Green synthesis of silver nanoparticles using honey from the *Meliponinae* stingless bee species and antibacterial studies.

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By

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Declaration

I hereby declare that this thesis entitled "**Green synthesis of silver nanoparticles using honey from the *Meliponinae* stingless bee species and antibacterial studies**" has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Semphete Leteba

Signature:  February 2023

Dedication

I am dedicating this thesis to my beloved family for the support they have shown me while pursuing my studies. My father, Mohobelo Leteba and my mother Nkabiseng along with my two siblings Katleho and my younger sister Retshepile.

Acknowledgements

I would like to express my gratitude to my loving family for the support they have shown me while pursuing my studies. Thank you for the constant reminder of why I began this journey and for pushing me to never give up when things did not go well or when I felt that I did not have it in me. My mother has been my pillar of strength since day one, she has been reminding me how much potential I have and the many sacrifices that her and my father made so I can get the best education. To my father, he encouraged me to always follow my dreams and that when an opportunity presents to myself, I should take it no matter the circumstances at home.

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Abstract

Green synthesis of silver nanoparticles (AgNPs) is an environmentally friendly approach for preparing nanoparticles using biological sources as the reducing agent. Apart from using plant extracts, honey, that is used in this study affords to act as a reducing and capping agent. Silver nanoparticles have been reported to show good antibacterial and anti-inflammatory properties and can be potentially applied as antibacterial agents. Additionally, to their attractive characteristics, they are biocompatible which makes them safe for therapeutic applications. Similarly, stingless bees honey has been reported to have anti-inflammatory properties and have been historically used to heal wounds.

AgNPs with an average size of 60 nm were synthesized using stingless bee honey following a method previously reported in the literature with slight modifications. Optimization was performed to find the suitable conditions for the green synthesis of AgNPs. The synthesis occurred at a temperature of 75°C and at constant stirring speed of 450 rpm for 3 hours. Then ultraviolet visible spectroscopy (UV-vis) was used to confirm the AgNPs followed by dynamic light scattering (DLS) analysis which evaluated the hydrodynamic size, PDI and the zeta potential of the nanoparticles. The UV-vis confirmed that SPR peak characteristic of silver nanoparticles was visible at 414 nm with a PDI of 0.36 and the zeta potential had a value of -15 mV. The morphology and core size were determined using high resolution transmission electron microscopy (HRTEM) in conjunction with energy dispersive energy-dispersive x-ray spectroscopy (EDS) for the elemental composition and finally the fourier transform infrared spectroscopy (FTIR) was used to identify the functional groups responsible for the reduction of silver ions. The AgNPs were tested for their antibacterial activities. An agar well diffusion method was used. The synthesized silver nanoparticles showed moderate antibacterial activity against the tested bacterial strains. However, the stingless bee honey per se showed no activity in all the selected bacteria. Hence it was concluded that the stingless bee honey probably did not enhance the overall activities of the AgNPs as was anticipated.

Keywords

Green synthesis

Stingless bees

Honey

Silver nanoparticles

Antibacterial

Anti-inflammatory

List of abbreviations

AgNPs	Silver nanoparticles
SBH	Stingless bee honey
NPs	Nanoparticles
Ag^+	Silver ion
Ag	Silver atom
$AgNO_3$	Silver nitrate
UV-vis	Ultraviolet Visible Spectroscopy
HRTEM	High Resolution Transmission Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
DLS	Dynamic Light Scattering
EDX	Energy-dispersive X-ray spectroscopy
SPR	Surface Plasmon Resonance
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MHB	Mueller Hinton Broth
LB	Luria-Bertani
ATCC	American Type Culture Collection
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
HS-SPME	Head Space-Solid Phase Micro Extraction
HPLC	High-Performance Liquid Chromatography
AFM	Atomic Force Microscopy
PMA	Poly (isobutylene-alt-Maleic Anhydride)
MUA	Mercapto-Undecanoic Acid

PEG	Poly-Ethylene Glycol
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectroscopy
ROS	Reactive Oxidative Species
DNA	Deoxyribose Nucleic Acid
HRSEM	High Resolution Scanning Electron Microscopy
IC	Inhibitory Concentration
LOQ	Limits Of Quantification

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Chapter 1

1.1 Introduction

Since ancient times, honey has been a significant natural food product, valued for both its healing and nutritional properties. It is made from liquid plant exudates that various species of bees collect, alter, and store (Namias, 2003). It has been utilized in ethno-medicine since the ancient times of history, and its significance in the treatment of burns, gastrointestinal diseases, asthma, and infected wounds has been more prominent in recent years and cannot be overemphasized (Küçük et al., n.d.).

Stingless bees like other bees, are members of the class Insecta, and family Apidae. They are the largest group of eusocial insects with no functional sting but take part in an inevitable plant process of pollination. Pollination is an important ecological service that contributes to improved food security and livelihoods. This service is provided by a variety of species, including bees. Stingless bees can be found all over the world in tropical and subtropical climates. Africa, Australia, Central and South America are among these regions recorded. However, the stingless bees are believed to have originated from Africa (Kwapong et al., 2010). In many localities, they are the most abundant bees, making them presumably the most important pollinators (Vit et al., 2013). The stingless bees are not only exceptional pollinators, which are crucial to tropical and subtropical environments, but they also store and chemically alter floral nectars to produce honey, which is prized for its particular flavor and aroma, more fluid texture, and gradual crystallization. Consumers all over the world enjoy this honey, which increases its premium in the market (Biluca et al., 2016). Across the world, various cultures use stingless bees for honey and wax production, as well as for the bees' outstanding pollination capacity. Numerous studies describe indigenous cultures and tribes' usage of stingless bees (Figure 1) in the increase of crop yields which are at the heart of the community's subsistence (Cockburn et al., 2014).

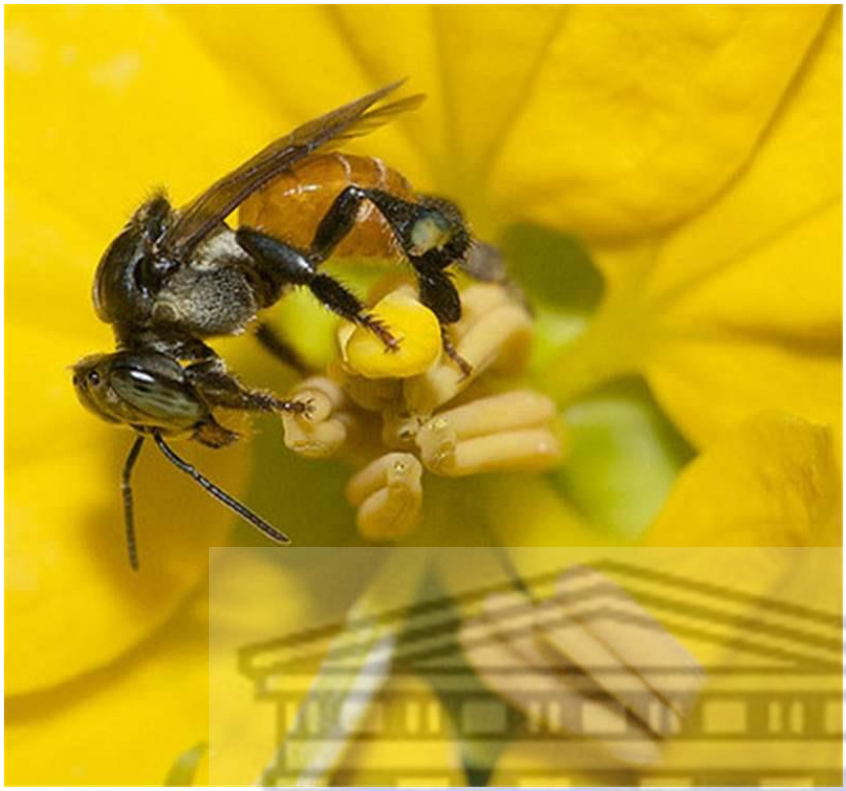

Figure 1*: Meliponula ferruginea* stingless bee (Théodore, et al., 2011).

Stingless bee honey (SBH), pot-honey, and Kelulut honey are some of the names given to the honey made by stingless bees (in Malaysia). It is one of the valuable bee products, which ancient people believed to have some medicinal properties (Souza et al., 2006). It has been reported that traditional doctors used Stingless bees honey as a remedy for high fever, treatment of wounds and burns as well as the cure for poisonous stings (Rosales, 2013). In the past, the Russians during World War 1 used honey to treat wound infections and speed up wound healing. The Germans discovered that a mixture of honey and cod liver oil was useful in treating burns and ulcers (Bansal et al., 2005). It has been reported that nearly all types of wounds such as burns, abrasion, amputation, septic wounds and diabetic ulcers, etc have been found to be responsive to honey treatment. When honey is used as a wound treatment, the healing process is accelerated, and the infection is quickly eradicated. As a non-adhesive tissue dressing, honey-impregnated pads are used to dress the wound. They have a cleansing effect on wounds, promote tissue regeneration, and reduce inflammation (Molan, 1999). The efficacy of honey and cod liver oil mixture have been experimentally evaluated in the treatment of wounds found in metacarpus of donkeys. Metacarpi wound surfaces were treated with gauze soaked in a blend of honey and cod liver oil, and the results showed complete wound healing after 30 days (Ali & Radad, 2011).

Studies have found that there are chemical content differences between the stingless bees' honey and the modern bee honey. The former has been found to possess extraordinarily higher concentration of flavonoids and polyphenols than honey made by the modern bee (Biluca et al., 2016). Researchers have discovered hundreds of bioactive compounds from the honeys made by the Melipona species in different countries such as organic acids, phenolic acids, flavonoids, proteins, amino acids, enzymes, vitamins, and minerals (Samarghandian, et al., 2017). Due to their function in preventing diseases linked to oxidative stress, research groups have paid particular attention to the biologically active substances found in honeys that exhibit antioxidant capacity, such as phenolic acids, flavonoids, and the enzymes glucose oxidase and catalase (Aljadi & Kamaruddin, 2004). Concerning the chemical composition of honey from the African *Meliponula ferruginea*, reporters considered trehalulose as a potential marker for African stingless bee honey (Popova et al., 2021). A study by (Biluca, et al., 2016) conducted research on the physiochemical characteristics of Meliponinae stingless bee honey. Thirty-three samples from 10 different species were examined. In their findings, the moisture content of the honey ranged from 23.1 to 43.5% (w/w). Some of the reducing sugars that have been shown to be present in stingless bee honey include fructose, glucose, and sucrose. In the samples that were analyzed, the amounts of fructose and glucose ranged from 30.4 to 46.1 and 8.21 to 31.3% (w/w), respectively. However, in all samples tested, sucrose content was under LOQ (LOQ = 0.074 mg L^{-1}). This leads to the conclusion that fructose levels are high in stingless bee honeys, which may be the cause of the honey's intense sweetness and high hygroscopicity, which keeps the honey liquid for a very long period without crystallizing (Biluca, et al., 2016).

One of the primary characteristics of these kinds of honeys is that it contains more moisture, which is frequently found in stingless bee honey compared to others such as the exotic Apis mellifera honey. Multiple sources, including the habitat and climate, storage, and level of maturation in the hive, may be involved in this. The acidity of SBH has been found to be higher as well. The aforementioned characteristics of SBH has been believed to be responsible for its antibacterial properties. Honey's physicochemical properties, such as its high sugar content, low pH, and hydrogen peroxide level, are what primarily account for its antibacterial properties. Although other non-peroxide molecules, including phenolic compounds, also play a significant role in antibacterial action, the latter is reported to be the predominantly an antibacterial compound (Carina Biluca et al., 2020).

Honey's antibacterial properties have long been studied scientifically and utilized medically. Up until recently, relatively little attention was given to honey produced by stingless bees; instead, most of the research has concentrated on honey produced by the European honeybee Apis mellifera (Boorn et al., 2010b). In a study by Temaru et al, (2007) they investigated the antibacterial activity of the honey of the Stingless bees. They discovered that many honey samples of stingless honeybees inhibited the growth of test strains of *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa*. In addition to that they possessed no-peroxide antibacterial activity against those strains. Staphylococcus aureus ATCC 9144 appeared to be more susceptible to honey than the other test strains. They discovered for the first time that non-peroxide antimicrobial activity was demonstrated by honey from several stingless bee species (Temaru, et al., 2007).

These antibacterial activities showed great potency, when compared to those of manuka honey from Apinae honeybees which have been vastly studied and reported to have antimicrobial activity (Boorn et al., 2010). According to (Temaru, et al., 2007), their findings led to conclusion that the major antibacterial activity of stingless honeybees lies in the non-peroxide activity. Hydrogen peroxide present in living tissues get degraded or reduced to water and molecular oxygen by using either iron or manganese as a cofactor (Goyal & Basak, 2010). It has been described that the potency of the antibacterial activity is likely to be reduced by the action of catalase present in the human body tissue.

In a study by (Rosli et al., 2020) , they investigated the antimicrobial ability of honey from eight different stingless bee species. The studied bacterial strains were *Serratia marcescens*, *Escherichia coli*, *Bacillus subtilis*, *Alcaligenes faecalis* and *Staphylococcus aureus*. Honey samples from Homotrigona fimbriata bee showed the highest antimicrobial activity with minimum inhibition zone diameter up to 28 mm in size. On the contrary, *H. erythrogastra* had shown no inhibitory effect at all (Rosli et al., 2020).

Table 1: Zone of inhibitions of antibacterial activity from several stingless bee honey against different bacterial strains.

Stingless Bee Honey Samples of Different Species & Concentration	pH	Inhibition Zone Measurement Against Different Bacteria (mm)				
		S. aureus	*A. faecalis*	*S. marcescens*	*B. subtilis*	*E. coli*
H. fimbriata	2.64 ± 0.17					
50%		28 ± 0.58	21 ± 1.00	18 ± 1.00	9 ± 3.72	12 ± 0.58
25%		19 ± 0.58	15 ± 0.58	12 ± 0.58	0 ± 2.40	11 ± 0.00
12.5%		16 ± 0.58	0 ± 0.00	11 ± 0.00	0 ± 8.59	9 ± 0.58
T. melanoleuca	2.89 ± 0.20					
50%		22 ± 0.58	21 ± 1.00	14 ± 0.58	9 ± 0.82	9 ± 1.00
25%		18 ± 0.58	0 ± 0.00	11 ± 1.53	11 ± 9.88	0 ± 0.00
12.5%		0 ± 0.00	0 ± 0.00	10 ± 1.53	0 ± 0.00	0 ± 0.00
H. itama	2.57 ± 0.19					
50%		13 ± 0.00	0 ± 0.00	0 ± 0.00	12 ± 0.00	0 ± 0.00
25%		11 ± 0.58	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
12.5%		10 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
T. apicalis	2.20 ± 0.21					
50%		13 ± 0.58	0 ± 0.00	0 ± 0.00	12 ± 0.00	0 ± 0.00
25%		9 ± 1.53	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
12.5%		8 ± 0.58	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
L. terminata	2.36 ± 0.20					
50%		11 ± 0.58	0 ± 0.00	0 ± 0.00	12 ± 1.53	0 ± 0.00
25%		10 ± 0.58	0 ± 0.00	0 ± 0.00	9 ± 0.58	0 ± 0.00
12.5%		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
G. thoracica	1.95 ± 0.13					
50%		11 ± 1.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
25%		9 ± 1.53	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
12.5%		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
T. binghami	2.19 ± 0.19					
50%		10 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
25%		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
12.5%		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
H. erythrogastra	1.82 ± 0.12					
50%		0 ± 0.00	0 ± 0.00	0 ± 0.00	9 ± 1.00	0 ± 0.00
25%		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
12.5%		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

Antimicrobial results obtained from a study by (Rosli et al., 2020) as shown in Figure 1 indicated that all honey samples with 50% (means that in a 100 ml solution, for every 50 ml of honey, there is 50 ml of water) concentration showed positive antagonism effect against *S. aureus* except for *H. erythrogastra* honey sample which did not exhibit any inhibition zone. Meanwhile at 25% concentration, *T. apicalis*, *T. binghami*, *G. thoracica* and *H. erythrogastra* honey samples did not exhibit any inhibitory activity towards all five bacteria. At 12.5% concentration, only three honey samples showed inhibitory effect against one or more bacteria at the range of 10 to 16 mm in diameter of the inhibition zone. Collectively, *H. fimbriata* had the highest antimicrobial activity compared to other honey samples with

inhibitory zone diameter up to 28 mm in size. This is evidence that stingless bee honey does exhibit antibacterial activity however honey from other stingless bee species can possibly not possess antimicrobial activity.

On the other hand, several studies have reported the antibacterial activity of honey originating from the traditional modern bee. In a study by Mokaya et al, (2020) they reported the antibacterial activity of several *Apis mellifera* honey from Kenya against *E. coli*. Agar well diffusion assay was carried out to test the potency of their selected honey samples. In their findings, all the honey samples at concentration of 25% w/v did exhibit antibacterial properties against *E. coli* and the largest zone of inhibition diameter was reported to be 33.3 mm and the minimum was 29.0 mm (Mokaya et al., 2020). In the majority of the reported studies MIC and MBC activity of honey samples are not carried out and it should be noted that it is not completely reliable to conduct agar diffusion assay for testing antibacterial activities of honey samples. In another study by (Haiza, et al., 2013) when they investigated the antimicrobial potential of honey on common human pathogens, they found relatively lower activity of honey samples on the agar diffusion assay compared to the MIC and MBC results.

Silver nanoparticles (AgNPs) are one of the most important nanometals due to their wide range of uses in biotechnology and biomedicine. Chemical and physical approaches were often used to produce AgNPs. Various toxic compounds are utilized in chemical processes, which are damaging to the health of living beings. These include the use of toxic precursor chemicals such as sodium borohydride, hydrazine and potassium bitartrate. In addition, the use of toxic solvents such as sodium dodecyl benzyl sulphate and polyvinyl pyrrolidone. As a result, in order to reduce the harmful substances, the AgNPs were manufactured from the utilization of biological processes based on green chemistry. Given the accessibility of plant resources, using fruit, vegetable, or plant extracts in the biological production of nanoparticles (NPs) is a useful approach (Zulkhairi Amin, et al., 2018). Other materials of a biological origin such as honey can also synthesize Ag, Au, Pd and Pt nanoparticles (Ghaffari-Moghaddam et al., 2014). Green synthesis is simple, inexpensive, and environmentally benign, and it can be easily scaled up for large-scale synthesis without the use of harmful and redundant ingredients in solid, liquid, and gaseous forms (Ashokkumar et al., 2015). Even though nanoparticles can be created using a variety of physicochemical techniques, their synthesis by using non-toxic and environmentally friendly biological techniques is appealing, particularly if they are meant for invasive medical purposes (Sharmila et al., 2021), (Cooper, et al., 2002). Green synthesis of silver (Philip, 2010), (Balasooriya et al., 2017a), platinum and palladium (Balasooriya et al., 2017a) nanoparticles

using honey has been previously reported. In a study by Philip, (2010) green synthesis of silver nanoparticles using honey was reported. They managed to synthesize 4 nm AgNPs at a pH of 8.5 in the absence of heat and the reaction was completed in one minute. The obtained AgNPs were also found to be stable for about 6 months, and it was suggested that proteins present in the honey are the possible capping agents responsible for the stability of their AgNPs.

Another study reported the eco- friendly synthesis of palladium nanoparticles using honey for a catalytic application in the synthesis of biaryls. The synthesized nanoparticles had a size ranging from 5 to 40 nm. However, in this synthesis procedure, the course of the reaction was completed in 5 hours with stirring. The palladium nanoparticles showed good catalytic activity with a yield of 75% even after four cycles of reusability (Reddy, et al., 2012). In similar application, a bio-directed synthetic method of platinum nanoparticles using honey has been reported. In this method, different incubation times of 2, 4, 10, 15 and 20 hours were carried out for each reaction vessel containing honey and H_2PtCl_6 aqueous solution. The synthesized nanoparticles had an average size of 2.2 nm at incubation of 2 hours whereas at more than that they formed nanowires with an average length of 15 nm and diameter of 2.2 nm. Their honey produced platinum nanoparticles and nanostructures were found to be stable in water for longer than 4 months. It was demonstrated that the honey-mediated platinum nanoparticles and nanowires have effective catalytic capabilities for the reaction of aniline and 4-aminoantipyrine in an acidic aqueous medium to produce the antipyrilquinoneimine dye (Venu et al., 2011a).

