BIOGENIC SILVER NANOPARTICLES SYNTHESIZED USING *EUCOMIS AUTUMNALIS* BULB AQUEOUS EXTRACT, THEIR CHARACTERIZATION AND *IN VITRO* ANTIBACTERIAL ACTIVITY

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A mini thesis submitted in partial fulfilment of the requirements for the degree - Magister Scientiae in Nanoscience

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KEYWORDS

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ABSTRACT

Biogenic silver nanoparticles synthesized using *Eucomis autumnalis* bulb aqueous extract, their characterization and *in vitro* antibacterial activity

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Antimicrobial resistance (AMR) is a growing global problem that poses a significant threat to public health. AMR arises when microorganisms become resistant to drugs that were initially designed to kill them. The application of antibiotics has proven effective in eliminating or inhibiting the growth of harmful microorganisms. Conversely, the primary cause of AMR is overprescribing and the misuse of antibiotics which is further increased by a reduced novel antibiotics discovery rate. AMR bacteria lead to hard-to-treat infections, causing longer hospital stays and higher healthcare costs, and is associated with a substantial risk of morbidity and mortality. Consequently, the development of infections caused by multi-drug resistant (MDR) microorganisms has resulted in a considerable reduction in the number of effective treatment options available. Thus, novel therapies are urgently required, considering the ineffectiveness of current therapy, moreover, further exacerbated by a reduced discovery rate of novel antibiotics. Alternative strategies are therefore urgently needed to treat AMR bacterial infections.

Nanotechnology has revolutionized the nanomedicine field owing to its potential to eradicate the burden of AMR by providing alternative novel or improved therapeutic strategies for AMR infections, while preventing further microbial resistance. Biodegradable metallic nanoparticles (MNPs), particularly silver, gold, cobalt, and zinc, have significantly transformed medicine by serving as antimicrobial, anticancer, drug delivery, contrast, and bioimaging agents. Silver nanoparticle (AgNPs) have been shown to exhibit antimicrobial properties which aid in the prevention and treatment of infections, are deemed less toxic, cost-effective, energy-efficient, and environmentally friendly, compared to the physical and chemical methods that were commonly used for the synthesis of MNPs. The green synthesis of AgNPs employs sustainable green chemistry principles, makes use of bioactive molecules containing medicinal properties. Previous

literature has demonstrated that *Eucomis autumnalis* can facilitate AgNPs synthesis while both extract and nanoparticles (NPs) exhibit antibacterial, anti-inflammatory, anticancer, antioxidative, and antihistaminic activities. Biogenic AgNPs with antibacterial efficacy may serve as a novel antibacterial agent which provides optimism in developing an alternative strategy to treat and prevent AMR infections. This study reports on the green synthesis and characterization of biogenic AgNPs, using *Eucomis autumnalis* bulb aqueous extract (EABE) and the antibacterial effects of the EABE-AgNPs against AMR pathogens such as *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*.

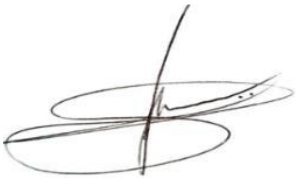
For the synthesized AgNPs, several parameters (i.e., pH, temperature, EABE concentration, and silver nitrate ($AgNO_3$) concentration) were optimized to obtain desirable physicochemical properties. The AgNPs were successfully synthesized and characterized by Ultraviolet-visible (UV-vis) Spectroscopy, Dynamic Light Scattering (DLS), High-Resolution Transmission Electron Microscopy (HR-TEM), and Fourier-Transform Infrared (FTIR) Spectroscopy. EABE-AgNPs antibacterial activity was assessed *in vitro* by agar well diffusion, microdilution assay to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

EABE-AgNPs exhibit a Surface Plasmon Resonance (SPR) of 404 nm with an OD value of 1.960, a hydrodynamic size of 126.4 ± 29.4 nm and an average core size of 13.03 ± 2.04 nm. EABE-AgNPs were spherical in shape with a PDI of 0.396 ± 0.07, stable in various physiological media, exhibiting a ζ-potential of − 25.2 ± 8.48 mV. The AgNPs were found to be relatively monodispersed and isotropic in nature. EABE-AgNPs were reduced and capped by phytochemical functional groups identified and confirmed in the EABE. All the bacterial strains studied exhibited dose-dependent inhibitory action against EABE-AgNPs. The EABE-AgNPs were equally bactericidal against all the bacterial strains tested. Furthermore, EABE has the potential to produce biogenic AgNPs with remarkable antibacterial activity. The results presented in this study demonstrated that, EABE-AgNPs may serve as a novel antibacterial agent to help in the prevention and treatment of AMR infections, and potentially aid in the global eradication of AMR pathogens.

DECLARATION

I declare that "**Biogenic silver nanoparticles synthesized using *Eucomis autumnalis* bulb aqueous extract, their characterization and *in vitro* antibacterial activity"** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Letoya Sheila Williams



Signature

Date: 04 January 2023

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Firstly, I would like to thank God, He has provided me the strength and wisdom to get through this chapter. I will always be eternally grateful for His unending love, mercy, and grace. Philippians 4–13: "***I can do all things through Him who gives me strength***."

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"It always seems impossible until it's done."
- Nelson Rolihlahla Mandela -

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°C	Degrees Celsius or degree centigrade
%	Percentage
β	Beta
λ_{max}	maximum absorbance/ absorption maximum
µl	Microliter
ζ	potential Zeta potential
Abs.	Absorbance
Ag	Silver
Ag^{+}	Silver ion
Ag^{0}	Silver atom
AgNP(s)	Silver nanoparticle(s)
$AgNO_3$	Silver nitrate
Al	Aluminium
AMR	Antimicrobial resistance
AMU	Antimicrobial use
ATCC	American Type Culture Collection
Au	Gold
AuNP(s)	Gold nanoparticle(s)
BSIs	Bloodstream infections
CAM	Complementary and alternative medicine
CDC	Centers for Disease Control and Prevention
CDI	*Clostridium difficile* infection

CFU	Colony forming units
COVID-19	Coronavirus disease 2019
Cu	Copper
DDD	Defined Daily Dose
ddH_2O	Deionized distilled water
dH_2O	Distilled water
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substances
ESBL	Extended spectrum β-lactamases
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter*
Fe	Iron
FTIR	Fourier-transform Infrared spectroscopy
g	Gram(s)
HAI(s)	Hospital-Acquired Infections
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HNO3	Nitric acid
hr(s)	Hour(s)
HR-TEM	High Resolution-Transmission Electron Microscopy
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IDs	Infectious diseases

KBr	Potassium bromide
L	Liter
LMICs	Low- and middle-income countries
LSPR	Localized surface plasma resonance
M	Molar
MBC	Minimum bactericidal concentration
MDR	Multi-drug resistant
mg	Milligram(s)
mg/ml	milligram(s) per millilitre
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
min(s)	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
MNPs	Metal nanoparticles
NaOH	Sodium hydroxide
NDoH-RSA	National Department of Health Republic of South Africa
NHLS	National Health Laboratory Service
nm	Nanometre
NP(s)	Nanoparticle(s)
OD	Optical density

PDI	Polydispersity index
pH	Potential of hydrogen
Pt	Platinum
QS	Quorum sensing
RFU	Relative Fluorescence Units
ROS	Reactive oxygen species
RSA/ SA	Republic of South Africa/ South Africa
RT	Room temperature
rpm	Revolutions per minute
SD	Standard deviation
SPR	Surface Plasmon Resonance
Ti	Titanium
UK	United Kingdom
U.S./ USA	United States/ United States of America
UTIs	Urinary tract infections
UV-vis	Ultraviolet-visible spectroscopy
v/v	Volume per volume
WHO	World Health Organization

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Antibacterial resistance is a growing concern worldwide, posing a threat to public health. The ability of bacteria to develop resistance to antimicrobial drugs is a complex issue, influenced by various factors (Farfán-García *et al*., 2023; Jonas *et al*., 2017; Murray *et al*., 2022). This exacerbates microbial infections and reduces the likelihood of successful treatment. In recent years, novel resistance mechanisms have posed serious threats to the treatment of many common bacterial diseases, resulting in treatment failures, bacterial sepsis and septic shock, or even death (Bennani *et al*., 2020; Kharga *et al*., 2023; O'Neill, 2016). The lack of new antibiotic development has allowed bacteria to evolve and develop resistance faster than new drugs can be introduced. Additionally, antibiotic residues from agriculture and pharmaceutical manufacturing can contaminate water sources and soil, further promoting the survival and spread of resistant bacteria not only in humans and animals, but also in the environment (Kharga *et al*., 2023; Vij *et al*., 2018).

Antimicrobial resistance (AMR) is a growing global problem that poses a significant threat to public health (Ferri *et al*., 2017; Tang *et al*., 2023). AMR arises when microorganisms become resistant to drugs that were initially designed to kill them (Serwecińska, 2020). The application of antibiotics has proven effective in eliminating or inhibiting the growth of harmful microorganisms (Salam *et al*., 2023; WHO, 2020). Conversely, the primary cause of AMR is overprescribing and the misuse of antibiotics which is further increased by a reduced novel antibiotics discovery rate (Chaw *et al*., 2018). AMR bacteria lead to hard-to-treat infections, causing longer hospital stays and higher healthcare costs, and is associated with a high risk of morbidity and mortality (Parmanik *et al*., 2022). Consequently, the development of infections caused by multi-drug resistant (MDR) microorganisms has resulted in a considerable reduction in the number of current traditional antimicrobial treatment options available and due to side effects, including other limitations associated with it. Therefore, there is an urgent need to develop alternative strategies to treat AMR bacterial infections (Garcia *et al*., 2021). Nanotechnology has revolutionized the field, enabling alternative therapeutic strategies against AMR infections, including biodegradable metal nanoparticles (MNPs). Green synthesized silver nanoparticles (AgNPs) have been shown to exhibit antimicrobial properties and are deemed less toxic, cost-effective, and eco-friendly compared to the physical and chemical methods that were commonly used for the synthesis of MNPs (Sidhu *et al*., 2022). The green

synthesis of AgNPs employs sustainable green chemistry principles, makes use of bioactive molecules containing medicinal properties. Biogenic AgNPs with antibacterial efficacy may serve as a novel antibacterial agent which provides optimism in developing an alternative strategy to treat and prevent AMR pathogens (Fadaka *et al*., 2021; Garza-Cervantes *et al*., 2020; Raj *et al*., 2021; Salazar-Bryam *et al*., 2021).

The rapid emergence of drug-resistant microbial strains is posing a significant threat to the effectiveness of current antibiotics in combating microbial infections (Makvandi *et al*., 2023). Despite the availability of various antimicrobial drugs, the misuse and overprescription, as well as microbial mutation have led to the development of new drug-resistant strains (Fadaka *et al*., 2021; Smith *et al*., 2015). As such, the healthcare system faces significant challenges due to the emergence of antibiotic-resistant bacteria, which could possibly lead to another global pandemic (Abdellatif & Mohammed, 2023; Abushaheen *et al*., 2020). The global increased mortality rates are associated with emergence and spread of MDR bacteria and a major health concern that necessitates the development of alternative therapies (Allahverdiyev *et al*., 2011; Årdal *et al*., 2020; Giráldez-Pérez *et al*., 2022; Mühlen & Dersch, 2016). Naturally derived products have shown potential in combating the most dreadful diseases, and therefore serve as an effective source of bioactive compounds that can be used as antibacterial agents (Majoumouo *et al*., 2019; Ngouana *et al*., 2015; Tchuenmogne *et al*., 2017). These compounds are able to reduce metal ions and cap nanoparticles (NPs) to form biogenic nanoparticles with remarkable anti-bacterial activities (Akhtar, Naeem, *et al*., 2023; Akhtar, Shahid, *et al*., 2023; Majoumouo *et al*., 2019).

Biogenic MNPs, such as silver nanoparticles (AgNPs), have been found to exhibit antibacterial and antibiofilm properties against bacterial and fungal pathogens (Abul Qais *et al*., 2018; Sánchez-López *et al*., 2020). Several studies report the green synthesis of AgNPs and their antimicrobial activity (Abdelghany *et al*., 2018; Kharissova *et al*., 2013; Mittal *et al*., 2013). Qais *et al*., was the first to report a study using *Carom copticum* (*C. copticum*) seed extract, demonstrating antibacterial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Salmonella typhi* (Raghunandan *et al*., 2011; Sharma *et al*., 2009). The *C. copticum* extract MnFe2O4 NPs has shown remarkable antibacterial action against *Staphylococcus epidermitis* (*S. epidermitis*), *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), Methicillin resistance in *S aureus* (MRSA), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). Biogenic NPs have shown their ability to suppress quorum sensing-mediated

virulence factors and biofilms, in addition proved to be effective therapy against intrinsically resistant biofilm infections by MDR quorum pathogens (Ghosh *et al*., 2015; Meeker *et al*., 2016). Thus, demonstrating its promising antibacterial activity not only on MDR pathogens but variety of diseases (Esmaeili & Ghobadianpour, 2016; Qais *et al*., 2020; Rozhin *et al*., 2021; Sharma *et al*., 2015; Truchado *et al*., 2012). In addition, a few papers have demonstrated the efficiency of AgNPs in anti-sensing (Anju & Sarada, 2016; Arunkumar *et al*., 2013; Singh *et al*., 2022; Srinivasan *et al*., 2018). While a particular study also reported an eco-friendly approach for the synthesis of AgNPs from *C. opticum* aqueous seed extract with broad-spectrum anti-quorum and antibiofilm capabilities. The NPs displayed antibiofilm and anti-quorum sensing effects against Gram-negative bacteria (*Chromobacterium violaceum*, *Serratia marcescens* and *P. aeruginosa*), as well as lowering pathogenicity and inhibiting biofilm formation in these pathogens (Qais *et al*., 2020).

1.2 Bacterial infections

Infectious diseases (IDs) caused by bacteria are one of the leading causes of chronic infections and mortality, globally (Ikuta *et al*., 2022; Ye *et al*., 2023). In 2019, ~7.7 million deaths globally were linked to bacterial infections, both resistant and susceptible to antimicrobials (Ikuta et al., 2022). ID caused by bacteria (Varela *et al*., 2021) often require antibiotics for treatment, but improper use has led or exacerbated antibiotic resistance (Jernigan *et al*., 2020). This then weakens the host immunity and potentially leads to severe complications, sepsis, and even death (Rawson *et al*., 2020).

All the human organs are susceptible to bacterial infections (Doron & Gorbach, 2008), resulting in serious bacteria-induced diseases shown in **Figure 1.1** including bacterial pneumonia (meningitis), diarrhoea, urinary tract infections (UTIs), tuberculosis (TB), wound infections, and sexually transmitted infections (STIs) (Abdellatif & Mohammed, 2023; Hutchings *et al*., 2019). Bacteria possess the ability to invade the host through various mechanisms, including the production of toxins, tissue invasion, and the formation of biofilms (Penesyan *et al*., 2019). Bacterial biofilms are formed by adhering to each other or on inert surface in an extracellular polymeric substance (EPS) (Penesyan *et al*., 2019). The EPS enables bacteria to communicate through quorum sensing (QS), a process that activates the immune system, causing inflammation and potentially sepsis in biofilms resistant to antimicrobial agents (Flemming & Wingender, 2010; T. Wang *et al*., 2023).

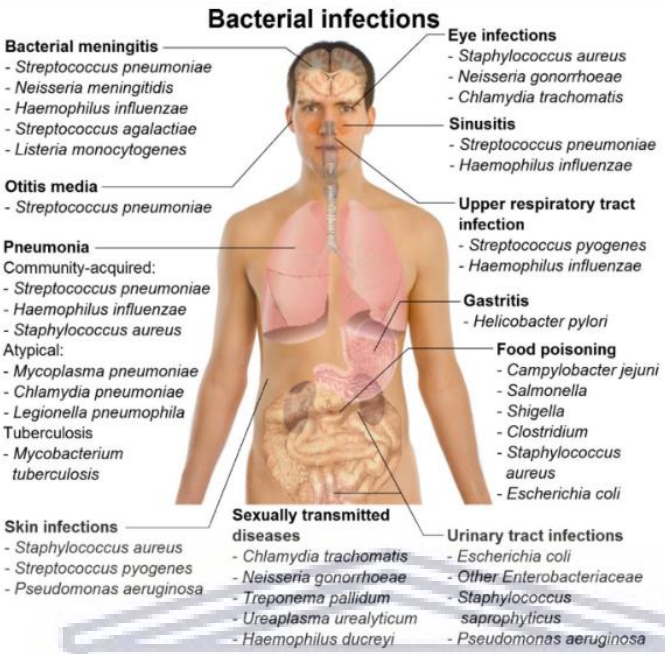


Figure 1.1: Organs which are more susceptible to bacterial infections and the various associated species involved (Adapted from Harvey, 2007).

Antibiotic abuse and overuse in these situations, where more than one antibiotic was used (Morrison & Zembower, 2020) have led to the development and spread of MDR microorganisms (Parmar *et al*., 2022). Thus, AMR pose a significant challenge to the medical industry (Akintelu *et al*., 2020; Holmes *et al*., 2016). In hospital settings, the urinary tract, respiratory tract, bloodstream (sepsis), post-surgical (wound) and pneumonia infections are common (Lanks *et al*., 2019; Liu & Dickter, 2020; Markwart *et al*., 2020). Bacterial drug resistance leads to higher treatment dosage, longer hospital stays, and increased mortality rates. In 2017, 48.9 million sepsis cases were reported globally, with 19.7% (11 million) global deaths. More of these cases and deaths (85%) occur in low-to-middle-income countries (LMICs), and 88% of infected patients develop sepsis (Rudd *et al*., 2020; Tancharoen *et al*., 2022). In African countries, hospital settings are increasingly contaminated with MDR pathogens, leading to pneumonia and UTIs, highlighting the need for improved healthcare systems to combat these diseases (Agyepong *et al*., 2018; Asante *et al*., 2021; Kayode *et al*., 2020; Odoi *et al*., 2022; Zachariah *et al*., 2021).

Currently, available antibiotics for combating IDs are rapidly becoming ineffective because of the development of drug-resistant microbial strains and remains a consistent global challenge (Makvandi *et al*., 2023). A study found 385 examples of various antibiotic misuse, including

335 prescription errors and 50 unfinished treatments, with the most common error being related to duration of treatment (Garcia-Vello *et al*., 2020). The Coronavirus disease 2019 (COVID19) pandemic has exacerbated the spread of AMR (~ 72%), due to the increased misuse of antibiotics (Karbelkar & Furst, 2020; Rawson *et al*., 2020), with an increased number of hospitalized patients receiving antibiotics for secondary infections (Rawson *et al*., 2020). During COVID-19 admission, about 132 respiratory and bloodstream pathogens were identified, with *S. aureus* causing the most infections (Wu *et al*., 2023).

Although antibacterial agents have been effectively used to treat bacterial infection, the emergence and spread of AMR, has weakened their effectiveness, making prevention and treatment of drug-resistant bacterial infections difficult to treat (Tang *et al*., 2023). MDR bacteria have now become a global issue of public health. Additionally, public health interventions like antibiotic stewardship programs (ASPs) and surveillance systems played a significant role in controlling the spread of pathogenic bacterial infections (Abushaheen *et al*., 2020). This will increase awareness and knowledge about MDR, improve public health conditions and hygiene. However, the lack of new and improved drug development by the pharmaceutical industry due to challenging regulations and reduced economic incentives for drug development must also be addressed (Hegemann *et al*., 2023).

1.2.1 Antimicrobial resistance (AMR)

AMR is a growing global concern and a major threat to human and animal health (Laxminarayan *et al*., 2020; Salam *et al*., 2023). AMR occurs when microorganisms, such as bacteria, fungi, parasites, and viruses, develop resistance to drugs used to treat infections (Friday *et al*., 2020). This natural genetic evolution is affecting the efficiency of antibiotic use in healthcare (Abushaheen *et al*., 2020; Alam *et al*., 2019). Moreover, the misuse, along with a reduced drug discovery rate of novel antibiotics increases the prevalence of IDs. The discovery of penicillin by Fleming in 1928 revolutionized bacterial research, significantly impacting both human and animal health by introducing antibiotics for treating IDs (Dyary *et al*., 2023; Reygaert, 2018; Zaman *et al*., 2017). Within 12 years, this discovery led into an incredible era of development of drugs capable of eradicating bacterial infections. Additional antibiotics followed, and continued to revolutionize healthcare, serving as the basis of many of the most significant medical developments of the 20th century (Rhee *et al*., 2019; Zhang & Cheng, 2022). Infections like pneumonia and TB, which are common but usually fatal, might

be easily treated. The hazards of ordinary surgery and childbirth were greatly diminished, and even a tiny incision no longer had the potential to be lethal if it became infected (Abdellatif & Mohammed, 2023; Rhee *et al*., 2019). Despite the significant development of new antibacterial drugs, bacterial infections continue to be a growing concern even though these drugs have been in use many years since their discovery (Hutchings *et al*., 2019; Iskandar *et al*., 2022; Salam *et al*., 2023).

In the 21st century, antimicrobial resistance has emerged as one of the major public health concerns (Balboa & Hicks, 2022) and has resulted in increased healthcare costs. The effectiveness of many antimicrobials (73%) used in human healthcare has declined, (Van Boeckel *et al*., 2015), thus reducing treatment options against pathogenic microorganisms (F. N. Idris & M. M. Nadzir, 2023). The emergence and spread of novel resistance mechanisms threatens the treatment of many common infectious diseases resulting in treatment failure, severe impairment, or even death. AMR will substantially compromise the efficacy of cancer treatment, transplantation surgery, and even simple dental operations, unless novel drugs with new mechanisms are developed (Anderson *et al*., 2019; Salam *et al*., 2023; Tenover, 2006). Centers for Disease Control and Prevention (CDC) indicated that half of the antibiotics prescribed were unnecessary and/or misused (Maddox, 2022), thus contributing to recalcitrant bacterial infections (Chopra *et al*., 2002). According to current estimates, at least 30% and up to 50% of all antibiotics administered are unnecessary (Salam *et al*., 2023; Saunders, 2020). The overuse of antibiotics and genetic mutations have altered their tolerance of antibiotics that were once effective, leading to drug inactivation, modification of critical biosynthetic pathways, changes in target sites (cell wall synthesis, folic acid biosynthesis, protein biosynthesis), or drug expulsion from cells (Kavya *et al*., 2023).

The resistance of bacteria to antibiotics can reduce the risk of preventing and managing immune-compromised health conditions like Human immunodeficiency virus (HIV), cancer, surgical procedures, and diabetes (Bartlett *et al*., 2013; Hegemann *et al*., 2023). A continuation of this situation could lead to a "post-antibiotic era" where minor injuries and common infections could become the leading causes of death (Alam *et al*., 2019). Nevertheless, several studies have shown that these microorganisms can evolve and adapt, which is crucial for their survival and enables them to resist any therapy (Fisher *et al*., 2018; Lee & Jun, 2019). The problem of AMR has become urgent in recent years, with rates of resistance rising at an ever-increasing rate (Abdellatif & Mohammed, 2023; Alam *et al*., 2019; Bell *et al*., 2014; Coppola

et al., 2022). Consequently, some new modified strains appear to reduce the chances of effective treatments in patients. Thus, alternative strategies must be developed to combat rapidly evolving pathogens and existing bacterial infections associated with AMR, which can reduce bacterial coinfections associated with MDR (Khameneh *et al*., 2016; Loyola-Cruz *et al*., 2023; Tenover, 2006; Zhou *et al*., 2015).

1.2.2 Prevalence and burden of AMR

The emergence and spread of bacterial MDR remains a global threat among people of all ages (Fernandez, 2022; Murray *et al*., 2022; Vos *et al*., 2020). An estimated 4.95 million deaths associated with bacterial AMR were reported in 2019 (Getahun *et al*., 2020; Murray *et al*., 2020; Murray *et al*., 2022). These rates are projected to increase by 2050, leading to AMR becoming the world's primary cause of death (O'Neill, 2016). The most prevalent locations for bacterial AMR infections, accounting for 78.8% of directly related AMR mortalities are the chest, bloodstream, and abdomen (Murray *et al*., 2022; Vos *et al*., 2020). As shown in **Figure 1.2**, 33 bacterial pathogens were responsible for 1.27 million deaths in 11 infectious syndromes in 2019 (Ikuta *et al*., 2022).

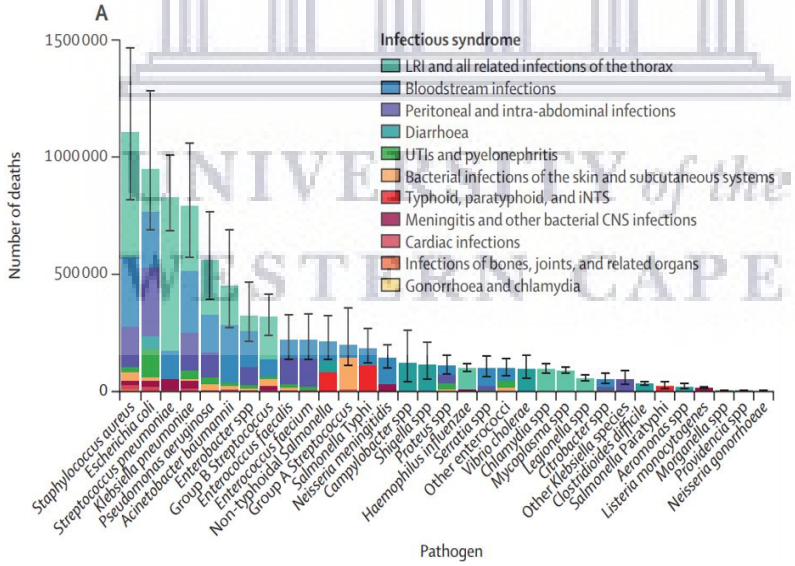


Figure 1.2: Bacterial-induced global mortalities in infectious syndrome (Adapted from Ikuta *et al*., 2022).

Currently, drug-resistant infections (**Figure 1.3 A**) are responsible for no less than 700,000 deaths each year (O'Neill, 2016; O'Neill, 2014). If preventative and control measures are not implemented, drug-resistant organisms may increase annual mortality, with an estimated $100

trillion in economic losses by 2050, mostly in LMICs (Bhattarai *et al*., 2021; Pullon *et al*., 2016). Drug-resistant infections are predicted to cause 10 million deaths annually (**Figure 1.3 B**), with 90% of these occurring in Asia and Africa alone (Islam *et al*., 2021; Jain *et al*., 2021; Safain *et al*., 2020). Furthermore, this would make it the primary cause of mortality among humans, surpassing heart disease, diabetes, and cancer (Belete *et al*., 2023; Morrison & Zembower, 2020; Murray *et al*., 2022).

