# Biogenic Synthesis of Silver Nanostructures from Propolis and Bee Pollen: Antibacterial, Antioxidant, and Biosensing Applications

**Gemechis Waktole Bayisa**

**A Dissertation Submitted to the Department of Applied Biology**
**College of Applied Natural Sciences**

**Presented in a Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Applied Biology (Specialization in Health Biotechnology)**

**School of Postgraduate Studies**
**Adama Science and Technology University**

**June, 2025**

**Adama, Ethiopia**

# Biogenic Synthesis of Silver Nanostructures from Propolis and Bee Pollen: Antibacterial, Antioxidant, and Biosensing Applications

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# DECLARATION

I declare that this dissertation entitled "**Biogenic Synthesis of Silver Nanostructures from Propolis and Bee Pollen: Antibacterial, Antioxidant, and Biosensing Applications**" is my own work and has not been submitted to any university for similar purpose. The references used in this dissertation are duly recognized by proper citations.

| Name of student | Signature | Date |
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## RECOMMENDATION OF ADVISORS

I, the major supervisor of this PhD Dissertation, hereby certify that I have closely supervised the student while developing this proposal and read the dissertation entitled **"Biogenic Synthesis of Silver Nanostructures from Propolis and Bee Pollen: Antibacterial, Antioxidant, and Biosensing Applications"** prepared under my guidance by **Gemechis Waktole**. Therefore, we recommend the submission of the dissertation to the department for further review and evaluation.

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## APPROVAL SHEET

We hereby certify that the recommendation and suggestion given by the dissertation review committee are appropriately incorporated into the final dissertation proposal entitled **"Biogenic Synthesis of Silver Nanostructures from Propolis and Bee Pollen: Antibacterial, Antioxidant, and Biosensing Applications"** by **Gemechis Waktole**

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# APPROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the dissertation open defense by **Gemechis Waktole Bayisa** have read and evaluated the dissertation entitled "**Biogenic Synthesis of Silver Nanostructures from Propolis and Bee Pollen: Antibacterial, Antioxidant, and Biosensing Applications"** and examined the candidate during open defense. This is therefore to certify that the dissertation is accepted for the partial fulfillment of the requirement of the degree of Doctor of Philosophy in Applied Biology (Specialization in Health Biotechnology).

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Final approval and acceptance of the dissertation is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC)

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| School of Postgraduate Studies, Dean | Signature | Date |

# DEDICATIONS

This dissertation is dedicated to the memory of my beloved father, **Waktole Bayisa**. He was more than a father to me; he was my first teacher, mentor, and greatest inspiration. His sudden death from glioblastoma multiforme left a deep wound, but his legacy continues to fuel my drive.

From the start of my academic journey, he was my guiding light. He instilled a love of learning, a drive for excellence, and a strong belief in my abilities. His encouragement was crucial to my decision to pursue this doctorate, making those early years particularly significant. Although he didn't live to see its completion, his teachings and values are fundamental to all I've accomplished.

Tragically, my dedication to this degree prevented me from being at his side during his final moments and attending his funeral. This absence is a heavy burden, but I find comfort in knowing that this work is a testament to his lasting influence. This dissertation is as much a reflection of his dedication as it is of my own.

To my beloved father, my guiding light, mentor, and greatest inspiration: Your wisdom, dedication, and unwavering support were essential to my growth and fueled my academic pursuits. I dedicate this work to you, with deepest gratitude and respect.

Rest in eternal peace, Abbakoo.

## BIOGRAPHICAL SKETCH

The candidate, Gemechis Waktole Bayisa, was born in March 1988 to his parents, Shibire Gudeta and Waktole Bayisa, in Gimbi, West Wollega, located 441 kilometers west of Addis Ababa, Ethiopia. His educational journey began at Walo Yesus and Melka Bidiru Primary Schools, where he completed his primary education between 1995 and 2002. He then attended Gimbi Comprehensive High School for his secondary education from 2003/4 to 2007/8, laying a strong foundation for his future academic pursuits.

In 2008/2009, he joined Wollega University, where his dedication and hard work led him to earn a Bachelor of Science (BSc) degree in Applied Biology in 2010/2011. After graduating, he began his professional career as a Junior Researcher I at the Oromia Agricultural Research Institute, marking the start of his journey in research and academia. Driven by a passion for learning, he pursued a Master of Science (MSc) in Biotechnology, enrolling in the postgraduate program in 2014/15 from Haromaya University. Upon completing his MSc, he served as the Livestock Research Process Owner at the Fadis Agricultural Research Center, of the Oromia Agricultural Research Institute.

Afterward, he became a lecturer and Director of Undergraduate Programs at Dambi Dollo University. In 2020/21, he embarked on his PhD journey at Adama Science and Technology University, dedicating himself to advanced research in Biotechnology. Over the past few years, he has worked tirelessly to refine his expertise and contribute to his discipline. His PhD dissertation, the product of years of dedicated work, is now ready for presentation, showcasing his commitment to biotechnology.

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My deepest appreciation goes to my family my wife, children (Nathan and Elmaya), mother, brothers, and sisters for their patience, love, and encouragement. I am also grateful to Adama Science and Technology University and Dambi Dollo University for their resources and support. Finally, I thank all my friends who contributed to this achievement, which is as much theirs as it is mine.

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# LIST OF ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| ATCC | American Type Culture Collection |
| Ag NS | Silver Nanostructure |
| CFU | Colony Forming Unit |
| CUPRAC | Cupric Ion Reducing Antioxidant Capacity |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl hydrate |
| EDS | Energy Dispersive X-ray Spectroscopy |
| FTIR | Fourier Transform Infrared Spectroscopy |
| HBRC | Holetta Bee Research Center |
| ICDD | International Centre for Diffraction Data |
| IZ | Inhibition Zone |
| JCPDS | Joint Committee on Powder Diffraction Standards |
| LSPR | Localized Surface Plasmon Resonance |
| MBC | Minimum Bactericidal Concentration |
| MDR | Multidrug Resistant |
| MIC | Minimum Inhibitory Concentration |
| Pol-Ag NS | Pollen Silver Nanostructure |
| Pro-Ag NS | Propolis Silver Nanostructure |
| SA | Scavenging Activity |
| SEM | Scanning Electron Microscopy |
| SPR | Surface Plasmon Resonance |
| ROS | Reactive Oxygen Species |
| TEM | Transmission Electron Microscopy |
| UV-Vis | Ultra Violet Visible Spectroscopy |
| VeA | *Vernonia amygdalina* |
| XRD | X-Ray Diffraction |
| ZP | Zeta potential |

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# ABSTRACT

*The increasing threat of antimicrobial resistance (AMR) demands innovative solutions against pathogenic bacterial infections and biofilms. Oxidative stress, linked to numerous chronic diseases, highlights the need for effective antioxidants. Additionally, early and accurate detection of hydrogen peroxide ($H_2O_2$) is crucial for disease diagnosis and environmental monitoring. This study aimed to develop an eco-friendly synthesis of silver nanostructures (Ag NSs) using honeybee-collected Pollen (Pol-Ag NSs) and Propolis (Pro-Ag NSs), characterize their physicochemical properties, evaluate their antimicrobial and antioxidant potential, and explore the sensing potentials of palladium-decorated Ag NSs (Pd@Ag NSs) for $H_2O_2$ detection. Pol-Ag NSs and Pro-Ag NSs were synthesized by reducing silver nitrate ($AgNO_3$) with ethanol extracts of pollen and propolis, respectively. Optimization experiments determined that a 1:10 ratio of 5 mM $AgNO_3$ to extract for Pol-Ag NSs and 1:1 for Pro-Ag NSs, reacted at 80°C for 2 hours, yielded stable nanostructures with sharp surface plasmon resonance (SPR) peaks at 411 nm for Pol-Ag NSs and 424 nm for Pro-Ag NSs, indicating narrow size distributions. Fourier-transform infrared (FTIR) spectroscopy elucidated key functional groups involved in reduction and stabilization. Pol-Ag NSs displayed characteristic peaks at 3410 $cm^{-1}$ (O-H, polyphenols), 1635 $cm^{-1}$ (C=O, flavonoids), and 1080 $cm^{-1}$ (C-O, polysaccharides), while Pro-Ag NSs showed additional peaks at 1510 $cm^{-1}$ (aromatic C=C) and 1380 $cm^{-1}$ (C-N, amines), confirming their roles as reducing and capping agents. X-ray diffraction (XRD) analysis verified a face- centered cubic (FCC) crystal structure, with crystallite sizes of 12.1 nm for Pol-Ag NSs and 33.76 nm for Pro-Ag NSs. Scanning electron microscopy (SEM) revealed distinct morphologies, with Pol-Ag NSs exhibiting spherical shapes and Pro-Ag NSs displaying rod-like structures. Antibacterial assays demonstrated robust activity against selected Gram-positive (Staphylococcus aureus, Enterococcus faecalis) and Gram-negative (Escherichia coli, Pseudomonas aeruginosa) bacteria via disc diffusion test and determination of minimum inhibitory concentration (MIC), and Minimum bactericidal concentration (MBC). Pol-Ag NSs and Pro-Ag NSs exhibited significant antimicrobial activity, with inhibition zones up to 17.33 ± 1.15 mm at concentrations (50-100 µg/mL). Antioxidant capacity was assessed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays. Both nanostructures demonstrated potent antioxidant effects, with half maximal inhibitory concentrations ($IC_{50}$) of 48.58 ± 1.04 µg/mL (Pol-Ag NSs) and 45.54 ± 0.57 µg/mL (Pro-Ag NSs). In the context of electrochemical applications, Pd@Ag NSs were synthesized, structurally* *characterized by XRD and SEM, and subsequently evaluated for non-enzymatic $H_2O_2$ detection via cyclic voltammetry (CV), chronoamperometry (CA), and electrochemical impedance spectroscopy (EIS). Pd@Ag NSs showed excellent electro catalytic performance, with a linear $H_2O_2$ detection range of 5-75 mM ($R^2$ = 0.9947) and efficient charge transfer in EIS. CV revealed a reduction peak at -0.5 V (vs. Ag/AgCl), where CA reveals a limit of detection (LOD) of 1.3 μM. Honeybee-derived Ag NSs offer a sustainable, multifunctional platform for antibacterial, antioxidant, and biosensing applications. Future research should focus on optimizing biocompatibility, scalability, and practical deployment in medical and environmental technologies.*

***Keywords:*** *Biosensor, Green synthesis, Honeybee Pollen, Hydrogen peroxide, Limit of Detection, Optimization, Propolis, Sensitivity.*

# 1. INTRODUCTION

## 1.1. Background

The escalating threat of antimicrobial resistance (AMR), oxidative stress, and the crucial need for early hydrogen peroxide ($H_2O_2$) detection, pose significant global health challenges. The World Health Organization estimates that AMR could cause 10 million deaths annually by 2050 if left unaddressed (Guevara, 2023), necessitating innovative solutions to combat bacterial infections and disrupt resilient bacterial biofilms implicated in chronic infections (Mikhailova, 2024). Concurrently, oxidative stress, driven by reactive oxygen species (ROS), contributes to a wide range of chronic conditions, including cardiovascular diseases, diabetes, and neurodegenerative disorders, underscoring the urgent need for effective antioxidant interventions (Urcan et al., 2023).

Furthermore, the early and precise detection of $H_2O_2$, a critical biomarker associated with oxidative stress and diseases such as Cancer, Alzheimer's, and Cardiovascular conditions, is vital for timely diagnosis, disease management, and environmental monitoring (Khalifa et al., 2024; Srikhao et al., 2022). The development of highly sensitive, selective, and cost-effective biosensing technologies is thus a priority to address these multifaceted health challenges. Nanobiotechnology, through the precise manipulation of matter at the atomic and molecular scale, has revolutionized healthcare, environmental monitoring, industrial processes, and energy applications (Khan et al., 2025).

Silver nanostructures (Ag NSs) stand out as a cornerstone of this field due to their exceptional antibacterial, antioxidant, and biosensing properties, positioning them as versatile tools for addressing pressing global health issues. Their potent antibacterial activity disrupts bacterial cell membranes and inhibits biofilm formation by interfering with quorum-sensing mechanisms, offering a promising strategy to combat AMR (Mikhailova, 2024). Ag NSs also exhibit antioxidant capabilities, neutralizing ROS and mitigating inflammation, which could alleviate the progression of chronic diseases (Urcan et al., 2023). Additionally, their high electrocatalytic activity enables sensitive and selective detection of $H_2O_2$, making them ideal for developing non-enzymatic electrochemical sensors for point-of-care diagnostics and environmental monitoring (Khalifa et al., 2024; Srikhao et al., 2022). These properties make Ag NSs invaluable for biomedical applications, including targeted drug delivery, wound healing, gene therapy, and real-time biomarker detection, with the potential to transform clinical and industrial practices (Zehra et al., 2025).

The unique physicochemical properties of Ag NS, such as high surface area-to-volume ratios, tunable size, and enhanced reactivity, underpin their suitability for diverse applications (Thakur & Kumar, 2022). However, conventional synthesis methods, including chemical reduction and physical vapor deposition, often rely on toxic reagents, and high-energy inputs, and produce hazardous byproducts, posing significant environmental and biocompatibility concerns (Naganthran et al., 2022). These drawbacks limit their use in sensitive biomedical contexts and conflict with the global push for sustainable technologies. To address these limitations, biogenic synthesis methods have gained traction, utilizing natural resources such as plant extracts, microorganisms, and agricultural waste to produce eco-friendly and biocompatible nanostructures (Rashidi et al., 2024). These methods leverage the inherent reducing and stabilizing properties of biomolecules, offering a cost-effective and environmentally benign alternative to traditional approaches (Liaqat et al., 2022).

Propolis and bee pollen, natural products derived from honeybees, have emerged as promising candidates for the biogenic synthesis of Ag NSs due to their rich bioactive composition (Khalifa et al., 2024). Propolis, a resinous substance collected by bees from plant exudates, contains flavonoids, phenolic acids, terpenoids, and alkaloids, which confer potent antimicrobial, antioxidant, and anti-inflammatory properties (Samanci et al., 2024). These compounds act as natural reducing agents, converting $Ag^{+}$ to $Ag^{0}$, and as capping agents, enhancing nanostructure stability (Singh et al., 2024). Studies on Saudi propolis indicate that propolis-mediated silver nanostructures (Pro-Ag NSs) possess elevated polyphenol levels compared to propolis extracts alone, which may improve their antioxidant and antimicrobial effectiveness (Al-Fakeh et al., 2021). Similarly, bee pollen, a nutrient-rich substance gathered from flowering plants, is abundant in flavonoids, phenolic acids, vitamins, and amino acids, which contribute to its antioxidant and antimicrobial activities and facilitate the formation and stabilization of Ag NSs (Rupanar et al., 2024). The synergy of these bioactive compounds not only ensures the production of stable nanostructures but also enhances their functionality for biomedical applications.

Despite their considerable potential, challenges remain in the biogenic synthesis of Ag NSs from these two honeybee products. The chemical composition of propolis and bee pollen varies due to regional biodiversity, seasonal differences, and bee species, affecting the reproducibility and properties of the resulting nanostructures (Singh et al., 2024). This variability makes it challenging to achieve consistent nanostructure quality and necessitates further research to standardize synthesis protocols despite compositional differences. Additionally, the optimization of synthesis parameters such as pH, temperature, reaction time, and precursor concentration are critical for achieving consistent nanostructure size, morphology, and functionality, yet these factors are often underexplored. Thorough optimization is crucial for developing robust and scalable production methods.

While preliminary studies have demonstrated the antimicrobial and antioxidant properties of Pro-Ag NSs and bee pollen-mediated Ag NSs, their full potential in advanced applications, such as $H_2O_2$ biosensing, remains insufficiently characterized. More comprehensive studies are needed to fully explore their capabilities in this area. The integration of bimetallic systems, such as Ag NSs functionalized with palladium (Pd), could further enhance their electrocatalytic performance for $H_2O_2$ detection, offering improved sensitivity and selectivity compared to monometallic systems (Srikhao et al., 2022). This is an important area for future review and exploration, as combining Ag NSs with other noble metals can lead to synergistic effects that boost their catalytic activity. Finally, while biogenic methods offer environmental benefits and cost-effectiveness at a lab scale, reviews indicate that challenges related to scaling up production for industrial applications, while maintaining desired nanostructure properties and cost-efficiency, still need to be thoroughly addressed.

This study leverages Ethiopia's rich biological resources, specifically propolis and bee pollen from the Dambi Dollo University Integrated Agricultural Research Apiary, to investigate the biogenic synthesis of Ag NSs. The research focuses on optimizing synthesis parameters to produce Ag NSs with tailored characteristics, followed by comprehensive characterization using techniques such as UV-Vis spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier-transform infrared spectroscopy (FTIR). The antibacterial activity of the synthesized Ag NSs was evaluated against different bacterial strains, their antioxidant potential was assessed through ROS scavenging assays, and their $H_2O_2$ biosensing capabilities were tested using electrochemical methods. By addressing the variability in bee product composition and optimizing synthesis conditions, this work aims to develop sustainable, biocompatible, and cost-effective nanomaterials with enhanced antibacterial, antioxidant, and biosensing properties, contributing to advanced healthcare solutions and aligning with global demands.

## 1.2. Statement of the Problem

The global rise of AMR, responsible for millions of deaths annually, poses a significant threat to public health (Murray et al., 2022), necessitating innovative solutions such as biocompatible nanostructures with broad-spectrum antimicrobial activity. Additionally, the lack of sensitive and eco-friendly methods for detecting $H_2O_2$, critical for diagnostics, environmental monitoring, and industrial applications, highlights the need for advanced nanomaterials (Yang et al., 2021), like bimetallic palladium-silver nanostructures (Pd-Ag NPs) with enhanced electrocatalytic properties.

Ethiopia's rich biodiversity, with over 7,000 species of flowering plants, and its well-established beekeeping traditions provide abundant yet underutilized resources like propolis and bee pollen, which are rich in bioactive compounds such as flavonoids and phenolic acids (Rushdi et al., 2014). These compounds show potential as natural reducing and stabilizing agents for the biogenic synthesis of Ag NSs and Pd-Ag NPs, but their application for these purposes remains largely unexplored. The variability in the chemical composition of Ethiopian bee products due to diverse agro-climatic conditions complicates achieving consistent nanostructure properties, and the absence of standardized protocols hinders reproducible synthesis.

Furthermore, while the bioactive compounds in propolis and bee pollen may enhance nanostructure stability and biocompatibility, their potential as reducing and capping agents remains underappreciated and warrants further exploration. Conversely, conventional nanostructure synthesis methods, such as chemical reduction and physical vapor deposition, rely on toxic reagents and high energy inputs, producing hazardous byproducts that limit their suitability for biomedical applications and conflict with global demands for sustainable technologies (Brown et al., 2012). The lack of research on leveraging Ethiopian bee products for eco-friendly synthesis of Ag NSs and Pd-Ag NPs, combined with insufficient characterization of their physicochemical properties and performance in combating AMR and enabling $H_2O_2$ detection, represents a critical gap in addressing pressing healthcare and environmental challenges.

This study aims to address these gaps by harnessing Ethiopia's propolis and bee pollen for the biogenic synthesis of Ag NSs and Pd-Ag NPs. The research focuses on optimizing synthesis conditions to achieve consistent nanostructure characteristics, characterizing their physicochemical properties using advanced techniques, and evaluating their performance in antibacterial activity against *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), and *Enterococcus faecalis* (ATCC 29212) pathogens, and $H_2O_2$ sensing through voltammetric and amperometric methods. By developing sustainable, biocompatible, and cost-effective nanomaterials, this work seeks to contribute to the global fight against AMR and advance eco-friendly biosensing technologies, leveraging Ethiopia's natural resources to address pressing healthcare and environmental challenges.

## 1.3. Objectives of the Study

### 1.3.1. General Objective

To synthesize and characterize silver nanostructure employing honey bee propolis and pollen as reducing and capping agents, and evaluate their potential for antibacterial, antioxidant and sensing applications.

### 1.3.2. Specific Objectives

- To determine qualitative phytochemical compositions of propolis and bee pollens extracts
- To determine optimal synthesis parameters of Propolis and bee pollen silver nanostructures
- To synthesize silver nanostructures by using honeybee propolis and pollen as reducing agents
- To characterize physicochemical properties of synthesized pollen and propolis silver nanostructures
- To examine the *in-vitro* DPPH scavenging activity of propolis and bee pollen extract with respective silver nanostructures.

- To assess the effectiveness of synthetic propolis and pollen silver nanostructures against selected gram-positive and gram-negative pathogenic bacteria
- To evaluate the performance of palladium-decorated silver nanostructures as biosensor to detect hydrogen peroxide

## 1.4. The Scope of the Study

This study centered on the collection and extraction of bee pollen and propolis, followed by the eco-friendly synthesis of Ag NSs using these natural extracts. The synthesis process was carefully optimized to control the size, shape, and properties of the resulting Ag NSs. The synthesized nanostructures were thoroughly characterized using advanced spectroscopic and microscopic techniques to analyze their physicochemical properties. The antimicrobial activity of the Ag NSs was evaluated against various bacterial strains, while their antioxidant potential was assessed to determine their health benefits.

Expanding on these findings, the study further functionalized the Ag NSs by decorating them with palladium (Pd) to synthesize Pd-Ag NSs. These Pd-decorated nanostructures were then utilized for the electrochemical detection of $H_2O_2$, a molecule of significant importance in biological and environmental processes. The sensitivity, and stability of the Pd-Ag NS -based $H_2O_2$ sensor were systematically evaluated. By exploring the dual potential of these bio-inspired nanomaterials combating microbial infections and oxidative stress while advancing sensing technologies, this research contributes to the development of innovative solutions for healthcare and environmental monitoring. The study highlights the versatility of Ag NSs and Pd-Ag NSs, offering sustainable and efficient approaches to address pressing global challenges.

## 1.5. Significance of the Study

The urgent need for environmentally friendly nanostructure production methods has intensified due to the ecological and biomedical limitations of conventional synthesis approaches that depend on hazardous chemicals and excessive energy consumption. Nature-inspired synthesis using biological compounds presents a viable solution, producing nanostructures with superior biocompatibility and minimal environmental footprint (Bhuyar et al., 2020; Zhao et al., 2022). Silver nanostructures demonstrate particular promise with their versatile antimicrobial, antioxidant, anticancer, and sensing properties (Brown et al., 2012), though current production techniques still face significant biocompatibility challenges. Ethiopia's exceptional floral diversity, featuring more than 7,000 flowering plant species and abundant apicultural products such as propolis and bee pollen (Rushdi et al., 2014), represents an underutilized yet valuable resource for eco-friendly nanostructure synthesis. Despite this potential, significant gaps remain regarding the process optimization of biologically synthesized Ag NSs from these natural sources, especially for addressing the critical global health threat of antimicrobial resistance.

Additionally, although Ag NSs have demonstrated effectiveness in hydrogen peroxide detection, the advantages of bimetallic Pd-Ag NPs through their enhanced catalytic properties (Yang et al., 2021) have not been sufficiently explored in biologically synthesized systems. This investigation addresses these research deficiencies by employing Ethiopian bee products to fabricate carefully designed and comprehensively characterized Ag NSs and bimetallic Pd-Ag NPs. The study includes a rigorous assessment of the nanostructures' antimicrobial performance and antioxidant activity, complemented by systematic electrochemical analysis of their responsiveness and specificity for $H_2O_2$ detection. By advancing these sustainable nanomaterial production techniques, this work contributes to the global fight against drug-resistant infections while promoting the development of green synthesis technologies.

## 1.6. Document Organization

This dissertation is organized into five chapters; Chapter 1 introduces the subject matter giving the background on the synthesis and characterization of Ag NPs and examining their significance in antibacterial, antioxidant, and $H_2O_2$ detection applications. This chapter also identified key research gaps and articulated the motivations for this study, concluding with a clear statement of objectives. Chapter 2 presents a comprehensive related literature review, encompassing the general characteristics of Ag NSs, various synthesis methods, and their antioxidant, antibacterial, and sensing applications. Chapter 3 covers the materials and methods employed in this study, outlining the synthesis procedures, characterization techniques, and application assays. Chapter 4 presents the results obtained, providing insights into the physicochemical properties of the synthesized materials, their antioxidant and antibacterial activities, and their performance in sensing applications. This chapter further discusses the implications of these findings. Finally, Chapter 5 summarizes the key findings, highlighting their significance and proposing recommendations for future research directions.

# 2. LITRATURE REVIEW

## 2.1. Brief Overview of Honeybees and Its Products

Honeybees are members of the *Apidae* family (superfamily *Apoidea*, order *Hymenoptera*) and have over 20,000 species (Engel et al., 2021). However, honey-producing bees are members of two of the four known bee subfamilies: the *Apinae* subfamily (honeybees) and the *Meliponinae* subfamily (stingless bees). There are four main species of honeybees namely: *Apis mellifera*, *Apis florea, Apis cerena* and *Apis dorsata*. *Apis mellifera* originates from Africa, a continent with a high honeybee population compared to Europe (Ruttner, 2013).

A multivariate morphometric analysis in Ethiopia revealed five different *Apis mellifera* clusters, each occupying a specific ecological niche and display unique characteristics. *Apis mellifera monticola*, which lives in the northern highlands and is less aggressive. Thriving in the moist, central highland regions of the country, the Bandansi bee represents a distinct subspecies of honeybee (*Apis mellifera bandansi*) specifically suited to these conditions. *Apis mellifera scutellata* lives in the humid tropical forests and is aggressive. *Apis mellifera jemenetica* inhabits the lowlands and is more defensive than other Ethiopian honeybees. Lastly, *Apis mellifera woyi-gambella*, the world's smallest honeybee, is endemic to Ethiopia and colonizes the semi-arid to sub-humid lowlands at the southwest. *Apis mellifera* is the most beneficial to humans as a provider for pollination service and also honey, pollen, venom, royal jelly and wax. We will focus on two of these products in this study, propolis and pollen (Amssalu et al., 2004).

Bees that produce propolis are abundant, and Ethiopia as a "cradle of bees" can be a potent source of propolis based on its diverse ecological zones and long history of beekeeping. So, it's very likely that the propolis here, made from plants that only occur in Ethiopia, contains a different composition of bioactive compounds than the propolis from other regions including rare flavonoids and terpenes. This has brought Propolis (it is natural antibiotic used by bees to balanced their hives) into the limelight in homeopathic treatment. In fact, honeybees harvest the resin from multiple plant species but are selective in the species they choose. They then process these resins, mixing them with their saliva to make propolis. This complex substance contains more than 300 components and its composition depends on the time and place of harvest (Barbosa et al., 2019).

Flavonoids, aromatic acids, diterpenoid acids, triterpenoids, and phenolic compounds are the key components that contribute to the various biological effects of propolis (Rushdi et al., 2014).

## 2.2. Honeybee collected Pollen and Propolis

Honey bee pollen and propolis are remarkable natural substances produced by honey bees, each offering distinct health benefits despite their shared origin. While both are recognized for their medicinal properties, they differ significantly in their origins, compositions, and primary uses.

Bee pollen, a mixture of flower pollen, nectar, enzymes, honey, and bee secretions, serves as the primary sustenance for a bee colony. Its rich and diverse nutritional makeup, which varies based on the plant and geographical source, typically includes proteins, carbohydrates, lipids, a wide array of vitamins (A, C, D, E, and B vitamins), and essential minerals such as calcium, magnesium, and zinc. This nutritional density, combined with high levels of antioxidants like flavonoids and carotenoids, has led to bee pollen being considered a "superfood." It is valued for its ability to supplement overall nutrition, provide antioxidant and anti-inflammatory effects, boost the immune system, increase energy, support liver health, and potentially assist with wound healing and menopausal symptoms.

The term "pollen," derived from the Latin word for fine powder or flour, was established in the 17th century, though this plant material has been consumed as food for centuries (Bogdanove, 2017). For all Apidae and some other insects, pollen is crucial for development. Currently, pollen is an important ingredient in animal feed, primarily recognized as an excellent protein source and a natural alternative to antibiotics (Abdelnour et al, 2019).

Regarding human consumption, the first comprehensive review of pollen as a dietary supplement was likely published in the 1970s. Stanley and Linskens (1970) reviewed existing data and concluded that pollen was valuable for weight management and preventing certain respiratory infections. Since then, pollen has often been lauded as "the world's best food product" and the "only perfectly complete food" (Ares et al, 2018). Consequently, bee pollen-based products, such as those found in bakeries, confectionery, juices, meat products, and fermented foods, are widely popular in developed nations. Various pollen products are available on the market, including granules, capsules, tablets, pellets, and powders. The recommended daily intake for adults typically ranges from 20 to 40 grams (Li-Q et al, 2018). The extensive industrial applications of pollen have also been thoroughly reviewed by Aleksandar et al (2020).

Honeybees collect and mix the male reproductive component, called flower pollen, with their digestive enzymes. Bees pack it into the cells, creating a nutrient-rich substance known as "bee bread," which is a crucial food source in the hive. Bioactive components such as polyphenols, flavonoids, vitamins and amino acids (El Ghouizi et al., 2023). It is also made up of sugars, proteins, minerals, vitamins, fatty acids, etc. Bee pollen has traditionally been used in the treatment of a wide range of health problems and is gaining considerable commercial attention because of its nutritional, antioxidant and antibacterial properties. Honey bee venom is another product derived from honey bee with a potential medicinal and nutritional substance, honey bee venom has proven to be a valuable agent in apitherapy with considerable action spectrum (Algethami et al., 2022) demonstrating properties like local anesthetic, antifungal, antibacterial, antiviral, anti-inflammatory, hepatoprotective and anticancer immunostimulant. In addition, it includes triterpene linkages, particularly oleanolic acids, 3-ursolic acid, and betulin alcohol (Komosinska-Vassev et al., 2015). Further studies are required to standardize extraction methods, develop optimal synthesis parameters, and study long-term stability and toxicity.

Notably, Ethiopia's exceptional biodiversity, characterized by a vast array of flowering plants across diverse agro-climatic zones, contributes significantly to the unique and rich composition of its bee pollen. Studies on Ethiopian bee pollen consistently reveal high levels of essential nutrients and strong antioxidant activity, with specific mineral and phenolic content varying based on the abundant local flora, such as *Guizotia* sp., *Brassica* sp., *Trifolium* sp., and *Eucalyptus* sp. (Addi et al., 2017; Damto et al., 2024; Yadeta et al., 2024).

Propolis, often referred to as "bee glue," is a resinous substance bee collect from plants, tree buds, and sap, which they then mix with beeswax and salivary enzymes. Bees utilize propolis to seal, strengthen, and sterilize their hive, protecting it from pathogens. Its chemical composition, highly variable by origin and plant source, primarily consists of resins and vegetable balsam (rich in polyphenols and flavonoids), waxes, essential oils, and a small amount of pollen. The therapeutic properties of propolis are largely attributed to its high polyphenol and flavonoid content, making it a powerful antimicrobial agent against bacteria, viruses, and fungi. It also exhibits strong anti-inflammatory and antioxidant activities, promotes wound healing, modulates the immune system, and is widely used for oral health. Furthermore, some research indicates that propolis may possess potential anti-cancer properties (Forma and Bryś,2021). In essence, while bee pollen shines as a comprehensive nutritional supplement supporting vitality and immunity, propolis stands out for its potent antimicrobial, anti-inflammatory, and wound-healing capabilities, making both invaluable natural remedies from the beehive. Given Ethiopia's remarkable biodiversity, which includes diverse flora and varied agro-climatic conditions across regions, the propolis collected here exhibits unique chemical profiles, often rich in saponins, tannins, flavonoids, steroids, and triterpenes, as reported in various studies (Ayad et al., 2025). This distinct composition underscores the significant potential of Ethiopian propolis for further research and development in pharmaceuticals, traditional medicine, and food industries, leveraging its varied bio-active compounds to address pressing health and wellness needs.