Antibacterial resistance (AR) has grown significantly around the globe in recent years and is now identified as a significant medical concern in the majority of healthcare settings. Resistance is mostly responsible for mortality, which raises the burden of infectious diseases (Theuretzbacher, 2013), (Theuretzbacher, 2012b) & (de Kraker et al., 2011b). The discovery of bacteria in the late 19th century sparked a hunt for effective therapeutic and preventive regimens, but it wasn't until the discovery and use of antibiotics half a century later that successful treatment became possible. Numerous lives have been saved thanks to the many ways in which antibiotics have altered medicine; their discovery marked a significant milestone in human history. Unfortunately, the fast emergence of resistance strains has coincided with the usage of these wonder therapeutics and suppressed their importance (Davies et al., 2010).

Infections with multi drug resistant pathogens whether in hospitals or in the community increase morbidity, decrease treatment success, reduce hospital turn-over rate and increase cost of patient care. The presence of drug resistant bacteria in the hospital environment and in

patients is a great threat for public health and because of the ever-increasing member of resistant strains with time. More than 90% of *S. aureus* produce penicillinase and are resistant to penicillin (Alebachew et al., 2012). Antibiotic-resistant illnesses have been shown to significantly increase the costs incurred by healthcare systems worldwide. Antibiotic-resistant infections typically demand extended treatments and cause more death and disability than non-antibiotic-resistant diseases. It has been challenging to calculate the total cost of AR to the US economy in affluent nations such as the US. However, it is currently thought that direct healthcare expenses may have exceeded $20 billion USD (World Health Organization, 2020).

Studies show that more than 80% of European intensive care unit (ICU) doctors responding to a poll said that antibiotic-resistant bacteria infections in the ICUs where they work were a serious or substantial problem (Theuretzbacher, 2012). The traditional antibacterial agents have indicated some degree of being ineffective in treating infections. This has led to the emergence of finding improved antibacterial agents against this worldwide challenge. From historical uses, silver has been known to exhibit good antibacterial properties and has been used for treating wounds. With the improving research on silver as an antibacterial agent, nanotechnology has been developing novel particles of silver that are promising in solving this demise.

In this study, AgNPs are selected to help tackle this serious problem due to its broad-spectrum antimicrobial capabilities and strong antimicrobial efficiency against a variety of bacteria, viruses, and fungi of their antibacterial properties. With the use of stingless bee honey, which is also known to possess antimicrobial properties, it is hoped that their antibacterial effect as well as its stability can be enhanced. Therefore, our problem statement is as given below.

1.2 Problem statement and rationale

Drug resistance poses a growing issue to global public health that affects all significant microbial infections and antimicrobial medications (Dhingra et al., 2020). Antibacterial resistance (AR) has grown significantly around the globe in recent years and is now registered as a significant medical concern in the majority of healthcare settings. Resistance is mostly responsible for mortality, which raises the burden of infectious diseases (resistance & 2013, n.d.), (Theuretzbacher, 2012a), (de Kraker et al., 2011a). The surging rise in infectious diseases leads to community morbidity, decrease treatment success, reduce hospital turn-over rate and cost of patient care.

In the development of nanotechnology-based medicines for challenging multidrug resistant microorganisms, several studies have investigated the green synthesis of silver nanoparticles using bees honey against the most common bacterial strains (Youssef et al., 2019), (Philip, 2010), (Khorrami et al., 2019). In a study by Youssef et al, (2019) they successfully synthesized silver nanoparticles with size ranging from 9-22nm using honey and their synthesized nanoparticles showed good antibacterial activity against *E. coli*, *Staphylococcus aureus*, *Streptococcus mutans*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *K. pneumonia*. In this study our focus will be on only the honey from stingless bees that will be used as both the reducing and stabilizing agent on the silver nanoparticles. Through this method we aim to investigate its antibacterial activity effect when combined with the honey and further evaluate the stability of the synthesized nanoparticles which is hypothesized that it might be improved given the characteristics of both silver and traditional antibacterial uses of honey. A formulation of this unique antibacterial agent is predicted to add promising treatment to tackle the ever-increasing antibacterial resistance crisis.

The use of honey in the synthesis of silver nanoparticles in water has been reported recently. However, to the best of our knowledge there has been no report on the synthesis of silver nanoparticles using honey of the *Meliponinae ferruginea* black stingless bee to be used as an antibacterial agent against several pathogens which leads us to our aims and objectives as shown in section 1.3.

The findings of this study will contribute to the green synthesis methods of nanoparticles using biological reducing agents as well as providing information about the medicinal application of stingless bee honey in the communities since little information about it is known.

1.3 Aims and Objectives

The main purpose of this study is to synthesis silver nanoparticles using a honey extract of the *Meliponinae* stingless bee which is proven to have antibacterial uses and to analyse its antibacterial activity.

Specific objectives are:

a) To synthesize the silver nanoparticles (AgNPs) using honey extract as the reducing and stabilizing agent.

b) To characterize the size, optical properties, surface morphology, structural composition and stability of H-AgNPs using different analytical techniques such as UV-vis, FTIR, HRTEM, and DLS.
c) To investigate the antibacterial properties of the stingless bee honey synthesized nanoparticles (AgNPs) using agar well diffusion method on gram-positive and gram-negative bacterial strains, i.e., *S. aureus, S. epidermidis*, MRSA, *K. pneumoniae*, *E. coli* and *P. auruginosa*.

This thesis is organized as shown below in the given thesis overview:

Chapter 1

This introductory chapter provides an explanation of stingless bees and the benefits of their honey throughout history. A detailed description of the chemical makeup of honey as well as the physiochemical characteristics that may be the basis for its medicinal use. The rising issue of antibacterial resistance, leading factors and its implications are discussed. The history of silver and silver-based therapies for application in the treatment of infections leading up to AgNPs is also discussed herein. The chapter closes off with the aims and objectives of the study.

Chapter 2

This chapter provides an in-depth assessment of the literature starting with the world statistical reports of Antibacterial resistance, the cause of this global issue and current antibacterial agents. Nanotechnology and nanomaterials are also briefly introduced, with the role of AgNPs as antibacterial agents as the central point of this chapter. Also, the synthesis, and properties that make AgNPs excel in wound healing are discussed in detail.

Chapter 3

This chapter reports the materials, reagents and analytical techniques used throughout this study. There is a description of a detailed procedure to synthesize AgNPs using stingless bee honey as a reducing agent. The analytical techniques employed to characterize and monitor the behaviour of the AgNPs are also explained. Lastly, the chapter describes the techniques of the

bioactivity assays of the AgNPs used to study their anti-bacterial effects through agar well diffusion method and inhibitory studies.

Chapter 4

This chapter begins with the synthetic results for the SBH-AgNPs and ends with their characterization utilizing multiple methods, including UV-vis, FTIR, HR-TEM, and DLS. This chapter also covers the antibacterial activity of SBH-AgNPs against various bacterial strains. The evaluation of SBH-AgNPs' growth inhibitory kinetics experiments is also discussed here as the chapter ends.

Chapter 5

The results of the synthesis, characterisation, and biological applications of SBH-AgNPs were summarized in this chapter. Additionally, it establishes a connection between the study's precise objectives and the results. Moreover, recommendations and future plans are briefly mentioned.

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Chapter 2

Literature review

2.1 Antibacterial resistance

According to the Centre of Disease Control (CDC) antimicrobial resistance poses a serious danger to global public health, causing at least 1.27 million deaths globally and and with recent covid-19 pandemic, approximately 5 million fatalities in 2019 (Centers for Disease Control and Prevention, 2013). It has been pointed out that the world is running out of effective ways to treat these diseases, as seen by the high rates of resistance among antimicrobials often used to treat common infections, such as urinary tract infections or various types of diarrhoea. For instance, ciprofloxacin, a common antibiotic used to treat urinary tract infections, had resistance rates ranging from 8.4% to 92.9% in 33 reporting nations (World Health Organization, 2020). Antibiotic resistance risk caused significant economic and human losses. Around 700,000 individuals worldwide pass away each year as a result of improper antibiotic use that leads to the development of resistance to conventional therapy(Shlaes et al., 1997). According to the information that is currently available, there are more than 2 million AMR infections with a death toll of 29,000 each year in the United States, with a corresponding health care cost of more than $4.7 billion (World Health Organization, 2020).

Antimicrobial Resistance (AMR) is a phenomenon in which bacteria, viruses, fungi, and parasites evolve over time and cease to respond to antibiotics, making infections more difficult to cure and raising the risk of disease transmission, life-threatening sickness, and death (Dadgostar, 2019). Through mutation or the acquisition of defence genes from other bacteria, the microorganisms compete for survival. Bacterial resistance to antibiotics also increases with the strength and use of antimicrobial medications, which can result in the development of "superbugs," or untreatable bacteria. Drug resistance makes it harder or impossible to treat illnesses and renders antibiotics and other antimicrobial medications ineffective (World Health Organization, 2020). In contrast to infections caused by methicillin-susceptible *S. aureus*, bacteraemia and other serious infections caused by MRSA have been shown to have a much higher resistance. In another study, non-duplicate MSSA and MRSA isolates from various clinical samples were examined for antimicrobial resistance patterns to 13 different antibiotics (i.e. urine, pus, HVS, blood, tissue, wound and ear swabs). The majority of cultures were found to be multi-drug resistant (MDR). The two antibiotics with the highest rates of resistance were

ampicillin and erythromycin (88% each), however resistance to oxacillin, fosfomycin, cefoxitin, and ciprofloxacin was also concerning. Antibiotic sensitivity varied widely among strains. MSSA also shown high levels of resistance to ampicillin, erythromycin, fosfomycin, and fusidic acid. Vancomycin displayed the lowest level of resistance. Only 12% of isolates, including 24 MRSA and 6 MSSA, were resistant to vancomycin (Hassan et al., 2019).

Pathogenic bacteria are those that cause bacterial infections and sicknesses. A class of substances known as antimicrobial therapy agents work to combat pathogenic bacteria by either killing them or inhibiting their metabolic activities (D. Li et al., 2020), (Neu & Gootz, 1996). The ability of antibiotics to treat and cure infection has dramatically reduced the number of incidences of infection, significantly improving the quality of life for numerous patients, reducing childhood mortality, increasing life expectancy, and saving numerous lives (B. Li et al., 2018). Regardless, one of the leading causes of AMR is the excessive and unnecessary use of antimicrobial medications, including antibiotics, which over time reduces their effectiveness against the bacteria, viruses, fungi, and parasites they are intended to treat. These resistant microbes are then more easily spread in environments with poor water quality, sanitation, and hygiene, whether in hospitals or in regular settings (World Health Organization, 2020), (B. Li et al., 2018). In relation to the poor water quality environments, a study in Brazil was carried out to provide information and raise awareness on the antibiotic resistance situation relationship between poor sanitation and public health. A total of 312 Gram-negative bacilli was reported from the hospital sewage (n = 130, 41.6%) and from the wastewater treatment plant (182, 58.4%). The most represented family in the hospital sewage was Enterobacteriaceae (61.2%), followed by Aeromonadaceae (18.6%) (Picão et al., 2013).

It has been suggested that gram negative bacteria are more resistant than gram positive because Gram-negative bacteria's outer membrane is frequently covered by a slime layer, which in turn conceals the cell's antigens, they are more resistant to antibiotics than Gram-positive bacteria. Gram-negative bacteria's distinctive outer membrane prevents some medications and antibiotics from entering the cell, increasing their drug resistance and making them more deadly disease-causing agents. (D. Li et al., 2020). The evolution and spread of multidrug-resistant organisms have reportedly accelerated rapidly during the past 50 years, according to epidemiological statistics and organism analysis. The development of antibacterial drugs and their expanding use within this time frame are coinciding (Shlaes et al., 1997).

Although many people believe that the abuse of antimicrobial medications is the main cause of AMR, environmental factors may actually be more responsible, especially in poorer countries with insufficient access to clean water, inadequate sanitation, and poor waste management (World Health Organization, 2020). In order to reduce resistance, the Center for Disease Control proposed that it is therefore essential to use fewer antimicrobial drugs on people, animals, and plants. In addition, it is also crucial to improve hygiene, provide safer water and sanitation, and better wastewater treatment on a global level. Otherwise, resistance may continue to rise, possibly causing the next pandemic.

2.1.1 Factors causing drug resistance

To discover any modifiable interactions to lessen or stop the continued spread of resistance throughout the ecosystem, it is necessary to understand the environmental factors that contribute to the development of resistant infections (World Health Organization, 1948). Antimicrobial resistance is primarily caused by the use and overuse of antibiotics, but it needs to be taken into consideration that other variables also play a role in its rising prevalence. Antimicrobial resistance is a natural occurrence, although "man-made" factors are primarily responsible for its emergence and spread. These factors include improper and excessive use of antibiotics in people, pets, and plants; lack of access to vaccines, accurate diagnoses, and appropriate treatment; poor access to clean water, sanitation, and hygiene; inadequate infection prevention and control; transmission of resistant pathogens through the food chain; and inadequate waste management systems (World Health Organization, 1948), (Laxminarayan et al., 2013), (Fletcher, 2015).

Many infectious and non-infectious organisms spread to people via water, which is a significant factor in this process. It was in the year 2014 when the WHO global report proposed that more emphasis should be aimed at public health interventions, such as improving hygiene and access to clean water. This section discusses some of the evidence around the dissemination of resistance elements and antibiotic-resistant organisms through water environments.

Drinking water derived from surface water sources, which serve as the means by which resistance genes are introduced in natural bacterial ecosystems, can spread antibiotic-resistant species. In this setting, non-pathogenic bacteria can serve as a source of platforms and resistance genes (Ding & He, 2010). The growth and spread of such resistant organisms in the water environment is made possible by the increasing build-up of antimicrobial agents,

detergents, disinfectants, and residues from industrial pollution, as well as heavy metals released into the environment (Baquero, et al., 2008).

Disinfectants are antimicrobial products that incorporate one or more active substances, such as chlorine, iodine, alcohols, hydrogen peroxide, silver, chlorhexidine, triclosan and quaternary ammonium compounds. They are indispensable in human and veterinary health care, the food industry and water treatment for the prevention of infections and intoxications (van Dijk et al., 2022). Disinfectants at sub-MIC concentrations can cause stress reactions in bacteria, which result in temporary, adaptive alterations to the structure and permeability of their cell envelopes. A study by Méchin et al., they found that the acquired resistance to didecyldimethyl ammonium bromide DDAB's bactericidal activity revealed that it was dependent on the presence of the biocide. The study showed that the more the cells were exposed with biocide, the more they acquired resistance to bactericidal activity (Méchin et al., 1999).

Even though human antimicrobial use accounts for the majority of the resistance problem in humans, there is growing concern that the use of antibiotics in food-producing animals, particularly the long-term use of antibiotics as prophylaxis or for growth promotion, significantly contributes to the emergence of antibiotic-resistant bacteria in animals. It has once been suggested that the use of growth promoters in animals to be banned as there is a high risk of transmission of antibiotic resistant pathogens from animals to humans (Phillips, et al., 2004). In countries like Asia, they have adopted a farming process which is the integrated agriculture–aquaculture farming system known as vegetation, aquaculture, and cage (VAC) (Fletcher, 2015). The VAC system is a recycling farm where livestock manure (often from pigs, chickens, and ducks) is directly transferred to fishponds as well as to vegetable and rice fields. This system normally consists of a vegetable field, an aquaculture pond, and caged animals. Fish farming and vegetable field fertilization both use this untreated sewage and wastewater from livestock activities. The VAC system is regarded as a particularly cost-effective approach of recycling farming (ALiSEA, 2015).

High levels of resistance to tetracycline, nalidixic acid, and enrofloxacin were found in intestinal bacteria in a study of pigs in VAC systems (Dang et al., 2011). This shows that the probability of colonization with antibiotic-resistant bacteria and genes increases in the intestinal tracts of animals as a result of exposure to high doses of different antibiotics. Then, through water and the food supply, the genes for antibiotic resistance can spread to humans and other habitats.

2.1.2 Current antibacterial agents

According to recent literature it has become noticeable that AR is one of the serious challenges faced globally and the implications caused by drug resistant pathogens has been causing a huge crisis and it needs to be solved. These are general therapies that have been developed to treat bacterial infections. Drugs that cure a variety of illnesses by eradicating or reducing the growth of the bacteria responsible for the infection are referred to as "antimicrobials" (Centers for Disease Control and Prevention, 2013).

Antibiotics remain the most common treatment for bacterial infections (Aksoy & Unal, 2008). While saving lives, antibiotics can also help to breed resistant bacteria. Previous studies have highlighted that the introduction of antibiotics has led to the increase in bacterial resistance (Shlaes et al., 1997). For common bacterial infections, including urinary tract infections (UTI), sepsis, sexually transmitted infections, and some forms of diarrhoea, high rates of resistance against antibiotics frequently used to treat these infections have been observed world-wide, indicating that we are running out of effective antibiotics. For example, a study in Brazil was carried out over a five-year period from 2010 to 2014. They found a high rate of resistance to antibiotics in patients with community-acquired UTI. In their findings *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus saprophyticus* were the most frequently found pathogens in urine samples. *E. coli* had the highest rate of ciprofloxacin resistance, with 36% of strains resistant to the antibiotic (Reis et al., 2016).

In February 2017, to focus and guide research and development related to new antibiotics, the WHO published its list of pathogens for which new antimicrobial development is urgently needed. Within this broad list, ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens were designated “priority status” (World Health Organization, 1948). Below we will discuss the traditional antibacterial agents used to treat infections associated with the ESKAPE pathogens.

The oxazolidinone class of antibacterial medications is regarded as the first truly novel class to be released in the previous twenty decades. The Food and Drug Administration (FDA) granted linezolid approval for adult usage in 2000 and pediatric use in 2005 (Colca, et al., 2003). The oxazolidinone linezolid is a synthetic antibiotic that, depending on the organism and the linezolid dose, either has a bacteriostatic or bactericidal effect. It does this by blocking the

development of the protein initiation complex and by inhibiting protein synthesis at the ribosome level (Swaney et al., 1998), (Aksoy & Unal, 2008). Vancomycin-resistant *Enterococcus faecalis* infections, *Staphylococcus aureus*-caused hospital acquired pneumonia, complicated skin and skin structure infections (SSSIs), uncomplicated SSSIs caused by methicillin-susceptible *S. aureus* or Streptococcus pyogenes, and community-acquired pneumonia caused by *Streptococcus pneumoniae* are all treatable with linezolid (Hashemian et al., 2018).

The second class of antibiotics is the Glycopeptides, which vancomycin and teicoplanin are still the most common drugs in the glycopeptide class. Biochemical studies indicate that glycopeptides inhibit the late stages of peptidoglycan synthesis (Van Bambeke, et al., 2004). Glycopeptides' range of activity mostly targets Gram-positive bacteria and a few anaerobes, while their activity against Gram-negative organisms is frequently insignificant.

Bacterial community-acquired respiratory tract infections (CA-RTIs) are a spectrum of infections including community-acquired pneumonia, acute bacterial exacerbations of chronic bronchitis, sinusitis, acute otitis media, and tonsillopharyngitis. These infections have been treated using Ketolides class of antibacterial, in addition, they extend the spectrum to cover antibiotic-resistant streptococci (Nilius & Ma, 2002). The ketolides inhibit protein synthesis by blocking elongation. In contrast to the previously mentioned class of compounds, another class of drugs named Streptogramins in its mechanism it inhibits protein translation rather than synthesis.

The streptogramins is another class of drugs used in bacterial treatments, an example of this is quinupristin–dalfopristin. It is a fixed mixture of semi-synthetic streptogramin derivatives. These drugs diffuse into bacterial cells where they attach to several locations on the 50S ribosomal subunit, irreversibly inhibiting bacterial protein synthesis. By preventing substrate attachment to the P-site and A-site of the ribosome, dalfopristin inhibits the process catalyzed by the peptidyl transferase catalytic center of the 50S ribosome. Peptide chain elongation is prevented by quinupristin. This medication combination's synergistic effect seems to be due to the fact that it affects both the early and late phases of protein synthesis (Aksoy & Unal, 2008). Quinupristin-dalfopristin is hence bactericidal to MSSA, MRSA, and Strep. pyogenes while being bacteriostatic to *E. faecium*. Against *Enterococcus faecalis*, it is ineffective.