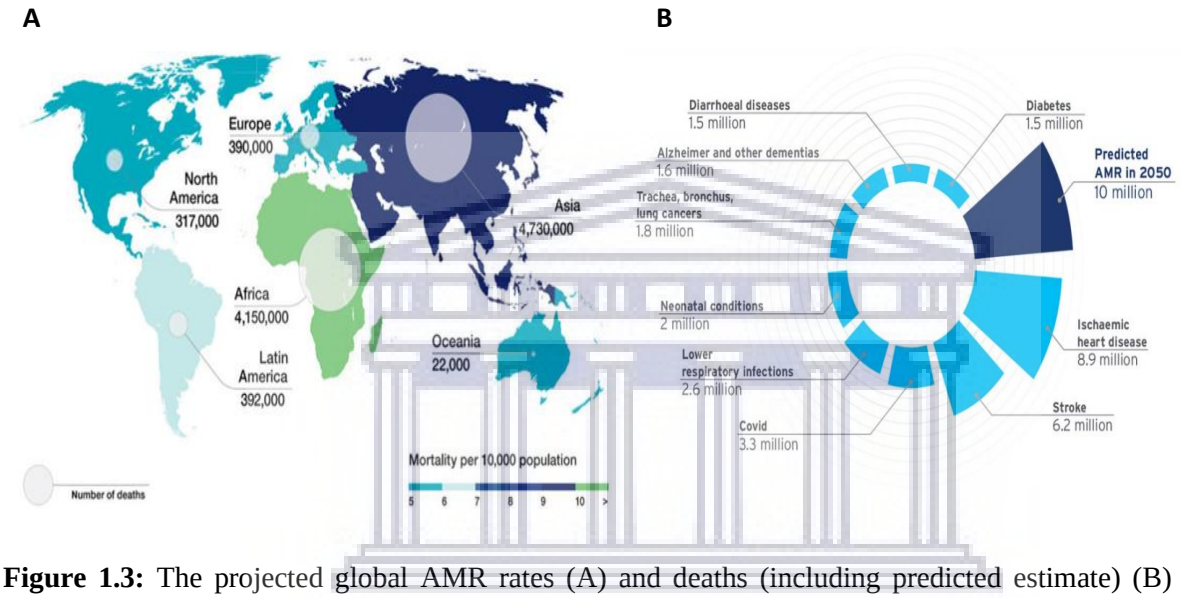


Figure 1.3: The projected global AMR rates (A) and deaths (including predicted estimate) (B) in different parts of the world by 2050 (Adapted from O'Neill, 2016; O'Neill, 2014).

Annually, a substantial amount of money is spent to address the impact of antibiotic-resistant bacterial infections around the world. In the European Union (EU) alone, a subset of drug-resistant bacteria are responsible annually for some 25,000 deaths, with extra health care costs and lost productivity due to AMR amounting to at least €1500 million (WHO, 2015). The EU invested approximately €1.5 billion each year to combat AMR infections, which are accountable for roughly 23,000 fatalities solely in Europe (Davies *et al*., 2013). The United States of America (USA) spent more than $20 billion to treat 20 million AMR patients each year (Hampton, 2013).

High-income nations are also impacted by the occurrence of AMR despite data suggesting that it is more prominent in LMICs (Ayobami *et al*., 2022; Rödenbeck *et al*., 2023). In 2019, the sub-Saharan Africa had the highest incidence with 23.5 million deaths per 100,000 attributed to AMR (Belete *et al*., 2023; Carlet *et al*., 2012; Murray *et al*., 2022). If precautionary and regulation measures are not implemented, the global landscape of disease burden, including

the highest-ranking IDs, may change due to the ongoing COVID-19 pandemic (Godman *et al*., 2021; Sulis *et al*., 2022).

1.3 Antibacterial resistance against ESKAPE pathogens

In 2017, the WHO issued a list of MDR microorganism posing a threat to human health, emphasizing the urgent need for developing novel antimicrobials and therapeutic modalities (WHO, 2017). These pathogens are known as "ESKAPE," an acronym for the group of bacteria, encompassing both Gram-positive and Gram-negative species. These include *Enterococcus faecium* (*E. faecium*), *S. aureus*, *Klebsiella pneumoniae* (*K. pneumoniae)*, *Acinetobacter baumannii* (*A. baumannii)*, *P. aeruginosa*, and *Enterobacter*, are common causes of life-threatening nosocomial infections among critically ill and immunocompromised individuals. These pathogens are characterized by high mortality and morbidity rates (Catalano *et al*., 2023).

Healthcare delivery and medical care are prone to 'nosocomial' or 'healthcare associated infections', which can occur during or after patient admission and even among medical staff and equipment, posing significant health risks (Kariuki *et al*., 2023; Shankar & Balasubramanium, 2014). The occurrence of infections with MDR, extensively drug-resistant (XDR), difficult-to-treat drug-resistant, carbapenem-resistant, and pan-drug-resistant is still increasing (Ardebili *et al*., 2023; Catalano *et al*., 2023; Quraini *et al*., 2023; WHO, 2022). Globally, 3.5% of active Tuberculosis (TB) and 18% of MDR-TB, with XDR-TB are a growing concern (Getahun, 2023; Salam *et al*., 2023). The majority of nosocomial infections are caused by the ESKAPE pathogens (Pendleton *et al*., 2013; H. Wang *et al*., 2023), further categorized into three groups based on their level of resistance: critical, high, and medium (Idris & Nadzir, 2023). Thus, there is a tremendous interest in developing novel agents, together with a gap in antimicrobial use (AMU), as alternative approaches for treating such infections (Hegemann *et al*., 2023; Mirghani *et al*., 2022).

The microorganisms that fall under the critical priority (**Table 1.1**) are Enterobacteriaceae (including *K. pneumoniae, E. coli, Enterobacter spp*., *Serratia spp*., *Proteus spp*., *Providencia spp*. and *Morganella spp*.), which are resistant to third-generation cephalosporin-resistant as well as carbapenem-resistant (including *A. baumannii* and *P. aeruginosa*) (Campos *et al*., 2020; Catalano *et al*., 2023; Pendleton *et al*., 2013; WHO, 2017). In hospital settings, *A. baumannii*, a bacteria found in medical equipment, poses a significant threat due to its resistance to

disinfectants, high temperature, and pH ranges (Antunes *et al*., 2014). It can survive on solid surfaces for up to 5 months, causing infections such as ventilator associated pneumonia, bacteraemia, endocarditis, meningitis, osteomyelitis, and skin, soft tissue, and UTIs (Campos *et al*., 2020; Nowak & Paluchowska, 2016; Peleg *et al*., 2008). *P. aeruginosa* on the other hand causes a variety of diseases including skin and gastrointestinal infections, pneumonia, bloodstream infections (BTIs), UTIs, and cystic fibrosis lung infections (Azam & Khan, 2019). Hospital-Acquired Infections (HAIs) caused by *P. aeruginosa* accounted for 9% in Europe, and 30% of these infections were resistant to carbapenems (Campos *et al*., 2020). In addition, Enterobacter *spp*. and *K. pneumoniae* that produce extended spectrum β-lactamase (ESBL) were also classified as severe risk and a critical priority if they are resistant to carbapenems (Garner *et al*., 2015). *K. pneumoniae* is the cause of 16% of UTIs in India (Niveditha et al., 2012), and is responsible for 61% of biofilm-related infections linked to medical devices, in UTIs; catheters in BSIs, and pneumonia connected to ventilator usage (Singhai *et al*., 2012).

Table 1.1: List of the different priorities given to ESKAPE pathogens (Adapted from Campos *et al*., 2020; Mogasale *et al*., 2021; Tacconelli, 2017).

Priority	**Pathogens**	**Reported antimicrobial resistances**
Critical priority	*A. baumannii*	Carbapenem resistance
	P. aeruginosa	3rd generation cephalosporin resistance
	Enterobacter spp.	
	K. pneumoniae	
High priority	*E. faecium*	Vancomycin resistance
	S. aureus	Methicillin resistance in *S aureus* (MRSA)
	Salmonella species	Fluoroquinolone-resistance
Medium priority	*S. pneumoniae*	Penicillin resistance
	Shigella species	Fluoroquinolone resistance

The high priority pathogens are the major causes of BTIs in Africa, which caused 58% gastroenteritis infections in both human and animal (Reddy *et al*., 2019). *S. aureus* was reported in 59–72% of BTIs in surgical sites and burnt wound infections, of which 38% were MRSA (Hasan *et al*., 2016; Tacconelli, 2017). The spread of MRSA infection is alarming, with deaths surpassing those of Acquired immunodeficiency syndrome (AIDS) and Parkinson's disease (Lessa *et al*., 2012). Recently, MRSA accounted for over 50% of *S. aureus* strains in the USA, causing 50% of all nosocomial *S. aureus* infections (Szabó *et al*., 2022).

Streptococcus pneumoniae (*S. pneumoniae*) and *Shigella spp*. are medium priority pathogens contracted by direct contact with an infected person or consumption of contaminated water or food (Alhumaid *et al*., 2023; Getahun, 2023). Most approved AMR drugs are no longer effective against ESKAPE pathogens (Haque *et al*., 2018; Tagoe & Desbordes, 2012).

1.3.1 Mechanisms of antibacterial resistance

Different molecular and cellular pathways for AMR in MDR pathogens exist. Active export systems within bacterial membranes, blocking antimicrobial cellular, enzymatic destruction of antimicrobial agents, production of thick biofilms, modified targets for antimicrobials, and bacteria with AMR sites of action, constitute to a few of the mechanisms aggravating AMR (Varela *et al*., 2021). Biofilms function as a physical barrier that prevents the transmission of antibiotics and upregulate certain pathogenic genes that are associated with biofilms and enhance antibiotic resistance (Bowler *et al*., 2020; Penesyan *et al*., 2019; Tuson & Weibel, 2013; Venkateswaran *et al*., 2023). Biofilm protects bacteria from both antimicrobials and host defence systems, they account for 80% of all infections (Şen Karaman *et al*., 2020; Tenover, 2006).

Understanding the molecular processes underlying AMR formation is crucial for developing innovative therapeutic techniques to prevent AMR linked to bacterial infections (Kok *et al*., 2022). Antimicrobial agents use different mechanisms against bacteria and are classified as either bactericidal or bacteriostatic. Bacteriostatic agents prevent bacteria's growth by keeping them in the stationary phase, while bactericidal agents kill the bacteria (Pankey & Sabath, 2004). Antibacterial agents includes both natural products produced by microbes to protect themselves and synthetic compounds with a wide range of actions. Antibiotics typically inactivate pathogenic bacteria through various genetic and phenotypic AMR pathways, with specifics varying from drug to drug (Kassinger & Van Hoek, 2021; Vikesland *et al*., 2019; Walsh & Wencewicz, 2020).

In general, AMR in bacteria may occur via four major mechanisms: (i) target alteration, (ii) drug inactivation, (iii) drug transport and (iv) inactivation of drug agent (Reygaert, 2018). Bacteria can also inactivate antibiotics by producing enzymes that recognize and destroy the structure of antibiotics (Egorov *et al*., 2018; Lambert, 2005). It has been demonstrated that post-translational processes are another way in which bacteria acquire resistance. Through

post-translational changes, bacteria are known to regulate their metabolism and pathogenicity (Broberg & Orth, 2010).

The fluoroquinolone resistance of ESKAPE pathogens is another example of AMR through modifying enzyme targets. Approximately 65 to 80% of bacterial and chronic infections are caused by biofilms (Jamal *et al*., 2018; Maillard & Centeleghe, 2023), especially chronic infections (such as cystic fibrosis, endocarditis, and osteomyelitis) (Chiș *et al*., 2022; Sharma *et al*., 2019). Additionally, biofilms cause persistent infection and antibiotic treatment failure probably due to specific interactions between the drug, pathogen and host (Mishra *et al*., 2023; H. Wang *et al*., 2023).

1.4 Current treatments for AMR infections and their limitations

Currently, antibiotics (e.g., disrupting their biofilm) and vaccines (e.g., immunization) have been traditionally utilized to manage bacterial infections, resulting in substantial improvements in health and longevity (Yang & Yang, 2019). However, vaccine therapy often requires booster shot negatively weakening the immune response, as the innate immune system recognizes ubiquitous molecular patterns in all microorganisms, preventing erroneous attacks on host cells, leading to pores in the membrane (Clem, 2011). While antibiotic therapy often leads to AMR (Chiș *et al*., 2022; Uruén *et al*., 2020), antibiotic therapy has been linked to adverse events in up to 20% of patients receiving systemic treatment (Tamma *et al*., 2017).

Biofilm-forming microorganisms cause a variety of infections, requiring treatment methods to remove and eradicate the biofilm for effective infection management (Mishra *et al*., 2023). There is a correlation between biofilms and IDs outbreaks which are exclusively induced by biofilm infections (Auinger *et al*., 2003; Diaz *et al*., 2011; Høiby *et al*., 2010; Olson & Hunstad, 2016). The most prevalent biofilm-forming species include *P. aeruginosa*, *S. aureus*, and *S. epidermidis*. Chronic osteomyelitis, periprosthetic joint infections, and implant-associated osteomyelitis of long bones are the three most typical orthopaedic biofilm infections (Rao *et al*., 2011; Zimmerli & Sendi, 2017).

The conventional/traditional antibiotic approach to treating infections caused by gram-positive or gram-negative bacteria is to find small molecules that inhibit the growth or kill one or the other, the latter are known as broad-spectrum antibiotics (Hauser *et al*., 2016). The years between 1940 and 1960 are regarded as the "golden age" of antibiotic research, during this time, the following were discovered: (i) natural antibiotics isolated from actinomycetes, e.g.,

aminoglycosides, tetracyclines, amphenicols, macrolides, glycopeptides, ansamycins, lincosamides, streptogramins and cycloserine; (ii) antibiotics of fungal origin, e.g., penicillins and cephalosporins; and (iii) synthetic antibiotics, such as sulfones, nitrofurans, quinolones, azoles, phenazines, ethambutol and thioamides (Pancu *et al*., 2021). The great majority of these antibiotics are still used as treatments, although their efficiency has reduced due to rising AMR (Hutchings *et al*., 2019; Pancu *et al*., 2021).

Antibiotic therapy, despite being abused for managing bacterial infections and their biofilms, remains a crucial method for effective infection management (Deusenbery *et al*., 2021; Mishra *et al*., 2023; Ye *et al*., 2022). The main agents in bacteria include those that inhibit cell wall synthesis, depolarize the cell membrane, inhibit protein synthesis, nuclei acid synthesis, and metabolic pathways (Bbosa *et al*., 2014; Reygaert, 2018). Broad-spectrum antibiotics are likely to cause adverse effects such as gastrointestinal effects due to disturbance of gut flora. *Clostridium difficile* (*C. difficile*) infections are more likely to be caused by ampicillin, clindamycin, third-generation cephalosporins, and fluoroquinolones (Mohsen *et al*., 2020).

The current non-antibiotic therapy is the only non-traditional alternative treatment for bacterial infections and associated biofilms (Guh & Kutty, 2018; Yi *et al*., 2018). These substances do not function by directly targeting bacterial components required for bacterial growth. Non-traditional approaches explore ways to influence disease beyond inhibiting or killing pathogens or imply an alternative therapy to antibiotics (Theuretzbacher & Piddock, 2019). Non-antibiotic approaches, such as probiotics, phages, and phytomedicines, are being explored for the treatment and prevention of infections. Probiotics like *Lactobacillus spp*. and *Saccharomyces boulardii* are effective in preventing and treating diarrhoea, particularly *C. difficile*-associated diarrhoea. Bacteriophages are being used to control staphylococcal and gastrointestinal infections. Phytomedicines like artesunate, tea tree oil, honey, mastic gum, and cranberry juice are also being used for various infections (Carson & Riley, 2003).

Alternative therapies have emerged in recent years, including antimicrobial peptides, phage therapy, efflux pump inhibitors, antibodies, and immunomodulatory agents, which have produced impressive results in both laboratory and in clinical trials (Konwar *et al*., 2022). Emerging non-antibiotic therapeutic strategies include chemical modification of antibiotics, antimicrobial peptides, engineered microorganisms, bacteriophage therapy, antimicrobial adjuvants, fecal microbiota transplant, genetically modified probiotics and postbiotics,

phototherapy, cationic polymers, natural active substances, and nanotherapy in combating AMR (Cotter *et al*., 2013; Garcia-Gutierrez *et al*., 2019; Gebreyohannes *et al*., 2019; Kumar *et al*., 2021; Nordmann *et al*., 2012). Moreover, combinatorial therapies that combine these new approaches have been efficient enough to get approval for clinical use and have accelerated the discovery of novel combination approaches that enhance the performance of already in-use antibiotics (Konwar *et al*., 2022). **Table 1.2** summarizes the advantages and disadvantages of non-antibiotic therapies (Yang & Yang, 2019).

Table 1.2: Advantages and disadvantages of different non-antibiotic therapies (Adapted from Yang & Yang, 2019).

Non-antibiotic therapies	**Advantages and disadvantages**	**Main mechanism**
Fecal microbiota transplantation (FMT)	Despite its high cure rate and rapid curative effect, rCDI has drawbacks such as inflated cost, complicated operation, not suitable for infants, and mild side effects.	Restore the normal microbiome and metabolites, thereby achieving homeostasis.
Probiotics	Affordable, safe for all ages, no side effects, effortless operation, aids drug effects, dietary supplement while slow curative effect, limited in rCDI.	Probiotics have numerous antibacterial properties, which activate the immune system and aid in the restoration of the normal microbiome.
Engineered microorganisms	High precision targets often have inadequate mechanisms of action, inflated costs, complicated manufacturing processes, and lack of clinical validation.	Direct genetic modification involves the direct action of microorganisms on targeted pathogens.
Bacteriophage	The treatment effectively combats MDR, but it lacks a clear mechanism of action, safety assessment, and clinical validation.	Bacteria lysis
Natural active substances	Affordable, easily accessible, and can be obtained from numerous natural plants, but it lacks a clear mechanism of action and clinical validation.	*C. difficile* can be inhibited by effective antibacterial constituents.
Nanoparticles and compounds	The inhibitor is C. difficile, causing minimal body damage and showing remarkable therapeutic effects in vitro, but lacks clinical validation.	Directly causing cell damage on pathogen cells, it effectively destroys pathogens without disrupting the normal microbiome.

rCDI: recurrent *C. difficile* infection; CDI: *C. difficile* infection.

Novel branded antibiotics (e.g., besifloxacin, ceftobiprole, ceftaroline, dalbavancin, delafloxacin, omadacycline, ozenoxacin, oritavancin, telavancin, and tedizolid) are effective against drug-resistant Gram-positive infectious bacteria (Koulenti *et al*., 2019). Although non-antibiotic therapies are effective in treating infectious pathogens, but overtime developed resistance. Thus, making it crucial to use alternative antibiotic compared to these therapies (Kumar *et al*., 2021). Drug-resistant bacteria are rendering antibiotics ineffective,

prompting a shift towards alternative therapies. Advancements in biotechnology, genetic engineering, and synthetic chemistry have opened new avenues in finding therapies that can substitute for antibiotics.

1.5 Complementary and Alternative Medicine (Phytotherapy)

Complementary and alternative medicine (CAM) refers to a broad spectrum of health-care methods that are not part of the country's own tradition or conventional medicine and are not completely incorporated into the prevailing healthcare system (Abdullahi, 2011; Mothibe & Sibanda, 2019; WHO, 2000; Yuan *et al*., 2016). The terms are used interchangeably with traditional medicine (TM) in some countries. Traditional medicines (TMs), including herbal medicines, are a significant part of traditional healthcare practices and often referred to as natural medicine, non-conventional medicine, and holistic medicine. Medicinal plants are utilized in both traditional and modern health management systems. Traditional systems have relied on plant-based medicines for thousands of years, with recent drugs containing active ingredients from plants. Before the advent of synthetic drugs, humans were entirely dependent on plant-based medicines for primary healthcare (Shi *et al*., 2010; Yuan *et al*., 2016).

TMs, one of the oldest forms of healthcare, has been used since ancient times to treat various life-threatening diseases, utilizing medicinal plants to prevent and treat physical and mental health issues across different cultures (Alam *et al*., 2021; Kurhekar, 2021; Schultz *et al*., 2020; Shi *et al*., 2010; Yuan *et al*., 2016). Currently, plants are now considered valuable resources of natural compounds (secondary metabolites with numerous biological activities) that may be exploited in the development of antidiabetic, anti-inflammatory, anticancer, and antimicrobial drugs (Angiolella *et al*., 2018; Friday *et al*., 2020). Approximately 80% of the patient population in Africa alone and globally, use TM – either alone or in conjunction with conventional medicine (Bhatia *et al*., 2021; Boakye *et al*., 2015). While in Nigeria, TMs system has shown medicinal plants as potent antimicrobial agents against MDR pathogens, such as *E. coli*, VRE, and *Candida albican* (*C. albican*) (Friday *et al*., 2020; Iwu, 2014). In general, natural compounds are obtained from plants and they serve as the favoured basis for new drug development (Lahlou, 2013). It has also been established that secondary compounds typically hold extremely intricate stereo structures, and these bioactive molecules are being established to possess medicinal function (Atanasov *et al*., 2015; Moiketsi *et al*., 2023; Moses *et al*., 2013).

Phytotherapy or phytomedicine, continue to fulfil therapeutic requirements in both conventional and clinical settings (Bone & Mills, 2012; Busia, 2016; Hooper *et al*., 2023). Phytotherapy, a traditional Western medicine practice, utilizes plants as health-promoting agents for medicinal purposes, preserving their composition and integrity, allowing for the use of the entire plant or a desired percentage. Plant-based therapies are utilized in various medical traditions such as anthroposophic medicine, naturopathic medicine, traditional Chinese medicine (TCM), Ayurvedic medicine, and allopathic medicine (Falzon & Balabanova, 2017). In Southern Africa, more than 10% of the plant species are utilized in TMs, with over 15 species experiencing commercialization (Ibrahim & Kebede, 2020; Moiketsi *et al*., 2023). While local populations in Latin America, Asia, and Africa have historically relied on traditional plant-based treatments for safe, cost-effective, and culturally appropriate primary healthcare (Friday *et al*., 2020).

According to the WHO, in developing countries, approximately 80% of primary health care needs are met by traditional and herbal medicines, mostly plant medicines (Modak *et al*., 2007; Mordeniz, 2019; Oliveira *et al*., 2006). TMs derived from medicinal plants are used by about 60% of the world's population (Modak *et al*., 2007). Over 85% of people in Africa, Asia, and the Middle East use herbal medicine as their first line of defence (Adeleye *et al*., 2022; Sen *et al*., 2011; Shareef *et al*., 2016; Singh *et al*., 2023). The global demand for herbal drugs are increasing due to their non-toxic, non-susceptible nature, affordability, and potential as a primary healthcare source for the poor (Friday *et al*., 2020).

The rapid increase in the consumption of herbal products, are widely advertised as safer, more natural, and healthier alternatives to conventional medicines, demonstrating the growing popularity of these herbal remedies worldwide (Mordeniz, 2019). Traditional herbal medicine, relies on phytochemicals found in plant extracts to treat and prevent diseases, potentially leading to the development of novel antimicrobials (Ivanišová *et al*., 2021; Liberal *et al*., 2020). African phytomedicines, such as *Cinchona*, which produces quinine, an antiplasmodial, are widely recognized on international markets for malaria treatment, with their annual market value of nearly $43 billion exceeding African budgets (Mordeniz, 2019).

Medicinal plants have long been used as medicines to treat microbial infections, among other human diseases; hence, their antibiotic development should now be focused on, as they have

proved to be the best alternatives for novel antibacterial targets and can be effective against MDR bacteria (Moiketsi *et al*., 2023).

The WHO has identified over 20,000 medicinal plant species, with approximately 374,000 plants and 28,187 medicinal species used by humans (Christenhusz & Byng, 2016; Han *et al*., 2023). These plants are considered potential sources of new drugs, with over 1340 plants having defined antimicrobial activity and over 30,000 compounds isolated from them (Srinivasan *et al*., 2001; Yadav & Agarwala, 2011). Over 100 countries have developed regulations for medicinal plants, with 14 − 28% of higher plant species being medicinal and 74% of bioactive plant-derived compounds discovered for ethnomedicinal uses (Miri *et al*., 2013; Talapko *et al*., 2018; Yadav & Agarwala, 2011). Numerous studies have demonstrated the efficacy of CAM phytotherapy against antimicrobial resistant bacteria, such as MRSA and ESBL. Plant extracts and phytochemicals, including essential oils from oregano, tea tree, and thyme, have shown potent antimicrobial activity, inhibited bacterial growth and disrupted biofilms (Hossain *et al*., 2016; Moiketsi *et al*., 2023). Additionally, plant extracts from garlic, turmeric, and ginger target different bacterial resistance mechanisms, making it challenging for bacteria to develop resistance against them (Moiketsi *et al*., 2023; Sultan *et al*., 2014).

The Nigerian TMs systems has shown many medicinal plants as potent antimicrobial agents against MDR pathogens such as *E. coli*, vancomycin-resistant *enterococci* (VRE), and *C. albicans* (Iwu, 2014). India's national economy has been significantly impacted by the collection and processing of medicinal plants, which play a major part in the country's health management. These plants are used in both traditional and modern systems of medicine, with some recent drugs containing active ingredients from plants (Singh *et al*., 2023). Plant compounds offer a sustainable alternative to synthetic antibiotics, as they are well-tolerated and have fewer side effects (Li et al., 2023). CAM phytotherapy, enhances antimicrobial activity and reduces resistance risk (Cheesman *et al*., 2017; Teja *et al*., 2022). The worldwide demand for plant-based antimicrobials is increasing due to their therapeutic potential and ability to mitigate side effects associated with synthetic agents (Teja *et al*., 2022). These non-toxic and affordable products are becoming the primary source of healthcare for the poor (Bonifacio *et al*., 2014). The need for intensified research in phytomedicine is crucial to conquer disease-causing organisms and ailments and eliminating the burden of AMR.