## 2.3. Overview of Nanotechnology

Nanotechnology focuses on the design and manipulation of systems at the nanoscale (1-100 nm), for advances in medicine, industry, and agriculture. At this scale, materials exhibit unique properties, such as increased surface area and quantum effects, enabling innovative applications like targeted drug delivery and advanced diagnostic techniques, and regenerative therapies (Brindhadevi et al., 2023). Pourmadadi et al., (2023) showed that nanotechnology facilitates precise drug delivery for cancer treatment and enables highly sensitive disease detection. Additionally, nano-silver enhances the fight against antimicrobial resistance, while other nanomaterials improve antioxidant properties (Zhou et al., 2024). Nanoscale biosensors also allow real-time monitoring of biomarkers (Hemdan et al., 2024). In agriculture, nanotechnology enhances biofertilizer applications by creating nano-biofertilizers, which combine the benefits of nanostructures and biofertilizers. This leads to improved nutrient availability, enhanced crop growth, and increased resilience to environmental stresses like drought and salinity (Diksha Grag et al., 2023).

In electronics, it drives device miniaturization, enhancing speed and energy efficiency, and advancing computing and communication technologies (Malik et al., 2023). In the energy sector, nanomaterials improve the efficiency of solar cells, batteries, and fuel cells, potentially reducing the environmental impact of energy production. Nanotechnology also offers innovative solutions for water purification and contaminant removal at the nanoscale (Ajith et al., 2021).

Through nanotechnology, materials science is yielding superior materials, characterized by increased strength, reduced weight, and enhanced durability, which are now seamlessly incorporated into daily life. Nanomaterials are increasingly incorporated into consumer products such as cosmetics, textiles, and other goods, significantly enhancing their performance and functionality (Abbasi et al., 2020). Beyond consumer applications, nanotechnology holds transformative potential across various industries, demonstrating its capacity to address complex challenges.

## 2.4. Nanoparticles

Metallic nanostructures have excellent properties due to their unique electronic structures and surface plasmon resonance (Khurana& Jaggi, 2021). Surface plasmon resonance refers to the collective oscillation of electrons at the surface of the nanostructures, enabling strong interactions with light and distinctive optical features. These nanostructures are, therefore, excellent conductors of electricity and heat, versatility that is critical in electronic equipment for use in conducting inks, interconnect and sensors, etc. Optical properties of those are utilized in surface-enhanced Raman spectroscopy (SERS) for the detection at a highly sensitive level and in plasmonic devices to control light at a nanoscale. Ag NSs, having antimicrobial characteristics, are usually located in film dresses, coatings, and water microfilter systems. Platinum and palladium nanostructures show great catalytic activity for industrial applications and clean energy technologies (Alaqarbeh et al., 2023).

Oxide nanostructures (ONPs), metal atoms bound to oxygen display semiconducting behavior often influenced by the electron bandgap (BG) of fillers. They have a large surface area and their natural stability, wonderful characteristics for catalysis, allow many chemical reactions depending on the environment (Mitchell et al., 2021). Titanium dioxide ($TiO_2$) nanostructures act as photocatalysts, degrading organic pollutants upon irradiation with ultraviolet (UV) light. This ability makes them beneficial in applications like self-cleaning surfaces, air purification systems, and UV-protective sunscreens (Nagaraj et al., 2025). Zinc oxide (ZnO) nanostructures are also UV protectants with applications in cosmetics, sensors, and transparent electronics. Ferromagnetic iron oxide nanostructures, notably magnetite ($Fe_3O_4$), are therefore attractive candidates for magnetic data storage devices, Magnetic Resonance Imaging (MRI) contrast agents, and targeted drug delivery due to these magnetic properties (Irede et al., 2024). Cerium oxide nanostructures have antioxidant properties, which signifies possible therapeutic applications in neurodegenerative diseases (Sundararajan et al., 2021). Oxide nanostructures play an important role in various fields and are widely used in different industries to solve environmental, medical, and technological problems.

Polymer-based nanostructures consist of large molecules containing repeating units, and this property allows them to provide ample functionalities due to their tunable properties whch are the ability to adjust or modify a material's characteristics in a desired way, often in response to external stimuli or changes in the environment (Geszke-Moritz & Moritz, 2024). Since many of these nanostructures are biocompatible and biodegradable, they are well-suited for biomedical applications, especially for drug delivery. Liposomes are composed of lipid double layers, and they can encapsulate and encode hydrophilic and hydrophobic drugs with a certain controllability. Dendrimers with a highly branched structure and a well-defined size can be adequately functionalized with targeting ligands that allow for site-specific delivery and lower off-target effects (Kedar et al., 2024). Polymeric micelles, consisting of self-assembled structures composed of amphiphilic block copolymers, improve the solubility of hydrophobic drugs and thus, increase their bioavailability. Chitosan is a naturally occurring polymer derived from crustacean shell, which is biodegradable and possesses inherent antibacterial properties, making it an ideal candidate for wound healing and tissue engineering applications (Ul-Islam et al., 2024). Polymers can be synthesized as nanostructures with customizable size, shape, and surface chemistry, allowing them to be tailored for diverse therapeutic applications, enhancing treatment efficacy and reducing side effects.

Additionally, carbon exists in numerous forms, and carbon-based nanostructures exhibit a remarkable variety of properties, which vary depending on the specific arrangement of their constituent carbon atoms. Carbon-based nanostructures are powering innovation across sectors like materials science and biomedicine, thanks to their diverse structures and properties (Parvin et al., 2024).

Core shell nanostructures are a class of biphasic nanomaterials characterized by an inner core structure surrounded by an outer shell, each made from different materials or compositions (Nomoev et al., 2015). The core and shell can be composed of various combinations, including inorganic/inorganic, inorganic/organic, organic/inorganic, and organic/organic materials (Tiwari et al., 2021). These nanostructures are designed so that the shell material can improve the reactivity, thermal stability, or oxidative stability of the core, or allow the use of a less expensive core material coated with a thin, more expensive or functional shell. The shell can also provide biocompatibility, protect the core from oxidation, and serve as anchor sites for molecular linkages, making core-shell nanostructures highly versatile in fields such as biomedicine, catalysis, electronics, and drug delivery (Chiozzi& Rossi, 2020).

The synthesis of core-shell nanostructures can be controlled by varying experimental parameters such as temperature, time, and concentration, which influence the size, shape, and end-use properties of the particles (Tiwari et al., 2021). Their unique structure enables them to exhibit properties that are distinct from those of their individual core or shell materials, making them highly functional and adaptable for advanced technological applications (Chiozzi and Rossi, 2020).

## 2.5. Nanostructures

A nanostructure, for example silver nanostructure is any material or object possessing at least one dimension on the nanoscale, typically ranging from 1 to 100 nanometers. This minute size is critical because it's where materials often exhibit fundamentally new and enhanced properties compared to their bulk counterparts, primarily due to quantum mechanical effects and a significantly increased surface area-to-volume ratio. Nanoparticles represent a specific type of nanostructure where all three dimensions fall within this nanoscale range, making them zero-dimensional (0D) nanostructures. While all nanoparticles are, by definition, nanostructures, the reverse isn't true; nanostructures also encompass other forms, such as nanowires (1D, extended in one dimension) and nanofilms (2D, extended in two dimensions), each with distinct properties and applications emerging from their unique dimensional confinement (Paras et al., 2022).

Nanostructures, owing to their unique size-dependent properties, such as quantum confinement and an elevated surface-to-volume ratio, possess remarkable characteristics that render them invaluable across numerous sectors, particularly within biomedical applications. In the field of medicine, these materials are revolutionizing drug delivery by enabling the accurate and controlled administration of therapeutic substances, including anticancer drugs encapsulated in polymeric nanostructures or liposomes. This precision significantly curtails systemic toxicity while simultaneously boosting treatment efficacy. Beyond their role in targeted delivery, nanostructures such as quantum dots and magnetic nanostructures function as superior contrast agents for cutting-edge bioimaging techniques, including magnetic resonance imaging (MRI) and fluorescence imaging. This capability enables earlier and more accurate disease diagnosis, paving the way for timely medical interventions (Wu et al., 2017).

Moreover, the inherent antimicrobial properties of nanostructures, notably silver and titanium dioxide nanostructures, are opening up new frontiers in the fight against infections. These properties allow for the development of advanced wound dressings and enhanced medical implants that actively resist microbial colonization. In the field of tissue engineering, silver nanostructure provides an optimal microenvironment that supports cell proliferation and tissue regeneration, offering promising solutions for repairing damaged tissues and organs (Kim et al., 2014). Concurrently, advancements in biosensing are harnessing the power of nanostructures to detect biomarkers and pathogens with unparalleled sensitivity (Hemdan et al., 2024). This heightened detection capability is instrumental in developing highly personalized and effective healthcare solutions, ultimately improving patient outcomes.

## 2.6. Silver Nanostructure Properties

Nanostructures, including Ag NSs, exhibit unique properties due to their high surface area-to-volume ratio. The larger surface area enhances the chemical reactivity of Ag NS which influences the antioxidant and antimicrobial properties of Ag NSs. This high surface area-to-volume ratio contributes to the greater reactivity of smaller Ag NSs (Duman et al., 2024). Quantum effects in materials become more prominent as the materials minimize in size (Khan & Hossain, 2022), and manipulation of nanostructure size allows for fine-tuning of properties such as melting point, fluorescence and conductivity. These attributes enable the fabrication of composites designed with specific functions, opening the door to a wide range of potential uses from electronics to localized drug delivery. Silver nanostructures, as a broad class of nanomaterials, are extensively studied due to their unique physical, chemical, and biological properties that correlate with their size, shape, and surface properties (Duman et al., 2024). The performance of Ag NSs is greatly influenced by their shape. Different shapes spheres or rods, for instance modify their optical and electronic properties, which in turn influences their interactions with bacteria and free radicals.

Moreover, Ag NSs surface chemistry is of great importance for their stability, compatibility with living tissues and their effectiveness in biological applications. A well-known phenomenon associated with Ag NSs, surface plasmon resonance, allows for rational light absorption and scattering at certain wavelengths, which are determined by size, shape and environmental parameters (Khurana & Jaggi, 2021). Whereas SPR is critical for biosensors, SERS, and numerous other applications, its contribution to antimicrobial and antioxidant activity is less direct.

When materials shrink in size, size-depend effects known as quantum effects begin to dominate. The electronic structure and energy levels become quantized, resulting in size-dependent properties for optical, electrical and magnetic (Gautam et al., 2024). For example, the electrical conductivity of nanowires can be fine-tuned by adjusting their diameter, paving the way for nano electronic applications. These size-dependent properties supply a useful way to tailor nanomaterials for specific applications (Ghobashy et al., 2024). These unique features can be grouped into modified melting points, mechanical features, increased chemical reactivity, unique optical interactions (for instance, SPR), and tunable electrical conductivity (Saha et al., 2022). Ag NSs with Variety of Functions Ag NSs have a range of functions due to the interaction of size, shape and surface properties and possibly SPR (surface plasmon resonance).

The distinct properties of nanomaterials, such as Ag NSs, will only be understood when the science behind these materials is unlocked, as they offer innovative applications across many fields. In medicine, such materials enable targeted drug delivery, improved diagnostics, and even regenerative therapies (Wang et al., 2024). In the field of electronics, they are vital to enable miniaturization, higher speed, and reduced power consumption for devices (Zhu et al., 2021). Nanomaterials are also extensively utilized in energy applications (Gohar et al., 2024) for improving solar cell and battery performance. They are also key in environmental science, helping to purify water and remediate pollution. The distinctive properties of nanomaterials mean that ongoing research into them will likely suggest even more novel applications, shaping many technologies and industries for decades to come.

## 2.7. Synthesis Methods for Ag NSs

The synthesis of silver nanostructures can be broadly classified into three principal approaches: chemical methods, physical methods, and green (biogenic) methods. Each of these methodologies presents distinct advantages and limitations that influence their applicability, scalability, and environmental impact.

### 2.7.1. Chemical Synthesis

Chemical synthesis represents a prevalent approach for Ag NS production, typically involving the reduction of silver ions ($Ag^+$) to elemental silver ($Ag^0$) using various chemical reducing agents such as sodium borohydride ($NaBH_4$) and hydrazine ($N_2H_4$). To prevent the agglomeration of the nascent nanostructures due to their high surface energy, stabilizing or capping agents like polyvinylpyrrolidone (PVP) or cetyltrimethylammonium bromide (CTAB) are commonly employed (Duman et al., 2024). While chemical methods can often yield large quantities of Ag NSs with controlled size and morphology, a significant drawback lies in the inherent toxicity and non-biodegradability of many chemicals utilized. These substances can pose substantial hazards to human health and the environment, raising concerns about their sustainability and long-term impact. Furthermore, residual chemicals adsorbed on the Ag NS surface may limit their biocompatibility and applicability in certain areas, such as biomedical applications.

### 2.7.2. Physical Synthesis

Physical synthesis methods offer an alternative route to Ag NS production, employing high-energy processes such as laser ablation, magnetron sputtering, and thermal decomposition. Laser ablation, in particular, has demonstrated the capability to generate highly pure Ag NSs, primarily because it avoids the use of chemical reducing and stabilizing agents, thereby minimizing the risk of chemical residues (Abbas et al., 2024). These methods often allow for precise control over the size and shape of the synthesized nanostructures by adjusting the operational parameters. However, physical synthesis approaches are not without their limitations. The requirement for extreme conditions, such as high temperatures or vacuum environments, typically leads to significant energy consumption and high operational costs (Rangasamy and Vo, 2025). Consequently, these methods are often more suitable for the synthesis of Ag NSs in small quantities and may not be economically viable for large-scale industrial production. The high energy input also raises questions about their overall environmental footprint, despite the absence of direct chemical pollutants.

#### 2.7.3. Green (Biogenic) Synthesis

In contrast to traditional physical and chemical methods, which can be energy-intensive and environmentally detrimental, green synthesis presents a more sustainable and eco-friendlier paradigm for nanostructure production. Biogenic synthesis, a cornerstone of green nanotechnology, harnesses the reducing and stabilizing capabilities of naturally occurring, non-toxic, and biodegradable agents derived from a variety of biological sources, including plant extracts, microorganisms (bacteria, fungi), and algae (Singh et al., 2023; Sabira et al., 2024). By operating under mild reaction conditions (e.g., ambient temperature and pressure, neutral pH), biogenic synthesis significantly reduces environmental impact and energy consumption (Liaqat et al., 2022). Furthermore, the biomolecules present in these natural extracts often act as both reducing and capping agents, simplifying the synthesis process and potentially enhancing the biocompatibility of the resulting Ag NSs. Green synthesis offers several key advantages, including the use of renewable resources, reduced toxicity, cost-effectiveness, and the potential for large-scale production. The inherent biodiversity of natural sources also allows for the synthesis of Ag NSs with diverse physicochemical properties, opening up possibilities for tailoring nanostructures for specific applications in medicine, agriculture, and environmental remediation. This approach aligns with the growing global emphasis on sustainable nanotechnology and minimizing the ecological footprint of nanomaterial production.

## 2.8. Need for Green Synthesis Approaches

Traditional techniques used in the synthesis of Ag NSs mostly use harmful chemicals and high energy, making them environmentally unsafe and harmful to human health. This therefore necessitates the need for better alternative techniques. Because the need to protect the environment is becoming increasingly important, and the limitations of conventional chemical and physical methods of synthesis are more obvious, the green synthesis technique is seen as a more viable alternative (Liaqat et al., 2022). Among the options considered, the biogenic method is of particular interest, as it uses natural reducing agents, and the process is ecologically friendly. In this approach, Ag NSs are synthesized using plant extracts, microorganisms, fungi, or algae, which are nontoxic and biodegradable. Phytochemicals in the plant extracts, including flavonoids, terpenoids, and phenolic compounds, have been found to be effective in reducing $Ag^+$ to metallic $Ag^0$ without the use of harmful chemicals (Sabira et al., 2024).

Biogenic synthesis is much safer than the chemical method because there are no toxic materials and high energy. This method is carried out at room temperature and a neutral pH value, which significantly reduces energy needs and lessens environmental pollution. Biogenic synthesis products are more biocompatible and stable with biomolecule coatings, so they can be used in biomedicine and pharmaceuticals for drug delivery and wound healing (Almatroudi, 2024). Materials for the biogenic method are usually agricultural waste, medicinal plants, and microorganisms, which are available and cost-effective for large-scale synthesis (Sharma et al., 2019). In general, the biogenic method provides opportunities to adjust Ag NS properties, such as size, shape, and surface functionality, which enables the design of nanostructures for specific purposes.

Biogenic synthesis is in line with green chemistry. It applies the use of natural reducing agents to prevent environmental pollution and to promote sustainability (Khosravi et al., 2024). The use of plant extracts, microbial cultures, and other renewable resources will ensure a continuous supply of the agent without depletion of natural reserves. They are biodegradable, and their accumulation is minimal in the environment, therefore reducing long-term ecological risks. Microbial synthesis is the best method for large-scale Ag NS production because bacteria, fungi, and yeast grow fast and are easy to cultivate (Huq et al., 2023). Algal synthesis, on the other hand, uses the polysaccharides and proteins available in algae for Ag NS synthesis. Carbon sequestration occurs during the cultivation of algae and is another benefit of algal synthesis (Hamida et al., 2022).

Biogenic synthesis utilizing these natural resources significantly reduces the dependency on hazardous chemicals and also promotes the concepts of circular economy through the recycling of waste products and use of renewable resources. As a part of the broader movement of green synthesis, these biogenic methods are revolutionizing nanotechnology towards a sustainable future. This approach overcomes the limitations of the traditional methods by providing eco-friendly, cost-effective, and scalable solutions for the synthesis of Ag NSs. With further advancements in research, biogenic synthesis has great potential for meeting the growing demand for nanostructures without any harm to the environment and human beings. Implementing these innovative strategies can thus pave the way for a sustainable future for nanomaterial production

(Liaqat et al., 2022). Biogenic synthesis thus provides a solution to the problems of traditional methods and allows for the production of sustainable and environmentally friendly nanostructures.

## 2.9. Parameters Influencing Ag NS Synthesis

The synthesis of Ag NSs is a sensitive procedure, with several critical factors influencing their properties, including size, shape, stability, and function. Some of these factors include temperature, pH, the ratio of precursor to extract, and reaction time.

### 2.9.1. Temperature Effects

Temperature in the process of Ag NS synthesis plays a significant role in the kinetics. The temperature is a key factor in the reduction and nucleation processes. Higher temperatures speed up the process of reduction of silver ions $Ag^{+}$ to $Ag^{0}$. This in turn leads to a more intensive formation of nuclei of nanostructures. The rapid nucleation, in turn, leads to a large number of smaller and more uniformly sized particles. This is beneficial for many applications (Ansari et al., 2023). The increased kinetic energy at higher temperatures leads to more effective collisions and aggregations of silver atoms, which in turn leads to a more efficient process of formation of nanostructures and a potential maximization of the yield in a shorter period of time (Dutta et al., 2016). However, the balance in this process should be maintained very carefully. Excessive heat can disrupt the balance and lead to uncontrolled agglomeration, which results in the reduced stability.

Lower temperatures, on the other hand, slow the reduction process, which leads to more gradual nucleation and growth (Guilger-Casagrande and Lima, 2019). In a slower kinetic environment, silver atoms have more time to diffuse and merge with existing nuclei, which results in the formation of larger nanostructures (Chakraborty et al., 2024). Although such an approach may provide better control over particle size distribution and reduce aggregation, it requires careful attention to the reducing and stabilizing agents used (Nurdin, 2016). In green synthesis, where biological extracts are used, maintaining the optimal temperature is essential to prevent the denaturation or degradation of these agents, which could lead to unpredictable nanostructure formation (Kredy, 2018). As a result, choosing the right temperature range that is finely tuned to the specific synthesis conditions is crucial to the production of Ag NSs with properties tailored to the desired application (Altammar, 2023).

### 2.9.2. pH Influence

The pH level of the solution during Ag NS synthesis is a critical factor, as it directly impacts the rate at which silver ions are converted to nanostructures and also determines how stable those nanostructures remain. According to Sati et al. (2025), the reduction process takes place faster in basic media. According to Melkamu and Bitaw (2021), the intensity of the SPR peak increases with an increase in pH and decreases in acidic media. Moreover, they also reported that the size and shape of the nanostructures are more uniform at higher pH values.

On the other hand, the nanostructures are stable in acidic media, which are explained by protonation of surface groups (Enayati Ahangar et al., 2022). At low pH the reduction of metal ions occurs slowly, which leads to the appearance of larger nanostructures with irregular shapes. Weak positive charges on reduced metal ions at an acidic pH led to a small electrostatic repulsion; as a result, the nanostructures aggregate and form larger particles. In case of near-neutral to slightly alkaline pH conditions, the ratio of the pH value at the beginning of the formation of nuclei of the particles to the pH value at the time of their growth results in smaller and more uniform nanostructures. Highly alkaline media leads to an acceleration of the formation of nuclei, which results in even smaller and more uniform nanostructures, but due to the high pH of the medium, unwanted reactions with metal oxides or hydroxides are possible, which leads to the appearance of nanostructures of different sizes.

### 2.9.3. Reaction Time

The length of the reaction is a key parameter that significantly affects the synthesis of Ag NSs, determining both their formation and stability. At the beginning of the reaction, a longer time will result in a higher yield of Ag NSs, as more silver ions will be reduced. However, an excessively long time can have negative consequences. In the case of the reaction, long times will lead to the aggregation of nanostructures and the formation of larger particles. This will worsen the properties of the Ag NSs.

In the case of green synthesis methods employing biological agents for reduction, the role of reaction time becomes more complicated. Prolonged reaction times could enhance the reduction of metal ions, thus leading to the increased production of Ag NSs (Azad et al., 2023). However, if reaction times are not carefully controlled, the increased production of larger aggregates might compromise the stability and performance of the synthesized Ag NSs.

Finding the optimal reaction time is important for the creation of stable and well-dispersed nanostructures and to reduce agglomeration (Jamil et al., 2024). The right reaction time should be long enough to create and reduce nanostructures but not so long as to cause significant aggregation. Factors such as temperature, pH, and the specific reducing agent used also depend on the time of the reaction to determine the final characteristics of the Ag NSs. Therefore, the accurate determination of the reaction time and these factors are necessary to make the Ag NSs with the necessary size, shape, and stability.

### 2.9.4. Precursor Concentration and Stabilizers

The concentration of precursor materials, the ratio of precursors to plant extract, and the reaction temperature have a remarkable impact on the size and uniformity of nanostructures. These species are key to the stage of nucleation (which is the formation of nuclei), followed by crystal growth of the nanostructures. Normally, increasing the precursors concentration provides more material for nucleation and growth, thus larger nanostructures are formed. But the relationship between starlight and dust is not always simple. For example, studies on iron oxide nanostructures have demonstrated that increasing precursor concentrations produce larger particle sizes until a certain point when particle size decreases due to other factors such as the ratios of solvent and surfactant (Al-Hada et al., 2021). In contrast, slower growth rates can lead to a more homogeneous population of smaller nanostructures at low precursor concentrations.

The control of nucleation and stabilization processes was also carried out by the stabilizers, for example, the biological sample extracts also affect the size of the nanostructures (Al-Hada et al., 2021). The extracts can bind to the growing nanostructures and prevent them from agglomerating which affects their final size. Temperature is another very important factor. The increase in temperature generally leads to the formation of larger particles due to a higher reaction rate (Ahmad et al., 2018). Now this faster growth can mean less control over the ultimate size distribution. Precursors and template method Synthesis of nanostructures can be prepared by varying the ratio of the precursors, where optimal concentration of the precursors needs careful control, and materials templates are assumed to obtain the desired properties. Therefore, an important requirement for controlled nanostructure synthesis is systematic parameter optimization. Ag NS properties can be tailored to specific applications by careful control of temperature, pH, reaction time, and precursor concentration to yield the desired size, shape, stability, and functionality.

## 2.10. Principles of Biogenic Synthesis

Taking the general principles of green synthesis into consideration, the following sections will briefly apply to biogenic synthesis principles. The current study provides an eco-friendly means of cell-free biological nanostructure production by exploiting the natural capabilities of living systems, including plants, bacteria, fungi, and algae. It is being an eco-friendlier approach than the traditional chemical and physical methods (Ahmad et al., 2024). The interaction of bioactive compounds, such as proteins, enzymes, and polyphenols, that these biological systems contain is the prevailing aspect in this procedure. These compounds serve not only as reducing agents that reduce $Ag^+$ ions into $Ag^0$ atoms that aggregate into nanostructures, but also as stabilizing agents. These improvements were attributed to less aggregation which was limited by capping the nanostructures and regulating their growth (Heinemann et al., 2021).

The properties of Ag NSs, such as their size, shape, and stability, are influenced by various factors. The selection of biological origin, be it plant extract, bacteria or fungi, is crucial since each organism contains distinct bioactive compounds and further differ in their ability to reduce and stabilize nanostructures. Additionally, the reaction conditions (e.g., the concentration of $AgNO_3$, pH, temperature, and reaction time) greatly influences the formation and properties of the nanostructures (Chugh et al., 2021; Azad et al., 2023). For example, increased temperature can increase the process of reduction resulting in smaller particle sizes due to quick nucleation. Moreover, the charge and function of biomacromolecules can be influenced by the pH of the reaction medium, which affects their binding with silver ions, and stimuli their stability (Rangasamy and Vo, 2025).

The biogenic synthesis process utilizes different natural resources, which come with their own sets of advantages and challenges. Such resources are generally classified into plant extracts, microbial systems, and animal sources. The synthesis of silver nanostructures using plant extracts is especially rewarding as they are easy to obtain in large quantities and contain lots of polyphenols and flavonoids, enabling a cost-effective and environmentally friendly synthesis method that utilizes native reducing and stabilizing process (Dhir et al., 2024; Shahzadi et al., 2025). In contrast, microbial systems are embraced for their optimized enzyme(s) driven synthesis providing precision and tunability albeit typically requiring specific growth conditions (Sheershwal et al., 2025). The choice of the source is also determined by the application and properties of interest of the nanostructures; thus, a careful assessment of parameters such as efficacy, cost and sustainability is needed (Liaqat et al., 2022; Vadakkan et al., 2024).

## 2.11. Characterization Techniques

### 2.11.1. X-Ray Diffraction Analysis

X-ray diffraction (XRD) spectroscopy is employed to examine the crystal structure, crystallite size, phase identification, and lattice parameters of the powder sample. This technique is highly effective for characterizing crystalline materials, offering essential insights into their atomic arrangement and structural characteristics. The crystallite size (D) of the powder was calculated using the Debye-Scherrer equation (Debye and Scherrer,1916). This equation connects the broadening of diffraction peaks to the average size of the coherently scattering domains (crystallites) present in the material, making it a commonly used approach for estimating crystallite size in powder samples.

$$D = \frac{K\lambda}{\beta \cos \theta}$$

Where, $\lambda$ represents the wavelength of the X-rays used, $\beta$ represents the width of the diffraction peak measured at full width at half maximum (FWHM), and $\theta$ represents the angle of diffraction (Bragg angle).

### 2.11.2. Microscopic Techniques

Microscopy, the process of visualizing and analyzing materials at the micro and nanoscale, has become one of the most important techniques in fields such as materials science, nanotechnology and life sciences. SEM and TEM are two well-established microscopy methods. The introduction of Scanning Electron Microscopy (SEM) enables imaging of nanostructures using a pencil-like focused electron beam scanning both the surface of a sample to generate signals (e.g., secondary electrons, backscattered electrons, and X-rays) (Agrawal et al., 2024). These signals generate detailed images of the surface, and SEM can also be used in conjunction with energy-dispersive

X-ray Spectroscopy (EDS) to assess the elemental composition of the sample (Girão et al., 2017). In contrast, TEM (Transmission Electron Microscopy) shines a high-energy beam of electrons through a thin specimen. The obtained images reflect the underlying distribution of electron density inside the material and thus provide information about the internal structure of the material (Li et al., 2023). TEM for nanostructures is well established, providing size, morphology, and crystallinity information, and can achieve sub-atomic resolution of internal structure.

SEM and TEM both demand meticulous preparation of samples. SEM requires samples to be coated with conductive material, and TEM requires that samples be thin and prepared by specialized means. Both approaches work under high-vacuum conditions. SEM generally provides nanometer-level of resolution, while TEM can deliver sub-nanometer resolution. SEM is frequently used for examining surface characteristics and fractures, while TEM is essential for studying the crystal structures and their defects (Vos et al., 2014). Importantly, SEM only images surfaces, whereas TEM requires thin slices. Both methods are prone to artifacts and require specialized training to use.

### 2.11.3. Spectroscopy Techniques

Spectroscopy is a core for material analysis to identify its electronic, vibrational, and chemical features (Khan et al. 2018). In all employed methods, UV-Vis spectroscopy has a fundamental role in nanostructures characterization and in optimizing their synthesis process (Akintelu et al., 2020). This technique is where you shine ultraviolet and visible light on a sample and see how much light the sample absorbs and how much it raises electrons up to higher energy states. UV-Vis spectroscopy is a strong aid for understanding nanostructure synthesis and obtained properties (Arif et al., 2024). Through the absorption spectrum, the relationship between light absorption and wavelength is determined, providing valuable information about the nanostructures, such as size and other relevant characteristics (Ríos-Reina and Azcarate, 2022).

Fourier transform infrared (FTIR) spectroscopy is a complementary and powerful analytical method for the analysis of molecular specifics of chemical structure (Mohamed et al., 2017). FTIR operates by analyzing the absorption of infrared radiation to evoke characteristic molecular vibrations and rotations, resulting in a distinct “fingerprint” spectrum (Khan et al., 2018) that assists in functional group identification, making FTIR a powerful analytical tool. FTIR spectroscopy is an effective method for analyzing natural extracts (e.g., pollen, propolis) and synthesized materials (e.g., Ag NSs). FTIR detects the main active components in pollen and propolis, such as flavonoids, phenolic acids, and terpenes. It plays a critical role in determining the stability of Ag NSs, as well as the types of functional groups that bind to atoms, which is important in evaluating the bioavailability of the Ag NSs in organisms and the interactions of the Ag NSs with other substances. FTIR can verify the existence of organic matter in extracts for pollen and propolis and confirm the chemical conjugation and functionalization performed with Ag NSs, as well as characterize the interaction and coating of biomolecules from extracts, which is set to important biocompatibility and applications in medicine and nanotechnology. It is also able to characterize materials efficiently due to its rapid analysis, minimal sample preparation, and non-destructive nature.

