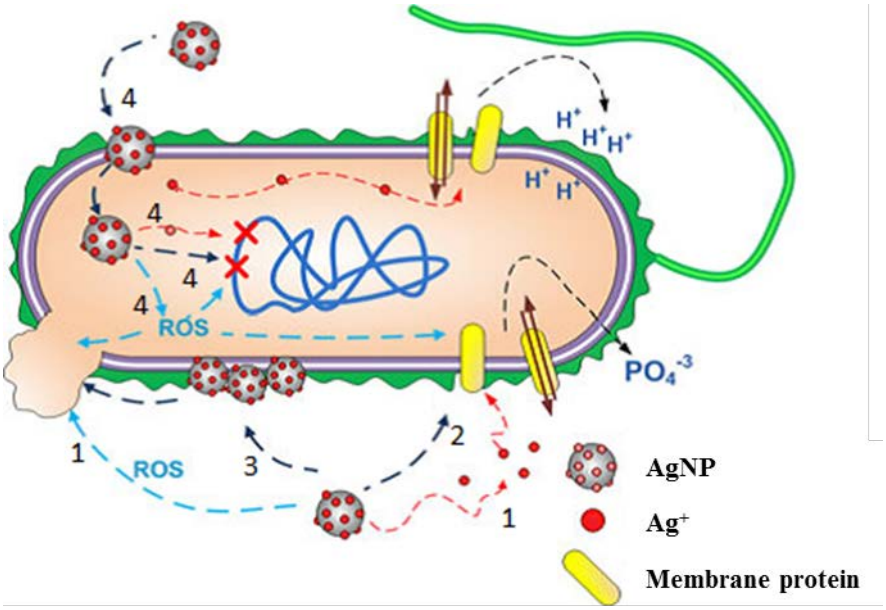

Figure 2.5 Interactions of AgNPs with bacterial cells: (1) release of Ag^+ and generation of ROS; (2) interaction with cell membrane proteins; (3) accumulation in cell membrane and disruption of permeability; (4) entry into the cell and release of Ag^+, leading to generation of ROS and damage of cellular DNA. In turn, generated ROS may affect DNA, cell membrane and membrane proteins whilst released Ag^+ may affect cell membrane proteins and DNA. Adapted from (Marambio-Jones and Hoek, 2010).

2.7.1.1 Uptake of Ag^+

Since Ag^+ are known to possess antibacterial activities, their release from AgNPs may potentially aid to the bioactivity of the nanoparticles. It is therefore fitting to consider the mechanistic action of Ag^+ on bacterial cells.

The NADH-ubiquinone reductase has been established as one of major targets for Ag^+. Specifically the binding of Ag^+ to this enzyme may be responsible for their bactericidal effect even at minute concentrations (Semeykina and Skulachev, 1990). Later, Dibrov *et al*., (2002) reported the binding of Ag^+ to transport proteins leads to the leakage of protons and ultimately induces the collapse of the proton motive force (Dibrov, *et al.*, 2002). Such interactions with transport proteins may be attributed to the strong affinity of Ag^+ to thiol groups found on cysteine residues of these molecules (Liau, *et al.*, 1997). Ag^+ has also been reported to inhibit phosphate uptake and additionally causes an efflux of intracellular phosphate (Schreurs and Rosenberg, 1982). It has also been hypothesised that the

antimicrobial effect of Ag^+ is correlated with the disruption of DNA replication. DNA molecules in a relaxed conformation can be replicated effectively. However, when Ag^+ are present in bacterial cells, DNA molecules enter a condensed form and replicating ability diminishes which ultimately leads to cell death (Rai, *et al.*, 2009).

2.7.1.2 Generation of ROS

The exposure of bacterial cells to AgNPs leads to the generation of ROS (Ninganagouda, *et al.*, 2014). Naturally, ROS are metabolic by-products of respiring beings. Whist low levels of these species are skilfully controlled by various antioxidant defence mechanisms, high levels of ROS results in oxidative stress which is detrimental to any living organism. Metals can serve as catalysts and produce ROS in an oxygen containing environment (Stohs and Bagchi, 1995). AgNPs are therefore likely to catalyse reactions with oxygen leading to the production of excess free radicals. Kim *et al*., (2007) demonstrated the generation of free radicals from AgNPs by means of spin resonance measurements. Toxicity of AgNPs and $AgNO_3$ diminished upon addition of an antioxidant suggesting that the mechanism of action against bacterial strains was associated with the formation of free radicals from AgNPs. The generation of excess free radicals attack membrane lipids resulting in the breakdown of the membrane and also cause damage to DNA (Kim, *et al.*, 2007).

The release of Ag^+ from nanoparticles attached to the membrane and nanoparticles inside the cell also play a role in the generation of ROS. Ag^+ released on the membrane are capable of ROS generation by acting as electron acceptors whilst those present inside the cell more likely to interact with thiol groups of respiratory chain enzymes as previously stated, or scavenging superoxide dismutase enzymes (Park, *et al.*, 2009). The effect of ROS scavengers on *E. coli* cells was reported by Inoue *et al*., (2002). Specifically ROS such as superoxide anions, hydroxyl radicals, hydrogen peroxide and singlet oxygen contributed to the bactericidal activity against *E. coli* (Inoue, *et al.*, 2002).

2.7.1.3 Cell membrane disruption

According to literature, the bactericidal effect of AgNPs may also be the result of damage to the outer membrane of bacterial cells. Previous studies by Sondi and Salopek-Sondi (2004) suggested that treatment of *E. coli* cells with AgNPs induced changes in the membrane morphology **(Fig. 2.6a)**. This resulted in increased membrane permeability

and shifts in normal transport through the plasma membrane (Sondi and Salopek-Sondi, 2004). Morones *et al*., (2005) hypothesised that these mechanisms could explain the number of nanoparticles found inside *E. coli cells* **(Fig. 2.6b)**. AgNPs with oxidised surfaces were also reported to induce the formation of holes on the surface of *E. coli* cells and portions of the cellular surface were observed to be eaten away (Smetana, *et al.*, 2008). The attachment and penetration of AgNPs has also been observed in *P. aeruginosa* **(Fig. 2.6c)**, *V. cholera* and *S. typhus* (Morones, *et al.*, 2005).

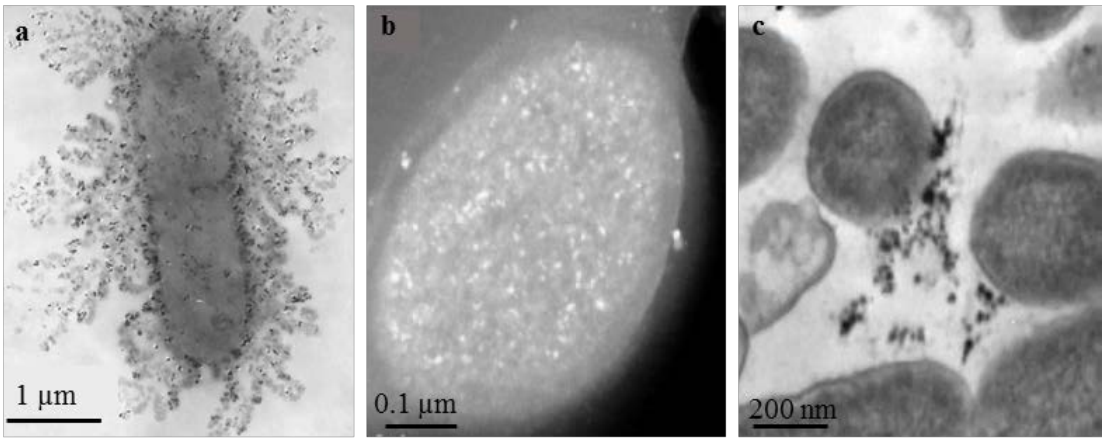

Figure 2.6 Transmission Electron micrographs of (a) *E. col*i cell after 1 h treatment with 50 µg cm^{-3} AgNPs; (b) *E. col*i cell after 30 min treatment with 100 µg ml^{-1} AgNPs (c) *P. aeruginosa* cells after 30 min treatment with 100 µg ml^{-1} AgNPs (Morones, *et al.*, 2005, Sondi and Salopek-Sondi, 2004).

The mechanism of AgNP adhesion and penetration of bacterial cell membranes remains to be elucidated. Literature reports indicate that electrostatic interactions between positively charged particles and negatively charged cell membranes is essential for the bioactivity of these particles (Hamouda and Baker, 2000, Stoimenov, *et al.*, 2002). However, this strategy does not validate the adhesion and penetration abilities of negatively charged nanoparticles (Sondi, *et al.*, 2003). The researchers argued that

although the particles were negatively charged, interactions between the particles and building elements of the membrane are likely to have occurred causing structural changes and degradation of the membrane. Morones *et al.,* (2005) proposed that the interaction of AgNPs and bacterial membranes could be the attributed to the strong affinity of the particles to sulphur containing proteins present on the membrane. These interactions are thought to be conserved in the interaction of Ag^{+} and thiol groups on respiratory enzymes and transport proteins (Marambio-Jones and Hoek, 2010, Morones, *et al.*, 2005).

Sondi and Salopek-Sondi (2004) further reported that damage to *E. coli* cell membranes might also occur due to the incorporation of AgNPs into their membrane structure. Scanning electron microscopy revealed the formation of "pits" on the surface of the membrane (Sondi and Salopek-Sondi, 2004). Similar findings were observed by (Stoimenov, *et al.*, 2002). Amro *et al.*, (2000) additionally reported the formation of irregularly shaped "pits" on the outer membrane of *E. coli* cells through the progressive release of lipopolysaccharides molecules. This release of LPS molecules was induced by metal depletion in the cells (Amro, *et al.*, 2000). A membrane with such morphological changes would display a high increase in permeability, rendering the cell incapable of regulating proper transport through the membrane as previously described.

Although these studies have been conducted on Gram-negative bacteria, AgNPs have also been reported to exert inhibitory activities against Gram-positive bacteria which differ from their counterparts based on differences on in cell wall structure (Dipankar and Murugan, 2012). It can be tentatively suggested that AgNPs may form interactions with Gram-positive bacteria through surface proteins present on the cell wall. Once penetrated, the mechanisms of bacterial activity are conserved with that of Gram-negative bacteria.

2.7.2 Antifungal mechanisms

A relatively confined amount of literature focuses on the mechanisms of antifungal activity exerted by AgNPs. However, based on the studies that have been reported, it seems that inhibition of fungal growth by AgNPs may be the result of damage to fungal cellular membranes.

Kim *et al.,* (2009) demonstrated the effect of AgNPs on *Candida albicans*. Transmission electron microscopy (TEM) analysis revealed that the treatment of cells with AgNPs lead to the formation of "pits" on the cell membrane which ultimately disrupts membrane potential (Kim, *et al.*, 2009). A similar finding was made by Nasrollahi *et al.,* (2011) who reported that AgNP incubation with *C. albicans* led to damage of the cell membrane (Nasrollahi, *et al.*, 2011). Endo *et al.*, (1997), reported that disruption of membrane integrity inhibits the normal budding process of daughter cells. Therefore the authors suggested that AgNPs exert their inhibitory activity by inhibiting the budding of daughter cells due to the destruction of the cell membrane (Endo, *et al.*, 1997).

AgNPs may also disrupt antioxidant defences in fungal cells. Eukaryotic cell studies suggest that AgNPs directly interact with gluthathione, gluthathione reductase or enzymes responsible for maintaining proper levels of gluthathione (Carlson, *et al.*, 2008). With respect to fungal cells, it has been hypothesised that Ag^+ largely effect the function of membrane bound enzymes such as those in the respiratory chain. It has also been reported the exposure of fungal cells to Ag^+ led to the loss of DNA replication ability. This results in the deactivation of ribosomal subunit protein expression and synthesis of non-functional enzymes and cellular proteins (Elgorban, *et al.*, 2016).

From these findings it can be tentatively suggested that bactericidal mechanisms of AgNPs are conserved in their inhibition of fungal cells. In summary, AgNPs exert their antimicrobial effects by releasing Ag^+, disrupting the cell membrane/wall, generating ROS and inhibiting proper DNA replication.

2.8 CYTOTOXICITY OF AGNPS

The unique physico-chemical and biological properties of AgNPs have extremely promising industrial and medical applications, as previously mentioned. However, there exists a dearth of knowledge regarding the effects of prolonged exposures to nanoparticles on human health and the environment (Ravishankar Rai and Jamuna Bai, 2011). It is therefore imperative to establish that the *in vitro* and *in vivo* cytotoxic effect of AgNPs in mind for therapeutic purposes.

2.8.1 *In vivo* studies

Human contact with nanoparticles occurs in the form of intravenous injection, oral administration, inhalation and dermal contact (Chen and Schluesener, 2008). Injection of AgNPs *in vivo* results in short circulation times and broad tissue distribution. Target sites often include the liver (main target), spleen, lungs and kidneys (Ahamed, *et al.*, 2010). Inhalation studies suggest that AgNPs become deposited in the olfactory mucosa and olfactory nerves which can potentially induce impairment and dysfunction of brain cells (Oberdörster, *et al.*, 2004) in addition to immunotoxicity (Rahman, *et al.*, 2009). With regard to oral administration, migration of AgNPs to the gastrointestinal tract promotes dissolution of the particles which subsequently releases Ag^+ (Zhang, *et al.*, 2014). A recent study on oral exposure to Ag^+ indicated that these ions interact with sulphur leading to the formation of sulphur containing Ag granules in the intestinal epithelium (Loeschner, *et al.*, 2011). The authors suggested that during intestinal digestion, Ag^+ give rise to particle formation, possibly in the form of Ag_2S or AgCl salt. They further added that this formation might influence their uptake and reduce the toxic effects of Ag^+, however the effects of Ag salts on the intestine are yet to be elucidated (Loeschner, *et al.*, 2011, Walczak, *et al.*, 2012). Reports on the exposure of workers to low doses of Ag dust indicated no significant changes in health status.

2.8.2 *In vitro* studies

Many researchers have demonstrated the cytotoxic effects of AgNPs *in vitro*, however there is still a lack of consistent and reliable data amongst publications. For example, in a recent review, Kim and Ryu (2013) attributed oxidative stress, apoptosis and genotoxicity to be the main *in vitro* outcome of AgNP exposure (Kim and Ryu, 2013). Later, Gliga *et al.*, (2014) identified a major drawback of this review, highlighting that the AgNPs were different in each study, *i.e.* synthesised by different techniques, of varying size distributions and coatings, tested on different cell lines under different cell culture conditions and often without the use of appropriate controls (Gliga, *et al.*, 2014).

Additionally, Hackenberg *et al*., (2011) reported cytotoxicity of human mesenchymal stem cells at a concentration of 10 µg ml^{-1} AgNPs (<50 nm), whereas Samberg *et al*., (2012) reported no toxicity of progenitor human adipose-derived stem cells at concentrations up to 100 µg ml^{-1} AgNPs (10-20 nm) (Hackenberg, *et al.*, 2011, Samberg, *et al.*, 2012). To determine the effect of size on cytotoxicity, Liu *et al*., (2010) compared the cytotoxicity of AgNPs ranging in size from 5-50 nm on four different cell lines (A549, HepG2, MCF-7 and CGC-7901) and reported that 5 nm AgNPs were most toxic (Liu, *et al.*, 2010). On the contrary, Kim *et al*.,(2012) reported the enhanced release of lactate dehydrogenase (LDH) and reduced cell viability in the presence of 100 nm sized AgNPs when compared to smaller AgNPs (10-50 nm) (Kim, *et al.*, 2012). It can be noted that the variation in parameters in these studies makes it difficult to observe trends and come to accurate assumptions. To achieve some consensus in this regard, Gliga *et al*., (2014) studied the cytotoxic effect of varying sized AgNPs capped by various agents on the normal bronchial epithelial cell line (BEAS-2B). They reported that 10 nm sized AgNPs induced cytotoxicity irrespective of the capping agents, at high concentrations (20-50 µg ml^{-1}), whilst larger AgNPs did not display significant cytotoxic effects at all tested concentrations. The group additionally reported that at non-cytotoxic concentrations (10 µg ml^{-1}), significant DNA damage was observed for all AgNPs independent of size and coating. In contrast, panda *et al*.,(2011) reported no genotoxicity of AgNPs capped with protein at 20-80 µg ml^{-1} for 24-55 nm sized particles (Panda, *et al.*, 2011).

Overall, it is difficult to establish the cytotoxic effect of AgNPs due to the differences in nanoparticle synthetic methods, their various sizes and capping agents and lastly the diverse evaluation tests used to determine toxicity. In fact, by using different organisms and/or culture cells there is no conclusive evaluation of AgNP toxicity (Lima, *et al.*, 2012). However, bearing in mind the results presented in this review, it can be tentatively suggested that smaller sized AgNPs are more cytotoxic than larger sized particles at higher concentrations.

2.9 SELECTED PLANT AND BACTERIUM USED IN THIS STUDY

2.9.1 *Moringa oleifera*

Moring oleifera LAM is a highly valued tree species belonging the family Moringaceae and is commonly referred to as the drumstick or horseradish tree. The species is indigenous to northwestern India and southern Asia and is also regarded as an important crop in several other countries such as the Philippines, Sudan and Ethiopia (Fahey, 2005). In South Africa, the tree is widely distributed and can be locally found in suburbs and back yards **(Fig. 2.7)**. It is well known by the Indian community for its drumsticks and leaves which are popularly consumed as a vegetable dish (Ramachandran, *et al.*, 1980).