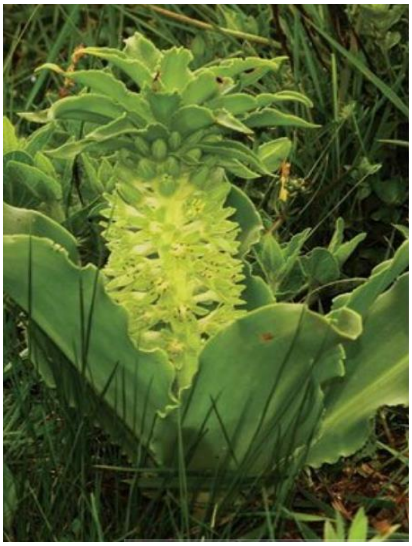
The oldest written evidence of medicinal plant was found on Sumerian clay slab from Nagpur used for drug preparation, dating back 5000 years (Castiglioni, 2019; Duffin, 2021; Guthrie, 1958; Kelly, 2009; Kurhekar, 2021; Major, 1954; Sigerist, 1987). It includes 12 recipes for over 250 plants, including alkaloids like poppy, henbane, and mandrake. Chinese book "Pen T'Sao" by Emperor Shen Nung treats 365 drugs (Bottcher, 1965; Kelly, 2009; Kurhekar, 2021; Petrovska, 2012; Wiart, 2007).

As more antibiotics are rendered ineffective by drug-resistant bacteria, focus must be shifted towards alternative therapies for treating infections. Although several alternatives already exist in nature, the challenge is to implement them in clinical use. Advancements within biotechnology, genetic engineering, and synthetic chemistry have opened up new avenues towards the search for therapies that can substitute for antibiotics (Ghosh *et al*., 2019).

1.5.1 *Eucomis autumnalis*

E. autumnalis, also known as "pineapple flowers" or uMathunga, is a native flora of South Africa with medicinal value and known for its antibacterial and antifungal properties (Alaribe *et al*., 2018; Grace *et al*., 2003; Hutchings, 1996). The bulbs have been and are used in TMs to reduce fever and for UTIs, stomach pains, lower backaches, syphilis, as well as the induction of labour (Alaribe *et al*., 2018; Masondo *et al*., 2015; McMaster, 2007; Salachna & Zawadzińska, 2015). *E. autumnalis* belongs to the Asparagaceae family, a deciduous bulb plant with decorative raceme inflorescences. It is composed of star-shaped, greenish-white, and sweet-scented flowers. Produced in mid to late summer, the bulbs are 8-10 cm in diameter and ovoid in shape (**Figure 1.4**). The trilocular capsule fruit contains shiny, black rounded seeds, and its name *E. autumnalis* refers to its flowering and fruiting time (Alaribe *et al*., 2018; Masondo *et al*., 2014; Salachna & Zawadzińska, 2015; Taylor *et al*., 2002). They are a group of 11 species found in various habitats in Southern Africa (Eastern Cape, Northern Province, Zimbabwe, Botswana, and Malawi), including forests, grasslands, wetland areas (Alaribe *et al*., 2018; Shuttleworth & Johnson, 2009) and mountain slopes (Alaribe *et al*., 2018; Salachna & Zawadzińska, 2015).

A



B

Figure 1.4: *E. autumnalis* plant (A) and bulb (B). The bulbs were used in green synthesis of EABE-AgNPs (Adapted from Alaribe *et al*., 2018).

1.5.2 Medicinal uses of *Eucomis autumnalis*

E. autumnalis are significant medicinal and horticultural plants produced for commercial cultivation. The Zulu people in South Africa utilize *E. autumnalis* for gastrointestinal illnesses, backache, enemas, teething, inflammatory diseases, postoperative recovery, and other ailments (Alaribe *et al*., 2018). According to literature, its terpenoids are beneficial for treating various wounds, including burns, and its decoction aids in postoperative recovery (Hutchings, 1989, 1996; Van Wyk *et al*., 1997). *E. autumnalis* possess anti-inflammatory, antibacterial, anticancer, anti-oxidative, and anti-histaminic properties (Bisi-Johnson *et al*., 2011; Diederichs, 2012; Joffe, 2012; Koorbanally *et al*., 2006; Van Wyk *et al*., 1997). The bulbs contain various phytoconstituents such as homoisoflavanones and triterpenoid glycosides in the waxy layer between storage leaves, and terpenoids in the bulb's tissue, highlighting the importance of these compounds in plant growth (Alaribe *et al*., 2018; Bisi-Johnson *et al*., 2011; Masondo *et al*., 2015; McGaw *et al*., 2007; Mulholland *et al*., 2013). *E. autumnalis*, a traditional therapeutic plant extract, has been shown *in vitro* to selectively inhibit cyclooxygenase-2 (COX-2), making it an effective treatment for wound healing and post-surgery recovery (Masondo *et al*., 2015; Mulholland *et al*., 2013; Street, 2012; Taylor *et al*., 2002). This unique property makes it a valuable addition to traditional healing practices (Alaribe *et al*., 2018).

The number of WHO member states with state-level laws regarding herbal medicines has increased significantly, from 45 to 109 between 1999 and 2018 (WHO, 2019a, 2019b). This

trend is attributed to the increasing number of countries implementing regulations on herbal medicines, and ethnopharmacological analysis being a crucial step in developing new pharmaceuticals, owing to their therapeutic benefits (Ionkova *et al*., 2022; Mei *et al*., 2023; Panda *et al*., 2022). A study demonstrated that the antibacterial activity of the *E*. *autumnalis* can be enhanced through green nanotechnology, as *E*. *autumnalis* AgNPs was more effective against clinical isolates (*Listeria monocytogenes*, *Enterococcus faecalis*, *K*. *pneumoniae* and *A. baumannii*) compared to their extracts (Lediga *et al*., 2018). This suggests that green nanotechnology could enhance the efficacy of medicinal plants against IDs, particularly AMR pathogens (Moradi *et al*., 2023a).

1.6 Nanotechnology

Nanotechnology is an interdisciplinary field of science, that involves the conversion of nanoscience theory into practical applications through the manipulation, assembly, control, and manufacturing of matter at the nanometre scale (Abushaheen *et al*., 2020; Bayda *et al*., 2019). The nanometre scale is defined as the size range of objects that are one billionth (10^{-9}) of a meter in size with at least one dimension in the 1 to 100 nm range (Abbas *et al*., 2022; Pabbati *et al*., 2021; Yin *et al*., 2020). Nanomaterials (NMs) are 80,000 times smaller than the diameter of a human hair as shown in **Figure 1.5** (Anbusagar *et al*., 2018).

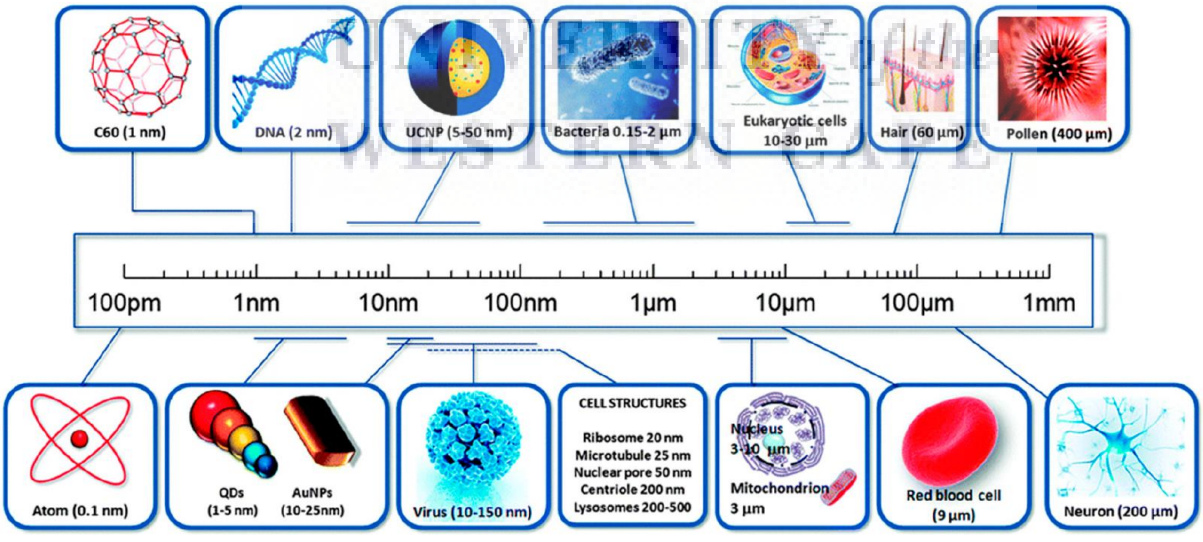


Figure 1.5: Schematic representation of materials in the nanoscale range (Adapted from Anbusagar *et al*., 2018).

The chemical structure, surface composition, and size of NPs contributes to their unique physiochemical properties and distinct applications (Bezza *et al*., 2020; Khan *et al*., 2019; Pabbati *et al*., 2021). These unique features are attributed to quantum confinement which is due to large surface-to-volume ratio (Patil & Burungale, 2020), in which quantum effects of nanomaterials can directly influence optical, thermal, electrical, mechanical, magnetic, and catalytic behaviour, compared to their bulk state counterparts (Ijaz *et al*., 2020; Oluwasanu *et al*., 2019). These properties can be utilized to overcome limitations faced by current therapeutic and diagnostic agents. The commercialization of nanomaterials in medicine and pharmaceuticals, including biomedical therapeutics is expanding rapidly and owns excellent potential (Kumar *et al*., 2023; Tiwari *et al*., 2023). In recent years, nanobiotechnology has emerged as an important branch of nanotechnology, focusing on the biosynthesis of NPs through innovative methods (Arroyo *et al*., 2020; Dhanjal *et al*., 2022).

NPs are categorized as either organic or inorganic based on their composition (Alshammari *et al*., 2023; Martinelli *et al*., 2019). Organic NPs are biodegradable and non-toxic. Examples include dendrimers, micelles, ferritin, and liposomes (Ealia & Saravanakumar, 2017; Rao & Geckeler, 2011). Inorganic NPs are synthesized using metal precursors (e.g., gold, silica, and iron oxide) (Ealia & Saravanakumar, 2017; Kumar *et al*., 2018). Inorganic NPs are further differentiated into noble MNPs such as, metal oxides or pure metals such as silver (Ag), aluminium (Al), gold (Au), copper (Cu), titanium (Ti), iron (Fe) and platinum (Pt) (Dhaka *et al*., 2023; Rosman *et al*., 2021). MNPs are widely utilized in biomedical applications due to their biocompatibility, low toxicity, excellent conductivity, and large surface area (Agarwal *et al*., 2019; Baygar *et al*., 2019; Chellapandian *et al*., 2019; Jebasingh & Arasu, 2020; Kang *et al*., 2020). The application of MNPs such as AgNPs, AuNPs, and CuNPs are different from non-metallic NPs, and have revolutionized medicine due to their antimicrobial, anticancer, drug delivery, contrast, and bioimaging properties (Aboyewa *et al*., 2021; Balamurugan *et al*., 2017). AgNPs are the most extensively studied and explored NPs among MNPs (Krishnanand & Sekhar, 2018; Rosman *et al*., 2021) in several industries, due to their antimicrobial properties (Akhtar, Naeem, *et al*., 2023; Ealia & Saravanakumar, 2017; Hong, 2019; Joudeh & Linke, 2022; Moreno-Vega *et al*., 2012; Nam *et al*., 2016; Rosman *et al*., 2021; Silva *et al*., 2019).

1.6.1 Silver nanoparticles (AgNPs) as antibacterial agents

Ag is very malleable and ductile, cost-effective and has the highest thermal and electrical conductivity (Krishnanand & Sekhar, 2018; Rosman *et al*., 2021). The usage of Ag dates back to ancient times where silver nitrate (lapis infernalis), a common silver salt has been utilized for medicinal purposes (Antonarakis & Emadi, 2010; Żyro *et al*., 2023), to prevent infections during wound healing because of their excellent antimicrobial properties (Konop *et al*., 2016; Medici *et al*., 2019; Nqakala *et al*., 2021; Żyro *et al*., 2023). Ag was used as jewellery, silverware, cutlery, preservative, and currency (Barillo & Marx, 2014; Talapko *et al*., 2018). In ancient times, Egyptians, Romans, Greeks and Phoenicians, utilized Ag as silverwares to store water, food, and wine to avoid spoilage, due to its antimicrobial properties (Barillo & Marx, 2014). The earliest medicinal use of Ag was recorded in 1500 BC, in China (Yamada, 1998). Silver nitrate ($AgNO_3$), first discovered in Rome in 69 BC, was used to prevent and treat wound infections (Calvery *et al*., 1941; Gao *et al*., 2018). Later in the 1800s, silvers antimicrobial properties have been used for over 200 years in treating burn injuries, eye infections (Barillo & Marx, 2014; Tarannum *et al*., 2019) and ulcers (Barillo & Marx, 2014; Pawlik *et al*., 2023). Ag wound dressings played a significant role in modern wound healing since the late 20th century (Ahmed *et al*., 2016; Barillo and Marx, 2014). Contrary to their historic oral administration, silver-based compounds are only effective externally (Żyro *et al*., 2023).

AgNPs, a rapidly researched nanomaterial, are in high demand in various industries such as pharmaceuticals, cosmetics, wound care, and food (Gherasim *et al*., 2020; Zhang *et al*., 2016). This is solely based on the fact that silver-based compounds and materials have been used for centuries due to their antimicrobial efficiency, versatility, and bio-functionality, leading to the development and clinical implementation of several commercial products (Gherasim *et al*., 2020).

1.6.2 Nanoparticle synthesis methods

Nanomaterials are synthesized using top-down and bottom-up approaches through various techniques like physical, chemical, hybrid (combination of the top-down and bottom-up approaches) and biological methods highlighted in **Figure 1.6** (Dhand *et al*., 2015; Rafique *et al*., 2019; Ramanathan *et al*., 2021). The top-down approach creates smaller structures from bulk material (Sudarman *et al*., 2023; Zamare *et al*., 2016) by using physical methods such as

mechanical machining, lithography, hydrothermal synthesis and thermal evaporation pyrolysis (Baptista *et al*., 2018; Khan *et al*., 2018; Sudarman *et al*., 2023).

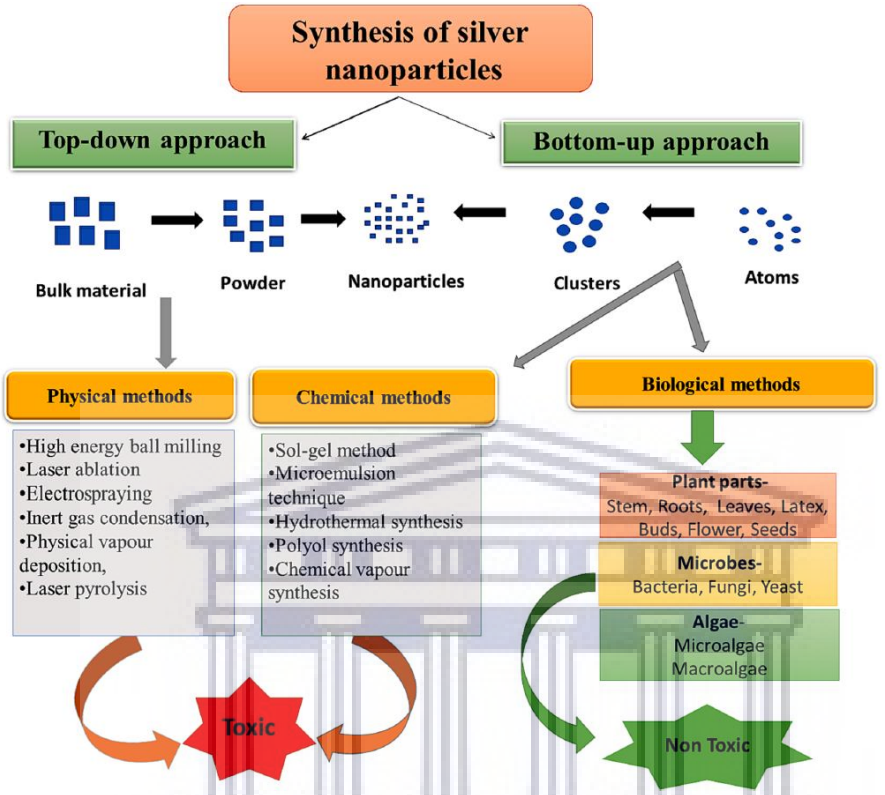


Figure 1.6: Various approaches and methods to nanoparticle synthesis (Adapted from Álvarez-Chimal & Arenas-Alatorre, 2023; Dhaka *et al*., 2023).

The bottom-up approach involves engineering large nanostructures from smaller atoms and molecules (Christian *et al*., 2008) using chemical reduction (Ahmed *et al*., 2016) and biological methods (Ramanathan *et al*., 2021). The bottom-up methods are simple, fast, and cost-effective, compared to the top-down methods. Furthermore, it has unique characteristics and excellent versatility such as the ability to control shape and size of the nanomaterial (Iqbal *et al*., 2012; Tripathy *et al*., 2023). Despite the advantages of chemical and physical methods, there are a few disadvantages such as high costs for equipment and operation, high energy consumption (Lin *et al*., 2021; Sudarman *et al*., 2023) and production of toxic by-products (Ramanathan *et al*., 2021). The production of NPs on a large scale is cost-effective, time-consuming, energy-intensive, and potentially harmful to the environment and living organisms due to its high temperatures and energy requirements (Anders, 2011; Bar *et al*., 2009; Bhagyanathan & Thoppil, 2018; Horwat *et al*., 2011; Madkour, 2017).

Chemical methods are preferred over physical methods; as chemicals can be used as reducing, capping and stabilizing agents to achieve AgNPs with excellent dispersion, stability and uniform size distribution (Díaz-Núñez *et al*., 2017). Due to the toxic nature of the chemicals, biological methods are emerging as an environmentally friendly, economical, and reliable alternative to both physical and chemical methods (Moradi *et al*., 2021; Rasheed *et al*., 2018; Tariq *et al*., 2022).

1.6.3 Biological synthesis methods

Green synthesis is a sustainable method for producing NPs, reducing the use of toxic solvents and hazardous chemicals. A developing area with substantial potential in environmental and biomedical fields (Khatiwara *et al*., 2023). This method utilizes organic substances as stabilizers and capping agents, influencing medical applications of AgNPs (Verma *et al*., 2022; Seerengaraj Vijayaram *et al*., 2023). It attempts to develop biocompatible NPs from biological resources such as plants and bacteria, as well as decreasing the utilization of toxic chemicals (Khatiwara *et al*., 2023). Biological synthesis of NPs can be achieved using a vast array of resources, including plant products, algae, fungi, yeast, bacteria, and viruses (**Figure 1.7**). The synthesis is initiated by mixing noble metal salt precursors (Ag, Au, Cu, Pt, Cd, Pt, Pd, Ru and Rh) with biomaterials as reducing and capping agents for the synthesis of MNPs. The biomaterials contain compounds such as proteins, alkaloids, flavonoids, reducing sugars, and polyphenols; all of which were involved in the reduction and stabilization of metal precursors. The reduction of these precursors to successive NPs can be confirmed by colour change of the colloidal solution (Sriramulu *et al*., 2020).

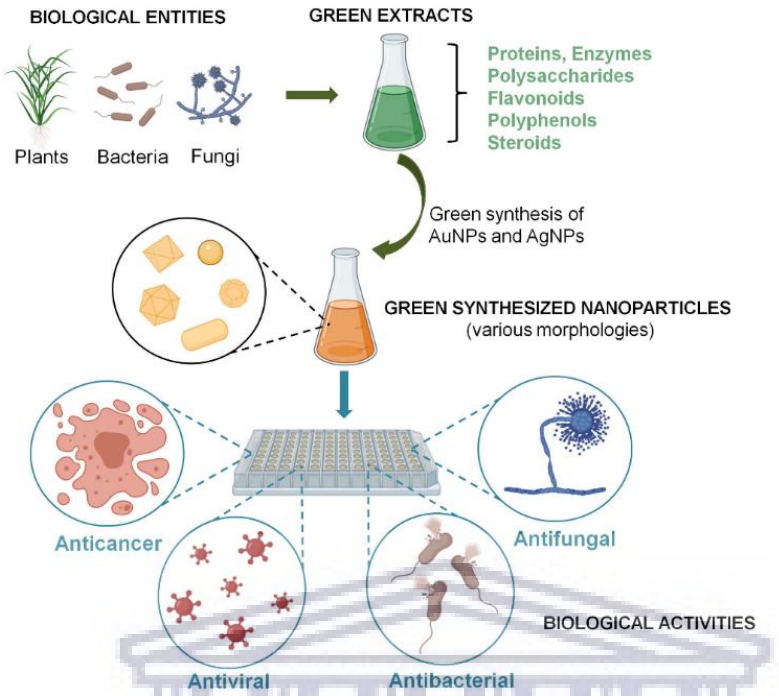


Figure 1.7: Biosynthesis of AgNPs utilising various natural biological resources (Adapted from Rónavári *et al*., 2021).

The synthesis of MNPs using biomaterials (microorganisms and plants), is widely recognized as a green and convenient approach (Singh *et al*., 2016; Verma *et al*., 2022). Microorganisms, ranging from prokaryotic simple bacterial cells to complex eukaryotes, utilize reductase enzymes to accumulate and detoxify heavy metals, converting metal salts into narrow-sized NPs with less polydispersity (Das *et al*., 2017; A. Dhaka *et al*., 2023; Singh *et al*., 2016).

Bacterial-mediated synthesis of AgNPs facilitated by *Pseudomonas stutzeri* (*P. stutzeri*), was first demonstrated in a silver mine (Ajaz *et al*., 2021; Klaus *et al*., 1999). *Pseudoduganella eburnean* (*P. eburnean*) mediated synthesized AgNPs demonstrated strong antibacterial efficacy against pathogenic microorganisms resistant to several drugs, including *S. aureus* and *P. aeruginosa* (A. Dhaka *et al*., 2023; Huq, 2020). In addition to bacteria, fungi have received increasing attention for their ability to synthesize NPs due to the ease of scale-up and downstream processing, and economic feasibility (Ottoni *et al*., 2017). Fungi (e.g., *Penicillium polonicum*, *Phomopsis liquidambaris* and *Trichoderma harzianum*) with unique metal bioreducing abilities, were used for biosynthesis of AgNPs (Ahmed *et al*., 2018). *Aspergillus brunneoviolaceus* (*A. brunneoviolaceus*), a marine-derived fungus, was used for synthesis of AgNPs with antibacterial and antioxidant properties. The biosynthesized AgNPs effectively inhibited both Gram-positive and Gram-negative bacteria and demonstrated potent radical

scavenging activity against 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Mistry *et al*., 2021). However, some fungi, like *Foxysporum*, are potentially pathogenic, posing health risks in subsequent future applications (Xu *et al*., 2020). Overall, microbial synthesis process is complex and involves numerous steps, such as isolation, culturing (time consuming) and maintenance, which is not feasible on an industrial scale (Dhaka *et al*., 2023; Ramrakhiani & Ghosh, 2018).

1.6.3.1 Green synthesis methods using plant extracts

Plants are safer, and biocompatible compared to microorganisms (Szczyglewska *et al*., 2023) and their use in the synthesis of plant-mediated MNPs will be cost-effective, renewable, ecofriendly and time-efficient (Ahmed *et al*., 2016; Ahmeda *et al*., 2020). Plant-mediated synthesis is a simple one-pot reaction that is easy to scale (Ahmed *et al*., 2016; Ahmeda *et al*., 2020). Plants have the potential to bioaccumulate heavy metals, enabling the transformation of metal ions into MNPs (Martínez Espinosa *et al*., 2020). This method has been used to synthesize MNPs from noble metals, metal oxides, and bimetallic alloys such as Cu, Ag, and Au (Ahmad *et al*., 2019). Alfalfa (*Medicago sativa*) sprouts were the first to synthesize AgNPs, and various plant species have been explored for MNPs production, examples include Au, Ag, Zn, Fe, Cu, and Pt. Plant extracts from aloe vera (*Aloe barbadensis* Miller), oat (*Avena sativa*), alfalfa, tulsi (*Osimum sanctum*), and lemon (*Citrus limon*), possess exemplary potential in reducing metal salt into NPs, such Ag-Au-Cu alloy NPs (Marchiol, 2012; Parveen *et al*., 2016; Singh *et al*., 2018).

The mechanism involved in the formation of AgNPs is displayed in **Figure 1.8**. In general, there are three phases in the production of MNPs from plant extracts: (i) activation stage: bioreduction and nucleation of metal ions/salts (Ag^0), (ii) Growth and coalescence phase: separation of small particles into large ones, creating clusters which continue to form until the particles are stable in shape and size, and (iii) termination phase: capping and stabilization of MNPs by plant biomolecules (Dias *et al*., 2021; Glusker *et al*., 1999). MNPs are reduced and stabilized by phytochemicals such as proteins, amino acids, polysaccharides, flavonoids, alkaloids, tannin, and polyphenols (Akintelu *et al*., 2021; Dawoud *et al*., 2021). These organic components contain a variety of functional groups, such as hydroxyl, carbonyl, and amidogen (Rengasamy *et al*., 2016; Xu *et al*., 2020), which can donate electrons to silver ions, resulting in the reduction of Ag^+ to Ag^o, thereby generating stabilized AgNPs (Alamier *et al*., 2022; Naseem *et al*., 2020; Vanlalveni *et al*., 2021; Vijayan *et al*., 2018). The plant-derived approach

offers a quicker and easier method for synthesis including the purification of MNPs compared to the microbial mediated approach (Martínez Espinosa *et al*., 2020; Salem & Fouda, 2021).