## 2.12. Applications of Ag NSs

Silver nanostructures present a multifaceted approach to addressing critical challenges in healthcare and environmental monitoring. Their antimicrobial potential stems from a variety of mechanisms, including cell membrane disruption, DNA replication inhibition, ROS generation, and microbial cell wall disruption, positioning them as a promising alternative to conventional antibiotics, especially in the fight against AMR. Beyond their antimicrobial properties, Ag NSs exhibit significant antioxidant activity, offering potential therapeutic benefits in preventing and treating chronic diseases linked to oxidative stress and aging. This is achieved through direct ROS scavenging, enhanced antioxidant enzyme activity, and free radical neutralization. Furthermore, the catalytic capabilities of Ag NSs, combined with their unique optical and electrical properties, make them exceptionally well-suited for the development of rapid and precise sensors. These sensors can effectively detect a wide range of targets, including biological markers, pathogens, and environmental toxins, utilizing techniques such as surface plasmon resonance. Thus, Ag NSs offer a combined solution to antimicrobial resistance, oxidative stress related diseases, and environmental monitoring.

### 2.12.1. Antimicrobial Activity of Silver NSs

Silver nanostructures have been emerged as revolutionary antibacterial agent, combining historical knowledge of silver's antimicrobial properties with modern nanotechnology. Their efficacy stems from multiple mechanisms: ion release, ROS generation, and direct physical interactions with bacterial cells. When Ag NSs release $Ag^+$ ions, these ions disrupt microbial DNA replication, enzyme function, and cell membrane integrity by binding to thiol groups in proteins (Le Ouay and Stellacci, 2015). Concurrently, ROS production induces oxidative stress, damaging lipids, proteins, and nucleic acids, which amplifies bactericidal effects. The nanostructures' small size and high surface-area-to-volume ratio enhance their adsorption to bacterial membranes, causing structural deformation and leakage of cytoplasmic content (Ansari et al., 2019). This multi-target action reduces the likelihood of resistance development, a critical advantage over traditional antibiotics.

Ag NSs exhibit broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi, and enveloped viruses (Tse et al., 2021). Their versatility extends to applications in wound dressings, medical implant coatings, and water purification systems, where they prevent biofilm formation and infections. Studies highlight their synergy with antibiotics, enabling lower antibiotic doses and overcoming resistance (Hamouda et al., 2021). For instance, Ag NSs combined with β-lactams or quinolones show enhanced efficacy against MDR strains.

Despite their benefits, safety considerations remain paramount. While Ag NSs are generally less toxic to human cells than bacteria, prolonged exposure may cause cellular stress or argyria (skin discoloration). Advances in surface functionalization with polymers or biomolecules (e.g., chitosan, peptides) improve targeting and biocompatibility, minimizing off-target effects (Keshari et al., 2020). Additionally, green synthesis methods using plant extracts or microbes reduce reliance on harsh chemicals, aligning with eco-friendly goals (Tripathi et al., 2019).

Looking ahead, Ag NSs hold promise for personalized antimicrobial therapies and smart drug delivery systems. Researchers are exploring stimuli-responsive Ag NSs that activate only in bacterial microenvironments, further reducing toxicity. However, challenges like standardized synthesis protocols, long-term environmental impact, and regulatory hurdles should be addressed to fully harness their potential as a cornerstone of post-antibiotic medicine.

#### *2.12.1.1. Antimicrobial Activity of Propolis Silver Nanostructures*

Honeybees produce a sticky substance called propolis to safeguard their hives from microbial threats, several studies showed the antibacterial properties of propolis and by modulating with silver NPs because it contains bioactive components that have therapeutic benefits. Mendez-Pfeiffer *et al*. (2022) tested the antibacterial and antibiofilm activities of Soronan Propolis-Ag NSs against clinical isolates of MDR bacteria. They demonstrated Soronan Propolis' effectiveness as a reducing and stabilizing agent for the synthesis of Ag NSs and their antibacterial properties.

Propolis silver NPs are a promising nanobiotechnological product that can be generated from a natural source and used for several antimicrobial applications, including the protection of medical equipment and wound dressings. Their synergism has significant antibacterial activity and shows promise as an efficient antimicrobial product against infections (Barbosa et al., 2019). Compared with silver nitrate, propolis extract, and chemically synthesized NPs, the Ag NS propolis extract exhibited a much better and selective effect on fungal cells. An ethanolic extract of propolis Ag NSs has exhibited antifungal, antibiofilm, and non-mutagenic properties (Kischkel et al., 2020).

Al-Fakeh et al. (2021) examined the antimicrobial effects of the aqueous and methanolic extracts of propolis and Propolis-Ag NSs on two fungal strains, *Helminthosporium* sp., and *Fusarium oxysporum*, as well as three bacterial strains, *Listeria monocytogenes, Salmonella typhimurium*, and *Escherichia coli*. Propolis-Ag NSs' aqueous extract's antibacterial activity was enhanced but its antiradical activity was not (Al-Fakeh et al., 2021).

Propolis can improve the bioactive characteristics of a silver nanostructure in addition to its synergistic effect. Consequently, it may represent a unique strategy for using biological materials as antimicrobial agents in the treatment of surgical site infections (Selvaraju et al., 2022). Ghramh et al. (2019) reported on the biogenic synthesis of silver NPs using propolis. They also discussed the silver NPs' hemolytic toxicity, immunological modulation, and antibacterial activity. Ag NSs found in the extract had a noticeable impact on the tested bacterial strains. The results also showed that propolis extract had only weakly stimulating effects on cells in the rat spleen that had not been activated and only moderately stimulating effects on cells stimulated by phytohemagglutinin (Ghramh et al., 2019).

most of the studies on propolis silver NPs synthesis, characterization, antimicrobial and antioxidant activity have been performed by using propolis from Latin and Europe whereas few have reported on propolis from north Africa mostly from Egypt (Mohamed *et al.*, 2022). In Ethiopia, a few information is available on antibacterial and antioxidant properties of propolis (Mitiku and Jobir, 2020. Yet, there is a need for more works to fully realize honey products in general including propolis and honeybee collected pollen.

##### *2.12.1.2. Antimicrobial Activity of Pollen Silver Nanostructures*

Pollen's nutritional richness, vital for plant growth, is highly variable. The composition of pollen is determined by the source plant and its geographical origin. However, environmental factors such as climate, soil composition, and bee activity significantly influence its chemical makeup (Bakour et al., 2021). Due to its diverse beneficial effects, including antifungal, antibacterial, antiviral, anti-inflammatory, immunostimulant, and wound-healing properties, bee pollen is widely used in apitherapy (Komosinska-Vassev et al., 2015). Research has explored the antimicrobial potential of silver nanostructures synthesized using pollen.

Al-Yousef et al. (2020) demonstrated the antibacterial efficacy of green-synthesized pollen-based silver nanostructures against several test bacterial pathogens, compared to chemically synthesized ones and ampicillin as positive control. Similarly, Khatami et al. (2017) found that silver nanostructures produced using pine pollen exhibited potent antimicrobial activity. However, despite the significance of bee pollen as a natural resource and the promise of silver pollen nanostructures, research in this area remains limited. Existing studies often focus on pollen from single tree species, rather than the diverse pollen collected by honeybees (Karimi et al., 2015; Namvar et al., 2016). This gap in research highlights the need for further investigation into the bioactivity of silver nanostructures synthesized from honeybee-collected pollen, which is why this study focuses on this specific source.

### 2.12.2. Antioxidant Activity of Silver Nanostructures

Any substance that may shield DNA, cell membranes, and other biological elements from the harmful effects of unstable molecules known as free radicals is an antioxidant. They guard against the harm that free radicals might cause to our cells. Endogenous antioxidants are those that our systems spontaneously manufacture. Antioxidants that come from outside the body are known as exogenous antioxidants. Free radicals are the results of food digestion and environmental interactions produced by cells. Oxidative stress may occur if our body is unable to effectively eliminate and manage these free radicals. As a result, cells and biological processes might be compromised (Lobo et al., 2010).

Oxidative stress is a disruption in the equilibrium between the generation of reactive oxygen species (free radicals) and antioxidant defenses. Free radicals are those compounds that can cause harm if their levels become too high in a body. They can cause multiple illnesses, including diabetes, heart disease, and cancer. This situation happens when oxidative chemicals are excessively generated or stored and defensive mechanisms are ineffective. Antioxidants or free-radical scavengers are substances that can prevent or slow the damage to cells caused by free radicals. The sources of antioxidants can be natural or even artificial (Kumar *et al.,* 2022). Thus, antioxidants are the molecules that fight free radicals in a biological system.  The most significant class of radical-containing oxidants is represented by reactive oxygen species (hydroxyl •OH, superoxide ion $O_2{\bullet}^-$), as well as non-radicals (hydrogen peroxide, organic peroxides) (Bedlovičová et al., 2020). Superoxide and hydroxyl radical are products of oxygen reduction by electrons.

Silver NSs are one of many well-known variables that can cause oxidative stress, including chemical, physical, and biological. It is important to remember that oxidative stress affects more than just the human body because free radicals are essential to all living things. The process of phagocytosis, which includes the defense against harmful microbes, is unquestionably one of the most important biological mechanisms in which free radicals play a significant role (Akter et al., 2018). The cell creates some internal or extracellular molecules acting as antioxidant defense systems in response to the molecular damage brought on by oxidative stress. The substance must have the following properties to be classified as an antioxidant: it must be active at low concentrations (phenolic antioxidants frequently lose their activity at high concentrations and act as prooxidants), be present in sufficient amounts to deactivate the target molecule, react with oxygen or nitrogen free radicals, and produce a product that is less toxic than the removed radical. There is no one size fits all antioxidant since different antioxidants interact with diverse reactive species in different ways and at various sites while defending particular biological targets (Bourgonje et al., 2020).

The antioxidant activity of materials from various origins can be assessed using different methods and assays. The 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) tests are the most popular techniques for measuring antioxidant capability (Bedlovičová et al., 2020). The Trolox Equivalent Antioxidant Capacity (TEAC) or ABTS methods both rely on the production of the cation radical $ABTS^{•+}$, which is created by emitting one electron from the nitrogen atom. In the DPPH assay, a purple stable free radical called DPPH• reacts with a hydrogen donor. The molecule of DPPH possesses delocalized spare electrons throughout, which prevent dimerization and give the compound its color, with an absorption maximum in the UV/Vis spectrum at about 520 nm. After the reaction, the DPPH• radical produces the reduced form of DPPH (hydrazine form), which changes the color from purple to pale yellow. The amount of purple color loss is influenced by the antioxidant content. Typically, the scavenging capacity is assessed in organic solvents rather than aqueous media. It is a quick, easy, and affordable method for figuring out antioxidant capacity, but it has certain drawbacks. The temporal response curve is nonlinear due to the fluctuating antioxidant and DPPH ratio, and the DPPH radical interacts with other radicals (Munteanu and Apetrei, 2021). But still, regardless of its drawbacks many researchers determined antioxidant capacity of prepared silver NPs by DPPH assays (Khorrami et al*.,* 2018; Ahn et al.,2019).

Silver has attracted the attention of scientists because of its extraordinary antimicrobial and antioxidant efficacy. Utilizing the antioxidant capacities of various natural product extracts to synthesize nanomaterials is a recent concept that has become quite popular. Ag NSs are an antioxidant with activity against hydrogen peroxide, hydroxyl radicals, and superoxide. Functional groups on the surface of Ag NSs are responsible for antioxidant activity (Keshari et al*.,* 2020). Since honey bee products are rich in phenolic compounds like cinnamic and benzoic acid derivatives and flavonoids like quercetin, kaempferol, and naringenin, they are considered possible sources of antioxidants. These products include pollen, propolis, beeswax, honey, bee venom and royal jelly (Rzepecka-Stojko *et al*., 2015).

#### ***2.12.2.1. Antioxidant Activity of Propolis Silver NPs***

Bee propolis, a resinous honeybee product steeped in folk medicine tradition, boasts a complex chemical makeup influenced by diverse environmental factors. This intricate blend of resins, waxes, amino acids, polyphenols, and other compounds grants propolis a broad spectrum of biological activities, including antibacterial, antifungal, anticancer, and antioxidant effects (Corciova et al., 2019). Notably, propolis is renowned for its high phenolic content, driving extensive research into its potent antioxidant and radical-scavenging capabilities.

Given this rich antioxidant profile, nanomaterials synthesis using natural product extracts, such as propolis, has become a popular strategy. While studies have shown the ability of the Ag NSs synthesized with plant extracts to act as antioxidants, in general, the extracts themselves tend to display a higher scavenging activity (Ahn et al., 2019). P-Ag NSs exhibit anticancer activity (Tan et al., 2021), while studies based on other designs making use of propolis as a stabilizing or reducing agent, only increase the antioxidant capacity of the obtained Ag NSs. These results indicate that natural phytochemicals from propolis are successfully applied to synthesize Ag NSs with potentially intensified anticancer and antioxidant effects.

#### ***2.12.2.2. Antioxidant Activity of Pollen Silver Nanostructures***

The antioxidant capacity of bee pollen is mostly, but not entirely dependent on its chemical constituents. Geographic origin, biological habitat, and even the season might affect the honey bee pollen's chemical makeup (Velikova et al., 2000). Among the many distinct chemicals that make up bee pollen are nutrients (proteins, carbohydrates, lipids, and vitamins), fatty acids and their esters, minerals, and phenolic organic compounds comprising flavonoids, phenolic acids, and their derivatives (Kocot *et al*., 2018). The importance of phenolic compounds, such as flavonoids and phenolic acids, as natural antioxidants are well known. With properties like anti-inflammatory, anti-cancer, antibacterial, antiviral, anti-allergic, antithrombotic, and signaling molecules, among others, pollen also plays a vital part in many biological and/or pharmacological processes (Bridi et al., 2022).

Phenolic compounds are powerful free radical scavengers and may offer protection against cellular damage induced by free radicals, offering protection against different diseases. Phenolic substances can prevent DNA damage caused by free radicals and reduce inflammation (Li et al., 2018). The antioxidant, hepatoprotective, and anti-steatosis effects of bee pollen were described in an *in vitro* model by Oyarzún and his colleagues, who studied the antioxidant activity of Chilean honeybee pollen extracts (Oyarzún et al., 2020). Turunc et al. (2021) studied the antioxidant activity of pollen extract silver NPs and found that they had a notable DPPH free radical scavenging efficiency (Turunc et al., 2021). By setting the Pollen Ag NSs' antibacterial and anticancer properties to the test against several bacterial strains, Al-Yousef et al. (2020) were able to demonstrate the excellent antioxidant effects of the biosynthesized Pollen Ag NSs. The biosynthesized Pollen Ag NSs demonstrated excellent antioxidant properties, which were confirmed by testing the antibacterial and anticancer properties of NPs against various bacterial strains and cell lines (Al-Yousef et al., 2020). The antioxidant, anti-microbial, and photocatalytic properties of silver NPs produced by bee pollen extract-assisted biosynthesis were studied by Corciova et al. (2019). They established that propolis extract is a significant source of substances that can help synthesize Ag NSs with antibacterial, antioxidant, and photocatalytic degradation capabilities (Corciova et al., 2019). Antioxidants are crucial molecules in the human body, responsible for fighting free radicals. They play indispensable role to prevent chronic diseases, as well as improve overall health. Therefore, screening of antioxidant properties of plants and plant-derived compounds from bee pollen and propolis is very important.

### 2.12.3. Sensing Applications of Ag NSs

Silver nanostructures are pivotal in sensing applications, significantly impacting healthcare and environmental monitoring. Their inherent optical, electrical, and catalytic properties enable the rapid, sensitive, and selective detection of diverse analytes at low concentrations. A key mechanism driving their sensing performance is SPR, where the oscillation of free electrons on Ag NS surfaces in response to light results in shifts in SPR signals. These shifts facilitate the label-free detection of target biomolecules, pathogens, and environmental contaminants by indicating changes in the local refractive index upon analyte complexation (Rossi et al., 2021; Sati et al., 2025). This capability allows for the identification of proteins, nucleic acids, and early-stage disease biomarkers, as well as the detection of toxic substances in air and water, and the rapid identification of bacteria, viruses, and fungi.

The extensive efforts made in the development of Ag NS research, particularly in biogenic synthesis using natural sources such as propolis and bee pollen, also have brought several challenges. Ag NSs exhibit unique physicochemical properties that result in their versatility in different arenas, especially in antimicrobial, antioxidant, and sensing applications. Potential toxicity effects at high concentrations, aggregation problems leading to loss of effectiveness and increase of toxicity, and environmental risk assessing their releases pinpoint the importance of joint safety evaluation approach and cumulated management. Surprisingly, propolis and bee pollen are among the promising precursors and capping agents for green synthesis of Ag NSs, which contributes to the in situ-generate stable and functional Ag NSs with potential therapeutic effects and sensing through known rich phytochemical profiles. The realization of better-quality Ag NS production, Ag NS functionality, availability of Ag NS safety data, and integration and collaborations in cross-disciplinary research can aid in the safe and effective use of Ag NSs. A deep understanding of the behavior of Ag NSs in real-world conditions along with further innovations will be important to realize the full potential of Ag NSs to aid in solving global health, environmental, and industrial challenges.

### 2.12.4. Hydrogen Peroxide Sensing

Hydrogen peroxide has been remarkably progressing due to its central importance in cellular signaling, immune response, and disease pathology, as well as its relevance in food safety and environmental monitoring, where its accurate quantification is critical for diagnostics and regulatory compliance (Srikhao et al., 2022). Various detection strategies have evolved, with electrochemical and optical methods leading the field by offering high sensitivity and selectivity, as evidenced in recent comprehensive reviews.

While enzymatic sensors, particularly those employing horseradish peroxidase (HRP), have been widely used, they are hampered by limitations such as enzyme instability, sensitivity to environmental conditions, and a limited shelf-life, which restricts their practical utility in real-world applications (Giaretta et al., 2022). To address these challenges, non-enzymatic electrochemical sensors leveraging nanomaterials have emerged as promising alternatives. Among these, bimetallic nanostructures like Pd@Ag NSs are particularly noteworthy. These materials exhibit enhanced electrocatalytic activity, stability, and reproducibility, enabling reliable detection of $H_2O_2$ across a broad range of concentrations and environmental conditions (Singh et al., 2023).

Meanwhile, optical sensing approaches, including fluorescence-based methods, have also advanced by incorporating nanomaterials to improve sensitivity and reduce background interference. Traditional techniques such as titration, chemiluminescence, and spectrophotometry remain in use but are often less practical for rapid or on-site detection due to their complexity and slower response times. The ongoing development and integration of novel nanomaterials, including carbon-based structures, noble metals, and metal oxides, have further enhanced the performance of $H_2O_2$ sensors, making them more robust, sensitive, and suitable for a diverse array of applications (Singh et al., 2023).

Notably, Ag NS -based sensors effectively address the detection challenges associated with $H_2O_2$, a crucial molecule in biological systems. These sensors provide direct, fast, and cost-effective $H_2O_2$ detection, eliminating the need for enzyme catalysis and thereby overcoming limitations inherent in traditional methods, such as low sensitivity and lengthy procedures (Teleanu et al., 2022; Giaretta et al., 2022). Furthermore, the high sensitivity of silver nanostructure-Schiff base sensors in biomarker detection, including $H_2O_2$-related biomarkers, promises to revolutionize disease diagnosis and personalized treatment for conditions like cancer, diabetes, and cardiovascular disorders (Hassan, 2022). The biomedical significance of Hydrogen Peroxide is depicted in Figure 1.

Yang et al. (2020) showed that transistors functionalized with Ag NSs can rapidly detect viruses such as Zika or breast cancer, potentially through the detection of $H_2O_2$ related biomarkers. In environmental monitoring, Ag NS -based sensors play an important role in detecting pollutants, including heavy metals, pesticides, and greenhouse gases, which can induce $H_2O_2$ production in biological systems (Thakur and Kumar, 2022). Moreover, the synergistic effect of adding palladium to silver nanostructures enhances catalytic activity, leading to the formation of palladium-doped silver nanoalloys and gold-palladium alloys, further improving catalytic performance, especially in $H_2O_2$ detection (Zhang et al., 2019; Zhu et al., 2019).

The extensive Ag NS research, particularly in biogenic synthesis using natural sources such as propolis and bee pollen, also have brought several challenges. These include. potential toxicity effects at high concentrations, aggregation problems leading to loss of effectiveness, and environmental risk Surprisingly, propolis and bee pollen are among the promising precursors and capping agents for green synthesis of Ag NSs, which contributes to the in situ-generation of stable and functional Ag NSs with potential therapeutic effects through known rich phytochemical profiles. The realization of better-quality Ag NS production, Ag NS functionality, availability of Ag NS safety data, and integration and collaborations in cross-disciplinary research can aid in the safe and effective use of Ag NSs. A deep understanding of the behavior of Ag NSs in real-world conditions along with further innovations will be important to realize the full potential of Ag NSs to aid in solving global health, environmental, and industrial challenges.

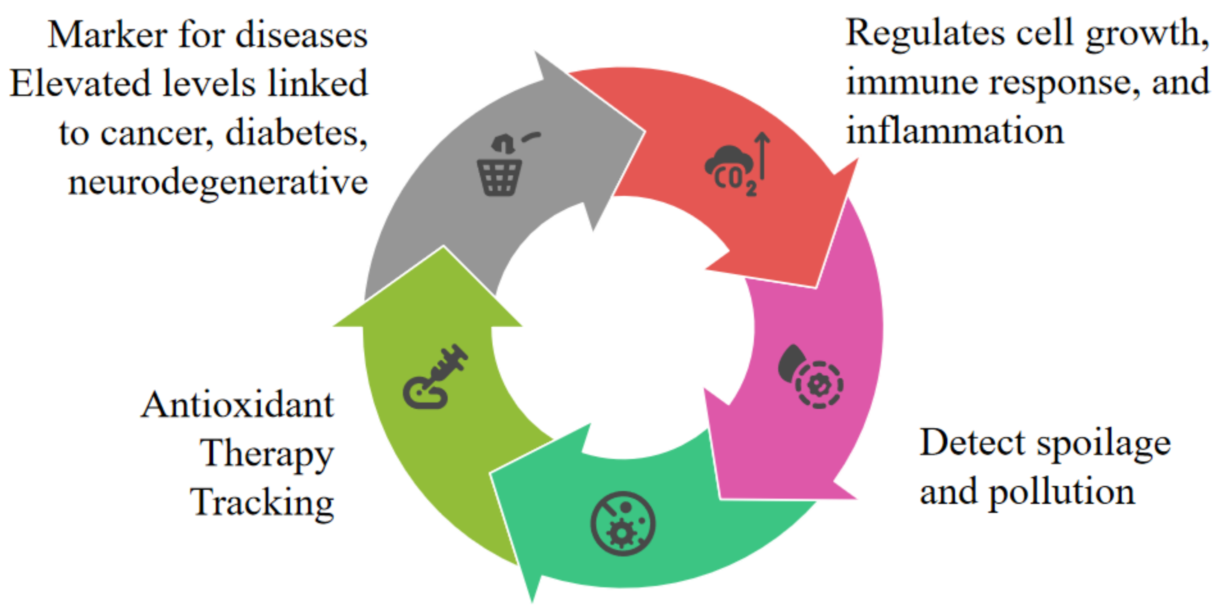


Figure 1. Biomedical importance of Hydrogen Peroxide

**2.12.5. Toxicity of Silver Nanostructures**

Silver nanostructures are widely used in consumer and industrial products due to their unique physical and chemical properties, especially their strong antimicrobial activity. However, growing concerns indicates that exposure to Ag NSs can pose significant toxicological risks to both human health and the environment. In humans, Ag NSs can accumulate in organs such as the liver, kidneys, lungs, and brain, where they may induce oxidative stress, DNA damage, and inflammation, potentially leading to diseases including cancer, neurological disorders, and respiratory illnesses (Zhang et al., 2022). There are also concerns about allergic reactions and the possibility that extensive use of Ag NSs as antibacterial agents could contribute to the development of antibiotic-resistant bacteria. From an environmental perspective, Ag NSs released into waterways and soil can bioaccumulate in aquatic organisms like fish and algae, disrupting their motility, reproductive capacity, and survival, which can cause ecological imbalances and threaten entire aquatic ecosystems McNeilly et al., 2021).

Additionally, Ag NSs can harm beneficial microorganisms, affecting nutrient cycling and wastewater treatment processes (McGee, 2020). The toxicity and bioaccumulation of Ag NSs are influenced by factors such as particle size, concentration, exposure duration, and their tendency to form aggregates, which can enhance their persistence and toxicity. Given these potential hazards, it is crucial to regulate the use and disposal of Ag NSs to minimize their impact on both human health and the environment (Rai et al., 2021).

While silver nanostructures offer valuable benefits in various applications, their potential to cause organ toxicity, environmental harm, and bioaccumulation raises significant health and ecological concerns. Regulatory oversight, safe handling, and proper disposal are essential to minimize these risks and ensure the responsible use of Ag NSs.

# 3. MATERIALS AND METHODS

## 3.1. Description of Study Site and Sample Collection

Organic honeybee propolis and pollen samples were carefully gathered from the Dambi Dollo University apiary, which is conveniently located in the midland region of western Ethiopia, about 645 kilometers west of Addis Ababa. This site, positioned at 8°32′N 34°48′E and ranging in altitude from 1701 to 1827 meters above sea level, boasts a vibrant array of plant life. Notable species include Coffee arabica, *Croton macrostachyus, Vernonia amygdalina, Ficus vasta, Olea africana, Cordia africana*, and various *Acacia* species, along with the agricultural staple of maize (*Zea mays*). To maintain the integrity of the samples, propolis was collected with sterile stainless-steel spatulas, ensuring minimal contamination risk. Figure 2 shows the geographical area covered in this study.

Pollen was gathered using traps strategically placed at the entrances of active beehives during the peak foraging season, effectively capturing a representative sample of the bees' foraging behavior. Propolis collected from traditional hive and pollen samples collected through Pollen traps were transferred into sterile glass bottles and stored right away to keep their chemical makeup and biological activity intact for future analyses. This thorough collection and preservation process are designed to support accurate and reproducible research, allowing for a deep dive into the chemical, nutritional, and potential medicinal benefits of these important bee products.

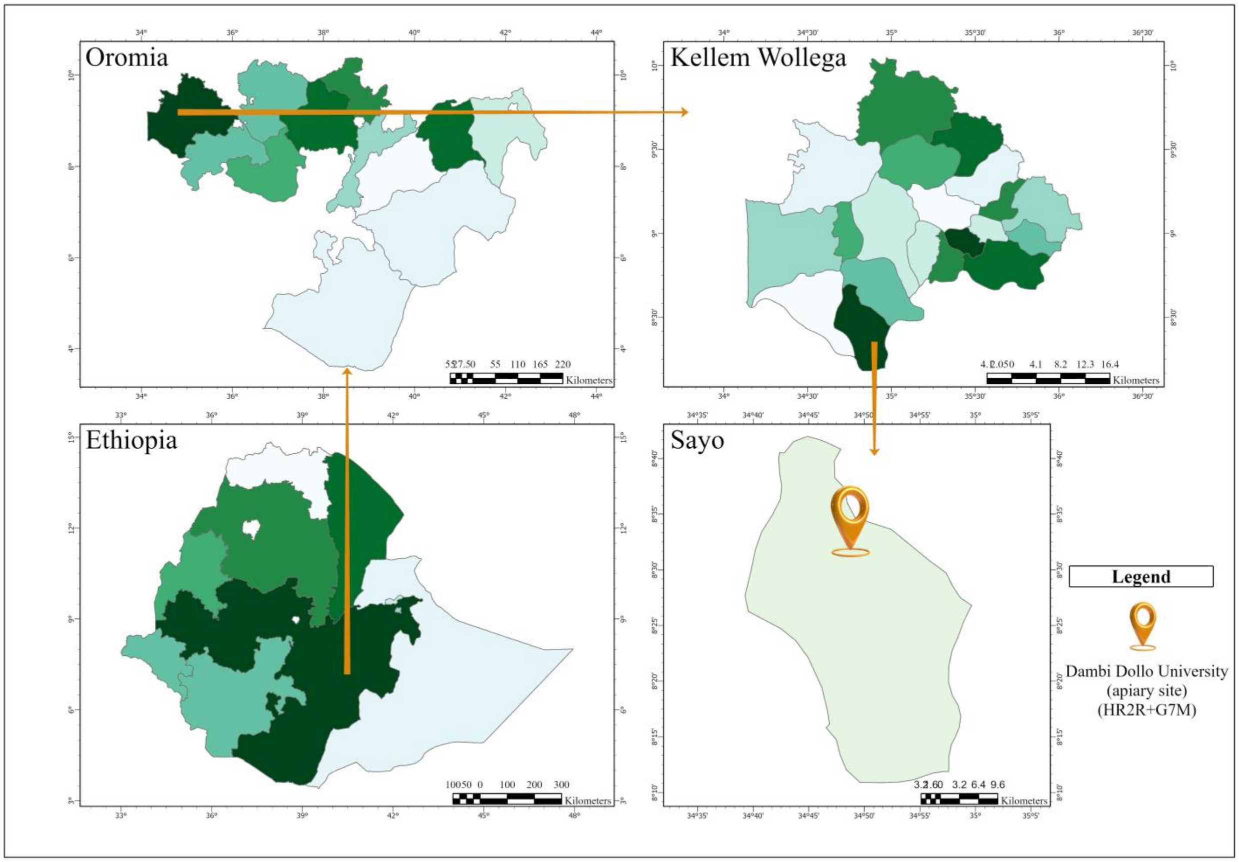


Figure 2**.** Map of the study area (ArcGIS Pro)

### 3.2. Preparation of Pollen and Propolis Extracts

The pollen extraction process involved using ethanol, following a modified technique of Turunc et al. (2021). First, the pollen gathered from *Apis mellifera* L. hives was left to dry naturally for three weeks at room temperature. Once dried, it was ground into a fine powder to achieve a consistent texture through sieving. Next, 20 grams of this powdered pollen were mixed with 200 mL of 70% ethanol and heated to 60 °C in a 500-mL round-bottom flask for one hour. After that, the mixture was centrifuged at 5000 rpm for 10 minutes and filtered using Whatman No.1 filter paper. The extract obtained was then stored at 4 °C for future use.

Following the procedure outlined by Rezk et al. (2022), propolis was gathered using a stainless-steel spatula. Once collected, it was meticulously cleaned by hand to eliminate any debris and insect parts, then frozen at -20°C for 24 hours to maintain its quality. To enhance homogenization and surface area, the frozen propolis was ground into a fine powder. Exactly 20 grams of this powdered propolis were weighed and combined with 200 mL of a 70% ethanol solution. This blend was heated for two hours at 60°C, followed by an additional two-hour incubation at a higher temperature of 80°C. After the extraction, the solution was centrifuged at 5,000 rpm for 10 minutes to remove any excess alcohol. The resulting supernatant was then filtered through Whatman No. 1 filter paper and stored in a refrigerator at 4°C for later use.