The leaves in particular are reported to possess several nutritional benefits. Research has unveiled that the leaves of *M. oleifera* contain higher levels of Vitamin A, Vitamin C, iron, potassium as well as protein found in carrots, oranges, spinach, bananas, milk and eggs (Fahey, 2005). In addition, they are also a rich source of the amino acids lysine, methionine, tryptophan and cysteine (Makkar and Becker, 1997). Studies have also revealed that woman who consume the leaves show enhanced breast milk production (Estrella, *et al.*, 2000). Importantly, the leaves have also demonstrated attractive medicinal properties such as anti-oxidizing, anti-hypertension and antimicrobial activities. Phytocompounds such as phenolics and carotenoids have been reported in the preservation of ghee from animal fats. In addition, antioxidant studies have identified the major bioactive compounds of phenolics to be flavonoid groups such as quercetin and kaempferol. The antimicrobial constituents of the leaves are yet to be identified (Fahey, 2005), however leaf extracts of *M. oleifera* display promising antimicrobial activities against microorganisms such as *Micrococcus pyogenes, E. coli, P. aeruginosa, Salmonella typhimurium, S. aureus* as well as *Bacillus subtilis* (Kumari, 2014, Siddhuraju and Becker, 2003).

In the search for capping agents for AgNP preparation for the purpose of antimicrobial application, the leaves of this tree display attractive qualities that make them suitable candidates. Previously, AgNPs (57 nm) from the leaves have been prepared in a heat driven process. However, their characterisation in terms of their minimal inhibitory concentration values remains undetermined. It is therefore fitting to establish these concentrations so as to determine their potential in antimicrobial development.

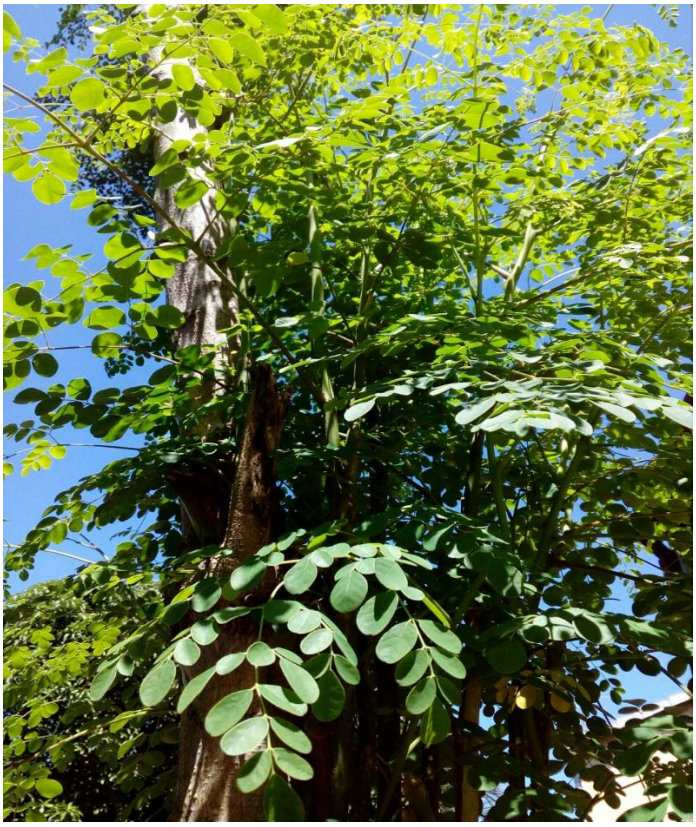

Figure 2.7 *M. oleifera* tree growing in a local suburb.

2.9.2 *Pediococcus acidilactici*

P. acidilactici PAC1.0. is a Gram-positive homofermentive microbe belonging to the family Lactobacillaceae and is renowned for the production of the antimicrobial peptide pediocin PA-1, belonging to class IIa of bacteriocins. Pediocin PA-1 is a small (<10 kDa) heat-stable peptide that is produced extracellularly and primarily displays substantial inhibition of the pathogenic bacterium, *Listeria monocytogenes*. However, this inhibition spectrum is commonly directed towards several other Gram-positive bacteria. (Eijsink, *et al.*, 1998, Ennahar, *et al.*, 1999, Marugg, *et al.*, 1992). It has therefore been actively researched for its application in food preservation (Ennahar, *et al.*, 1999).

In the context of this study, production of AgNPs from a bacterium with such antimicrobial potential seems interesting. Particularly, because the peptide is produced extracellularly, it may therefore be exploited as a capping agent in AgNP production by the use of spent cultures. To the best of our knowledge, production of AgNPs from this bacterium has not been reported.

2.10 CONCLUSION

Given the literature presented, it can be concluded that green synthetic strategies by the use of plant and bacterial based extracts are promising alternatives for the production of AgNPs. However, for the production of AgNPs with enhanced bioactivities, morphological characteristics such as size and shape need to be finely tuned. In this regard, sunlight constitutes a promising candidate compared to the use of alternative reaction parameters. Furthermore, the use of extracts with known medical value provides with attractive capping substrates that may potentially enhance the bioactivities if the produced particles.

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Chapter 3

RESEARCH RESULTS I

Bioactivity of silver nanoparticles derived via green technology from *Moringa oleifera* leaf extracts

Bioactivity of silver nanoparticles derived via green technology from *Moringa oleifera* leaf extracts

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3. 1 ABSTRACT

The increasing incidence of multi-drug resistant organisms has led to the resurgence in application of Ag-based compounds. Accordingly, increased efforts have been made in the field of nanotechnology to develop silver nanoparticle (AgNP) synthetic strategies that are inexpensive and environmentally friendly without the use of hazardous chemicals. To this end, sunlight irradiation, a relatively new primary source of energy was used instead of heat to drive the synthesis of AgNPs. Biologically-derived capping substrates were independently extracted from fresh (F) and freeze-dried (FD) leaf samples of the medicinal tree species, *Moringa oleifera*. Importantly, the extracted substrates advantageously displayed a dual functionality by reducing Ag^{+} as well as stabilising the resulting AgNPs. Yield analysis indicated a recovery ranging from 0.81 mg and 0.91 mg AgNPs per gram dry mass F and FD leaf tissue, respectively. AgNPs from both sample types were consistent in terms of their spherical shape with average diameters of 9 nm and 11 nm for F and FD samples, respectively. Fourier transform infrared (FTIR) spectroscopic analysis suggested that flavones; terpenoids and polysaccharides predominate and are primarily responsible for the reduction and subsequent capping of AgNPs. Interestingly, bioactivity screening revealed that AgNPs exhibited broad-spectrum antimicrobial activity against both bacterial and fungal strains. The results suggest that AgNPs derived from *M. oleifera* leaf extracts exhibit potential for application as broad-spectrum antimicrobial agents.

3.2 INTRODUCTION

Nanotechnology is a science centered on atomic, molecular and supramolecular molecules aiming to create nano-structures with enhanced functionalities (Roco, 2005), and the term nanoparticle describes particulate matter ranging in size from 1-100 nm (Christian, *et al.*, 2008). Bearing a nano scale size offers the advantage of having a significantly large surface area to volume ratio (Iravani, 2011). Increased surface area, in combination with nanoparticle conformation and distribution in solution contribute to their enhanced physical and chemical properties which are useful in a variety of fields such as antimicrobial development (Rai, *et al.*, 2009), bio-molecular detection, diagnostics (Schultz, *et al.*, 2000), catalysis (Crooks, *et al.*, 2001), micro-electronics (Gittins, *et al.*, 2000), sensing devices, targeting of drugs to cancer cells (Sengupta, *et al.*, 2005) and yet to be realised applications (Zhang, *et al.*, 2010). These wide ranging applications and the increasing ability to manipulate nanoparticle form and function has sparked high interest in nanotechnology over the past decade, particularly in terms of the development of efficient and environmentally friendly synthetic methods.

Conventional physical and chemical methods presently have limited use to prepare metal nanoparticles. This to a large extent, stems from the use of hazardous chemicals, which may become adsorbed onto the surface of the particles (Bhattacharya and Mukherjee, 2008). Moreover these methods are associated with high-energy input and costly downstream processing (Awwad, *et al.*, 2013). Finally, conventional chemically synthesised nanoparticles show decreased stability and statically agglomerate within a short period (Kapoor, *et al.*, 1994).

To date, AgNPs receive the most attention based on their desirable antimicrobial effects (Panáček, *et al.*, 2009) and especially with the ongoing emergence of multi drug-resistant pathogens. This has propelled an exponential increase in research activities pertaining to AgNPs as novel antimicrobial agents. Unlike conventional antibiotics which usually have a single mechanism of action, AgNPs display multi-mode structure activity mechanisms which importantly reduces the probability rate of microorganisms developing resistance (Jung, *et al.*, 2008). This has motivated researchers to formulate greener alternatives for AgNP synthesis.

Green synthesis is defined as the use of environmentally compatible materials such as bacteria, fungi and plants in the synthesis of nanoparticles (Patra and Baek, 2014). These attractive green strategies are free of the shortfalls aforementioned for conventional synthetic strategies, *i.e.* they are eco-friendly (Veerasamy, *et al.*, 2011). Green-synthesised nanoparticles are therefore suitable for pharmaceutical and medical applications (Ezhilarasan and Sahadevan, 2011). Additionally, precursor costs are generally low, waste products derived from these natural extracts are compatible with the environment (Kharissova, *et al.*, 2013), and synthesis can be easily up-scaled for increased production (Veerasamy, *et al.*, 2011). Intracellular biosynthesis of nanoparticles within plants, bacteria or fungi provides bioactive nanoparticles but at relatively slow rates (Nath and Banerjee, 2013). Alternatively, synthesis from biologically derived extracts offers several advantages such as rapid synthesis; high yields and importantly, the lack of costly downstream processing required to obtain to the produced particles (Das, *et al.*, 2014, Gannimani, *et al.*, 2014, Liu, *et al.*, 2005). Hence, nanoparticle synthesis from plant extracts tentatively offers a route for large scale production of commercially attractive nanoparticles.

Numerous studies report on the use of plant extracts to synthesise AgNPs with significant antimicrobial activities: leaf extracts of *Acalypha indica* (Krishnaraj, *et al.*, 2010); *Chenopodium album* (Dwivedi and Gopal, 2010); *Murraya koenigii* (curry leaf) (Christensen, *et al.*, 2011); *Ocimum sanctum* (Tulsi leaf) (Ahmad, *et al.*, 2010); *Garcinia mangostana* (mangosteen leaf) (Veerasamy, *et al.*, 2011); seed extracts of *Acacia farnesiana* (Yallappa, *et al.*, 2013); *Macrotyloma uniflorum* (Vidhu, *et al.*, 2011); root extracts of *Trianthema decandra* (Geethalakshmi and Sarada, 2012), stem extracts of *Ocimum sanctum* (Ahmad, *et al.*, 2010); and even fruit extracts of *Musa paradisiaca* (banana) peels (Bankar, *et al.*, 2010) and *Carica papaya* (Jain, *et al.*, 2009). In these studies, phytocompounds in the plant extract serve as reducing and/or capping agents in the reaction with silver nitrate ($AgNO_3$), a commonly used precursor in AgNP synthesis. High concentrations of the active ingredient polyphenol in the plant extracts are said to enhance reduction and provide stabilisation of the nanoparticles, preventing agglomeration (Kharissova, *et al.*, 2013).

Moringa oleifera Lam (drumstick tree) is a tree species indigenous to north-western India but is also regarded as an important crop in several other countries such as the Philippines, Sudan and Ethiopia. The tree is also widely distributed in tropical Asia, West, East and South Africa (Fahey, 2005, Siddhuraju and Becker, 2003). It belongs to the genus *Moringa* and the family Moringaceae (Kumari, *et al.*, 2006), and is highly sought after for its tender pods, flowers and leaves, all of which are safe for human consumption (Ramachandran, *et al.*, 1980). The leaves, in particular, are recognised for their natural healing properties and are popularly consumed in a variety of ways (Kumari, *et al.*, 2006, Makkar and Becker, 1997). Research to date has unveiled that extracts prepared from the leaves possess high natural antioxidant properties and some antibacterial activity against Gram-positive and Gram-negative bacteria (Rahman, *et al.*, 2009, Siddhuraju and Becker, 2003).

The present study was conceptualised on the basis that AgNP formulation of *M. oleifera* leaf extract may harbor the potential to increase its antimicrobial range and bioactivity. Furthermore, the study compared the yield, physical characteristics and bioactivity between nanoparticles synthesised using extracts prepared from freeze-dried (FD) and fresh (F) leaf material. The comparison between FD and F leaf extracts was inspired by the increasing amount of work being done on AgNP synthesis using either F (Veerasamy, *et al.*, 2011) or dry plant material (Prasad and Elumalai, 2011). There are few published comparisons on nanoparticle yield, quality and bioactivity for extracts prepared with dry and F tissue within individual species. However, using *Amaranthus dubius* leaf extracts, Sigamoney *et al*., (2016) have recently shown that the nature (dry versus F) of the starting plant material can influence the yield, morphology and bioactivity of AgNPs produced (Sigamoney, *et al.*, 2016). With their reported medicinal, antioxidant and antimicrobial activities, *Moringa* leaves represent a promising candidate for green synthesis of bioactive AgNPs that can be produced in an environmentally friendly manner.

3.3 MATERIALS AND METHODS

All chemicals, solvents and media used in this study were of analytical grade and purchased from Merck (Pty) Ltd, South Africa, unless stated otherwise. Antibiotics were purchased from Sigma Aldrich, Germany.

3.3.1 Materials

Fresh *M. oleifera* plant material was harvested from the south coast of KwaZulu-Natal (GPS co-ordinates: 30°0.51'33"S, 30°54.49'39"E). Leaf material was separated from the stems, washed with distilled water and air dried to remove residual debris. A portion of the leaf material was placed in a freeze dryer (Edwards Wirsam, England) for ±72 h until all moisture was removed. Thereafter it was stored at -16°C for further use.

3.3.2 Preparation of leaf extract samples

Methods used for the preparation of leaf extract samples and biosynthesis of AgNPs were adapted from Veerasamy *et al*., (2011). Extracts were prepared using 10 g of FD leaf material and the equivalent amount of F leaf tissue (in terms of dry weight), in replicates of four. Each replicate was homogenised thoroughly in 50 ml Millipore water (using a IKA T25 digital Ultra-Turrax-T 25 D, Germany) and the final volume was adjusted to 100 ml. Resulting homogenates were transferred to 250 ml Erlenmeyer flasks. Flasks were covered with foil and placed on a mechanical shaker (Labcon, Spo-MP8) at 115 rpm, for 24 h at room temperature. To obtain aqueous extracts, homogenates were subjected to vacuum filtration using Whatman No.1 filter paper (Whatman Limited, England) (Veerasamy, *et al.*, 2011).

3.3.3 Synthesis of AgNPs

An aliquot (5 ml) of aqueous plant extract sample was added to 50 ml of $1x10^{-3}$ M aqueous $AgNO_3$. To drive AgNP formation the reaction mixtures were exposed to direct sunlight. Colour change of the reaction mixtures were monitored to determine nanoparticle formation which is indicated by a dark brown colour. Once colour intensities of the solutions reached a maximum, the vessels were removed from sunlight and stored in darkness at room temperature to prevent agglomeration of the nanoparticles. A 50 ml aliquot of $AgNO_3$ containing 5 ml distilled water was processed as described above and used as a negative control.

3.3.4 UV-Vis Spectral analysis

Further confirmation of AgNP formation by the reduction of Ag^{+} from $AgNO_3$ was achieved by UV-Vis Spectral analysis. Nanoparticle solutions were diluted 1:2 with distilled water (200 µl of nanoparticle sample and 400 µl of distilled water) for these measurements and distilled water was used as a blank. Nanoparticle solutions and the control were simultaneously analysed: absorbance was scanned from 190-900 nm using a UV-Vis spectrometer (Specord 210 Analytikjena, Germany).

3.3.5 Purification and concentration of AgNPs and leaf tissue

The purification and concentration of AgNPs from the final reaction mixture was adopted from the method available in literature (Dipankar and Murugan, 2012). The 55 ml reaction mixture (50 ml $AgNO_3$ + 5 ml leaf extract sample) (n=4) was split into two equal parts and transferred to pre-weighed sterile 50 ml centrifuge tubes (United Scientific, South Africa). The preparations were then centrifuged at 4000 rpm for 2 h (Eppendorf centrifuge 5810 R, Germany), at 4°C. Supernatants were discarded and the pellet washed in 10 ml of distilled water to remove any contaminating plant material before centrifugation for 1 h as per the conditions described above. This wash step was repeated twice to remove water soluble biomolecules such as proteins and cellular metabolites. One half of each replicate was then dried in an oven at 37°C for 24 h to determine the dry mass of the AgNPs (difference between mass of tube + nanoparticles, and mass of tube), whilst the other portion of each replicate was reconstituted in 1 ml of distilled water. The mass of each dried pellet was applied as the equivalent mass of its corresponding reconstituted pellet since each replicate was equally split. Thus, the concentration of AgNPs was determined on a mg/ml basis. This procedure was also used to determine the dry mass of the leaf tissue samples from 5 ml aliquots of the extracts. A comparison of nanoparticle yield between F and FD starting material was made using the mass of the AgNPs attained on a mg AgNP/g dry mass (DM) basis. Dry AgNP samples were kept at room temperature whilst reconstituted samples were stored at 4°C prior to use.