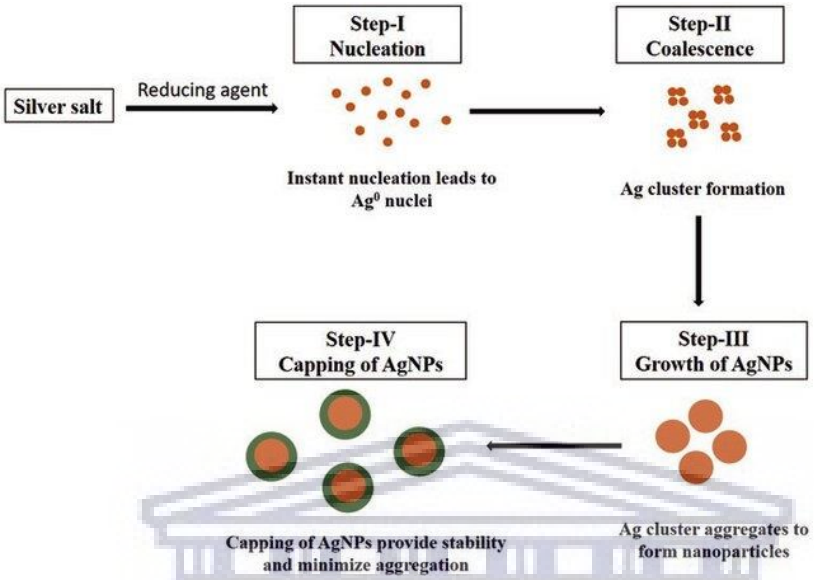


Figure 1.8: Steps involved in the formation of biogenic AgNPs (Adapted from Arya *et al*., 2019).

Synthesis of NPs based on biological precursors are influenced by reaction parameters such as solvent, temperature, pressure, pH, reaction duration, and reactant concentration (Seerengaraj Vijayaram *et al*., 2023; Zhang *et al*., 2020). Altering these parameters control the rate of NPs formation, and stability (Kaur *et al*., 2021; Yadav *et al*., 2015), resulting in production of MNPs with varying sizes and shapes (Jain *et al*., 2021). The adjustment and modification of MNPs shape and size enhances their functionality for a broad spectrum of applications (Kaur *et al*., 2021; Tsai *et al*., 2022). The shape, size, and surface composition of MNPs can significantly influence their entry into cells and their bio-application (Alsaiari *et al*., 2023). Previous studies have proven that MNPs synthesized using leaf extracts (*Ageratum houstonianum*, *Celastrus paniculatus*, *Hagenia abyssinica*, and *Jatropha curcas*) demonstrated photocatalytic, antifungal, and antibacterial properties (Chandraker *et al*., 2020; Ghosh *et al*., 2020; Kesharwani & Gajbhiye, 2023; Mali *et al*., 2020). Therefore, phytomedicine in combination with green nanotechnology can effectively eradicate AMR burden and improve bacterial infections by overcoming the limitations of conventional antibiotics (Choi *et al*., 2021).

1.7 Phytonanomedicine - Combatting Antibiotic-Resistant Bacteria using AgNPs

Plant-based MNPs have shown promising outcomes against life-threatening diseases like cancer, diabetes, neurodegenerative diseases, and cardiovascular diseases (Alabi & Badmus,

2023; Nampoothiri *et al*., 2023; Samrot *et al*., 2023). Biogenic MNPs are advantageous due to their enhanced bioactivity and biocompatibility (Kadu *et al*., 2023; Praveen *et al*., 2023), and have potential to overcome the limitations of traditional drug delivery systems and therapy (Kadu *et al*., 2023). Plants have long been used in TMs as antibacterial, anti-inflammatory, antipyretic, and analgesic agents; and many contemporary pharmaceuticals are derived from their secondary metabolites (Hamburger & Hostettmann, 1991; Hussein & El-Anssary, 2019; Teoh & Teoh, 2016). Plant-based natural medicines independently, and/or combined with conventional therapies, have the ability to alleviate the consequences of current therapies. These natural therapies are less expensive, widely available, safe, and have less side effects than conventional medicines, making them a viable alternative for disease therapy (Hoseinzadeh *et al*., 2014; Mirhosseini & Firouzabadi, 2015). The rise in synthetic compound has prompted the exploration and utilization of plant-based antimicrobial options (Fatima *et al*., 2023). Many studies have shown that plant extracts can be an alternative to chemical synthesis approach which requires toxic chemicals (Das *et al*., 2017; Dhaka *et al*., 2023; Lee & Jun, 2019).

Green nanotechnology continues to show considerable promise in this particular field, and many medicinal plants have reportedly been employed to produce MNPs with more favourable biological activity (Ahmed *et al*., 2016; Katas *et al*., 2019). The effectiveness of antimicrobial activity of MNPs are significantly influenced by the particle size (Adibkia *et al*., 2010; Buzea *et al*., 2007). Independent studies demonstrated that because of their small size, NPs may be rapidly absorbed into cells and organelles. They can readily pass through cellular barriers, including the placenta and the blood-brain barrier (Mody *et al*., 2009; Trickler *et al*., 2010). The use of combination therapy with MNPs has the potential to eradicate the emergence of bacterial resistance to multiple antibacterial agents (Hoseinzadeh *et al*., 2014; Mirhosseini & Firouzabadi, 2015).

Plant-synthesized MNPs, may have the potential to inhibit various resistant microbial species including both gram-negative (*E. coli*, *P. aeruginosa*, and *K. pneumoniae*) and gram-positive (*S. aureus* and *B. subtilis*) bacteria (Al Jahdaly *et al*., 2021; Y.-G. Yuan *et al*., 2017). Ag, Au, Pd, and Se NPs synthesized with diverse plant extracts (*Rosmarinus officinalis* leaf, *Moringa oleifera* petals, *Rosa hybrida* petal, Banana peel, *Asparagus racemosus* root, *Ocimum tenuiflorum* leaf) displayed significant antibacterial and antifungal activity against various bacteria and fungi (P. Dikshit *et al*., 2021; Rabiee *et al*., 2020). AgNPs are by far the most

widely studied antimicrobial agents. AgNPs synthesized from *Cymbopogan citratus* (lemon grass) leaf extract with sizes ranging from 15 to 65 nm, had a high antibacterial efficacy against *K. pneumoniae* (22 mm), *Shigella somenesis* (*S. somenesis*), *P. aeruginosa*, *E. coli*, *Proteus mirabilis* and *Shigella flexaneri* (*S. flexaneri*) (Geetha *et al*., 2014; Masurkar *et al*., 2011). *Phyllanthus pinnatus* (*P. pinnatus*) AgNPs demonstrated significant antibacterial activity against *Vibrio cholerae* and *S. flexneri* (Balachandar *et al*., 2019). There are numerous studies on MNPs synthesized using indigenous South African plants (e.g., *Salvia africana-lutea, Sutherlandia frutescens, Galenia africana*) that exhibited high antibacterial activities compared to their respective plant extracts (Aboyewa *et al*., 2021; Dube *et al*., 2020; Elbagory *et al*., 2016).

These findings suggested that the antibacterial activity of NPs is significantly influenced by their size. Numerous other studies have proven that NPs capacity to penetrate bacteria increases with decreasing NPs size (Bruna *et al*., 2021; Loo *et al*., 2018; P. R. More *et al*., 2023; Yin *et al*., 2020).

1.7.1 Biomedical applications of AgNPs

Historically, Ag was known as a broad-spectrum antimicrobial agent, which pilot to its use in many medical fields (Kumar & Yadav, 2009; Parak *et al*., 2003; Sotiriou & Pratsinis, 2011), as catalytic, anti-viral, anti-bacterial, and anti-fungal agents (P. K. Dikshit *et al*., 2021; Goyal *et al*., 2019). Although still in use today, Ag have limitations such as high cost, skin discoloration, nervous system disturbance, pneumothorax, and partial healing (Mihai *et al*., 2019). MNPs pose a significant threat to human health and the environment due to their potential cytotoxic effects. These NPs can accumulate in various organs, including the liver, lungs, spleen, and kidneys (Luceri *et al*., 2023; Zhang *et al*., 2014). Ag, in its ionic form, is more toxic than NPs, possibly due to different biokinetics. AgNPs and ions exhibit similar toxicity patterns, with AgNPs primarily mediated by released ions (Hadrup *et al*., 2020; Hyun *et al*., 2008; Mathur *et al*., 2018). The toxic effects depend on time of exposure, dose (Luther *et al*., 2011), NP size (Liu *et al*., 2010), shape (Liu *et al*., 2010), and surface coating present (Ahamed *et al*., 2008; Luceri *et al*., 2023; Stoehr *et al*., 2011).

In recent years, properties of Ag can be made selective by reducing their size to nanoscale. This size allows them to pass through various barriers, enhancing their pharmacological activities (Elsaesser & Howard, 2012) and higher activity compared to pure silver metal (Ali

et al., 2022; Qamer *et al*., 2021). These properties can overcome drug resistance in pathogenic bacteria (Jiang & Pinchuk, 2015). AgNPs were reported to have bactericidal effect on 650 pathogens, without promoting resistance mechanisms (Talapko *et al*., 2018; Wang *et al*., 2017).

The main applications of AgNPs in medicine are in diagnostics and therapy (Mathur *et al*., 2018). Clinical trials are underway for over 26 nanovaccine candidates, with 60 more in preclinical advance (Vu *et al*., 2021). NPs are being used in COVID-19 diagnostic testing to enhance medication transfer efficiency and eliminate waiting periods (Chowdhury *et al*., 2021). This technology may prove beneficial in time-sensitive COVID-19 testing (Kusumoputro *et al*., 2020; Norouzi *et al*., 2019; Yasamineh *et al*., 2022). According to literature, ESKAPE pathogens, including Gram-negative *P. aeruginosa*, are causing a growing need for novel antibiotic alternatives. Nanotechnology has enabled researchers to use AgNPs as antibacterial agents, as demonstrated by Bankalgi *et al*. (2016), which showed strong antibacterial effects against Gram-negative *P. aeruginosa* and *Enterobacter aerogenes* (Bankalgi *et al*., 2016; Jiang *et al*., 2018; Klasen, 2000a, 2000b). Ag is utilized in health sector facilities and agriculture for disinfection, while AgNPs are utilized in orthopaedics, dentistry, intelligent food packaging, and textiles as coatings, additives, including textiles (Bakis *et al*., 2002; Butola, 2018; Deshmukh *et al*., 2019; E. A. Kukushkina *et al*., 2021; Tjong, 2006).

Fennel's green-synthesized AgNPs exhibited potent antibacterial properties against MRSA clinical isolates (Fatima *et al*., 2023). Their smaller size allows for easier penetration through cell membranes, altering bacterial cell processes. AgNPs with diameters ranging from 38 to 72 nm and 17 to 29 nm can be synthesized using *Chrysanthemum indicum* or *Acacia leucophloea* extract, with excellent antibacterial activity (Arokiyaraj *et al*., 2014; Murugan *et al*., 2014). Both samples had excellent antibacterial effects. Similarly, *Ganoderma neojaponicum Imazeki* AgNPs showed potential in chemotherapeutics against breast cancer cells (Gurunathan *et al*., 2014; Qamer *et al*., 2021).

A study reported, biogenic synthesis using *Origanum majorana* (*O. majorana*) leaf extract (Yassin *et al*., 2022a). AgNPs with an average particle size of 26.63 nm demonstrated antibacterial efficacy against MDR bacterial strains (Yassin *et al*., 2022b). Earlier, another study published in 2018 by Shaik *et al*. confirmed the green synthesis of AgNPs, demonstrating antibacterial activity against both Gram-negative (e.g., *E. coli*, *P. aeruginosa*, *S. typhi*, and *S. sonnei*) and Gram-positive (e.g., *M. luteus*, *S. epidermidis*, MRSA and *S. aureus*) bacterial strains and pathogenic fungi (*A. flavus*, *A. alternate*, *P. alba*, and *P. variotii*). The bacterial

strains had inhibition zones ranging from 12 to 19 mm in diameter (Shaik *et al*., 2018; Yassin *et al*., 2022b). These NPs effectively coated hospital and critical care unit surfaces, limiting the level of MDR nosocomial bacterial infection, demonstrating their potential in antimicrobial applications (Alsaiari *et al*., 2023).

Biomedical research utilizes green-synthesized NPs for drug delivery, bioimaging, biosensors, and biomolecular recognition. These bioactive materials can be incorporated into everyday products like humidifiers, water purification systems, deodorant, toothpaste, and cosmetics (Alsaiari *et al*., 2023). Numerous independent studies have reported the application of MNPs, particularly Ag, Au, Co, and Zn, as wound healing, tissue treatment, immunotherapy, regenerative medicine, dentistry, antibacterial, anticancer (e.g., chemotherapy), drug delivery, contrast, and bioimaging agents has revolutionized medicine (Gupta *et al*., 2023; Pandit *et al*., 2022; Sibuyi *et al*., 2021).

The recent advances in the field of green synthesis have inspired scientists and researchers to explore its potential against pathogenic microbes (Nisar *et al*., 2019). These properties include improved bioavailability, solubility, toxicity safeguard, and increased drug stability, making them a highly researched domain (Zoroddu *et al*., 2014). Therefore, it is necessary to create innovative, green synthetic antimicrobial agents that are efficient against bacteria that are resistant to antibiotics, inexpensive, non-toxic to normal cells with minimal resistance in AMR. Recent studies have shown AgNPs, synthesized via green processes, have gained popularity in the biomedical field for healing skin wounds due to their versatile activity against pathogenic bacteria and minimal toxicity towards mammalian cells (Haghniaz *et al*., 2021; Pangli *et al*., 2021).

Nanosilver-based materials such as dressings, antimicrobial gel formulations, medical catheters, instruments, implants, contact lens coatings; are used in orthopaedics and wound healing to prevent microbial colonization (Nandhini *et al*., 2023; Pangli *et al*., 2021). Moreover, surface modification is easily achievable, and modified for targeted drug delivery and high antibacterial efficacy, making them ideal for various applications (Jana & Pal, 2007; Nandhini *et al*., 2023). Several clinical trials on AgNPs-based products exist for various applications, including dental applications (Ahmed *et al*., 2022; Simon *et al*., 2022).

1.8 Mechanisms of antibacterial effects of silver nanoparticles (AgNPs)

The bacteria may develop resistance towards antibiotics following several mechanisms, as discussed in section 1.3.1 (Chiș *et al*., 2022; Uruén *et al*., 2020). Gram-positive bacteria exhibit intrinsic resistance through: (i) restrictive drug uptake, (ii) inactivation of drug, (iii) active drug efflux, and (iv) reactive oxygen species (ROS) production. While acquired resistance may involve (v) modification of the drug target of the microbial cell, inactivation of drug molecule, and drug efflux pump (Blair *et al*., 2014; Rehman, 2023). Gram-negative bacteria may opt for all four main mechanisms, while Gram-positive bacteria mostly adopt restrictive drug uptake due to their lack of the peptidoglycan layers, which causes a barrier to various drug molecules resulting in innate drug resistance (Chancey *et al*., 2012; Rehman, 2023).

AgNPs have previously been extensively researched as novel antimicrobial agents and exhibited broad-spectrum antibacterial action against multiple Gram-positive and Gram-negative bacteria (Cavassin *et al*., 2015; Ekaterina A Kukushkina *et al*., 2021). Surface charge and size play a significant role in their bactericidal effects. AgNPs with a positive charge are most potent against all bacterial species (Abbaszadegan *et al*., 2015), with smaller AgNPs in the 1 to 10 nm range showing increased activities (Dakal *et al*., 2016; Talapko *et al*., 2020).

The antibacterial mechanisms of action of AgNPs are still not clear although a number of potential interactions between AgNPs and pathogens have been proposed (E. A. Kukushkina *et al*., 2021). The four main actions of AgNPs are displayed in **Figure 1.9**, (1) AgNPs adhere to a cell surface via interactions with thiolated molecules, damaging its membrane and allowing their uptake (Prabhu & Poulose, 2012), (2) AgNPs penetrate the cell, interacting with various cellular components, altering organelles and cellular function, (3) they generate ROS, inducing cellular damage and genotoxicity, (4) release of Ag^+ induce the genotoxicity and disrupt cellular signals (Prabhu & Poulose, 2012). Previous studies have implicated Ag^+ as the primary cause of AgNPs antibacterial activity. Ag^+ interact with DNA and peptides by binding to amines, phosphates, and thiols (Le Ouay & Stellacci, 2015; Lee & Jun, 2019; Menichetti *et al*., 2023).

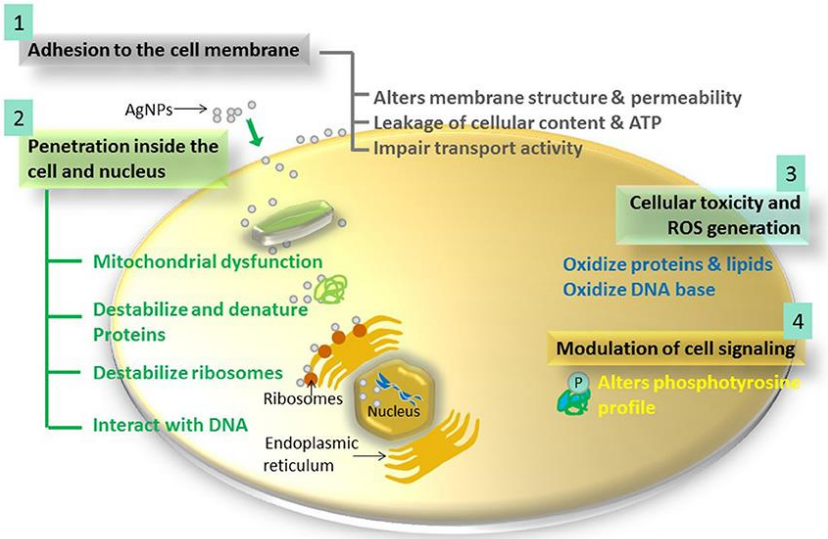


Figure 1.9: The proposed mechanisms of action of AgNPs against bacteria (Adapted from Dakal *et al*., 2016).

The bacterial envelope is disrupted when Ag^{+} attach to the cell wall or cytoplasmic membrane, thus enhancing the permeability of the cell. When free Ag^{+} are taken up by cells, they deactivate respiratory enzymes, generating ROS and interrupting adenosine triphosphate (ATP) production (Arya *et al*., 2023; P. R. More *et al*., 2023; Pragati Rajendra More *et al*., 2023). ROS is the principal species that provokes the activity of DNA modification and cell membrane disruption. Interaction of AgNPs with sulfur and phosphorus containing biomolecules alters DNA replication, cell reproduction and inhibition of protein synthesis resulting in bacterial death (Dakal *et al*., 2016; Pragati Rajendra More *et al*., 2023). This suggests that AgNPs are likely to have unique targets or ability to override the mechanisms that promote the MDR (Lee, 2019; Prasher *et al*., 2018; Y. G. Yuan *et al*., 2017).

AgNPs can inhibit and/or kill a variety of microorganisms following different mechanisms to conventional therapy, and a potential to escape microbial resistance and treat biofilm (Essghaier, Ben Khedher, *et al*., 2022; Essghaier, Toukabri, *et al*., 2022; Gomaa, 2017; Ravindran *et al*., 2018; Yener *et al*., 2020). AgNPs inhibited *P. aeruginosa* biofilm formation and showed bactericidal effects on existing biofilm further confirming that these NPs may find application in the prevention and treatment of biofilm-related infections, as well as ESKAPE pathogens (Talapko *et al*., 2020). Plant-synthesized AgNPs can be an alternative approach to combat AMR, offering a cost-effective and environmentally safe option to circumvent limitations of current treatments (Essghaier, Ben Khedher, *et al*., 2022;

Prabhakar & Doble, 2011). Natural agents have shown potential in synthesizing NPs with antibacterial activity against AMR pathogens and bacterial biofilms (Moradi *et al*., 2023b). This motivated the choice of plant species (*Eucomis autumnalis*) used in this study.

1.9 Aim and objectives

The aim of this study was to synthesize biogenic AgNPs using aqueous *E. autumnalis* bulb extract and investigate their antibacterial activity.

The objectives of this research were to:

1. Prepare an aqueous extract from *E. autumnalis* bulb
2. Optimize the reaction conditions and evaluate the effect of the pH, temperature, extract concentration, $AgNO_3$ concentration, and reaction time on the synthesis of AgNPs using the aqueous bulb extract
3. Synthesize AgNPs using all the optimized conditions for EABE-AgNPs synthesis
4. Characterize the biogenic EABE-AgNPs
5. Determine the stability of the EABE-AgNPs in various media
6. Assess the antibacterial activity of the EABE-AgNPs and EABE against ESKAPE pathogens

1.10 Hypothesis

Eucomis autumnalis possess phytochemicals that can reduce, stabilize, and produce AgNPs with antibacterial properties.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials – Reagents, Equipment, and Suppliers

Table 2.1: Materials and reagents used and their suppliers.

Materials and reagents used	Supplier	Company location
AlamarBlue™ cell viability reagent	Thermo Fisher Scientific	Massachusetts, United States of America (USA)
Ciprofloxacin	Sigma-Aldrich	Missouri, USA
Conical tubes (15 ml and 50 ml)	SPL Life Sciences	Kyonggi-do, South Korea
Disposable cuvette (DTS0012)	Malvern Instruments	Worcestershire, United Kingdom (UK)
Disposable folded capillary cell (DTS1070)	Malvern Instruments	Worcestershire, UK
Hydrochloric acid (HCl)	Merck	New Jersey, USA
Millipore 0.45-micron filter paper	Sigma-Aldrich	Missouri, USA
Millipore Ultra-purified distilled water (18.2 MΩ cm at 25 °C)	Thermo Fisher Scientific	Massachusetts, USA
Mueller Hinton agar	Sigma-Aldrich	Missouri, USA
Mueller Hinton broth	Sigma-Aldrich	Missouri, USA
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich	Missouri, USA
Nitric acid (HNO_3)	Kimix	Cape Town, Republic of South Africa (RSA)
Polystyrene 96-well microtiter™ plates	Greiner Bio-One (Lasec)	Cape Town, RSA
Silver nitrate ($AgNO_3$)	Sigma-Aldrich	Missouri, USA
Sodium hydroxide (NaOH)	Sigma-Aldrich	Missouri, USA
Sterile cotton swabs	Lasec	Cape Town, RSA
Sterile loops	Lasec	Cape Town, RSA
Whatman No. 1 filter paper	Sigma-Aldrich	Missouri, USA

Table 2.2: Equipment used and their suppliers.

Equipment	Supplier	Company location
Analytical weighing balance	Ohaus Adventurer	New Jersey, USA
Blender	Panasonic	Osaka, Japan
Centrifuge 5415D	Eppendorf	Hamburg, Germany
High-Resolution Transmission Electron Microscope (FEI Tecnai G2 20 FEG)	Thermo Fisher Scientific	Massachusetts, USA
IncoTherm Oven	Labotec	Cape Town, RSA
Incubator	Thermo Fisher Scientific	Massachusetts, USA
Fourier-Transform Infrared Spectrophotometer Perkin Elmer Spectrum 400	Perkin Elmer	Waltham, USA
Laminar flow hood	Thermo Fisher Scientific	Massachusetts, USA
pH meter – Crison Basic 20	Lasec	Cape Town, RSA
POLARstar Omega Plate Reader	BMG Labtech	Ortenberg, Germany
Sorvall Lynx 6000 Centrifuge	Thermo Fisher Scientific	Massachusetts, USA
Stuart Heat-Stir CB162 Hot Plate	Lasec	Cape Town, RSA
Thermomixer Comfort	Eppendorf	Hamburg, Germany
Varian 710-ES Inductively Coupled Plasma Optical Emission Spectrometer	Varian	California, USA
Zetasizer – Nano-ZS90 System	Malvern Instruments	Worcestershire, UK

Table 2.3: Bacterial strains used in this study and their suppliers.

Bacterial strains	Gram reaction	ATCC number
Staphylococcus aureus	Gram-positive	25923
Methicillin-resistant *Staphylococcus aureus*	Gram-positive	33591
Streptococcus pyogenes	Gram-positive	19615
Escherichia coli	Gram negative	35218
Klebsiella pneumoniae	Gram negative	27853
Pseudomonas aeruginosa	Gram negative	13883
Acinetobacter baumannii	Gram negative	19606

2.2 Research Methodology

2.2.1 Preparation of aqueous extract of *E. autumnalis*

E. autumnalis bulbs were collected by Prof. Abram Madiehe in Kuilsriver, Cape Town, SA. The bulbs were peeled, then cleaned to remove soil and other impurities. The bulbs were cut into small pieces, using a sterile blade. The plant material was dried in an oven for 24 hrs at 70 °C. The dry plant material was homogenized using a blender (Panasonic, Osaka, Japan). Five grams (5g) of the plant powder was added to 100 ml of boiling distilled deionized water (ddH_2O) and stirred for 18 hrs at room temperature (RT). The mixture was centrifuged at 9 000 revolutions per minute (rpm) for 10 mins at RT. The supernatant was vacuum filtered using a Whatman No. 1 filter paper and further micro-filtered using Millipore 0.45 µm filter paper. The *E. autumnalis* aqueous bulb extract (EABE) was stored at − 20 °C in aliquots for further use.

2.2.2 Optimization and synthesis of EABE-AgNPs

EABE and 1 mM $AgNO_3$ were mixed in a volume ratio of 1:10 (v/v) in a final reaction volume of 400 µl and placed in an Eppendorf thermomixer for the optimization of synthesis conditions. The original pH of EABE was 5.2. The influence of various reaction parameters were investigated namely, pH, temperature, the concentration of EABE, and the concentration of $AgNO_3$, on the synthesis of EABE-AgNPs. These parameters were altered for each set of reactions, and the optimum conditions for EABE-AgNPs synthesis were determined. The experiments were performed in triplicate.

2.2.2.1 Effect of pH vs. temperature on EABE-AgNPs synthesis

A stock of 1 % EABE was prepared in sterile ddH_2O and adjusted to a range of pH values (pH 8, 9, 10, 11 and 12). First, the biological pH (unpH) of the EABE was determined (pH 5.2), then the pH was adjusted with 0.1 M NaOH. The final concentration of all newly prepared samples was, 0.5% EABE. The EABE samples were centrifuged at 13 200 rpm for 10 min and the pellet was discarded. The supernatants were used in the synthesis of the EABE-AgNPs. Subsequently, 40 µl of 1% EABE concentration at the various pH values were mixed with 360 µl of 1 mM $AgNO_3$ in 2 ml tubes. The samples were placed in an Eppendorf thermomixer comfort, shaking at 750 rpm for 1 hr (exposure time) at 100, 90, 80 and 70, expect at 25 °C, no shaking. After

synthesis at each temperature, the EABE-AgNPs were cooled to RT and centrifuged for 15 min at 14 200 rpm. The supernatants were removed, and the pellets were resuspended in 400 µl of sterile ddH_2O. The experiments were performed in triplicate.