## 3.3. Qualitative Phytochemical Screening of Pollen and Propolis Extracts

The ethanol extracts of pollen and propolis were subjected to preliminary phytochemical screening following standard methods. The presence or absence of phytochemicals in the ethanol extracts of pollen and propolis was tested. Phenols (detected using ferric chloride test), alkaloids (using Mayer's test), flavonoids (using alkaline reagent test), anthraquinones (using 5 ml of chloroform and 5 ml of ammonia solution), terpenoids and steroids (using 2 ml of chloroform and 3 ml of concentrated sulphuric acid solution), tannins (using 1 ml distilled water and 2 drops of ferric chloride solution and 10% lead acetate solution were used alternatively), saponins (0.2 ml of the plant leaf extracts were shaken with 5 ml of distilled water and then heated to boil) (Gul et al., 2017; Shaikh and Patil, 2020).

## 3.4. Optimization of Synthesis Parameters

Key synthesis parameters were examined within reaction times of 30, 60, 90, 120, and 150 minutes; varying volume ratios of silver nitrate ($AgNO_3$) to propolis and pollen extract at ratios of 1:1, 1:5, and 1:10; reaction temperatures set at 25°C, 50°C, 70°C, 80°C, and 90°C; and pH levels adjusted to 5, 7, 9, 11, and 13. The formation and the growth of Ag NSs was monitored throughout the optimization process using UV-visible spectroscopy, which enabled real-time tracking of surface plasmon resonance (SPR) peaks characteristic of silver nanostructures.

## 3.5. Synthesis of Pol-Ag NSs and Pro-Ag NSs

This section presents the synthesis of Ag NSs using two separate methods, one of them using propolis extract (Pro-Ag NSs) and another with pollen extract (Pol-Ag NSs). For Pro-Ag NSs, a 1:1 volume ratio between 5 mM $AgNO_3$ and propolis extract was applied. Pro-Ag NSs synthesis entailed the slow addition of 50 mL of propolis extract to 50 mL of 5 mM $AgNO_3$ solution. The pH of the mixture was adjusted to 11 using 1 M NaOH, followed by stirring at 80°C for two hours, as shown in Figure 3. Magnetic stirring ensured constant mixing. Completion of the reaction and successful synthesis were visually ascertained by color changes from dark yellow to dark brown. The as-synthesized nanostructures were then centrifuged with high-speed centrifugation, washed multiple times with distilled water and ethanol, and oven-dried. Subsequently, Pro-Ag NSs were prepared for characterization and analysis of their biological activity.

For Pol-Ag NSs, a 1:10 volume ratio of 5 mM $AgNO_3$ to pollen extract was applied, with pollen extract serving as both capping and reducing agent, while $AgNO_3$ served as the precursor. Pol-Ag NSs synthesis entailed the slow addition of pollen extract to 5 mM $AgNO_3$ solution (maintaining the 1:10 ratio). The mixture's pH was then raised to 11 with 1 M NaOH and was subsequently stirred at 80°C for two hours under dark conditions, covered with aluminum foil. Magnetic stirring ensured constant mixing throughout the reaction. Completion of the reaction and successful Ag NSsynthesis was visually confirmed by color changes from a lighter yellow to a dark brown. A double-beam UV–Vis spectrophotometer was used to measure the reaction progress to optimize the $AgNO_3$ solution concentrations, reaction time, the volume ratio of $AgNO_3$ to extracts, reaction mixture temperatures, and pH effects. The as-synthesized nanostructures were then centrifuged with high-speed centrifugation, washed multiple times with distilled water and ethanol to remove impurities, and finally oven-dried. Subsequently, Pol-Ag NSs were prepared for characterization and analysis of their biological activity (Figure 3).

The molar ratio of the precursor silver nitrate to the pollen extract was 1:10, where the pollen extract acted as the reducing and capping agent. Optimization of the reaction involved varying various parameters, including concentration, temperature, pH, and reaction time, with the reaction progress monitored using a dual-beam UV-Vis spectrophotometer. The first preparation was by adding 10 mL of the pollen extract and 90 mL of a solution of 5 mM $AgNO_3$. This was adjusted to pH 11 using 1 M NaOH, and under continuous stirring, it was homogenized and heated at 80°C for 2 hours. The process was carried out in the dark and under a cover of aluminum foil with ongoing homogenization. Similar to the Pro-Ag NSs synthesis, Ag NSs formation was evidenced by a color change from deep yellow to dark brown. Pol-Ag NSs were purified by centrifugation at 20 minutes, washed multiple times with distilled water and ethanol, and oven-dried for further characterization and biological evaluation.

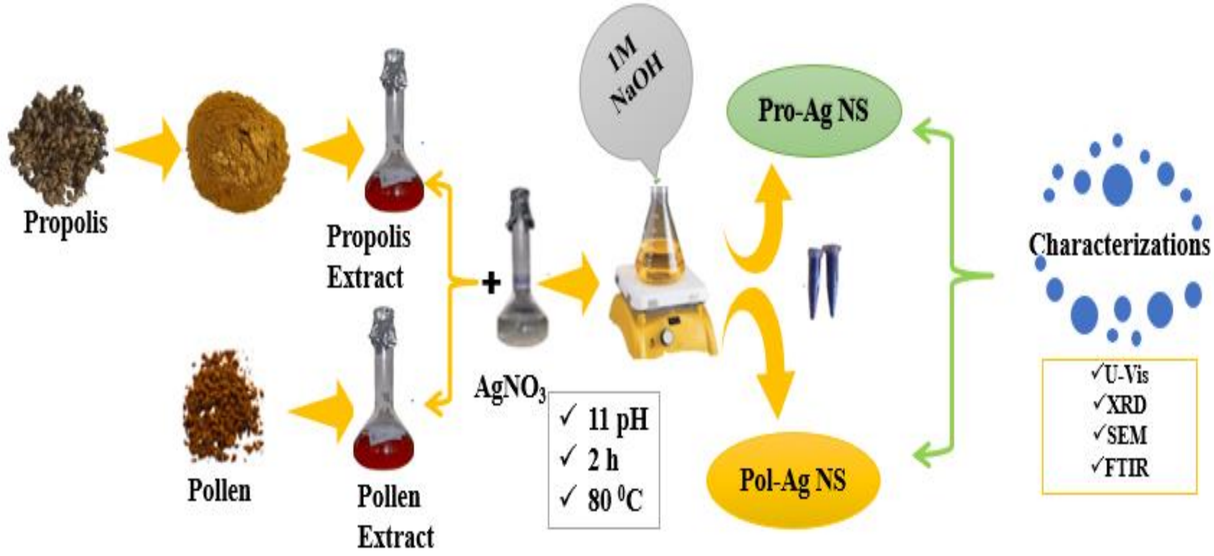


Figure 3. Pol and Pro-Ag NSs Synthesis and Characterization Scheme.

### 3.6. Synthesis of Pd@Ag NSs

Following an adapted method from Wicaksono et al. (2020), Pd@Ag NSs were synthesized by coating Pro-Ag NSs with palladium. A palladium chloride solution was prepared by dissolving 0.1 g of $PdCl_2$ in 10 mL of distilled water, which was then mixed with an equal volume of Ag NSs suspension. To optimize the coating process, the pH of the mixture was adjusted to 11 using 1 M NaOH. Propolis extract, serving as a green reducing agent, was added dropwise to facilitate the reduction and deposition of palladium. The mixture was stirred gently on a magnetic stirrer for 1 hour at 80°C to ensure a uniform palladium layer on the Ag NSs. The resulting Pd@Ag NSs were centrifuged, thoroughly washed with a water-ethanol (1:1) mixture to remove residual chemicals and impurities, and dried in a vacuum oven at 60°C for 12 hours. Finally, the product was stored for further electrochemical testing.

### 3.7. Characterization of Bio-synthesized Silver Nanostructures

### 3.7.1. UV-Spectrophotometer of Pol-Ag NSs

UV–Vis absorption measurements were conducted using a UV-visible spectrophotometer (DU800R DRAWELL, China) to confirm the biosynthesis of Ag NSs through surface plasmon resonance. Absorbance spectra were recorded in the wavelength range of 200-800 nm.

### 3.7.2. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy EVO18 (CARL ZEISS, Germany) was used to examine the surface morphology of the synthesized Pro-Ag NSs, providing high-resolution images to analyze their shape, structure, and distribution. Samples were prepared by depositing a small amount of Pro-Ag NSs on adhesive conductive carbon tape attached to an aluminum stub, followed by a 3-minute gold-sputtering process for SEM analysis at a magnification of 510x and an electron high tension (EHT) of 10.00 kV (Balamurugan et al., 2024).

### 3.7.3. X-Ray Diffraction

The X-ray diffractometer (XRD-7000), Shimadzu Corporation, Japan) was employed to analyze the crystalline properties, particle sizes, and structural characteristics of synthesized Ag NSs. The oven-dried nanostructures were obtained as a fine powder. This powder was then prepared for various characterizations, including X-ray Diffraction (XRD) analysis. For this purpose, the powdered samples were carefully packed into a standard aluminum sample holder using a front-loading method, ensuring a smooth and flat surface. The average crystallite size was estimated using the Debye–Scherrer equation (Debye and Scherrer,1916). XRD spectra were recorded using a CuKα filter ($\lambda = 0.15418$ nm) and scanned from 10° to 90° (2θ/θ).

$$D = \frac{K\lambda}{\beta \cos\theta} \qquad (1)$$

Where, $\lambda$ represents the wavelength of the X-rays used, $\beta$ represents the width of the diffraction peak measured at full width at half maximum (FWHM), and $\theta$ represents the angle of diffraction (Bragg angle).

### 3.7.4. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR was performed using a Nicolet iS10 FT-IR Spectrometer from Thermo Fisher Scientific, USA. The spectrometer operated at a resolution of 4 $cm^{-1}$ across a scanning range from 400 to 4000 $cm^{-1}$. Before analysis, powder Pol-Ag NSs and Pro-Ag NSs samples were purified to remove any unbound substances. The pollen and propolis extract samples were then analyzed as a liquid using the Attenuated Total Reflectance (ATR) method (Makauki et al., 2023).

## 3.8. Antibacterial Activity of Silver Nanostructure

### 3.8.1. Disc Diffusion Method

The antimicrobial activity of Pol-E, Pro-E, Pro-Ag NSs, and Pol-Ag NSs was evaluated using the disc diffusion method against test bacterial pathogens: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), and *Enterococcus faecalis* (ATCC 29212), obtained from the Institute of Pharmaceutical Sciences (Adama Science and Technology). These strains, representing Gram-negative (*E. coli, P. aeruginosa*) and Gram-positive (*S. aureus, E. faecalis*) bacteria, were selected because they are standard test microorganisms.

Bacterial strains were sub-cultured in Mueller-Hinton Broth (MHB), prepared according to manufacturer guidelines, and sterilized at 121°C, 0.11 MPa for 15 minutes (Zonkia, LX-B75L, China). Bacterial suspensions were adjusted to $1.5 \times 10^8$ CFU/mL using 0.8% NaCl, with absorbance (625 nm) set to 0.08-0.10, corresponding to the 0.5 McFarland standard (prepared by mixing 0.05 mL of 1% $BaCl_2$ with 9.95 mL of 1% $H_2SO_4$). Mueller-Hinton Agar (MHA) (HIMEDIA, M173-500G, India) was prepared per manufacturer's instructions, poured into petri dishes to a uniform 3 mm thickness, and sterilized. Sterile 6 mm Whatman No. 1 filter paper discs, prepared using a paper puncher, were sterilized under the same conditions.

Test solutions of Pol-E, Pro-E, Pro-Ag NSs, and Pol-Ag NSs were prepared at concentrations of 12.5, 25, 50, and 100 µg/mL in 10% dimethyl sulfoxide (DMSO). Sterile filter paper discs were impregnated with these solutions and placed on MHA plates uniformly inoculated with bacterial suspensions. Plates were incubated at 37°C for 24 hours, and inhibition zone diameters were measured in millimeters using a ruler. Ciprofloxacin (5 µg/disc) served as the positive control, and

10% DMSO-impregnated discs were the negative control. Experiments were conducted in triplicate, with results reported as mean ± standard deviation (Tahan et al., 2024).

### 3.8.2. Minimum Inhibitory Concentration Assay

The Minimum Inhibitory Concentrations (MICs) of propolis, pollen, and their respective Ag NSs toward *E. coli, P. aeruginosa, S. aureus*, and *E. faecalis* were elucidated as follows. The antimicrobial effectiveness of silver nanostructures was assessed using the standard broth dilution technique following the CLSI M07-A9 guidelines, by monitoring the presence or absence of visible microbial growth in the broth medium. MHB (HIMEDIA, M173-500G, India) was prepared per the manufacturer's guidelines by considering 5mL MHB per test tube. It was sterilized at 121°C 0.11Mpa for 15 min. Test tubes filled with 5 mL of nutrient broth were prepared for bacterial culture, adjusted to the 0.5 McFarland standard (~$1.5 \times 10^8$ CFU/mL), and then diluted 1:100 in nutrient broth to achieve approximately $1.5 \times 10^6$ CFU/mL. The serial dilution method was used to prepare different concentrations of each test solution, ranging from 1.56-100 µg/mL. Then, 1 mL of the already prepared concentrations were added to their respective tubes. Tubes were incubated at 37°C for 24 hours. Bacterial growth was determined by measuring optical density on UV-visible spectroscopy at 600 nm after 24 hours, with MIC defined as the lowest concentration inhibiting visible growth, after subtracting the OD of uninoculated broth containing the test substance (Al-Saggaf et al., 2021). All experiments were performed in triplicate.

### 3.8.3. Minimum Bactericidal Concentration

Following MIC testing, a loopful of broth from each test tube not showing growth, was inoculated into Muller Hinton agar plates. Thereafter, equal volumes of sterile nutrient broth were added into the test tube cultures and incubated further for 24 h at 37°C. The MBC endpoint was defined as the lowest antimicrobial concentration that was required for ≥99.9% eradication of the bacterial population, as confirmed by the absence of colony formation on post-incubation agar plates

## 3.9. Antioxidant Activity of Silver NPs

The antioxidant activity of both propolis and pollen with their respective Ag NSs was characterized utilizing DPPH assay. A stock solution of DPPH in methanol was prepared. The DPPH in methanol 0.1 mM solution was made, and 1.0 mL of this solution was added to 2.0 mL of prepared sample solutions at various concentration. Various concentrations of honeybee Propolis extract, Pollen extract, their respective Ag NSs and standard Ascorbic Acid (ranging from 25 to 400) was prepared at µg/mL. The negative sample (containing all reagents except the test sample) was also prepared in the same conditions. After incubation for 30 min, the absorbance was measured at 517 nm using UV-Visible spectrophotometry. Lower absorbance of the reaction mixture indicated higher radical scavenging activity. Ascorbic acid was used as positive control. Antioxidant activity was estimated by calculating the % inhibition by the following formula as recently described by (Essghaier et al., 2023).

$$SA(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (2)$$

## 3.10. Modification of Working Electrodes

In the electrochemical studies, a nickel foam (NF) substrate (99.99% pure) with a surface density of 346 g/cm² and 1.6 mm thickness was used. Before use, the nickel foam was chemically cleaned through immersion in 0.1 M hydrochloric acid for 30 minutes to dissolve surface oxides and impurities. After the acid treatment, the NF was thoroughly washed with distilled water to remove any remaining HCl or loosened contaminants, ensuring a clean surface for precise electrochemical testing. The substrate was then dried in a sterile environment to avoid recontamination before further use. Next, an 8-µg sample of the synthesized Ag@Pd NPs was mixed with 400 µL of distilled water and 400 µL of polyvinyl alcohol (PVA), and the mixed components were allowed to homogenize at room temperature for 24 hrs. Following this, the working electrode was modified by applying 10 drops of the NPs mixture to improve its electrochemical properties. A high surface area working electrode, modified with Pd@Ag NPs deposited onto cleaned nickel foam, was constructed for $H_2O_2$ detection. This modified electrode was incorporated into a standard three-electrode electrochemical system.

## 3.11. Electrochemical Measurements

The detection of hydrogen peroxide using Pd@Ag NSs modified working electrode was assessed through CV and CA. In the CV experiments, the electrode potential was swept from 0 to 0.65 V at different scan rates for each concentration of $H_2O_2$ to evaluate the electrode's response more effectively. For CA, the electrode was held at a constant potential of -0.5 V, and after stabilizing the background current, known amounts of $H_2O_2$ were added to the stirred 10 mL phosphate buffer solution (5mM, pH 7). The changes in current over time were recorded, with all experiments conducted at room temperature. The limit of detection (LOD) for the Ag@Pd NSs sensor was determined by calculating the ratio of the standard deviation of the blank (bare electrode) response to the slope of calibration curve. This calculation follows the formula as outlined by (Armbruster and Pry, 2008).

$$\mathrm{LOD} = 3.3 * (\sigma / S) \quad (3)$$

Where: σ represents the standard deviation of replicate measurements at the lowest concentration. S is the slope of the calibration curve. Essentially, this equation determines the minimum concentration of a substance that can be reliably detected by the test.

### 3.12. Data Analysis

All experiments were conducted in triplicate to ensure reliability and reproducibility. Data points were expressed as means ± standard deviations (SD) to summarize variability across replicates. Statistical analysis was performed using SPSS software (Version 22). Two-way analysis of variance (ANOVA) was employed to assess the effects of test substances and concentrations on bacterial growth inhibition, followed by post-hoc pairwise comparisons. Additionally, independent t-tests were conducted to determine statistical significance between specific groups, with a significance level set at $p \leq 0.05$.

# 4. RESULTS AND DISCUSSION

## 4.1. Phytochemical Analysis of the Propolis and Pollen Extracts

Phytochemical screening of ethanol extracts from propolis and pollen revealed distinct secondary metabolite profiles. Propolis extracts contained all of the tested secondary metabolites; whereas pollen extract failed to produce anthraquinones, saponins, or steroids (Table 1).

**Table 1.** Phytochemical analysis for the ethanolic extract of Propolis and Pollen

| S/N | **Phytochemical Compounds** | **Method of Test** | **Propolis** | **Pollen** |
|---|---|---|---|---|
| 1 | Phenols | Ferric Chloride | + | + |
| 2 | Alkaloids | Mayer's Reagent | + | + |
| 3 | Flavonoids | Alkaline Reagent | + | + |
| 4 | Anthraquinones | Bontrager's | + | - |
| 5 | Terpenoids | Salkowski Test | + | + |
| 6 | Steroids | Salkowski Test | + | - |
| 7 | Tannins | Ferric Chloride | + | + |
| 8 | Saponins | Frothing Test | + | - |

**Key:** $^{+}$Indicates Presence, and $^{-}$Absence

Geographical differences significantly influence the phytochemical profiles of propolis and pollen. Propolis from tropical regions, such as Brazil, often contains higher levels of prenylated flavonoids and diterpenes due to the abundance of resinous plants like *Baccharis dracunculifolia* (Santos et al., 2021). In contrast, propolis from temperate regions, like Europe, is richer in phenolic acids and flavonoids derived from poplar trees (Populus spp.), reflecting local flora (Widelski et al., 2023). Pollen’s phytochemical composition also varies, with Mediterranean pollen showing elevated levels of quercetin and kaempferol due to diverse flowering plants, while pollen from arid regions may have lower phenolic content but higher concentrations of specific alkaloids (Campos et al., 2022). These variations stem from differences in climate, soil composition, and plant species, impacting the bioactivity of both propolis and pollen.

The Phytochemical compositions identified align with prior study with pollen and propolis, supporting their reported bioactivities. Propolis's antioxidant activity, attributed to its phenolic and flavonoid content, was confirmed through ferric chloride and alkaline reagent tests, consistent with previous findings (Bayram et al., 2020). These compounds likely contribute to its robust antioxidant properties. Pollen, with phenolic profile, exhibits stronger antioxidant activity, which may be linked to its increased secondary metabolite variety.

Tannins, detected via Ferric Chloride tests, are known for antimicrobial and anti-inflammatory properties. The synergistic action of tannins, phenols, and flavonoids in propolis likely enhances its broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria (Alcivar-Saldaña et al., 2024). Saponins, identified by froth formation, contribute to antimicrobial and immune-modulating effects in propolis. Their absence in pollen aligns with its primary role as an energy source rather than a therapeutic agent like propolis, a hive defense product. The lack of detectable steroids in pollen contrasts with propolis, where steroids, possibly from plant resins or beeswax, may drive its anti-inflammatory and tissue-regenerative properties (de Almeida-Junior et al., 2023).

## 4.2. UV-Visible Spectroscopy Analysis

The synthesis of Pro-Ag NSs effectively utilizes propolis extract as both a reducing and capping agent, crucial for the formation and stability of silver nanostructures. The visual indication of successful $Ag^+$ reduction to elemental silver ($Ag^0$) is the development of a dark brown color in the solution (Mulvaney,1996). This is further substantiated by UV-Vis spectrophotometric analysis, which reveals a characteristic Surface Plasmon Resonance (SPR) peak at 424 nm (Figure 4, red line). This SPR peak, arising from the collective oscillation of conduction electrons on the nanostructure surface upon light exposure, is a well-established signature of Ag NSs. The observed SPR peak at 424 nm is consistent with findings from other studies employing various propolis extracts for Ag NS synthesis (Barbosa et al., 2019; Al-Saggaf et al., 2021; Tiri et al., 2021), highlighting the reliability of this indicator for successful synthesis.

Furthermore, the presence of a peak in the 320-330 nm range (Figure 4, black line) within the propolis extract's UV-Vis spectrum is indicative of flavonoids, particularly flavones and flavanols. This observation is consistent with existing research on the chemical composition of propolis, which frequently highlights these specific flavonoid classes as major constituents (Ferreira et al., 2024). The consistency of the 424 nm SPR peak across different studies underscores the effectiveness of using propolis for Ag NS synthesis. It emphasizes its potential as a sustainable and natural resource for this purpose. The results are comparable to those achieved with established methods, further validating the proposed approach.

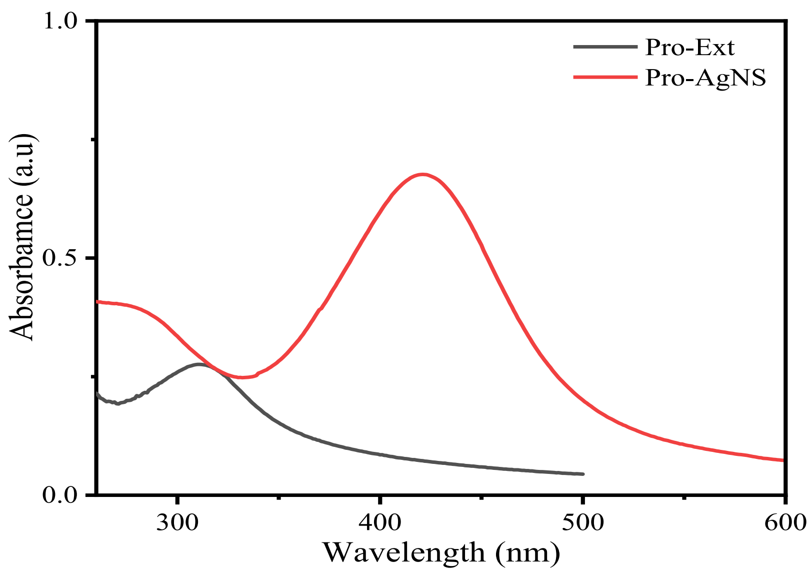


Figure 4. UV-Vis absorption Spectra of Propolis Extract and Propolis nanostructure.

Similarly, the synthesis of Pol-Ag NSs was monitored using UV-Vis spectrophotometry (200-800 nm), with the formation of a dark brown color signaling electron excitation and the reduction of $Ag^+$ to Ag0 (Mulvaney,1996). This environmentally friendly method employs pollen extract, collected by honeybees, as a natural reducing and capping agent for Ag NS synthesis. The formation of Ag NSs was confirmed by an SPR peak within the 400-500 nm range (Rezaei Somee et al., 2018), with Pol-Ag NSs exhibiting a distinct UV-Vis absorption peak at 411 nm (Figure 5 red). This finding is consistent with other reported SPR peaks for pollen-derived Ag NSs, which fall within a similar range, such as 414 nm (Khatami et al., 2017), 440 nm (Al-Yousef et al., 2020; Kumar et al., 2022), and 420 nm (Essghaier et al., 2022). This sustainable approach, utilizing bee bread extracts, effectively supports the synthesis of plasmonic Ag NSs within the 400-450 nm range (Urcan et al., 2023).

A UV-Vis absorption peak at 288 nm (Figure 5, black line) in a pollen extract suggests the presence of phenolic compounds. These include flavonoids like rutin and quercetin, and phenolic acids such as vanillic, chlorogenic, and ferulic acids, all of which characteristically absorb within the 250-290 nm range. Research, including studies on Betula and Pinus pollen (Kerienė et al., 2023), consistently demonstrates that these compounds are the primary contributors to the UV-Vis spectra observed in pollen extracts.

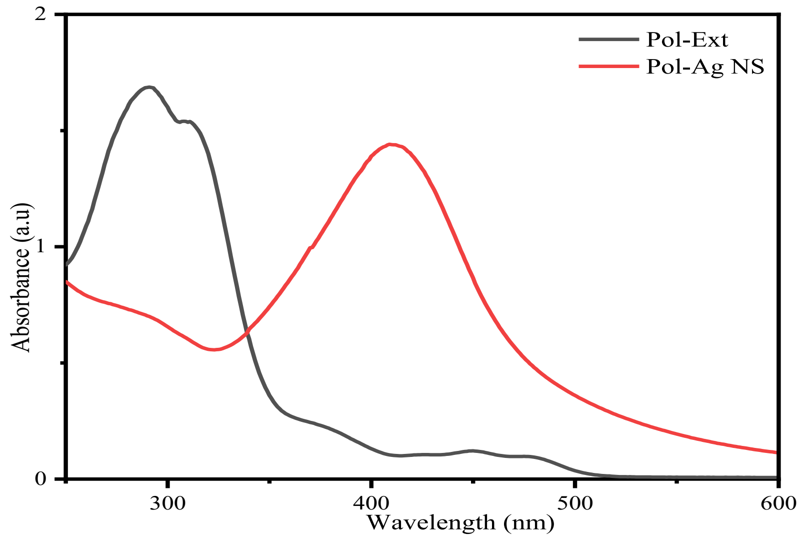


Figure 5. UV-Vis absorption Spectra of Pollen Extract and Pollen nanostructure.

## 4.3. Optimization of Synthesis Parameters

### 4.3.1. Effects of Precursor Concentration Ratio on Pro-Ag NSs

The ratio of biological extract to metal precursor significantly influences the successful synthesis of Ag NSs (Soni et al., 2021). This study explored the impact of varying volume ratios of $AgNO_3$ to propolis extract (1:1, 1:5, and 1:10) on Pro-Ag NS synthesis. All tested ratios resulted in nanostructure formation, as evidenced by surface plasmon resonance (SPR) absorption peaks (Figure 6a). The 1:1 ratio produced the most intense SPR peak, indicating the highest yield of nanostructures. This optimal result is likely due to a balanced concentration of bioactive molecules in the propolis extract, which effectively reduces silver ions and stabilizes the resulting nanostructures (Liu et al., 2023).

At the 1:5 ratio, a sharper and more defined SPR peak was observed compared to the 1:10 ratio (Zulfajri et al., 2024), suggesting improved nanostructure size and shape uniformity. However, the SPR peak intensity at 1:5 was lower than at 1:1, possibly due to reduced availability of reducing agents as the extract proportion increased. At the 1:10 ratio, the SPR peak intensity further decreased, likely because the precursor concentration became too dilute to fully utilize the extract's reducing capacity. This trend suggests that an excess of precursor relative to reducing agents can lead to inefficiencies, such as self-quenching, resulting in a weaker SPR peak (Zulfajri et al., 2024). Thus, the 1:1 ratio was identified as the most effective for Pro-Ag NS synthesis, achieving an optimal balance that maximizes nanostructure yield within the tested range.

### 4.3.2. Effects of Reaction Temperature on Pro-Ag NSs

Temperature plays a crucial role in determining the size, morphology, and stability of nanostructures during synthesis. For Pro-Ag NSs, a two-hour reaction was optimized across a temperature range from room temperature to 90°C. UV-Vis spectroscopy revealed 80°C as the optimal temperature, producing Pro-Ag NSs with the highest stability and concentration (Figure 6 b). This temperature provides sufficient thermal energy to facilitate silver ion reduction while preventing excessive heat that could destabilize the system or degrade propolis-derived capping agents. Beyond 80°C, improvements in yield and stability were minimal, emphasizing the need for precise temperature control to avoid issues like nanostructure agglomeration, degradation of bioactive molecules, or altered surface chemistry (Rashidi et al., 2024). Higher temperatures accelerate reaction kinetics, promoting faster nucleation and yielding smaller, more uniform AgNSs (Sonawane et al., 2023). Conversely, lower temperatures slow the reaction, resulting in larger nanostructures with increased polydispersity and reduced uniformity. Maintaining 80°C ensures stable, well-dispersed Pro-Ag NSs with minimal aggregation, making them ideal for applications such as antimicrobial agents, antioxidants, and catalysts.

Temperature also affects the functional performance of Pro-Ag NSs. Smaller nanostructures, formed at higher temperatures due to rapid nucleation, exhibit a higher surface-area-to-volume ratio, enhancing antibacterial efficacy by improving penetration through bacterial cell walls (Smiechowicz et al., 2021). Additionally, temperature influences antioxidant capacity by modulating surface reactivity, with elevated temperatures introducing surface defects that enhance free radical scavenging (Khan et al., 2022). Temperature also impacts the interaction between nanostructures and stabilizing agents, affecting biocompatibility and environmental stability, which are critical for biomedical and industrial applications. Optimizing temperature is thus essential to tailor Pro-Ag NSs for specific applications, balancing size, stability, and reactivity to maximize their antimicrobial, antioxidant, and catalytic potential.

### 4.3.3. Effects of Reaction pH on Pro-Ag NSs

The pH of the reaction significantly affects the synthesis and properties of Pro-Ag NSs. Higher pH levels, particularly alkaline conditions, promote greater uniformity in size and shape, enhancing dispersion and suspension stability. This is attributed to the enhanced stabilizing role of propolis-derived capping agents, such as flavonoids and phenolic acids, which exhibit increased electron-donating capacity and free radical neutralization at higher pH (Kurek-Górecka et al., 2022). Nanostructures synthesized at pH 9 show increased surface reactivity, improving radical scavenging efficiency and antioxidant performance. Additionally, alkaline pH increases the negative zeta potential of Pro-Ag NSs, reducing aggregation and enhancing colloidal stability (Patil et al., 2023).

For Pro-Ag NSs, alkaline conditions at pH 11 optimize reduction efficiency (Figure 6c), morphological uniformity, and colloidal stability, driven by the enhanced activity of bioactive molecules in propolis extracts that facilitate effective silver ion reduction and nanostructure stabilization (Ramos et al., 2023). Higher pH enhances silver ion reduction, reflected in stronger SPR peaks, with Pro-Ag NSs at pH 11 showing increased surface reactivity (Mittal et al., 2017). Acidic conditions (pH $< 7$) hinder silver ion reduction due to protonation of bioactive molecules, reducing their electron-donating capacity, leading to larger, irregularly shaped nanostructures with broader size distributions and aggregation (Verma et al., 2019). The identification of pH 11 as the optimal condition highlights its role in producing high-quality Pro-Ag NSs with enhanced stability and functionality.