The glycylcycline was reported as the first class of antibiotics which exhibits powerful in-vitro action against numerous multidrug-resistant Gram-negative pathogens and anaerobes, as well as a variety of Gram-positive and Gram-negative bacteria, including MRSA and tetracycline-resistant *S. aureus*. A study reported the in vitro efficacy of glycylcycline which was assessed against gram-positive bacteria: *S. aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *S. pneumoniae* ATCC 49619 and *S. pneumoniae* ATCC 49619. The MICs in (µg/ mL) found for the respective organisms were as follows (subscript indicates number of times that the value was observed): 0.12_1, 0.25_{10}, 0.5_5, and 1.0_2; 0.25_1, 0.5_8, and 1.0_9; 0.25_1, 0.5_8, and 1.0_9; 0.06_2; and 0.03_2.A common derivative of this class of compounds is the tigecycline antibacterial agent. Tigecycline works by suppressing protein translation in bacteria by reversibly binding to the 30S ribosomal subunit. This blocker stops aminoacyl tRNA molecules from entering the ribosome's A-site, which limits the production of peptides (Chopra, 2001).

Another class of antibiotics which possess extensive antibacterial action against anaerobes and both gram positive and negative bacteria is the carbapenems. It is reported that they have typically been kept in the hospital for the therapy of the most relentlessly ill patients. Their mechanism involves binding and inactivating the penicillin-binding proteins which are involved in the crosslinking and elongation of the peptidoglycan of the bacterial cell wall. Most carbapenems are effective against clinically important, Gram-negative, non-fermenters (such as *P. aeruginosa*, *Burkholderia cepacia*, and *Acinetobacter spp*.) and anaerobes, in contrast to the majority of other antibiotics (Nicolau, 2007). Most anaerobes are susceptible to the carbapenems' activity. In a study by Sheikh et al., they determined that the carbapenems were the most effective antibiotic class after comparing their activity against over 1000 clinical anaerobic isolates with those of other active drugs (cefoxitin, clindamycin, and metronidazole). (Sheikh, et al., 1993).

The quinolones are a bactericidal class which in their mechanism hinder bacterial DNA gyrase, also known as topoisomerase II, which interferes with bacterial DNA synthesis, repair, recombination, and transposition. Commercially available products include ciprofloxacin, norfloxacin, lomefloxacin, ofloxacin, and enoxacin. The second method, in contrast to the primary one, seems to kill stationary-phase bacteria, or those lacking active RNA or protein production. In gram-negative bacteria, both ciprofloxacin and ofloxacin exert this mechanism, whereas only ofloxacin does so in gram-positive bacteria (Just, 1993).

Table 2: Existing classes of antibacterial drugs (Devasahayam et al., 2010).

Class of compound	Phase of development	Analogues	Mechanism of action	Spectrum of activity	Resistance mechanism	Drug company
Oxazolidinones	FDA approved 2000	Linezolid, Radezolid, Torezolid, RWJ-416457	Inhibits protein translation (initiation/ elongation)	Gram-positives, some anaerobes, *M. tuberculosis*	rRNA mutations	Pfizer, Rib-X, Trius Therapeutics, Johnson & Johnson
Glycopeptides	Phase III	Oritavancin, Dalbavancin, Telavancin	Inhibit PG biosynthesis/ transglycosylation	Gram-positive	Unidentified	Targanta/The Medicines Co., Pfizer, Theravance
Ketolides	Phase III	Cethromycin	Inhibits protein synthesis	Gram-positive	rRNA dimethylation, ribosomal protein mutations	Advanced Life Sciences
Glycylcyclines	FDA approved 2005	Tigecycline, PTK0796	Inhibits protein synthesis	Gram-positive, Gram-negative, aerobes, anaerobes	Efflux pumps	Wyeth, Paratek Pharmaceuticals
Carbapenems	FDA approved 2007	Doripenem, Razupenem	Inhibits PG biosynthesis	Gram-positive, Gram-negative, anaerobes	Carbapenemases, Efflux pumps, Porin mutations	Johnson & Johnson, Protez Pharmaceuticals
Streptogramins	Phase II	NXL103/XRP2868	Inhibits protein translation	Gram-positive, Gram-negative	Unidentified	Novexel
Fluoroquinolones	Preclinical	JNJ-Q2, finafloxacin	Inhibits type II topoisomerase	Gram-positive, Gram-negative	gyrA, parC mutations	Johnson & Johnson, MerLion Pharmaceuticals

The majority of antimicrobial medications either work statically (for example, bacteriostatic) or cidally to exert their effects (bactericidal). Cidal medications typically kill the bacterium at doses that can be attained clinically; static drugs generally stop the organism from developing but do not necessarily kill it (Purssell, 2020).

Of all the above-mentioned antibacterial drugs that are being employed as depicted in Table 1, one of the alarming concerns about them are their effects on the environment upon disposal (Bbosa et al., 2014). There is also another issue of potentially worrying adverse effects which arise for long course usage. This excludes the complexity of the synthesis, longer reaction time as well as the cost and energy involved in such reactions. Additionally, the traditional antibacterial agents have indicated some degree of being ineffective in treating bacterial infections. This has led to the emergence of finding improved antibacterial agents against this worldwide challenge. Alternative therapies that are currently in practice or under trial include the use of antibiotics in combination or with adjuvants, bacteriophage therapy, use of antimicrobial peptides, photodynamic therapy, antibacterial antibodies, phytochemicals and nanoparticles as antibacterial agents (Mulani et al., 2019).

2.2 The development of Nanoscience

Richard P. Feynman prophesied that one day we would be producing things at the atomic level in 1959 in his well-known lecture "There is plenty of room at the bottom." This lecture has since been associated with the reason behind the development of nanoscience and nanotechnology. Nanoscience has been established recently as a new interdisciplinary science. It can be defined as a whole knowledge on fundamental properties of nano-size objects. The

prefix ‘nano’ indicates one billionth or 10^{-9} units. Nanoparticles with a diameter of less than 100 nm are currently gaining more attention due to their numerous novel applications in several industrial sectors. Attributed to properties including small particle size, large surface area, quantum confinement, and other characteristics, such powders may have characteristics that are significantly different from those of bulk materials (Solomon et al., 2007). In the current situation, nanoscale materials have developed up as novel antimicrobial agents due to their high surface area to volume ratio and the unique chemical and physical properties. With its use in science and technology to create novel materials at the nanoscale, nanotechnology is emerging as a rapidly expanding area (Rai, et al., 2009). Atomic clusters between 1 and 100 nm in size are known as nanoparticles. Nanomaterials can be created with a variety of physicochemical and biological properties on both their surface and core due to their small size. These characteristic features make nanotechnology facilities useful for application in medicine and biology (Sindhwani et al., 2021), (Zhu et al., 2012).

The modulation of metals into their nano-size has been evidently the growing focus in the present century since the nanoscale metals have unique physical, chemical and optical properties compared to bulk metals. Metallic silver in the state of silver nanopartilces is one of the notable metals being researched as potential antimicrobial agents (“The Bactericidal Effect of Silver Nanoparticles,” 2005). Jain et al., synthesized spherical AgNPs with a size range of 7-20 nm and their synthesized AgNPs were found to be an effective antibiotic against *E. coli*, *S. typhi*, *Staphylococcus epidermidis* and *S. aureus* (Jain et al., 2009). Particle size, surface charge, and solubility are the primary physicochemical characteristics of nanosystems that regulate critical activities such as intracellular absorption, biodistribution, or clearance. In order to prepare metallic nanoparticles, a variety of techniques are used. Depending on the starting material, these techniques can be divided into two broad categories: bottom-up methods and top-down methods (Jamkhande et al., 2019).

2.2.1 Synthetic methods of nanoparticles

Synthesis of nanoparticles can be categorized into top-down or bottom-up approaches displayed in Figure 3 (Sepeur, 2008). When using a top-down strategy, large structures are broken down into smaller ones. Among the most often used top-down approaches are physical techniques including lithography, laser ablation, sputtering deposition, pulsed electrochemical etching, and vapor deposition these methods are also known as physical approaches. Whereas in bottom-up techniques also known as “chemical approaches”, atom by atom, molecule by

molecule, or cluster by cluster, material is synthesized using techniques such sol-gel processing, chemical vapor deposition, plasma or flame spraying synthesis, laser pyrolysis, and microemulsion (Balasooriya et al., 2017). However, top-down production methods introduce imperfections in the surface structure of the product, and this is a major limitation because the surface chemistry and the other physical properties of nanoparticles are highly dependent on the surface structure (Mittal et al., 2013). Previous literature has mentioned that these top-down methods are not suitable for formulating evenly shaped nanomaterials and in certain cases it becomes challenging to get very small sizes although high energy is employed (Khan, 2020).

Apart from the hazardous issues, methods such as lithography, laser ablation, aerosol technologies and ultraviolet irradiation remain expensive. For an example, in laser ablation there must be a lot of energy used to achieve a high ablation efficiency (Abid et al., 2022). Therefore, there is an increasing interest in the development of simple, eco-friendly, biocompatible, practical, affordable, and sustainable solutions to enhance and/or safeguard our planet's ecosystem.

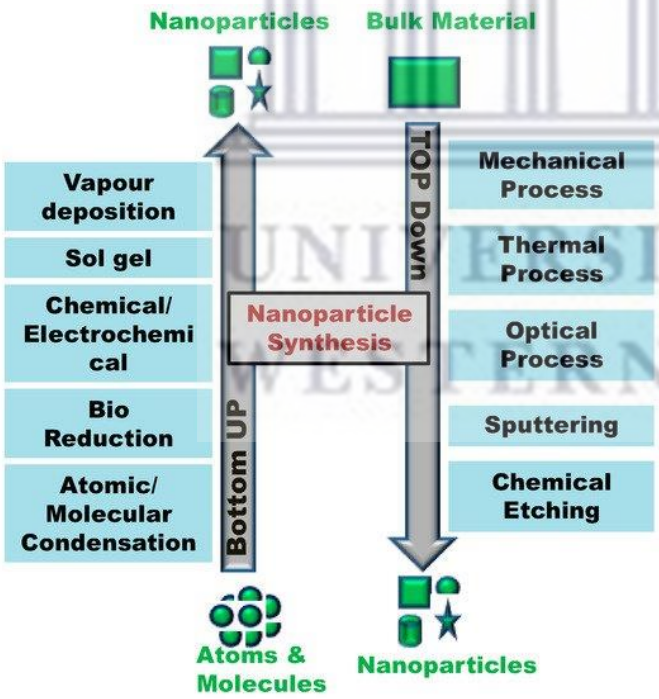

Figure 2: Top-down and bottom-up approaches of nanoparticles synthesis (Bayda et al., 2019).

Regardless, bottom-up approaches also have numerous downfalls which also makes them no longer a suitable synthetic route. The majority of these typically demand physical conditions like high temperatures, vacuum conditions in addition to toxic and harsh chemical additions

like dimethyl formamide, hydrazine, and sodium borohydride. Due to the high surface charge and high surface area of nanoparticles, harsh chemicals may stay adsorbed onto nanoparticles, resulting in poisonous and harsh compounds that may pose biological dangers to the environment. These substances may have negative impacts on a variety of trophic levels of organisms, including microbes, plants, invertebrates, and vertebrates, including humans (Balasooriya et al., 2017), (Rana et al., 2013).

The choice of a metallic nanoparticle preparation technique is crucial because processes used in nanoparticle synthesis, such as the kinetics of metal ions' interactions with reducing agents, the adsorption of stabilizing agents onto metal nanoparticles, and various experimental techniques, have a significant impact on the stability, morphology (structure and size), and physicochemical properties of the final product (Jamkhande et al., 2019), (Vijayakumar, et al., 2013). Chemical reduction is the most frequently applied method for the preparation of silver nanoparticles as stable, colloidal dispersions in water or organic solvents (Vijayakumar, et al., 2013).

As it has been detailed that various toxic compounds are utilized in chemical processes, which are damaging to the health of living beings. Green synthesis has a number of benefits over chemical and physical methods, including the ability to produce large quantities of nanoparticles quickly and cheaply. In this regard, reducing agents and stabilizers can be made from bacteria (Huq, 2020), plant extracts (Gopinath et al., 2012), fungi (Vigneshwaran et al., 2007), polysaccharides, and their derivatives (Liao et al., 2019). Therefore, it is essential to develop green methods for nanoparticle synthesis. To reduce the harmful substances, the AgNPs can be manufactured utilizing biological processes based on green chemistry. Green synthesis of AgNPs can be accomplished using a variety of resources, including bacteria, fungus, enzymes, and plant extracts. Other materials of a biological origin such as honey (Figure 4), can also synthesize Ag (Philip, 2010), Au (Chandran et al., 2006), Pd (Reddy et al., 2012) and Pt nanoparticles (Ghaffari-Moghaddam et al., 2014). Natural products are frequently utilized as capping and reducing agents to strengthen the stability of produced nanoparticles and prevent agglomeration (Majoumouo et al., 2019). Recent developments in nanotechnology focus on environmentally friendly, cost-effective synthesizing methods. Green synthesis of nanoparticles is an ecofriendly and safe mode of synthesis of nanomaterial using biological resources (Balasooriya et al., 2017).

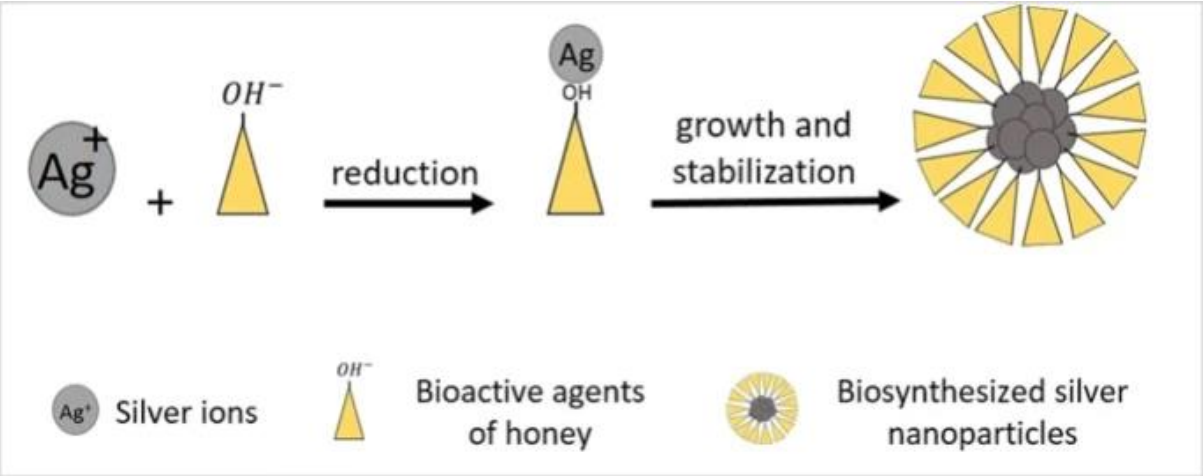

Figure 3: Schematic representation of honey reduced silver nanoparticles. (Khorrami et al., 2019).

Generally, the synthesis process of the Ag-NPs in solution usually employs the following three main components: (i) metal precursors, (ii) reducing agents and (iii) stabilizing/capping agents. The formation of colloidal solutions from the reduction of silver salts involves two stages of nucleation and subsequent growth (Tran & Le, 2013), (Prabhu & Poulose, 2012).

When it comes to green chemistry, biological methods outperform physical and chemical approaches. Biological processes can show to be more effective for large-scale synthesis since they are a cleaner method of synthesis, offering a sustainable way to utilize their antibacterial action. Although many different metals can be used to create nanoparticles, silver nanoparticles hold the top spot in nanotechnology due to their versatility.

2.2.2 Properties of silver nanoparticles and their applications

When it comes to biological systems, living things, and medicine, silver (Ag) is the noble metal of preference (Parashar et al., 2009). Silver has been utilized for treating bacterial infections throughout history, from Hippocrates' "the father of modern medicine" on early treatment of ulcers through the treatment of gonococcal infections in infants (Chaloupka et al., 2010), (Rai, et al., 2009). This is evidence that silver has long been known to have antibacterial activity.

In comparison to their bulk counterparts, nanoparticles have different physical and chemical properties which include catalytic activity, thermal and electrical conductivity, optical absorption, and melting point due to their small size and high surface to volume ratios (Vishwanath & Negi, 2021). When high surface areas are essential for realization, these characteristics can be used. Due to their significant potential for use in plasmonics,

antimicrobial materials, sensing, and spectroscopy, Ag nanoparticles and their related nanostructures have been the subject of much research (Shenashen et al., 2014). In plasmonics nanoparticles can rise in temperature when exposed to irradiation at their resonance wavelength which is applicable for a wider range of therapies such as targeting tumour tissues (Wang et al., 2020). Antimicrobial use of nanoparticles has been demonstrated by Garibo et al., where they synthesized silver nanoparticles using a plant and studied its antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) and one yeast (*Candida albicans*) (Garibo et al., 2020). In the sensing application of nanoparticles, gold nanoparticles are used as a colorimetric technique for the detection of small concentrations of aqueous heavy metal ions, including toxic metals such as lead, cadmium, and mercury in water (Y. Kim et al., 2001).

Ag NPs stand out among other metal nanoparticles for having enhanced innate physical, chemical, and biological properties that greatly increase their value (Vishwanath & Negi, 2021). There are many consumer products and applications utilizing nanosilver; nanosilver-related applications currently have the highest degree of commercialization. A wide range of nanosilver applications has emerged in consumer products ranging from disinfecting medical devices (Deshmukh et al., 2019) and home appliances to water treatments (Tran et al., 2013). Wound dressing serves as another application for nanocrystalline silver as a commercial product. In a clinical investigation, the effectiveness of the new chitosan-nanocrystalline silver and the existing 1% silver sulfadiazine were evaluated for the treatment of wounds. Chitosan-nanocrystalline silver had a 13-day healing rate that was 98.98% greater than the control and 6.09% higher than that of 1% silver sulfadiazine. Additionally, for the chitosan-nanocrystalline silver dressing group and the 1% silver sulfadiazine group, the healing periods were 13.51 4.56 days and 17.45 6.23 days, respectively. It was discovered that the chitosan-nanocrystalline silver combination had very high antibacterial activity and wound healing properties (Deshmukh et al., 2019).

Lv et al., synthesized silver nanoparticles functionalized with porous ceramic composite with the presence of an aminosilane coupling agent, 3-aminopropyltriethoxysilane as a connecting molecule. The Ag-NPs-porous ceramic composite was used as an antibacterial water filter against *E coli* and it was found the output count of *E coli* was zero after it was previously measured to have a bacterial load of approximately 105 CFU (colony forming unit) ml^{-1} (Lv,

et al., 2009). This shows that the AgNPs-porous ceramic composite is a good antibacterial agent in water treatment.

AgNPs were used in medical devices as antimicrobial coatings to lower nosocomial infections in hospitals. In the study of Roe et al., they coated catheter with AgNPs which were prepared by heating a solution containing silver nitrate, sodium saccharine and tetramethyl-ethylenediamine in a microwave. Catheters with a silver coating demonstrated considerable invitro antibacterial efficacy and reduced pathogen biofilm development against *E. coli*, *Enterococcus*, *S. aureus*, *coagulase-negative staphylococci*, *P. aeruginosa* and *C. albicans*. They prevented the development of biofilms and cell growth for at least 72 hours (Table 1). With the exception of *P. aeruginosa*, all microorganisms had complete growth inhibition (67% growth inhibition at t = 72 h). After 72 hours, the biofilm-forming ability of *E. coli*, *S. aureus*, and *C. albicans* was almost completely inhibited, while that of *Enterococcus*, coagulase-negative *staphylococci*, and *P. aeruginosa* was more than 50% inhibited. They may be helpful in lowering the risk of infection problems in patients with indwelling catheters due to their proven antibacterial capabilities (Roe, et al., 2008).