3.3.6 Characterisation and analysis of AgNPs

Characterisation techniques were adopted from Gannimani *et al.*, (2014). Dry nanoparticle preparations were used for Scanning electron microscopy (SEM), Energy dispersive x-ray (EDX) and Fourier transform infrared (FTIR) spectroscopy analysis whereas reconstituted samples were used in Transmission electron microscopy (TEM) (Gannimani, *et al.*, 2014).

3.3.6.1 SEM and EDX analysis

Morphological characteristics of AgNPs were determined using SEM (FEGSEM Zeiss Ultraplus). Purified AgNP mass was placed on a carbon tape stuck to a clean aluminium stub. Samples (n=3) were then coated with gold followed by SEM analysis and simultaneous EDX analysis at 20 kV to determine elemental composition of the particles. AZTEC software (Oxford Instruments NanoAnalysis, Ver 1.2) was used for the analysis.

3.3.6.2 TEM

Transmission electron microscopy was employed to determine concise nanoparticle characteristics such as shape, size and size class distribution. Reconstituted, pure AgNP samples were first sonicated to maintain an even distribution of the nanoparticles in solution prior to TEM analysis. Samples (n=3) were prepared by placing a small amount of nanoparticle solution onto a carbon-coated copper grid which was then dried under a lamp. Sample images were obtained using a JEOL TEM (1010) at an accelerating voltage of 100 kV. The sizes of the nanoparticles and data analysis of the sizes was accomplished using ITEM (Soft imaging system, Germany Ver 5.0**).**

3.3.6.3 FTIR

The purified dry mass of AgNPs were subjected to FTIR analysis (ALPHA-BRUKER, Germany) to identify functional groups capping the surface of the nanoparticles. To determine which of these groups belong to the plant, purified leaf tissue samples were also analysed. Software used for analysis was OPUS Version 6.5.

3.3.7 Biological Assays

3.3.7.1 Strains

Varying concentrations of the reconstituted nanoparticle solutions were screened for bioactivity against the following strains which were from the American Type Culture Collection (ATCC): *Staphylococcus aureus* (43300); *Enterococcus faecalis* (5129) (Gram-positive); *Escherichia coli* (35218); *Pseudomonas aeruginosa* (27853); *Klebsiella pneumoniae* (700603) (Gram-negative); *Candida albicans* (10231); *Candida krusei* (6258) and *Candida parapsilosis* (22019).

3.3.7.2 Antimicrobial agents

Reconstituted AgNPs derived from FD and F leaf extracts were sonicated to maintain an even distribution of the nanoparticles in solution prior to use in the bioassays. Testing was conducted in 96-well flat-bottom microtitre plates (Greiner bio-one, Germany) using final AgNP and leaf tissue concentrations ranging from 6.25 to 200 µg ml^{-1}. The first row (row A) of the plates were reserved for blank/negative and uninhibited growth controls as the wells were filled with media only (Mueller Hinton (MH) for antibacterial testing and Sabouraud-2% dextrose (SD) for antifungal testing, respectively). Aliquots of 100 µl of twofold serial dilutions of AgNPs and leaf tissue (2 x final concentrations) were added for analysis. The commercially available antibiotics: neomycin; gentamicin; ciprofloxacin hydrochloride (bacteria) and amphotericin B (fungi) were used as positive antibiotic controls and assessed in the concentration range of 0.0012 to 200 µg ml^{-1}. Since nanoparticles themselves possess absorption characteristics, a background control plate was set up, in which varying concentrations of AgNPs were plated with the respective media and the absorbance was used for removal of AgNP background absorbance by reducing the results of their corresponding test replicates in sample antibacterial and antifungal assay plates.

3.3.7.3 Antibacterial assay

Bacterial susceptibility was determined according to the broth microdilution method (Schwalbe, *et al.*, 2007). The bacterial strains were freshly inoculated into 50 ml of MH

broth. The cultures were incubated overnight at 37°C in an Infors HT Multitron environmental shaker (United Scientific, South Africa) at 150 rpm. To obtain working suspensions with optical densities in the range of 0.08-0.10 which conform to McFarland's constant, the absorbance of overnight starter cultures was determined spectrophotometrically at a wavelength of 625 nm. The absorbance of experimental suspensions were adjusted by dilution with MH broth. Aliquots (100 µl) of experimental cultures were dispensed into microtitre plates containing 100 µl samples (AgNPs; leaf tissue and antibiotics) as prepared in the previous section and uninhibited growth control wells. The microtitre plate cultures were incubated aerobically for 16-18 h at 37°C. Subsequently, 40 µl of freshly prepared iodonitrotetrazolium chloride (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, INT, 200 µg ml^{-1}) solution was added to all wells and the plates incubated in the dark for 45 minutes at 37°C. The INT reagent, initially colourless, was reduced to a red product following incubation. This reduction was the result of persistent bacterial growth, whilst no colour change denoted the inhibition or lack of bacterial growth. The absorbance was determined at 630 nm using a multimodal plate reader (Biotek Synergy HT, USA) with Gen 5 software (Biotek Synergy HT, USA Ver 2.01.14). The experimental design included two biological replicates and in each instance three technical replicates per sample were used.

3.3.7.4 Antifungal assay

The antifungal activity of AgNPs was determined using the broth microdilution method as prescribed by the NCCLS guidelines (NCCLS, 2002). Strains were inoculated into freshly prepared SD broth (50 ml) and grown aerobically overnight at 30°C in an Infors HT Multitron environmental shaker (United Scientific, South Africa) at 150 rpm. The cells were harvested by centrifugation at 4°C for 5 minutes at 4000 rpm and thereafter re-suspended in 1% saline (10 ml). The absorbance of resuspended starter cultures was determined spectrophotometrically at 600 nm. To conform to McFarland's standards, the cells were diluted using SD broth to achieve optical densities in the range of 0.08-0.10. Once achieved, the working suspension was diluted (1:20) in RPMI 1640 medium (with

L-glutamine, without bicarbonate and phenol red, Biochrom, Berlin). The working suspension was further diluted (1:50) with RPMI 1640 to obtain the final test inoculum concentration of 1-5 x 10^3 CFU ml^{-1}. Aliquots (100 µl) of inoculum were added to all wells of the test microtitre plates except the blank/negative control wells and nanoparticle control plate. The plates were incubated aerobically at 30˚C for 16-20 h. After incubation, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H terazolium salt (MTS, Promega Corporation, Madison, USA) was added to each well and incubated in the dark at 30˚C for 4 h. Thereafter the absorbance was determined at 490 nm. The experimental design included two biological replicates and in each instance three technical replicates per sample were used.

3.3.8 Data analysis

All data were statistically analysed using SPSS version 21. All percentage data obtained from the biological assays were arcsine transformed and analysed for normality (p>0.05) using a Kolmogorov-Smirnov test. Parametric data (which in some cases the consequence of transformation) were subjected to an analysis of variance (*ANOVA*). Differences were considered significant at the 0.05 level and where possible, means were separated by a Tukey post-hoc test. Nanoparticle yield and size were analysed for normality as above and subjected to an Independent sample t-test.

3.4 RESULTS

3.4.1 Confirmation of AgNP synthesis

A primary indication of AgNP formation is represented by a reaction solution colour change to dark brown (Veerasamy, *et al.*, 2011). Addition of $AgNO_3$ to FD and F *M. oleifera* leaf extract samples produced an instantaneous colour change from an initial, yellow solution for both FD and F samples **(Fig. 3.1a & Fig. 3.1b)**, to dark brown solutions **(Fig. 3.1c & Fig. 3.1d)** within 1 h of reaction time.

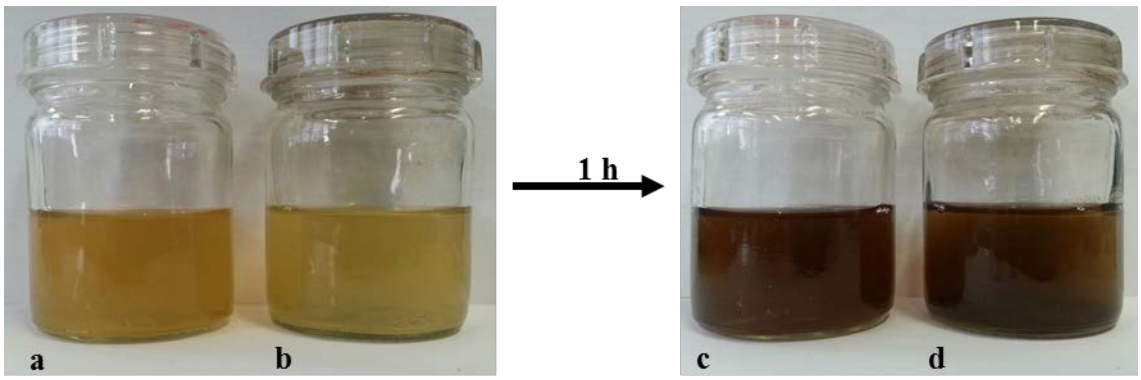

Figure 3.1 Colour change of reaction solutions containing FD (a & c) and F (b & d) leaf extract samples with $AgNO_3$ at 0 h and 1 h.

3.4.2 Characterisation of AgNPs

3.4.2.1 UV-Vis Spectral analysis

The UV-Vis spectral profiles using AgNPs prepared from both leaf sample types displayed absorbance peaks at 440 nm and 450 nm which is characteristic of AgNP containing solutions (Logeswari, *et al.*, 2012). The absence of an absorbance peak at these wavelengths for the control solution containing $AgNO_3$ and distilled water confirmed the involvement of leaf compounds in the reduction of Ag^+ and subsequent generation of AgNPs **(Fig. 3.2)**.

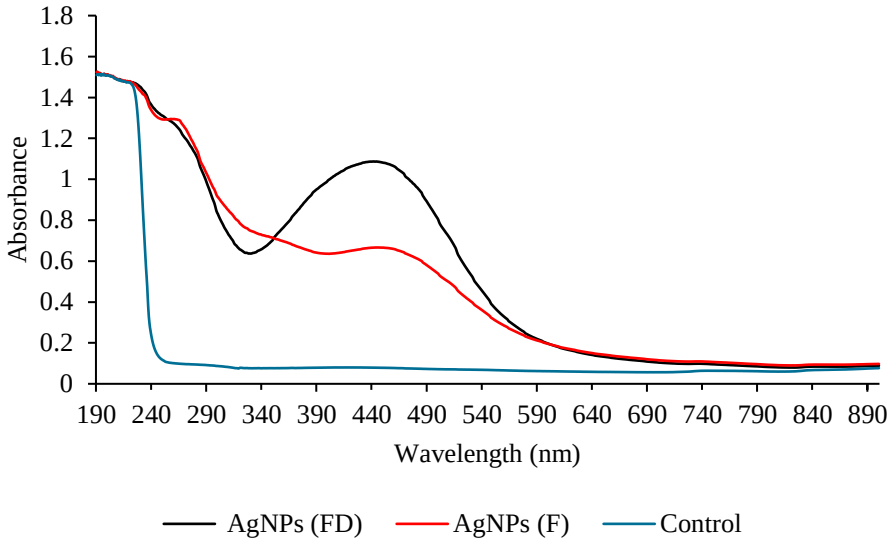

Figure 3.2 UV-Vis spectral profiles of AgNP containing solutions synthesised from FD and F *M. oleifera* leaf extract samples and control solution after 1 h of reaction.

3.4.2.2 Yield analysis

Nanoparticle yield for AgNPs prepared from FD leaf samples (0.91±0.4 mg AgNPs/g leaf tissue dry mass) was statistically comparable (p>0.05) to that obtained for AgNPs prepared from F samples (0.81±0.2 mg AgNPs/g leaf tissue dry mass) **(Fig. 3.3)**.

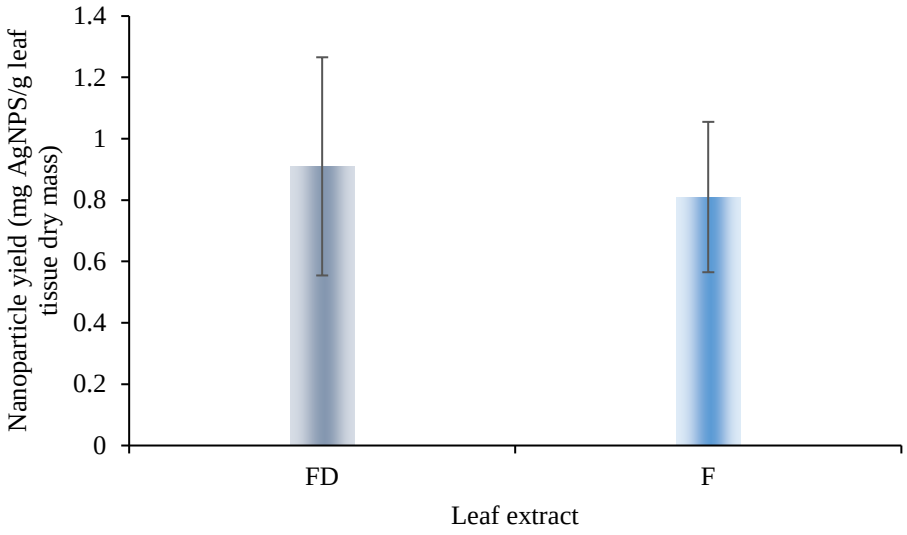

Figure 3.3 AgNP yield from FD and F *M. oleifera* leaf extract samples (mg AgNPs/g leaf tissue dry mass). Values represent mean±SD (n=4). p>0.05 when yield was compared between FD and F leaf extract samples (t-test).

3.4.2.3 SEM and EDX analysis

SEM analysis of purified nanoparticle solutions revealed that the particles were roughly spherical in shape **(Fig. 3.4a & Fig. 3.4c)**. Additionally, the particles were observed to be highly agglomerated, possibly an artefact of the centrifugation and subsequent drying required to prepare AgNP samples for SEM analysis. EDX analysis demonstrated a strongly well defined silver signal along with weak carbon, oxygen and nitrogen peaks, with the latter weaker signals probably representing surface biomolecule capping structures originating from the leaf extracts **(Fig. 3.4b & Fig. 3.4d)**.

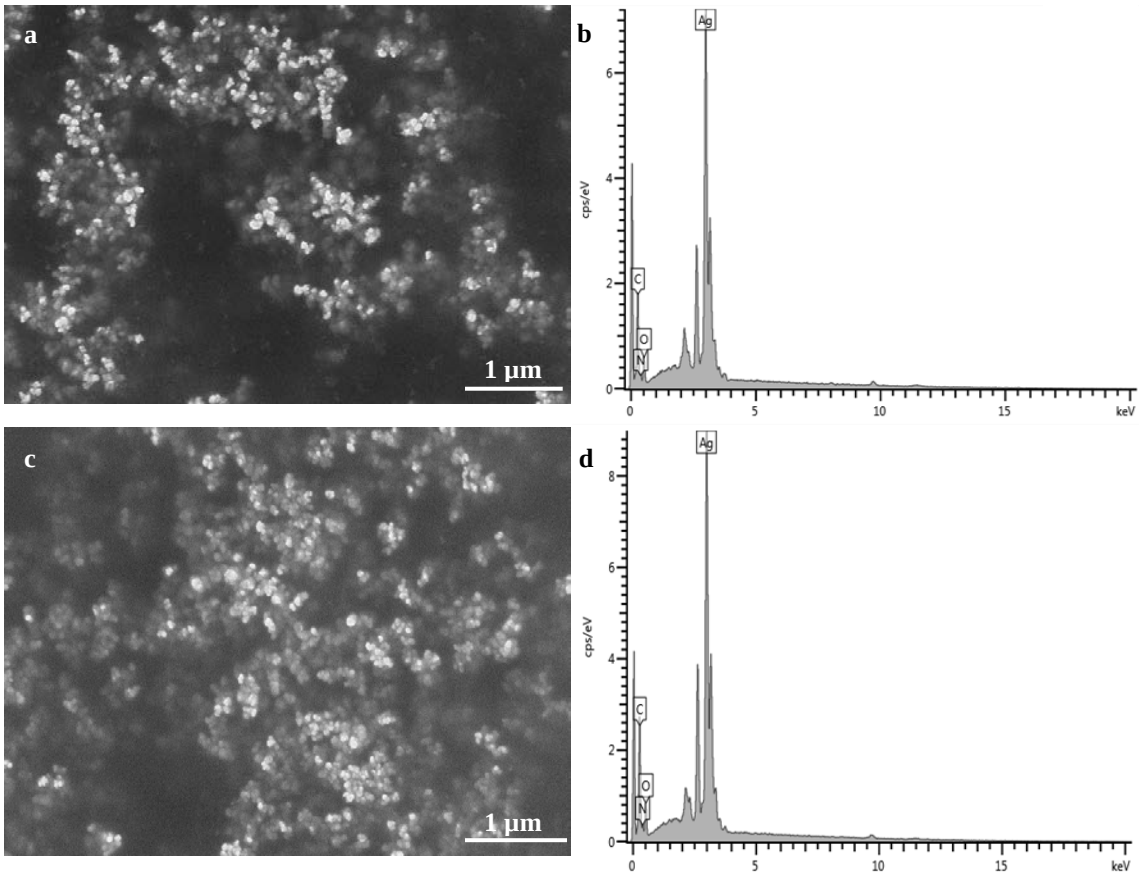

Figure 3.4 SEM images and EDX spectra of AgNPs prepared from FD (a and b) and F (c and d) leaf extract samples, respectively.