### 4.3.4. Influences of Reaction Time on Pro-Ag NSs

Reaction time significantly impacts the yield, size, morphology, and stability of Pro-Ag NSs. The optimal reaction time was determined by monitoring nanostructure formation using UV-Vis spectroscopy at 30-minute intervals from 30 to 150 minutes (Figure 6d). A reaction time of 120 minutes produced the highest absorbance peak, indicating maximum nanostructure yield, stability, and uniformity. Shorter durations (30, 60, and 90 minutes) resulted in lower yields and compromised stability due to incomplete silver ion reduction and insufficient time for well-defined nanostructure formation. Extending the reaction beyond 120 minutes led to aggregation and increased polydispersity, reducing nanostructure quality (Sonawane et al., 2023). Short reaction times produced inconsistent shapes and sizes, underscoring the need for an optimal temporal window.

The 120-minute reaction time allowed complete silver ion reduction and effective stabilization by propolis-derived bioactive molecules, such as flavonoids and phenolic compounds, resulting in uniform, well-dispersed nanostructures. This duration balance’s reaction kinetics and prevents overgrowth or aggregation, ensuring high-quality, often spherical or near-spherical, Pro-Ag NSs ideal for applications like antimicrobial coatings, antioxidant therapies, and catalysis (Patil et al., 2023). Uniform nanostructures at 120 minutes exhibit enhanced antimicrobial efficacy due to their high surface-area-to-volume ratio and improved antioxidant activity due to optimized surface reactivity (Miranda et al., 2022). Precise temporal control also minimizes energy consumption, supporting cost-effective and environmentally sustainable large-scale production.

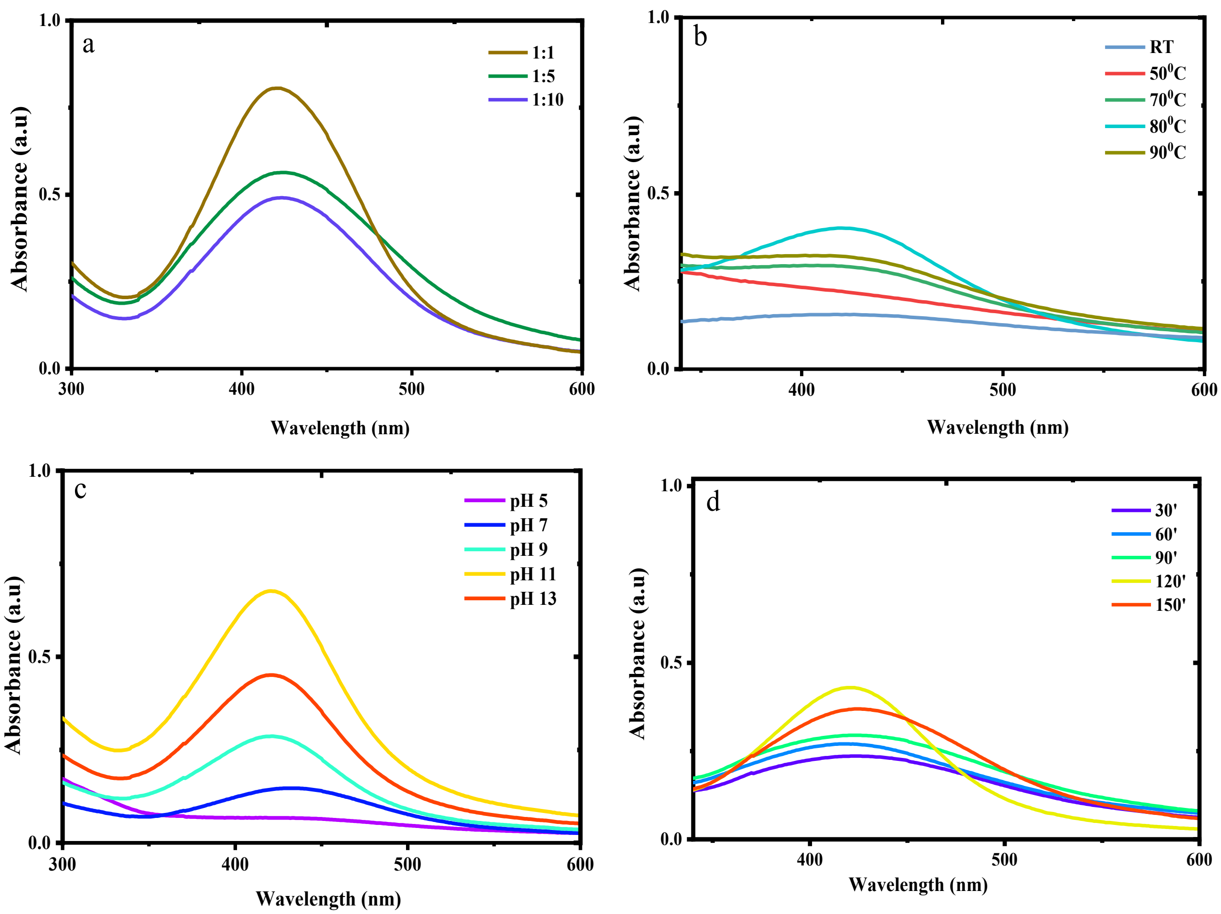

Figure 6. UV-Vis Spectra of Pro-Ag NSs under different synthesis conditions: (a) varying $AgNO_3$: propolis ratios (fixed $AgNO_3$), (b) temperature optimization, (c) pH optimization(d) reaction time optimization.

### 4.3.5. Effects of Precursor Concentration Ratio on Pol-Ag NSs

The ratio of biological extract to metal precursor is a key factor in the synthesis of Pol-Ag NSs (Soni et al., 2021). This study investigated the effect of varying $AgNO_3$ to pollen extract ratios (1:1, 1:5, and 1:10) on Pol-Ag NS synthesis. Unlike Pro-Ag NSs, the 1:10 ratio yielded the most promising results, showing a highly intense SPR peak indicative of substantial nanostructure formation (Figure 7a). The higher proportion of pollen extract likely provided an abundant supply of biomolecules, enhancing both silver ion reduction and nanostructure stabilization, leading to a higher yield. The 1:5 ratio produced a sharper SPR peak compared to the 1:1 ratio, suggesting improved nanostructure characteristics, but its intensity was lower than at 1:10, possibly due to a suboptimal concentration of reducing agents. The 1:1 ratio resulted in the weakest SPR peak, indicating inefficiencies, such as self-quenching, due to an excess of precursor relative to the extract's reducing capacity (Sahu et al., 2024). Thus, the 1:10 ratio was the most effective for Pol-Ag NS synthesis, providing a strong foundation for future optimization.

These findings highlight the importance of balancing precursor and extract concentrations in biogenic Ag NS synthesis. The optimal ratio of 1:10 for pollen extract contrasts with the 1:1 ratio for propolis extract, reflecting the unique properties of each extract. This underscores the need to tailor synthesis conditions to the specific characteristics of the biological extract to achieve optimal nanostructure production.

### 4.3.6. Effects of Reaction Temperature on Pol-Ag NSs

Temperature significantly influences the size, morphology, and stability of Pol-Ag NSs during synthesis. A two-hour reaction was tested across a temperature range from room temperature to 90°C. UV-Vis spectroscopy confirmed 80°C as the optimal temperature, producing Pol-Ag NSs with superior stability and high yield (Figure 7b). This temperature activates bioactive molecules in the pollen extract, facilitating efficient silver ion reduction without compromising molecular integrity. Temperatures above 80°C showed only slight increases in absorption band intensity, indicating diminishing returns, consistent with previous findings (Turunc et al., 2021; Shumi et al., 2023). Higher temperatures enhance nucleation by increasing kinetic energy, yielding smaller, more uniform nanostructures (Hernández-Pinero et al., 2016). Lower temperature slow reaction kinetics, producing larger nanostructures with higher polydispersity, which may limit their suitability for precision applications. Maintaining 80°C minimizes aggregation, enhances reproducibility, and supports scalability for large-scale Pol-Ag NS production.

The consistent optimality of 80°C for both Pro-Ag NS and Pol-Ag NS synthesis highlights its versatility across different biological extracts in green synthesis protocols. This temperature balances reduction and stabilization while preventing aggregation, biomolecule degradation, or inconsistent morphology. Precise temperature control ensures energy efficiency, reduces production costs, and supports eco-friendly synthesis, enhancing the shelf-life and performance consistency of Pol-Ag NSs for commercial applications.

### 4.3.7. Effects of Reaction pH on Pol-Ag NSs

The pH of the reaction plays a critical role in Pol-Ag NS synthesis. The pH was varied from 5 to 13 using 1 M NaOH (Figure 7c), with absorbance measured via UV-Vis spectrophotometry (200-800 nm). At pH 11, Pol-Ag NSs exhibited distinct absorption spectra, indicating successful formation and robust stability under alkaline conditions (Melkamu and Bitew, 2021). SPR peak intensity increased with rising pH and decreased in acidic environments, reflecting more efficient silver ion reduction and nanostructure stabilization in alkaline conditions. Higher pH levels yielded Pol-Ag NSs with uniform size and shape, improving suspension stability and reducing aggregation, attributed to enhanced interactions between silver ions and bioactive compounds in pollen extracts, such as polyphenols, acting as reducing and capping agents (Kumar et al., 2024).

Alkaline conditions at pH 11 optimize reduction efficiency, morphological uniformity, and colloidal stability for Pol-Ag NSs, driven by the enhanced activity of bioactive molecules like polyphenols (Kumar et al., 2024). Higher pH increases the negative zeta potential, reducing aggregation and enhancing stability (Melkamu and Bitew, 2021). Acidic conditions ($pH < 7$) hinder silver ion reduction due to protonation of bioactive molecules, leading to larger, irregularly shaped nanostructures with broader size distributions and aggregation (Srikar et al., 2016). The optimal pH of 11 for Pol-Ag NSs underscores its role in producing high-quality nanostructures with enhanced stability and functionality.

#### 4.3.8. Influences of Reaction Time on Pol-Ag NSs

Reaction time profoundly affects the yield, size, morphology, and stability of Pol-Ag NSs. The synthesis was evaluated over 30 to 150 minutes, with UV-Vis spectroscopy measurements taken every 30 minutes (Figure 7d). The highest absorbance intensity, indicating optimal nanostructure yield and stability, was observed at 120 minutes. Shorter durations (30, 60, and 90 minutes) produced lower absorption peaks, reflecting slower formation kinetics, incomplete reduction, and reduced stability. Extending the reaction beyond 120 minutes led to agglomeration, resulting in larger, less uniform nanostructures with increased polydispersity. Shorter reaction times yielded irregular or non-spherical particles, highlighting the need for an optimal duration to achieve well-defined morphologies, such as spheres, rods, or cubes (Restrepo & Villa, 2021). At 120 minutes, Pol-Ag NSs exhibited uniform size and shape, enhancing their suitability for applications like biosensors, antimicrobial surfaces, and drug delivery systems.

The 120-minute reaction time allows sufficient time for the pollen extract's bioactive compounds to reduce silver ions and stabilize nanostructures, avoiding aggregation from excessive durations or incomplete formation from insufficient times. This uniformity improves reproducibility, enhances shelf-life by reducing aggregation tendencies, and supports scalability for industrial applications (Kumar et al., 2024). The alignment of 120 minutes as the optimal duration for both Pro-Ag NSs and Pol-Ag NSs demonstrates its robustness in green synthesis protocols, providing a reliable framework for producing high-quality Ag NSs with tailored properties.

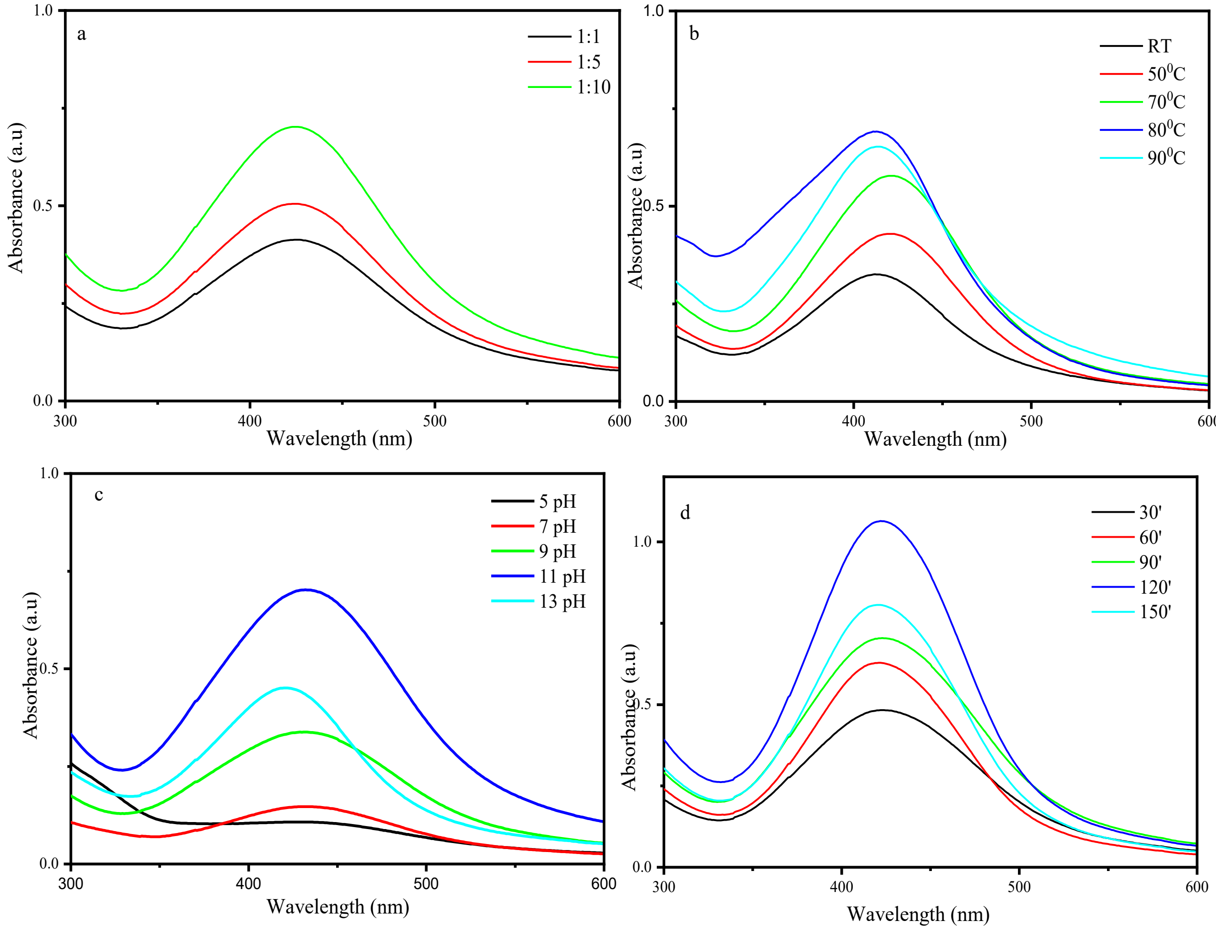


Figure 7. UV-Vis Spectra of Pol-Ag NSs under different synthesis conditions: (a) varying $AgNO_3$: Pollen ratios (fixed $AgNO_3$), (b) temperature optimization, (c) pH optimization (d) reaction time optimization.

## 4.4. X-Ray Diffraction Analysis

X-ray diffraction (XRD) analysis (Figure 8) confirmed the crystalline structure of Pro-Ag NSs, revealing distinct peaks at 2θ values of 38.1°, 44.3°, 64.5°, and 77.4°. These peaks correspond to the (111), (200), (220), and (311) planes of a face-centered cubic (FCC) silver lattice, consistent with the standard reference (JCPDS No. 04-0783). The absence of additional or impurity-related peaks in the XRD pattern underscores the high purity of the synthesized Pro-Ag NSs, indicating a clean synthesis process free from secondary phases or contaminants.

The sharp, well-resolved XRD peaks reflect the crystalline nature of the Pro-Ag NSs, suggesting a minimal lattice defects. The lack of peak broadening or shifting indicates low lattice strain, which is critical for maintaining structural integrity and consistent nanostructure properties. This high crystallinity enhances the stability of the nanostructures (Patil et al., 2023). By confirming the FCC structure and high crystallinity, the XRD analysis provides a robust foundation for understanding the structural characteristics of Pro-Ag NSs.

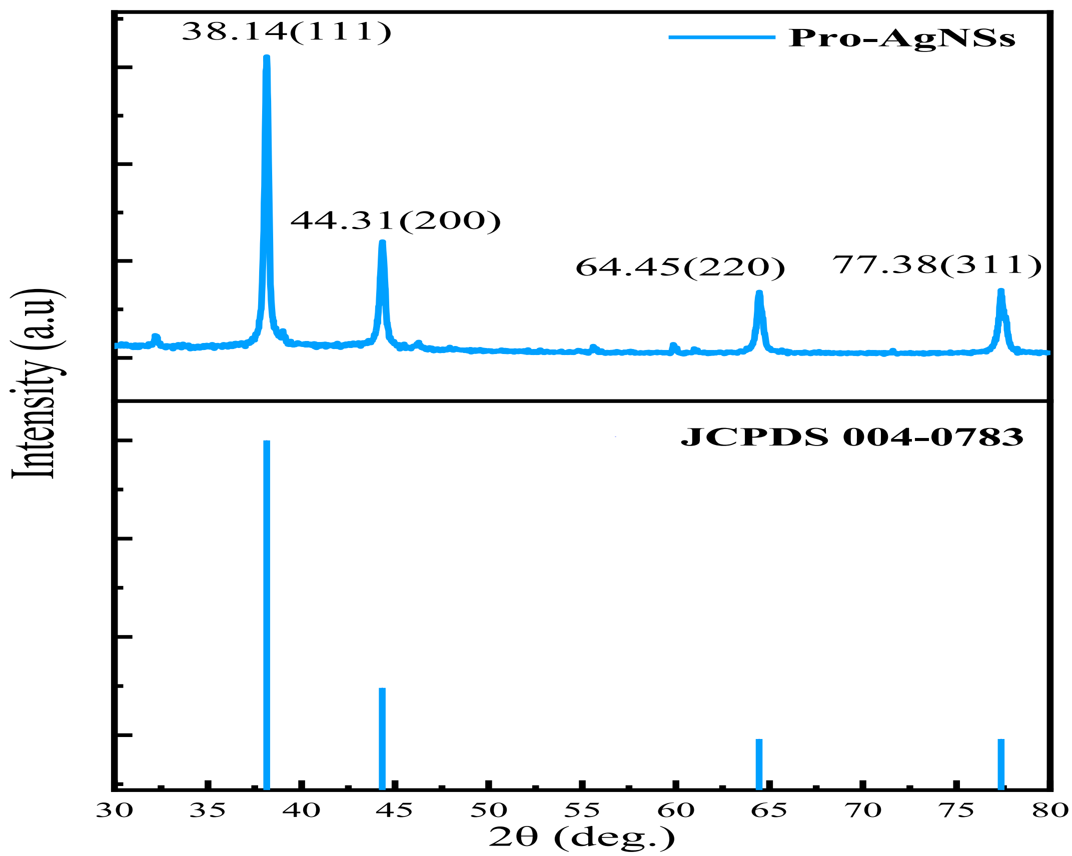


Figure 8. XRD spectra of Propolis mediated Ag NSs (Pro-Ag NSs).

The crystal size of Pro-Ag NSs results from bioactive compounds in propolis extract, such as flavonoids, phenolic acids, and terpenes, which promote metal ion reduction and stabilize nanostructures, ensuring a narrow size distribution (Huo et al., 2022). These compounds inhibit excessive nanostructure growth, enhancing their stability and efficacy in applications like antimicrobial therapies, where uniform size facilitates consistent microbial interactions (Acharya et al., 2021). The unique properties and biological activities of Pro-Ag NSs make them valuable across diverse fields, including biomedicine and nanotechnology. XRD analysis, with specific average crystal sizes reported in Table 2, highlights the critical role of crystal size in their performance. Importantly, the controlled size distribution also reduces aggregation, improving reproducibility and scalability for industrial applications, while the eco-friendly nature of propolis-mediated synthesis enhances their appeal for sustainable nanotechnology.

Table 2. Average Crystal Size of the Pro-Ag NSs as Calculated from XRD Spectra

| | **Lattice Plane** | **2θ(deg.)** | **FWHM (radian)** | **Size** | **Average** |
|---|---|---|---|---|---|
| **Pro-Ag NSs** | (111) | 38.14 | 0.25 | 33.62 | |
| | (200) | 44.31 | 0.2841 | 30.16 | 33.76 |
| | (220) | 64.45 | 0.2653 | 35.37 | |
| | (311) | 77.38 | 0.2836 | 35.92 | |

Similarly, Pol-Ag NSs displayed prominent diffraction peaks at these same planes (Figure 9), with an additional (222) reflection noted, reinforcing the FCC structure of metallic silver. The alignment of these Miller indices (hkl) values with standard references confirms the high purity of nanostructure, as no extraneous peaks were observed, indicating minimal impurities or secondary phases. In Pol-Ag NSs, the FWHM values, ranging from 0.6933 to 1.1067, further affirm good crystallinity, with crystal sizes calculated between approximately 7.78 nm and 14.54 nm. In contrast, Pol-Ag NSs, may present varied crystallographic facets that influence biological interactions, such as enhanced antimicrobial effects through sharp-edged facets disrupting cell membranes (Akhtar et al., 2024). The well-defined crystalline structure in both reduces the release of toxic silver ions, ensuring bioactivity with minimal adverse effects.

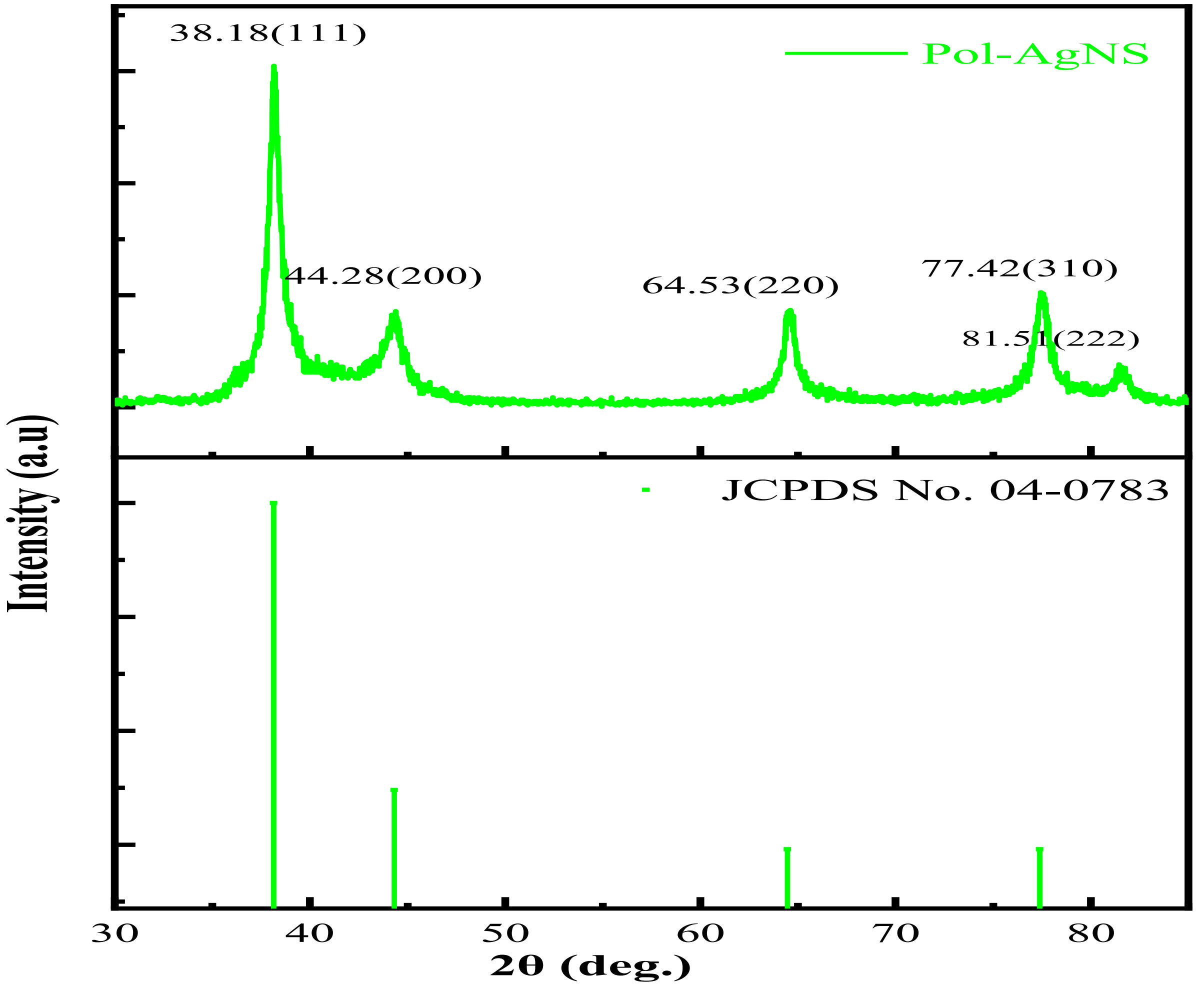


Figure 9. XRD spectra of Pollen mediated Ag NSs.

Table 3 provides crystal size estimates for Pol-Ag NSs, including corresponding 2θ values and FWHM values from XRD analysis. The analysis verifies the crystalline structure of Pol-Ag NSs, with an average crystallite size of 12.1 nm calculated using the Debye-Scherrer equation. Prominent diffraction peaks at specific 2θ values align with distinct crystallographic planes, confirming the nanostructures' crystallinity.

Table 3. Average Crystal Size of the Pol-Ag NSs as Calculated from XRD Spectra

| **Lattice Plane** | **2θ (deg.)** | **FWHM (radian)** | **Size** | **Average** |
|---|---|---|---|---|
| (111) | 38.18 | 0.7529 | 11.16 | |

| | | | | | |
|---|---|---|---|---|---|
| **Pol-Ag NSs** | (200) | 44.28 | 1.1067 | 7.78 | 12.1nm |
| | (220) | 64.53 | 0.6933 | 13.63 | |
| | (311) | 77.42 | 0.76 | 13.39 | |
| | (222) | 81.51 | 0.72 | 14.54 | |

**Key:** FWHM=full width at half maximum

The sharp, intense diffraction peaks of both Pro-Ag NSs and Pol-Ag NSs indicate high crystallinity and uniform crystallite size distribution. For Pro-Ag NSs, the absence of peak broadening or shifting suggests minimal lattice strain and a consistent FCC structure, for maintaining stability and reactivity. Pol-Ag NSs, with FWHM values between 0.6933 and 1.1067, exhibit good crystallinity, with calculated crystal sizes ranging from 7.78 nm to 14.54 nm. The prominent (111) reflection in both nanostructures confirms a preferred FCC orientation, enhancing structural stability and contributing to a high surface area-to-volume ratio, vital for applications like antimicrobial action.

While both nanostructure types possess an FCC structure, their morphology presents subtle differences with functional implications. The highly ordered crystalline arrangement of Pro-Ag NSs is ideal for sustained stability and reactivity. Conversely, the occasional rod-like shape of Pol-Ag NSs may lead to varied crystallographic facets, potentially enhancing biological interaction such as antimicrobial effects through membrane disruption by sharp edges (Akhtar et al., 2024). The well-defined crystalline structure in both types minimizes the release of toxic silver ions, ensuring bioactivity with reduced adverse effects. Thus, XRD analysis confirms the purity and crystalline nature of both Pro-Ag NSs and Pol-Ag NSs, demonstrating how their structural integrity, influenced by synthesis conditions, underpins their functional effectiveness.

### 4.4.1. X-Ray Diffraction Analysis of Pd@Ag NS

Figure 10 displays the XRD pattern of the synthesized Pd@Ag core shell structures. The XRD analysis of these Pd@Ag core-shell nanostructures reveal clear structural characteristics, confirming the coexistence of silver and palladium in a face-centered cubic (FCC) configuration. The pattern exhibits four prominent diffraction peaks at 2θ values of 38.05, 44.33, 64.52, and 77.31 for silver, and 39.39, 46.11, 67.24, and 81.21 for palladium. These peaks align with the Ag (111), Ag (200), Ag (220), and Ag (311) planes, as well as the Pd (111), Pd (200), Pd (220), and Pd (311) planes, respectively, indicating high crystallinity for both metals. The lack of overlapping peaks points to a core-shell architecture rather than an alloyed structure, as alloys typically show shifted peaks, according to research on Ag-Pd alloys (Zamora Zeledón et al., 2021). The minor deviations in the Pd peak positions relative to pure Pd could be attributed to lattice strain or electronic effects between the Ag core and Pd shell.

The XRD results align with prior reports on core-shell systems, where distinct peaks for each metal were observed without significant overlap. For instance, studies on mechanochemically synthesized Pd-Ag catalysts often show additional features from oxide supports or impurities (Kley et al., 2023). In contrast, the present pattern indicates high purity and a well-defined core-shell morphology. The observed lattice strain and electronic effects are consistent with findings in nanoscale bimetallic systems, where interfacial interactions between core and shell metals influence structural properties (Belser et al., 2024). Furthermore, the clear separation of Ag and Pd peaks underscores the precision of the synthesis method.

The XRD analysis confirms the successful formation of Pd@Ag core-shell nanostructures with distinct crystalline phases for both metals. The results agree with previous studies on bimetallic systems, particularly those highlighting strain effects and electronic interactions in core-shell nanostructures (Ntemogiannis et al., 2021). The absence of alloying and the well-resolved peaks demonstrate the effectiveness of the synthesis approach, offering insights into the structural characteristics of such nanostructures (Maity et al., 2023).