Recent studies have indicated that the extensive research being done on silver nanoparticles is mostly for application of these nanoparticles as antibacterial agents. Before determining toxicity or biocompatibility, it is crucial to analyse the distinctive properties of nanomaterials, such as size, shape, size distribution, surface area, form, solubility, aggregation, etc (Murdock et al., 2008) since the application of the nanoparticles is determined by these features. Numerous analytical methods, such as UV-vis spectroscopy, X-ray diffractometry, Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy, dynamic light scattering, scanning electron microscopy, transmission electron microscopy, atomic force microscopy, and others, have been used to assess the synthesized nanomaterials (Sapsford et al., 2011).

However, it is the exceptional antibacterial activity exhibited by AgNPs that has focused the attention of researchers and industries on this nanomaterial (Bruna et al., 2021). Colloidal silver nanoparticles with sizes ranging from 20 to 45 nm were synthesized by sol-gel method and their antibacterial activity was determined using the broth microdilution method. The antibacterial activity of these synthesized silver nanoparticles was evaluated against *E. coli*, *S. aureus*, *C. albicans*, *B. subtilis*, *Salm. typhimurium*, *P. aeruginosa* and *K. pneumoniae*. The MIC of the silver nanoparticles was found to be 2-4 µg/mL (Lkhagvajav, et al., 2011).

In another study by Zarei et al., they investigated the antibacterial activity of silver nanoparticles of four pathogens found in food. The bacterial strains tested were *E coli*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Vibrio parahaemolyticus*. The nanoparticles showed great antibacterial effects with MIC values ranging from 3.12 µg/mL to 6.25 µg/mL. *E coli*, *Salmonella typhimurium* and *Vibrio parahaemolyticus* all had the same MIC value of 3.12 µg/mL while *Listeria monocytogenes* showed a higher MIC of 6.25 µg/mL (Zarei et al., 2014). As an antibiotic alternative, this application has been broadly studied in recent years with the objective of developing new bactericidal products for decontamination or infection treatments taking advantage of the already established knowledge about their efficiency even against multidrug resistant organisms (Lee et al., 2019). In all the reported properties of AgNPs, one which is of interest for the scope of this study is the antibacterial characteristics that AgNPs possess. This property makes them attractive for application in the medical field for developing new antibacterial agents because they are biocompatible.

2.3 Application of silver nanoparticles for antibacterial uses

As it has been highlighted that one of the significant properties that silver nanoparticles hold is their ability to behave as antimicrobial agents they have emerged as the most researched metal nanoparticle in the drug industry. Many studies have investigated the antibacterial properties that silver nanoparticles possess which renders them as potential antibiotics for use in the pharmaceuticals. A one-step synthesis protocol of silver nanoparticles was reported by Panacek et al. (2006). The synthesized silver nanoparticles possessed high antimicrobial and bactericidal activity against both Gram-positive and Gram-negative bacteria as well as drug-resistant strains such as methicillin resistant *S. aureus* (Panáček et al., 2006) .In another study by Leaper, (2006), they investigated the use of nanosilver in wound dressings and their role in wound healing. They reported that the topical application of silver nanoparticles promoted healing in burn wound with better scarring (Leaper, 2006).

The silver nanoparticle's antibacterial effect is thought to be achieved via oxidation. When silver nanoparticles (silver NPs) are administered to the body as antimicrobials, they become oxidized and release the Ag+ ion, which causes the bacterium to die (Vishwanath & Negi, 2021). According to a study by Morones et al. (2005) on the antibacterial action of silver nanoparticles against Gram-negative bacteria, silver nanoparticles disrupt cell function by adhering to the cell membrane's surface, entering bacteria, and then releasing silver ions. They confirmed this by analysing the electrochemical behaviour of the nanoparticles using stripping

voltammetry. In their findings they found that the concentration of Ag^{+} decreased over a period of 24 hours compared to the initial known concentration (Morones et al., 2005).

AgNPs' ability to exert antibacterial activity through a variety of mechanisms—including possible effects on the cell membrane, intracellular components, and the respiratory chain—is one of its distinguishing features (Dakal et al., 2016). This feature is viewed as a key benefit because it would be necessary for bacteria to target numerous simultaneous modes of action in order to build resistance against AgNPs. Because of these factors, AgNPs have also been proposed as an antibiotic alternative. In this regard, there has been numerous publications reporting on potential use of AgNPs as an antibiotic agent. The covered studies are those where green synthesis methods are implemented to produce these nanoparticles, particularly where honey is used as a reducing agent. In comparison to other biological approaches, honey-mediated biological synthesis has several advantages, including the avoidance of complex procedures like drying plant materials when using plants as the reducing agent (Venu et al., 2011). The drying process of plant material is laborious as highlighted in previous studies for example, in a study by Wang et al. (2019) they washed the flowers and leaves of plants using double-distilled water which was followed by drying at room temperature before they weighed and crushed the dried sample (Wang, et al., 2019).

A study by (Khorrami et al., 2019) they studied the antibacterial effect of Astragalus gossypinus honey synthesised silver nanoparticles against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* bacterial strains. In their findings, the honey synthesized AgNPs had an MIC of 100, 24.5 and 24.5 µg/mL against *S aureus*, *P aeruginosa* and *E coli* respectively. These nanoparticles inhibited the growth of Gram-negative strains at the lower concentrations of 24.5 µg/mL. However, the biological activity of the honey by itself did not show any activity against all the tested strains.

In another study, they investigated the antimicrobial activity of silver nanoparticles synthesized using honey and further evaluated their activity against different silver-resistant clinical bacterial isolates obtained from wounds and burns and their MIC for each strain was determined. These bacterial isolates were identified as *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Acinetobacter* spp. and *Enterobacter* spp. The results reveal that the AgNPs showed good antibacterial activity against the tested strains, the lowest MIC of the AgNPs against *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Acinetobacter* spp.

and *Enterobacter* spp. were found to be 6.25, 3.375, 3.75, 3.375, 3.375 and 3.375 µg/mL respectively (Hosny et al., 2017).

The antimicrobial properties of colloidal silver nanoparticles prepared using an environmentally friendly procedure employing honey as a reducing agent was investigated against both gram positive and negative microbial such as *Bacillus cereus, Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus* (Siddiqui et al., 2018). Upon testing their susceptibility on the selected strains, the % growth of *Bacillus subtilis* and *Bacillus cereus* appeared to be completely suspended at concentration of AgNPs higher than 200 µL/2mL. Towards the % growth of *Escherichia coli* and *Staphylococcus aureus* and the minimum concentration of 300 µL/2mL was required to inhibit the growth of these bacteria.

It is evident that in the process of green synthesis of silver nanoparticles using honey, the reaction is driven by the presence of OH^- because in the most reported literature the reaction is feasible under alkaline conditions, and this is presumed that it also shortens the reaction time from several hours to minutes. In addition, the higher pH values have been reported to yield smaller sizes of nanoparticles (Siddiqui et al., 2018).

In a previous study by (Khalil, et al., 2014), they reported the green synthesis of silver nanoparticles with the assisted bio-reduction using an aqueous leaf extract. In their evaluations, they investigated the effect of pH on the formation of silver nanoparticles using UV-vis analysis. According to their findings, the absorbance was found to be increasing with increasing pH from 2 to 8. Furthermore, using TEM for size analysis they observed that the particles became larger in acidic medium than in basic medium. Hence it can be suggested that the alkine pH environment improved the reduction process (Khalil, et al., 2014).

AgNPs are shown to have a strong antibacterial effect on both Gram-positive and Gram-negative bacteria. The underlying mechanisms by which they exert bactericidal or growth-inhibitory effect has not yet been fully explored. Various theories that take into account AgNPs' physical characteristics, such as size and surface, which enable them to interact with or even pass-through cell walls or membranes and directly alter intracellular components, are supported by the experimental data currently available (Bruna et al., 2021).

Several studies have shown that AgNP activity is strongly dependent on the size (Wu et al., 2014). In fact, the bactericidal activity of AgNPs of smaller dimensions was found to be optimal against several most common pathogens (Hosny et al., 2017). A study by Ethiraj et al, (2016)

confirmed that smaller silver nanoparticles possess stronger bactericidal activity. They synthesized silver nanoparticles using three different leaf extracts of *Alstonia scholaris*: fresh, sun-dried and sun-dried. Using SEM analysis, they confirmed that the size of the AgNPs synthesized from the different leaf extracts increased in the order: fresh< oven-dried<sun-dried. Antibacterial studies showed that the AgNPs synthesized from the fresh leaves had the highest activity (Ethiraj et al., 2016).

Smaller nanoparticles seem to have a superior ability to penetrate into bacteria. In fact, the interactions with the membranes and any resulting damage, which may lead to cell death, are certainly more evident in the case of nanoparticles with smaller diameter and a positive zeta potential. Electrostatic forces that develop when nanoparticles with a positive zeta potential encounter bacteria with a negative surface charge promote a closer attraction and interaction between the two entities and possibly the penetration in bacterial membranes. Indeed, the zeta potential along with the size of the nanoparticles is a fundamental parameter for controlling the antimicrobial activity and more effective nanoparticles have a positive zeta potential and a reduced size (Franci et al., 2015).

The three main methods by which AgNPs exert their antibacterial action have been seen together or independently and are now supported by the literature(Marambio-Jones & Hoek, 2010). Figure 4 gives a brief summary of all the suggested mechanisms of AgNPs against pathogens. According to the first theory, AgNPs work at the membrane level because they can pass through the outer membrane and accumulate in the inner membrane, where their adhesion causes the cell to become damaged and destabilized. This increases membrane permeability, causes cellular content to leak out, and eventually causes the cell to die. Additionally, there is evidence that AgNPs can interact with sulfur-containing proteins in bacterial cell walls, which may result in structural damage and cell wall rupture (Bruna et al., 2021).

The antimicrobial activity of silver nanoparticles against *E. coli* was investigated as a model for Gram-negative bacteria in a study by (Deshmukh et al., 2019). Bacteriological tests were performed in Luria–Bertani (LB) medium on solid agar plates and in liquid systems supplemented with different concentrations of nanosized silver particles. These particles were shown to be an effective bactericide. Scanning and transmission electron microscopy (SEM and TEM) were used to study the biocidal action of this nanoscale material. The results confirmed that the treated *E. coli* cells were damaged, showing formation of "pits" in the cell wall of the bacteria, while the silver nanoparticles were found to accumulate in the bacterial

membrane. A membrane with such a morphology exhibits a significant increase in permeability, resulting in death of the cell (Deshmukh et al., 2019).

The second mechanism suggests that nanoparticles can enter cells, where it has been suggested that due to AgNPs' characteristics, they will have an affinity to interact with sulfur or phosphorus groups present in intracellular content such as DNA and proteins, changing their structure and functions. Nanoparticles can also break and cross the cell membrane, altering its structure and permeability. Likewise, they can damage intracellular machinery, activate the apoptotic pathway, and change the respiratory chain in the inner membrane by reacting with thiol groups in the enzymes and creating reactive oxygen species and free radicals (Gomaa, 2017). Kim et al., (2008) demonstrated that silver ions do cause critical cell damage by complexing with thiol groups of the structural or enzymatic proteins of the microorganisms. They performed spectrophotometric and MALDI-TOF mass analysis to support this fact (J. Y. Kim et al., 2008).

The release of silver ions from the nanoparticles, which because of their size and charge can interact with biological components to change metabolic pathways, membranes, and even genetic material, is a third mechanism that is proposed to happen simultaneously with the other two. Amother study by Feng et al., (2000) supported the fact that when silver nanoparticles release the silver ions inside the bacteria, these ions altered their deoxyribonucleic acid (DNA) molecules. Two strains of bacteria, Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*, were treated with $AgNO_3$ and examined using combined electron microscopy and X-ray microanalysis to determine the mechanism of inhibition of silver ions on microorganisms. Both *E. coli* and *S. aureus* cells underwent comparable morphological alterations following Ag^+ treatment. The cell wall and the cytoplasm membrane were visibly separated and in the centre of the cells, a striking electron-light area was visible that included condensed DNA molecules. The antibacterial mechanism of silver was revealed by the presence of silver and sulfur elements in the electron-dense granules and cytoplasm discovered by X-ray microanalysis: DNA lost its ability for replication, and the protein became inactive after Ag^+ treatment (Feng, et al., 2000).

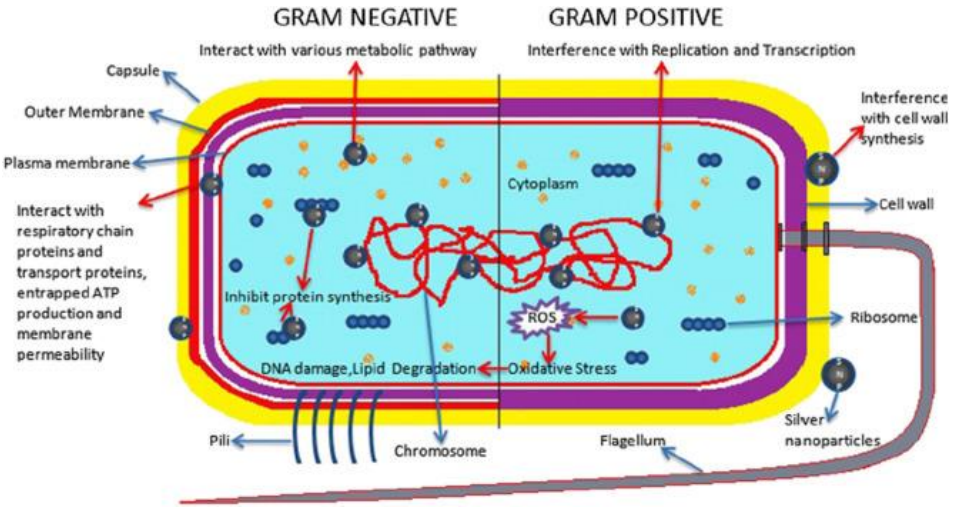

Figure 4: Antibacterial mechanisms of AgNPs (Deshmukh et al., 2019) .

Undoubtedly, one mechanism cannot individually be considered as responsible for the antibacterial behaviour of silver nanoparticles. All these mentioned factors can interfere with the death of bacteria. However, depending on the physicochemical properties of nanoparticles and the biological features of bacteria, one of these mechanisms is dominant.(Khorrami et al., 2019). The biological activity of AgNPs depends on factors including surface chemistry, size, size distribution, shape, particle morphology, particle composition, coating/capping, agglomeration, and dissolution rate, particle reactivity in solution, efficiency of ion release, and cell type, and the type of reducing agents used for the synthesis of AgNPs are a crucial factor for the determination of cytotoxicity.

One of the factors of major consideration is the toxicity that nanoparticles could have for human and environmental health, given that their size, which can be considered their main advantage, is also what attributes to them the possibility of crossing the defense barriers in organisms, being able to induce mild to chronic toxic effects after their accumulation.

2.4 Toxicity of silver nanoparticles

Numerous research has been conducted with the aim of comprehending the potential harmful effects of exposure to large dosages of AgNPs due to the quick advancement of new items and technologies utilizing AgNPs. Given that the usage of medical goods containing AgNPs may result in the ingesta, inhalation, or cutaneous exposure to this nanomaterial, the in vitro effects of various dosages and sizes of AgNPs in mammalian cells will be addressed in order to establish antecedents about these effects in this section (Bruna et al., 2021). Nanoparticles are extremely small and have a large surface area, which allows for potential interactions with

membrane surfaces as well as facile translocation and distribution throughout the body (Panyala et al., 2008). The fate of nanoparticles introduced into the environment, whether on purpose or accidentally, is another crucial factor; this is typically controlled by interactions with the surrounding environment as well as the surface characteristics of AgNPs (Tortella et al., 2020). The intrinsic properties of the AgNPs such as concentration, size and the presence of capping agents, are also included in these parameters along with pH. Toxicity of AgNPs are linked with several physicochemical properties such as size, the chemical nature, surface area, reactivity and charge and ease of aggregations (Korani et al., 2015). An in-vitro research study was carried out by Liu et al., (2010) to evaluate how the size of silver nanoparticles affects its toxicity. Using three different sizes of AgNPs (5, 20 and 50 nm). Human cell models were exposed to the different silver nanoparticles and assessed their cell viability, cellular membrane integrity, cell cycle progression and oxidative stress. They found that the cells which were exposed to 5 and 20 nm sized nanoparticles were prone to elevated ROS levels, had lost cellular membrane integrity and shown no cell progression. In addition, apoptosis occurred on 4-9% of the cells. According to an elemental analysis of silver in cells by ICP-MS, it showed that smaller nanoparticles enter cells more readily than larger ones, which could account for the higher toxicity effects (Liu et al., 2010).

To evaluate the effect of surface chemistry on the toxicity of silver nanoparticles, a set of three types of silver nanoparticles with the same core but different surface chemistry was prepared. The Ag cores were stabilized with a polymer shell [poly (isobutylene-alt-maleic anhydride) (PMA)] or with mercaptoundecanoic acid (MUA). Then the polymer coated AgNPs were further functionalized with polyethylene glycol (PEG) to reduce their cellular uptake. It was found that the PMA coating did not reduce the release of Ag ions compared to the MUA coating. They found that pH environment of the cell did influence the release of silver ions because more Ag(I) ions are released inside endosomal/lysosomal compartments as a result of faster corrosion of all Ag NPs in an acidic environment. In addition, their colloidal stability was investigated, and they found that the MUA coated nanoparticles had a lower stability and were more agglomerated than the PMA coated ones. Finally, they discovered that the PEG coat did reduce their cellular uptake which resulted in lower toxicity (Caballero-Díaz, et al., 2013). In another study, Kittler et al., (2010) demonstrated the release of Ag ions from the AgNPS due to oxidation. They found that at pH 7, after 7 days of incubation, the amount of Ag ions released was extremely low. However, after seven days, at pH 3, about 1% og the Ag ions from the silver nanoparticles was detected in solution. This is in agreement with the fact that, Ag

NPs inside acidic endo/lysosomal compartments release more Ag(I) than NPs in the extracellular neutral medium (Kittler et al., 2010).

According to previous studies, it is claimed that AgNPs and Ag^+ released from the nanoparticles both exert toxicity by encouraging DNA damage, mitochondrial dysfunction, protein oxidation and denaturation, membrane damage, reactive oxygen species (ROS) formation, and cell proliferation suppression (Durán et al., 2010), (Yin et al., 2020). Concerns have been expressed about AgNPs' potential release into the environment through a number of industrial processes, including particle creation during production and absorption into goods, recycling, and disposal. Even during industrial manufacturing and laboratory synthesis of silver nanoparticles, they may enter the body by inhalation and skin contact (Tran et al., 2013), (Durán et al., 2010), (Liao et al., 2019).

Tonnes of silver are reportedly released into the environment through industrial waste, and free silver ions in the aqueous phase are presumed to be primarily responsible for silver's environmental toxicity. The harmful effects of these free silver ions on people and all other living things include permanent bluish-gray discoloration of the skin or eyes (argyria), and exposure to soluble silver compounds may result in toxic effects such as liver and kidney damage and respiratory insults (Panyala et al., 2008). In a detailed nanotoxicology study of silver nanoparticles, it was found that intracellular release of silver is responsible for the toxicity to human lung cells (Gliga, et al., 2014). In a separate study, (Han et al., 2014) showed that oxidative stress and ROS formation caused by the toxicity of AgNPs in alveolar basal epithelial cells was dose dependent. In addition, green AgNPs were more hazardous than chem-AgNPs at lower doses. Biosynthesized AgNPs and chemically synthesized AgNPs had IC50 values of 25 g/mL and 70 g/mL, respectively. This suggested that the chemically synthesized nanoparticles were more suitable for therapeutic applications than the biosynthesized ones.

According to a number of cross-sectional research, argyria is the most typical unfavorable result of silver exposure (Chang et al., 2006). Several studies reveal that continuous consumption of drugs containing silver results in skin colour changes and a blue-grey appearance on the face (Han et al., 2014), (Liao et al., 2019). A study was carried out by (Parang & Moghadamnia, 2018) to assess the toxicological effect of silver nanoparticles on liver cells of mice. They observed and compared the changes of serum levels of hepatic enzymes through the induction of oxidative stress. There were three experimental groups and two of them which were receiving silver nanoparticles showed a noticeable increase in the

serum of albumin levels. Silver nanoparticles at a dose of 100 mg / kg caused a significant rise in the ROS thus stimulating the elevation of serum levels of hepatic enzymes and other biochemical factors associated with liver which eventually leads to hepatic injuries (Parang & Moghadamnia, 2018). After the effective review of the topic of research, we shall report on how the problem statement was investigated so as to achieve the desired results. This involved several techniques which are outlined in the next chapter.