3.4.2.4 TEM

TEM was used to characterise the morphology of AgNPs in terms of their shape, size and size class distribution. Sample preparation for TEM analysis did not require physical dehydration stated for SEM studies above. As mentioned previously, the latter procedure may promote agglomeration thereby limiting concise characterisation. TEM analysis clearly showed that the particles are spherical in shape and appear well dispersed **(Fig. 3.5a & Fig. 3.5b).** Comparing the morphological characterisation techniques used in this study, it seems that TEM analysis strongly suggests that compounds originating from leaf extracts exhibit the dual role of reducing Ag^{+} as well as stabilising synthesised particles. Additionally TEM analysis revealed the particles possess narrow size distributions with average sizes of 11±4.3 nm and 9±4.2 nm for FD and F AgNPs, respectively. **(Fig. 3.6a & Fig. 3.6b)**. $p>0.05$ when sizes were compared between FD and F leaf extract samples (t-test).

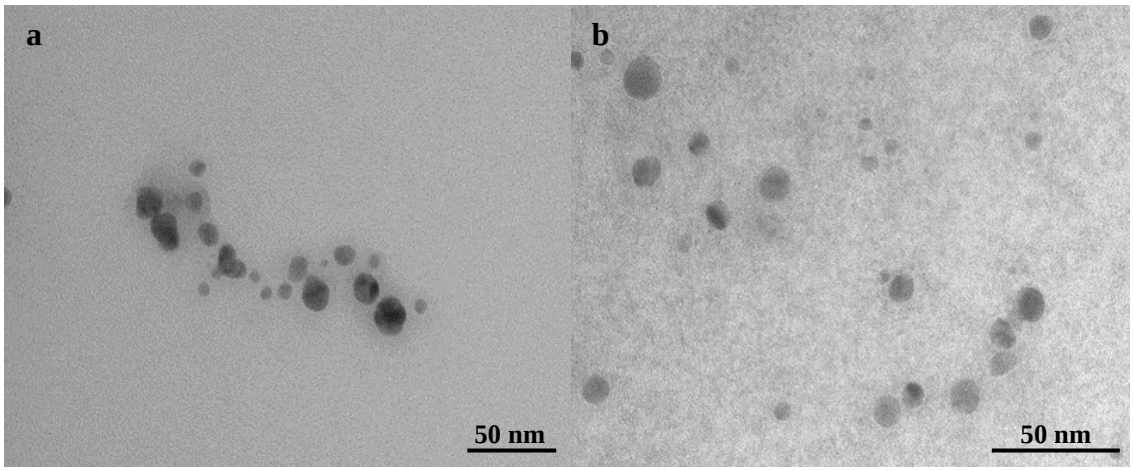

Figure 3.5 TEM images of AgNPs produced from (a) FD and (b) F leaf samples.

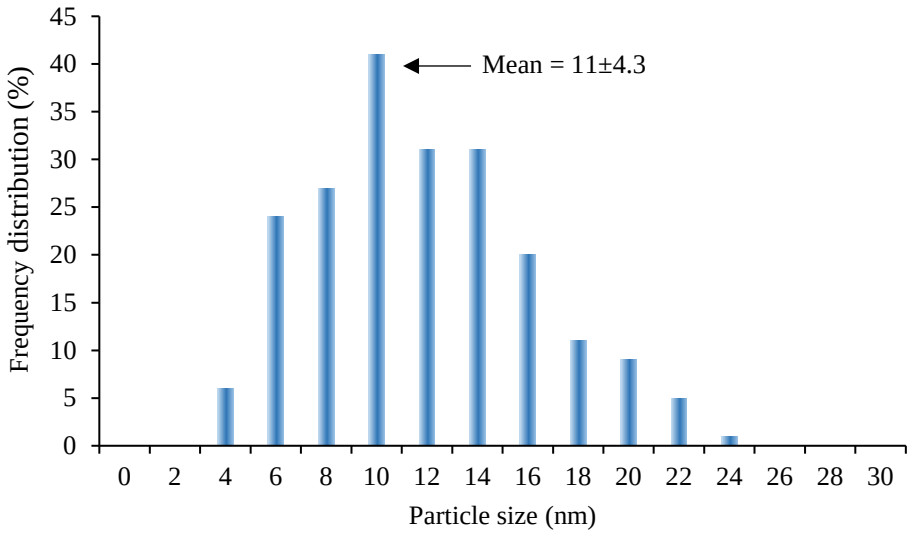

Figure 3.6a Size distribution of AgNPs synthesised from FD leaf samples of *M. oleifera.* (n=100, $p>0.05$ when compared with AgNPs prepared from F samples, t-test).

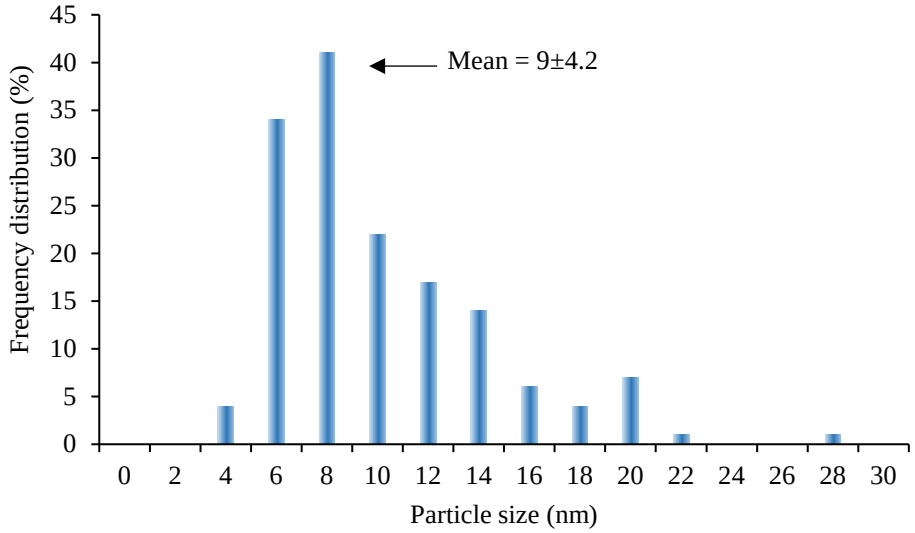

Figure 3.6b Size distribution of AgNPs synthesised from F leaf samples of *M. oleifera.* (n=100, $p>0.05$, when compared with AgNPs prepared from FD samples, t-test).

3.4.2.5 FTIR

FTIR analysis of FD and F leaf material and the corresponding AgNPs **(Fig. 3.7)** revealed that similar or identical functional groups in both leaf sample types were responsible for binding and reducing Ag^{+}. Functional groups corresponding to absorbance peaks in the range of 3000-3300; 2800-3000; 1626; 1400-1550; 1380-1403 and 1000-1100 cm^{-1} were observed. These peaks are known to be associated with stretching vibrations of hydroxyl groups in alcohols or phenolic compounds (Chandran, *et al.*, 2006); CH_2 and CH_3 functional groups; C=C groups of aromatic compounds or C=O groups of carboxylic acids (Shankar, *et al.*, 2004); Amide I ($CONH_2$) and Amide II (CONH) groups (Awwad, *et al.*, 2013); geminal methyls; ether linkages and C-O or C-O-C functional groups, respectively (Shankar, *et al.*, 2004). In contrast these peaks, were either absent or displayed drastically reduced intensities in the corresponding AgNP spectra. This seems to suggest that functional groups in this region are involved in the reduction and stabilisation of the AgNPs by their subsequent oxidation. Additionally, ether linkages, C-O and C-O-C functional groups are thought to be groups of flavones, terpenoids and polysaccharides, in the leaf biomass that are adsorbed on the metal surface (Shankar, *et al.*, 2004).

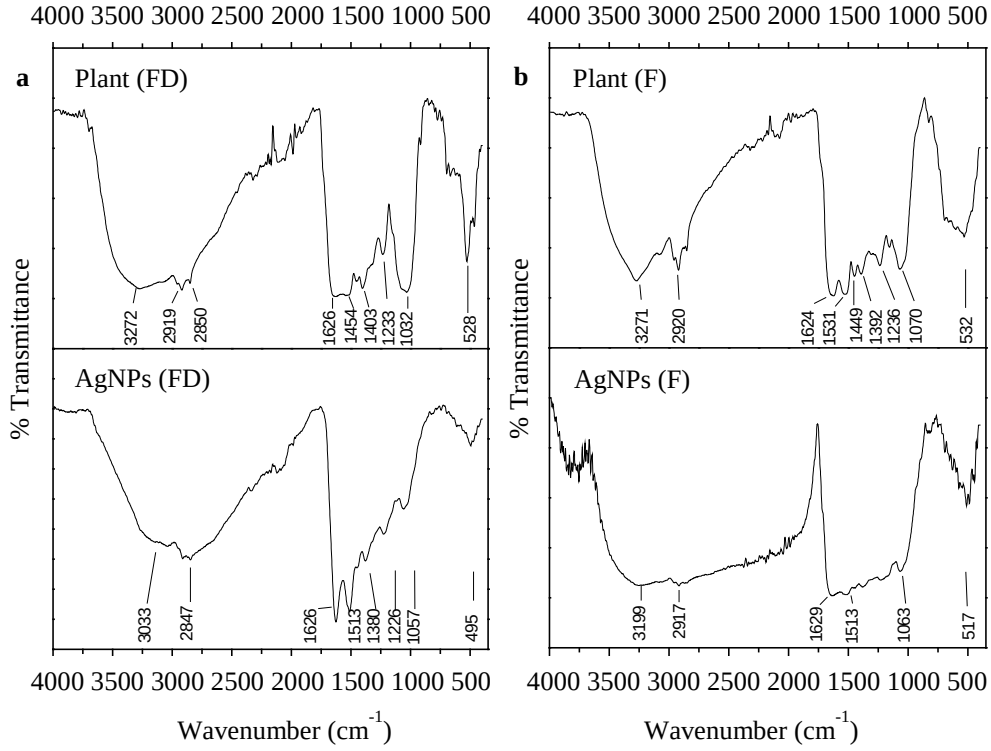

Figure 3.7 (a) FTIR spectrum of FD leaf sample (plant) and AgNPs derived from FD leaf sample; (b) FTIR spectrum of F leaf sample (plant) and AgNPs derived from F leaf sample.

3.4.5 Bioactivity of AgNPs

Bioactivity against microbes in this study was determined in terms of the minimal inhibitory concentration (MIC) *i.e.* the lowest concentration of an AgNP solution to inhibit 80 to > 80% growth of a microorganism was considered as a positive inhibition result.

3.4.5.1 Antibacterial studies

The AgNPs synthesised from both leaf sample types displayed inhibition of Gram-negative and Gram-positive-bacteria **(Table 3.1)**. Solutions of AgNPs from both leaf preparations at a concentration of 25 µg ml^{-1} inhibited the growth of *K. pneumoniae, P. aeruginosa and S. aureus* strains. Interestingly, a two-fold decrease in concentration (12.5 µg ml^{-1}) of both AgNP preparations was observed to inhibit the growth of *E coli, and E.*

faecalis. Importantly, no significant differences were noted for the antibacterial activities of AgNP preparations within each bacterial strain (p>0.05). Both *Moringa* leaf extract samples did not display inhibitory activities throughout the concentration range (200-6.25 µg ml^{-1}) evaluated in this study (data not shown). Commercially available antibiotics namely: neomycin, ciprofloxacin hydrochloride and gentamicin displayed MIC's ranging from 0.39 to 200 µg ml^{-1} and in certain instances displayed no inhibition. Attractively, the data seems to suggest that AgNPs prepared in this study are not only more effective at lower concentrations but also display a broader susceptible bacterial spectrum range. It is noteworthy that *E. faecalis* was insensitive to three commercial antibiotics but was susceptible to the AgNPs prepared from both leaf extracts.

Table 3. 1 MIC_{80} of AgNPs synthesised from *M. oleifera* leaf extract samples and commercial antibiotics against bacterial strains

	MIC (µg ml^{-1})				
Organism	**AgNPs synthesised from FD tissue**	**AgNPs synthesised from F tissue**	**Neomycin**	**Ciprofloxacin hydrochloride**	**Gentamicin**
E. coli	12.5±0.01	12.5±0.01	6.25±0.04	0.39±0.01	1.56±0.07
E. faecalis	12.5±0.01	12.5±0.00	NI*	NI*	NI*
K. pneumoniae	25±0.01	25±0.02	25±0.04	25±0.02	6.25±0.07
P. aeruginosa	25±0.01	25±0.01	NI*	1.56±0.05	200±0.06
S. aureus	25±0.03	25±0.04	NI*	1.56±0.09	NI*

Data are reported as the mean ± SD. (p>0.05, for FD and F AgNP preparations within each microorganism tested, *ANOVA*) NI = No inhibition. *Commercial antibiotics showed no inhibition in terms of effecting 80 % kill at their evaluated concentrations.

3.4.5.2 Antifungal studies

Three *Candida* reference strains were employed to evaluate the antifungal potency of the AgNP preparations **(Table 3.2)**. It was observed that both AgNP preparations displayed similar inhibitory activities (p>0.05) for each fungal strain. A concentration of 6.25 µg ml^{-1} AgNPs inhibited the growth all fungal strains whilst the leaf extract samples did not display inhibition (data not shown). The pharmaceutical bioactive amphotericin B displayed a MIC of 0.02 µg ml^{-1} across all evaluated fungal strains.

Table 3. 2 MIC_{80} of AgNPs synthesised from *M. oleifera* leaf extract samples and a commercial antifungal preparation against fungal strains

	MIC (µg ml^{-1})		
Organism	**AgNPs synthesised from FD tissue**	**AgNPs synthesised from F tissue**	**Amphotericin B**
C. albicans	6.25±0.03	6.25±0.02	0.02±0.00
C. krusei	6.25±0.02	6.25±0.02	0.02±0.00
C. parapsilosis	6.25±0.02	6.25±0.01	0.02±0.00

Data are reported as the mean ± SD. (p>0.05, for FD and F AgNP preparations within each microorganism tested, *ANOVA*).

3.5 DISCUSSION

Given the wide ranging of applications touted for AgNPs in recent years, many researchers have focused on the development of modified or novel synthetic strategies for AgNPs as opposed to the use of conventional methods which are strongly associated with toxic environmental footprints (Patra and Baek, 2014). This study reports on the antimicrobial activities of AgNPs prepared from leaf extract samples of the medicinal tree species, *M. oleifera* by a clean green cost effective synthetic method.

Up until now, green synthesis of AgNPs from leaf extract samples at room temperature were shown to yield AgNPs at relatively slow rates. For instance, complete bioreduction of Ag^+ from *Aloe vera* and *Iresine herbstii* leaf extracts occurred at 24 h and 168 h, respectively (Chandran, *et al.*, 2006, Dipankar and Murugan, 2012). The aforementioned disadvantage of certain green synthetic routes requires optimisation to be a viable alternative to the use of conventional chemical methods for AgNP synthesis. With this in mind, several researchers have implemented physical parameters such as heat to drive green nanoparticle formation. Prasad and Elumalai (2011) reported rapid synthesis (10 minutes) of AgNPs from *M. oleifera* leaf powder at a reaction temperature range of 60-80˚C (Prasad and Elumalai, 2011). In a separate study, Veerasamy *et., al* (2011) reported the temperature profile (37-90˚C) of AgNP synthesis from mangosteen leaf extracts. In their study, maximum AgNP formation occurred after 1 h at 75˚C. In addition, the group also reported that incubation time is an important consideration in AgNP synthesis since extended exposure to heat promotes agglomeration of AgNPs to yield undesirable particles of larger size (Veerasamy, *et al.*, 2011). As suggested by previous studies, sunlight irradiation, a freely available bio-energy resource, was used to drive nanoparticle

formation (Annadhasan, *et al.*, 2012, Karimi Zarchi, *et al.*, 2011, Rastogi and Arunachalam, 2011). Reduction of Ag^{+} by *M. oleifera* leaf extracts occurred instantaneously in the presence of direct sunlight and complete reduction was observed after 1 h. This was indicated by colour change of the reaction solutions to dark brown as it has previously been reported that AgNPs display a brown colour in aqueous solution due to their surface plasmon phenomenon (Veerasamy, *et al.*, 2011). Additionally, this phenomenon also facilitates their detection in the UV-region and hence, provided a further indication of their formation (Kim, *et al.*, 2007). Previous studies on sunlight driven AgNP synthesis using *Allium sativum* (garlic extract); *Andrachnea chordifolia* ethanol leaf extract and the sodium salt of *N*-cholyl amino acids revealed that sunlight rapidly enhanced nanoparticle formation with reduction times observed in the range of 5-15 minutes (Annadhasan, *et al.*, 2012, Karimi Zarchi, *et al.*, 2011, Rastogi and Arunachalam, 2011). This seems to suggest that the time required for nanoparticle formation varies according to the nature of reducing agent. However, considering the reducing times reported for the use of sunlight irradiation as a primary energy source, this technology constitutes a promising alternative to the use of non-renewable energy sources for AgNP production.

Yield analysis indicated no significant differences between AgNPs prepared from FD or F leaf sample types. Although, it has previously been reported that the dehydration of plant material increases the availability of phytocompounds and would therefore increase the bioactivity of the plant material (in terms of their reducing ability) (Larson, 1988). To date, AgNP recovery from leaf samples is relatively unreported. However, AgNPs produced in this study are comparable to previously derived AgNPs prepared from various organ extracts of *Amaranthus dubius* (Sigamoney, *et al.*, 2016).

