RESEARCH ARTICLE
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Antibacterial and Antifungal Activities of Silver Nanoparticles Synthesized Using Neem (*Azadirachta indica*) Leaf Extract

ABSTRACT

Background: The current investigation was conducted to illustrate the antifungal and antibacterial activity of non-toxic and environmental friendly biosynthetic nanoparticles of silver, which were prepared from the extract of *Azadirachta indica* leaf.

Methods: Synthesis of silver nanoparticles (AgNPs) was estimated by identifying variations in color pattern from yellow (bright) to brown (darkish). Five distinct techniques were used to investigate the characteristics of AgNPs, including an ultraviolet (UV)-Vis Spectrophotometer, Energy Dispersive X-ray Spectroscopy, X-ray diffractometer (XRD), Fourier Transform Infrared Spectroscopy (FT-IR), and a scanning electron microscope (SEM). The disc diffusion method was used to investigate microbial activity. The color shift in the solution suggested the formation of AgNPs, which was validated using a UV-Vis spectrophotometer.

Results: Energy Dispersive X-ray Spectroscopy examination showed the purity of AgNPs, and FT-IR analysis indicated the functional group of photo-chemicals in plant extract involved in decreasing and stabilizing AgNPs. The XRD examination showed that AgNPs are crystalline, and the nanoparticle size was determined to be 21.64 nm on average for *A. indica*. The rectangular segments fused together in *A. indica* were depicted by SEM images. About 200 µL concentration of AgNPs exhibited the most significant antifungal activity and was evaluated against *Fusarium oxysporum* and *Aspergillus flavus*. Similarly, 200 µL of AgNPs showed significant antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobacter aerogenes*, and *Pasteurella multocida*, with a maximum inhibition of growth in the zone of 13 mm.

Conclusion: In conclusion, biosynthesized AgNPs derived from *A. indica* leaf extract demonstrate significant potential as an eco-friendly and non-toxic alternative for antimicrobial applications. The remarkable antifungal and antibacterial activities exhibited by these *A. indica* AgNPs underscore their potential for developing innovative therapeutic agents against microbial pathogens.

Keywords: AgNPs, antimicrobial activity, *Azadirachta indica*, FT-IR analysis, scanning electron microscope, silver nanoparticles, UV-Vis spectrophotometer

What is already known on this topic?

- Silver nanoparticles (AgNPs) have a large surface area that allows interaction with various ligands, making them promising candidates for drug delivery and therapeutic applications.
- Biosynthesis of AgNPs using plant extracts is an emerging green technology, offering an eco-friendly alternative to conventional chemical synthesis methods.

What this study adds on this topic?

- This study highlights the successful green synthesis of AgNPs utilizing *Azadirachta indica* (Neem) leaf extract, presenting a sustainable and non-toxic method for nanoparticle production.
- Advanced characterization confirmed the crystalline structure, purity, and functional stabilization of the AgNPs, with an average size of 21.64 nm.
- The AgNPs exhibited significant antibacterial and antifungal activity, reinforcing their potential as alternative therapeutic agents.

INTRODUCTION

Nanotechnology is a rapidly advancing scientific discipline, offering immense opportunities for research and development in creating and applying innovative nanoscale materials.[1,2] Nanomaterials are particles having a size less than 100 nanometers,[3] with characteristics based on size, usage, and form. The purpose of nanoparticle characterization is to gain access to various properties.[4] The literature states that a variety of nanoparticles could be used for achieving different goals such as curing diseases and disorders, but silver nanoparticles have the strongest antibacterial potential.[5]

The Neem tree *(Azadirachta indica)* belongs to the Meliaceae family and is one of the most adaptable and diverse tropical trees with enormous potential.[6] Its bark, oil, and extracts of leaves have been utilized for the treatment of leprosy, intestinal helminthiasis, respiratory disorders, constipation, and as a health regulator. Its oil is used to treat a number of skin problems.[7] By mixing the bark, leaves, root, flower, and fruit, one can treat a variety of ailments including phthisis, blood sickness, biliary ailments, itching, skin ulcers, and

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burning sensations.[8] A variety of climatic, geographic, and edaphic conditions can be accommodated by the tree. It develops on shallow, hard calcareous or clay pan soils that are dry, stony, and have little water and lots of sunlight requirements.[9]

Neem leaves (*A. indica*) are widely available and highly valued for their medicinal properties, making them an ideal choice for this investigation.[10] Their natural synergistic effects eliminate the need for external agents during synthesis, potentially enhancing the antibacterial characteristics of the resulting silver nanoparticles.[11,12]

Several synthetic approaches have been employed, including the chemical reduction of silver ions in aqueous solutions, either in the presence or absence of stabilizing agents,[13] thermal decomposition in organic solvents,[14] and photochemical reduction in reverse micelles.[15] However, most of these methods are expensive and involve the use of toxic and hazardous substances, posing significant environmental and biological risks.