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Chapter 3

Methods and materials

3.1 Materials

All the reagents purchased were of good analytical grade and quality and were used without any further modifications.

The *Meliboca meliponae* honey was obtained from Kakamega Tropical Rain Forest in Kenya (Center for African Medicinal and Nutritional Flora and Fauna (CAMNFF) supplied by Professor Maurice Omolo at (Masinde Muliro University of Science and Technology). Silver nitrate ($AgNO_3$), 99% purity was obtained from Merck (Daemstadt, Germany).

Table 3: Lists of all the instrumentation and equipment used for the synthesis, analysis and application of the silver nanoparticles.

Technique	Common name	Machine	Supplier	Location
Ultraviolet Visible Spectroscopy	UV-vis	POLARstar Omega Plate reader	BMG Labtech	Offenburg Germany
Fourier Transform Infrared Spectroscopy	FTIR	PerkinElmer Spectrometer	Anton Paar	Graz Austria
Dynamic Light Scattering	DLS	Malvern Zetasizer Nano-ZS90	Malvern Instruments	Malvern, UK
High Resolution Transmission Electron Microscopy	HRTEM	F20 Tenai EDAX G^2	FEI	Eindhovea, Netherlands
Thermo-shaker	Thermo-shaker	Thermoshaker, model DBS-001	MRC Lab	-

3.2 Methods

3.2.1 Synthesis of AgNPs

Silver nanoparticles were synthesized following a method by (Sreelakshmi et al., 2011) with slight modifications, such as using stingless bee honey as a reducing agent. The H-AgNPs were synthesized using stingless bees honey (SBH) from the *Meliponula feriguenea* bee specie and the method is defined below.

To synthesise the AgNPs, 1M standard solution of silver nitrate ($AgNO_3$) was prepared by dissolving 1.70g of silver nitrate (crystal pure purchased at Merck) in 10mL of deionized water and this $AgNO_3$ standard was used to prepare 10, 5, 1, 0.75, 0.5 and 0.25mM $AgNO_3$ solution. Then the following concentrations of honey solutions were prepared 100, 50, 25, 12.5, 6.25 and 3.125 mg/mL using the stingless bees honey of *Meliponula feriguenea*. A stock solution was prepared by diluting the honey using deionized water to make a solution of 200mg/mL and the other concentrations were achieved by serial dilution. The honey solutions pH was adjusted using 0.1M NaOH to obtain the following pH 4, 6, 7, 8, 9 and 10. The synthesis of AgNPs was carried out by adding 40µL of honey and 360 µL of $AgNO_3$ to make a ratio of (1:9) in a 2mL Eppendorf and placed in a thermo-shaker and the synthesis was done at different temperatures of 100, 75, 50, 25˚C in 1 hour for the various pH of the honey extracts.

3.2.2 Storage of the AgNPs

The AgNPs synthesized were covered with foil and stored in the cupboard at room temperature.

3.3 Characterization of the AgNPs

It is important to analyse and understand the physiochemical properties of silver nanoparticles to better understand their behaviour. Characterization techniques are employed to investigate their size, shape, optical properties, stability, and chemical composition. In this study, Ultraviolet Visible Spectroscopy (UV-vis), Fourier Transform Infrared (FTIR) spectroscopy, High Resolution Transmission Electron Microscopy (HRTEM), Dynamic Light Scattering (DLS) spectroscopy and X-ray diffraction (XRD) were used to analyse the H-AgNPs.

3.3.1 UV-vis spectroscopy

UV-vis spectroscopy from POLARstar Omega plate reader at the Life Sciences building, University of the Western Cape (UWC) was used to analyse the absorption spectra of the prepared H-AgNPs. The absorbance measurements were read at the wavelength range of 220-700 nm at 100 µL of each sample added in a 96 non-sterile well plate.

UV-Vis spectroscopy is a quantitative technique used to investigate the optical absorbance of thin or thick films or any chemical substance, as well as the system's light absorbance. This is accomplished by comparing the intensity of light passing through a sample to the intensity of light passing through a reference sample or blank. An Ultraviolet-Visible (UV-Vis) absorption spectrometer is used for the optical characterization of materials based on light attenuation, which can be the absorption or reflection of light from the sample under measurement. The schematic for the UV–Vis absorption spectroscopy is shown in Figure 6. UV-vis spectroscopy from POLAR star Omega plate reader at the Life Sciences Building, University of the Western Cape (UWC) was used to analyse the absorption spectra of the prepared H-AgNPs. The absorbance measurements were read at the wavelength range of 220-700 nm at 100 µL of each sample added in a 96 non-sterile well plate.

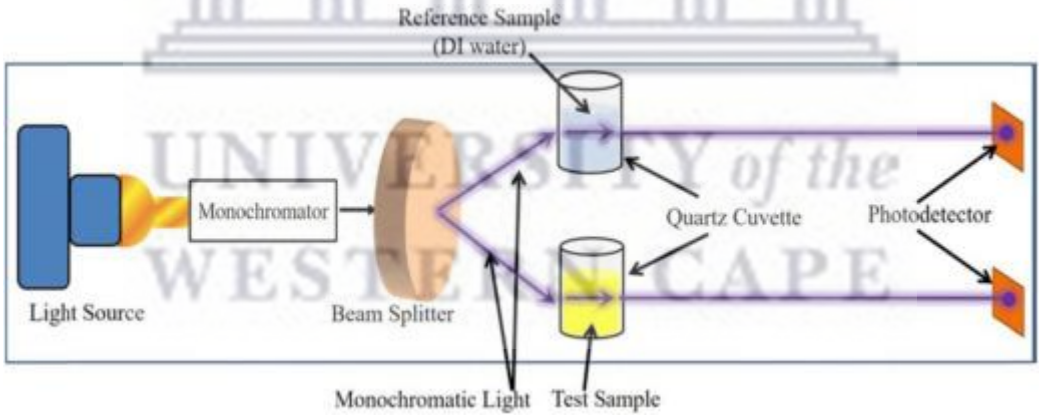

Figure 5: UV-vis spectroscopy principle (Sharma & Sharma, 2021).

3.3.2 FTIR spectroscopy

Structural compositions of H-AgNPs were assessed using Fourier Transform Infrared (FTIR) spectroscopy by identification of functional groups present in the sample at an absorption spectral range of 4000-600 cm^{-1}. A pellet was prepared by grinding a KBr powder and mixing it with a dried sample of the H-AgNPs and converted into a pellet form by using bench press.

FTIR analysis determines the spectrum of infrared wavelengths that a substance absorbs. The schematic for the FTIR is shown in Figure 6. To do this, samples of the material are exposed to infrared radiation (IR). To ascertain the material's molecular makeup and structure, the sample's capacity to absorb energy from infrared light at various wavelengths is measured. An extensive database of reference spectra is searched against the IR spectrum to identify unknown materials. As long as it is possible to create a standard curve of known concentrations of the component of interest, materials can be quantified using the FTIR materials characterization technique. Unknown materials, additives found in polymers, surface contamination on a material, and more can all be found using FTIR analysis. The results of the tests can identify the molecular structure and composition of a sample. To identify samples, a straightforward tool called an interferometer creates an optical signal that has all the IR frequencies encoded into it. It is quick to measure the signal. The signal is then decoded using a mathematical process called the Fourier transformation. The spectral data is then mapped using this computer-generated process. The spectrum that results from this graph is then identified by searching it against reference libraries.

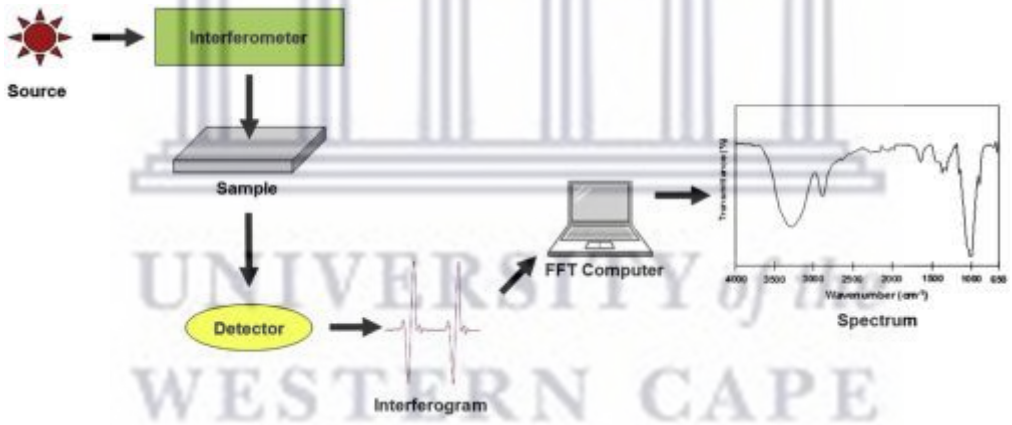

Figure 6: FTIR spectroscopy principle (Mathias, 2022).

3.3.3 HR-TEM

The morphology and the particle size distribution of *Cotyledon*-AgNPs were determined by HR-TEM analysis using a FEI Tecnai G2 F20 field-emission. TEM samples were prepared by drop-casting the AgNPs on carbon-coated grids and allowed to dry at room temperature, prior to measurement. The same samples were also examined using HR-TEM for Selected Area Electron Diffraction (SAED) and Energy-dispersive X-ray spectroscopy (EDX) studies. Software called Image J was used to determine the particle size distribution.

Electrons are made to pass through the specimen, and the image is formed on the fluorescent screen using either the transmitted or diffracted beam. It is made up of an electron gun that generates electrons. A magnetic condensing lens is used to condense the electrons and to control the size of the electrons that fall onto the specimen. As shown in Figure 7, the specimen is placed between the condensing and objective lenses. The magnetic objective lens is used to block the high-angle diffracted beam, and the aperture is used to eliminate any diffracted beam, increasing image contrast. To achieve higher magnification, the magnetic projector lens is placed above the fluorescent screen. A fluorescent screen or a camera can be used to capture the image (CCD – Charged Coupled device). The morphology and the particle size distribution of *Cotyledon*-AgNPs were determined by HR-TEM analysis using an FEI Tecnai G2 F20 field emission.

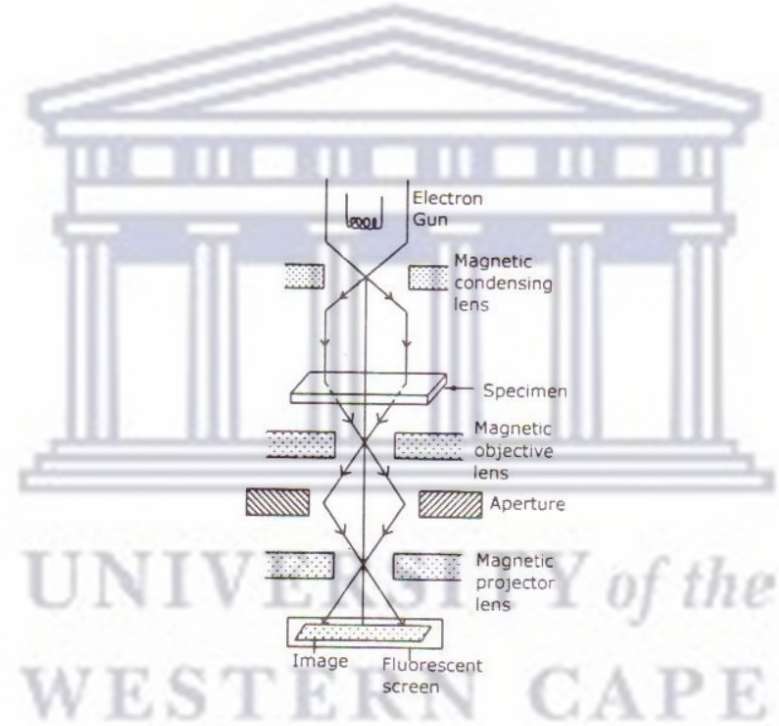

Figure 7: HRTEM working principle (BrainKart, 2018).

3.3.4 DLS

The particle size, the size distribution, zeta-potential, and the peak intensity of the AgNPs was determined by DLS using Zetasizer nano series Nano ZS, Malvern Instrument, UK. The DLS measurements were carried-out for size ranges from 0.1 to 100 nm.

Dynamic Light Scattering (DLS) is a popular light scattering technique because it allows for particle sizes as small as 1 nm in diameter. Emulsions, micelles, polymers, proteins, nanoparticles, and colloids are common applications. A laser beam illuminates the sample, and the fluctuations of the scattered light are detected by a fast photon detector at a known scattering

angle. Simple DLS instruments with a fixed angle of measurement can determine the mean particle size in a limited size range. Multi-angle instruments with greater sophistication can determine the entire particle size distribution. From a microscopic perspective, the particles scatter light, imprinting information about their motion. As shown in Figure 8, analysing the fluctuation of the scattered light yields information about the particles. The particle size, size distribution, zeta-potential, and peak intensity of the AgNPs were determined by DLS using Zetasizer nano series Nano ZS, Malvern Instrument, UK. The DLS measurements were carried out for size ranges from 0.1 to 100 nm.

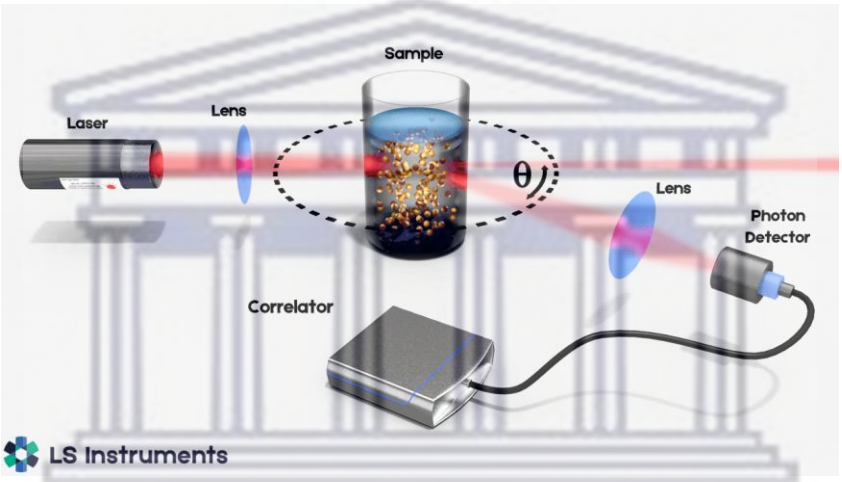

Figure 8: DLS working principle (LS Instruments, 2022).

3.4 Biological activity of the AgNPs

The biological activity of the AgNPs was assessed in agreement with biosafety level 2 guidelines at the Biolabels Node research labs (Department of Biotechnology, UWC). The laboratory disposed of the biological waste in accordance with the disposal technique outlined in their normal operating procedures. All the bacterial strains were obtained from ATCC (Manassas, USA).

3.4.1 Stability testing of SBH-AgNPs

The stability of the *Cotyledon*-AgNPs was evaluated in different biological media and buffers used in this study were Mueller Hinton broth (MHB) and LB broth. MHB and LB are used for determining minimal inhibitory concentrations (MICs), commonly used for antibiotic

susceptibility testing, specifically disk diffusion tests. In glass test tubes, 250 µL of aqueous solutions of *SBH*-AgNPs were mixed with the same volume of the medium or buffer. The tubes were incubated at 25 °C or 37 °C for 24 h. The stability of these nanoparticles was evaluated by measuring changes in their Ultraviolet-Visible spectroscopy (UV-Vis) spectra.

3.4.2 Antibacterial activity of AgNPs

The agar well diffusion method was used as described by (Boorn et al., 2010a) where the antibacterial activity of the synthesized AgNPs was evaluated on six bacterial strains of *S. aureus, S. epidermidis,* MRSA, *K. pneumoniae*, *E. coli* and *P. aeruginosa*.

Microbial suspensions of the bacteria were cultured in MHB broth and diluted until they reached a 0.5 McFarland standard (standard is approximately 1×10^8 CFU/mL). A volume of 50 µL of the microbial suspensions was added to each well containing MHB and AgNPs of 63 µg/mL concentration. The plates were sealed and incubated for 24 h at 37 °C and the zone of inhibitions were observed and determined. Ciprofloxacin (2 µg/mL) was used as positive control for the bacteria, while sterile deionized water was used as the negative control. An inhibition zone with diameter greater than 10 mm is considered as positive antagonism effect.

3.4.3 Determination of minimum inhibition concentration

The microbial suspensions were applied in volumes of 50 µL to each well containing MHB broth and AgNPs. The plates were sealed and kept at 37 °C for 24 hours. The negative control was sterile deionized water, while the positive control for the microorganisms was ciprofloxacin (2 g/mL).

After the initial incubation, in each well 10 µL of Alamar Blue was added, and the plate underwent a further three hours of dark incubation. The non-fluorescent Alamar Blue dye (resazurin) was transformed into the extremely fluorescent pink chemical resofurin in the presence of live bacteria. A spectrophotometer was then used to determine the MIC at a wavelength of 530-560/590 nm for fluorescence excitation and emission. Three duplicates of the screening were conducted. The MIC was reported as the lowest Ag-NP concentration that inhibited bacterial growth and MBC as the Ag-NP concentration that showed no bacterial growth.

Chapter 4

Results and discussion

4.1 Synthesis of SBH-reduced AgNPs

The synthesis of AgNPs using honey is suggested to be plausible due to the presence of phenolic substances and sugars present in the honey which reduces the Ag^{+} ions into metallic silver (Haiza, et al., 2013). In a study by Haiza and colleagues, they reported the green synthesis of silver nanoparticles using honey as the reducing agent. They suggested that the sucrose, glucose and even proteins and enzymes play a role in the reduction process. The synthesised AgNPs exhibited dark brown colour (See Figure 9) due to an excitation of the surface plasmon vibrations of the ionic AgNPs and the reduction of silver ions by phenolics and sugars to form metallic AgNPs (Hasim et al., 2020), (Khorrami et al., 2019).

Figure 9: Visual representation of the synthesis of silver nanoparticles, indicated by the colour change of the solution from colourless to brown.

According to an earlier research, honey has a significant amount of phenolic chemicals, reducing sugars, proteins, and vitamins as organic and natural molecules. The reduction of silver nitrate, as suggested above, may involve phenolic and reducing substances like fructose and glucose (Khorrami et al., 2019). The schematic depicting how honey's bioactive ingredients help reduce silver ions to silver nanoparticles is shown in Figure 4. In a different study by Khorrami and colleagues they also synthesized AgNPs using honey and observed a colour change from light yellow to brown in the solution containing honey and silver nitrate.

Similarly, they reported that the compounds responsible for the silver nitrate reduction process were fructose, sucrose and the phenolic compounds. These studies support the fact that honey through its constituents is responsible for the reduction of silver ions to silver atoms. Additionally, the colour change observed in the reported literature is similar to the one obtained in this study.

4.2 Optimization of synthesis

In every synthesis protocol, it is essential that the parameters necessary for generating a desired product need to be optimized for better results. An optimization is carried out to determine the optimal experimental conditions for a reaction synthesis. The $AgNO_3$ concentration, reaction time, pH, and the involvement of reducing agents in promoting a reaction are some of the variables that affect the formation of Ag-NPs. Therefore, the direct control of these parameters is a critical factor that affects the biosynthesis and characteristics of the nanoparticles. In the literature, Ghafari et al., (2014) reported the synthesis of silver nanoparticles using a plant extract. When the synthesis was carried out in various reaction conditions such as the extract concentrations, pH and mixing ratio of the reactants were investigated (Ghafari-Moghaddam & Hadi-Dabanlou, 2014).