2.2.2.2 Effect of EABE concentration on EABE-AgNPs synthesis

Various extract concentrations ranging from 0.5 – 3% of the EABE for each sample were used for AgNPs synthesis. Forty microlitres (40 µl) EABE of each concentration, at pH 12, were mixed with 360 µl of 1 mM $AgNO_3$. The synthesis was conducted as before for 1 hr at 90 °C.

2.2.2.3 Effect of $AgNO_3$ concentration on EABE-AgNPs synthesis

Up until this point, all AgNPs were synthesized using 1mM $AgNO_3$. Forty microlitres (40 µl) of EABE (0.5 %, pH 12) and various concentrations of $AgNO_3$ (0.5 1, 2 and 3 mM) were prepared in a volume ratio of 1:10 (v/v) in a final reaction volume of 400 µl. The samples were placed in an Eppendorf thermomixer comfort and subjected to similar synthesis parameters using optimised, shaking at 750 rpm for 1 hr at 90 °C.

2.2.2.4 Effect of EABE vs $AgNO_3$ volume ratios on EABE-AgNPs synthesis

Up until this point, all AgNPs were synthesized in a volume ratio of 1:10 (v/v), in a final reaction volume of 400 µl. Synthesis was performed with various volume ratios [1:1; 1:4; 1:7 and 1:10 (v/v)] using optimised pH, temperature, concentrations of precursor and extracts.

2.2.2.5 Effect of reaction time on EABE-AgNPs synthesis

EABE-AgNPs synthesis was carried out using the following optimum conditions: pH 12 extract, 90 °C with 750 rpm shaking, using 0.5% EABE (2mM $AgNO_3$) and 1:7 (v/v) ratio. The samples were placed in an Eppendorf thermomixer comfort and UV-vis spectra were measured after 0, 1, 2.5, 5, 10, 15, 20, 30, 60, 90, 120, 150, 180, 240, 300, 360, 720 and 1440 min.

2.2.2.6 Upscaling of EABE-AgNPs synthesis using optimal conditions

The EABE-AgNPs synthesis was upscaled to a final volume of 50 ml, briefly: 7.14 ml of 2mM $AgNO_3$ was heated to 90 °C then 42.86 ml of 0.5% EABE (pH 12) was added, and the reaction was allowed to continue for 90 min at 750 rpm. After synthesis, the EABE-AgNPs samples were washed in ddH_2O and recovered by centrifugation at 14 200 rpm for 15 min. The pellet was

resuspended in sterile ddH_2O to a final volume of 50 ml. This experiment was performed in triplicate.

2.2.3 Characterization of EABE-AgNPs synthesis

2.2.3.1 Ultraviolet-visible spectroscopy (UV-vis)

Ultraviolet-visible spectroscopy (UV-vis) spectroscopy was used to determine the optical properties of the biogenic AgNPs, by observing the Surface Plasmon Resonance (SPR) of the synthesized EABE-AgNPs. The EABE-AgNPs were diluted in a 1:10 (v/v) ratio with sterile ddH_2O in a final volume of 300 µl in a 96 well plate, the absorbance spectra were measured on a POLAR star Omega microplate reader at a wavelength (λ) range of 300 to 800 nm. The generated data was analysed using Microsoft Excel software.

2.2.3.2 Dynamic Light Scattering analysis (DLS)

EABE-AgNPs were characterized using Malvern Zetasizer Nano-ZS90 System to measure the particle hydrodynamic size, size distribution, polydispersity index (PDI) and zeta potential (ζ-potential) values at RT. To determine the hydrodynamic size and PDI, 1 ml of EABE-AgNPs were diluted 1:10 (v/v) in ddH_2O and transferred into a 10 mm optical density square polystyrene cuvette. Three measurements were read per samples and averaged to obtain the mean hydrodynamic size of the NPs. For ζ-potential measurements, 0.7 ml of the AgNPs was transferred into a disposable folded capillary DTS1070 cell, and then analysed at a voltage of 4 mV at RT at 90^o angle. The ζ-potential was determined in triplicate.

2.2.3.3 High-Resolution Transmission Electron Microscopy analysis (HR-TEM)

HR-TEM was used to characterize the morphology and core size of EABE-AgNPs. The analysis was performed at the University of Cape Town (UCT). The sample was diluted with ddH_2O at a 1:10 ratio and one drop of the sample was placed onto a carbon-coated copper grid. Samples were then allowed to dry under a xenon lamp for 10 min before being analysed and viewed using an FEI Tecnai G2 20 field-emission gun HR-TEM microscope operated in bright field mode at an accelerating voltage of 200 kV. The particle size distribution was determined using Image J software (Version 1.54g, National Institute of Health, USA).

2.2.3.4 Fourier-transform infrared spectroscopy (FTIR)

The functional groups found in the EABE-AgNPs were determined using FTIR. The EABE and EABE-AgNPs were dried and taken for FTIR analysis at the Department of Chemical Science, (University of the Western Cape). The EABE-AgNPs were placed at 90 ºC for 1 hr, then allowed to cool at RT. The EABE-AgNPs were centrifuged at 14 200 rpm (RT) for 15 min. The AgNPs were air dried. Potassium bromide (KBr) powder was used for background correction. The EABE and EABE-AgNPs powders were individually mixed and ground with KBr in a pestle and mortar. The resultant powdered mixtures were pressed into a pellet and analysed using a Perkin Elmer Spectrum 400 FTIR spectrometer. The spectra were collected in the 4 000 – 400 cm^{-1} at 2 cm^{-1} resolution. The measurements were done using attenuated total reflectance (ATR) accessory. The data was analysed using OriginLab 8 Pro 2023b (Northampton, USA) software.

2.2.3.5 Induced coupled plasma optical emission spectrometry (ICP-OES)

Induced coupled plasma optical emission spectrometry (ICP-OES) (Agilent 5900 ICP-OES), was used to quantify the concentration of EABE-AgNPs. The Ag content in EABE-AgNPs was determined by ICP-OES following HCl/nitric acid digestion. EABE-AgNPs (1 ml) were centrifuged at 13 200 rpm for 15 mins at RT. The concentrated pellet was transferred to a clean glass vial, then lysed with 2 ml of aqua regia (3 HCl:1 HNO_3) solution. The sample was digested by incubation at 90 °C for 90 min. After incubation, the samples were then diluted with 2% HCl to a final volume of 10 ml. These samples were then analysed for total silver content by a Varian 710-ES ICP-OES (Varian) at the Chemistry Department (University of the Western Cape). The experiments were performed in triplicate, the values reported were based on a calibration curve using a silver ICP standard (Sigma- Aldrich). The concentration of silver in the sample was calculated using the below formula:

$$Concentration\ of\ silver\ (\mu g/ml) = Amount\ of\ silver\ detected \times dilution\ factor$$

2.2.3.6 Stability analysis of EABE-AgNPs

The stability of the EABE-AgNPs were evaluated in various physiological mediums, namely Mueller Hinton broth (MHB), ddH_2O, and dH_2O. Briefly, 1ml of EABE-AgNPs were mixed with ddH_2O, dH_2O and MHB [1:1 (v/v)] respectively, in a final volume of 2 ml. The tubes were incubated at 37 °C, 100 µl aliquots were added in a 96-well plate at 0, 1, 3, 6, 24 and 48 hours.

The stability of the EABE-AgNPs was determined by observing any changes (SPR wavelength) in the UV-vis spectra (POLARstar Omega microplate reader, BMG Labtech, Germany) measurements in a range of 300-900 nm. The experiment was performed in triplicate.

2.2.4 Antibacterial activity of EABE-AgNPs

The antibacterial activity of the synthesized EABE-AgNPs was evaluated by using agar well diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Seven bacterial strains (**Table 2.3**) were used for this study. All the bacterial strains used in this study were purchased from American Type Culture Collection (ATCC) (Virginia, USA) and used as representative bacterial strains for the testing of the antibacterial activity of the EABE-AgNPs.

2.2.4.1 Bacterial culture

Individual bacterial strains were cultured on Mueller Hinton agar (MHA) plates and incubated at 37 °C for 24 hrs. Single colonies from the individual bacterial culture were inoculated in 2 ml of MHB mixed well and incubated for 2 hours at 37 °C in a shaking Thermo Fisher Scientific incubator (Massachusetts, USA) at 200 rpm. Following incubation, the bacterial suspensions were cultured until they read an absorbance unit (a.u) or optical density (OD) of 0.08 - 0.12 at a wavelength of 600 nm and diluted 1:150 in fresh MHB which is equivalent to 0.5 McFarland turbidity standard (~ 1.5×10^8 CFU/ml).

2.2.4.2 Determination of antibacterial activity using agar well diffusion method

The antibacterial activity of the EABE and EABE-AgNPs was studied against various Gram-positive and Gram-negative bacterial strains (**Table 2.3**), on MHA plates using the agar well-diffusion assay according to the method of (Dhand *et al*., 2015). The assay was used to evaluate the inhibition of bacterial growth by the EABE-AgNPs. The bacterial suspensions were adjusted to 0.5 MacFarland, using a sterile cotton swab, the cultures were uniformly spread onto MHA plates. Subsequently, seven wells (6 mm in diameter) were punched into the MHA plates using a sterile P200 yellow tip. The wells were then filled with 50 µl of five different concentrations of EABE-AgNPs. For the negative control, 50 µl of MHB was added to the well. While Ciprofloxacin (standard antibiotic) was used as a positive control, 50 µl of 15 µg/ml Ciprofloxacin was used for all strains, except for *E. coli* where 10 µg/ml Ciprofloxacin was used. The MHA plates were allowed to dry for 1 hr before being inverted, then incubated at 37 °C for

24 hours. The zone of inhibition (ZOI) was measured using a Vernier caliper. Antimicrobial effects of EABE and EABE-AgNPs were indicated by the formation of a clear zone around the wells. The experiment was performed in triplicate.

2.2.4.3 Microdilution assay for minimum inhibitory concentration and minimum bactericidal concentration (MIC)

The MIC, defined as the lowest concentration of treatments required to inhibit the visible growth, was evaluated by microdilution assay using the resazurin microtiter assay (Serker *et al*., 2020). The bacterial suspensions of the strains were adjusted to a 0.5 MacFarland turbidity standard and diluted in a 1:150 (v/v) ratio with MHB as described in **2.2.4.1**. Fifty microliters (50 µl) of the bacterial suspensions were added to a Greiner 96-well plate containing 50 µl of treatments (EABE, EABE-AgNPs and Ciprofloxacin, serially diluted in MHB) into 64.5, 32.3, 16.1, 8.0, 4.0 2.0, 1.0 and 0.5 µg/ml for EABE-AgNPs; 5 – 0.03% for EABE; and 15 – 0.01 µg/ml Ciprofloxacin. All treatments were done in triplicate. For the negative control, 50 µl of MHB was added to the well. The plates were incubated at 37 °C for 24 hrs. Subsequently, 10 µl of AlamarBlue™ dye (resazurin) was added in each well, covered in foil and incubated for 4 hours. The MIC was measured by spectrophotometry at 570 nm and a fluorescence excitation/emission wavelength of 530-560/590 nm. The experiment was performed in triplicate. The OD was measured before the AlamarBlue™ dye, data not shown.

2.2.4.4 Determination of Minimum bactericidal concentration (MBC)

The MBC was determined by sub-culturing a loopful of bacterial culture/treatment mixture from the wells that showed no microbial growth of microorganisms in the microdilution assay onto MHA plates. The plates were incubated at 37 °C for 24 hrs. The MBC was recorded as the lowest concentration at which no growth was observed on the MHA plates. The experiment was performed in triplicate.

CHAPTER 3: RESULTS AND DISCUSSION

Eucomis autumnalis has a long history in TMs, and their ability to bio-accumulate and reduce Ag^{+} has generated interest in their usage as an alternate technique for manufacturing AgNPs (Maroyi, 2017). Green nanotechnology is a rapidly growing research field that aligns with the sustainable development goals (SDG), that focuses on sustainable green (biosynthesis) methods (Matlin *et al*., 2015; Seerengaraj Vijayaram *et al*., 2023). The major advantages of plant-mediated synthesis of MNPs includes accessibility, abundance, safety and a broad variety of metabolites; thus, making them a sustainable and economical source of bioreducing agents (Gorlenko *et al*., 2020). In recent years, plant-synthesized (biogenic) AgNPs have gained attention due to their antibacterial properties. Moreover, plants are a safer and more efficient method for AgNPs synthesis, as phytochemicals serve as both reducing and stabilizing agents (Soltani *et al*., 2023; Soni *et al*., 2021; Subramanian *et al*., 2015). It has been established that secondary metabolites (phenolic and flavonoid compounds) found in plant stabilize and prevents aggregation of MNPs (Narayanan & Sakthivel, 2011; Roy & Das, 2015).

Over the past few decades, researchers have studied AgNPs potential in many biological applications. In wound healing, specifically on prostheses and catheters, were shown to prevent bacterial colonization and reduce infection in burn wounds. Other potential applications include anticancer, antimicrobial, and antidiabetic treatments (Raskin, 1996; Salabat & Mirhoseini, 2018, 2022). They are increasingly used in these treatments due to their ability to penetrate diseased cells (Asimuddin *et al*., 2020) based on their size, shape, chemical composition, morphology, surface charge, and stability. Understanding and modifying reaction parameters directly affect nanomaterial behaviour in downstream applications, making it essential for effective therapeutic applications (Kim *et al*., 2007; Shukla & Iravani, 2018). Stable and monodispersed (uniform size and shape) biogenic AgNPs can be produced by controlling different factors such as pH, temperature, reaction time, concentration of plant extract and concentration of metal precursor (Benelmekki, 2015; Li *et al*., 2014; S. Vijayaram *et al*., 2023). Thus, modifying and optimizing the parameters of biogenic EABE-AgNPs can potentially enhance its antibacterial efficacy, thus reducing the development of AMR (Kim *et al*., 2007; Shukla & Iravani, 2018). This chapter reports on green synthesis, characterization, and antibacterial effects of EABE-AgNPs against seven pathogenic bacteria associated with AMR: *S. aureus*, MRSA, *S. pyogenes*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*.

3.1 Visual observation of synthesis of EABE-AgNPs

Green synthesis of AgNPs was mediated by EABE as the reducing agent and $AgNO_3$ as the Ag^+ precursor. As shown in **Figure 3.1**, the bioreduction reaction was visually observed after the EABE and $AgNO_3$ reaction mixture changed from translucent yellow to a brownish colour, indicating the successful formation of biogenic EABE-AgNPs. The colour change can be attributed to the SPR and bioreduction of pure metal Ag^+ by phytochemicals present in the EABE (Panigrahi, 2013). Previous studies reported colours ranging from yellow and brown as a characteristic colour that confirms the formation of AgNPs (Aisida *et al*., 2019; Patel *et al*., 2023). The colour change is commonly visualized as a preliminary screening technique by researchers (Tesfaye *et al*., 2023). AgNPs exhibit a specific colour change in solution due to SPR in the visible region of the electromagnetic spectrum, as measured using UV-visible spectrometry, attributed to the SPR in the visible region (Amendola *et al*., 2010). A significant method of characterizing AgNPs is the distinctive absorption band, which varies in wavelength from 400 to 500 nm and is produced by the collective oscillation of free electrons in AgNPs during exposure of the electromagnetic radiation.

Figure 3.1: Schematic diagram of EABE-mediated synthesis of AgNPs.

3.2. Characterization of synthesized EABE-AgNPs

3.2.1. Ultraviolet-visible Spectroscopy of EABE-AgNPs

The application and physicochemical properties of EABE-AgNPs are influenced by various reaction conditions like pH, temperature, extract, and concentration of the precursor (Dhanjal *et al*., 2022; Srikar *et al*., 2016). Synthesis of AgNPs during optimization of these parameters were visually detected. The UV-Vis absorbance spectra readings were confirmed in the wavelength

range of 300 to 800 nm. The SPR effect, a phenomenon involving the refraction of light, has been a key component in assessing the formation of AgNPs, which exhibit a distinct absorption band in the visible region of the electromagnetic spectrum (Liaqat *et al*., 2022). The UV-Vis spectra or SPR band provide a qualitative understanding of the concentration and shapes of AgNPs (Ndikau *et al*., 2017; Riaz *et al*., 2021). When particle size increases, a red shift to a longer wavelength is observed, while smaller sizes will have a blue shift (El-Sherbiny *et al*., 2016; Shukla & Iravani, 2018). The size and shape of the NPs are crucial for downstream applications (Khan *et al*., 2019). The SPR band of EABE-AgNPs was monitored in all optimization steps to confirm the successful synthesis.

3.2.1.1 Effect of pH vs. temperature on EABE-AgNPs

The effect of varying pH of EABE and synthesis temperatures on the formation of EABE-AgNPs are displayed in **Figure 3.2**. Each experiment was performed for 1 hr at 750 rpm, at the respective temperatures. The SPR peak in the UV-vis absorption spectra indicated successful formation of EABE-AgNPs, and the influence that pH and temperature had on the shape, size, and concentration of EABE-AgNPs. According to literature, the SPR band of AgNPs can be observed in the range of 380-500 nm (Ashraf *et al*., 2016; Sastry *et al*., 1997), AgNPs with a colour range from yellow to dark brown usually have a spectra at 400-550 nm. These findings also align with previous studies on plant synthesized AgNPs which resulted in pale yellow to brown colours (Aromal & Philip, 2012; Moond, Singh, Sangwan, Devi, Beniwal, *et al*., 2023).

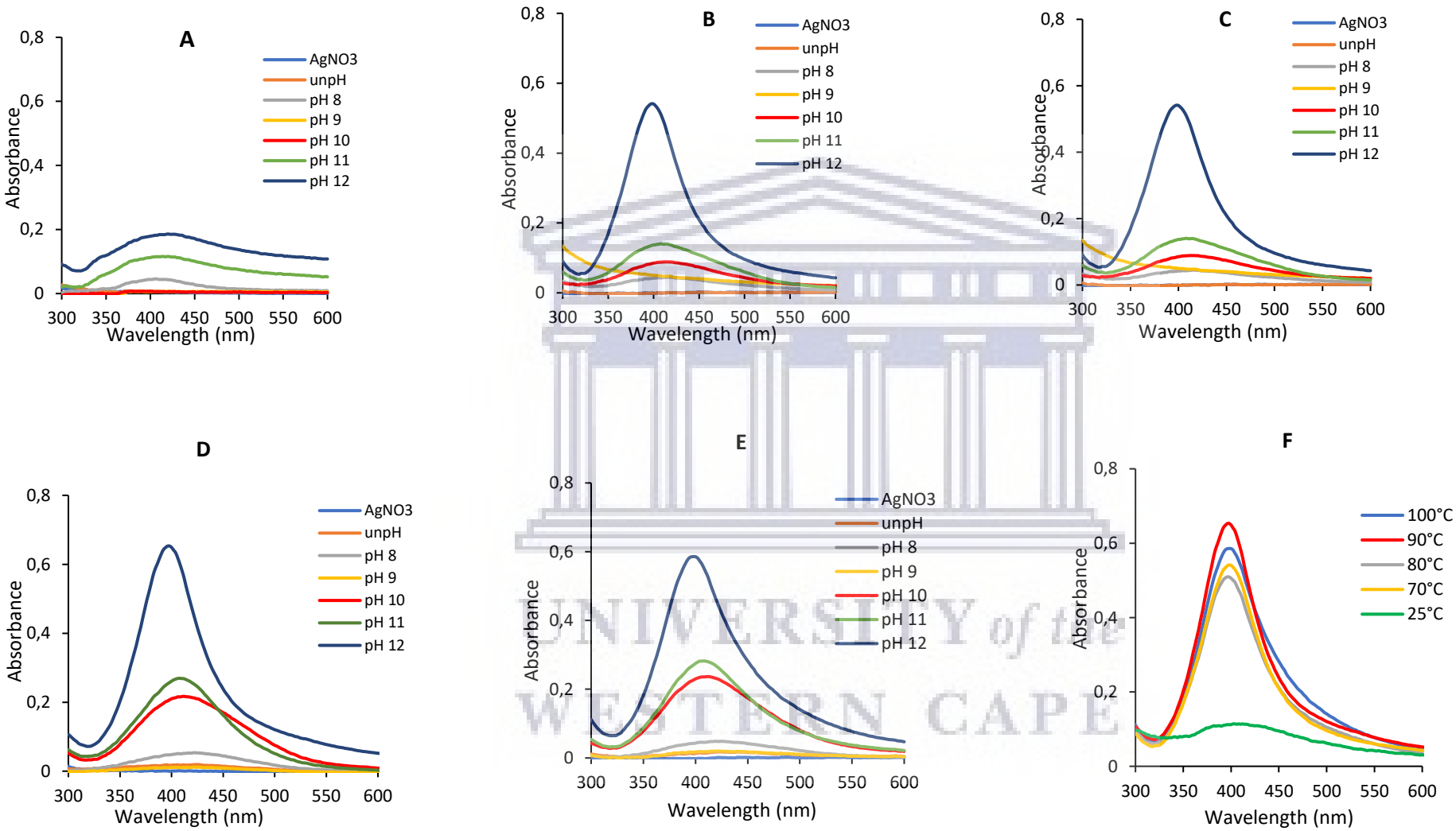


Figure 3.2: UV-vis absorption spectra for various reactions showing the effect of varying pH and temperatures on the formation of EABE-AgNPs (A: 25, B: 70, C: 80, D: 90, E: 100 °C and F: EABE-AgNPs at optimal pH 12 and various temperatures).

Synthesis of EABE-AgNPs was pH and temperature dependent, at RT (25 ºC) broad spectra was seen only at higher extract pH 10 to 12 as depicted in **Figure 3.2 A**. The EABE was not able to reduce Ag^+ to AgNPs at 25 ºC for pH 8 and 9. The reaction mixtures in these conditions showed no colour change and were further confirmed by a flat spectrum. The SPR peaks (λ_{max}) at 25 °C were either flat or broad with a λ_{max}, below 0.2 with no significant colour change in the reaction mixture. The synthesis at this temperature was not favourable for the optimal synthesis of EABE-AgNPs, due to the low OD observed. Thus, indicating the synthesis was unsuccessful at low temperatures.

The reduction of the Ag^+ to Ag^0 occurred at 70 °C, with a sharp narrow SPR peak observed for pH 12 as shown in **Figure 3.2 B**. The EABE-AgNPs synthesized at pH 8, 9, and 11, had broader peaks with SPR (λ_{max}) at 414, 415, 414 and 408 nm corresponding to OD values of 0.446, 0.047, 0.088 and 0.140, respectively. This indicated at lower pH values, acidic conditions are not favourable for the synthesis of AgNPs. According to literature the size of NPs are significantly influenced by pH, with acidic media resulting in larger particle sizes (Gontijo *et al*., 2020; Khalil *et al*., 2014). These results indicated that pH 12 had a narrow λ_{max} at 398 nm with an OD of 0.541 which was significantly higher than the lower pH values. This indicated that monodispersed and more EABE-AgNPs were synthesized at pH 12 compared to lower pH values. The blue-shift in the SPR suggested smaller and more uniform sized EABE-AgNPs were formed at pH 12 (Jayapriya *et al*., 2019). Higher temperatures were demonstrated to increase the rate at which AgNPs are formed (Liaqat *et al*., 2022).

It is worth mentioning that reaction pH alters the electrical charges of biomolecules, impacting their capacity for capping and stabilizing, and the growth of NPs (Liaqat *et al*., 2022). The pH of the medium influences the protonation of amino acids, which contributes to the stability and production of NPs (Gericke & Pinches, 2006; Liaqat *et al*., 2022). Literature suggests that biological components facilitate the conversion of Ag^+ to neutral silver atoms and aid in stabilizing the growth of AgNPs (Chutrakulwong et al., 2020).

A similar trend was observed for EABE-AgNPs synthesized at 80, 90 and 100 °C (**Figure 3.2 C**), indicating successful synthesis of EABE-AgNPs at pH 10, 11 and 12. The SPR bands at 80 °C for pH 10 and 11 were 414 and 406 nm with OD values of 0.121 and 0.156, respectively. This pH range had broader peaks indicating that polydisperse AgNPs formed at 80 °C for pH 10

and 11 (Busi *et al*., 2014; Mat Yusuf *et al*., 2020). While EABE-AgNPs synthesized at pH 12 had a λ_{max} at 396 nm and OD value of 0.509.

At 90 °C (**Figure 3.2 D**), EABE-AgNPs were synthesized at all pH values except pH 8 and 9. At pH 10 and 11, EABE-AgNPs had a λ_{max} at 412 and 408 nm with OD values of 0.217 and 0.269, respectively. Notably, at pH 12 an intense narrow and sharp peak was observed at 398 nm and OD value of 0.653. The OD at this temperature was significantly higher than the lower temperatures, this indicated that the EABE-AgNPs became smaller as temperatures increased. At 100 °C (**Figure 3.2 E**), the SPR bands for EABE-AgNPs at pH 10 and 11 were 412 and 402 nm with OD values of 0.236 and 0.278, respectively. At pH 12 EABE-AgNPs had a λ_{max} at 398 nm and OD value of 0.585. Thus, pH 12 was selected as an optimal pH for synthesis of EABE-AgNPs at all temperatures (**Figures 3.2 A - E**).

From the summary in **Figure 3.2 F**, it was evident that EABE-AgNPs had increased yield and formed monodisperse NPs with increased temperature (Benelmekki, 2015; Li *et al*., 2014; S. Vijayaram *et al*., 2023). The results indicated that pH 12 at all temperatures excluding 25 °C had an effect on EABE-AgNPs synthesis, indicating that temperature influenced the reaction mixture. In comparison to 90 °C, the AgNPs formation rate decreased (100 °C) with increasing temperatures indicating that higher temperatures enhance the reduction process of Ag^+ to Ag^0 and particle aggregation, leading to a decrease in the number and size of AgNPs, as a result of fast nucleation of silver from the reduction of Ag^+. The highest OD 0.653 value was observed at 90 °C which indicated that smaller monodisperse (uniform size and shape) and potentially uniform AgNPs formed. Moreover, increased temperatures enhance molecule kinetic energy, facilitating the rapid reduction of Ag^+ and reducing the potential for particle size growth (Verma & Mehata, 2016). This study showed that with increase in temperature, a higher yield and uniform size distribution of AgNPs were obtained, as Ag^+ are consumed faster, thereby reducing the size (Shukla & Iravani, 2018). This indicated that higher temperatures were favourable for synthesis of higher yields.