Antibacterial activity of *A. indica*: The Neem plant is widely used in pharmaceutics and medicine. Methanolic Neem extracts exhibit antibacterial activity against *Vibrio cholerae*, whereas chloroform extracts are effective against *Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalis*, and *Streptococcus faecalis*.[16] Neem oil effectively eliminates both Gram-positive and Gram-negative bacteria, including *Mycobacterium tuberculosis* strains and streptomycin-resistant bacteria. Additionally, Neem crude extract exhibits antibiotic properties against ear and eye infections.[17]

Antifungal action of *A. indica:* When tested against dermatophytes, the ethanol and aqueous extracts of *Azadirachta indica* leaves showed anti-dermatophyte activity. In some experiments, ethanolic extracts outperformed aqueous extracts in terms of perceived efficacy. The antifungal activities of *A. indica* methanolic and acetone extracts were evaluated against 2 fungal strains, *Aspergillus fumigatus* and *Aspergillus niger.*[18] When compared to acetone extracts, plant extracts in methanol demonstrated the strongest antifungal effects. The antifungal efficacy of Neem leaf and seed extracts against dermatophytes was also investigated.

The continuous release of silver ions from silver nanoparticles serves as an antimicrobial mechanism.[19] A strong affinity for sulfur-containing proteins is exhibited by silver ions, which electrostatically adhere to the bacterial cell wall and cytoplasmic membrane. This interaction increases membrane permeability, ultimately leading to bacterial envelope rupture.[19]

Upon exposure to free silver ions, bacterial cells generate reactive oxygen species (ROS) instead of ATP due to the inhibition of respiratory enzymes.[20] These ROS contribute to DNA damage and membrane disruption. Since phosphorus and sulfur are crucial for DNA replication and cell growth, their interaction with silver ions can hinder these processes, potentially leading to microbial cell death. Furthermore, silver ions can inhibit protein synthesis by denaturing ribosomes within the cytoplasm, further disrupting bacterial function.[21]

Green chemistry techniques are recommended because they are eco-friendly.[22] Recent advances in green synthesis research have been driven by the utilization of plants to produce metal nanoparticles.[23] Nanoparticles are formed by a variety of synthetic techniques such as lithography, ball milling, etching, and sputtering.[24] Thus, in the present study, the aim was to investigate the antibacterial and antifungal activity of Neem (*A. indica)* through the synthesis and characterization of AgNPs.

MATERIAL AND METHODS

Extraction of *Azadirachata indica*

After washing the *A. indica* leaves with distilled water, an electric blender was used to crush the dried leaves. A total of 20 g of leaf powder was agitated with 300 mL of distilled water. The mixture was heated using a magnetic stirrer on a hot plate. After heating, the solution was allowed to cool before being centrifuged at 7000 rpm for 15 minutes to separate the supernatant, which was then filtered through Whatman No. 1 filter paper. The pH of the extract ranged from 7.3 to 7.4, and it was stored at 4 °C.

Synthesis of Silver Nanoparticles

To reduce silver ions, 90 mL of 1 mM silver nitrate ($AgNO_3$) solution was mixed with the leaf extract. The mixture was then heated at 100°C for a specified duration with continuous agitation until a color change from light green to yellow was observed, indicating the formation of silver nanoparticles.[25]

Scanning Electron Microscope

The sample surface was observed by a scanning electron microscope (SEM), and Fourier Transform Infrared Spectroscopy (FT-IR) was performed with the FT-IR RX1-Perkin Elmer across a wavelength range of 4000-400 cm^{-1} to determine the biomolecules responsible for the reduction process.

Nanoparticles Characterization

The SEM analysis was conducted to characterize the morphology of the synthesized silver nanoparticles, and a UV-visible spectrophotometer was used to confirm their presence. The crystalline nature of the bio-fabricated silver nanoparticles was analyzed using an X-ray diffractometer (XRD) by Scherrer's equation, and their purity was assessed through Energy Dispersive X-ray Spectroscopy (EDX).