Microscopic analysis (SEM) provided an inconclusive indication that AgNPs were spherically shaped. In addition, the particles appeared to be highly agglomerated, possibly owing to the physical dehydration exerted during the SEM sample preparation procedure (Sadeghi and Gholamhoseinpoor, 2015, Sigamoney, *et al.*, 2016). In contrast, TEM analysis of aqueous AgNP samples provided unequivocal evidence that the prepared AgNPs were spherical in shape. Furthermore, the particles were observed to be stable and dispersed, even within aggregates. This finding is in accordance with previously derived AgNPs from *M. oleifera* leaf extracts (Prasad and Elumalai, 2011, Sathyavathi, *et al.*,

2011) suggesting that the leaf extract of *M. oleifera* presents a dual functionality of reducing Ag^+ and subsequently stabilising nascent AgNPs. Interestingly, a previous study on the influence of AgNP shape on bioactivity suggested that spherically shaped AgNPs display superior antimicrobial activities when compared to rod shaped AgNPs (Pal, *et al.*, 2007).

Characteristically, nanoparticles are small in size (1-100 nm) and harbor a large surface area that constitutes their reactivity (Christian, *et al.*, 2008). In recent years however, studies have suggested that the bioactivity of AgNPs occur in a size-dependent manner with smaller particles exerting better bioactivities than larger particles (Jeong, *et al.*, 2014, Martinez-Castanon, *et al.*, 2008). Size class distribution studies of the AgNPs prepared in this study indicate that the particles had narrow size distributions with average diameters of 9±4.2 nm and 11±4.3 nm for AgNPs prepared from the respective F and FD leaf extract samples. No significant differences were noted for AgNP preparations in terms of their size. Previously synthesised AgNPs from the leaf extracts of *Ziziphora tenuior* and *Mimusops elengi* at room temperature were found to range in size of 20-83 nm (Prakash, *et al.*, 2013, Sadeghi and Gholamhoseinpoor, 2015). The introduction of heat in AgNP synthesis has been reported to significantly decrease the size of synthesised AgNPs (Veerasamy, *et al.*, 2011). However, AgNPs synthesised from *M. oleifera* leaf powder extract in a heat driven process produced particles with an average diameter of 57 nm (Prasad and Elumalai, 2011). It can be suggested that incubation at room temperature or heat strongly influences the size of generated nanoparticles according to the nature of reducing agent. Attractively, the use of sunlight in the aforementioned AgNP synthesis from *A. sativum* and *A. chordifolia* extracts were reportedly 7.3 nm and 3.4 nm, respectively. These findings and the data reported in this study strongly favour the use of sunlight for the production of AgNPs with small sizes.

EDX analysis revealed that in addition to silver, oxygen; carbon and nitrogen were also present on the nanoparticle surface. Similar results were reported by Song and Kim (2009) and believed to originate from biomolecule capping structures (Song and Kim, 2009). Importantly, AgNPs with oxidised surfaces have been reported to induce the formation of “holes” on the surface of bacteria (Smetana, *et al.*, 2008), which suggests that the presence of oxygen on the surface of the nanoparticles prepared here may enhance their bactericidal activities.

FTIR analysis suggest the presence of phenolic compounds, flavones, terpenoids and polysaccharides in the leaf biomass as potential Ag^{+} capping agents. Interestingly, *M. oleifera* leaves are known to be a rich source of the antioxidant phenolic compounds: quercetin and kaempferol (Siddhuraju and Becker, 2003). In particular, quercetin belonging to the flavonoid group of phenolic compounds has previously been shown to possess strong chelating ability (Makarov, *et al.*, 2014). This tentatively suggests that the reducing of Ag^{+} by quercetin may potentially reduce the cytotoxic effects of AgNPs produced in this study.

This is the first report on characterisation of *M. oleifera* leaf derived AgNPs using sunlight in terms of their MIC bioactivities. Antimicrobial screening of AgNPs revealed strong inhibition of Gram-negative and Gram-positive bacteria as well as various fungal species, irrespective of whether they were derived from F or FD leaf sample material (p>0.05). More specifically, MIC values were recorded in the range 12.5-25 µg ml^{-1} for bacterial strains and 6.25 µg ml^{-1} for fungal strains.

The effective inhibition of both Gram-negative and Gram-positive bacteria by AgNPs derived from *M. oleifera* leaf extracts is of great significance as it demonstrates their broad-spectrum antibacterial activity. It also indicates that the mode of action is not affected by the difference in membrane stabilities of the bacteria since Gram-positive bacteria contain a thick peptidoglycan layer whilst Gram-negative bacteria possess a rigid outer membrane structure composed of lipids and lipoproteins. Contrasting results were observed in a similar study where AgNPs (5-35 µg ml^{-1}) synthesised from reducing agents such as D-glucose and hydrazine, displayed effective activity towards Gram-negative bacteria but minimal activity against Gram-positive bacteria (Shrivastava, *et al.*, 2007). Additionally, in the previously mentioned study on AgNPs derived from *M. oleifera* leaf powder, no activity against *K. pneumoniae* was noted. This might be due to the fact that AgNPs in the present study are ± 6 times smaller than those in the previous study (Prasad and Elumalai, 2011). It is worth mentioning that *E. faecalis* displayed resistance to all three commercial antibiotics but was susceptible to both AgNPs prepared in this study. This is significant since this bacterium has previously been reported for its intrinsic resistance and further acquisition of resistance genes leading to the emergence of *E. faecalis* as a nosocomial pathogen that is a refractory to most therapeutic agents (Paulsen, *et al.*, 2003). In addition, the conjugative transfer of high levels of resistance genes from

E. faecalis to *S. aureus* has been long established and may probably explain the increased incidence of *S. aureus* resistance to both neomycin and gentamicin observed in this study (Noble, *et al.*, 1992).

Both AgNP preparations displayed good antimicrobial activities against all fungal test strains. These findings are contradictory to what has been suggested by Nabikhan *et al*., (2010) that AgNPs are better antibacterial agents rather than antifungal agents. This notion was supported by the finding that AgNPs are able to freely adhere to and penetrate bacterial cells, whilst they are unable to enter fungal cells at low concentrations (Nabikhan, *et al.*, 2010). The AgNPs produced in this study show no alteration in bioactivity between bacterial and fungal organisms, suggesting that their mode of action remains unaffected by the difference in the cell wall structures of these organisms. Importantly, AgNPs produced in this study display bioactivities at concentration doses that are generally associated with limited or no cytotoxic potential (Dipankar and Murugan, 2012) and therefore provide promising broad-spectrum antimicrobial alternatives to orthodox antibiotics which may provide much needed relief to the health care delivery system.

3.6 CONCLUSION

Development of greener alternatives for AgNP synthesis has emerged as an important branch of nanotechnology due to the urgent need for novel, bio-compatible antimicrobial agents. In the present study, AgNPs with desirable physical attributes were produced equally well from FD and F leaf extract samples of *M. oleifera* by the use of sunlight irradiation as an alternative energy source. The produced AgNPs were comparable in terms of their yield, morphology and bioactivities and seemingly suggests that freeze-drying of the leaf material may be useful in their preservation for future nanoparticle synthesis. Importantly, AgNPs displayed a broad-spectrum antimicrobial susceptibility range and therefore represent as promising antimicrobial agents with potential applications in pharmaceutical industries for controlling pathogenic microorganisms. However, more research needs to be done on characterising the capping agents that may harbor potential antioxidant activities. Issues of safety and toxicity around their use for treatment in humans also needs to be considered but this nevertheless presents a simple,

economical and environmentally friendly method for the rapid synthesis of bioactive AgNPs from the leaves of one of the most widely investigated and used medicinal tree species, *M. oleifera*.

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Chapter 4

RESEARCH RESULTS II

Antimicrobial activities of silver nanoparticles derived from bacterial extracts using green synthesis

Antimicrobial activities of silver nanoparticles derived from bacterial extracts using green synthesis

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4.1 ABSTRACT

The development and adaptation of green synthetic methods for silver nanoparticle (AgNP) synthesis has flourished into a widely researched area of nanotechnology. Particularly, in the development of AgNPs for application as antimicrobial agents. The current study encompasses the synthesis, characterisation and antimicrobial evaluation of AgNPs derived from the cell free supernatant (CFS) of the bacteriocin producer *Pediococcus acidilactici* and its comparison to AgNPs derived from CFS of a non-bacteriocin producing *Bacillus subtilis* strain. Interestingly, sunlight irradiation, a relatively unexplored primary energy source was able to photo-catalyse nanoparticle formation completely within 1 hour of exposure as observed by reaction color change and UV-Vis spectral analysis. Transmission electron microscopy (TEM) confirmed that both AgNP preparations were consistent in their spherical shape, with average diameters of 16 nm and 12 nm for AgNPs derived from CFSs of *P. acidilactici* and *B. subtilis*, respectively. Fourier transform infrared spectroscopy (FTIR) and Energy dispersive X-ray (EDX) analysis identified the presence functional groups on the surface of both AgNPs which may originate from protein molecules. This seemingly suggests that AgNP formation from both extracts occurred via the oxidation of proteins by Ag^{+}. Bioactivity screening revealed that AgNPs were generally comparable and exhibited broad-spectrum antimicrobial activity against both bacterial and fungal strains. Importantly, our findings suggest that AgNPs derived from *P. acidilactici* and *B. subtilis* extracts exhibit potential for application as broad-spectrum antimicrobial agents. Moreover, this approach seems to be cost-effective, eco-friendly and can be easily up-scaled for large production.

4.2 INTRODUCTION

The increase in antimicrobial resistance to commercial antibiotics is of tremendous concern that threatens the well-being of global populations. In fact, the problem has escalated to an extent that a post-antibiotic era, one in which fatalities could potentially arise from common infections and minor injuries, is a scary reality for the 21st century (World Health Organisation, 2014).

Microbial development of drug resistance is a complex phenomenon driven by various intricate factors, of which the foremost is through the improper use of antibiotics (World Health Organisation, 2001). This is due to the inherent ability of microbes to rapidly develop resistance mechanisms when a subset of the microbial population survives exposure to the drug (Seil and Webster, 2012). In addition, microbial exogenous gene transfer mechanisms further accelerate the spread of resistance genes between microorganisms (Tomasz, 2006). Although the development of novel antimicrobial agents is a daunting and economically draining exercise, nevertheless an urgent uncovering of such antimicrobial agents is becoming increasingly unavoidable. (Ventola, 2015).

The antimicrobial effects of silver salts although not well understood has been in use since ancient times (Kim, *et al.*, 2007). However with the discovery of the antibiotic penicillin, the use of silver salts and other antimicrobial preparations became obsolete (Alexander, 2009). As mentioned above, with the increasing prevalence of microbial drug-resistance, the application of this metal using modern technologies to generate its nano-particulate form has resurfaced as a promising antimicrobial candidate (Kim, *et al.*, 2007, Rai, *et al.*, 2009). Currently, a host of methodologies for AgNP synthesis comprises various physical and chemical strategies (El-Nour, *et al.*, 2010, Iravani, *et al.*, 2014). However, the experimental nature of these methods (*i.e*. the use of toxic chemicals and/or adverse reaction conditions) is much cause for debate which limits the application of produced nanoparticles (Thakkar, *et al.*, 2010). In recent years, ongoing advancements in nanotechnology have led to the development of greener alternatives for AgNP synthesis wherein environmentally inert capping agents are utilised in the reduction of metal ions (Dauthal and Mukhopadhyay, 2016, Rajput, *et al.*, 2016).

Developing biological approaches for AgNP synthesis is an ongoing branch of nanotechnology and researchers are currently focused on identifying new reducing or capping agents for production of AgNPs with superior bioactivities (Hazarika, *et al.*, 2016). To this end, a variety of capping substrates from diverse biological sources have been explored for AgNP synthesis including plants, algae, fungi, yeast, bacteria, mammalian cells and viruses (Makarov, *et al.*, 2014, Pantidos and Horsfall, 2014, Thakkar, *et al.*, 2010). Incidentally, laboratory based nanoparticle synthesis from extracts of a biological resource is favored over their biosynthesis in living organisms due to several distinct advantages including: short reaction times; ease of handling extracts compared to whole organisms; high yield of nanoparticles and importantly, the lack of costly downstream processing required to obtain to the produced particles (Das, *et al.*, 2014, Gannimani, *et al.*, 2014, Liu, *et al.*, 2005).

The use of bacterial extracts as reducing agents in the production of AgNPs have been reported with optimised reaction times that are comparable to chemical approaches for AgNP synthesis (Shahverdi, *et al.*, 2007). Furthermore, the abundance and rapid growth rate of bacteria coupled to the possibility of their genetic modification for increased production of a well-defined capping substrate marks these organisms as promising candidates for AgNP production (Pantidos and Horsfall, 2014). In this regard, the present study evaluates the bio efficacies of AgNPs generated from an antimicrobial peptide producing bacterium to their counterparts generated from a non-producing strain.

Pediococcus acidilactici PAC1.0. is a Gram-positive homofermentive microbe belonging to the family Lactobacillaceae and is renowned for its inhibitory effects against the pathogenic bacterium, *Listeria monocytogenes*. This inhibition attributed to the plasmid-encoded production of the bacteriocin pediocin PA-1, a small (<10 kDa) heat-stable peptide produced extracellularly. More concisely, the current study was conceptualised on the basis that nanoparticle formation using capping structures present in spent culture supernatants of *P. acidilactici* may potentially produce AgNPs with higher antimicrobial efficacies as those compared to AgNPs from a non-bacteriocin producing strain (Marugg, *et al.*, 1992).

To date, the use of sunlight irradiation, a relatively new source of energy has been reported to drive AgNP formation using plant extracts and to a lesser extent, bacterial extracts (Karimi Zarchi, *et al.*, 2011, Rastogi and Arunachalam, 2011, Wei, *et al.*, 2012). In this study, the use of this energy source to drive nanoparticle formation from bacterial extracts was explored and the resulting AgNPs characterised in terms of their morphology and bioactivity. The data presented in this study seemingly suggest that AgNPs produced from both bacteriocin containing and non-containing extracts are interesting candidates for further development towards controlling drug-resistant microbial infections.

4.3 MATERIALS AND METHODS

All chemicals, solvents and media used in this study were of analytical grade and purchased from Merck (Pty) Ltd, South Africa, unless stated otherwise. Antibiotics were purchased from Sigma Aldrich, Germany.

4.3.1 Strains

The bacteriocin producing strain *P. acidilactici* PAC1.0.was kindly provided Professor L.M.T Dicks from the Department of Microbiology, Stellenbosch University. A non-producing *Bacillus subtilis* strain was obtained from the Department of Biochemistry, University of Kwa-Zulu Natal. Strains used in bioactivity assays were from the American Type Culture Collection (ATCC): *Staphylococcus aureus* (43300); *Enterococcus faecalis* (5129) (Gram-positive); *Escherichia coli* (35218); *Pseudomonas aeruginosa* (27853); *Klebsiella pneumoniae* (700603) (Gram-negative); *Candida albicans* (10231); *Candida krusei* (6258) and *Candida parapsilosis* (22019).

4.3.2 Preparation of CFS

Preparation of CFSs was conducted by the method of Gonzalez and Kunka (1987). Briefly, 100 ml of de Man, Rogosa and Sharpe (MRS) broth was individually inoculated (1%) with overnight cultures of *P. acidilactici* and *B. subtilis* grown in the same medium. Cultures were incubated statically at 35˚C for 24 h. Thereafter, the pH of the cultures were adjusted to pH 6.0 and the cells were removed by centrifugation (Eppendorf centrifuge 5810 R, Germany) at 4000 rpm for 10 min. The CFSs were filter sterilised (0.45 µm filters, (GVS, USA) and subsequently stored at 4˚C (Gonzalez and Kunka, 1987).

4.3.3 Assay for bacteriocin production

The spot on lawn method was used to confirm bacteriocin production by *P. acidilactici* and non-production by *B. subtilis* (Pucci, *et al.*, 1988). Mueller Hinton (MH) plates were overlaid with room temperature equilibrated soft agar (0.75% w/v) that was seeded with test culture cells (≈ 10^6 cells ml^{-1}). An aliquot (10 µl) of CFS was spotted onto overlaid MH plates. Inoculated plates were incubated at 37˚C for 18 h. A clear zone of inhibition around spotted inoculum areas indicated a positive result for the presence of bacteriocins in the CFS.

4.3.4 Protein determination of CFSs

The total protein content of CFSs was determined using the microplate procedure of the BCA Protein Assay (Pierce 23225, Thermo Scientific, USA) according to the manufacturer's specifications. Distilled water was used as a solvent to make up dilutions of the CFSs. Absorbance of test wells at 562 nm were determined using a multimodal plate reader (Biotek Synergy HT, USA) with Gen 5 software (Biotek Synergy HT, USA Ver 2.01.14). Subsequently, the protein concentrations of both CFSs were equilibrated to contain a final protein content of 50 mg ml^{-1}.