The physicochemical properties of EABE-AgNPs and AgNPs in general, can be influenced by various reaction conditions affecting their size, shape, reaction rate, and activity (Dube *et al*., 2020; Manosalva *et al*., 2019; Singh *et al*., 2013). Temperature of the reaction influences the rate at which AgNPs grow (Shukla & Iravani, 2018) and their sizes. It was also evident that at low pH

values larger NPs formed, depicted by a significant red-shift in the absorption spectra and broad SPR bands. This demonstrated that lower pH, EABE were not favourable for the synthesis of AgNPs, which suggests that successful synthesis of EABE-AgNPs were favoured by more basic conditions (Alqadi *et al*., 2014; Dong *et al*., 2009) and higher temperatures. Qin *et al*. found that as the pH of a solution increase, the size of AgNPs became smaller and spherical in shape (Qin *et al*., 2010). In another study, Dong *et al*., 2009 demonstrated the shape of AgNPs was significantly influenced by the pH, with high pH resulting in a mixture of spherical and rod-like particles, while low pH resulted in triangular and polygonal particles (Alqadi *et al*., 2014; Dong *et al*., 2009). Gericke and colleagues demonstrated that pH also influences the protonation of amino acids, affecting the conformation of peptides or proteins, resulting in variations in shape and size during NPs synthesis (Gericke & Pinches, 2006; Shukla & Iravani, 2018). Similarly, the composition of EABE-AgNPs were significantly influenced by pH and temperature (Gericke & Pinches, 2006; Hulkoti & Taranath, 2014; Shukla & Iravani, 2018). Studies reveal that higher temperatures increase the rate of reaction, leading to the formation of smaller nanoparticles due to the increased kinetic energy of molecules (Dwivedi & Gopal, 2010; Philip, 2009).

3.2.1.2 Effect of EABE concentration on EABE-AgNPs synthesis

Figure 3.3 displays the effect of varying EABE concentrations on the formation of EABE-AgNPs. AgNPs were successfully synthesized with all tested EABE concentrations. The EABE-AgNPs synthesized with 0.5, 1, 2 and 3 % of EABE had SPR peaks at 404, 398, 400 and 396 nm with OD values of 0.929, 0.651, 0.463 and 0.418, respectively. Notably, 0.5 % EABE concentrations had the highest and sharpest SPR peak, compared to the higher EABE concentrations. Moreover, the UV-vis spectra for the EABE concentrations of 1, 2 and 3 % were lower and broader than the one for 0.5% EABE-AgNPs, and absorption tails towards longer wavelengths were present which indicated aggregation of the AgNPs at higher concentrations.

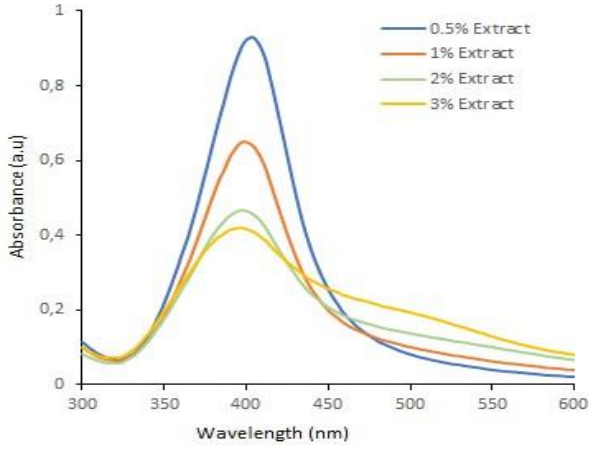


Figure 3.3: UV-vis absorption spectra showing the effect of varying EABE concentration on the formation of EABE-AgNPs at 90 °C for 90 minutes using 1 mM $AgNO_3$. The experiment was performed in triplicate (n=3) and the graphs are presented as average results.

The spectra also suggested that higher EABE concentrations produced less and polydisperse AgNPs, confirmed by longer tails that formed at 450 – 600 nm. This was a clear indication of aggregation, which occurs when NPs cluster together to produce bigger particles (Polte *et al*., 2010). The broader spectra observed (longer wavelengths) at higher extract concentrations is familiar in green nanotechnology and was reported for AgNPs synthesized from *Amomum Subulatum* fruit extract (Dhir *et al*., 2023). This suggests that there could be more extracts and a low amount of $AgNO_3$. The presence of different phytochemicals in plants have been shown to reduce and stabilize NPs and protect them against sedimentation, agglomeration, and loss of surface characteristics (Anil Kumar *et al*., 2007; Mohanpuria *et al*., 2008; Mukherjee *et al*., 2001). However, optimization is required to determine sufficient extract and metal ions concentrations that can produce stable NPs at a given reaction. For this study, 0.5% EABE produced stable with higher yield of EABE-AgNPs and was selected as the optimum concentration for further synthesis reactions.

3.2.1.3 Effect of $AgNO_3$ concentration on EABE-AgNPs synthesis

The effect of the precursor concentration ($AgNO_3$) was evaluated using optimal EABE concentration (0.5%) and temperature (90 °C). Prior to this, all the optimal conditions were evaluated with 1 mM $AgNO_3$. **Figure 3.4** shows that $AgNO_3$ from 0.5 – 3 mM can synthesize EABE-AgNPs with varying optical properties and yields. The SPR of the AgNPs showed that with increasing $AgNO_3$ concentration, there was an increase in the OD values corresponding to the

concentration of the EABE-AgNPs. The smaller SPR peak simply means a lower absorbance and therefore a lower concentration of the EABE-AgNPs at lower $AgNO_3$ concentration (Mondal *et al*., 2020). The SPR for AgNPs synthesized with 0.5, 1, 2 and 3 mM $AgNO_3$ were 399, 404, 404 and 400 nm, respectively. The OD values for 0.5 and 1 mM $AgNO_3$ were below 1, while the OD values for 2 and 3 mM $AgNO_3$ were 1.866 and 1.933, respectively. Although 3 mM $AgNO_3$ had the highest OD, a tail formed at longer wavelengths, indicating the presence of larger NPs or clusters, due to aggregation, thereby creating instability of the AgNPs. In previous studies, larger NPs were generated by the secondary reduction process of Ag^+ adsorbed on built-in nuclei surfaces at higher concentrations (Moond, Singh, Sangwan, Devi, Rani, *et al*., 2023). Absorption spectra of MNPs, including AgNPs are influenced by particle size and dielectric medium, with plasmon absorption shifting towards longer wavelengths as particle size increases (Mulvaney, 1996; Zuber *et al*., 2016). Previous studies also demonstrated bioreduction of Ag^+ using *Coleus aromaticus* leaf extract at 1 mM or less, while at higher concentrations aggregated NPs formed (Vanaja *et al*., 2013). In this study, 2 mM $AgNO_3$ was chosen as the optimum $AgNO_3$ concentration.

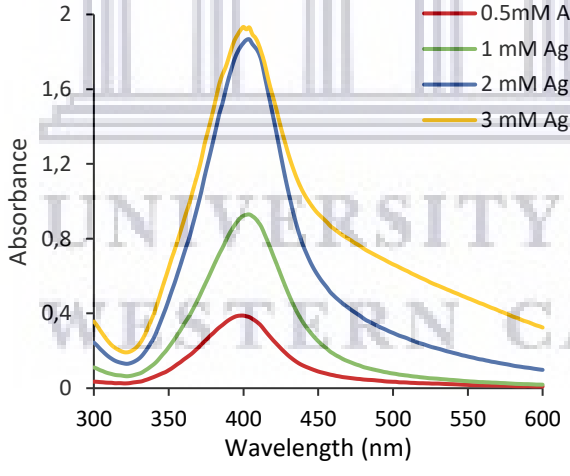


Figure 3.4: UV-vis absorption spectra showing the effect of varying $AgNO_3$ concentration on the formation of EABE-AgNPs. The experiment was performed in triplicate (n=3) and the graphs are presented as average results.

3.2.1.4 Effect of 2 mM $AgNO_3$ and 0.5% EABE concentration on EABE-AgNPs synthesis

At 2 mM $AgNO_3$, EABE-AgNPs with a narrow spectrum and no sign of aggregation formed, indicating that this concentration is optimum for synthesis of uniform and spherical NPs. Previous studies reported that $AgNO_3$ at 1 and 2 mM are the optimal concentrations are able to produce

AgNPs with physicochemical properties that are suitable for biological applications (Gurunathan *et al.*, 2009). Higher concentrations up to 5mM produce lower yields and aggregated AgNPs. Thus, 2 mM $AgNO_3$ was selected as the optimum concentration for further EABE-AgNPs synthesis. **Figure 3.5** shows that the concentration (OD) of EABE-AgNPs synthesized with 0.5 – 3% EABE at 2 mM $AgNO_3$, reduce as the EABE concentrations increases. AgNPs synthesized at 0.5 % EABE had a λmax of 400 nm and an OD value of 1.847 indicating the highest yield compared to increased EABE concentrations. The NPs aggregated at higher concentrations, indicated by the absorption tail that formed at longer wavelengths. This indicated that the NPs were polydisperse and larger in size. The width of the peak suggested that the AgNPs were monodisperse at 0.5% (Kirubaharan *et al.*, 2012).

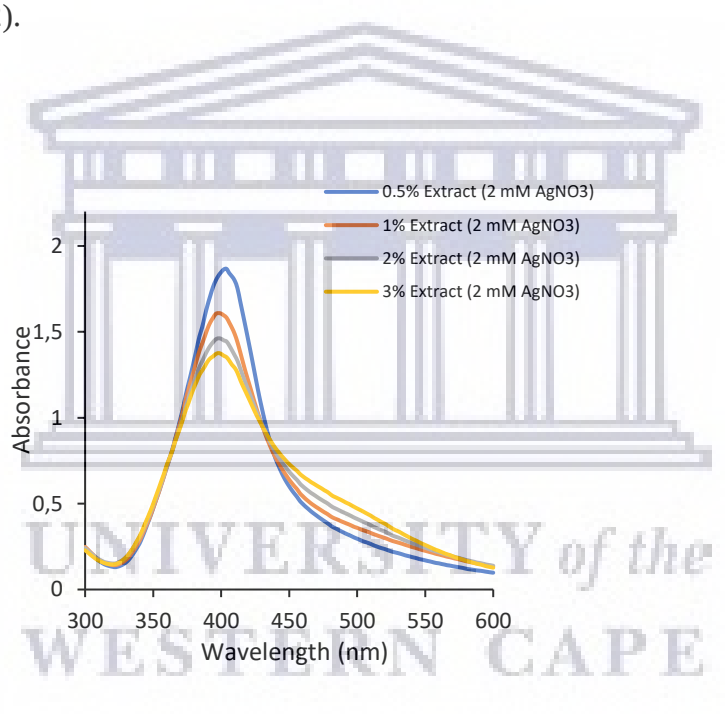


Figure 3.5: UV-vis absorption spectra showing the effect of varying EABE concentration on the formation of EABE-AgNPs. The experiment was performed in triplicate (n=3) and the graphs are presented as average results.

3.2.1.5 Effect of reaction time on the synthesis of EABE-AgNPs

The rate of reaction was investigated using optimal conditions, **Figure 3.6** show that the synthesis was observed to reach saturation at approximately 10 minutes. Though from this point there was a further increase in EABE-AgNPs synthesis, the 30 min time interval showed (**Figure 3.6 A**) a shift in SPR from 404 nm to 406 nm, which may be attributed to the nucleation process and instability of the NPs. After 90 min, the synthesis reached complete saturation (**Figure 3.6**

B), similar to 10 min. In this study, complete synthesis was achieved within 10 min. However, the optimum time chosen was 90 min to allow enough time to add capping agents and enhance stability.

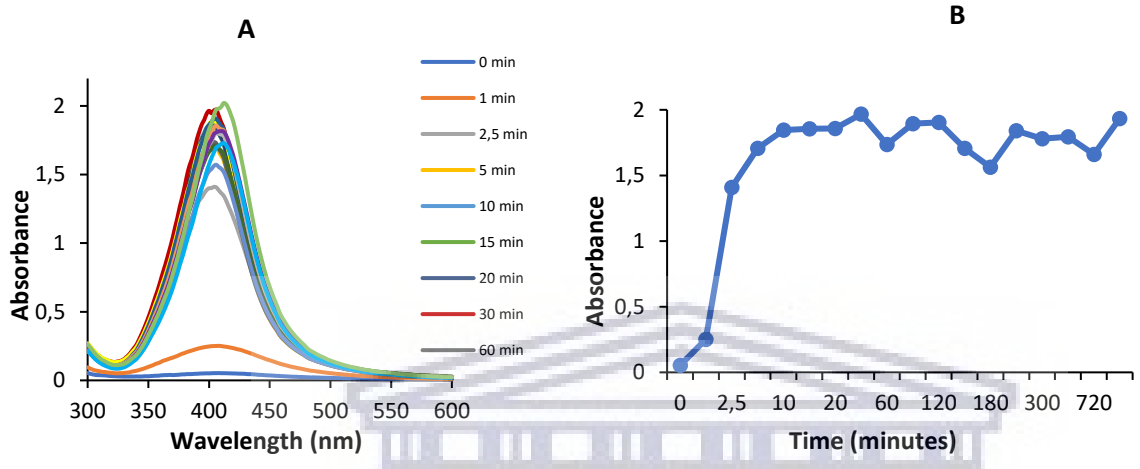


Figure 3.6: UV-vis absorption spectra of EABE-AgNPs at various time points (A) and the reaction rate at their SPR (B) using all optimum reaction conditions.

3.2.1.6 Upscaled synthesis of EABE-AgNPs with all optimum synthesis conditions

EABE-AgNPs were upscaled using optimum conditions for downstream applications; 0.5 % EABE concentration, 2 mM $AgNO_3$, 90 °C temperature, pH 12, 90 min of reaction time, shaking at 750 rpm in a final reaction volume of 50 ml. The UV-vis absorption spectra of EABE-AgNPs in **Figure 3.7** displays the SPR at 404 nm with an OD value of 1.960. The AgNPs were subjected to further characterization to assess their size, shape, polydispersity index (PDI), ζ-potential and functional groups contained in the AgNPs.

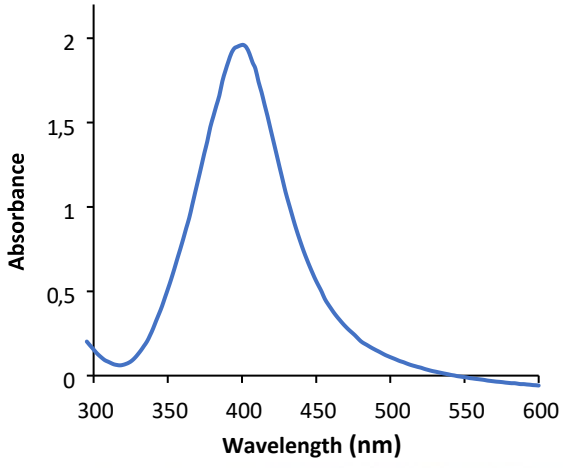


Figure 3.7: UV-vis absorption spectra showing the upscaled synthesis of EABE-AgNPs using all optimum conditions (EABE concentration: 0.5 %, $AgNO_3$ concentration: 2 mM, temperature: 90 °C, pH 12, time of reaction: 90 min, shaking at 750 rpm). The experiment was performed in triplicate (n=3) and the graphs are presented as average results.

3.2.2 Dynamic light scattering analysis of EABE-AgNPs

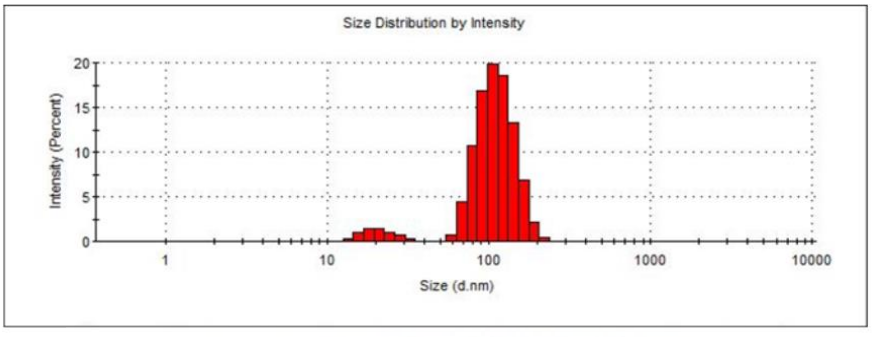
The hydrodynamic size and PDI of EABE-AgNPs were evaluated by using DLS. DLS measures the hydrodynamic size, and the surface charge (Linkov *et al*., 2013). As shown in **Figure 3.8 A**, EABE-AgNPs had a hydrodynamic size of 126.4 ± 29.4 nm, which measure both core size and the surface composition (Souza *et al*., 2016).

The PDI values were measured to predict the uniformity of EABE-AgNPs in solution (broadness of the size distribution), values ranging from 0 to 1, indicated highly homogenous to highly polydisperse molecules (Abdella *et al*., 2023). A PDI above 0.5 indicates that the NPs are polydisperse, while PDI values exceeding 0.7 indicate a broad particle size distribution (Danaei *et al*., 2018). The PDI for EABE-AgNPs was 0.396 ± 0.07, indicating monodispersed NPs, even though plant-mediated synthesized NPs are expected to be polydisperse due to the presence of multiple phytochemicals. These findings agree with previous studies indicating that stable, uniform and monodisperse NPs can be produced using plant extracts. Similar to another study that demonstrated the PDI of AgNPs produced from *Tecomella undulata* (*T. undulataI*) leaf extract of 0.378, which indicated their uniformity (Dhaka *et al*., 2023).

A

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 126.4	Peak 1:	113.0	93.8	29.40
PdI: 0.396	Peak 2:	21.06	6.2	4.833
Intercept: 0.887	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report



B

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -25.2	Peak 1:	-25.2	100.0	8.48
Zeta Deviation (mV): 8.24	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.00809	Peak 3:	0.00	0.0	0.00

Result quality : Good

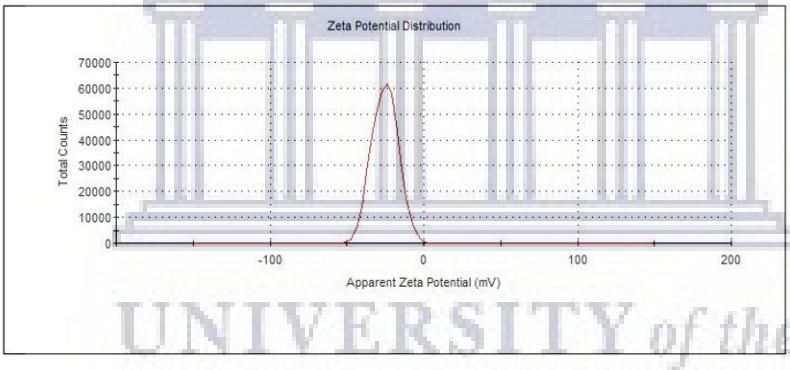


Figure 3.8: Hydrodynamic size distribution of the biogenic EABE-AgNPs (A) and ζ-potential (B).

In **Figure 3.8 B**, the EABE-AgNPs had a ζ-potential of − 25.2 ± 8.48 mV which indicated that they were highly stable and anionic in nature. Biosynthesized AgNPs, unlike chemical-synthesized NPs, relies on phytochemicals (as reducing and capping agent) for stabilization in a one-step reaction. The capping agents are implicated in their bioactivities as anticancer (Cho *et al*., 2013; Sankar *et al*., 2014), antioxidant activity, antidiabetic, antidiarrheal, hepatoprotective activity and antimicrobial agents (Mohanta & Behera, 2014). A ζ-potential range from ±30 mV have high electrical charge on their surface and due to their strong repellent force among them, agglomeration can be prevented (Manikandan & Sathiyabama, 2015). EABE-AgNPs, due to their high stability, exhibit physiochemical properties that make then suitable for *in vitro* studies. Therefore, EABE-AgNPs could potentially be useful for biomedical applications.

3.2.3 High-resolution transmission microscopy analysis of EABE-AgNPs

HR-TEM was used to analyze the shape, size, morphology (dispersion) and the distribution (uniformity) of EABE-AgNPs. In **Figure 3.9 A**, the particles were spherical in shape and are monodispersed. In **Figure 3.9 B**, the particles had an average core size of 13.03 ± 2.04 nm in diameter. The discrepancy between DLS and TEM analysis exists because DLS measures the hydrodynamic diameter and the core size, while TEM only accounts for the core size (Eaton *et al*., 2017). DLS provides information about particle solution dynamics, while HR-TEM measurements calculate the size of the metal without solvent layers (Baraka *et al*., 2017; Elakraa *et al*., 2022). Ultrasmall AgNPs (size range 1.59 − 3.91 nm) synthesized by Ji and colleagues (2020) obtained with a thermosensitive copolymer had antibacterial activity against *E. coli* and *S. aureus*, as well as the highest polymer content. Thereby highlighting the significance of physiochemical characteristics of AgNPs (Ji *et al*., 2020). Furthermore, it has been observed that isotropic forms of AgNPs have been found to effectively combat bacterial strains, with visible zones of inhibition (ZOI) indicating the impact of the size and morphology of the AgNPs on bacterial activity (Kumar *et al*., 2014). In isotropic forms, MIC values also decrease as the particle size decreases. While anisotropic particles, MIC values change steadily due to their different shapes, indicating that the shape and size of the NPs affects the antibacterial activity (Sathishkumar *et al*., 2010; Sondi & Salopek-Sondi, 2004). Similar observations were made with biogenic AgNPs, where size, shape, and surface composition associated with their enhanced antimicrobial efficacy (Bhuyan *et al*., 2015; Khatami *et al*., 2015; Mostafa *et al*., 2015).

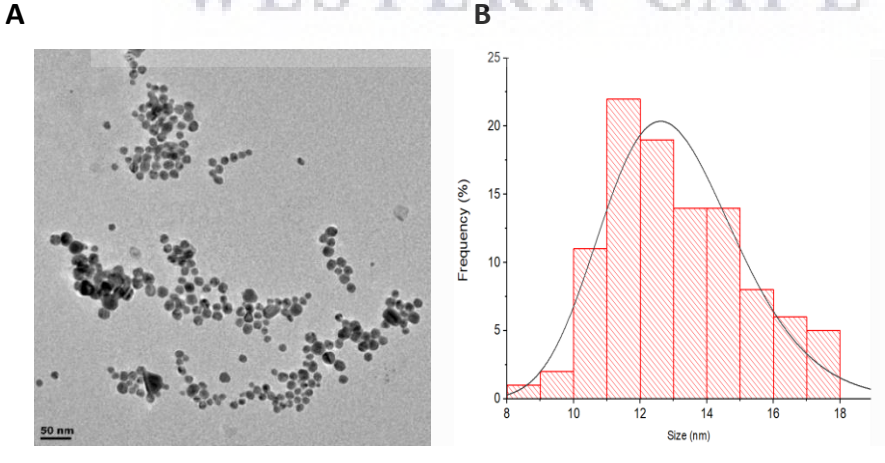


Figure 3.9: HR-TEM analysis of EABE-AgNPs showing their morphology and size: (A) Micrograph and (B) size distribution of EABE-AgNPs.

3.2.4. Fourier-transform infrared spectroscopy (FTIR) of EABE-AgNPs

FTIR spectroscopy was used to identify the surface structural composition (biomolecules) of the EABE and EABE-AgNPs. The structural composition may provide information on organic functional groups (hydroxyl groups, carbonyl groups, and other stabilizing molecules) that are present on the surface of EABE-AgNPs. Literature suggests that functional groups of aromatic amines, amide groups, and secondary alcohols may serve as reducing agents (Anandalakshmi *et al*., 2016; Elavazhagan & Arunachalam, 2011; Jassim *et al*., 2016; Jayaseelan *et al*., 2013; Narayanan & Sakthivel, 2010). Several functional groups in the synthesis of EABE-AgNPs were identified as shown (**Figure 3.10**) by the differences between the spectra for EABE and EABE-AgNPs. The infrared radiation (IR) spectrum can distinguish biomolecules such as alkanes, alkenes, and alkynes and predict the type of samples being analyzed (Harris *et al*., 2023; Liu, 2021).

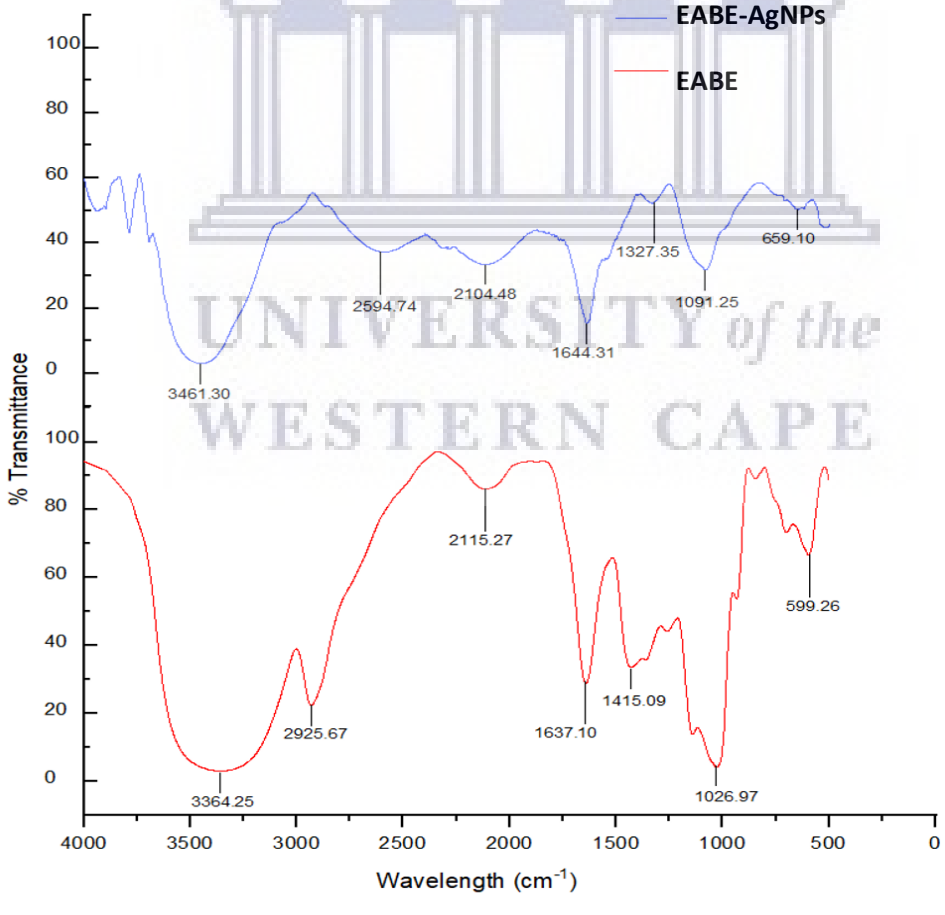


Figure 3.10: FTIR spectra of EABE and EABE-AgNPs.