$t = k\lambda / B \cos\theta$,

where K = (0.92), t = crystal size, and A = (1.5418A°)[26]

Green Synthesis of Silver Nano-Particles

During the synthesis of silver nanoparticles, *A. indica* extracts serve as the primary reducing and capping agents. Their bioactive compounds, present in specific concentrations, play a crucial role in the eco-friendly synthesis of nanoparticles.[27] Silver nitrate ($AgNO_3$) is the most commonly used salt in the synthesis of AgNPs. Maintaining a neutral pH is essential for the successful preparation of green-synthesized AgNPs.[28] The reaction time between the plant extract and silver salt plays a crucial role in AgNPs synthesis. Additionally, the duration of their interaction is a significant factor in the biosynthesis of AgNPs[29] (Figure 1).

Antibacterial Activity

In this study, the antibacterial activity of biosynthesized Neem AgNPs was evaluated using the Kirby-Bauer standard disc diffusion method.[26,30] This assay was used against 4 bacterial strains: *Bacillus subtilis, Staphylococcus aureus, Enterobacter aerogenes*, and *Pasteurella multocida*. The bacterial strains were cultured in Luria–Bertani broth and incubated at 37°C for 24 hours. Mueller–Hinton Agar plates were prepared to uniformly inoculate with the bacterial suspension. Sterile filter paper discs (6 mm in diameter) were impregnated with varying volumes of Neem AgNP solution (50 µL, 100 µL, 150 µL, and 200 µL). A Gentamicin disc served as the positive control, while a distilled water disc was used as the negative control. The plates were incubated at 37°C for 24 hours. Following incubation, the zones of inhibition surrounding each disc were measured in millimeters (mm) using a ruler. All measurements were performed in triplicate, and the mean values were taken to assess the antibacterial efficacy of the Neem AgNPs.

Antifungal Activity

The antifungal activity of *A. indica* leaf extract-mediated AgNPs was evaluated using the disc diffusion method[31] against *Fusarium oxysporum* and *Aspergillus flavus*. Fungal strains were cultured on Sabouraud Dextrose Agar and incubated at 28°C for 4-5 days. In 1 set, Disc A served as the negative control containing only distilled water, while Disc B and Disc C contained 200 µL and 150 µL of AgNP solution, respectively. In a second set, Disc A was again the negative control, with Disc B containing 100 µL and Disc C containing 50 µL of AgNP solution.

RESULTS

Description of Silver Nanoparticles

Silver nanoparticles (AgNPs) were characterized using advanced techniques such as EDX analysis, X-ray Diffraction Spectroscopy (XRD), SEM, and Fourier Transform Infrared Spectroscopy (FT-IR) to assess their properties. The results are presented as follows:

Ultraviolet-Visible Spectroscopy: The UV-visible spectroscopy was employed to analyze the nanoparticles synthesized through the reaction between a silver nitrate solution and the plant extract. The UV-visible spectra were recorded at the International Islamic University Islamabad to confirm the formation of AgNPs mediated by *A. indica* (neem) leaf extract. Prior to spectral analysis, the AgNPs were subjected to ultra-sonication for 15 minutes to ensure a uniform dispersion of nanoparticles within the sample. Subsequently, UV-visible spectra were recorded in the wavelength range of 300-700 nm. The spectral analysis revealed a prominent absorption peak at point "A," within the range of 420-440 nm, as illustrated in Figure 2.

Scanning Electron Microscopy: Scanning Electron Microscopy was employed at the institute to analyze the structural properties of AgNPs synthesized from *A. indica* leaf extract. The AgNPs were first suspended in water and then carefully placed onto a carbon-coated copper grid. To remove any excess solution, blotting papers were used, ensuring an even distribution of nanoparticles. The prepared film was then left to dry under a mercury lamp for 5 minutes to achieve optimal imaging conditions. Once dried, the film was analyzed using SEM, and images were captured at various magnifications to observe the

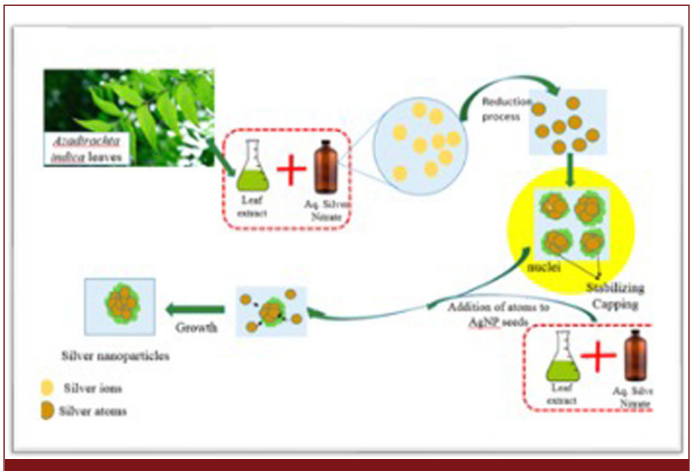

Figure 1. Green synthesis of silver nanoparticles.