4.3.5 Synthesis of AgNPs

Preparation of reaction solutions for AgNP synthesis was adapted from Shahverdi *et al*., (2007). An aliquot (5 ml) of *P. acidilactici* CFS extract was added to 50 ml of aqueous $1x10^{-3}$ M silver nitrate ($AgNO_3$) contained in a 100 ml clear glass reaction vessel. The aforementioned reaction was also performed using 5 ml of *B. subtilis* CFS extract. To drive formation of AgNPs, the reaction mixtures were exposed to direct sunlight. Colour change of the reaction mixtures were monitored to determine nanoparticle formation which is indicated by a dark brown colour. Once colour intensities of the solutions reached a maximum, the vessels were removed from sunlight and stored in darkness at room temperature to prevent agglomeration of the nanoparticles. A 50 ml aliquot of $AgNO_3$ containing 5 ml distilled water was processed as described above and used as a negative control (Shahverdi, *et al.*, 2007).

4.3.6 UV-Vis Spectral analysis

Preliminary characterisation of synthesised AgNPs was carried out by UV-Vis spectral analysis. Nanoparticle solutions were first diluted 1:2 with distilled water (200 µl of nanoparticle sample and 400 µl of distilled water). Thereafter the absorbance of diluted AgNPs and $AgNO_3$ was scanned from 190 to 800 nm using a UV-Vis spectrophotometer (Specord 210 Analytikjena, Germany). Distilled water was used as a blank to adjust the baseline.

4.3.7 Purification and concentration of AgNPs

The purification and concentration of AgNPs from the final reaction mixture was adopted from the method available in literature, (Dipankar and Murugan, 2012). Reaction mixtures were split into two equal parts and transferred to pre-weighed sterile 50 ml centrifuge tubes (United scientific, South Africa). The preparations were then centrifuged at 4000 rpm for 2 h (Eppendorf centrifuge 5810 R, Germany), at 4˚C. Supernatants were discarded and the pellet washed in 10 ml of sterile distilled water before centrifugation for 1 h as per the conditions described above. This wash step was repeated twice to remove water soluble biomolecules such as proteins and cellular metabolites. One half of each replicate was then dried in an oven at 37˚C for 24 h to determine the dry mass of the AgNPs (difference between mass of tube + nanoparticles, and mass of tube), whilst the other half of each replicate was reconstituted in 1 ml of distilled water. The mass of each dried pellet was applied as the equivalent mass of its corresponding reconstituted pellet since each replicate was equally split. Thus, the concentration of AgNPs was determined on a mg/ml basis. Dry AgNP samples were kept at room temperature whilst reconstituted samples were stored at 4˚C prior to use.

4.3.8 Characterisation and analysis of AgNPs

Characterisation techniques were adopted from Gannimani *et al.*, (2014). Dry AgNP samples were used for Scanning electron microscopy (SEM), Energy dispersive x-ray (EDX) and Fourier transform infrared spectroscopy (FTIR) analysis whereas reconstituted samples were used in Transmission electron microscopy (TEM) analysis (Gannimani, *et al.*, 2014).

4.3.8.1 SEM and EDX analysis

Morphological characteristics of AgNPs were determined using SEM (FEGSEM Zeiss Ultraplus). Purified nanoparticle mass was placed on a carbon tape stuck to a clean aluminium stub. Samples were then coated with gold and observed at 10000 X magnification followed by EDX analysis at 20 kV to determine the elemental composition of the particles. AZTEC software (Oxford Instruments NanoAnalysis, Ver 1.2), was used for the analysis.

4.3.8.2 TEM

Transmission electron microscopy was employed to determine concise nanoparticle characteristics such as shape, size and size class distribution. Reconstituted, pure AgNP samples were first sonicated to maintain an even distribution of the nanoparticles in solution prior to TEM analysis. Samples (n=3) were prepared by placing a small amount of nanoparticle solution onto a carbon-coated copper grid which was then dried under a lamp. Sample images were obtained using a JEOL TEM (1010) at an accelerating voltage of 100 kV. The sizes of the nanoparticles and data analysis of the sizes was accomplished using ITEM (Soft imaging system, Germany Ver 5.0).

4.3.8.3 FTIR

The purified dry mass of AgNPs were subjected to FTIR analysis (Perkin Elmer Spectrum One, USA) to identify biological functional groups capping the surface of the nanoparticles. The respective spectra were obtained using Spectrum Analysis Software.

4.3.9 Biological Assays

4.3.9.1 Antimicrobial agents

Reconstituted AgNPs derived from bacterial CFSs were sonicated to maintain an even distribution of the nanoparticles in solution prior to use in the bioassays. Testing was conducted in 96-well flat-bottom microtitre plates (Greiner bio-one, Germany) using final AgNP concentrations ranging from 6.25 to 200 µg ml^{-1}. The first row (row A) of the plates were reserved for blank/negative and uninhibited growth controls as the wells were filled with media only (MH for antibacterial testing and Sabouraud-2% dextrose (SD) for antifungal testing, respectively) Aliquots of 100 µl of twofold serial dilutions of AgNPs (2 x final concentrations) were added for analysis. The commercially available

antibiotics: neomycin; gentamicin; ciprofloxacin hydrochloride (bacteria) and amphotericin B (fungi) were used as positive antibiotic controls and assessed in the concentration range of 0.0012 to 200 µg ml^{-1}. Since nanoparticles themselves possess absorption characteristics, a background control plate was set up, in which varying concentrations of AgNPs were plated with the respective media. The absorbance was used for removal of AgNP background absorbance by reducing the results of their corresponding test replicates in sample antibacterial and antifungal assay plates.

4.3.9.2 Antibacterial assay

Bacterial susceptibility was determined according to the broth microdilution method (Schwalbe, *et al.*, 2007). The bacterial strains were freshly inoculated into 50 ml of MH broth. The cultures were incubated overnight at 37˚C in an Infors HT Multitron environmental shaker (United Scientific, South Africa) at 150 rpm. To obtain working suspensions with optical densities in the range of 0.08-0.10 which conform to McFarland's constant, the absorbance of overnight starter cultures was determined spectrophotometrically at a wavelength of 625 nm. The absorbance of experimental suspensions were adjusted by dilution with MH broth. Aliquots (100 µl) of experimental cultures were dispensed into microtitre plates containing 100 µl samples (AgNPs; antibiotics) as prepared in the previous section and uninhibited growth control wells. The nanoparticle control plate did not receive the bacterial inoculum but rather 100 µl of fresh MH broth was added to each well. The microtitre plate cultures were incubated aerobically for 16-18 h at 37˚C. Subsequently, 40 µl of freshly prepared iodonitrotetrazolium chloride (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, INT, 200 µg ml^{-1}) solution was added to all wells and the plates incubated in the dark for 45 minutes at 37˚C. The INT reagent, initially colourless, was reduced to a red product following incubation. This reduction was the result of persistent bacterial growth, whilst no colour change denoted the inhibition or lack of bacterial growth. The absorbance was determined at 630 nm using a multimodal plate reader (Biotek Synergy HT, USA) with Gen 5 software (Biotek Synergy HT, USA Ver 2.01.14). The experimental design included two biological replicates and in each instance three technical replicates per sample were used.

4.3.9.3 Antifungal assay

The antifungal activity of AgNPs was determined using the broth microdilution method as prescribed by the NCCLS guidelines (NCCLS, 2002). Strains were inoculated into freshly prepared SD broth (50 ml) and grown aerobically overnight at 30˚C in an Infors HT Multitron environmental shaker (United Scientific, South Africa) at 150 rpm. The cells were harvested by centrifugation at 4˚C for 5 minutes at 4000 rpm and thereafter re-suspended in 1% saline (10 ml). The absorbance of resuspended starter cultures was determined spectrophotometrically at 600 nm. To conform to McFarland's standards, the cells were diluted using SD broth to achieve optical densities in the range of 0.08-0.10. Once achieved, the working suspension was diluted (1:20) in RPMI 1640 medium (with L-glutamine, without bicarbonate and phenol red, Biochrom, Berlin). The working suspension was further diluted (1:50) with RPMI 1640 to obtain the final test inoculum concentration of 1-5 x 10^3 CFU ml^{-1}. Aliquots (100 µl) of inoculum were added to all wells of the test microtitre plates except the blank/negative control wells and nanoparticle control plate. The plates were incubated aerobically at 30˚C for 16-20 h. After incubation, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H terazolium salt (MTS, Promega Corporation, Madison, USA) was added to each well and incubated in the dark at 30˚C for 4 h. Thereafter the absorbance was determined at 490 nm. The experimental design included two biological replicates and in each instance three technical replicates per sample were used.

4.3.10 Data analysis

All data were statistically analysed using SPSS version 21. All percentage data obtained from the biological assays were arcsine transformed and analysed for normality (p>0.05) using a Kolmogorov-Smirnov test. Parametric data (which in some cases the consequence of transformation) were subjected to an analysis of variance (*ANOVA*). Differences were considered significant at the 0.05 level and where possible, means were separated by a Tukey post-hoc test. Nanoparticle size was analysed for normality as above and subjected to an Independent sample t-test.

4.4 RESULTS

4.4.1 Bacteriocin production

The extracellular release of pediocin PA-1 by *P. acidilactici* was confirmed by the presence of clear inhibition zones around areas spotted with CFS. Only *E. faecalis* displayed sensitivity to pediocin PA-1 (16 mm) (**Fig. 4.1a**) whilst no sensitivity was displayed by *E. coli, K. pneumoniae, P. aeruginosa* and *S. aureus.* As expected, CFS of *B. subtilis* which is a bacteriocin non-producer was inactive. The commercial antibiotic neomycin (200 µg ml^{-1}) was active against *E. coli* (11 mm) (**Fig. 4.1b**), and *K. pneumoniae* (9 mm) (result not shown) whilst no inhibition was displayed against *P. aeruginosa, S. aureus* and *E. faecalis.*

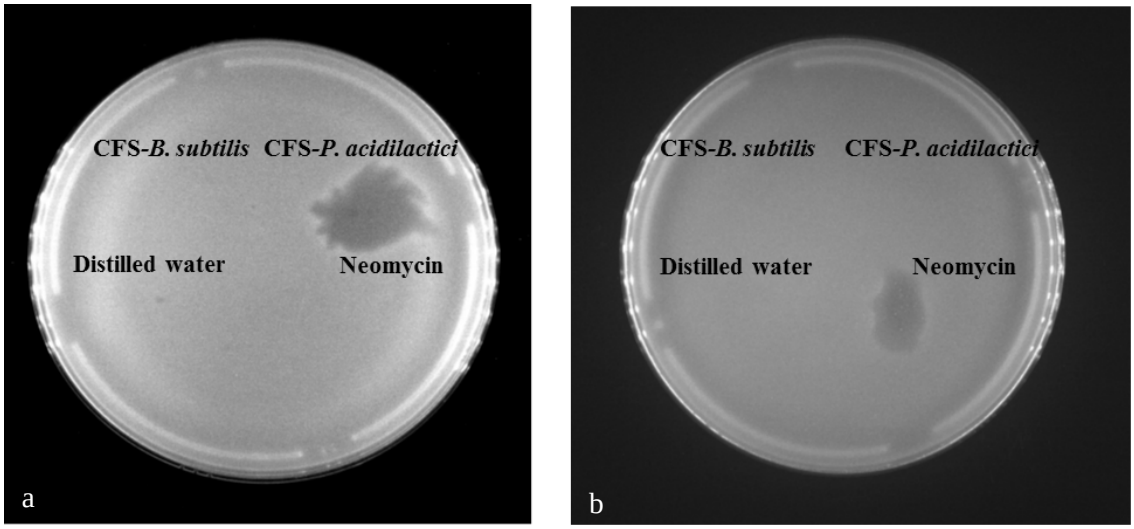

Figure 4.1 Spot-on-lawn assay plates of *E. faecalis* (a) and *E. coli* (b), respectively.

4.4.2 Preparation of AgNPs from CFSs

The reaction mixtures for AgNP synthesis from CFS of both bacterial strains initially displayed a faint yellow colour **(Fig. 4.2a & Fig. 4.2c)**. Upon exposure of reaction vessels to direct sunlight, a brown colour immediately started to develop which signified the reduction of Ag^{+} to the resulting AgNPs (Veerasamy, *et al.*, 2011). A maximum brown colouration intensity was observed at 1 hour indicating completion of AgNP synthesis **(Fig. 4.2b & Fig. 4.2d)**. No colour change was observed in the negative control solution (result not shown).

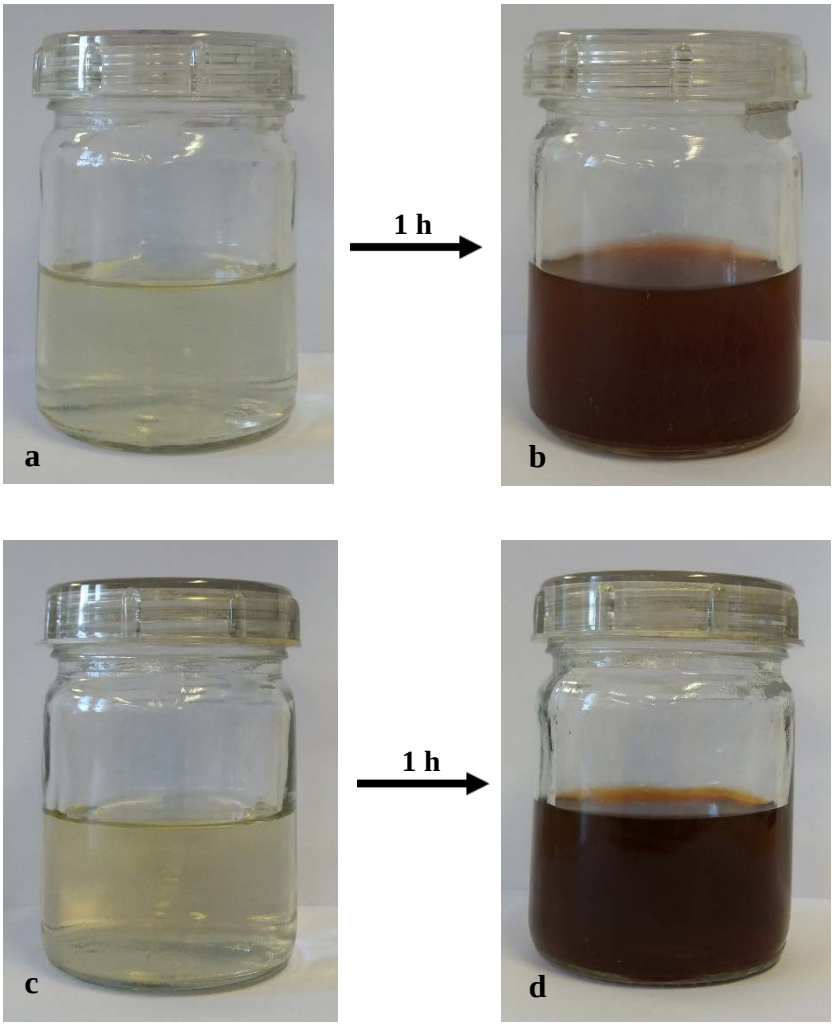

Figure 4.2 Colour change of reaction solutions containing CFSs of *P. acidilactici* (a & b) and *B. subtilis* (c & d) with $AgNO_3$ at 0 h and 1 h, respectively.

4.4.3 Characterisation of AgNPs

4.4.3.1 UV-Vis Spectral analysis

The spectral profiles of both AgNP containing solutions displayed broad peaks in the range of 340-530 nm **(Fig. 4.3)**. Absorbance associated at wavelengths in this range are typical of spherical AgNPs due to their surface plasmon (Kim, *et al.*, 2007). Furthermore, broadened peaks indicated that the AgNPs are polydispersed (Peddi and Sadeh, 2015). There were no peaks observed for the negative control. This confirms that the CFS of each bacterium reduced Ag^+ for the subsequent generation of AgNPs.

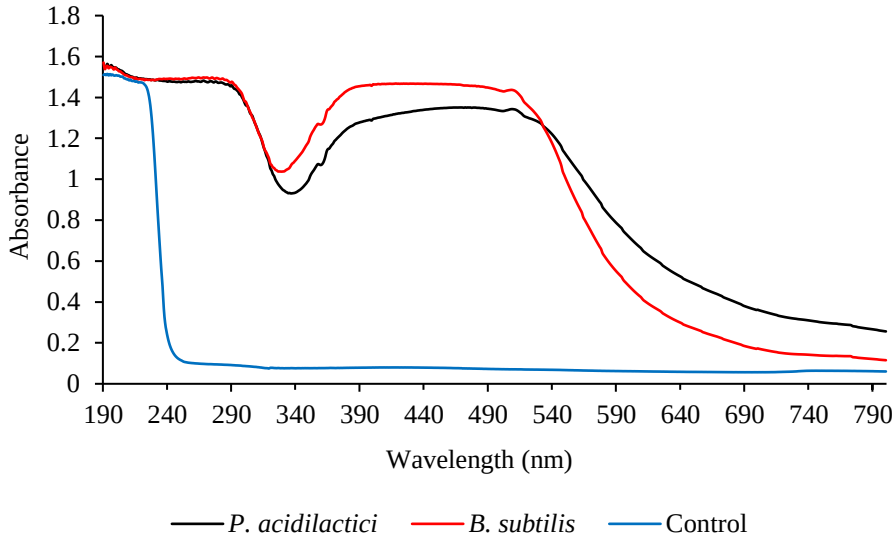

Figure 4.3 UV-Vis spectral profiles of AgNP containing solutions derived from CFSs of *P. acidilactici* and *B. subtilis* and control solution after 1 h of reaction.