The FTIR analysis identified some of the functional groups of organic compounds in EABE responsible for reducing Ag^+ ions and capping the EABE-AgNPs. The FTIR spectra of the EABE and EABE-AgNPs demonstrated absorption peaks at 3461.30 cm^{-1}, corresponding to the O-H stretching vibrations in alcohols and phenolic compounds (Annamalai & Nallamuthu, 2016); 3364.25 cm^{-1} are related to the C-H stretching vibrations of methyl groups (Devi *et al*., 2016); 2959.74 cm^{-1} assigned to the H-C=O stretching vibrations in aldehydes; 2925.67 cm^{-1}, corresponding to the C-N stretching vibrations of nitriles (Keshari *et al*., 2020); 2104.48 cm^{-1}, is related to the C=O stretching vibrations in proteins (Velgosova & Veselovský, 2019); 2115.2 cm^{1}, assigned to the C-C stretching vibrations in the aromatic compounds (Velgosova & Veselovský, 2019); 1644.31, 1327.35 and 1092.25 cm^{-1}, corresponding to the C-C stretching vibrations in alcohols, carboxylic acids, ethers, and esters (Naseer *et al*., 2020); 1637.10, 1415.09 and 1026.97 cm^{-1}, corresponding to C-O stretching vibrations in 3° alcohols and phenols (Selvaraj *et al*., 2019); and the shift from 599.26 (EABE) to 659.10 cm^{-1} (EABE-AgNPs) corresponds to the C-H bonding vibrations of the aromatic compounds (Muthukumar *et al*., 2020). These functional groups are common to AgNPs synthesized using plant extracts.

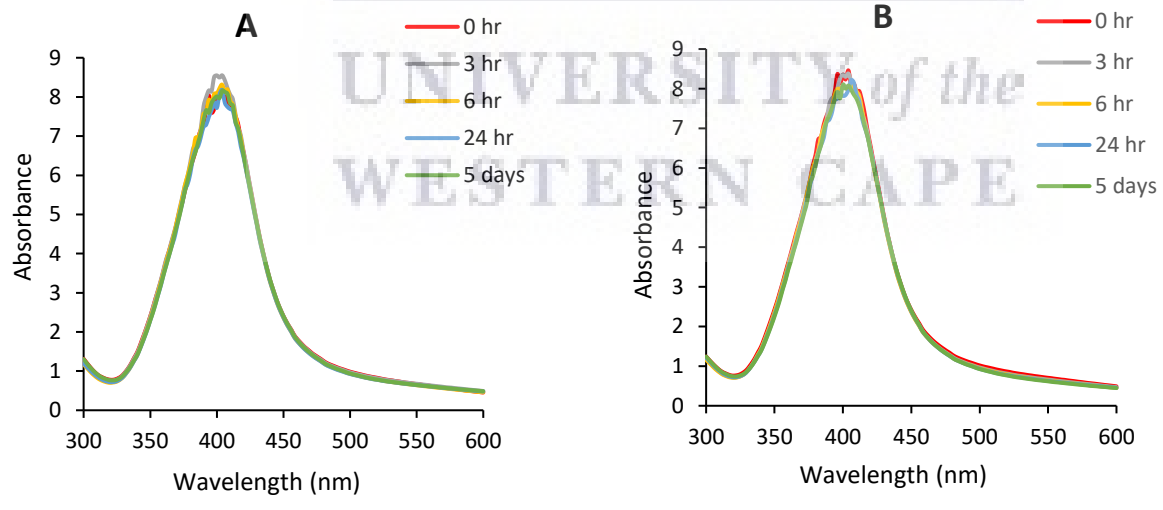
Plants contain phytochemicals with different active groups such as hydroxyl, carboxyl, and amine, which serve as reducing and capping agents (Badru *et al*., 2023; Castro *et al*., 2013; Shankar *et al*., 2016) which were identified in MNPs. *Astragalus tribuloides* root extract AgNPs had peaks corresponding to the O-H stretching vibrations in alcohols and phenolic compounds (Annamalai & Nallamuthu, 2016). The FTIR results of *Ananas comosus L*. (pineapple) AgNPs confirmed the presence of O-H stretching of a phenolic compound, N-H stretching of a 1° amine, C-H stretching of amides, N-H stretch stretching of 1° amine and alkyne (Mohanta & Behera, 2014; Subhankari & Nayak, 2013). The presence of O-H, C-H, H-C=O, C=O, C-C, C-C, C-O, and C-H bonds in EABE-AgNPs spectra suggests that the EABE secondary metabolites (phenolic and flavonoid chemicals) were involved in reduction and capping of the NPs.

The FTIR spectra of EABE-AgNPs were discovered to have similar peaks to those found in EABE, while some were shifted. The absorption peaks in the EABE spectra at 2104.48 and 1327.35 cm^{-1} shifted to 2115.27 and 1092.25 cm^{-1}. The peaks at 900 and 600 cm^{-1} shifted to 659.10 and 599.26 cm^{-1} corresponded to polysaccharides, vitamins, nucleic acids (i.e., phenylalanine, tyrosine, tryptophan, and various nucleotides), proteins, and amino acids (Shukla & Iravani, 2018; Sukprasert *et al*., 2020; Venkatpurwar & Pokharkar, 2011; Yang *et al*., 2015).

Peaks at 3461.30, 2959.74, 2104.48, 1644.31,1327.35, 1092.25 and 659.10 cm^{-1} shifted to 364.25, 2925.67, 2115.27, 1637.10, 1415.09, 1026.97 and 599.26 in the EABE-AgNPs. These shifts demonstrated that the functional groups associated with these bands were responsible for the bioreduction of Ag^{+} and the stability of EABE-AgNPs (Devi *et al*., 2016; Sharifi-Rad *et al*., 2020). Flavonoids and phenolic acids such as eucomic acid, are major bioactive compounds in the EABE (Ami *et al*., 2013; Lediga *et al*., 2018), suggesting their involvement in the reduction, capping and stability of EABE-AgNPs.

3.2.5. Stability analysis of EABE-AgNPs

Stability of EABE-AgNPs in dH_2O, ddH_2O and MHB were evaluated over a 24-hour period. As indicated in **Figure 3.11**, there were no changes in the UV-vis spectra of the EABE-AgNPs in solution. The EABE-AgNPs stability was evaluated at 37 °C since most *in vitro* and *in vivo* applications are performed at this temperature. The interactions between the media components and biomolecules on the surface of EABE-AgNPs did not affect their stability and might not influence their bioactivity. The stability of chemically synthesized AgNPs was reduced overtime after incubation with various media suggesting that biogenic AgNPs will be best suited for downstream applications.



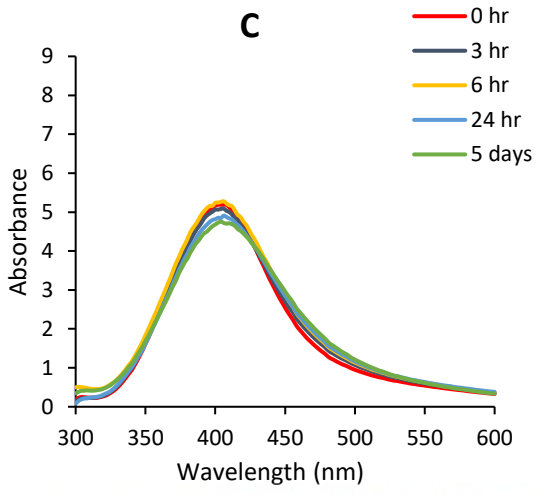


Figure 3.11: UV-vis absorption spectra showing the stability of the biogenic EABE-AgNPs in various media during incubation at 37 °C for 24 hrs (A: dH_2O, B: ddH_2O, C: MHB).

3.3. Antibacterial activity of EABE-AgNPs

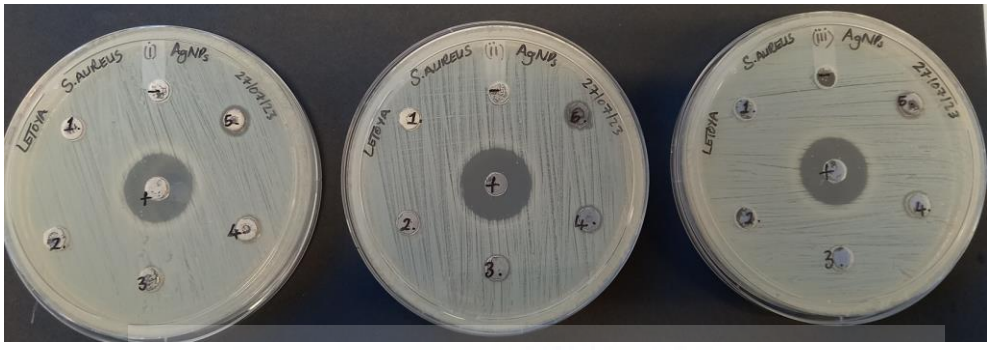
3.3.1. Antibacterial activity of EABE-AgNPs using agar well diffusion assay

The antibacterial activity of EABE-AgNPs and EABE were tested *in vitro* against seven pathogenic bacterial strains; *S. aureus*, *S. pyogenes*, MRSA, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* using agar well diffusion method. Ciprofloxacin and MHB were chosen as the positive control and negative control, respectively. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for Ciprofloxacin were also determined in this study, it is notable that the concentration of Ciprofloxacin used was 15 µg/ml for all the microorganisms tested, except for *E. coli* where 10 µg/ml Ciprofloxacin was used. The circular area, known as the zone of inhibition (ZOI) around the antibacterial treatment in which bacteria colonies do not grow, can be used to measure the susceptibility of the bacteria towards the antibacterial treatment. The ZOI surrounding each well for the various concentrations of EABE-AgNPs and EABE was measured to determine the antibacterial effect of the EABE-AgNPs and EABE on the microorganisms of interest.

EABE-AgNPs demonstrated broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria (**Figure 3.12** and **Table 3.1**). Notably, EABE-AgNPs was able to efficiently inhibit the growth of both gram-positive and gram-negative bacteria in a dose-

dependent manner. The EABE did not exhibit any antibacterial activity against all the bacteria as there was no ZOI on the agar plates, except for the positive control (data not shown).

A



B

C

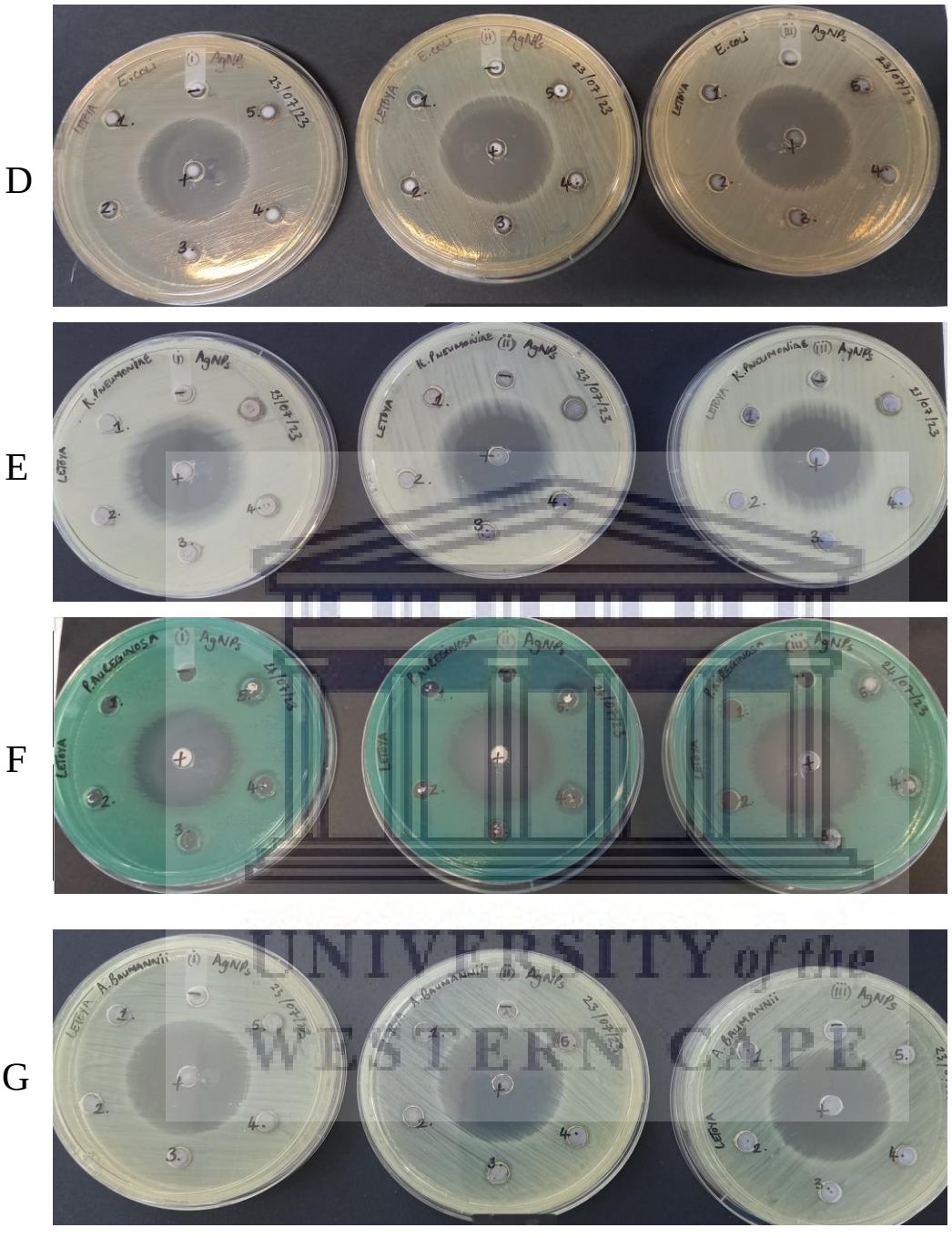


Figure 3.12: Antibacterial activity of EABE-AgNPs. The zone of inhibition (ZOI) of synthesized EABE-AgNPs (mm) on (A) *S. aureus*, (B) MRSA, (C) *S. pyogenes*, (D) *E. coli*, (E) *K. pneumoniae*, (F) *P. aeruginosa* and (G) *A. baumannii*. Note: Concentrations of EABE-AgNPs - 1: 4.04 µg/ml, 2: 8.07 µg/ml, 3: 16.15 µg/ml, 4: 32.30 µg/ml and 5: 64.59µg/ml EABE-AgNPs; +: Ciprofloxacin; –: MHB).

Table 3.1: The antibacterial effects of biogenic EABE-AgNPs against seven human pathogenic bacteria tested at different doses ranging from 4.0 – 64.5 µg/ml (n=3).

Treatments (µg/ml)	Zone of inhibition ± SDM (mm)						
	S. aureus	MRSA	*S. pyogenes*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*A. baumannii*
4.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16.1	0 ± 0	0 ± 0	7.66 ± 0.47	0 ± 0	0 ± 0	7.16 ± 0.23	7.33 ± 0.29
32.3	7.16 ± 0.23	0 ± 0	8.66 ± 0.47	0 ± 0	7.66 ± 0.47	10.83 ± 0.23	8.67 ± 0.58
64.5	8.5 ± 0.47	7.5 ± 0.40	10.66 ± 0.47	7 ± 0	9.16 ± 0.23	11.5 ± 0.40	10.17 ± 0.29
Ciprofloxacin	20.66 ± 0.23	26.66 ± 0.47	26.66 ± 0.47	36 ± 0.81	29.16 ± 0.23	34 ± 0.81	37.33 ± 0.47
MHB	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

The Gram-negative bacteria were more susceptible to the EABE-AgNPs than MRSA and *S. aureus* (**Table 3.1**). The lowest concentration of EABE-AgNPs that was able to inhibit growth of these microorganisms (*S. pyogenes*, *P. aeruginosa*, *A. baumannii*) was 16.1 µg/ml whilst the lowest concentration of EABE-AgNPs that inhibited growth of the others was 32.3 µg/ml except MRSA at 64.5 µg/ml. Antibacterial activity of plant mediated NPs has been reported on various strains (Saravanan *et al*., 2013). *V. amygdalina* AgNPs showed significant inhibitory activity against *S. pyogenes* and *S. aureus*, with 17, 15, 13 mm and 17, 14, 12.5 mm ZOIs at 75, 50, 25 µg/ml, respectively (Gonfa *et al*.; Tesfaye *et al*., 2023). According to Krychowiak *et al*., combining plant compounds with AgNPs can be an alternative to antibiotics. This combination was able to kill 99.9% of bacteria in burnt victims, including drug resistant organisms such as MRSA (Krychowiak *et al*., 2014; Wypij *et al*., 2021). MRSA accounts for ≤80% of all nosocomial infections causing postsurgical wound infections (Fomda *et al*., 2014; Krishnamurthy *et al*., 2014). In 2015, 17.2 billion cases of upper respiratory tract infection caused by *S. pyogenes* occurred, resulting in approximately 3000 deaths (Sahoo *et al*., 2020; Vos *et al*., 2017; Vos *et al*., 2016; Vos *et al*., 2015).

All strains tested in this study were susceptible to the antibacterial action of EABE-AgNPs, although Ciprofloxacin (15 – 0.1 µg/ml, positive control) had stronger activity. This confirmed that the EABE-AgNPs were responsible for the antibacterial activity observed in these pathogens, rather than the phytochemicals by themselves. These phytochemicals have medicinal properties and controls physiochemical properties of NPs and their activities. The results obtained in this study are in agreement with a previous study in which Lediga *et al*. (2018) also showed that AgNPs functionalized with *Sclerocarya birrea* and *E. autumnalis* extracts also showed microbial

activities against Gram-negative and Gram-positive microorganisms with negligible or low cytotoxicity (Lediga *et al*., 2018). AgNPs from aqueous sumac extract treated bacterial canker disease in stone fruit plants caused by *Pseudomonas syringae* (*P. syringae*). The study found that varying NP concentrations significantly reduced the severity of the disease, with the highest decrease observed at 100 µg/ml NPs concentration (Castillo-Henríquez *et al*., 2020; Castillo *et al*., 2020; Shahryari *et al*., 2020).

A study involving isolates from hospitalized patients revealed that the biosynthesized AgNPs nanocomposite, were susceptible to MDR strains (*P. aeruginosa* and A. *baumannii*), including other Gram-positive and Gram-negative bacteria tested (Silva Santos *et al*., 2016). AgNPs synthesized from *Caesalpinia pulcherrim* extract have shown antimicrobial, antibiofilm, and antioxidant properties against various bacterial strains. These NPs have been found to inhibit pyocyanin and extracellular polysaccharide production, as well as biofilm formation by *P. aeruginosa* (Dhaka *et al*., 2023; Moteriya & Chanda, 2018). AgNPs from *Salvadora persica* (*S. persica*) root extract demonstrated antibacterial effect in Gram-negative and Gram-positive bacteria (Shaik *et al*., 2016; Shukla & Iravani, 2018).

3.3.2 Antibacterial activity of EABE-AgNPs using microdilution assay

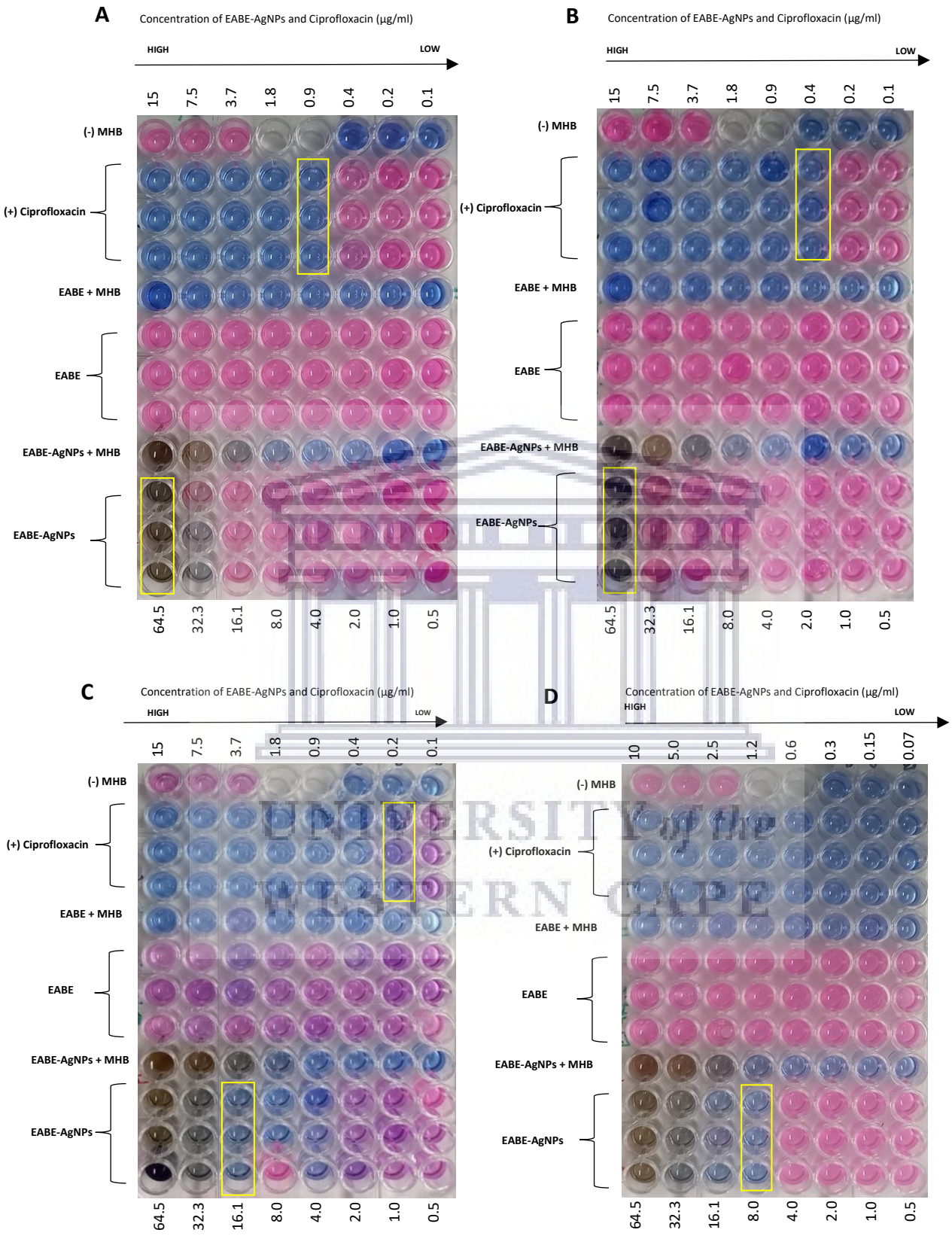
The MIC and MBC of EABE-AgNPs and Ciprofloxacin were tested against the seven bacterial strains by the microdilution assay in a wide range of concentrations to identify values capable of inhibiting bacterial growth. The MIC is the lowest concentration that effectively prevents visible growth, while the MBC kills at least 99.9% of the original inoculum (EUCAST, 1998; Phillips *et al*., 1998; Tahan *et al*., 2023; Taylor *et al*., 1983; Testing, 1998).

The lowest concentration that inhibited visual growth of the bacteria (MIC) were visually assessed and recorded after 24 hours of treatment. EABE-AgNPs had broad-spectrum antibacterial activity against all tested microorganisms, as shown in **Table 3.3**. The inhibitory and bactericidal concentrations of the EABE-AgNPs against the microorganisms are depicted in **Figures 3.13** to **3.15**. The presence of viable bacteria in a culture was quantified using AlamarBlue™ dye, a stable, non-toxic, water-soluble dye. In the presence of viable bacteria, the non-fluorescent AlamarBlue™ dye was reduced to resorufin, a highly fluorescent pink compound, which was used as a colorimetric indicator. Thus, enabling continuous monitoring of cellular activity

permeable to cell membrane of the bacteria (Elshikh *et al*., 2016; Kreft & Kreft, 2009; Page *et al*., 1993). The fluorescent signal is proportional to the number of viable cells (Riss *et al*., 2016).

Table 3.2: The MIC and MBC of EABE-AgNPs and Ciprofloxacin against seven human pathogenic bacteria.