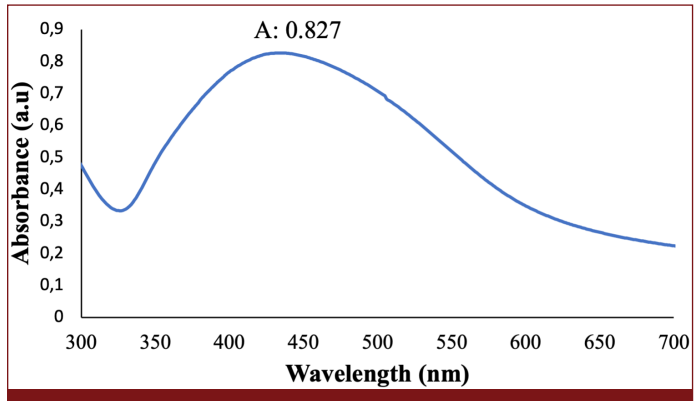

Figure 2. The ultraviolet-visible spectrum of green-synthesized silver nanoparticles (AgNPs) from *Azadirachta indica* displayed surface plasmon resonance (SPR) at A in the wavelength range of 420-440 nm.

morphological features of the synthesized AgNPs, which ranged from 10 to 50 nm (Figure 3).

X-Ray Diffraction Spectroscopy: X-ray diffraction (XRD) spectroscopy was utilized to analyze the diffractogram of AgNPs synthesized from *A.indica* within the angular range of 50° to 500°. The resulting data revealed non-crystalline peaks at 35.63°, 38.23°, and 47.25° (Figure 4). These non-crystalline diffraction peaks suggest the presence of phytochemicals, which play a key role in both the reduction process and the stabilization of the nanoparticles. The existence of phytochemicals that are involved in the reduction and stability of nanoparticles.

Energy Dispersive X–Ray Spectroscopy: To prepare the suspension of AgNPs, the green-synthesized AgNPs were carefully deposited onto a carbon-coated copper grid and left to dry. The EDX spectra revealed distinct absorption peaks corresponding to silver nanocrystals in the range of 2.5-4 keV. These peaks are indicative of the characteristic energy levels of silver, confirming the presence of silver nanoparticles. More specifically, the absorption peaks observed between 3 and 4 keV (Figure 5) further highlight the presence of silver nanocrystals, providing additional evidence of the successful synthesis and characterization of AgNPs.

Fourier Transform Infrared Analysis: The Fourier Transform Infrared (FT-IR) spectroscopy technique was employed to identify the functional groups involved in the stabilization and reduction of the AgNPs. The FT-IR spectrum of the AgNPs synthesized using *A. indica* exhibited prominent peaks at 3329 cm^{-1}, 2941 cm^{-1}, 1759 cm^{-1}, 1067 cm^{-1}, and 731 cm^{-1}. These peaks correspond to specific functional groups: the peak at 3329 cm^{-1} is associated with the stretching vibration of the hydroxyl group from intermolecularly bonded alcohols; the peak at 2941 cm^{-1} corresponds to the CH stretching vibration of alkanes; the 1759 cm^{-1} peak indicates C=O stretching, which is characteristic of carboxylic acid groups; the 1067 cm^{-1} peak is related to the C-O stretching of primary alcohols; and the peak at 731 cm^{-1} is attributed to C=C bending of alkenes with disubstitution. These functional groups play crucial roles in the reduction of silver ions and the stabilization of the resulting nanoparticles, as shown in Figure 6.

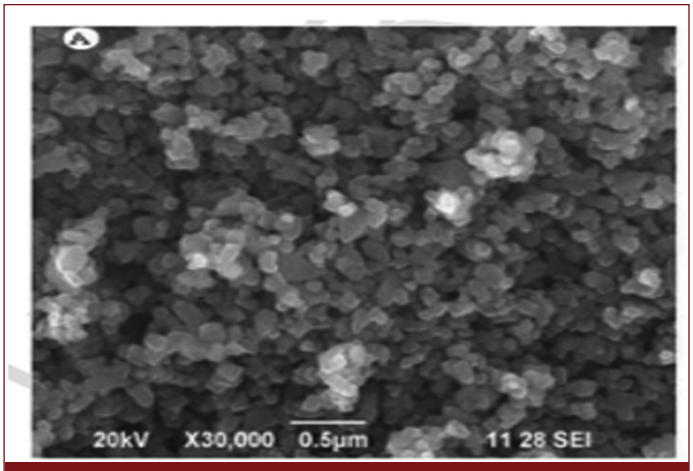

Figure 3. Scanning electron microscopy (SEM) of *Azadirachta indica* nanoparticles.