An UV–visible spectrophotometer was employed for the spectrometric analysis of honey synthesized silver nanoparticles. Free electrons in the silver nanoparticles evoked by absorbing visible light and transmitted to a higher energy level is unstable at the excited state, therefore has to return to the base energy level simultaneously emitting a photon (Behravan et al., 2019). This phenomenon gives rise to the surface plasmon resonance (SPR) peak commonly known as the absorption band. Many previous literature reports indicate that the absorption band of AgNPs are visible at around 400-490 nm in the UV-visible spectra. According to a study by Gonzalez and colleagues, an ecologically friendly method for generating silver nanoparticles in water under ambient conditions using natural honey was reported. The obtained silver nanoparticles were characterised by using UV-vis spectroscopy giving the SPR band at around 413 nm (Gonzalez, et al., 2017). The SPR peaks and line widths are descriptive of the size and shape of the nanoparticles and their height provides information on the concentration of AgNPs since the maximum SPR is directly proportional to the concentration of AgNPs. The UV-vis absorption peak is widely known for providing information on the degree of dispersion of silver nanoparticles. The greater the degree of dispersion of nanoparticles, the narrower the absorption peak (Philip, 2010). We shall now look at the pH as one of the important parameters.

4.2.1 Effect of pH

A pH of a solution is a measure of how basic or acidic a solution is. The pH scale ranges from 0 to 14. A pH value of 7 is considered neutral on this scale, meaning it is neither acidic nor basic. It is more acidic if the pH value is below 7, and more basic if the pH value is above 7 (Yuqing, et al., 2005). Previous studies have pointed out that, reduction of Ag^{+} to Ag is assisted by a high pH which was always achieved by the addition of NaOH. The alkaline environment facilitates the opening of the glucose ring by the abstraction of the α-proton of the ring oxygen and subsequently glucose is oxidized to gluconic acid (Siddiqui et al., 2018), (Philip, 2010). The effect of pH on the synthesis of honey reduced AgNPs was evaluated, and Figure 10 shows the different absorption bands obtained from using different pHs values. As depicted in figure 10 at lower pH, the SPR band is not visible but at pH 9 and 10 the SPR characteristic of AgNPs is present which suggests that nanoparticles are formed at higher pH values. Thus, it can be concluded that the effect of pH plays a vital role in AgNPs formation for green synthesis of SBH reduced nanoparticles. According to previous studies it was found that nanoparticles form at higher pH values which explains why there are no observed SPR peaks of AgNPs at acidic pH values.

In a study done by Hosny and colleagues, they synthesized AgNPs using honey at different pH and were able to successfully obtain the nanoparticles at an alkaline pH= 10 (Hosny et al., 2017). Another study by Philip, (2010) investigated the preparation of silver nanoparticles using honey. In their findings, they observed that the SPR peak was smoother and narrower as the pH was increasing. The desired uniformly distributed peak was observed at pH of 8.5 whereas, at lower pH values the absorbance peak was broader and less intense (Philip, 2010). In a different study, Youssef et al, 2019 reported an eco-friendly synthesis of AgNPs from an Egyptian honey. They evaluated the effect of pH on the formation of the nanoparticles at pH values of 5, 6, 7, 8 and 9. In their findings, a sharp peak with the maxima absorbance was observed at the pH value of 9 (Youssef et al., 2019).

Therefore, it can be concluded that the best pH for synthesizing these nanoparticles under these conditions is generally at pH 10. Contrastingly, another study by (González Fá et al., 2017) synthesized AgNPs using honey at pH values of 5 and 10. They both gave surface plasmon resonance bands at about 400 nm. However, the former pH was used under an increased honey concentration, the plasmon spectral band changed to a shorter wavelength, indicating a reduction in particle size. This observation does suggest that in some instances the reduction

of silver ions may take place at pH values lower than 7. The explanation behind this observation indicated that there is a possibility of the reactive fructose component taking part in the reduction reaction alongside the glucose, and vitamin C which are all reducing agents found in honey. The addition of the alkaline NaOH serves to induce the Ag ions reduction. The metal ions oxidize glucose to gluconic acid, while the base aids the opening of the glucose ring by abstracting the α-proton of the sugar ring oxygen (Philip, 2010). According to (Philip, 2010) as the pH is increased, the SPR band gets sharpened.

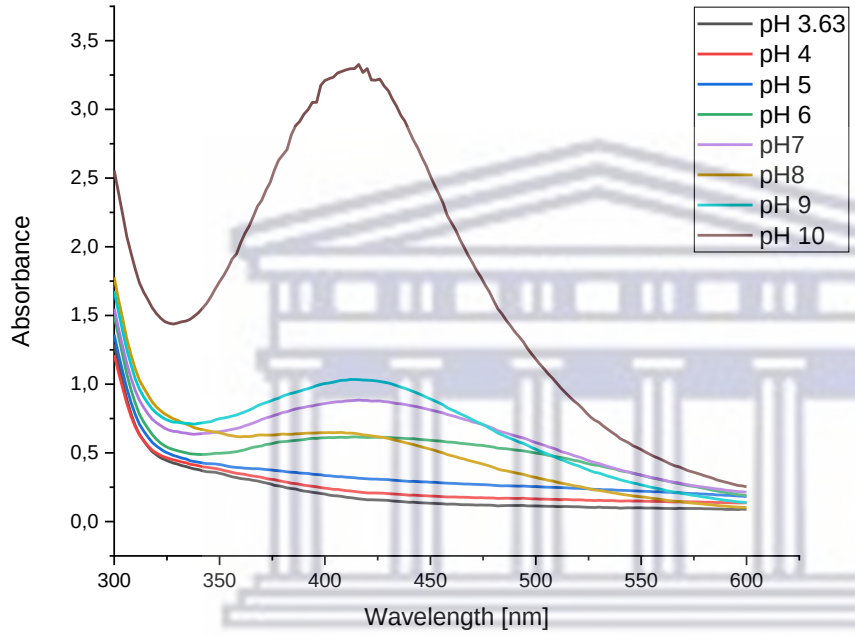

Figure 10: UV-Vis absorbance spectra of AgNPs at 100 °C and 50mg/mL Honey concentration at different pH values.

4.2.2 Effect of temperature

It has been long established that when the reaction temperature rises, the reaction rate and particle production rate increase in a reaction. Due to this fact, the temperature in which a reaction occurs plays a huge role on the production of nanoparticles (Shah et al., 2015). To investigate the effect of temperature, the reaction mixture of SBH and $AgNO_3$ were allowed to react at different heating temperatures of 25°C, 50°C, 75°C and 100°C. The selected temperatures for the study were comparable previously reported by other researchers in the literature. For example, for a start, Haiza et al, 2013 successfully synthesized silver nanoparticles using honey at room temperature (Haiza, et al., 2013). In a different study, the synthesis of AgNPs was carried out 70 °C (Czernel et al., 2021). The samples were removed from the thermo-shaker and the UV-vis spectra analysed. Figure 11 shows that, the changes in

the intensity of the SPR band are strongly affected by the synthesis temperature. It is hypothesized that high temperatures will enable faster production of nanostructures due to Ostwald's ripening. Additionally, it has been discovered that the temperature at which nanoparticles made from plant extracts are synthesized can influence their size, shape, and production (Shah et al., 2015), (Liu et al., 2011). For instance, the synthesis of Ag nanoparticles using *Citrus sinensis* (sweet orange) peel extract at a reaction temperature of 25 °C resulted in particles with an average size of about 35 nm. However, the average particle size dropped to 10 nm when the reaction temperature was raised to 60 °C. According to their findings, at room temperature, the reaction took up to 75 minutes to complete. Contrastingly, at 60 °C, the reaction was completed in 45 minutes saving a whole half an hour period. The rise in temperature was responsible for this observation while smaller nanoparticles eventually form as a result of the reactants being consumed quickly. (Kaviya, et al., 2011). A further support was shown by the SPR peak obtained from the UV-vis spectra of the synthesized AgNPs at 60 °C which gave an intense peak than that of the 25 °C reaction.

At 75°C, the fast formation of AgNPs was observed, with the resonance absorption intensity being increased with the reaction temperature. For lower temperatures of 50 °C and and even lower 25 °C, the intensity peak was absent at the region of 400 nm which shows that no AgNPs were formed.

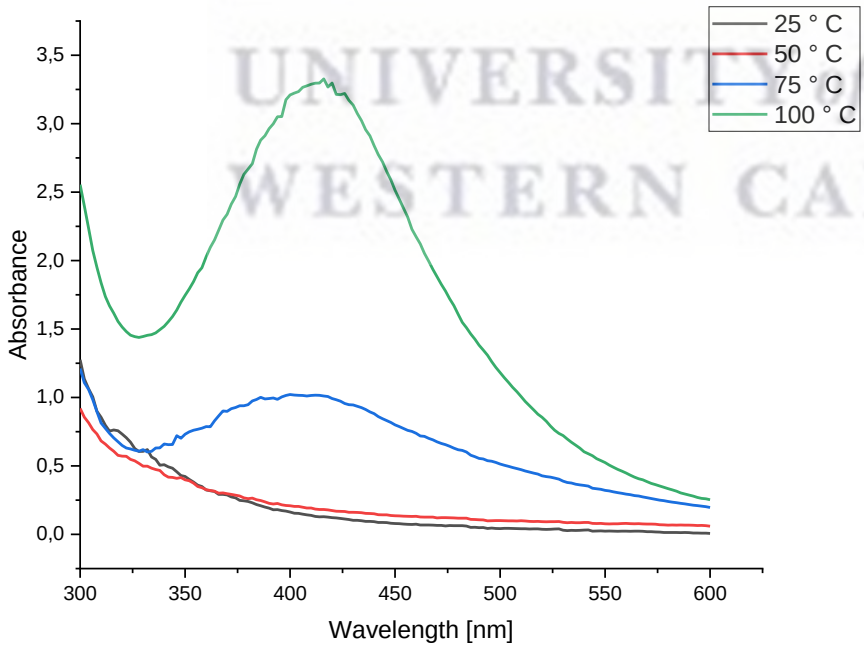

Figure 11: UV-Vis spectra of AgNPs at pH 10 and 50mg/mL Honey concentration under different temperatures.

Comparing the absorbance peak intensity at 100 ˚C and the one at 75 ˚C it can be clearly seen that the most favourable conditions for this synthesis are at higher temperatures. The intensity of the SPR peak increases with increasing temperatures which agrees with the study already reported above by Kayiva et al., (2011). Similarly, the immediate previous literature report does support this observation. In a study conducted by Stavinskaya and colleagues, where they synthesized silver nanoparticles by using *Vitex agnus-cactus* extract and investigated the effect of temperature on their synthesis by varying the temperatures from 40-80 °C (Stavinskaya et al., 2019). As expected, they found out that the intensity of the SPR peak increased with increasing temperature. It was observed that at 80 °C, fast formation of AgNPs had occurred indicating a higher SPR peak intensity. Whereas, at lowest study temperatures of 40 °C the SPR band was lower and did not produce a pronounced signal at the region of 400 nm.

Similarly, in another study AgNPs were synthesized using honey while varying the reaction temperatures from 35 °C to 70 °C. The UV-vis results of AgNPs synthesized at the temperature of 70 °C, had a band with the highest intensity (Czernel et al., 2021). Hence it was deduced that when the temperature reaches 70 °C, specific interactions, mainly hydrogen and van der Waals bonds, break, and initiates the monomerization processes. Additionally, a higher temperature significantly speeds up the Ag^{+} ion reduction reaction.

Therefore, it can be concluded that higher temperatures speed up the formation of AgNPs hence there will be more nanoparticles produced at higher temperatures compared to lower temperatures. Conclusively, the temperature range which gave favourable results ranges between 50 °C to 80 °C alongside a supporting pH. Our synthesis is about developing friendly conditions which abide by the green synthesis guidelines, the 100 ˚C temperature was out of our selection because it was still plausible to obtain AgNPs at lower temperatures of 75 ˚C hence it was the working temperature of choice.

4.2.3 Effect of SBH concentration

In order for the green synthesis of nanoparticles to take place, Ag^{+} ions require a biological reducing agent. Most of the time, reducing agents also function as coating and stabilizing agents. In this method, nanoparticles are synthesized using stingless bee honey (SBH), which is reported to possess antimicrobial properties, thereby enhancing the effect of the nanoparticles through synergistic effects. The concentration of the reducing agent has an effect on the properties, rate and quantity of nanoparticles produced in a reaction (Behravan et al., 2019).

The effect of SBH concentration on the formation of AgNPS was studied by varying the concentration of the honey solutions as shown in Figure 12.

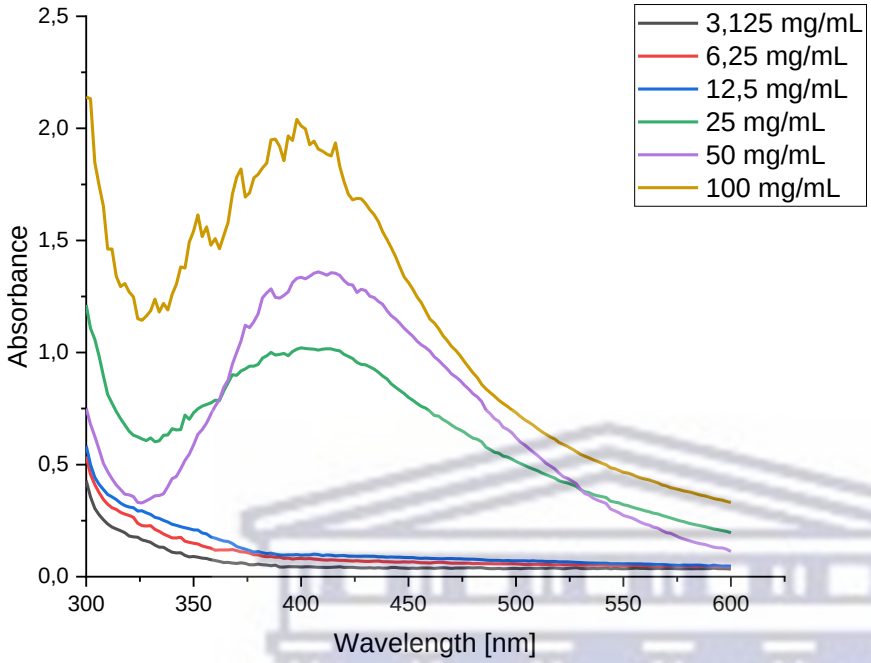

Figure 12: UV-Vis spectra of AgNPs at 75 °C and pH 10 at varying SBH concentrations.

The synthesis at pH 10 at 75 °C temperature was carried out using different SBH concentrations in order to assess the impact of the SBH concentration on the formation of silver nanoparticles. In each instance, surface plasmon resonance bands were identified and recorded.

The UV-vis spectra shown above (Figure 12) is the intensity of SPR peak obtained by changing the concentration of the honey solution while keeping the reaction temperature at 75 ˚C. The results showed that the higher the concentrations of the SBH the higher the absorbance. It should be noted that with increased honey concentration, the plasmon spectral band changed to a shorter wavelength, indicating a reduction in particle size. Theoretically and experimentally, it is found that when size decreases, the SPR peak shifts towards shorter wavelength side (Haiza, et al., 2013). Haiza et al., (2013) reported the synthesis of silver nanoparticles using honey and studied the effects of different honey concentrations (10g and 40g) at a fixed pH value. In their findings, the particles sizes obtained for the 10 g honey were ranging from 18.98 nm to 26.05 nm and from the range of 15.63 nm to 17.86 nm for 40 g of honey which is four-fold of the former. Therefore, it can be concluded that honey concentrations do have an effect on the particle size of silver nanoparticles generated.

In the case of SBH concentrations lower than 25 mg/ml, there was no SPR peak observed. Thus, this is an evidence that no nanoparticles were formed at low SBH concentrations. The

highest absorbance peak is observed at SBH concentration of 100 mg/mL. Nevertheless, the peak does not have a uniform and smooth distribution. Therefore, the SBH concentration of 50 mg/mL was selected as the optimal honey concentration for the synthesis of AgNPs in this study because it gave an acceptable uniform smooth absorbance peak.

4.2.4 Effect of $AgNO_3$ concentration

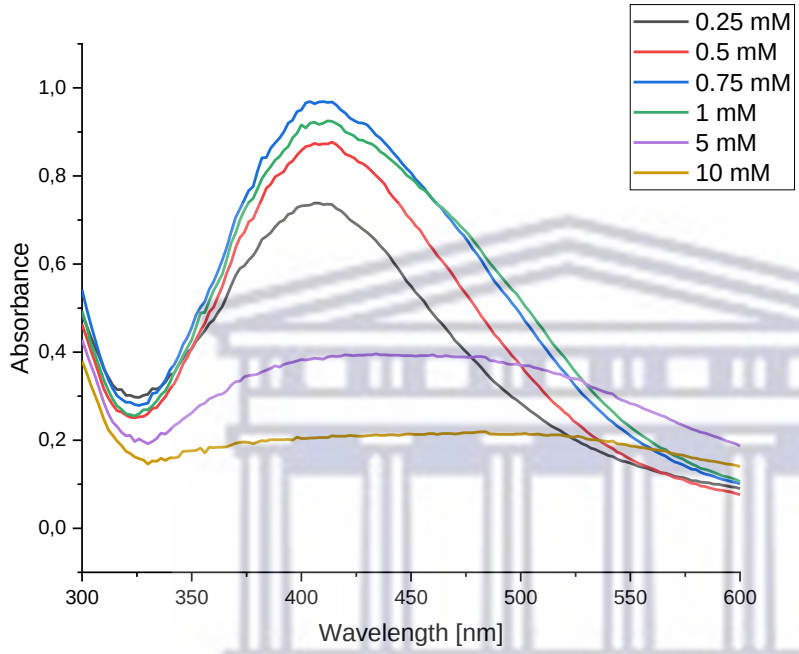

Figure 13: UV-Vis spectra of AgNPs at 75 °C, pH 10 and 50 mg/mL Honey concentration at different $AgNO_3$ concentration.

Upon investigating the effect of $AgNO_3$ concentration on the synthesis of SBH-AgNPs, it was observed that higher concentrations of $AgNO_3$ led to a decrease in recorded absorbance (See Figure 13). The 0.25 mM, 0.5 mM, 0.75 mM, 1 mM, 5 mM, and 10 mM AgNPs had a peak at 406 nm, 414 nm, 404 nm, 412 nm, 420 nm, and 434nm with absorbances of 0.738, 0.877, 0.969, 0.925, 0.364, and 0.212 respectively. The results obtained in this study are contradictory to those reported by Behravan and colleagues where they found that increasing the concentration of silver nitrate also increased the absorption peak. They concluded that increasing the $AgNO_3$ concentration led to more Ag^+ being converted to Ag thereby increasing the amount of silver nanoparticles (Behravan et al., 2019). However, in their study they synthesized silver nanoparticles using a plant extract which is different from the reducing agent used in this study. In other literatures utilizing honey as the reducing agent, they did not investigate the effect of silver nitrate concentration and thus does not report it.

The change in amount of $AgNO_3$ brought variations in the observed SPR band of the SBH-AgNPs. Furthermore, as expected the SPR band was shifted to shorter or longer wavelengths with a change in $AgNO_3$ concentration. It was found that at higher concentrations of $AgNO_3$ at 5 mM and 10 mM the absorbance peak was significantly reduced and had a very broad distribution as well as a red shifted wavelength. The latter was similarly observed by Htwe, et al (2019) where they synthesized silver nanoparticles by green method but with varied concentrations of $AgNO_3$. The absorption of silver nanoparticles at 0.5 mM showed a peak at 417 nm which then shifted to 424 nm at 0.7 mM, as was observed by HRSEM micrograph, that indeed the particles were increasing in size. The solution containing 0.75 mM $AgNO_3$ was used for subsequent synthetic purposes in that study (Htwe, et al., 2019).

4.3 Characterization of AgNPs

4.3.1 UV-vis spectroscopy analysis of the AgNPs

UV-vis spectroscopy is an analytical tool used to monitor the formation of silver nanoparticles. In metal nanoparticles such as in silver, the conduction band and valence band lie very close to each other in which electron moves freely. These free electrons give rise to a SPR absorption band (Taleb et al., 1998). It has been previously reported that the plasmon resonance peaks and line widths are sensitive to the shape and size of nanoparticles (Abou El-Nour et al., 2010). Hence, the existence of SPR peak is the primary signature of metal nanoparticle formation (Das et al., 2010), (Haiza, et al., 2013). The results obtained from the UV-vis absorption spectra of silver nanoparticles is shown in Figure 14. It gave a uniformly distributed absorption peak with a maximum wavelength of 414 nm.