Microorganisms	MIC (μg/ml)		MBC (μg/ml)	
	EABE-AgNPs	Ciprofloxacin	EABE-AgNPs	Ciprofloxacin
S. aureus	64.5	0.9	64.5	0.9
MRSA	64.5	0.4	64.5	0.4
S. pyogenes	16.1	0.2	16.1	0.4
E. coli	8.0	<0.1	32.3	<0.1
K. pneumonia	64.5	<0.1	64.5	<0.1
P. aeruginosa	32.3	0.2	64.5	0.4
A. baumannii	64.5	<0.1	64.5	<0.1



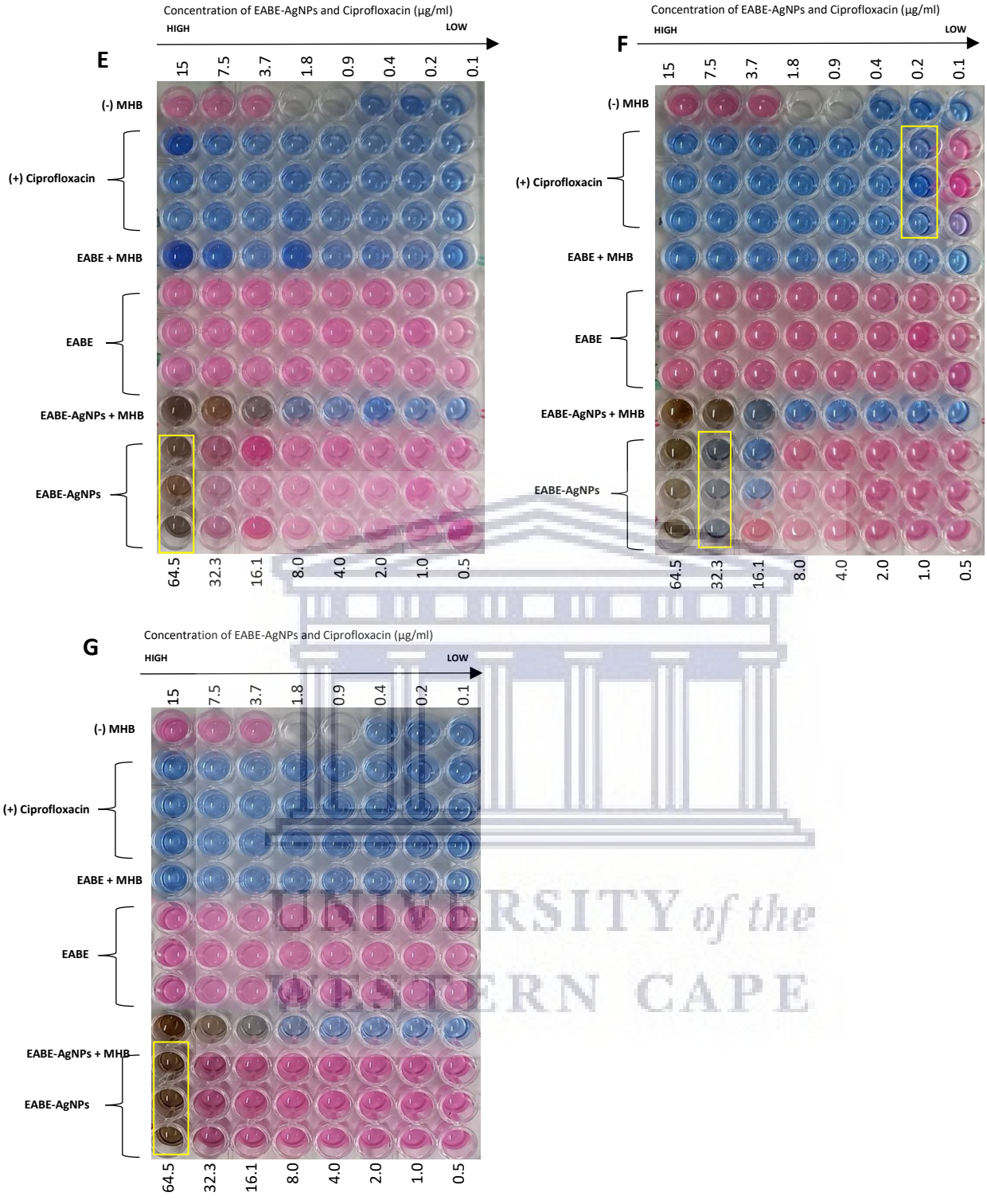
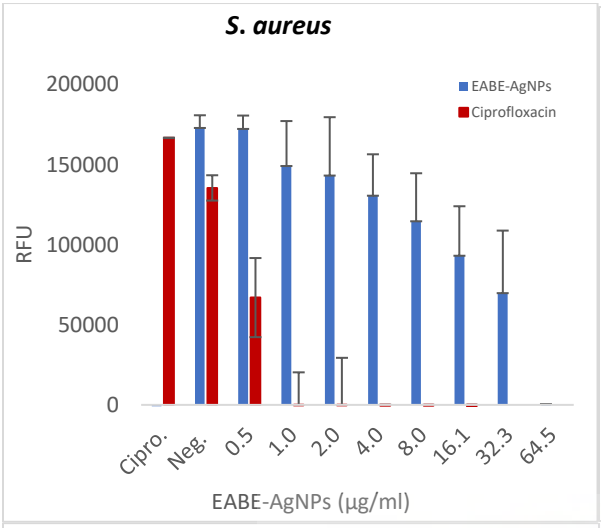
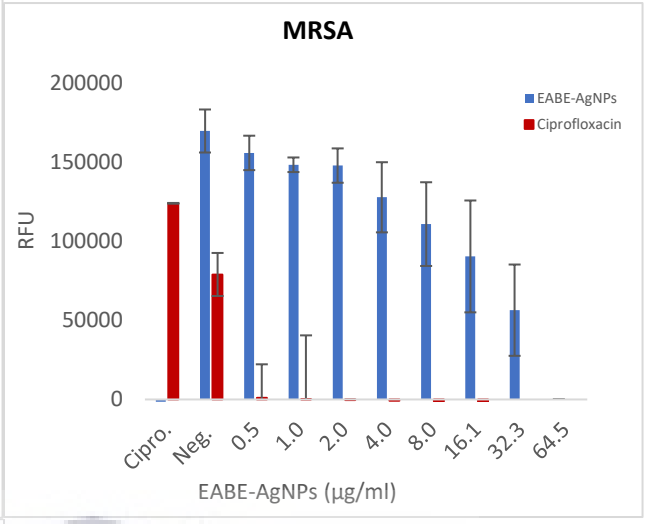
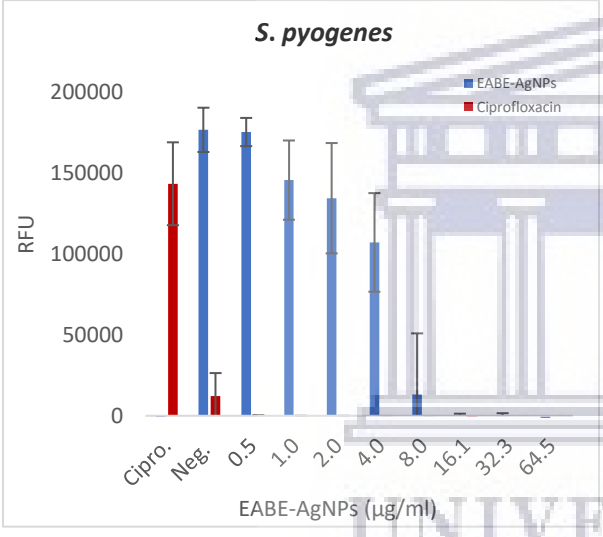
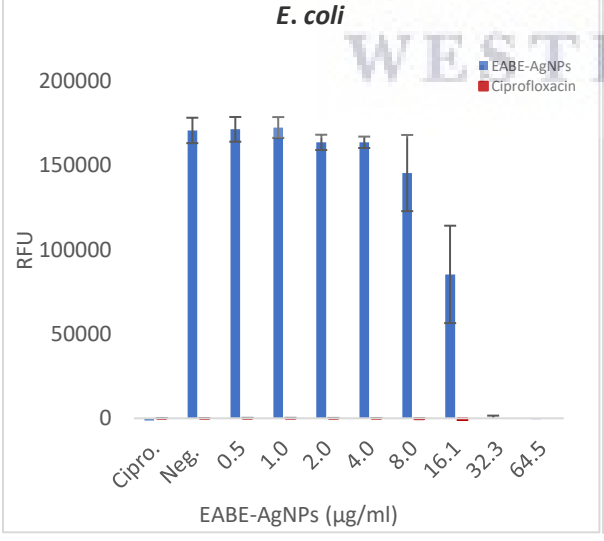


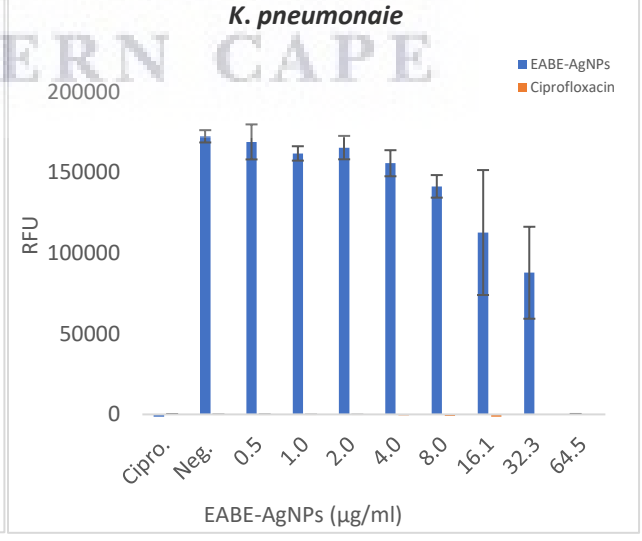
Figure 3.13: The inhibitory effects of EABE-AgNPs on bacterial growth using AlamarBlue™ assay. A colour change from blue to pink is observed in the presence of viable bacteria A) *S. aureus*, B) MRSA, C) *S. pyogenes*, D) *E. coli*, E) *K. pneumoniae* F) *P. aeruginosa* and G) *A. baumannii*.











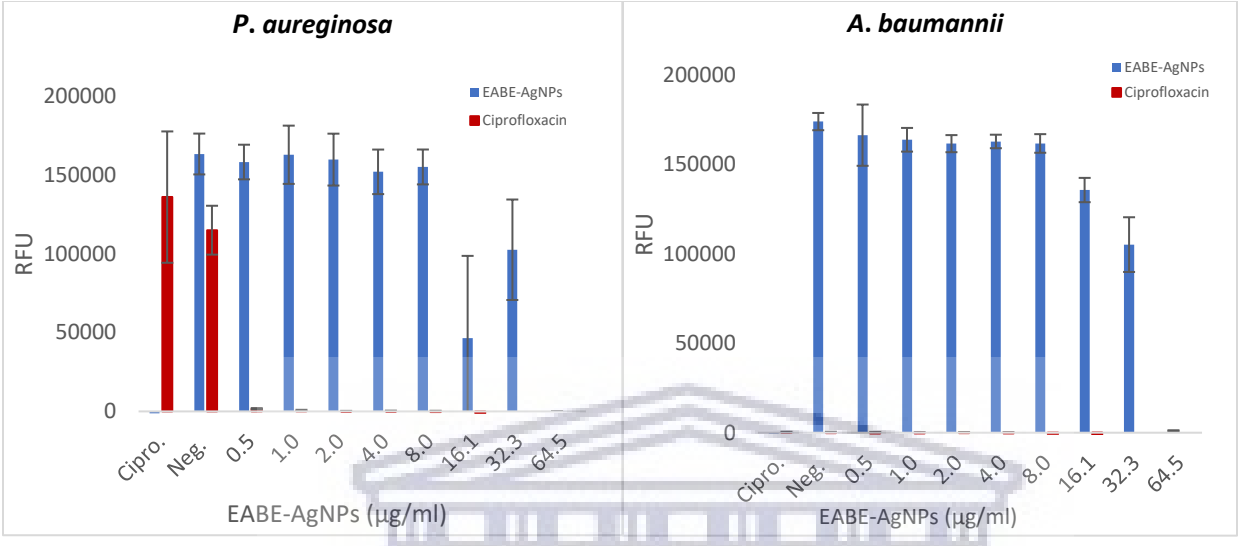


Figure 3.14: Inhibitory effect of EABE-AgNPs and ciprofloxacin on seven bacterial strains. The fluorescence of AlamarBlue™ was measured as a bacterial viability indicator and is directly proportional to the number of viable cells present (RFU: Relative Fluorescence Units).

The MIC varied between 16.1 and 64.5 µg/ml for Gram-positive bacteria and from 8.0 and 64.5 µg/ml for Gram-negative bacteria, respectively. This suggested that Gram-negative bacteria were more susceptible to inhibitory and bactericidal actions of EABE-AgNPs. Although the complete detailed mechanisms are still under debate, it has been proposed that the possible bactericidal activity of AgNPs is due to the release of Ag^{+} from the NPs. Antibacterial activity is more evident in Gram-negative bacteria due to their thicker, denser cell wall and thick layer of peptidoglycan. Gram-negative bacteria exhibit resistance to antibiotics due to their unique envelope structures. According to literature, antimicrobial compounds, such as fluoroquinolones, can penetrate these bacteria through three pathways: hydrophilic, hydrophobic, and self-promoted pathways, although the exact mechanisms of these pathways remains unclear (Chapman & Georgopapadakou, 1988; Marshall & Piddock, 1994). Drug resistance is influenced by the differences in envelope structures among different bacteria, which makes it easier to penetrate cells (Berlanga *et al*., 2004; Hancock, 1997). Thus, AgNPs have an advantage (Abalkhil *et al*., 2017) and interact with the bacterial

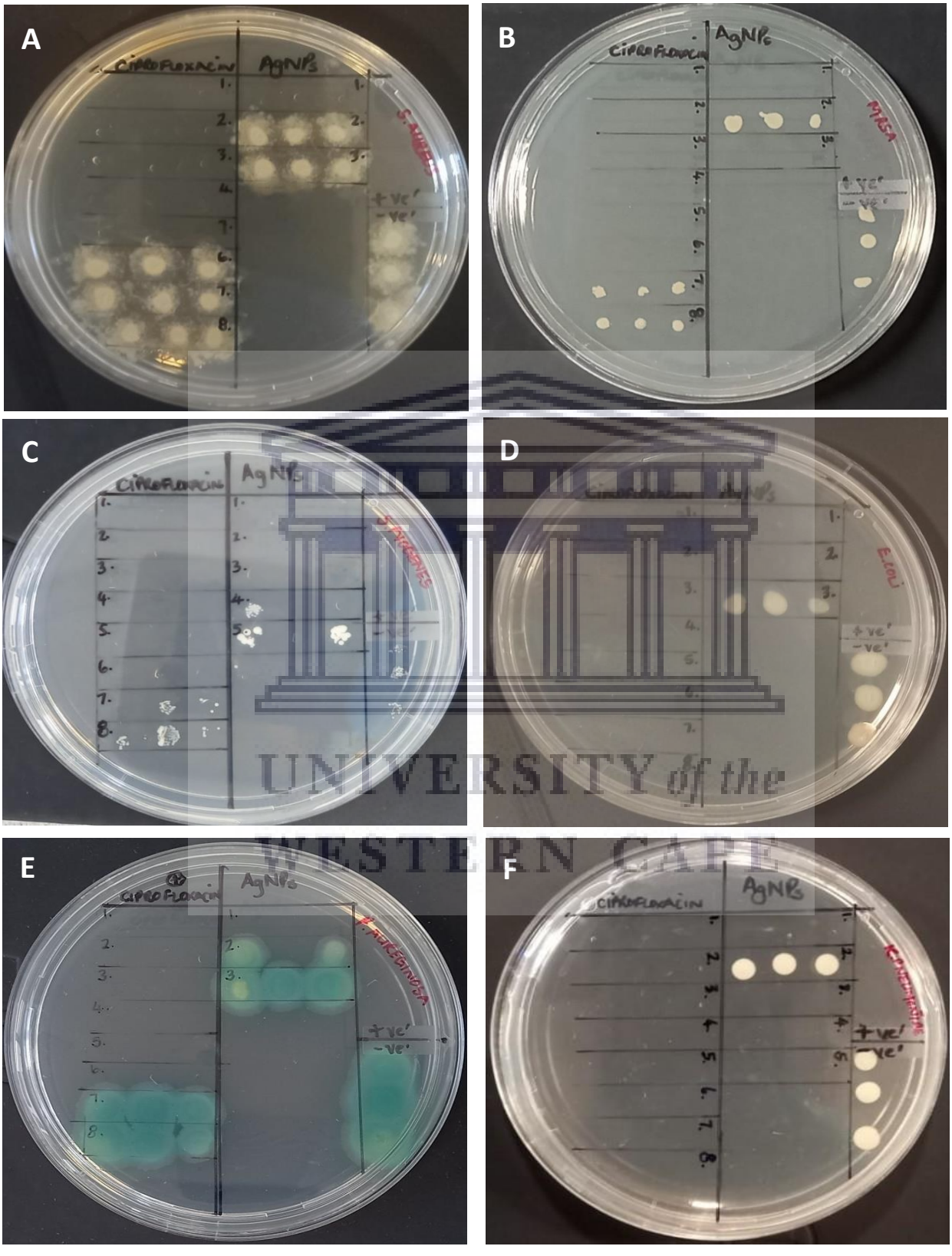
membranes (Dakal *et al*., 2016). The antibacterial activity of smaller AgNPs (<30 nm) showed optimal activity against *K. pneumonia* and *S. aureus*, suggesting that AgNPs may inactivate respiratory chain dehydrogenases killing the pathogens (Kim & Lee, 2012).

EABE-AgNPs exhibited the highest antibacterial activity against *E. coli*, with both MIC and MBC values of 0.8 and 32.3 µg/ml, respectively. According to a study by Fenq *et al*., *S. aureus* is less permeable to silver ions when compared with *E. coli* (Feng *et al*., 2000). These pathogens cause a major threat in non-social, increasing AMR. *E. coli* strains are the primary cause of complex UTIs. In particular, the uropathogenic strains accounts for approximately 50% of hospital acquired and 95% of community-acquired infections (Guiton *et al*., 2010). A recent study in Egypt found that 62% of *E. coli* strains from outpatients with community-acquired UTIs, were MDR (Farahat *et al*., 2021). Uropathogenic *E. coli* strains are projected to adversely impact 150 million people globally annually (Flores-Mireles *et al*., 2015). Furthermore, with a substantial increase in healthcare expenditures, reaching more than $6 billion dollars per annum (Akoachere *et al*., 2012; Yassin *et al*., 2022b).

Overall, the antibacterial effects of EABE-AgNPs did not differ amongst all the strains. The lowest antibacterial activity recorded for EABE-AgNPs, except on *E. coli* and *S. pyogenes*. The lowest MBC recorded for EABE-AgNPs was against *S. pyogenes* and *E. coli* at 8.0 and 32.3 µg/ml. While the MBC for *S. aureus*, MRSA, *K. pneumonia, P. aeruginosa* and *A. baumannii*, was 64.5 µg/ml. These results are similar to a study conducted by Ghaedi *et al*., (2015) who also showed that AgNPs synthesized from *Rosmarinus officinalis* leaf extract had a lower MIC against *P. aeruginosa* (193.31 µg/ml than *E. coli* (386.62 µg/ml), *S. aureus* (773.24 µg/ml) and *B. subtilis* (1546.4 µg/ml) (Li *et al*., 2017; Wypij *et al*., 2021).

The antibacterial effects of the EABE-AgNPs and Ciprofloxacin followed similar trends. The highest antibacterial activity recorded for Ciprofloxacin was against *S. pyogenes,* MRSA *and P. aeruginosa* with the same MIC value of 0.2 µg/ml and same MBC value of 0.4 µg/ml, except for MRSA that had an MIC value of 0.4 µg/ml. The broad-spectrum antibacterial effects of the EABE-AgNPs and Ciprofloxacin suggests that their inhibitory effects may follow similar mechanisms. The small size of EABE-AgNPs allowed easy penetration into microbial cells, disrupting cellular structure, and producing ROS (Hosseini Bafghi *et al*., 2021; Khorsandi *et al*., 2021; Naskar &

Kim, 2019). These substances can alter signal transduction pathways, bind to drugs, while delivering them into the bacterial cell. EABE-AgNPs may be an effective antibacterial agent against human infections. However, the problem with MDR bacteria emerging due to the overprescribing and/ or misuse finds biogenically produced NPs appealing owing to their potential efficacy with minimum adverse effects (Qidwai *et al*., 2018).



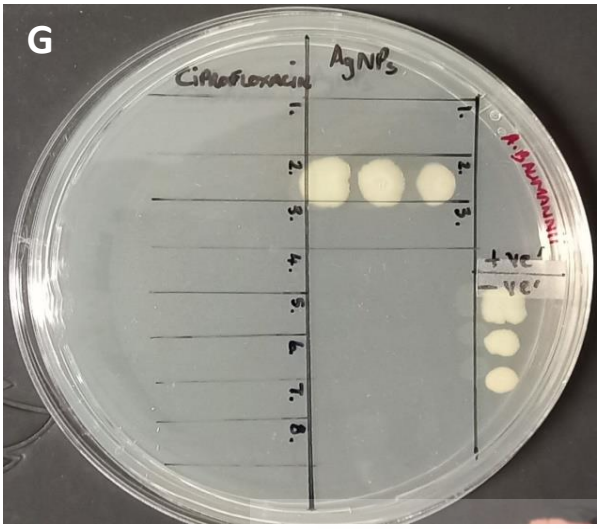


Figure 3.15: The bactericidal effects of EABE-AgNPs on seven different bacterial strains (A: *S. aureus*, B: MRSA, C: *S. pyogenes*, D: *E. coli*, E: *K. pneumoniae*, F: *P. aeruginosa* and G: *A. baumannii*). Row number 1 – 8.

In 2019, lower respiratory infections and diarrheal illnesses were among the top 10 causes of mortality globally, according to the WHO (Lugo & Rivera, 2023). While in LMICs, diseases were among the top ten primary causes of mortality (Mohan *et al*., 2023). In 2019, a study demonstrated that bacterial pathogens accounted for 55% of the 55.4 million deaths, occurred worldwide (Safiri *et al*., 2023). While Enterobacteriaceae family, primarily *E. coli*, *E. aerogenes*, *E. cloacae*, *K. pneumoniae*, and *Providencia stuartii*, are susceptible to drug-resistance. *K. pneumoniae* causing life-threatening infections, and carbapenem antibiotic resistance, which is common across the world. Because of the reported colistin and vancomycin resistance of ESKAPE pathogens, new antimicrobial combinations are needed to combat the high prevalence of the MDR bacterial strains in hospital settings and ICUs (Yassin *et al*., 2022b). Antibiotic such as, Colistin and vancomycin are the only last resort drugs currently available for fatal diseases (Kharaba *et al*., 2024; Tartor *et al*., 2021), resulting in infections for which there is currently no effective antibiotic treatment (Paneri & Sevta, 2023). *S. aureus* is prevalent in natural skin flora and is also responsible for diseases in the community and healthcare facilities. Individuals infected with MRSA are 64% more likely to die than those infected with drug-sensitive pathogens (Faheem *et al*., 2021; Ugwu *et al*., 2016). A recent report suggests that 40 – 60% of *S. aureus* strains in United States (US) hospitals are drug

resistant (Gupta *et al*., 2019). On the contrary, mortality in drug-resistant *P*. *aeruginosa* infections is growing, accounting for around 11% of hospital-acquired bacterial infections. Despite these challenges, antibiotic treatment remains a crucial aspect of managing infections (C Nwobodo *et al*., 2020; Kharaba *et al*., 2024). Research has shown that active botanicals and phytochemicals from African flora can effectively combat these bacteria. Traditional cutoff factors for categorizing antibacterial agents against Enterobacteria were identified from 179 crude plant extracts (Demgne *et al*., 2021; Dzotam *et al*., 2016; Ghisalberti *et al*., 2005; Kuete, 2023; Mohan *et al*., 2023; Seukep *et al*., 2020). The prevalence of antibiotic resistance worldwide, suggests that effective antibiotics for treating common bacterial infections (such as HAIs, UTIs, sepsis, STIs, and diarrhoea) are running out (Ranjbar & Alam, 2023; Salehi *et al*., 2022). Therefore, alternative therapeutic agents are urgently required to fight MDR bacteria and combat AMR pathogens.

CHAPTER 4: CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

Antimicrobial resistance (AMR) significantly remains a leading cause of death worldwide, with rates expected to rise by 2050. Conventional treatments for AMR infections are urgently needed due to their inefficiency, frequent dosages, and potential side effects. New, more effective therapeutic approaches are needed to address antibiotic-resistant bacteria and alleviate the need for alternative treatments. MDR strains and antibacterial drug use are increasing, with side effects being a significant concern. An alternative method is needed to effectively inhibit the growth of these microorganisms, as the use of antibacterial drugs is becoming more prevalent due to the side effects. The aim of this study was to investigate the antibacterial effects of biogenic silver nanoparticles (AgNPs) synthesized using *Eucomis autumnalis* and their potential as an effective antibacterial agent. Furthermore, this study successfully synthesized EABE-AgNPs through green synthesis using *E. autumnalis* bulb aqueous extract, followed by characterization and *in vitro* antibacterial activity assessment of EABE-AgNPs by demonstrating their potential as an effective antibacterial agent.

Overall, the optimized biogenic synthesized EABE-AgNPs exhibited antibacterial activity against all multidrug-resistant tested. From these findings, all seven strains showed growth inhibitory and bactericidal activity, with gram-negative bacteria being more susceptible to the antibacterial action of the EABE-AgNPs compared to gram-positive bacteria. The antibacterial effect of EABE-AgNPs was consistent with the standard concentration (natural agent if MIC value ≤1000 μg/ml) of an antibacterial agent, as it was able to inhibit bacteria growth in multiple wells (96 well plate). Although Ciprofloxacin outperformed EABE-AgNPs during this investigation, it is safe to say this biogenic AgNPs performed better than a clinal study that reported adverse effect of Ciprofloxacin after it was taken orally (Ball, 1986).

In conclusion, this study successfully synthesized, characterized, and evaluated the antibacterial activity of biogenic EABE-AgNPs, thereby achieving the main objectives. These findings have proven the hypothesis, that phytochemicals present in *E. autumnalis* bulb aqueous extract may act as reducing, stabilizing, and capping agents in the synthesis of AgNPs with antibacterial activities. Moreover, green synthesized EABE-AgNPs have shown potential

in preventing the spread of AMR infections, offering novel alternative treatment and the potential to eradicate the burden of multidrug-resistant bacterial infections.

4.2 Recommendations and Future work

Given that the findings of this study have shown that EABE-AgNPs has potential to be used as an antibacterial agent, the EABE-AgNPs can be utilized in other products with beneficial properties (i.e., medicinal). Numerous MNPs possess antimicrobial properties, their applications are not limited to biomedicine, but can also include water treatment, textiles, food packaging, cosmetics, agriculture, and self-cleaning coatings. Therefore, the various applications of EABE-AgNPs can be explored in these fields.

4.3 Study limitations

In order to interpret the results of the present study, it is important to consider various limitations. Some limitations include the handling and preparation of the EABE before synthesis of EABE-AgNPs. The handling of the EABE involved the continuous freezing and thawing of the EABE to preserve the EABE, however, these processes may have compromised the biochemical composition of the biomolecules found within the EABE. The EABE was not prepared using drying methods. The vast range of drying methods is used to prepare plant material and is expected to modify the particles of the material and facilitate the release of bioactive compounds. Therefore, the handling and preparation of the EABE may not have yielded the maximum quantity of bioactive compounds. This may have played a role in EABE-AgNPs being synthesized with biological activity that may have the potential to be enhanced.

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