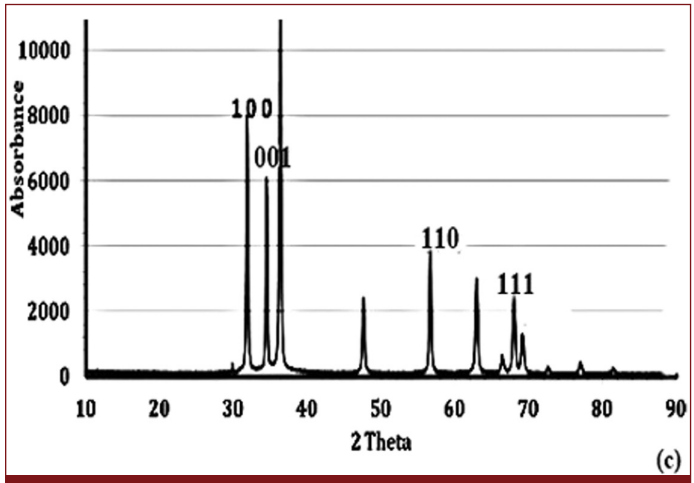

Figure 4. X-ray diffraction (XRD) pattern of the synthesized silver nanoparticles (AgNPs) from A*zadirachta indica*, showing diffraction peaks at 30.30°, 37.85°, and 55.55°, with additional non-crystalline peaks observed at 45.75°.

Antibacterial Activity

The antibacterial effectiveness of the synthesized AgNPs was assessed using the disc diffusion method. When a 200 µL solution of AgNPs was applied, significant anti-bacterial activity was observed against *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobacter aerogenes*, and *Pasteurella multocida*, with a maximum inhibition of growth in the zone of 13 mm. The antimicrobial action of the AgNPs is believed to be due to their ability to bind to the negatively charged surfaces of bacterial cells. This interaction disrupts

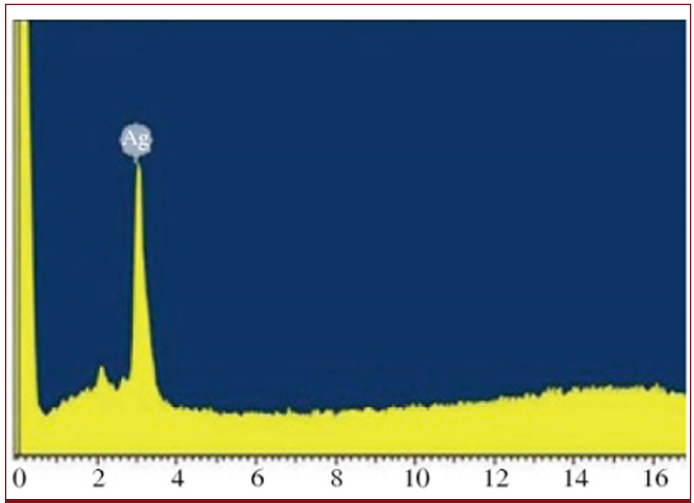

Figure 5. Energy Dispersive X-ray Spectroscopy (EDX) analysis of the green-synthesized silver nanoparticles (AgNPs) from A*zadirachta indica* demonstrates the presence of metallic silver ions along with impurities.

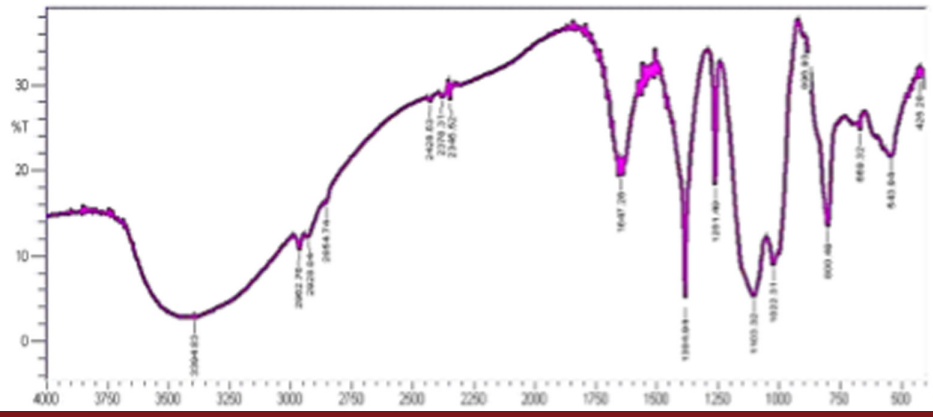

Figure 6. RTIR spectrum of *Azadirachta indica* medicated silver nanoparticles (AgNPs).