4.4.3.2 SEM and EDX analysis

SEM analysis of purified AgNP mass provided a vague indication that both nanoparticle preparations are spherical in shape **(Fig. 4.4a & Fig. 4.4c)**. Furthermore, the particles appear to be agglomerated, possibly because of the nature of sample preparation comprising of several centrifugation steps and their subsequent dehydration. As such, conclusive morphological characteristics could not be determined with SEM analysis. EDX spectral analysis of both AgNP preparations revealed a strong well defined silver signal at approximately 3 keV, typical for metallic silver (Jyoti, *et al.*, 2016). In addition, weaker signals corresponding to carbon, oxygen and nitrogen were observed and they possibly originate from biomolecule capping structures bound to the AgNP surface **(Fig. 4.4b & Fig. 4.4d)**.

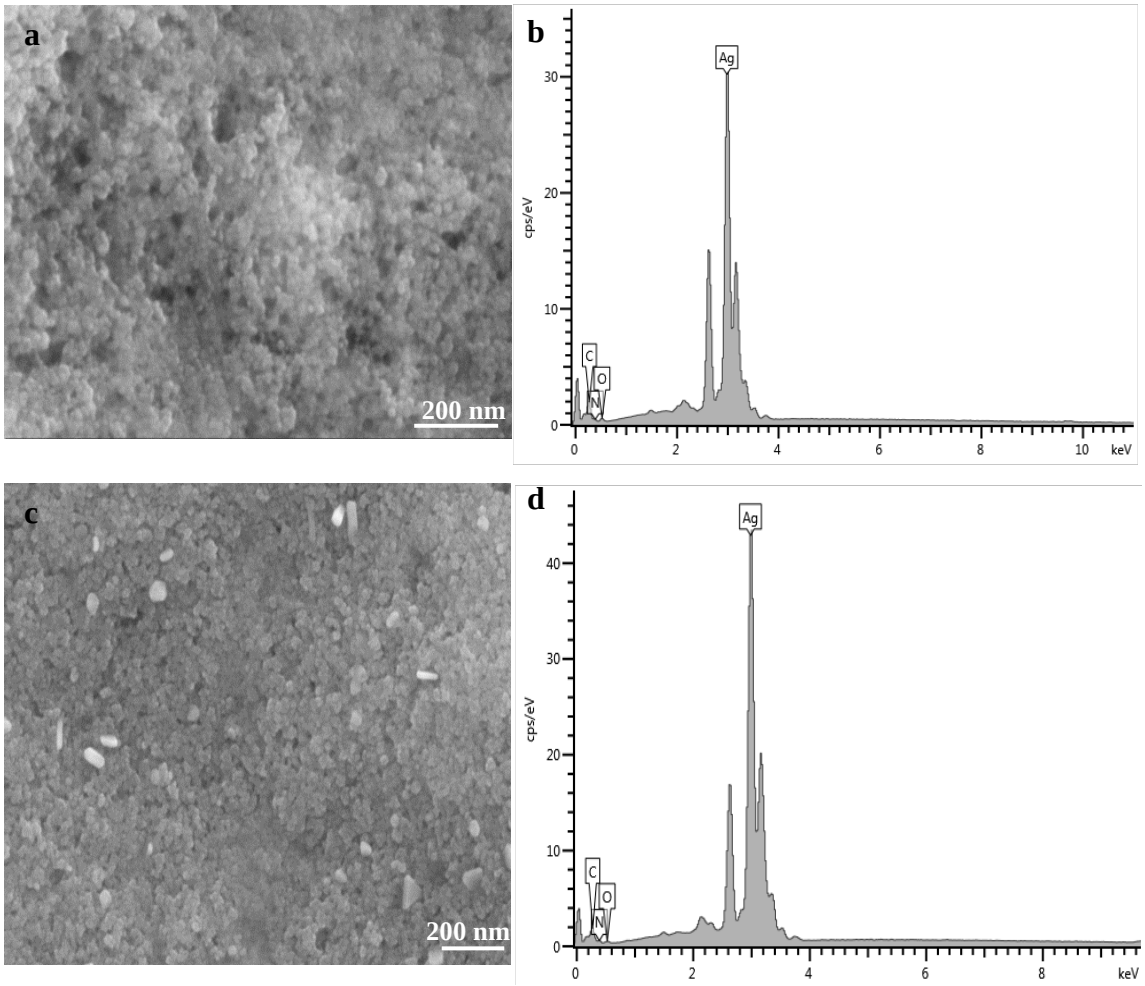

Figure 4.4 SEM images and EDX spectra of AgNPs derived from CFSs of *P. acidilactici* (a and b) and *B. subtilis* (c and d), respectively.

4.4.3.3 TEM

TEM analysis of AgNPs from both preparations confirmed that nanoparticles are spherical in shape and appear well dispersed (**Fig. 4.5a & Fig. 4.5b**). In addition, the analysis did not require samples to be subjected to physical dehydration, which as previously described in SEM analysis, could potentially lead to nanoparticle agglomeration thereby limiting their precise characterisation. Very interestingly, TEM analysis also revealed that the prepared AgNPs from both bacterial CFSs seem to have a surface coating. Size and size class distribution studies indicated narrow size distributions (**Fig. 4.6a & Fig. 4.6b**) with average diameters of 16±3.9 nm and 12±3.1 nm for AgNPs prepared from *P. acidilactici* and *B. subtilis* CFS (p>0.05, t-test), respectively.

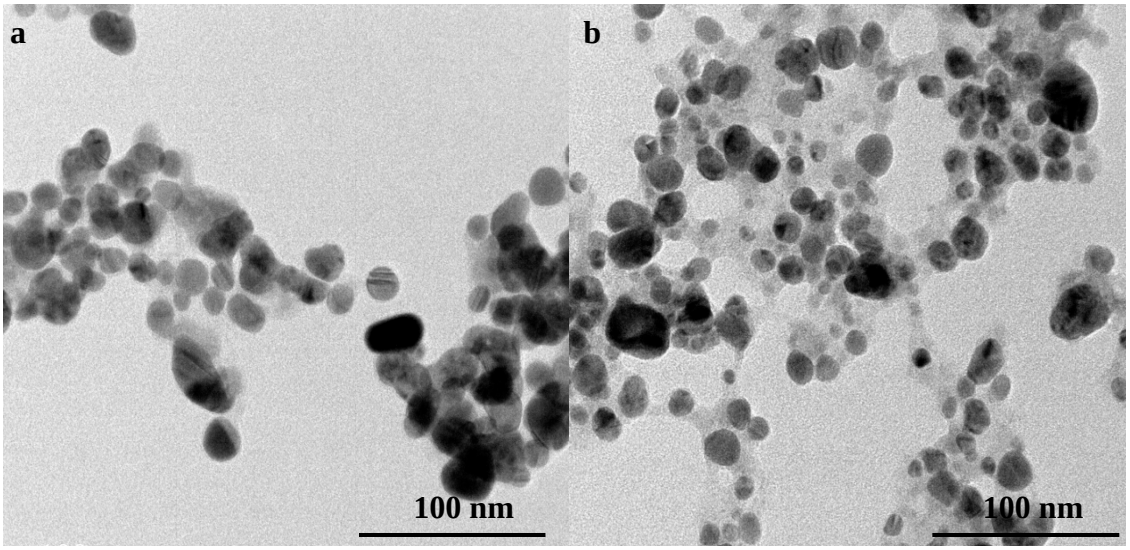

Figure 4.5 TEM images of AgNPs derived from CFS of (a) *P. acidilactici* and (b) *B. subtilis*.

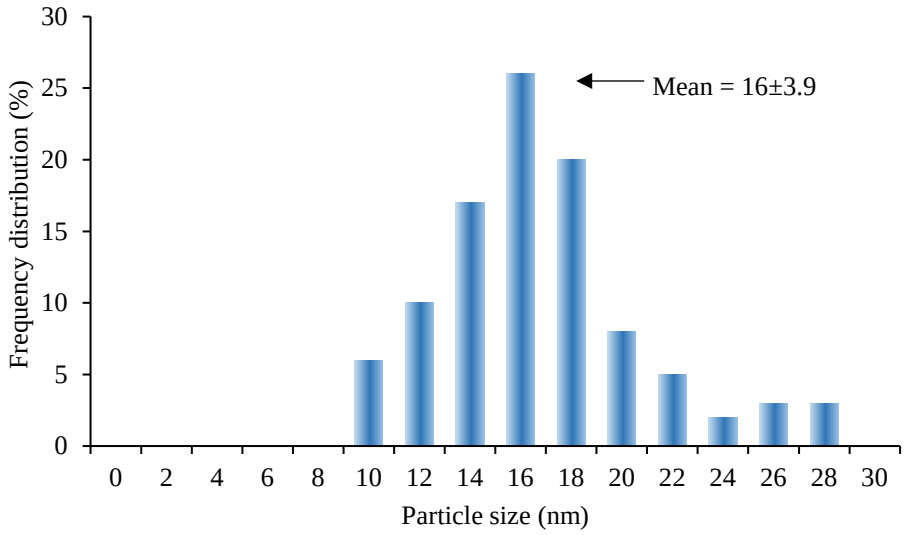

Figure 4.6a Size class distribution of AgNPs derived from *P. acidilactici* CFS (n=100, $p>0.05$ when compared with AgNPs prepared from *B. subtilis* CFS, t-test).

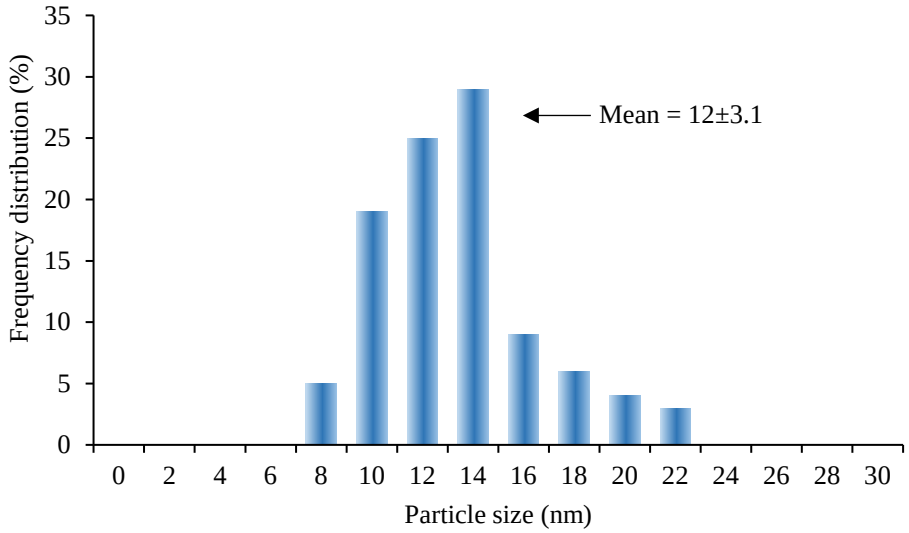

Figure 4.6b Size class distribution of AgNPs derived from *B. subtilis* CFS (n=100, p>0.05 when compared with AgNPs prepared from *P. acidilactici* CFS, t-test).

4.4.3.4 FTIR

FTIR analysis was conducted to determine the biomolecules responsible for capping and subsequent synthesis of AgNPs. For the analysis, dry preparations of purified AgNPs were used. AgNPs prepared from CFS of *P. acidilactici* indicated major absorbance peaks at 3095; 1564 and 1400 cm^{-1} (**Fig. 4.7a**). These peaks are believed to arise from N-H stretching vibrations of primary amines (NH_2) and secondary amines (NH) and carbonyl vibrations of amide I ($CONH_2$) and amide II (CONH) groups (Awwad, *et al.*, 2013, Gomaa, 2017, Jeevan, *et al.*, 2012). Likewise, analysis of AgNPs from CFS of *B. subtilis* indicated major peaks at 3448; 3146; 2875; 1574 and 1400 cm^{-1} which also correspond to amine and amide functional groups (**Fig. 4.7b**).

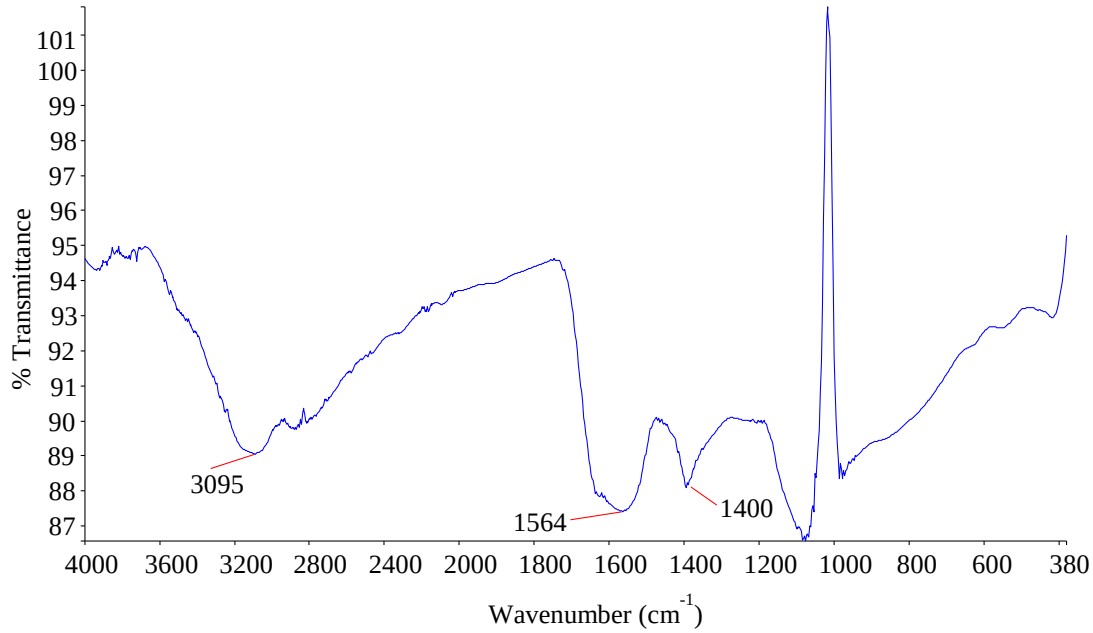

Figure 4.7a FTIR spectrum of AgNPs derived from *P. acidilactici* CFS.

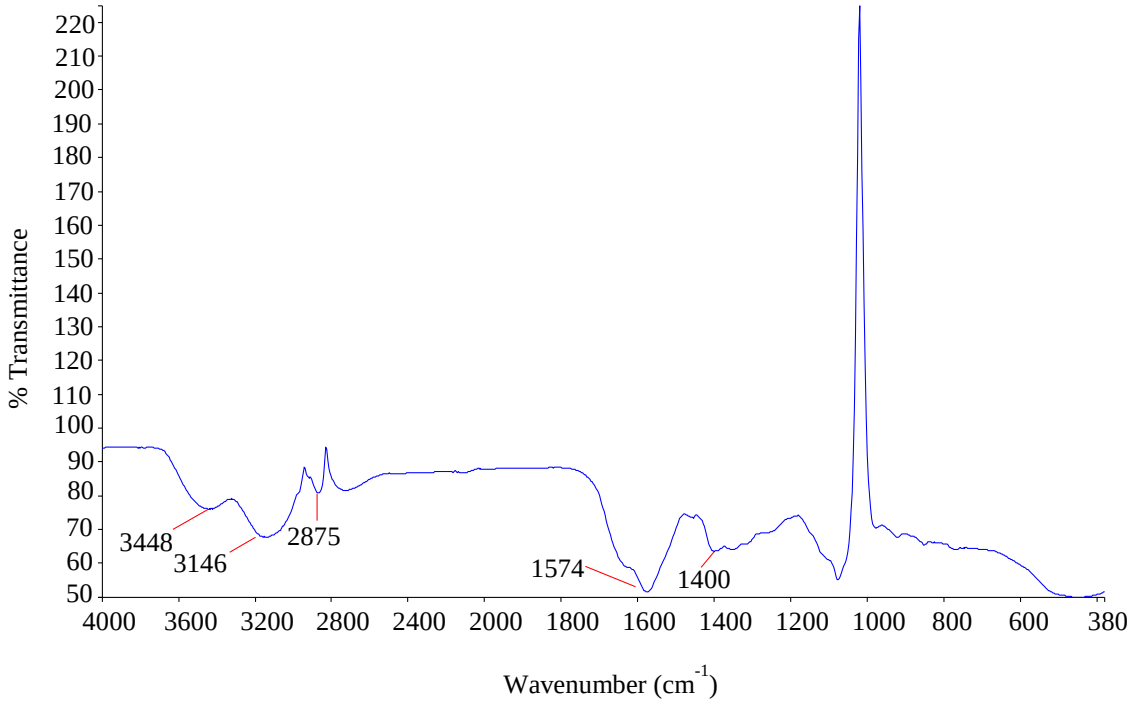

Figure 4.7b FTIR spectrum of AgNPs derived from *B. subtilis* CFS.

4.4.4 Bioactivity of AgNPs

In the current investigation, the antimicrobial effect of varying concentrations (6.25-200 µg ml^{-1}) of bacterial-derived AgNPs was quantitatively assessed based on the growth of cells treated with AgNPs in comparison to the growth of non-treated cells. Bioactivity was determined using the INT and MTS based microbroth dilution methods for bacteria and fungi, respectively (NCCLS, 2002, Schwalbe, *et al.*, 2007). The results are presented in terms of the minimal inhibitory concentration (MIC) *i.e*. the lowest concentration of an AgNP solution to inhibit 80 to > 80% growth of a microorganism was considered as a positive inhibition result.