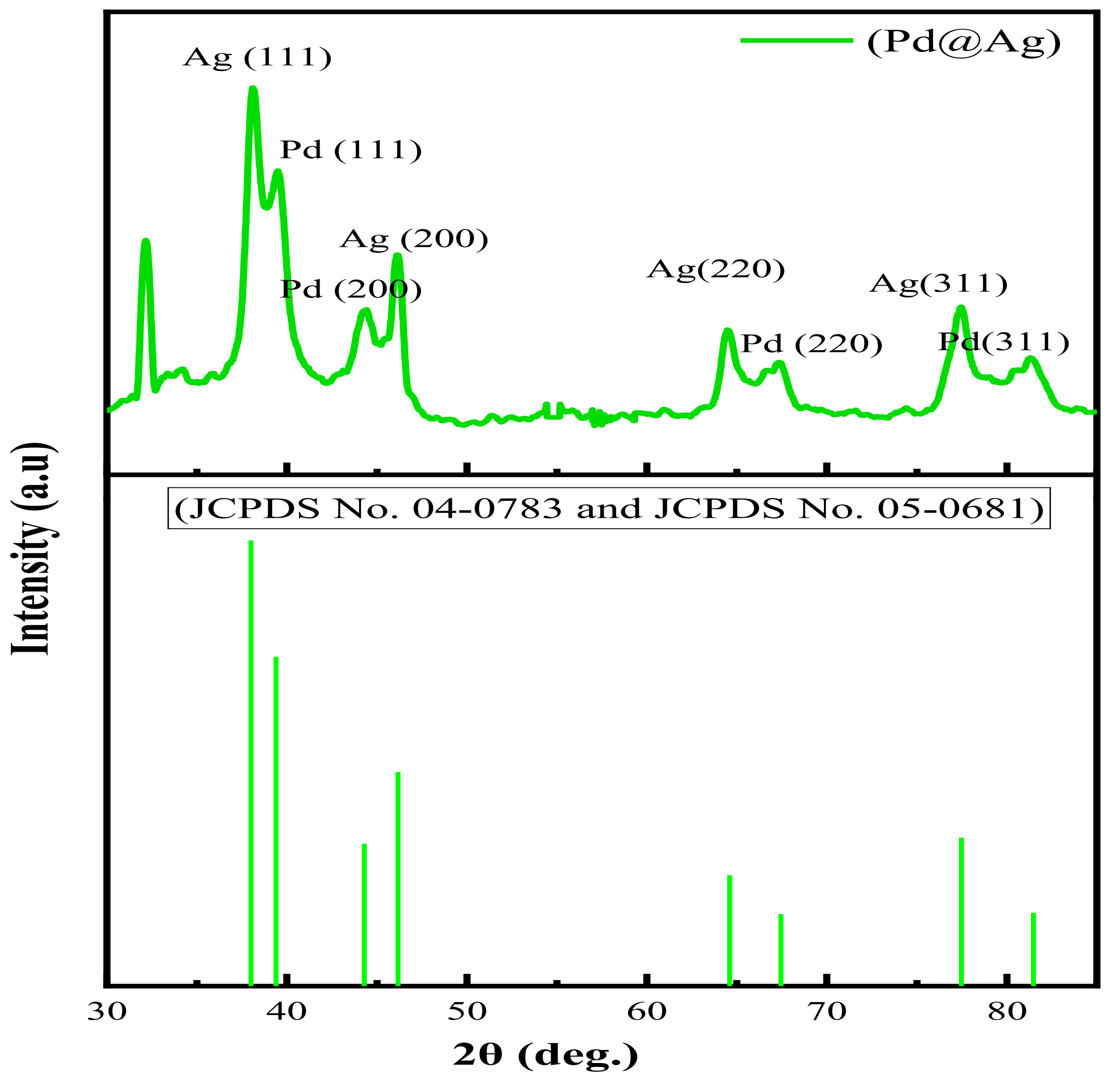


Figure 10. XRD spectra of Propolis mediated Pd@Ag NSs.

The observed 2θ peaks and corresponding crystallite sizes, calculated using the Scherrer equation Table 4, confirm the presence of nanoscale crystallites for both metals. Ag, likely forming the core, exhibits crystallite sizes ranging from 7.40 to 12.18 nm, suggesting a robust foundation with anisotropic growth. Conversely, Pd, the catalytically active component, displays sizes between 6.81and 12.5 nm, indicating a significant deposition rather than a mere monolayer. The variation in Pd crystallite size, along with the observed peak broadening, hints at potential strain or defects at the Ag-Pd interface, likely stemming from lattice mismatch (Wang et al., 2023). These structural features are crucial for the material's electrochemical performance. The nanoscale Pd crystallites offer a high surface area, enhancing catalytic activity and thus, sensor sensitivity. Meanwhile, Ag provides a conductive support, facilitating efficient electron transfer. The distinct crystallite sizes and peak separations underscore a core-shell or nanostructure-on-film morphology, where Pd forms a substantial layer or discrete nanostructures on Ag, rather than a homogeneous alloy (Maity et al., 2023).

Table 4. Average Crystal Size of the Pd@Ag core shell FWHM as Calculated from XRD Spectra

| No | Lattice Plane (hkl) | Material | 2θ(deg.) | FWHM (radian) | Average crystal size (D) | Average |
|---|---|---|---|---|---|---|
| 1 | (111) | Ag | 38.05 | 0.69 | 12.18 nm | 9.81 nm |
| 2 | (111) | Pd | 39.39 | 1.24 | 6.81 nm | |
| 3 | (200) | Ag | 44.33 | 1.16 | 7.40 nm | |
| 4 | (200) | Pd | 46.11 | 0.69 | 12.5 nm | |
| 5 | (220) | Ag | 64.52 | 0.80 | 11.7 nm | |
| 6 | (220) | Pd | 67.24 | 0.92 | 10.4 nm | |
| 7 | (311) | Ag | 77.31 | 1.09 | 9.3 nm | |
| 8 | (311) | Pd | 81.21 | 1.28 | 8.2 nm | |

**Key:** FWHM=full width at half maximum

## 4.5. Fourier Transform Infrared Spectroscopy Analysis

Fourier Transform Infrared (FT-IR) spectroscopy was employed to identify the functional groups involved in the reduction, stabilization, and capping of propolis-mediated silver nanostructure (Pro-Ag NSs), offering critical insights into the biochemical interactions that drive eco-friendly synthesis. The FT-IR spectrum of the propolis extract used for Pro-Ag NS synthesis revealed characteristic peaks at 3361 $cm^{-1}$ (O-H stretching, indicative of hydroxyl groups), 2976 $cm^{-1}$ (C-H stretching, associated with aliphatic hydrocarbons), and 1646.65 $cm^{-1}$ (C=O stretching, linked to carbonyl groups), as shown in Figure 11a (red line). These peaks confirm the presence of bioactive molecules, including flavonoids, phenolic compounds, lipids, and carbohydrates, which are essential for facilitating silver ion reduction and nanostructure stabilization. The involvement of such diverse functional groups highlights the complex, synergistic role of propolis constituents in forming stable, high-quality Ag NSs, aligning with green chemistry principles (Kulkarni et al., 2024).

In the FT-IR spectrum of Pro-Ag NSs, shifts in peak positions were observed, reflecting changes in the molecular environment during nanostructure synthesis. Notably, the C=O stretching peak shifted from 1646.65 $cm^{-1}$ in the propolis extract to 1737.11 $cm^{-1}$ in Pro-Ag NSs, suggesting interactions between carbonyl groups and silver ions that contribute to reduction and capping processes. Additional peaks at 1367 $cm^{-1}$ were attributed to phenolic biomolecules, while peaks at 1085, 1045, and 877 $cm^{-1}$ indicated the involvement of carbohydrate moieties, consistent with carbohydrate signatures observed in similar biogenic syntheses. These shifts and new peaks demonstrate that hydroxyl, carbonyl, and phenolic groups in propolis play pivotal roles in reducing silver ions and stabilizing the resulting nanostructures, preventing aggregation and enhancing colloidal stability (Patil et al., 2023). The pronounced influence of phenolic and lipid components in propolis further distinguishes the stabilization mechanism of Pro-Ag NSs, ensuring robust nanostructure formation.

The FT-IR analysis underscores the adaptability of propolis as a natural reducing and capping agent in eco-friendly Ag NS synthesis. The presence of flavonoids, phenols, lipids, and carbohydrates not only facilitates efficient silver ion reduction but also ensures long-term nanostructure stability, critical for applications such as antimicrobial coatings, antioxidant therapies, and biosensors (Miranda et al., 2022). Moreover, the molecular interactions revealed by FT-IR highlight the unique biochemical profile of propolis, which enables tailored synthesis outcomes compared to other biological extracts. By elucidating the functional groups responsible for synthesis, FT-IR analysis provides a foundation for optimizing synthesis protocols, enhancing reproducibility, and scaling up production while maintaining environmental sustainability. These insights into the chemical mechanisms of Pro-Ag NS formation emphasize the potential of propolis-derived nanostructures as versatile, high-performance materials for diverse biomedical and technological applications.

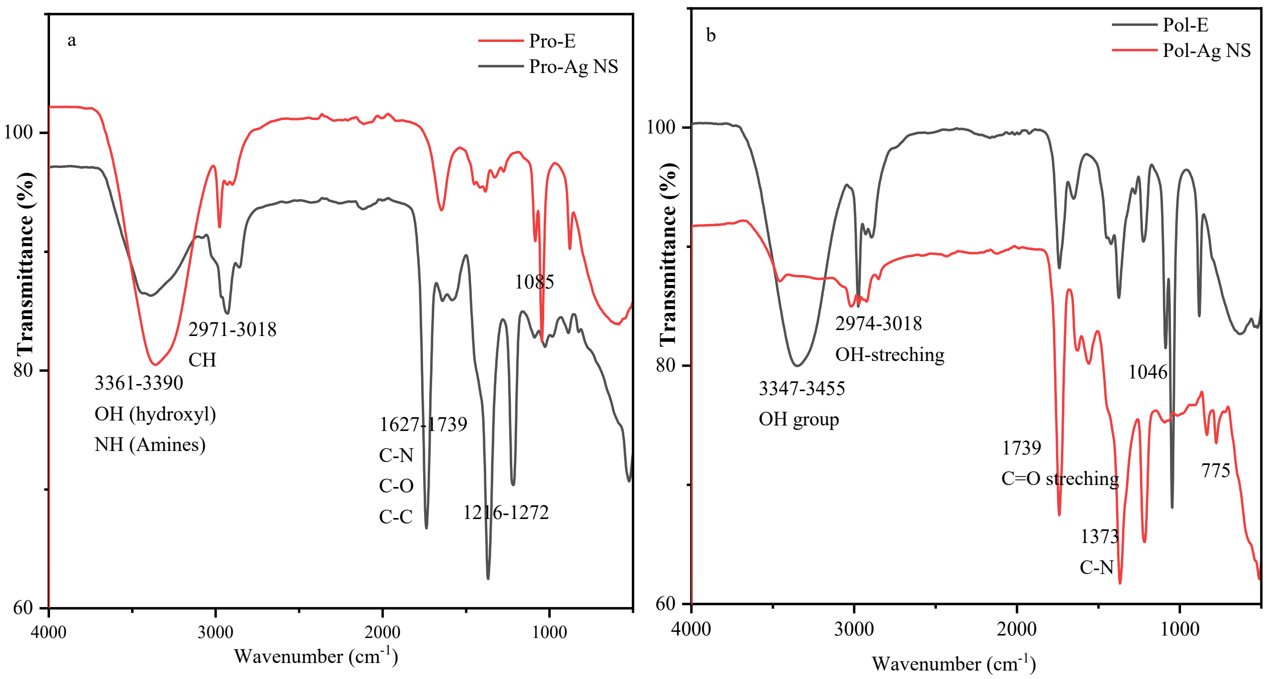


Figure 11. FT-IR spectra of Pol- E, Pro-E, Pol-Ag NS and Pro-Ag NSs.

The FT-IR analysis (Table 5) of Pro-Ag NSs shows that hydroxyl, carbonyl, and phenolic groups, indicated by peaks at 3361 cm⁻¹, 1646.65 cm⁻¹, and 1367 cm⁻¹, along with carbohydrate signatures (1085, 1045, 877 cm⁻¹), facilitate the reduction and capping of silver nanostructures, with shifts (e.g., C=O to 1737.11 cm⁻¹) highlighting propolis-derived biomolecules' role in ensuring stable, eco-friendly nanostructure synthesis.

Table 5. Interpretations of FTIR Data for Propolis Extracts with their respective Ag NSs

| Wave H Frequency Numbers ($cm^{-1}$) | | Functional Groups |
|---|---|---|
| **Pro-E** | **Pro-Ag NS** | |
| 3361.3 | 3390.29 | OH- stretching vibrations in phenols<br>N-H stretch (Amines) |
| 2976.72 | 2929.51 | C-H stretching vibrations |
| | 1737.11 | C=O stretch (Ester Fatty acid groups) |
| 1646.65 | 1573.65 | C=C stretch (Alkenes, Aromatic Compounds) |
| 1383.75 | | Vibrations related to C-N stretching in amines or |
| 1272.8 | 1216.5 | amides, or CH aliphatic groups<br>Vibrations associated with C-O stretching in alcohols, ethers, or carbohydrates |
| 1085.29 | | Bending vibrations of C-H bonds in aromatic |
| 1045.22 | 1026.52 | compounds<br>Bending vibrations of C-H bonds in alkenes |

| | | |
|---|---|---|
| 877.87 | 885.18 | Bending vibrations of C-H bonds in alkanes or methyl groups |
| 585.15 | 522.87 | Vibrations related to C-N stretching in amines or amides, or CH aliphatic groups |

Fourier Transform Infrared spectroscopy was utilized to identify the functional groups involved in the reduction, stabilization, and capping of pollen-mediated Pol-Ag NSs, shedding light on the biochemical interactions that underpin their eco-friendly synthesis. The FT-IR spectrum of the pollen extract displayed prominent peaks at 3347.77 $cm^{-1}$ (O-H stretching, indicative of hydroxyl groups in phenols), 3018 $cm^{-1}$ and 2974.14 $cm^{-1}$ (C-H stretching, associated with lipids, fatty acids, or carbohydrates), 1739.52 $cm^{-1}$ (C=O stretching, linked to carbonyl groups), 1046.16 $cm^{-1}$ (methyl $CH_3$ or methylene $CH_2$ groups), and 879.87 $cm^{-1}$ (O-H groups of carbohydrates), as shown in Figure 8b (black) (Zeghoud et al., 2021). These peaks reflect the presence of diverse phytochemicals, including phenols, carbohydrates, lipids, and fatty acids, which are critical for reducing silver ions and stabilizing the resulting nanostructures. The rich biochemical composition of pollen, influenced by factors such as soil geochemistry and floral diversity, underscores its effectiveness as a natural reducing and capping agent in green synthesis protocols.

In the FT-IR spectrum of Pol-Ag NSs, subtle shifts in peak positions and intensities were observed, indicating interactions between the pollen extract's functional groups and silver ions during nanostructure formation. While the core peaks remained consistent with the pollen extract, new peaks emerged in the 1215-1366 $cm^{-1}$ range, corresponding to hydroxyl group bending vibrations, which suggest their active role in silver ion reduction and nanostructure stabilization (Ullah et al., 2023). The slight shifts, particularly in hydroxyl and carbonyl-related peaks, confirm that these functional groups coordinate with silver ions, facilitating reduction and capping processes that prevent aggregation and enhance colloidal stability. The prominence of hydroxyl, carbonyl, and phenolic groups in the FT-IR spectra highlights their pivotal contributions to forming stable Pol-Ag NSs, with carbohydrates and lipids further supporting the stabilization mechanism (Zeghoud et al., 2021). These molecular interactions reflect the unique phytochemical profile of pollen, which enables tailored nanostructure synthesis compared to other biological extracts.

The FT-IR analysis (Table 6) emphasizes the versatility of pollen as a sustainable resource for eco-friendly Ag NS synthesis. The involvement of hydroxyl, carbonyl, phenolic, and lipid-related functional groups not only ensures efficient silver ion reduction but also imparts long-term stability to Pol-Ag NSs (Patil et al., 2023). By elucidating the chemical mechanisms driving synthesis, FT-IR analysis provides a foundation for optimizing reaction conditions, improving reproducibility, and scaling up production while adhering to green chemistry principles (Ramos et al., 2023). These insights into the molecular interactions of Pol-Ag NSs highlight their potential as high-performance, environmentally sustainable nanomaterials for advanced biomedical and technological applications.

Table 6. Interpretations of FT-IR Data for Pollen Extracts with their respective Pol-Ag NSs

| Wave Numbers ($cm^{-1}$) | | Functional Groups |
|---|---|---|
| **Pol-E** | **Pol-Ag NSs** | |
| 3347.77 | 3455.86 | O-H from Carbohydrates and Polyphenols |
| 2974.14 | 3018.34 | CH stretching alkane and alkene group |
| 1739.52 | 1739.61 | C=O stretch (Ester Fatty acid groups) |
| 1651.42 | 1627.65 | C=C stretch (Alkenes) |
| 1423.23 | 1558.19 | CH Aliphatic bending group, C=C stretch (Alkenes, Aromatic Compounds) |
| 1373.57 | 1366.68 | related to C-N stretching vibrations in amines or |
| 1223.1 | 1216.13 | amides, or CH aliphatic group |
| 1085.74 | | |
| 1046.16 | | C-N stretching vibrations in amines, aromatic C-H |
| 879.59 | 832.83 | out-of-plane bend |
| 630.01 | 775.74 | A bend involving aromatic C-H atoms out of the plane, and oxygen bonding with metal (Ag NS) |
| 518.76 | 511.19 | O bonding with metal (Ag NS) |

The FT-IR analysis of Pol-Ag NSs reveals that hydroxyl, carbonyl, lipid, and carbohydrate-related functional groups in pollen extract, evidenced by peaks at 3347.77 $cm^{-1}$, 1739.52 $cm^{-1}$, and 1046.16 $cm^{-1}$, drive the reduction and stabilization of silver nanostructures, with additional hydroxyl bending vibrations (1215-1366 $cm^{-1}$) confirming phytochemical involvement, ensuring stable, eco-friendly nanostructures.

## 4.6. Scanning Electron Microscopy Analysis of Ag NSs

### 4.6.1. Scanning Electron Microscopy Analysis of Pro-Ag NSs

Scanning Electron Microscopy analysis revealed that Pro-Ag NSs are predominantly spherical with a uniform size distribution ranging from 10 to 50 nm, as corroborated by X-ray diffraction

(XRD) analysis. The SEM micrographs (Figure 12) showed high dispersity and minimal aggregation, indicating monodispersed and consistent physicochemical properties.

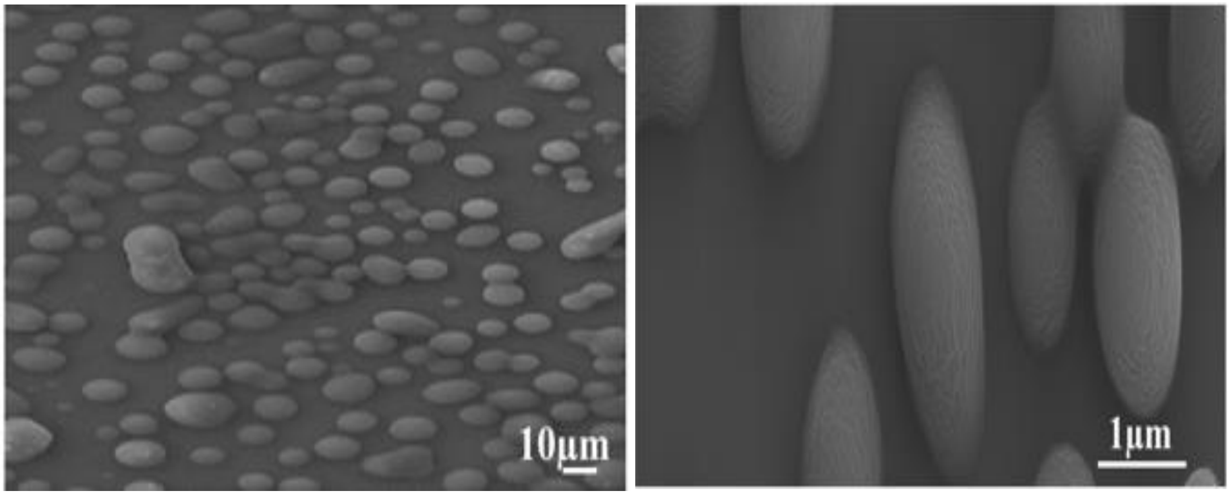


Figure 12. SEM images of synthesized Pro-Ag NSs.

The spherical morphology of Pro-Ag NSs, consistent with prior studies on Ag NSs synthesized using plant extracts like Vernonia amygdalina (Aisida et al., 2020) and *Tagetes erecta* (Katta and Dubey, 2020), maximizes the surface area-to-volume ratio, enhancing reactivity and interactions with biological targets. This uniform size distribution (10-50 nm) and ensure reliable performance in applications such as antimicrobial treatments, biosensing, and drug delivery. The high dispersity and minimal aggregation are attributed to the stabilizing effects of propolis-derived bioactive compounds, including flavonoids, phenolic acids, and terpenes (Samanci et al., 2024). These capping agents preserve a highly effective surface area, critical for efficient binding to bacterial cell walls and maintaining colloidal stability in aqueous or physiological environments.

The spherical shape and uniform size contribute to the potent antimicrobial activity of Pro-Ag NSs against pathogens such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Enterococcus faecalis.* This activity stems from ROS generation and controlled silver ion release, ensuring consistent dosing and interactions (Mikhailova, 2020). Additionally, the controlled ion release, modulated by propolis-derived capping agents, minimizes cytotoxicity in mammalian cells, improving biocompatibility for applications like wound dressings, tissue engineering, and targeted drug delivery (Brown et al., 2012; Bhuyar et al., 2020). The high surface area of non-aggregated Pro-Ag NSs facilitates strong interactions with biological molecules, such as enzymes or DNA, making them effective against biofilms (Akhtar et al., 2024).

The uniform spherical morphology also supports the antioxidant properties of Pro-Ag NSs by enabling consistent electron donation and enhancing electrocatalytic performance in electrochemical $H_2O_2$ biosensing (Srikhao et al., 2022). Their alignment with sustainable nanotechnology principles (Rashidi et al., 2024) underscore their potential to address critical challenges in healthcare and technology.

### 4.6.2. Scanning Electron Microscopy Analysis (Pol-Ag NSs).

Scanning Electron Microscopy imaging (Figure 13) revealed that Pol-Ag NSs, synthesized using pollen collected by honeybees, predominantly exhibit a rod-like or needle-like morphology. The SEM images showed a uniform size distribution within the nanoscale range, typically 7-15 nm, and minimal aggregation.

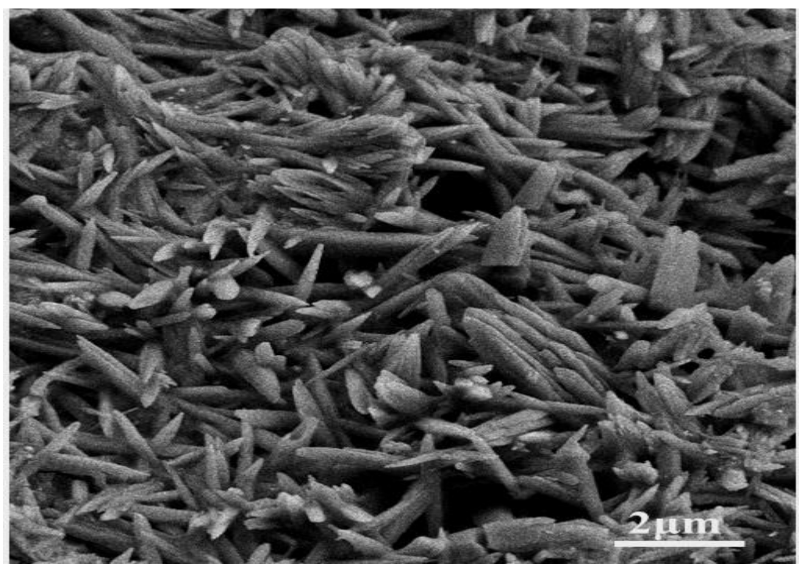


Figure 13. SEM images of synthesized honeybee collected Pol-Ag NSs.

Pol-Ag NSs, synthesized using pollen, exhibit a rod-like morphology, distinguishing them from the spherical shape of propolis-derived Ag NSs. This anisotropic structure confers several functional advantages, including enhanced optical properties, improved drug delivery, superior catalytic activity, heightened sensitivity in sensing, better electrochemical performance, and effective photothermal therapy (Goel et al., 2023). This anisotropic shape facilitates polarized light interactions and directional growth, amplifying plasmonic effects and enabling precise targeting in biomedical applications.

The structure of Pol-Ag NSs, likely arising from the pollen's complex biomolecular matrix acting as a natural template, increases the surface area (Zhang et al., 2023; Xue et al., 2024). This enhanced surface area boosts catalytic efficiency, antimicrobial activity, sensing capabilities, drug delivery efficiency, and electrochemical performance (Zhang et al., 2023; Xue et al., 2024).

Pol-Ag NSs demonstrate a uniform size distribution (7-15 nm). This uniformity ensures predictable physicochemical properties critical for applications like antimicrobial coatings, biosensors, and targeted therapeutics. Minimal aggregation is attributed to the stabilizing effects of pollen's bioactive compounds (proteins, carbohydrates, and polyphenols), which provide steric and electrostatic barriers to prevent clustering (Abbas et al., 2024).

The small size and high surface-to-volume ratio of Pol-Ag NSs enhance their interactions with biological systems, improving antimicrobial, catalytic, and other functional activities. However, this smaller size may also increase cellular uptake and potential cytotoxicity due to easier cell membrane penetration, necessitating thorough safety evaluations for therapeutic applications (Xue et al., 2024). Additionally, the high surface energy of smaller nanostructures could lead to aggregation if stabilization is not maintained, potentially compromising bioavailability and biological effects.

The rod-structure morphology further enhances stability and reactivity. The elongated shape may improve mechanical stability in solution, reducing sedimentation compared to spherical nanostructures. The uniform size distribution reflects precise control over synthesis conditions, optimizing silver ion reduction and minimizing polydispersity, which are essential for scalability and reproducibility in industrial or clinical settings. The stabilizing role of pollen's phytochemicals may also modulate silver ion release, potentially mitigating toxicity concerns in biomedical applications (Abbas et al., 2024). Their sustainable synthesis aligns with green nanotechnology principles, though their small size and unique shape require careful cytotoxicity assessments to ensure safety in biological systems.

## 4.7. Antibacterial Activity of Propolis Extract, Pollen Extract, Pollen Ag NS and Propolis Ag NSs.

### 4.7.1. Disc Diffusion Method

The antibacterial efficacy of Pol-E, Pro-E, Pro-Ag NSs, and Pol-Ag NSs was investigated against *E. coli*, *P. aeruginosa*, *S. aureus*, and *E. faecalis* using the disc diffusion method. Bacterial suspensions standardized to a 0.5 McFarland turbidity were treated with varying concentrations (12.5, 25, 50, and 100 µg/mL) of the extracts and silver nanostructures. Notably, at the highest concentration tested (100 µg/mL), Pro-Ag NSs demonstrated antibacterial activity against the selected bacteria, exhibiting the largest zone of inhibition (17.33 ± 1.15 mm) specifically against *Enterococcus faecalis* (Table 7). In comparison, propolis extract showed a ZOI of 13.67 ± 1.15 mm against Pseudomonas aeruginosa ATCC 27852. Notably, *E. faecalis* demonstrated the highest sensitivity to Pro-Ag NSs, with a ZOI at 100 µg/mL, suggesting that the nanostructures effectively disrupt bacterial membranes and induce oxidative stress. This strong effect underscores the potential of Pro-Ag NSs as a highly effective antimicrobial agent, particularly against Gram-positive bacteria, a finding consistent with studies on green-synthesized silver nanostructures (Ilk et al., 2020).

Even at a lower concentration of 50 µg/mL, Pro-Ag NSs maintained effective inhibition against all tested bacterial strains, indicating broad-spectrum antibacterial capabilities. The antimicrobial action of silver nanostructures is attributed to several mechanisms, including physical damage to bacterial cell membranes, interference with crucial enzyme functions, and the generation of ROS, leading to oxidative stress. Furthermore, $Ag^{+}$ released from the nanostructures contribute to their bactericidal effects by binding to essential bacterial components, disrupting energy production, and DNA replication (Dakal et al., 2016).

Table 7. Propolis extracts and Pro-Ag NSs' inhibitory zone diameters against *S. aureus*, *E. coli, P. aeruginosa*, and *E. faecalis*

| **Con. (µg/ml)** | **Pro-E** | | | | **Pro-Ag NS** | | | |
|---|---|---|---|---|---|---|---|---|
| | ***E. coli*** | ***P. aeruginosa*** | ***S. aureus*** | ***E. faecalis*** | ***E. coli*** | ***P. aeruginosa*** | ***S. aureus*** | ***E. faecalis*** |
| **12.5** | - | - | - | - | - | - | - | - |
| **25** | - | - | - | - | $10.33 \pm 0.57^{b}$ | $11 \pm 1^{b}$ | $10.33 \pm 0.57^{b}$ | $10.6 \pm 0.57^{b}$ |
| **50** | $10.33 \pm 0.57^{b}$ | $10.33 \pm 0.57^{b}$ | $9.33 \pm 0.57^{b}$ | $12.33 \pm 0.57^{b}$ | $12.67 \pm 1.15^{c}$ | $11.67 \pm 0.57^{b}$ | $13 \pm 1^{c}$ | $14.3 \pm 0.57^{c}$ |
| **100** | $11.33 \pm 1.15^{c}$ | $13.67 \pm 1.15^{c}$ | $12.33 \pm 0.57^{c}$ | $13.33 \pm 1.15^{c}$ | $15.67 \pm 0.57^{d}$ | $16.67 \pm 1.52^{c}$ | $16.33 \pm 1.52^{d}$ | $17.33 \pm 1.15^{d}$ |
| **Cipro** | $20.33 \pm 0.57^{d}$ | $20.67 \pm 1.15^{d}$ | $20 \pm 0^{d}$ | $23 \pm 0^{d}$ | $20.67 \pm 0.57^{e}$ | $19 \pm 0^{d}$ | $20 \pm 0^{e}$ | $23.67 \pm 0.57^{e}$ |
| **DMSO** | - | - | - | - | - | - | - | - |

**Key:** DMSO=Dimethyl sulfoxide, Pro-Ag NS = Propolis silver nanostructures, Pro-E=Propolis Extract, Cipro=Ciprofloxacin *E. coli=Escherichia coli*, *P. aeruginosa=Pseudomonas aeruginosa*, *S. aureus=Staphylococcus aureus*, *E. faecalis=Enterococcus faecalis*.

**Note:** Mean values in the same column with different letters differ significantly ($P < 0.05$).

A comparative analysis presented in Table 7, which includes propolis extract, Pro-Ag NSs, and the antibiotic ciprofloxacin, highlights the better performance of Pro-Ag NSs at higher concentrations. These findings suggest that propolis extract and Pro-Ag NSs hold considerable promise as antimicrobial agents, especially at higher concentrations, warranting further investigation to optimize their clinical use and explore their potential in addressing antibiotic-resistant infections.

Employing the disc diffusion method, the antimicrobial efficacy of Pol-E and Pol-Ag NSs was evaluated, with ZOI recorded after a 24-hour incubation at 37°C. Ciprofloxacin acted as a positive control, while DMSO served as the negative control. Across a concentration range of 12.5 to 100 µg/mL, neither Pol-E nor Pol-Ag NSs showed any inhibitory action against the tested bacteria at the lowest concentration. However, at the highest concentration (100 µg/mL), Pol-E exhibited ZOIs between 10.67 ± 0.57 and 12.33 ± 0.57 mm (Table 8). Notably, Pol-Ag NSs demonstrated greater antibacterial activity at this concentration, yielding ZOIs ranging from 13.33 ± 1.15 to 17.0±0 mm. These results corroborate earlier studies (Urcan et al., 2023; Santos et al., 2024), reinforcing the enhanced antimicrobial properties of Pol-Ag NSs compared to the pollen extract in isolation.