several essential cellular functions, including respiration, electron transport, membrane permeability, and osmoregulation. Furthermore, the silver ions (Ag+) released from the nanoparticles interact with bacterial proteins, particularly by binding to sulfhydryl groups, leading to protein denaturation. The silver ions also penetrate the bacterial cell wall, causing significant damage to the cell structure, which ultimately results in bacterial cell death. This mechanism highlights the potent antibacterial properties of AgNPs and their potential as effective antimicrobial agents.

Disc AB served as the positive control, containing the antibiotic Gentamicin, which demonstrated antimicrobial activity. In contrast, discs a, b, c, and d represented the negative controls, where 50 µL, 100 µL, 150 µL, and 200 µL solutions of AgNPs were tested, respectively. Among these, the 200 µL solution (disc d) exhibited the highest inhibitory effect against *Enterobacter aerogenes*, *Pasteurella multocida*, *Bacillus subtilis*, and *Staphylococcus aureus*, as shown in Figure 7. This suggests that the effectiveness of the AgNPs increased with the volume used, highlighting their potential as a strong antimicrobial agent in comparison to the antibiotic control.

In comparison, the 200 µL concentration of AgNPs demonstrated the highest antibacterial potential among all tested concentrations. However, it is noteworthy that all dilutions—50 µL, 100 µL, 150 µL, and 200 µL—displayed

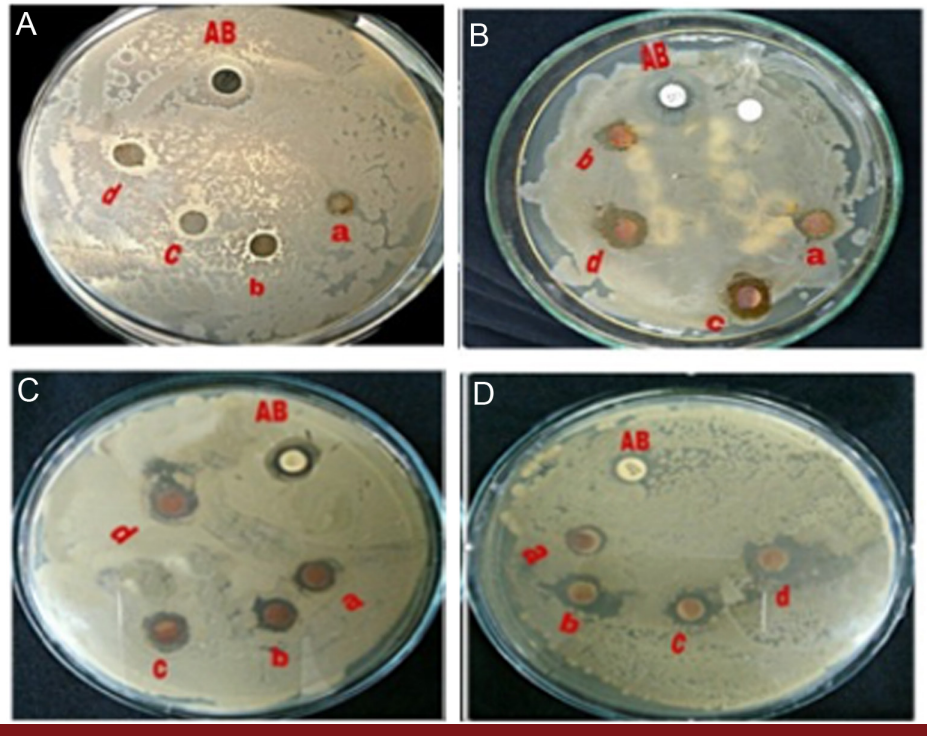

Figure 7. Antibacterial activity of AgNPs against *Enterobacter aerogenes*, *Pasteurella multocida*, *Bacillus subtilis*, *Staphylococcus aureus*.