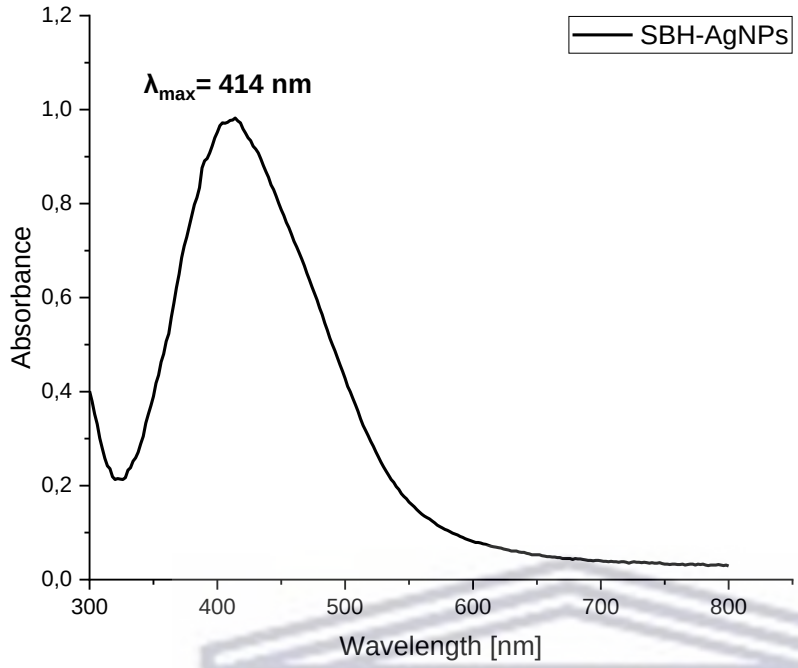

Figure 14: UV-vis spectra of SBH-AgNPs as synthesized at 75 °C, pH 10, 50 mg/mL Honey concentration and 0.75 mM of $AgNO_3$.

These results are in good agreement with previous evaluations done by Philip (2010). They synthesized AgNPs and obtained the SPR peak in the UV-vis spectra appearing at 413 nm (Philip, 2010). In a different study, Khorrami and colleagues synthesized silver nanoparticles and the SPR peak was visible at 426 nm (Khorrami et al., 2019). The SPR peak obtained from the UV-vis results on the study by Philip, (2010) is almost the same as the one obtained in this study (414 nm) and they also observed a smooth and narrow absorption band. The reaction of the silver nanoparticles was carried out in a controlled pH synthesis and the SPR they reported was found at a basic pH of 8.5. Similarly, in this study the smooth and uniformly distributed SPR band was observed in a basic pH of 10. It was previously reported that increasing the pH results in the greater wavelength of the absorbance peak which is the plausible explanation of the higher wavelength in our study (Fan, et al., 2015). However, in the study by Khorrami et al, (2019) they did not report the synthesis pH which makes it appear controversial.

4.3.2 FTIR analysis of the SBH and SBH-AgNPs

The FTIR measurements were carried out to identify the functional groups responsible for reducing capping and efficient stabilization of the AgNPs synthesized using stingless bee honey and $AgNO_3$. The comparison of the FTIR spectrum results of SBH sample, 50 mg/ml and SBH-AgNPs, synthesized using the optimal conditions, are displayed in Figure 15 below.

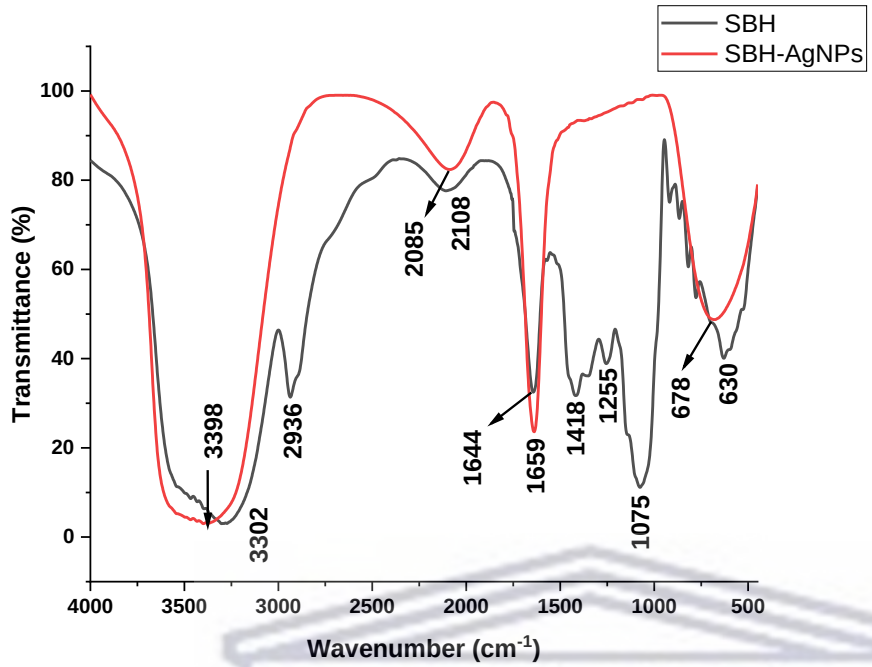

Figure 15: FTIR spectra of stingless bee honey and SBH-AgNPs.

According to Khorrami et al. (2019) honey has been found to contains a significant number of phenolic compounds, reducing sugars, proteins and vitamins. Based on the FTIR spectrum depicted in Figure 15, in the stingless bee honey spectrum appears a broad at 3302 cm^{-1} related to functional groups of phenols' OH. This peak is attributed to phenolic compounds found in the honey and this peak was also found in a study by Khorrami et al. (2019) when they were analysing the functional groups present in *Astragalus gossypinus* honey. A minor peak appeared in 2936 cm^{-1} is assigned to the CH groups of aldehydes. Aldehydes was among the compounds found in honey from *Melipona subnitida* and *M. scutellaris* by Costa et al. (2018) when they analysed honey samples using headspace-solid phase microextraction (HS-SPME) and gas chromatography coupled with mass spectrometry.

At around 1660 and 1535 cm^{-1} the amide I and II bands of proteins are expected to occur as prominent bands. In the present work, the band appears at 1644 cm^{-1} (Haiza, et al., 2013), (Khorrami et al., 2019). According to Kędzierska-Matysek et al. (2018) the vibration bands at around 1418 and 1347 cm^{-1} are characteristic for bending vibration of O-CH and C-CH in the carbohydrate structure which were also observed at 1418 cm^{-1} in this study. Another band appearing at 1255 cm^{-1} was observed which is comparable to a band around 1250 cm^{-1} that was also reported by Kędzierska-Matysek et al. (2018) is characteristic of the stretching vibration of C-H of the C-H in carbohydrates.

The strong peaks at 1075, and 630 cm^{-1} correspond to C-O and C=C stretching vibrations indicating the presence of vinyl ether and alkene compounds (Ghramh et al., 2020). The IR band present at 2108 cm^{-1} can possibly be characteristic of carbonyl group C=O stretching (Youssef et al., 2019). According to an investigation reported by Youssef et al (2019) they synthesized silver nanoparticles using honey and used FTIR to identify the functional groups present in honey responsible for the reduction of silver nitrate. They also observed a band at 2119 cm^{-1} which was characteristic of carbonyl group C=O stretching. The broad band found at around 3300- 3400 cm^{-1} arising from –OH stretching vibrations has been previously reported to be responsible for the presence of polyphenols on the honey. The –OH functional groups are the main components of phenols and flavonoids of the honey, and they play a major role in the formation of AgNPs (Hasim et al., 2020). Evidently, honey has been also reported to have some protein content in its composition and to confirm this, there is a band at 1644 cm^{-1} which has been indicated to be attributed to the presence of amides (proteins) (Hasim et al., 2020).

On the other hand, the FTIR spectrum of the SBH-AgNPs as shown in Figure 15 produced only 4 bands, at 3398, 2085, 2885, 1659, and 678 cm^{-1} indicating the presence of stabilizing and capping agent with the NPs. Some of the bands are similar to that obtained in the SBH spectrum while some peaks disappeared such as the band at 2936, 1418 and 1075 cm^{-1}. The broad band that appeared at 3398 cm^{-1} is assigned to the presence of the O-H stretch of alcohols. In a different study, Hasim et al. (2020) synthesized silver nanoparticles using Tualang honey. The FTIR spectra of the silver nanoparticles had a broad peak found at 3335 cm^{-1} attributed to (OH) vibrations of phenols (Hasim et al., 2020). Another peak observed at 2085 cm^{-1} may correspond to nitrile C≡N stretch or alkynyl C≡C stretch (Ghramh et al., 2020). While the peaks at 1659 and 678 cm^{-1} were assigned to the NH group of amines (Khorrami et al., 2019) and C=C stretching vibrations (Ghramh et al., 2020) respectively. Some peaks disappeared such as that corresponding to, CH functional groups of alkanes, and the CO group of carboxylic acids, respectively which could indicate that those functional groups were involved in the reduction and stabilization process of SBH-AgNPs. The existence of these identified functional groups then validated the successful reduction of $AgNO_3$ into biogenic AgNPs using honey.

The FTIR analysis confirms the idea that proteins (Hasim et al., 2020), polyphenols, amine groups (Khorrami et al., 2019), flavonoids, and other compounds reduce $AgNO_3$ to generate AgNPs. The molecules attached to the AgNPs appear to have free and bound amide groups, and these substances may be polyphenols with aromatic rings and bound amide regions, according to the IR spectra. Differences between the honey and AgNPs together with some

variance in the peak shift positions strongly suggest that the honey is what gives the AgNPs their stability.

4.3.3 HR-TEM analysis of AgNPs

The size and shape of nanoparticles synthesized using honey solution were measured using TEM instrumentation. To analyse the size distribution of SBH-AgNPs, ImageJ software was employed. EDS was also used to identify the elemental composition of the synthesized nanoparticles and an SAED pattern was obtained to analyse the crystallinity of the AgNPs. Figure 16 (A) shows a TEM micrograph of AgNPs synthesized using 50mg/mL of stingless bee honey solution.

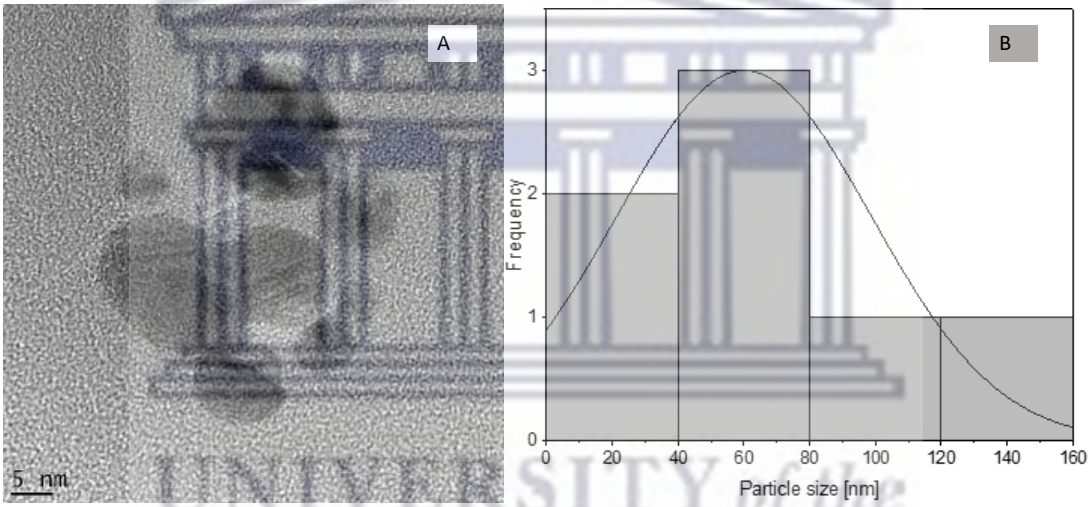

Figure 16: (A) HR-TEM image of SBH-AgNPs at 5 nm scale and (B) size distribution curve of the obtained SBH-AgNPs.

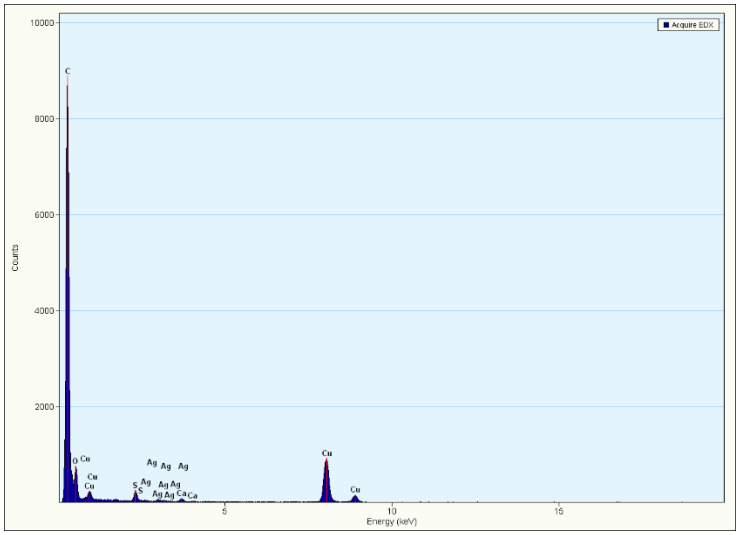

Figure 17: EDX characterization spectrum obtained for SBH-AgNPs. Visible peaks confirm the presence of silver, carbon and copper substances in the tested sample.

TEM image clearly shows the dispersion of silver nanoparticles supported (dark spherical particles) over honey matrix. The histogram indicated in Figure 16 (B) evaluated from the corresponding TEM image to determine average size of nanoparticles which was computed as 60 nm with broad size distribution. The TEM image showing the particles at 5 nm microscopic viewing shows darker spots and the particles appear to be agglomerating as there are some spherical nanoparticles joined together. The average size indicated by the TEM results is smaller than that obtained from the DLS analysis (79,67 nm) which was as expected. This is because the DLS measures the hydrodynamic size of the nanoparticles which encompasses the organic layer while TEM only measures the inorganic core size (Mourdikoudis et al., 2018). Coleman et al, (2011) compared several characterization techniques used to obtain data on particle size distributions. In their findings, DLS gave higher values than TEM. For instance, it gave 42 nm for a given silica reference sample which was reported to be 25 nm by TEM (Coleman et al., 2011). The quantitative analysis using EDX as depicted in Figure 17 showed relatively low silver content which confirms the presence of AgNPs. The spectrum also showed the presence of carbon and copper which results from the carbon-copper grid used for the analysis of the sample (Rodríguez-León et al., 2013), (Alsammarraie et al., 2018).

4.3.4 DLS analysis of AgNPs

A DLS analysis was used to measure size distribution, zeta potential and PDI values of the SBH-AgNPs as shown in Table 4. A particle size distribution's level of non-uniformity is referred to as "polydispersity." (Dos Santos, et al., 2012). The molecular weight distribution width is measured by the PDI, ranges from 0 to 1. Monodispersed nanoparticles are those with

a PDI value of less than 0.2. The broadening of the particle size distribution is shown by an increase in the PDI value (Bhattacharjee, 2016). The SBH-AgNPs have a PDI value of 0.36 and a hydrodynamic size about 79.67nm hence it can be concluded that these nanoparticles are polydispersed.

Table 4: Average size, PDI and zeta potential of the SBH-AgNPs synthesized using 50 mg/mL concentration of honey solution at 75 °C for 3 h.

Average size (nm)	PDI	Zeta potential (mV)
79.67	0.36	-15

Previous studies have described that the parameter that affects the characteristics of the silver nanoparticles is the PDI, which is largely influenced by the $AgNO_3$ concentration. Since monodisperse samples are more stable and have uniform morphologies, PDI values less than 0.3 are indicative of a monodisperse sample, which is strongly related to the zeta potential. In this sense, the reaction parameters' effects are uniform across all the examined characteristics. The size distribution, agglomeration, and shape of the resulting nanoparticles are all directly influenced by the reaction conditions used to synthesize Ag-NPs, which leads one to the conclusion that these results are very significant (Manosalva et al., 2019). The zeta potential of the SBH-AgNPs was determined and it was -15 mV. The negative charge suggests there are repulsive forces between the nanoparticles. In a different study, silver nanoparticles were synthesized using a leaf extract as a reducing agent (Anandalakshmi, et al., 2016). The DLS results indicated a broad spectrum of particle size distribution which had a value of 73.14 nm. Subsequently, the zeta potential of the AgNPs was reported to be -7.66 mV. It was suggested that the negative zeta potential indicates that the nanoparticles were stable, and their results agree with ours.

According to previous literature, it is indicated that metal nanoparticles that have a large negative zeta potential repel each other, hence they do not aggregate. Nevertheless, those with low zeta potential values can easily aggregate since there are no strong repulsive forces keeping them apart. Nanoparticles with negative or positive zeta potential values between ±10 to 20 mV are regarded as relatively stable (Ardani et al., 2017). A similar zeta potential value of 15.3 mV was reported by Jemilugba et al (2019) where they synthesized AgNPs using *Combretum erythrophyllum* plant leaves and they suggested that the mono-dispersity seen in their TEM

image indicates that the NPs have an appropriate surface charge for electrostatic stability to prevent aggregation. However, the latter is not true in our study as the TEM images show some degree of aggregation and thus it can be possibly that the honey is not a good stabilizing agent unlike in the case of plant extracts.

4.4 Antibacterial activity of AgNPs

4.4.1 Stability of SBH-AgNPs in biological media

Regarding AgNPs and other silver species, some of their interactions with culture media and their components have been reported by several authors. When culture media interact with AgNPs, they may affect their properties in different ways, such as the silver ion release rate, aggregation, or surface oxidation process (Vazquez-Muñoz, et al., 2020). The dispersion of nanosilver in a biological medium causes the surface-coating agents to re-establish equilibrium by mostly losing some of the coating molecules. When these coating agents are displaced by other molecules such as inorganic ions or water present in the media, the nanoparticles may no longer be stable therefore undergo aggregation. Additionally, in the presence of molecular oxygen, silver atoms can interact with molecular oxygen and become oxidized to silver ions (McShan, et al., 2014). The stability of SBH-AgNPs was evaluated in LB and MHB biological media over a 24h period as shown in Figure 18. These incubation temperatures are the most used for biological assays.

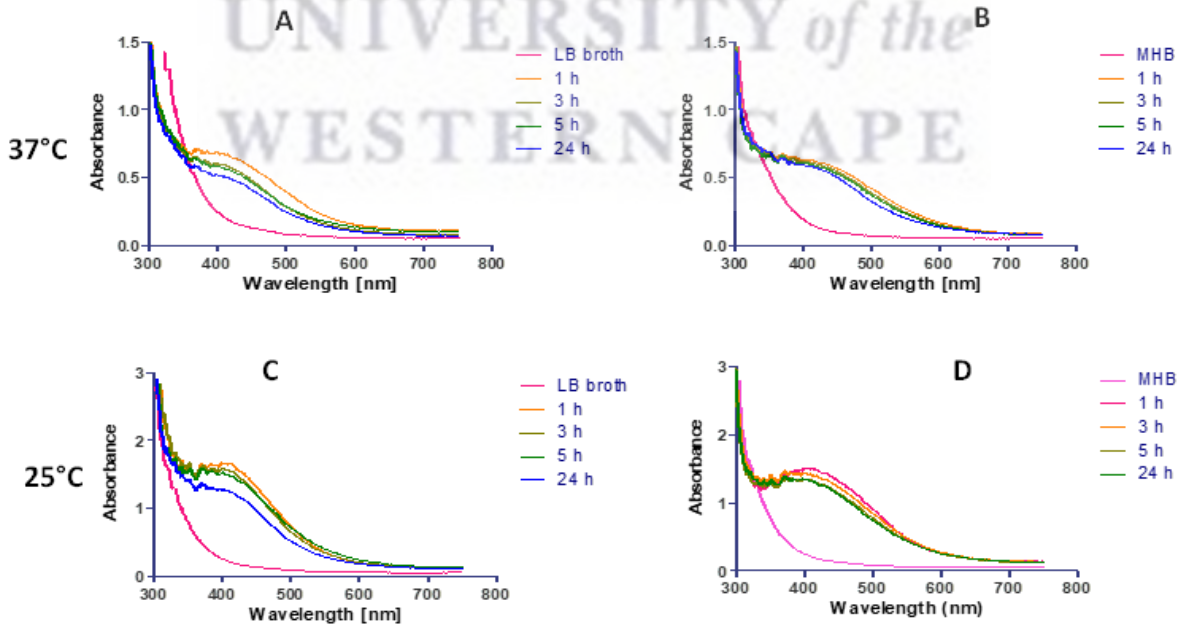

Figure 18: The UV-Vis spectra of SBH-AgNPs, recorded over a 24 h period (at 25 and 37 °C) after mixing with different biological media; MHB and LB broth.