**Table 8.** Pollen extracts and Pol-Ag NSs' inhibitory zone diameters against *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis*

| **Con.** | **Pol-E** | | | | **Pol-Ag NS** | | | |
|---|---|---|---|---|---|---|---|---|
| **(µg/ml)** | ***E. coli*** | ***P. aeruginosa*** | ***S. aureus*** | ***E. faecalis*** | ***E. coli*** | ***P. aeruginosa*** | ***S. aureus*** | ***E. faecalis*** |
| **12.5** | - | - | - | - | - | - | - | - |
| **25** | - | - | - | - | $8.33±0.57^{b}$ | $7±1^{a}$ | $7.33±1.15^{b}$ | $7.6±0.57^{b}$ |
| **50** | $8.67±1.15^{b}$ | $7.67±0.57^{b}$ | $9.33±0.57^{b}$ | $9.33±0.57^{b}$ | $10.67±1.52^{c}$ | $10±0^{b}$ | $12±2^{c}$ | $14.3±0.57^{c}$ |
| **100** | $10.67±0.57^{c}$ | $11.3±1.15^{c}$ | $11.67±0.57^{c}$ | $12.33±0.57^{c}$ | $13.67±1.15^{d}$ | $13.33±1.15^{c}$ | $14.67±1.15^{d}$ | $17.0±0^{d}$ |
| **Cipro** | $21.67±0.57^{d}$ | $20.33±0.57^{d}$ | $21.33±0.57^{d}$ | $23±0^{d}$ | $21.33±0.57^{e}$ | $20±0^{d}$ | $20.33±1.15^{e}$ | $23.67±0.57^{e}$ |
| **DMSO** | - | - | - | - | - | - | - | - |

**Key:** Con. concentration, Pol-E pollen extract, Pol-Ag NS =pollen-mediated silver nanostructure, Cipro=ciprofloxacin, DMSO= dimethyl sulfoxide, *E. coli=Escherichia coli*, *P. aeruginosa=Pseudomonas aeruginosa*, *S. aureus=Staphylococcus aureus*, *E. faecalis=Enterococcus faecalis*. **Note:** Mean values in the same column with different letters differ significantly ($P < 0.05$)

The antibacterial activity of Pol-E is concentration-dependent, aligning with research that attributes pollen's antimicrobial potential to its phenolic compounds, which vary with regional biodiversity (Singh et al., 2024). However, its limited effectiveness at lower doses suggests a threshold effect, a challenge also noted with conventional extracts by Naganthran et al. (2022).

The better performance of Pol-Ag NSs supports the idea that biogenic synthesis enhances nanostructure properties. The high surface area and reactivity of silver nanostructures, combined with the reducing and capping agents from pollen, improved their bacterial targeting capabilities (Zehra et al., 2025). This is further corroborated by Kumar et al. (2024), who highlighted the dual role of bee products in stabilizing Ag NSs. The varying susceptibility among bacterial strains, such as *E. faecalis* versus *P. aeruginosa*, can be attributed to differences in their cell wall composition. This phenomenon has been explored by Mikhailova (2024), whose work also emphasizes the effectiveness of Ag NSs against biofilms and resistant strains.

The antimicrobial activity of Pol-Ag NSs, particularly their sensitivity toward *E. faecalis*, indicates their potent effects against both Gram-positive and Gram-negative bacteria (Bruna et al., 2021). This is crucial for addressing the global challenge of antimicrobial resistance, where Ag NSs offer a promising alternative by disrupting bacterial membranes and biofilms (Mikhailova, 2024). While the exact mechanisms of their inhibitory action are not fully elucidated, the toxicity of Ag NSs is likely linked to their diminutive size, large surface area-to-volume ratio, and ability to generate reactive ROS. Research on other nanostructures, like copper nanostructures derived from *A. repens* extract, also suggests their potential as therapeutic agents by promoting oxidative stress and cell death via increased ROS production (Ahmeda et al., 2020). Proposed mechanisms for nanostructures generally include DNA binding and damage (Khina and Krutyakov, 2021), ROS generation (Huang et al., 2021), protein interaction and denaturation (Turbay et al., 2021), and cell membrane disruption (Mikhailova, 2020).

Furthermore, the anisotropic morphology and high dispersity of Pol-Ag NSs, achieved through eco-friendly pollen-mediated biosynthesis, position them as versatile nanomaterials for biomedical and biosensing applications. Optimization of synthesis parameters (e.g., reaction time, temperature) enhances the consistency and efficacy of Pol-Ag NSs, addressing gaps in earlier studies (Al-Yousuf et al., 2020). Ag NSs induce oxidative stress in bacterial cells by generating

ROS, which damages crucial cellular components like DNA, proteins, and lipids. This oxidative damage compromises bacterial membrane integrity, leading to the leakage of cellular contents and ultimately cell death (Adeyemi et al., 2020). Studies confirm that Ag NSs increase ROS levels in various bacterial strains, correlating with enhanced antimicrobial activity (Mammari et al., 2022). Ag NSs can also interact with thiol groups in membrane proteins, impairing respiratory enzymes and altering fatty acid structures within the membrane. This interaction increases membrane permeability, facilitating further ROS accumulation and disrupting essential cellular metabolic processes (Adeyemi et al., 2020; Mammari et al., 2022). The structural damage from Ag NSs can lead to cytoplasmic leakage and alterations in cell morphology, which are vital for bacterial survival (Arya et al., 2024; Jahan et al., 2024).

### 4.7.2. Minimum Inhibitory Concentration

The Minimum Inhibitory Concentrations of Pol-E, Pol-Ag NSs, Pro-E, and Pro-Ag NSs were determined against *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), and *Enterococcus faecalis* (ATCC 29212), with ciprofloxacin as the reference antibiotic. The MIC values (µg/mL) are presented in Table 9.

**Table 9.** Minimum Inhibitory Concentration Results (µg/ml)

| Bacterial Strain | Sources | Pol-Extract | Pol-Ag NSs | Pro-Extract | Pro-Ag NSs | Ciprofloxacin |
|---|---|---|---|---|---|---|
| ***E. coli*** | ATCC 25922 | 50 | 12.5 | 25 | 12.5 | 3.125 |
| ***P. aeruginosa*** | ATCC 27853 | 50 | 25 | 25 | 12.5 | 3.125 |
| ***S. aureus*** | ATCC 25923 | 25 | 25 | 25 | 6.25 | 3.125 |
| ***E. faecalis*** | ATCC 29212 | 25 | 12.5 | 12.5 | 6.25 | 3.125 |

**Key:** Con concentration, Pol-E Pollen extract, Pol-Ag NS =Pollen-mediated silver nanostructure, Pro-Ag NS =Propolis silver nanostructure, DMSO dimethyl sulfoxide, *E. coli=Escherichia coli*, *P. aeruginosa=Pseudomonas aeruginosa*, *S. aureus=Staphylococcus aureus*, *E. faecalis=Enterococcus faecalis*.

Thus, the nanostructure modulated pollen and propolis extracts showed a pattern of 2-4 times more effectiveness in suppressing the test pathogens than the free extracts, except the pollen extracts on E. *coli* (Table 9). Among the treatments, Pro-Ag NSs inhibited *S. aureus* and *E. faecalis* with the lowest concentration (6.25µg/ml) followed by the same extract inhibiting E. *coli* and *P.*

*aeruginosa*, at MIC of 12.5ug/ml. This indicates a lower MIC value indicates that less of the drug is required in order to inhibit growth of the organism, drugs with lower MIC scores are more effective antimicrobial agents.

The data didn't show significant difference between the two Ag NS nano-treated extracts to inhibit *E. coli* and within the treated and untreated pollen extracts to inhibit *S. aureus* with MIC (12.5). In all cases, the standard drug Ciprofloxacin displayed the lowest concentration (3.12) to inhibit the pathogens indicating that the Pro-Ag NSs and Pol-Ag NSs extracts were still 2-4 and 4-8 times less potent than the standard drugs indicating the significant difference between the bio synthesized antagonists and the standard drugs. *E. faecalis* was the most sensitive test pathogen followed by*S. aureus*that were inhibited by the low MIC tests (Table 9).

The MIC results demonstrate that silver nanostructure-based agents (Pol-Ag NSs and Pro-Ag NSs) generally exhibit a pattern more antibacterial potency than their extract counterparts (Pol-E and Pro-E), as evidenced by lower MIC values. This indicated enhanced efficacy of Pro-Ag NSs, which achieved MICs of 6.25-12.5 µg/mL across all tested strains, compared to 12.5-25 µg/mL for Pol-Ag NSs and Pro-E, and 25-50 µg/mL for Pol-E. The better performance of Ag NSs aligns with literature indicating that their small size, high surface area-to-volume ratio, and ability to generate ROS and release silver ions disrupt bacterial cell walls and metabolic processes (Chapa González et al., 2023; Aldosary and Abd El-Rahman, 2019).

Against Gram-negative bacteria (*E. coli and P. aeruginosa*), Ag NSs showed lower MICs (12.5-25 µg/mL) compared to extracts (25-50 µg/mL), highlighting their potential against resistant pathogens like *P. aeruginosa* (Alotaibi et al., 2022). The identical MICs of Pol-Ag NSs and Pro-Ag NSs (12.5 µg/mL) against *E. coli* suggest that nanostructure formulation enhances activity irrespective of the extract source. For *P. aeruginosa*, Pro-Ag NSs' lower MIC (12.5 µg/mL) compared to Pol-Ag NSs (25 µg/mL) may reflect propolis's richer phytochemical profile, including phenols and flavonoids, which synergize with silver ions (Alcivar-Saldaña et al., 2024).

Gram-positive bacteria (*S. aureus and E. faecalis*) exhibited greater susceptibility to Pro-Ag NSs (MIC 6.25 µg/mL), a four-fold improvement over Pro-E (25 µg/mL) for *S. aureus* and a two-fold improvement for *E. faecalis*. This suggests a specific interaction enhancing Pro-Ag NSs' efficacy against Gram-positive strains, possibly due to their thicker peptidoglycan layer facilitating nanostructure adhesion and ROS-mediated damage (Halkai et al., 2018). Pol-Ag NSs matched Pol-E's MIC (25 μg/mL) against *S. aureus*, indicating limited enhancement, possibly due to pollen's simpler phytochemical composition (Bayram et al., 2020).

The differential susceptibility between Gram-negative and Gram-positive strains reflects structural differences. Gram-negative bacteria, with their outer lipopolysaccharide layer, showed higher extract MICs but were more susceptible to Ag NSs, likely due to nanostructure penetration and disruption of the inner membrane (Adeyemi et al., 2020). Gram-positive strains, with lower extract MICs, benefited most from Pro-Ag NSs' enhanced activity, suggesting tailored applications for specific pathogens.

In essence, the inherent antimicrobial properties of Pro-Ag NSs and Pol-Ag NSs, coupled with the critical advantages conferred by their optimal shape and size distribution, make them exceptionally promising candidates for various applications. The research by Loo et al. (2018) further solidifies the understanding that nanostructure morphology and size are paramount for maximizing antimicrobial efficacy, while also reinforcing the safety and sustainability benefits of green synthesis. These advancements position Pro-Ag NSs and Pol-Ag NSs as key tools in addressing the ongoing global challenge of bacterial infections and antimicrobial resistance.

The broad-spectrum activity, of synthesized Ag NSs, warrants further investigation into biofilm inhibition, resistance mechanisms, *in vivo* efficacy, and potential toxicity (Alotaibi et al., 2022). While Pol-E and Pro-E are less potent, their cost-effectiveness makes them viable for Gram-positive infections. Although ciprofloxacin outperformed all agents (MIC 3.125 μg/mL), the promising activity of Ag NSs, especially Pro-Ag NSs.

### 4.7.3. Minimum Bactericidal Concentration

The minimum bactericidal concentration (MBC) results, as presented in Table 10, demonstrate the antibacterial activity of Pol-E, Pro-E, their silver nanostructure derivatives (Pol-Ag NSs and Pro-Ag NSs), and the standard antibiotic ciprofloxacin against four bacterial strains: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), and *Enterococcus faecalis* (ATCC 29212). The formation of silver nanostructures generally enhanced antibacterial activity relative to the crude extracts across most strains, with both Pol-Ag

NSs and Pro-Ag NSs exhibiting a two-fold reduction in MBC against the Gram-negative bacteria *E. coli* and *P. aeruginosa* compared to Pol-E and Pro-Extract, indicating enhanced efficacy. Pro-Ag NSs notably showed a four-fold decrease in MBC against the Gram-positive bacterium *S. aureus* (12.5 µg/ml compared to 50 µg/ml for Pro-E), highlighting an improvement, while also displaying the lowest MBC among the tested extracts and nanostructures against *E. faecalis* (12.5 µg/ml), demonstrating promising activity against this strain. Across all bacterial strains, ciprofloxacin consistently showed the lowest MBC values (3.125 µg/ml), underscoring its potent broad-spectrum antibacterial activity. In contrast, both crude extracts exhibited some antibacterial properties that were improved in their nanostructure forms, particularly with Pro-Ag NSs, which emerged as a promising candidate for complementing antibiotics in targeting resistant bacterial strains. These results suggest that Pro-Ag NSs could complement antibiotics like ciprofloxacin in targeting resistant bacterial strains, though further mechanistic studies are required to elucidate their interactions (Aabed and Mohammed, 2021).

**Table 10.** Minimum Bactericidal Concentration Results (µg/ml)

| Bacterial Strain | Sources | Pol-Extract | Pol-Ag NSs | Pro-Extract | Pro-Ag NSs | Ciprofloxacin |
|---|---|---|---|---|---|---|
| ***E. coli*** | ATCC 25922 | 50 | 25 | 50 | 25 | 3.125 |
| ***P. aeruginosa*** | ATCC 27853 | 50 | 25 | 50 | 25 | 3.125 |
| ***S. aureus*** | ATCC 25923 | 50 | 25 | 50 | 12.5 | 3.125 |
| ***E. faecalis*** | ATCC 29212 | 25 | 25 | 25 | 12.5 | 3.125 |

**Key:** Con concentration, Pol-E Pollen extract, Pol-Ag NS =Pollen-mediated silver nanostructure, Pro-Ag NS=Propolis mediated silver nanostructure, *E. coli=Escherichia coli*, *P. aeruginosa=Pseudomonas aeruginosa*, *S. aureus=Staphylococcus aureus*, *E. faecalis=Enterococcus faecalis*.

The MBC results reveal notable trends in the antibacterial efficacy of the tested extracts and their silver nanostructure derivatives compared to ciprofloxacin. The two-fold reduction in MBC for Pol-Ag NSs and Pro-Ag NSs against Gram-negative bacteria *(E. coli* and *P. aeruginosa*) aligns with the synergistic effects observed in biogenic synthesis, where natural extracts enhance the antimicrobial properties of silver nanostructures. The high surface area and reactivity of Ag NSs, combined with the reducing and capping agents in propolis and pollen, improve their ability to disrupt bacterial cell membranes (Zehra et al., 2025). Parvekar et al. (2020) similarly noted that plant-mediated Ag NSs exhibit enhanced antibacterial activity due to the combined effects of phytochemicals and silver ions, which aligns with the observed results.

The four-fold reduction in MBC for Pro-Ag NSs against *S. aureus* suggests a specific interaction with Gram-positive bacteria, likely due to their thicker peptidoglycan layer being more susceptible to nanostructure penetration and ROS generation (Mikhailova, 2024). Propolis's rich phenolic content, which acts as a reducing agent during synthesis, may further enhance this effect by increasing the polyphenol content in Pro-Ag NSs, as reported by Al-Fakeh et al. (2021). The low MBC of Pro-Ag NSs against *E. faecalis* (12.5 µg/ml) underscores their broad-spectrum potential, supporting their role in addressing resistant strains, a critical need in combating antimicrobial resistance (Aabed and Mohammed, 2021). The effectiveness of Pro-Ag NSs may also be attributed to their smaller size, which increase their surface area-to-volume ratio and facilitate better penetration into bacterial cells (Zehra et al., 2025).

The mechanisms underlying this enhanced activity likely involve multiple pathways. Ag NSs generate ROS, leading to oxidative stress that damages bacterial DNA, proteins, and lipids, ultimately causing cell death (Adeyemi et al., 2020). They also interact with thiol groups in membrane proteins, impairing respiratory enzymes and increasing membrane permeability, which facilitates further ROS accumulation (Mammari et al., 2022). These mechanisms are consistent with the findings of Kumar et al. (2024), who emphasized the stabilizing role of bee products in enhancing Ag NS efficacy.

Green synthesis for Pol-Ag NSs and Pro-Ag NSs offers additional advantages, including reduced toxicity and environmental impact compared to conventional methods that use toxic reagents (Rashidi et al., 2024). This aligns with the global push for sustainable nanotechnology, as highlighted by Naganthran et al. (2022). However, challenges such as variability in propolis composition due to regional biodiversity (Singh et al., 2024) and the need for scalable production methods must be addressed to ensure consistent therapeutic outcomes (Khalifa et al., 2024). Furthermore, the potential of Pro-Ag NSs to complement antibiotics like ciprofloxacin in clinical settings opens avenues for combination therapies, particularly against multidrug-resistant bacteria, but requires rigorous mechanistic and clinical studies to validate their efficacy and safety (Aabed and Mohammed, 2021). These results warrant deeper investigation into the mechanisms of action of Pol-Ag NSs and Pro-Ag NSs and their potential therapeutic applications in combating bacterial infections.

Table 11. MBC:MIC ratio of Pol-E, Pol-Ag NS, Pro-E and Pro-Ag NS

| **Strains** | **Pol-E** | | **Ratio MBC: MIC** | **Pol-Ag NS** | | **Ratio MBC: MIC** | **Pro-E** | | **Ratio (MBC: MIC)** | **Pro-Ag NS** | | **Ratio (MBC: MIC)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | MBC | | MIC | MBC | | MIC | MBC | | MIC | MBC | |
| *E. coli* | 50 | 50 | 1:1 | 12.5 | 25 | 2:1 | 25 | 50 | 2:1 | 12.5 | 25 | 2:1 |
| *P. aeruginosa* | 50 | 50 | 1:1 | 25 | 25 | 1:1 | 25 | 50 | 2:1 | 12.5 | 25 | 2:1 |
| *S. aureus* | 25 | 50 | 2:1 | 25 | 25 | 1:1 | 25 | 50 | 2:1 | 6.25 | 12.5 | 2:1 |
| *E. faecalis* | 25 | 25 | 1:1 | 12.5 | 25 | 2:1 | 12.5 | 25 | 2:1 | 6.25 | 12.5 | 2:1 |

**Key:** Pol-E=Pollen extract, Pol-Ag NS =Pollen-mediated silver nanostructure, Pro-Ag NS =Propolis silver nanostructure, *E. coli*=*Escherichia coli*, *P. aeruginosa*=*Pseudomonas aeruginosa*, *S. aureus*=*Staphylococcus aureus*, *E. faecalis*=*Enterococcus faecalis*.

The MBC:MIC ratios, generally around 2:1 (Table 11), indicate that the extracts and nanostructures are primarily bactericidal, as ratios ≤ 4:1 suggest both inhibitory and killing effects (Ranjita Misra and Saho, 2012). Low ratios are critical for severe infections requiring bacterial elimination. The consistent bactericidal activity across pathogens, similar to ciprofloxacin, underscores the therapeutic potential of Pro-Ag NSs and Pol-Ag NSs, particularly for resistant strains, warranting further mechanistic and clinical studies.

## 4.8. Antioxidant Activity of Propolis Extract, Pollen Extract, Pollen Ag NS and Propolis Ag NSs.

### 4.8.1. Antioxidant Activity of Propolis Extract and Pro-Ag NSs

Table 12 illustrates the DPPH radical scavenging activity of Pro-E, Pro-Ag NSs, and AA across various concentrations (25-400 µg/mL). The percentage inhibition of DPPH radicals is presented for each sample, alongside $IC_{50}$values, which indicate the concentration required to inhibit 50% of DPPH radicals.

Table 12**.** DPPH Radical Scavenging Activity of Pro-E, Pro-Ag NSs, and the standard ascorbic acid

| **Concentration** | **% Inhibition (DPPH Radical Scavenging Activity)** | | |
|---|---|---|---|
| **(µg/mL)** | **Pro-E** | **Pro-Ag NSs** | **AA** |
| **25** | 25.43±0.59 | 29.80±0.86 | 81.76±0.40 |
| **50** | 31.44±0.41 | 40.15±0.77 | 83.81±0.30 |
| **100** | 41.75±0.52 | 49.63±0.58 | 86.91±0.29 |
| **200** | 51.82±0.63 | 60.98±0.26 | 96.03±0.39 |
| **400** | 64.81±0.40 | 71.33±0.49 | 98.08±0.22 |
| **Mean** | 43 | 50.4 | 89.4 |

**Key:** Pro-E=Propolis extract Pro-Ag NSs =Propolis silver nanostructure AA=Ascorbic acid

The results demonstrate a positive correlation between concentration and DPPH radical scavenging activity for Pro-E, Pro-Ag NSs, and AA. The SA activity in general was slightly higher Pro-Ag NSs than Pro-E irrespective of the concentrations (Table 12). It was generally almost twice lower than the control highlighting their potential as natural antioxidants (Kurek-Górecka et al., 2022). The enhanced antioxidant activity of Pro-Ag NSs is likely due to the synergistic effects of silver nanostructures and propolis-derived bioactive compounds, such as flavonoids, phenolic acids, and terpenoids, which are known to effectively neutralize free radicals through electron donation (Chandraker et al., 2021).

These results highlight the promising potential of Pro-E and Pro-Ag NSs as effective natural antioxidants for use in pharmaceuticals, food preservation, and cosmetics (Al-Yousef et al., 2020; Javed et al., 2022). The eco-friendly synthesis of Pro-Ag NSs supports sustainable nanotechnology by providing a greener alternative to synthetic antioxidants. The enhanced performance of Pro-Ag NSs at higher concentrations indicates their potential for targeted therapeutic applications, such as reducing oxidative stress in chronic diseases or stabilizing industrial formulations. Nevertheless, further research is necessary to evaluate their stability, bioavailability, and safety within biological systems. Generally, these findings underscore the versatility and high performance of propolis-derived extracts and nanostructures as antioxidants, suggesting opportunities for their expanded use in industrial and biomedical fields.

### 4.8.2. Antioxidant Activity of Pollen Extract and Pol-Ag NSs

Table 13 demonstrates the DPPH radical scavenging capacity of honeybee-collected Pol-E, pollen-derived Ag NSs, and the reference antioxidant AA at concentrations ranging from 25 to 400 µg/mL. The results, presented as percentage inhibition values, were obtained from triplicate experimental measurements. To quantify the antioxidant potency of each sample, we calculated the $IC_{50}$ values, which represent the effective concentration required to neutralize 50% of DPPH free radicals.

Table 13. DPPH Radical Scavenging Activity of Pol-E, Pol-Ag NSs, and the standard ascorbic acid

| **Concentration** | **% Inhibition (DPPH Radical Scavenging Activity)** | | |
|---|---|---|---|
| **(µg/mL)** | **Pol-E** | **Pol-Ag NSs** | **AA** |
| **25** | 28.85±0.66 | 29.94±0.85 | 81.76±0.40 |
| **50** | 38.78±0.79 | 41.74±0.56 | 83.81±0.30 |
| **100** | 43.93±1.92 | 50.22±0.38 | 86.91±0.29 |
| **200** | 57.11±0.34 | 61.07±0.60 | 96.03±0.39 |
| **400** | 69.27±0.95 | 74.46±0.22 | 98.08±0.22 |

| | | | |
|---|---|---|---|
| **Mean** | 47.59 | 51.49 | 89.4 |

**Key:** Pol-E=Pollen extract   Pol-Ag NSs =Pollen silver nanostructure AA=Ascorbic acid

The DPPH radical scavenging activity results indicate a concentration-dependent antioxidant efficacy for Pol-E, Pol-Ag NSs, and AA. At lower concentrations (25 and 50 µg/mL), Pol-E showed lower scavenging activity than Pol-Ag NSs and AA, highlighting the superior antioxidant capacity of Pol-Ag NSs even at these levels. At higher concentrations (100-400 µg/mL), Pol-E's antioxidant activity was improved, approaching that of Pol-Ag NSs and AA, suggesting comparable efficacy at elevated concentrations. Pol-Ag NSs consistently exhibited robust antioxidant activity, closely rivaling AA, the standard antioxidant (Mani et al., 2021).

The antioxidant properties of Pol-Ag NSs are likely due to synergistic effects between silver nanostructures and pollen-derived bioactive compounds, such as polyphenols, flavonoids, and carbohydrates, which enhance free radical scavenging through electron donation or hydrogen donation mechanisms (Javed et al., 2022; Chandraker et al., 2022). Bee pollen directly scavenges ROS (Tutun et al., 2021), while Ag NSs neutralize ROS through electron donation and catalyze their conversion (Kumar et al., 2020). Pollen's phytochemicals likely enhance Ag NS antioxidant activity by acting as quenchers, donors, or reducing agents. The slightly lower $IC_{50}$ of Pol-Ag NSs compared to Pol-E suggests that the incorporation of silver nanostructures amplifies the antioxidant potential, a finding consistent with studies on green-synthesized Ag NSs, which often show enhanced radical scavenging due to nanostructure surface effects (Pirabassi et al., 2024).

### 4.8.3. Antioxidant Activity via $IC_{50}$ Values

The $IC_{50}$ values, as shown in Table 14, quantify the antioxidant potency of propolis and pollen extracts, their silver nanostructure derivatives, and AA. These values indicate the concentration required to inhibit 50% of free radicals, with lower values reflecting higher antioxidant activity.

Table 14. DPPH scavenging activity ($IC_{50}$ values) of Pol-E, Pro-E, Pol-Ag NSs, Pro-Ag NSs and the standard ascorbic acid

| | **Pro-E** | **Pol-E** | **Pro-Ag NSs** | **Pol-Ag NSs** | **AA** |
|---|---|---|---|---|---|
| **$IC_{50}$** | 61.56±0.47 | 48.62±0.99 | 45.54±0.57 | 48.58±1.04 | 5.49±0.31 |
| **$R^2$** | 0.9827 | 0.9764 | 0.9921 | 0.9828 | 0.9926 |

**Key:** Pol-E=Pollen Extract Pro-E = Propolis Extract Pol-Ag NSs =Pollen silver nanostructure Pro-Ag NSs Propolis Silver nanostructures AA=Ascorbic acid

Pro-Ag NSs demonstrated superior antioxidant activity with an $IC_{50}$ of 45.54 ± 0.57 µg/mL, outperforming Pro-E at 61.56 ± 0.47 µg/mL, though both were less potent than AA (5.49 ± 0.31 µg/mL). The enhanced efficacy of Pro-Ag NSs likely stems from the synergistic interaction between silver nanostructures and propolis-derived bioactive compounds, including flavonoids, phenolic acids, and terpenoids, which neutralize free radicals through electron donation (Chandraker et al., 2021). Pro-Ag NSs' $IC_{50}$ aligns with reported values for propolis-synthesized Ag NSs (40-60 µg/mL), attributed to the stabilizing role of phenolic compounds (Al-Fakeh et al., 2021). Compared to crude plant extracts, which often exceed 100 µg/mL, Pro-Ag NSs offer a promising natural alternative (Chandraker et al., 2022). Their potential applications span pharmaceuticals, cosmetics, and food preservation, supported by the eco-friendly synthesis process that minimizes environmental impact (Rashidi et al., 2024). However, optimizing nanostructure size or coating could further enhance potency to approach AA's efficiency (Javed et al., 2022). Variability in propolis composition due to regional biodiversity may affect consistency, necessitating further stability and safety studies for clinical or industrial use (Singh et al., 2024).

Pol-Ag NSs exhibited an $IC_{50}$ of 48.58 ± 1.04 µg/mL, slightly better than Pol-E at 48.62 ± 0.99 µg/mL, but both were significantly less potent than AA (5.49 ± 0.31 µg/mL). These values indicate moderate antioxidant activity, competitive with other biogenic Ag NSs, such as those from *Ziziphora clinopodioides* ($IC_{50}$: 30-50 µg/mL), due to pollen's phenolic stabilizers (Pirabassi et al., 2024). The marginal improvement of Pol-Ag NSs over Pol-E suggests that nanostructure synthesis enhances antioxidant capacity, though less dramatically than in propolis. Compared to crude plant extracts with $IC_{50}$ values often above 100 µg/mL, pollen-derived materials show promise (Chandraker et al., 2022). Pol-Ag NSs' high antioxidant activity at elevated concentrations suits applications in pharmaceuticals, cosmetics, and food preservation, particularly in formulations requiring sustained radical scavenging.

Green synthesis aligns with sustainable nanotechnology, reducing toxicity and environmental impact (Rashidi et al., 2024). However, further refinement of synthesis parameters, such as nanostructure size or coating, could boost potency closer to AA's level (Javed et al., 2022). In vivo stability, bioavailability, and safety require exploration, as regional pollen variability may impact reproducibility (Singh et al., 2024). Pol-Ag NSs thus offer a versatile, eco-friendly option for antioxidant systems, with potential for cost-effective solutions pending additional research.

Both Pro-Ag NSs and Pol-Ag NSs demonstrate enhanced antioxidant activity compared to their crude extracts, with Pro-Ag NSs showing greater potency. While less effective than AA, their $IC_{50}$ values position them as viable natural alternatives, particularly for sustainable applications. Further optimization and safety studies are essential to maximize their therapeutic and industrial potential.

## 4.9. Electrochemical Performance of the Fabricated Pd@Ag Nanostructures

### 4.9.1. Cyclic Voltammetry

Cyclic voltammetry (CV) was employed to thoroughly assess the electrochemical behavior of the fabricated Pd@Ag nanostructures. Initial CV analysis (Figure 14a) revealed a substantial enhancement in electron transfer at the Pd@Ag NP-modified nickel foam electrode compared to the bare electrode, signifying the catalytic efficacy of the bimetallic nanostructure. Subsequent experiments (Figure 14b) demonstrated a linear increase in anodic peak current with increasing $H_2O_2$ concentration (5 mM to 25 mM), confirming the sensor's enhanced activity towards $H_2O_2$ oxidation. Further investigation through CV at varying scan rates (5 to 110 mV/s) in 5 mM $H_2O_2$ (Figure 14c) showed an increase in redox peak current with scan rate, indicating a diffusion-controlled reduction process, which was corroborated by the linear relationship between the peak current and the square root of the scan rate (Figure 14d), yielding $R^2$ values of 0.999 and 0.988 for anodic (Ipa) and cathodic (Ipc) peak currents, respectively, confirming the diffusion-limited $H_2O_2$ redox at the Pd@Ag NPs.