Table 1. Comparisons of the Antibacterial Activity of Silver Nanoparticles Synthesized by *Azadirachta indica*

	Inhibition Zone Against Bacteria				
Bacteria	**Gentamicin**	**50 µL**	**100 µL**	**150 µL**	**200 µL**
Enterobacter aerogenes	13 mm	7 mm	9 mm	11 mm	13 mm
Pasteurella multocida	13 mm	8 mm	9 mm	12 mm	13 mm
Bacillus subtilis	11 mm	8 mm	10 mm	11 mm	12 mm
Staphylococcus aureus	8 mm	8 mm	9 mm	10 mm	13 mm

significant antibacterial activity, indicating that even lower concentrations of AgNPs possess strong potential as antimicrobial agents. These findings suggest that AgNPs, regardless of the concentration, can effectively serve as antibacterial agents, with the 200 µL concentration showing the most pronounced effect (Table 1).

Antifungal Activity

The antifungal activity of various dilutions (50 µL, 100 µL, 150 µL, and 200 µL) of *A. indica* leaf extract-mediated AgNPs was evaluated against 2 fungal species, *Fusarium oxysporum* and *Aspergillus flavus*. The results showed that the 200 µL concentration of AgNPs exhibited the most significant antifungal effectiveness, demonstrating a pronounced ability to suppress fungal growth. This suggests that higher concentrations of AgNPs synthesized with *A. indica* leaf extract possess enhanced antifungal properties, as illustrated in Figure 8.

DISCUSSION

In the present study, the biosynthesis of AgNPs using *A. indica* leaf extract was successfully carried out, with the process being marked by a noticeable color change from bright yellow to dark brown. This color transformation is a well-known indicator of the formation of silver nanoparticles, confirming the successful synthesis of AgNPs.

Characterization techniques further supported these findings. Ultraviolet-Vis spectrophotometry revealed a distinct peak characteristic of AgNPs, which is typically observed due to the surface plasmon resonance of silver nanoparticles (Figure 1). This peak is often used as a reliable indicator of nanoparticle formation, confirming the presence of AgNPs in the sample. Additionally, EDX analysis provided further validation by confirming the purity of the synthesized AgNPs, detecting silver as the primary element and eliminating the presence of any significant impurities (Figure 3). X-ray diffraction (XRD) analysis provided insights into the crystalline structure of the synthesized AgNPs, revealing an average particle size of 21.64 nm (Figure 4). The XRD pattern also showed non-crystalline peaks at 35.63°, 38.23°, and 47.25°, which are consistent with patterns reported in various studies (Figure 4). These peaks are likely associated with the phytochemicals in *A. indica* leaf extract that contribute to the reduction and stabilization of the nanoparticles, while the crystalline peaks indicate the formation of silver nanoparticles with a well-defined structure. The combination of visual, spectroscopic, and diffraction-based analyses in this study confirms the successful biosynthesis of AgNPs using *A.indica* leaf extract and provides a thorough characterization of their size, structure, and purity. These findings align with those from numerous other studies in the field, supporting the potential of *A. indica* as an effective green synthesis method for silver nanoparticles.[32] Scanning electron microscope images revealed fused rectangular segments, which further emphasized the unique morphology of the silver nanoparticles (Figure 2). Fourier Transform Infrared Spectroscopy analysis identified functional groups in the *A. indica* leaf extract that are responsible for both the reduction and stabilization of the nanoparticles (Figure 5). Furthermore, the AgNPs exhibited notable antibacterial and antifungal activity, as shown by the disc diffusion assay. The successful biosynthesis and characterization of AgNPs using *A. indica* leaf extract align with the increasing interest in plant-based products as sustainable alternatives to conventional therapeutics.