Upon the addition of SBH-AgNPs to the LB broth and MHB, the UV-Vis absorption spectra did slightly change as shown in Figure 18 (A-D). The stability of SBH-AgNPs was measured at 37°C and 25 °C to observe the effect of the biological assay conditions towards their stability. It was noted that in the LB broth, there was a significant decrease in the absorbance spectra for both temperatures, hence it could be concluded that the SBH-AgNPs are stable in the MHB media. Therefore, using LB broth in the biological evaluation assays was discontinued. According to some authors, the pH of the media affects the stability of AgNPs. The silver ion release rate may be favoured in alkaline environments, which leads to faster degradation of the AgNPs. Therefore, the AgNPs are more stable in the MHB than the LB broth since the pH in LB broth is more alkaline than MHB. A recent study by Vazquez-Muñoz et al. (2020) evaluated the effects of the physicochemical properties of the culture medium on AgNP stability and antibacterial activity. It was found that the SPR peak of AgNPs in an acidic pH (6.2) was similar to the profile of AgNPs in the control solution (pH 7.2). However, AgNPs in the basic pH (8.2) solution had shown a different SPR peak. There was also an increase in the peak wideness which suggested an increasing oxidation of the AgNPs (Vazquez-Muñoz, et al., 2020).

4.4.2 Agar well diffusion assay

Antimicrobial results obtained from this study are shown in Table 5. An inhibition zone with diameter greater than 10 mm is considered as positive antagonism effect (Kumar & Kumar, 2015). The synthesized AgNPs show positive antagonism effect against almost all the selected bacterial strains except for MRSA which has a zone of inhibition that is not greater than 10 mm. The low inhibitory activity against MRSA can be expected since this bacterial strain has been reported to have high antibacterial resistance (Davies & Davies, 2010). The highest zone of inhibition of 13mm was measured against *S. epidermidis* and *P. aeruginosa* followed by 12 mm towards *S. aureus*, *K. pnuemoniae* and *E. coli*. Meanwhile at 50 mg/mL concentration, the SBH did not show any inhibition for all the selected bacterial strains. This can be due to the fact that the honey solution has a very low concentration for it to show its antibacterial activity that is reported to be present in previous studies. In a study by Rosli and colleagues, they investigated the antibacterial activity of honey samples from eight stingless bees. The antibacterial studies indicated good antagonism effect against *S. aureus* at 50% concentration for all honey samples. At 12.5% concentration, only three honey samples showed inhibitory effect against one or more bacteria at the range of 10 to 16 mm in diameter of the inhibition zone. Thus, it can be suggested that the concentration of honey do have an effect its antibacterial activity (Rosli et al., 2020).

Additionally, in a different study, Temary et al, (2007) also investigated the antibacterial activity of honey sample from stingless bees. In their finding, the stingless bee honey did possess antibacterial activity against *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa*. Agar well diffusion test showed that the honey inhibited the growth of bacteria at a concentration of 50 % (w/v) which is equivalent to 500 (mg/mL) (Temaru, et al., 2007). However, this concentration is higher than that tested in this study (50 mg/mL) which also suggests that the honey used in this study was very diluted which may have decreased its antibacterial activity. Since the optimal honey concentration that was selected during the optimization process was 50 mg/mL it was quantitively correct to also use it for testing the antibacterial activity that it possesses at that particular concentration hence it was not increased to enhance its activity.

Table 5: The antibacterial effects of green synthesized SBH-AgNPs with the concentration of 63 µg/mL against six human pathogenic bacterial strains. (-) indicates no zone of inhibition.

Microorganisms	Zones of inhibition (mm)		
	SBH	SBH-AgNPs	Ciprofloxacin
S. aureus	-	12	22
S. epidermidis	-	13	27
MRSA	-	10	21
K. pnuemoniae	-	12	28
E. coli	-	12	34
P. aeruginosa	-	13	24

The obtained antibacterial findings of AgNPs are comparable with those obtained by Jemilugba et al (2019) where they synthesized AgNPs with an average size of 13.62 nm using plant leaves as a reducing agent. The zone of inhibition for their AgNPs against *S. aureus*, *E. coli* and *S. epidermidis* were found to be 15, 12 and 12 mm respectively (Jemilugba et al., 2019). Although they had synthesized smaller nanoparticles compared to the ones in our study with an average size of 60 nm, it is interesting to note that they showed similar antibacterial activity against those mentioned bacterial strains. In another study they reported a green synthesis of AgNPs

using honey and evaluated their antibacterial activity. To their findings, the AgNPs showed increasing susceptibility in the order of *S. aureus* < *K. pnuemoniae* < *P. aeruginosa* < *E. coli* with zone of inhibition diameter of 20.00, 23.33, 25.00 and 29.33 mm respectively (Youssef et al., 2019). According to the reported literature on the antibacterial activity of silver nanoparticles using green synthesis, they did not report the concentration of the AgNPs, but they do assess the antibacterial activity of silver nanoparticles against some of the bacterial strains which are selected in this study. Khorrami et al, (2019) reported the antibacterial activity of silver nanoparticles synthesized using *Astragalus gossypinus* honey from plants.

Agar diffusion assay was used to study the antibacterial activity of AgNPs and the honey against *S. aureus*, *P. aeruginosa* and *E. coli*. The activity of AgNPs was evaluated using different concentrations of 64, 125, 250, 500 and 100 µg/mL. The results revealed that the AgNPs at the concentration of 64 µg/mL, there was no activity against all the three tested strains. However, at AgNPs concentrations of 125 µg/mL some degree of inhibition was found but against only *S. aureus*, and it was 10 mm. In the other concentrations, AgNPs had significant antibacterial activity against all the three bacterial strains. The activity of these nanoparticles was found to be concentration dependent (Khorrami et al., 2019). According to the concentration of 64 µg/mL of the *Astragalus gossypinus* honey synthesized AgNPs, the activity of SBH-AgNPs in our study appears to be more significant.

4.4.3 MIC and MBC of AgNPs

The MIC is reported as the lowest Ag-NP concentration which inhibits bacterial growth and MBC as the Ag-NP concentration in which no bacterial growth occurs. The MIC and MBC of SBH-AgNPs was investigated against six pathogens, namely *S. aureus*, *S. epidermidis*, MRSA, *P. aeruginosa*, *E. coli* and *K. pnuemoniae* as indicated in Table 6. The SBH-AgNPs showed activity against all the microorganisms tested. In contrast, the honey solution did not display significant activity against all the selected strains. However, SBH-AgNPs were mostly active against *S. epidermidis*, with MIC and MBC values of 16 and 32 µg/mL, respectively. The lowest antibacterial activity recorded for SBH-AgNPs was against MRSA, with MIC and MBC values of 63 and 125 µg/mL, respectively. SBH-AgNPs exhibited the same inhibitory activity against *S. aureus*, *E. coli* and *P. aeruginosa*, with an MIC and MBC value of 63 µg/mL. Similarly, the zone of inhibition recorded against MRSA microorganism, the MBC was higher than all the other bacterial strains due to the resistant nature of this human bacteria. Contrastingly, Youseef et al (2019) reported better inhibitory activity with MIC values

against, *P. aeruginosa*, *E. coli* and *K. pnuemoniae* of 6.3, 6.3 and 12.5 µg/mL respectively. As revealed by the results shown in Table 5, there is no definite MIC value trend for both Gram-negative and Gram-positive bacteria. In the case of Gram-negative organisms, the silver nanoparticles must pass through the thin peptidoglycan layer and outer membrane. Gram-positive bacteria lack the outer membrane and have a thick (30 nm) peptidoglycan layer (Feng et al., 2000), (Slavin et al., 2017). Therefore, the physical barrier that the particles must pass does not vary significantly. In a different study, Khorrami and colleagues reported the MIC and MBC of their honey synthesized silver nanoparticles. It was discovered that this nanoparticle's MIC and MBC against *S. aureus* were 100 g/mL and 200 g/mL, respectively. In contrast better efficacy of the AgNPs was observed against Gram-negative microorganisms *P. aeruginosa* and *E. coli* at lower doses of 24.5 g/mL for both the MIC and MBC.

Table 6: The Minimum inhibitory assay and minimum bactericidal assay (µg/mL) of SBH-AgNPs and honey solution against the most common pathogens. (-) indicates no antibacterial activity.

Microorganisms	SBH		AgNPs		Ciprofloxacin	
	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	-	-	63	63	0.5	1
S. epidermidis	-	-	16	32	0.25	0.25
MRSA	-	-	63	125	0.5	2
K. pnuemoniae	-	-	32	32	0.02	1
E. coli	-	-	63	63	0.02	1
P. aeruginosa	-	-	63	63	0.5	2

In most cases, Ag-NPs' bactericidal activity is brought on by their adhesion to bacterial cell walls. As a result, this attachment generates an accumulation of envelope protein precursor, which in turn results in protein denaturation, proton motivation force reduction, and ultimately cell death (Yu-sen et al., n.d.). The size of the NPs has a significant impact on the bactericidal activity they exert against pathogens. Smaller NPs are more readily absorbed by the bacterial cell membranes. This is because smaller particles have higher surface area for interacting with

microbes and releasing Ag^{+} through oxidation. These speeds up the production of reactive oxidant species, which further compromises cellular structure and ultimately leads to cell death (Chudasama et al., 2010). In our study the nanoparticles had a larger average size of 60 nm which may have caused them to be slightly slower to be absorbed or penetrate the bacterial cell membrane.

4.4.4 Growth inhibitory kinetics of SBH-AgNPs

The bacterial growth kinetics of six bacterial strains was evaluated in response to treatment with the SBH-AgNPs. Figure 19 show the effects of SBH -AgNPs on the bacterial growth of six bacterial strains. Bacterial growth was evaluated using variations in optical density (OD). This offers a quick and effective approach to measure bacterial growth over time. Reduced absorbance shows that bacterial growth has been inhibited, which is a result of cell death being blocked during the stationary phase (Kumar & Kumar, 2015). As shown in Figure 19, SBH-AgNPs caused a time- and dose-dependent growth inhibition in the selected strains. All six bacterial strains tested were suppressed.

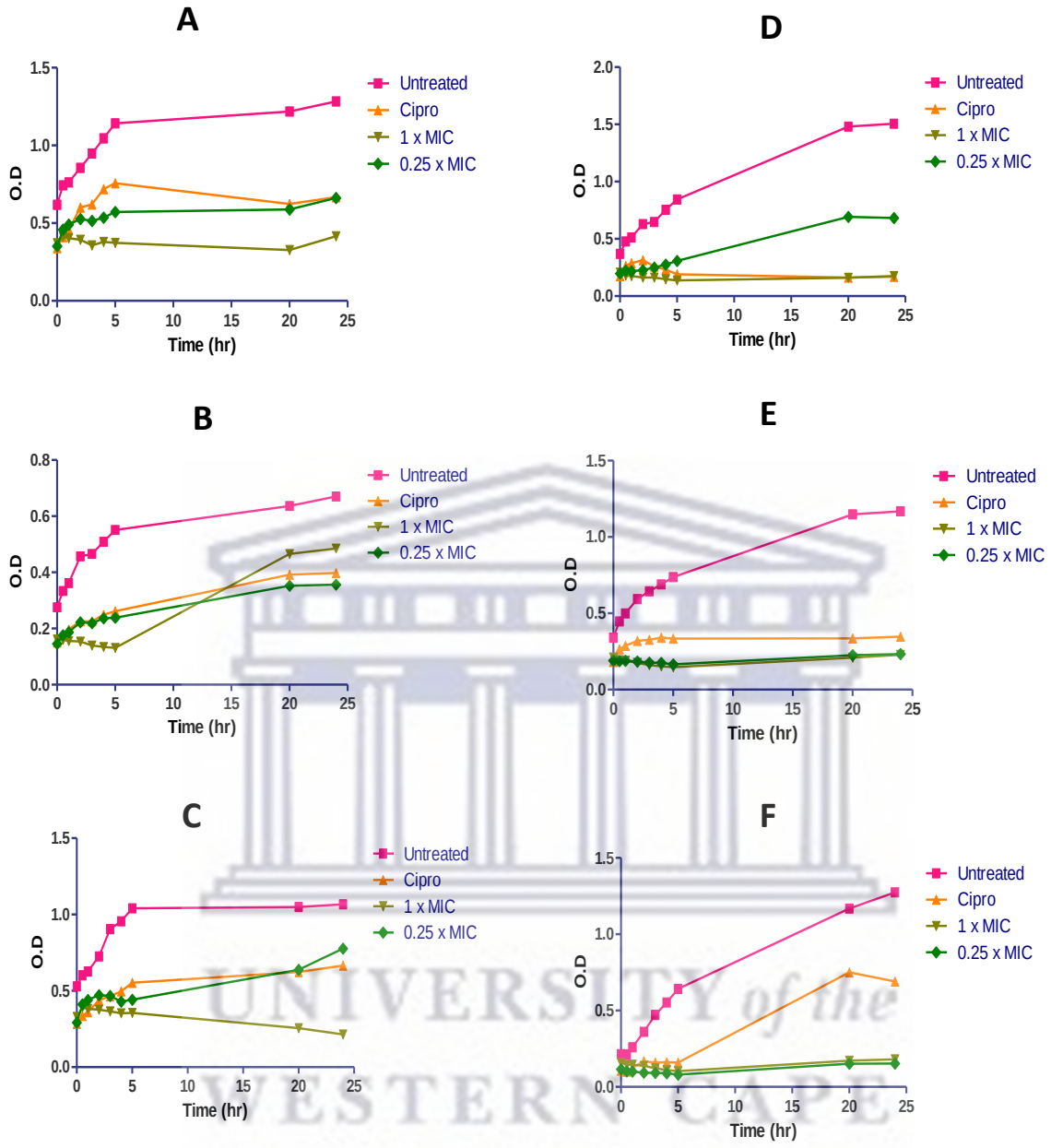

Figure 19: Growth inhibitory activity of AgNPs at 1X MIC (250 µg/mL) and 0.25X MIC (63 µg/mL) against selected Gram-positive and Gram-negative bacteria. (A) represents *S. aureus*, (B) *S. epidermidis*, (C) MRSA, (D) *K. pneumoniae*, (E) *E. coli* and (F) *P. aeruginosa*. The positive control ciprofloxacin was used at a concentration of 2 µg/mL.

The growth curve of *S. aureus* shows good inhibitory activity of the SBH-AgNPs for both MIC concentrations and interestingly both these curves are below the positive control (ciprofloxacin) from 0 to 24 hours. However, a varying trend is observed in the growth curve of *S. epidermidis* as the inhibitory effect at MIC of 250 µg/mL drastically diminish after 5 hours. A similar observation on the growth curve of MRSA occurred and the bactericidal effects of SBH-AgNPs at 63 µg/mL showed a decline in inhibitory effect after 5 hours. The

growth curves of (D) *K. pneumoniae*, (E) *E. coli* and (F) *P. aeruginosa* show that SBH-AgNPs are able to effectively inhibit the growth of these three organisms from 0 to 24 hours at MIC concentration of 250 µg/mL. Contrastingly, the growth curve on gram positive bacteria, *S. epidermidis* does not show any growth inhibitory effect at MIC concentration of 250 µg/mL after 5 hours while the other positive strains do display a growth decline. Overall, the SBH-AgNPs showed improved inhibitory effect against gram-negative bacteria than in gram-positive bacteria. The explanation behind this observation could be because Gram-negative bacteria lack the thick peptidoglycan layer found in Gram-positive bacteria, which may potentially act as a protective layer, the cell wall breakdown that results from physical interaction between NPs and the cell wall is more harmful for Gram-negative bacteria (Slavin et al., 2017).

This study, however, is subject to several limitations. The instrumentation used for analysing SBH-AgNPs was limited due to time constraints and unavailability of certain instruments. XRD analysis was not performed to study the crystallinity of the silver nanoparticles. It is possible that the stingless bee honey possess significant anti-inflammatory properties as stated in the previous literature hence exploring this anti-inflammatory characteristic could be beneficial to this research study. Lastly, there is limited research on the *Meliponula feriguenea* stingless bee honey composition and its properties.

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Chapter 5

Conclusions and recommendations

5.1 Conclusions

AgNPs were successfully synthesized using stingless bee honey, characterized, and evaluated for antibacterial activity for the very first time according to the best of our knowledge, the study's aims and objectives were met.

The optimum conditions for the synthesis of SBH-AgNPs were honey solution of 50 mg/mL (pH 10) at temperature of 75°C and $AgNO_3$ concentration of 0.75 mM over a period of 3 hours under dark. The SBH-AgNPs had an SPR peak at 414 nm after synthesis. The DLS analysis showed that the hydrodynamic size was 79.67 nm with a PDI of 0.36 and the zeta potential had a value of -15 mV which indicated moderate stability.

HR-TEM results confirmed that the SBH-AgNPs are spherical in shape with an average core diameter of 60 nm with a broad size distribution. FTIR analysis confirms that proteins, polyphenols, amine groups, flavonoids, and other compounds present in the honey were able to reduce $AgNO_3$ to generate AgNPs. The FTIR spectra obtained revealed similarities between the extracts and AgNPs along with some variation in peak shift positions, which sufficiently confirms the idea that phytochemicals are responsible for the reduction. The EDX confirms that there are Ag elements present in the sample.

After successful determination of the physiochemical properties of the SBH-AgNPs, biological studies were performed to assess their antibacterial activity. The antibacterial studies showed that the SBH-AgNPs do exhibit antibacterial effects. Agar well diffusion was carried out and SBH-AgNPs showed some degree of potency against the different bacterial strains within this study. The highest zone of inhibition of 13 mm was displayed against both *S. epidermidis* and *P. aeruginosa*. However, there was no inhibition zone visible for the stingless bee honey in all the tested strains. The MIC and MBC assays indicated that SBH-AgNPs were mostly active against *S. epidermidis*, with MIC and MBC values of 16 and 32 µg/mL, respectively. Although, it had been previously reported that agar well diffusion is not a reliable antibacterial activity test, but it was carried out in this study as the primary method for determining antibacterial susceptibility and it also aids in visualization of growth inhibition. The lowest activity recorded for SBH-AgNPs was against MRSA, with MIC and MBC values of 63 and 125 µg/mL,

respectively. Lastly, growth kinetics revealed that the SBH-AgNPs did inhibit the growth of the selected bacteria strains even at lower concentrations. However, against *S. epidermidis* at SBH-AgNPs concentrations equal to the MIC (250 µg/mL) there was no growth inhibition observed.

5.2 Recommendations

1. We highly recommend a further examination of the antibacterial activity of the stingless bee honey by using the concentrations higher than 50 mg/mL.

2. Secondly, the stability and the broad size distribution of SBH-AgNPs can be improved by introducing a capping or stabilizing agent such as Chitosan because it is biocompatible polysaccharide with positive Z potential which can stabilize negative charged nanoparticles.

3. It is very important to identify the compounds in the SBH and analyse those which are responsible for the capping of AgNPs.

4. It has been reported that stingless bees honey possess anti-inflammatory properties, therefore in the future, the anti-inflammatory tests need to be carried out.

5.3 Future work

To develop a method to stabilize the AgNPs produced. In order to acquire antimicrobial activity for the SBH alone, a higher concentration of stingless bee honey of more than 50 mg/ml can potentially be used in the agar well diffusion assay. Additionally, anti-inflammatory studies for the stingless bee honey can also be conducted.

To use HPLC on the analysis of honey and SBH-AgNPs for identifying the phytochemicals which are responsible for antimicrobial activity. Furthermore, these phytochemicals can be isolated and used for antimicrobial applications.

Studying the mechanism of interaction of SBH-AgNPs with biological systems to be able to develop nanoparticles with favourable physiochemical properties which will not produce negative effects.