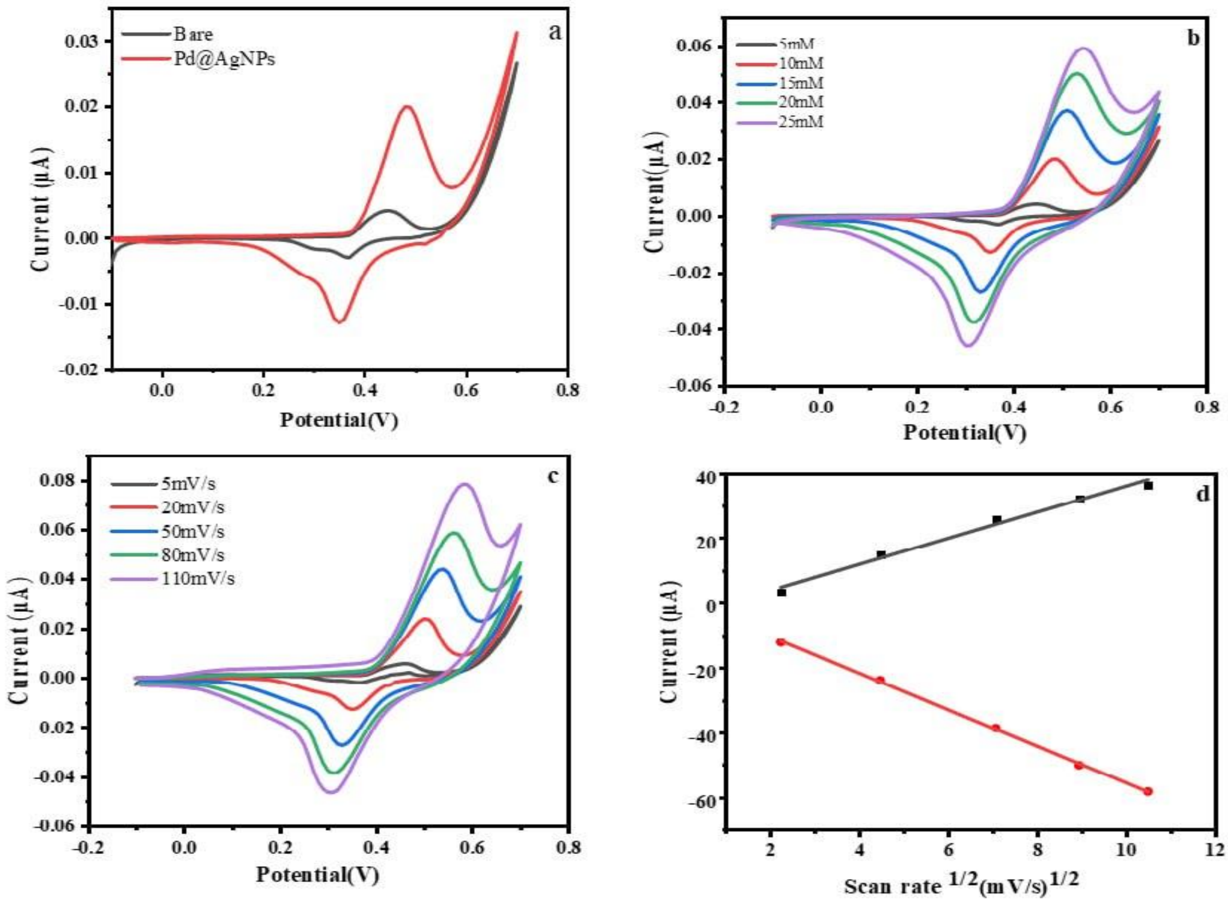


Figure 14. CV data for a modified Pd@Ag NPs electrode: (a). CV curves of bare and modified electrodes in 5 mM PBS solution. (b). Shows effect of varying $H_2O_2$ concentrations on CV response of Pd@Ag NPs electrode. (c). CV curves at different scan rates.

The observed enhancement in electron transfer at the Pd@Ag NP-modified electrode aligns with previous findings where synergistic metal interactions and unique surface properties of bimetallic nanostructures, such as Pt-Ag and Ag-ZnO, facilitated improved electron transfer kinetics (Bekmezci et al., 2023; Herbei et al., 2023). This behavior is consistent with the established understanding that Ag-based nanostructures, due to their high surface area and active sites, exhibit superior catalytic activity toward $H_2O_2$ oxidation (Wen et al., 2023). The linear increase in anodic peak current with $H_2O_2$ concentration (5 mM to 25 mM) further supports the sensor's sensitivity, a finding consistent with studies on hollow Ag-Pd nanostructures that have shown exceptional electrocatalytic properties for detecting analytes at trace concentrations (Wen et al., 2023). The diffusion-controlled nature of the redox process, evidenced by the linear relationship between peak current and the square root of the scan rate ($R^2$ = 0.999 for Ipa and 0.988 for Ipc), is well-established in electrochemical theory (Percival & Zhang, 2016), reinforcing the reliability of the Pd@Ag NPs as an effective electrocatalyst.

The increased energy required for $H_2O_2$ oxidation at higher concentrations arises from a combination of factors such as changes in surface interactions, including saturation and adsorption/desorption dynamics; localized oxygen-rich environments; the accumulation of intermediate species, such as hydroxyl radicals and superoxide anions, which compete for active sites and potentially passivate the electrode; diffusion limitations due to concentration gradients; and electrode fouling caused by by-product formation. Collectively, these factors elevate the energy barrier for $H_2O_2$ oxidation at higher concentrations. Additionally, the less distinct cathodic peak likely corresponds to the reduction of species generated during anodic oxidation. The strong linear correlation between peak currents and $H_2O_2$ concentrations confirms the high sensitivity of the Pd@Ag NP catalyst for $H_2O_2$ detection, a finding consistent with reports on other noble metal-based catalysts (Li et al., 2022). The low LOD of 1.306 µmol $L^{-1}$ highlights the sensor's capability to detect trace $H_2O_2$ levels, making it suitable for applications in monitoring oxidative stress-related diseases. Consequently, these results highlight the potential of Pd@Ag NPs as promising electrocatalysts for highly sensitive $H_2O_2$ detection, suggesting that further optimization is warranted for practical applications.

### 4.9.2. Electrochemical Impedance Spectroscopy

Electrochemical impedance spectroscopy (EIS) analysis of the Pd@Ag NS-modified electrode, presented as Nyquist plots (Figure 15), revealed a low solution resistance ($R_1$ = 1.292 ohms), a very low charge transfer resistance ($R_2 = 1.00 \times 10^{-4}$ ohms), a high double-layer capacitance ($C_1 = 1.384 \times 10^{-3}$ F), and a low Warburg impedance ($W_1$ = 0.0008591 $s^{1/2}/\Omega$).

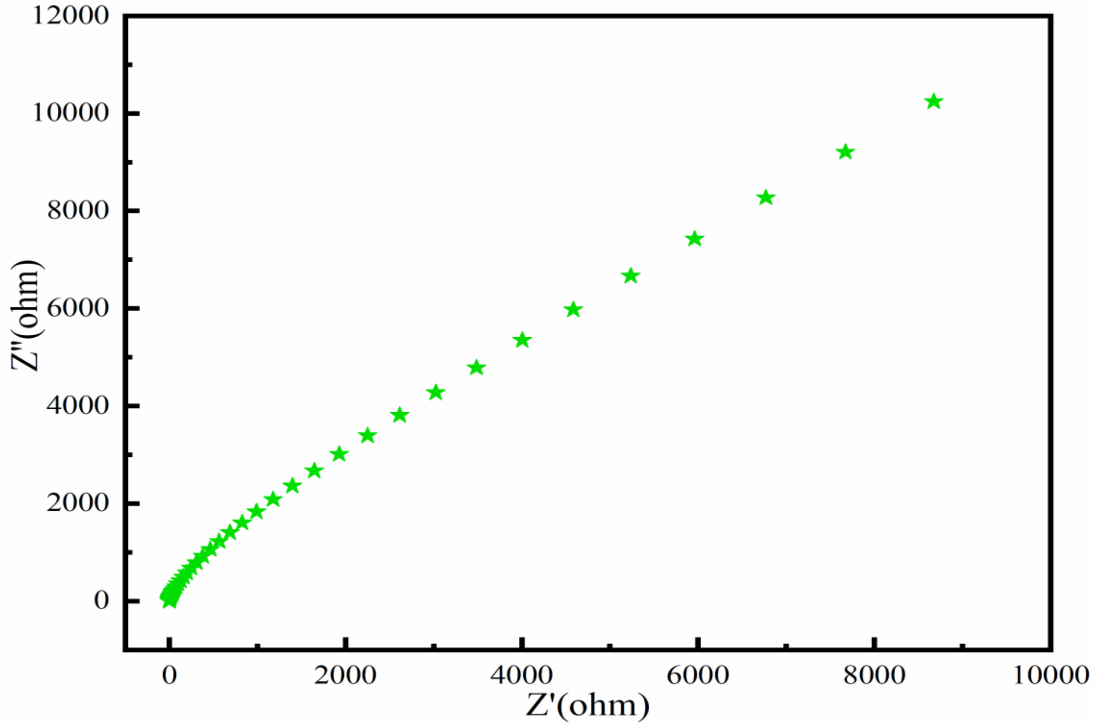


**Figure 15.** Electrochemical impedance spectroscopy spectra of the synthesized Pd@Ag NSs electrode.

The low solution resistance ($R_1$ = 1.292 ohms) indicates high conductivity within the medium, facilitating efficient $H_2O_2$ detection (Siracusano et al., 2018). The extremely low charge transfer resistance ($R_2 = 1.00 \times 10^{-4}$ ohms) reflects rapid charge transfer and strong catalytic interaction between Pd@Ag NSs and $H_2O_2$, underscoring the electrode's high catalytic activity. The high double-layer capacitance ($C_1 = 1.384 \times 10^{-3}$ F) further supports efficient charge transfer at the Pd@Ag NSs-$H_2O_2$ interface, enhancing sensor performance (Zhang et al., 2024). Additionally, the low Warburg impedance ($W_1 = 0.0008591$ $s^{1/2}/\Omega$) indicates minimal diffusional limitations, enabling efficient $H_2O_2$ diffusion to the electrode surface, which contributes to rapid electron transfer and sensor responsiveness (Nycz et al., 2019).

The combination of low $R_1$, $R_2$, and $W_1$ values, along with high $C_1$, highlights the Pd@Ag NSs' core-shell structure as a key factor in enhancing electron transfer and catalytic activity, making the electrode highly sensitive for $H_2O_2$ detection. While Warburg impedance is typically significant in diffusion-limited systems (Cruz-Manzo & Greenwood, 2020), its low value here suggests that the catalytic properties of the Pd@Ag NSs minimize diffusion resistance, likely due to the synergistic effects of the palladium core and silver shell. These characteristics position the Pd@Ag NS-modified electrode as a robust and efficient platform for $H_2O_2$ biosensing, with potential applications in biomedical fields, such as monitoring oxidative stress or $H_2O_2$-related therapeutics, and in industrial analytical systems requiring high sensitivity and rapid response.

### 4.9.3. Sensitivity Test

Amperometric measurements (i-t curve) conducted on the Pd@Ag NSs electrode at a constant potential of -0.5 V, with $H_2O_2$ additions every 60 seconds, showed a sharp initial current increase followed by decay and stabilization (Figure 16a). The electrode exhibited a linear current response across a $H_2O_2$ concentration range of 5 mM to 75 mM, with a high correlation coefficient of 0.994 (Figure 16b). The linear current response of the Pd@Ag NSs electrode across a wide $H_2O_2$ concentration range (5-75 mM) with a high correlation coefficient ($R^2 = 0.994$) demonstrates its sensitivity and reliability for quantitative $H_2O_2$ detection, making it suitable for biomedical applications such as assessing oxidative stress or monitoring $H_2O_2$-related therapeutic agents (Wang et al., 2024). The sensor exhibited a low detection limit (LOD) of 1.306 $\mu mol\ L^{-1}$, highlighting its capability to detect trace $H_2O_2$ concentrations. The sharp initial current increase upon $H_2O_2$ addition indicates efficient analyte adsorption and rapid detection of concentration changes, while the subsequent current decay likely results from active site saturation, diffusion limitations at higher concentrations (70 mM), and the kinetics of electrochemical reactions (Patel et al., 2020; Ahmad et al., 2022). The stabilization of the current at a steady-state value proportional to $H_2O_2$ concentration further confirms the sensor's robustness for precise measurements (Xing et al., 2022).

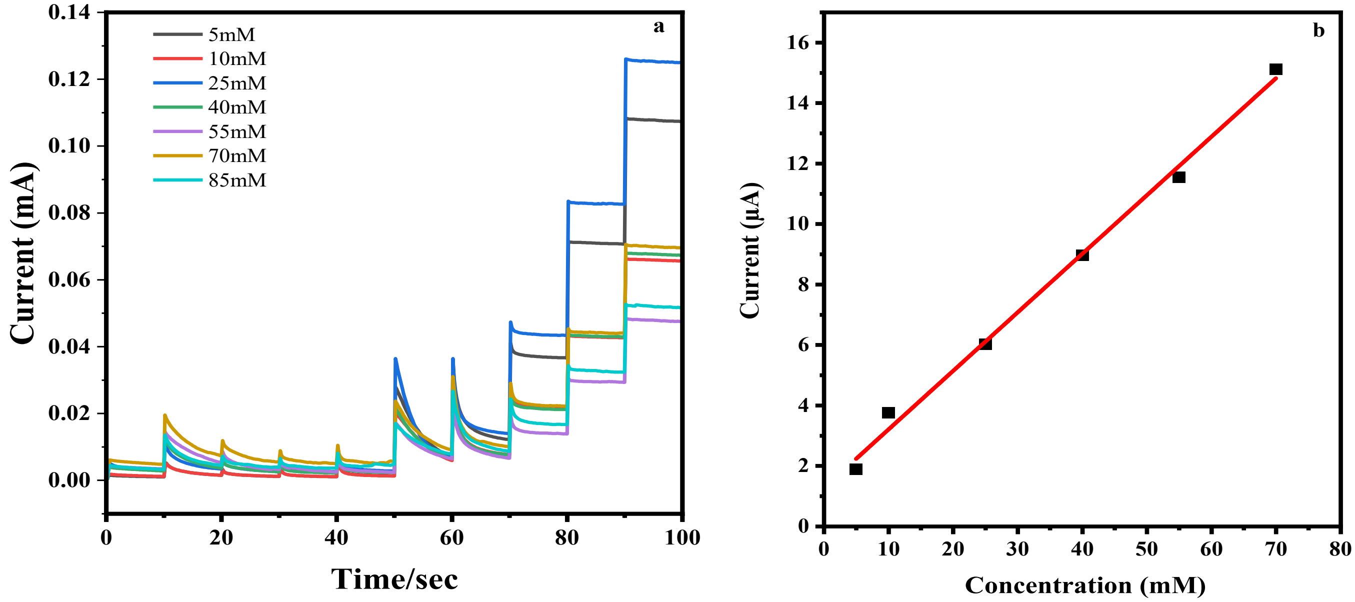


Figure 16. Electrochemical response of a Pd@Ag NSs electrode to $H_2O_2$ at -0.5 V. a) Current-time (i-t) curve in 5mM PBS (pH 7). b) Fitting curve for 5 mM to 70 mM $H_2O_2$ concentrations.

The high sensitivity and operational stability of the Pd@Ag NSs electrode underscore its potential as an effective $H_2O_2$ biosensor for research, industrial, and clinical applications. The core-shell structure of Pd@Ag NSs likely enhances electrocatalytic activity by improving electron transfer and analyte interaction compared to single-metal nanostructures, as the palladium core provides catalytic sites while the silver shell increases conductivity and surface area (Wen et al., 2023). This synergistic effect aligns with studies on bimetallic nanostructures like Pt-Ag and Ag-ZnO, which have shown improved electron transfer kinetics due to metal-metal interactions (Bekmezci et al., 2023; Herbei et al., 2023). Surface functionalization by propolis-derived biomolecules during synthesis may further enhance the electrode's performance by improving nanostructure stability and providing additional active sites for $H_2O_2$ interaction, a phenomenon observed in biogenically synthesized Ag NSs (Kumar et al., 2024). The strong linear correlation ($R^2$ = 0.994) between current response and $H_2O_2$ concentration validates the sensor's reliability, consistent with findings on noble metal-based catalysts for $H_2O_2$ detection (Li et al., 2022).

The low LOD of 1.306 µmol $L^{-1}$ and wide linear range (5-75 mM) position the Pd@Ag NSs electrode as a versatile tool for applications requiring accurate $H_2O_2$ detection, such as real-time monitoring of oxidative stress in clinical settings, where $H_2O_2$ serves as a biomarker for diseases like cancer and Alzheimer's (Khalifa et al., 2024). The rapid response to $H_2O_2$ concentration changes, as evidenced by the sharp initial current increase, supports its potential for point-of-care diagnostics, where quick and sensitive detection is critical (Srikhao et al., 2022). The cost-effectiveness of the sensor, due to the use of abundant materials like silver and propolis extract, makes it a viable option for widespread adoption, particularly in resource-limited environments, while the incorporation of propolis aligns with sustainable nanotechnology principles by reducing environmental impact compared to conventional synthesis methods (Rashidi et al., 2024). However, challenges such as long-term electrode stability and potential fouling at high $H_2O_2$ concentrations (e.g., 70 mM), as indicated by current decay, may limit its practical utility over extended periods, necessitating further optimization of surface properties and electrode design. Addressing these challenges could enhance the sensor's durability and ensure consistent performance in real-world applications, as noted in studies on electrochemical sensors (Ahmad et al., 2022).

A comparison of $H_2O_2$ biosensors, as shown in Table 15, highlights the competitive advantages of the Pd@Ag electrode. This new electrode shows promising performance regarding linear range, detection limit, and other crucial metrics. Importantly, it's also cost-effective due to the use of abundant materials like silver and propolis extract, making it relatively cheaper to produce than sensors using more expensive or intricate components. This affordability positions the Pd@Ag NSs electrode as a strong contender for widespread use in $H_2O_2$ sensing, especially where resources are limited. The incorporation of propolis, a natural substance, may also offer environmental benefits.

Table 15. Performance comparison of Pd@Ag NS with different electrochemical $H_2O_2$ sensors

| **Electrode** | **Potential (V)** | **Linearity** | **LOD** | **References** |
|---|---|---|---|---|
| HbGp/Nafion/GCE | -0.5 - +1.4 | 1.99-15.74 µM | 0.85µM | Nascimento et al., 2022 |
| Co@Ag/PPy | +0.4 | 0.1 µM - 1.8 mM. | 20 nM | Lamiri et al., 2024 |
| Ag-AuNPs/rGO | - 0.2 | 0.1-10 µM | 0.57 µM | Zhao et al., 2018 |
| Ag NSs-rGO | - 0.4 | 0.0015-100 mM | 1.90 µM | Bhangoji et al., 2021 |
| QDs/GO/PPy | -0.5 | 0.1-1 mM | 0.027 µM | Guan et al., 2023 |

| | | | | |
|---|---|---|---|---|
| Ag-Au/rGO/TiO2/GCE | -0.2 - +0.6 | 10-30,000µM | 3.0 µM | Han et al., 2020 |
| Pd@Ag NSs | -0.5 | 5-70 | 1.3 µM | This study |

# 5. CONCLUSIONS AND RECOMMENDATIONS

## 5.1. Conclusions

The presented study collectively showcases the remarkable potential of leveraging naturally derived biomolecules, specifically honeybee pollen and propolis, for the sustainable and eco-friendly synthesis of metallic nanostructures with implications across biological and sensing domains. This convergence of research underscores the burgeoning field of biomimetic nanotechnology, offering innovative pathways for creating sustainable solutions in medicine, environmental monitoring, and beyond.

The study synthesized propolis-mediated silver nanostructures, leveraging the dual role of propolis extract as both a reducing and stabilizing agent. Spectroscopic and microscopic analyses confirmed the formation of these nanostructures, which exhibited promising antibacterial activity against Gram-positive and Gram-negative bacteria. Notably, Pro-Ag NSs also demonstrated potent free radical scavenging activity, indicating their potential as natural antioxidants to combat oxidative stress in health, nutrition, or pharmaceutical applications. These dual antimicrobial and antioxidant properties suggest promising uses, particularly in antimicrobial coatings for medical devices and advanced wound dressings.

The green synthesis and biomedical potential of pollen-derived Ag NSs study demonstrates the successful biogenic fabrication of silver nanostructures using honeybee-collected pollen. Pollen enables a more environmentally friendly nanostructure synthesis by functioning as both a reducing and stabilizing agent. Comprehensive characterization through UV-visible spectroscopy, FT-IR, XRD, and SEM not only confirms the formation of Ag NSs but also elucidates their structural and functional attributes. Notably, the synthesized Ag NSs exhibit potent antimicrobial activity against pathogenic bacteria and antioxidant capacity, their therapeutic potential in combating infections and mitigating oxidative stress-related conditions. To fully realize this potential, future investigations should delve deeper into the precise mechanisms underlying their biological activities, rigorously evaluate their cytotoxicity in healthy cell lines and in living organisms, and conduct thorough preclinical studies to pave the way for safe clinical translation.

Building upon the demonstrated utility of biogenically synthesized silver nanostructures, the Pd-decorated Ag NSs for biosensing of $H_2O_2$ study explores the fabrication of palladium-decorated silver nanostructures using a green approach involving propolis extract. The introduction of palladium decoration enhanced the electrochemical sensing capabilities of the Ag NSs. Electrochemical characterization reveals markedly improved catalytic activity and efficient electron transfer, rendering Pd@Ag NSs-modified electrodes highly effective for the non-enzymatic detection of $H_2O_2$. The resulting sensor exhibits a wide linear detection range, high sensitivity, and excellent stability, with low charge transfer resistance confirmed through impedance spectroscopy. These attributes position Pd@Ag NSs as promising candidates for real-time $H_2O_2$ monitoring in diverse settings, including biomedical diagnostics, environmental quality control, and industrial process monitoring. Future research should focus on optimizing the electrode fabrication processes for enhanced performance, improving the long-term operational stability of the sensors, and rigorously validating their performance in complex real-world biological and environmental sample matrices.

## 5.2. Recommendations

- ✓ Firstly, a fundamental understanding of the intricate mechanisms driving the observed antimicrobial and antioxidant activities is paramount for optimizing their applications and predicting potential interactions.
- ✓ Secondly, comprehensive cytotoxicity assessments, employing both *in vitro* and *in vivo* models, are indispensable for establishing robust safety profiles and guiding dosage considerations.
- ✓ Thirdly, actively exploring and validating real-world applications, such as incorporating these nanostructures into medical coatings, wound healing formulations, and biosensing devices, will be essential for demonstrating their practical utility.
- ✓ Finally, rigorous validation of their performance in relevant clinical and environmental samples is necessary to ensure their effectiveness and reliability in intended use scenarios. Collaborative efforts across disciplines will be crucial to accelerate the translation of these exciting discoveries into tangible benefits for human health and environmental sustainability.

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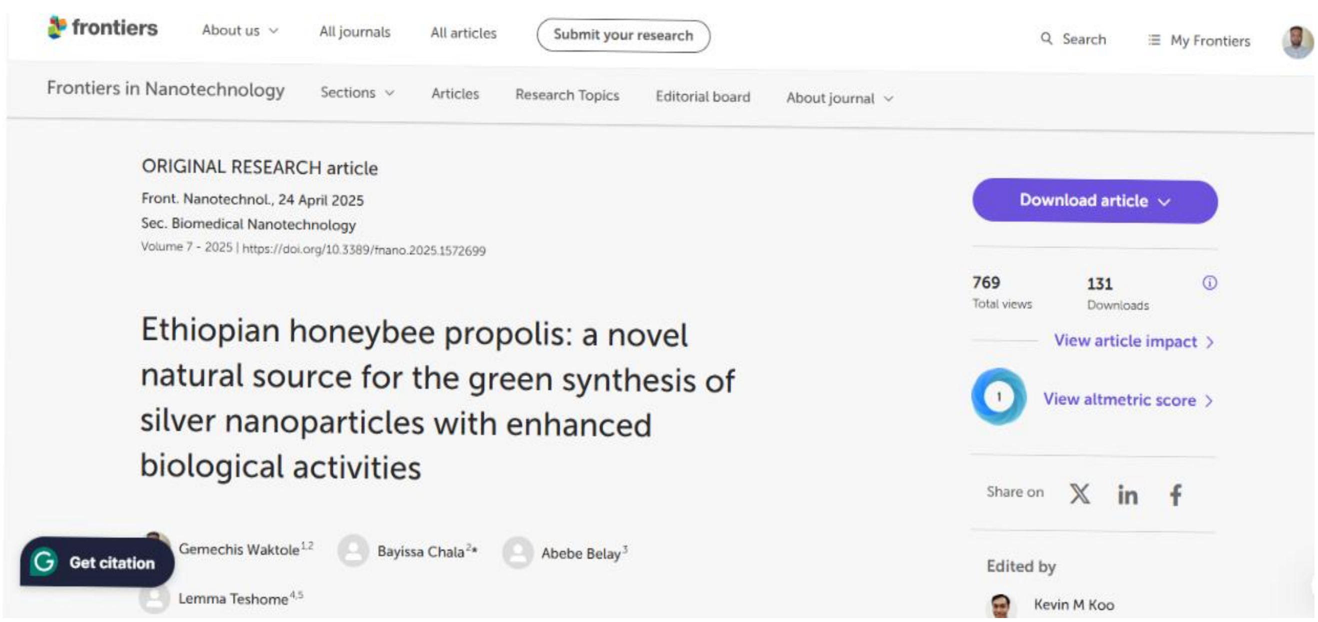
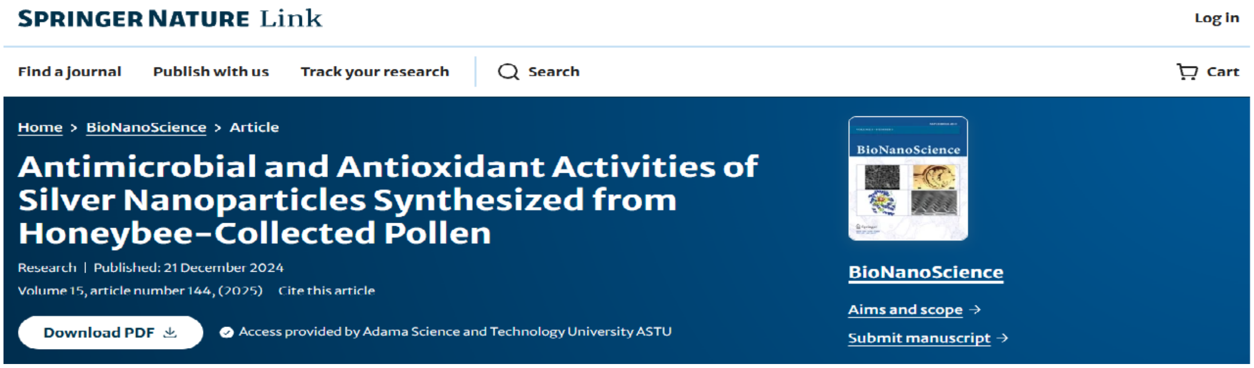
# 7. Appendices

### Appendix:1. List of Publications

1. Waktole, G., Chala, B., Belay, A., & Teshome, L. (2025). Ethiopian honeybee propolis: A novel natural source for the green synthesis of silver nanostructures with enhanced biological activities. *Frontiers in Nanotechnology*, *7*, 1572699. https://doi.org/10.3389/fnano.2025.1572699




2. Waktole, G., Chala, B., Belay, A., & Teshome, L. (2025). Antimicrobial and Antioxidant Activities of Silver Nanostructures Synthesized from Honeybee-Collected Pollen. *BioNanoScience*, *15*(1), 144.https://doi.org/10.1007/s12668-024-01766-6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**Antimicrobial and Antioxidant Activities of Silver Nanoparticles Synthesized from Honeybee-Collected Pollen**

Research | Published: 21 December 2024

Volume 15, article number 144, (2025) Cite this article

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BioNanoScience

Aims and scope →

Submit manuscript →

3. Palladium-Functionalized Silver Nanostructures for Non-Enzymatic Hydrogen Peroxide Biosensing (developed manuscript and ready to be submitted).

4. The Anti-Ageing Potential of Honeybee Pollen: A Focus on Its Antioxidant Properties and $H_2O_2$ Neutralization(Under Review,Journal of PharmaNutrition).


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Peer review status

The Anti-Ageing Potential of Honeybee Pollen: A Focus on Its Antioxidant Properties and H2O2 Neutralization

- Reviews completed: 0
- Review invitations accepted: 2
- Review invitations sent: 2+

Under Review

Last review activity: 16th May 2025

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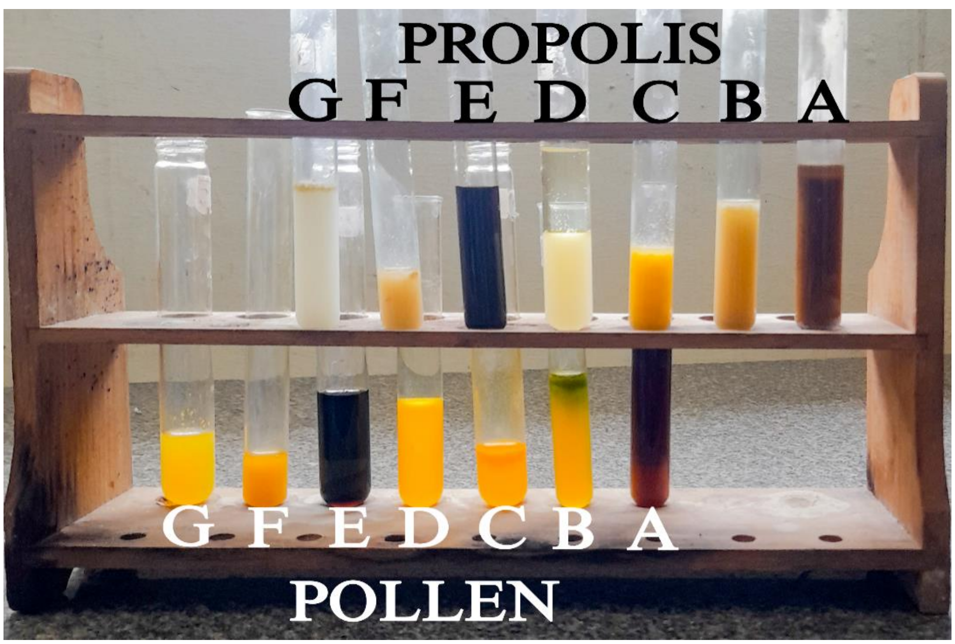
## Others

1. Chala, B., Tilaye, T., & Waktole, G. (2023). Re-emerging COVID-19: controversy of its zoonotic origin, risks of severity of reinfection and management. *International journal of general medicine*, 4307-4319.**DOI** https://doi.org/10.2147/IJGM.S419789.
2. Waktole, G., & Chala, B. (2023). The role of biosynthesized metallic and metal oxide nanostructures in combating anti-microbial drug resilient pathogens. Journal of Biomaterials and Nanobiotechnology, 14(1), 1-22.**DOI:** 10.4236/jbnb.2023.141001.

3. Waktole, G. (2023). Toxicity and molecular mechanisms of actions of silver nanostructures. *Journal of Biomaterials and Nanobiotechnology*, *14*(3), 53-70. **DOI:** 10.4236/jbnb.2023.143005.

**Appendix:2.**Supplementory Figures




**Supplementary Figure 1.**Phytochemical Analysis of the Propolis and Pollen Extracts


**Supplementary Figure 2.**Minimum Inhibitory Concentration