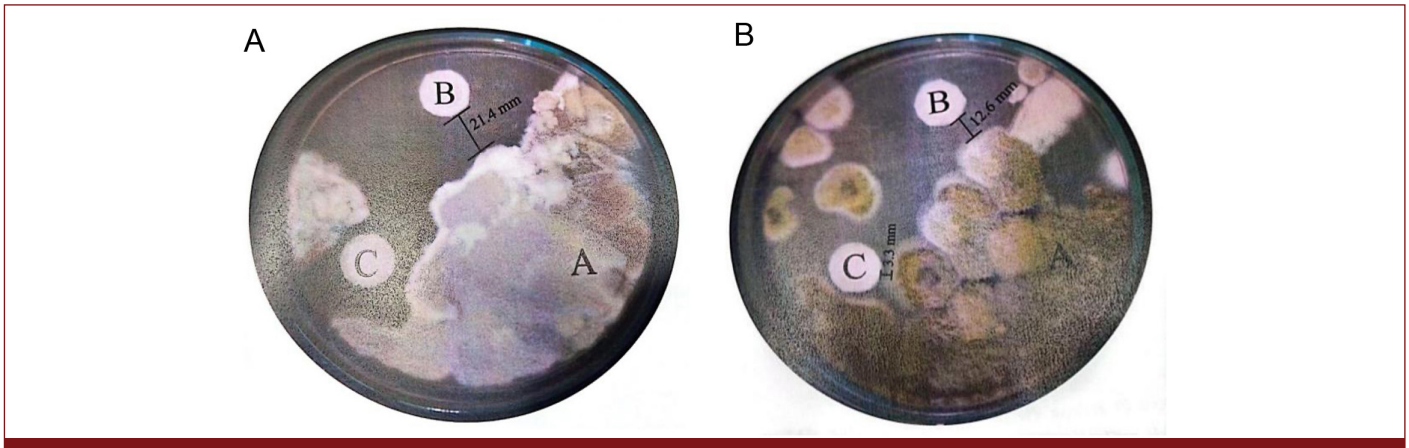

Figure 8. (A) Antifungal activity of AgNPs against *F. oxysporum*, (B) Antifungal activity of silver nanoparticles (AgNPs) against *Aspergillus flavus*.

The Centers for Disease Control and Prevention (CDC) has emphasized the urgent need to expand the pharmacological repertoire in response to the growing threat of antibiotic resistance, which contributes to over 35 000 deaths annually.[31,32] Despite the long-standing global use of herbal medicine, conventional antibiotics have historically dominated the search for therapeutic agents, leaving plant-based alternatives underexplored.[33] However, as the demand for new antimicrobial solutions continues to rise, an increasing number of researchers are now focusing on novel plant sources and other natural resources to identify potential new treatments.

Despite the widespread use of herbal medicine worldwide, traditional antibiotics have often taken precedence in antimicrobial research, limiting the exploration of plant-derived alternatives. However, the pressing need for new antimicrobial agents, fueled by challenges such as antibiotic resistance, has led to a growing interest in natural sources.[33] In this regard, *A. indica*, a medicinal plant known for its diverse therapeutic properties, stands out as a promising candidate (Figure 7). The notable antibacterial and antifungal activity exhibited by the synthesized AgNPs highlights the potential of neem-based nanotechnology in combating multidrug-resistant pathogens.

The results were in complete agreement with previous studies on the reduction of silver ions. The Surface Plasmon Resonance (SPR) of the silver nanoparticles, which is considered the primary indicator of AgNPs formation, caused a color shift in the reaction mixture from yellow to brown. This resonance was observed at wavelengths between 423 and 425 nm. The functional groups present in the phytomolecules of the leaf extract, which contribute to the bio-reduction of AgNPs, were successfully detected using FT-IR spectroscopy. The biosynthesized AgNPs appeared to inhibit the growth of *E. coli* at a concentration of 10 µg/mL (Figure 6, Table 1), while the growth of *S. aureus* was completely suppressed by AgNPs at a concentration of 5 µg/mL. These results align with previous studies that indicated *S. aureus* is more sensitive to AgNPs than *E. coli*.

These results highlight the potential of biosynthesized AgNPs as effective antimicrobial agents. Their environmentally friendly synthesis process, coupled with their strong biological activity, makes them promising candidates for future pharmaceutical and therapeutic applications, especially in combating the global issue of antibiotic resistance. Further research is needed to better understand the precise mechanisms driving their antimicrobial effects and to assess their biosafety in vivo models.

The present study demonstrates that the green synthesis of AgNPs offers several advantages over traditional chemical methods, including enhanced safety, environmental sustainability, and improved outcomes. Scanning electron microscope analysis confirmed that the AgNPs were spherical in shape, and FT-IR analysis indicated the involvement of biomolecules in both the reduction of silver ions and the stabilization of AgNPs. Biosynthesized AgNPs exhibited superior antibacterial and antifungal activity against pathogenic microorganisms, presenting a promising approach for a range of applications. The results also indicate that AgNPs can inhibit the growth of leukemic cells, suggesting potential for further testing against other cell lines to explore their viability as a therapeutic strategy. Additionally, both current and previous studies highlight the significant impact of the geographical origin of *A. indica* on the properties of the synthesized AgNPs.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: This laboratory-based study involved chemical and microbiological analyses, with no human participants; therefore, ethics committee approval was not required.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – M.Y.A.; Design – M.Y.A., R.L.; Supervision – M.Y.; Resources – M.Y.A., R.L.; Materials – R.L., M.Y.S.; Data Collection and/or Processing – R.L., M.Y.S.; Analysis and/or Interpretation – A.S., M.Y.A.; Literature Search – A.S., R.L., M.Y.S.; Writing Manuscript – A.S., M.Y.A, R.L.; Critical Review – M.Y.A.

Declaration of Interests: The authors have no conflicts of interest to declare.

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