4.4.4.1 Antibacterial studies

AgNP preparations from both bacterial CFSs displayed inhibition against Gram-positive and Gram-negative bacteria **(Table 4.1)**. The MIC's of the AgNP solutions ranged from 6.25-25 µg ml^{-1}. No significant differences were noted for the bioactivity of AgNPs (p>0.05). However, a noticeable exception was observed with *E. faecalis*, which was susceptible to AgNPs prepared from *P. acidilactici* CFS at a two-fold decreased concentration when compared to AgNPs prepared from *B. subtilis* CFS (p<0.05). It is noteworthy, that this bacterium, out of all test strains, displayed sensitivity to pediocin-PA1. This seemingly suggests that pediocin PA-1 may be a participating capping agent in Ag^{+} reduction and subsequently enhances the bioactivity of its nanoparticle formulation against sensitive strains. The commercially available antibiotics: neomycin; ciprofloxacin hydrochloride and gentamicin displayed MIC's ranging from 0.39 to 200 µg ml^{-1} and in certain instances displayed no inhibition. Attractively, the data seems to suggest that in certain instances, AgNPs prepared in this study are not only more effective at lower concentrations but also display a broader susceptible bacterial spectrum range. It is worth mentioning that *E. faecalis* was insensitive to three commercial antibiotics but was susceptible to the AgNPs prepared from both CFS extracts.

Table 4. 1 MIC_{80} of AgNPs synthesised from CFSs and commercial antibiotics against bacterial strains

	MIC (µg ml^{-1})				
Organism	**AgNPs from *P. acidilactici* CFS**	**AgNPs from *B. subtilis* CFS**	**Neomycin**	**Ciprofloxacin hydrochloride**	**Gentamicin**
E. coli	6.25±0.02^{a}	6.25±0.00^{a}	6.25±0.04	0.39±0.01	1.56±0.07
E. faecalis	6.25±0.04^{a}	12.5±0.02^{b}	NI*	NI*	NI*
K. pneumoniae	25±0.02^{a}	25±0.00^{a}	25±0.04	25±0.02	6.25±0.07
P. aeruginosa	12.5±0.02^{a}	12.5±0.03^{a}	NI*	1.56±0.05	200±0.06
S. aureus	25±0.02^{a}	25±0.02^{a}	NI*	1.56±0.09	NI*

Data are reported as the mean ± SD. Values followed by a or b superscript alphabets are significantly different. (p<0.05, for AgNP preparations within each microorganism tested, *ANOVA*) NI = No inhibition. *Commercial antibiotics showed no inhibition in terms of effecting 80 % kill at their evaluated concentrations.

4.4.4.2 Antifungal studies

Three *Candida* reference strains were employed to evaluate the antifungal potency of the AgNP preparations **(Table 4.2)**. MIC's for AgNPs from *B. subtilis* CFS ranged from 6.25-12.5 µg ml^{-1} whilst AgNPs from *P. acidilactici* CFS displayed a MIC of 6.25 µg ml^{-1} across all evaluated fungal strains. The bioactivities between AgNP preparations were statistically significant (p<0.05) in the case of *C. albicans* and *C. krusei*. The pharmaceutical bioactive amphotericin B displayed a MIC of 0.02 µg ml^{-1} across all fungal reference strains.

Table 4. 2 MIC_{80} of AgNPs synthesised from CFS and a commercial antifungal preparation against fungal strains

	MIC (µg ml^{-1})		
Organism	**AgNPs from *P. acidilactici* CFS**	**AgNPs from *B. subtilis* CFS**	**Amphotericin B**
C. albicans	6.25±0.03^{a}	12.5±0.01^{b}	0.02±0.00
C. krusei	6.25±0.02^{a}	12.5±0.04^{b}	0.02±0.00
C. parapsilosis	6.25±0.02^{a}	6.25±0.01^{a}	0.02±0.00

Data are reported as the mean ± SD. Values followed by a or b superscript alphabets are significantly different. (p<0.05, for AgNP preparations within each microorganism tested, *ANOVA*)

4.5 DISCUSSION

AgNPs destined for antimicrobial application require processing strategies that are clean and economical which can be easily up-scaled for large production. To this end, the use of nanotechnology to formulate AgNPs from a variety of biological capping substrates and non-renewable energy sources are well researched (Singh, *et al.*, 2016, Velusamy, *et al.*, 2016). In this study we report on the use of sunlight, as a viable and freely available bio-energy resource to drive AgNP formation from bacterial capping substrates.

Bio-reduction of Ag^+ into AgNPs by biological extracts can be easily followed by a colour change of reaction solution to dark brown (Dipankar and Murugan, 2012). Previous AgNP preparation from an *Enterobacteria* CFS extract has been reported for its exceptional reduction time, with complete colour change observed within 5 minutes at room temperature (Shahverdi, *et al.*, 2007). However, this largely depends on the mechanism of nanoparticle formation (enzyme-; protein-; or metabolite-mediated) and in certain instances require the use of an external energy source to drive nanoparticle formation. For example, Saifuddin *et al.*, (2009) reported that maximum AgNP formation from bacterial CFS at room temperature occurred at 120 hours whilst the application of microwave irradiation substantially decreased the duration of synthesis to 3 minutes (Saifuddin, *et al.*, 2009). Alternative physical parameters such as heat and agitation have likewise have been explored in the optimisation of AgNP production from bacterial CFSS (Gurunathan, *et al.*, 2009). In this study, AgNP formation was noted at 1 hour by the observed colour change of reaction solutions, irrespective of the bacterial CFS used. This was further confirmed by the presence of absorbance peaks at wavelengths associated with the surface plasmon vibrations of silver particles (Kim, *et al.*, 2007). This finding is in accordance with Wei *et al.*, (2012) who used a similar approach for AgNP production and reported a reduction time of 80 minutes using the CFS of *Bacillus amyloliquefaciens* (Wei, *et al.*, 2012). Our data suggests that sunlight irradiation may provide a viable alternative to non-renewable energy to drive AgNP formation at acceptable reaction times.

Morphological characterisation by SEM analysis provided inconclusive evidence that AgNPs were spherical in shape, due to the degree of agglomeration observed for the nanoparticles. This may have been a consequence of the sample preparation required for

the analysis which, in previous studies, display similar inadequacies (Sadeghi and Gholamhoseinpoor, 2015, Sigamoney, *et al.*, 2016). In contrast, TEM analysis displayed clear, unequivocal evidence that both nanoparticle preparations were spherical in shape. Interestingly, a previous study on the influence of AgNP shape on bioactivity suggested that spherically shaped AgNPs display superior antimicrobial activities when compared to rod shaped AgNPs (Pal, *et al.*, 2007). Attractively, TEM analysis further revealed that AgNPs prepared from both CFSs were stabilised by a surface coating. It can be tentatively suggested that the surface coating seems important in preventing their agglomeration as observed from the high incidence of mono-nanoparticles. This seemingly suggests that biomolecule capping structures present in both CFS extracts co-function as reducing and stabilising agents. Interestingly, previous studies using biological substrates have also reported this coating phenomenon (Ezhilarasan and Sahadevan, 2011, Sigamoney, *et al.*, 2016).

Intrinsically, AgNPs are highly reactive due to their nano-dimension (1-100 nm) and increased surface area (Christian, *et al.*, 2008). However, it has recently been demonstrated that nanoparticle size alters their antimicrobial efficacies, with smaller AgNPs displaying superior inhibitory activities compared to larger AgNPs (Jeong, *et al.*, 2014, Martinez-Castanon, *et al.*, 2008). In this study, AgNP sizes were statically comparable ($p>0.05$) with average diameters of 16±3.9 and 12±3.1 for *P. acidilactici* and *B. subtilis* derived AgNPs, respectively. In addition, size-class distribution studies indicated that the particles possessed narrow size distributions. Previously derived AgNPs that were produced at room temperature from *Enterobacteria* CFS, displayed a size range of 28-122 nm with an average diameter of 53 nm (Shahverdi, *et al.*, 2007). Similarly, Gurunathan *et al.*, (2009) reported average diameter of AgNPs derived from *E. coli* supernatants to be 50 nm with size distributions of 42-90 nm. In the same study, application of heat to the reaction mixture initially decreased nanoparticle size, however at temperatures above 60˚C larger nanoparticles were observed (Gurunathan, *et al.*, 2009). Attractively, Wei *et al.*, (2012) who used sunlight to drive AgNP formation, reported AgNPs with an average diameter of 15 nm. The findings and the data reported in this study strongly support the use of sunlight for the production of small sized AgNPs.

EDX analysis revealed that in addition to silver and although with weaker signals oxygen, carbon and nitrogen were present on the nanoparticle surface. Similar results were reported by Song and Kim (2009). It was proposed that they originate from biomolecule capping structures (Song and Kim, 2009). Importantly, AgNPs with oxidised surfaces have been reported to induce the formation of "holes" on the surface of bacteria (Smetana, *et al.*, 2008), which suggests that the presence of oxygen on the surface of the nanoparticles synthesised here may enhance their bactericidal activity.

FTIR analysis of AgNPs suggest that carbonyl groups of amino acids and protein linkages present in the CFSs have a strong metal binging ability. This may explain the presence of carbon, oxygen and nitrogen signals observed in EDX analysis. Consequently, it seems that AgNP formation occurred by the oxidation of protein molecules by Ag^+ (Awwad, *et al.*, 2013). Attractively, these protein molecules may be responsible for the protective covering observed on the surface of the nanoparticles as it has recently been suggested that protein capping molecules tend to form a surface coat on AgNPs which averts their agglomeration (Gomaa, 2017).

The antimicrobial effects of Ag and it's counterparts are known since antiquity (Maity, *et al.*, 2011). However, these agents have limited usefulness in a biological setting due to the interfering effects of salts and the discontinuous release of inadequate amounts of Ag^+ from the metal. In contrast, AgNP formulations have been shown to overcome these limitations and are therefore promising candidates in the development of novel antimicrobial agents (Kim, *et al.*, 2007). The antimicrobial activities of AgNPs derived from bacterial CFS extracts are relatively unexplored in terms of their MIC values. The AgNP preparations of this study displayed strong antimicrobial activities irrespective of the bacterial extract used and are comparable to AgNPs derived from other biological extracts (Dipankar and Murugan, 2012). The highest MIC values in this study were noted for *K. pneumoniae* and *S. aureus* (25 µg ml^{-1}) whilst *E. coli* displayed most sensitivity (6. 25 µg ml^{-1}) to both AgNP preparations. These findings are in accordance with previous AgNP studies in which activities for *K. pneumoniae* and *S. aureus* were either low or absent (Gannimani, *et al.*, 2014, Prasad and Elumalai, 2011). Interestingly, *E. faecalis* displayed sensitivity to AgNPs prepared from *P. acidilactici* CFS at a two-fold decreased concentration when compared to AgNPs prepared from *B. subtilis* CFS ($p<0.05$). Since *E. faecalis* initially displayed sensitivity to the pediocin PA-1 containing CFS, this may

tentatively suggest that pediocin PA-1 probably participated in Ag^{+} reduction and in so doing enhanced the bioactivity of its nanoparticle formulation. Importantly, this organism displayed resistance to all three commercial antibiotics and has previously been reported for its intrinsic resistance and further acquisition of resistance genes leading to the emergence of *E. faecalis* as a nosocomial pathogen that is a refractory to most therapeutic agents (Paulsen, *et al.*, 2003). Furthermore, the conjugative transfer of high levels of resistance genes from *E. faecalis* to *S. aureus* has been long established and may probably explain the increased incidence of *S. aureus* resistance to both neomycin and gentamicin in this study (Noble, *et al.*, 1992).

Both AgNP preparations displayed good antimicrobial activities against fungal strains with MIC values of 6. 25 µg ml^{-1} for AgNPs from *P. acidilactici* CFS and 6.25-12.5 µg ml^{-1} for AgNPs from *B. subtilis* CFS. The difference in MIC's is at present unclear and further studies need to be conducted to determine if capping structures present in the CFS constitute to the marked difference in bioactivities. These findings are in contrast to what has been suggested by Nabikhan *et al*., (2010), that AgNPs are better antibacterial agents rather than antifungal agents. This notion was supported by the finding that AgNPs are able to freely adhere to and penetrate bacterial cells, whilst they are unable to enter fungal cells at low concentrations (Nabikhan, *et al.*, 2010). The AgNPs produced in this study show no alteration in bioactivity between bacterial and fungal organisms, suggesting that their mode of action remains unaffected by the difference in the cell wall structures of these organisms. Importantly, AgNPs produced in this study display bioactivities at concentration doses that are generally associated with limited or no cytotoxic potential (Dipankar and Murugan, 2012) and therefore provide promising broad-spectrum antimicrobial alternatives to orthodox antibiotics which may provide much needed relief to the health care delivery system.

4.6 CONCLUSION

Nanotechnology provides a much needed platform for the development of new or improved antimicrobial agents. Physical characteristics of AgNPs such as shape and size are critical in the development of low concentration dose bioactive pharmaceuticals. The use of sunlight irradiation for AgNP production from *P. acidilactici* and *B. subtilis* CFSs

produced nanoparticles with desired physical attributes at favourable reaction times. Furthermore, capping structures present in the CFSs advantageously reduced Ag^{+} and stabilised the resultant AgNPs. Importantly, AgNPs displayed excellent antimicrobial activities and thus represent as promising novel Ag-based antimicrobial agents as alternatives to current antibiotics. For future studies, it would be interesting to isolate pediocin PA-1 and other bacteriocins from culture supernatants so as to explore their potential as a capping agents for development of novel AgNPs.

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Chapter 5

GENERAL DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION AND CONCLUSION

The development of novel antimicrobial therapeutic agents is a cumbersome, expensive, yet unavoidable exercise. On average, it takes at least a decade for a novel antimicrobial agent to succeed clinical trials and reach the market (Hughes, *et al.*, 2011). This may be owing the number of unwanted side effects exerted by a drug or the risk of antimicrobial resistance development. The use of nanotechnology to generate nanoparticulate forms of silver for antimicrobial applications is rapidly emerging into an important branch of science and technology (Sharma, *et al.*, 2009). In this regard, concerted research iniatives are being made in terms of identifying green synthetic strategies with optimal reaction conditions and novel capping agents for the production of AgNPs with enhanced bioactivities (Hazarika, *et al.*, 2016).

The first aspect of this study reports on the formulation AgNPs using freeze-dried and fresh leaf extract preparations of the medicinal tree species *M. oleifera* in a green synthetic protocol wherein sunlight irradiation was used to drive nanoparticle formation. AgNPs were produced equally well from both leaf samples and were comparable in terms of their morphology, yield and bioactivity. Attractively, capping structures identified to be flavones, terpenoids and polysaccharides in the leaf biomass displayed a dual role of reducing Ag^{+} and stabilising the resulting nanoparticles. Importantly, AgNPs displayed good antimicrobial activity against bacterial and fungal strains and in certain instances outperformed commercial antibacteral pharmaceuticals. It seems that AgNPs in this study were produced by a more efficient green technology, in addition this is the first report on the characterisation of *M. oleifera* leaf derived AgNPs in terms of their minimal inhibitory concentrations.

For the second aspect of the study, novel AgNPs were produced from bacterial extracts using the same energy source as mentioned above. AgNPs were prepared from the cell free supernatant of the bacteriocin (pediocin PA-1) producing strain, *P. acidilactici* and evaluated against AgNPs from the non-bacteriocin producing *B. subtilis* strain. The results suggest that for both AgNP preparations, protein capping molecules in the bacterial extracts were responsible for the formation and subsequent stabilisation of the

particles. AgNPs were generally consistent in terms of their morphology and bioactivity. However, it was noted with regard to *E. faecalis* that AgNPs produced from the bacteriocin containing extract displayed a two-fold increase in bioactivity when compared to AgNPs prepared from its non-bacteriocin containing counterpart. Since this strain initially displayed sensitivity to the pediocin PA-1 containing CFS, it is tentatively suggested that pediocin PA-1 probably participated in Ag^{+} reduction and in so doing enhanced the bioactivity of the nanoparticle formulation. Nevertheless, both AgNP samples showed good antimicrobial activities and in some instances, at concentrations at which the commercial antibiotics were inactive.

The results attained in this study suggest that sunlight irradation provides a viable alternative to the use of non-renewable energy sources for production of attractive AgNPs. Interestingly, using this energy source, production of AgNPs from extracts of two different phyla closely mirrored each other and were consistent in terms of their reduction time, morphology, stability and antimicrobial-spectrum range. These findings and previous reported findings seemingly suggest that the use of sunlight may be applied for the production of uniform AgNPs even from different capping substrates (Annadhasan, *et al.*, 2012, Karimi Zarchi, *et al.*, 2011, Rastogi and Arunachalam, 2011). Therefore, this method represents an attractive alternative to current nanoparticle syntheic routes which is cost-effective, eco-friendly and facilitates easy up-scaling for large production. Furthermore, with their observed antimicrobial potential, AgNPs dervied in this study contribute to the search for novel antimicrobial agents.

For future studies, it would be interesting to isolate pediocin-PA1, and other bacteriocins and assess their ability as Ag^{+} capping agents. Futhermore, the bioactivities observed in this study indicate that the derived AgNPs serve as promising antimicrobial alternatives and therefore warrants their cytotoxic screening. South Africa is blessed with an extensive indigenous flora used by traditional medicine and the nanoparticle synthetic strategy herein provides an attractive avenue for their protential development into antimicrobial AgNPs.

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