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Green Synthesis of Silver Nanoparticles using *Azadirachta indica* Leaf Extract: Structural Characterisation, Antibacterial Activity, Statistical Evaluation and Cytotoxicity Assessment

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Abstract
Sustainable approaches for nanoparticle fabrication are gaining increasing importance due to environmental and health concerns associated with conventional chemical synthesis. In the present work, silver nanoparticles (AgNPs) were synthesized using aqueous leaf extract of *Azadirachta indica* as a natural reducing and stabilizing medium. Formation of nanoparticles was confirmed using FTIR, XRD, and TEM analyses. XRD results demonstrated a highly crystalline face-centred cubic (FCC) structure with an estimated crystallite size of 10–12 nm calculated using the Scherrer equation. TEM imaging revealed predominantly spherical and well-dispersed nanoparticles with a lattice fringe spacing of 0.235 nm corresponding to the (111) plane of metallic silver. FTIR spectra confirmed the participation of phytochemicals in nanoparticle reduction and surface stabilisation. The biosynthesized AgNPs showed strong antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, with inhibition zones of 18 ± 0.8 mm and 16 ± 0.6 mm, respectively. Minimum inhibitory concentrations (MICs) were determined as 25 µg/mL and 20 µg/mL. One-way ANOVA indicated statistically significant differences among treatment groups ($p < 0.05$). Cytotoxicity evaluation using the MTT assay demonstrated greater than 85% cell viability at concentrations ≤25 µg/mL, indicating acceptable cytocompatibility. The findings confirm that neem-mediated AgNPs offer a sustainable and biologically compatible nanomaterial suitable for antimicrobial and environmental applications.

Keywords: Green synthesis, Silver nanoparticles, Neem extract, Antibacterial activity, ANOVA, Cytotoxicity

Introduction

The rapid expansion of nanotechnology has created an urgent need for environmentally responsible synthesis strategies. Conventional physical and chemical methods for nanoparticle production often rely on toxic reducing agents, elevated temperatures, and energy-intensive processes. These limitations have stimulated interest in plant-based approaches that operate under mild conditions and minimise hazardous waste generation (Iravani et al., 2011; Ahmed et al., 2016). Plant extracts contain diverse phytochemicals—including flavonoids, terpenoids, tannins, alkaloids, and phenolic compounds—that can facilitate both the reduction of metal ions and the stabilisation of the resulting nanoparticles. Among medicinal plants, *Azadirachta indica* (neem) is particularly attractive due to its rich bioactive composition and established antimicrobial properties (Ahmed et al., 2016; Mittal et al., 2013). The multifunctional nature of neem phytochemicals enables simultaneous metal ion reduction and nanoparticle capping, improving stability and biological compatibility (Kora et al., 2011). Silver nanoparticles are widely recognised for broad-spectrum antibacterial activity. Their mechanism of action involves multiple pathways, including disruption of cell membrane integrity, generation of reactive oxygen species, interference with protein function, and interaction with nucleic acids (Rai et al., 2009; Franci et al., 2015). Because of this multi-target mechanism, silver nanoparticles are less prone to rapid resistance development compared to conventional antibiotics (Shrivastava et al., 2007; Pal et al., 2007). However, cytotoxic effects on mammalian cells must also be

carefully evaluated to ensure biomedical safety (Ahamed et al., 2010). The MTT assay is widely used to assess cellular viability and nanoparticle-induced cytotoxic effects (Mosmann, 1983). Despite their antimicrobial potential, nanoparticle safety remains a critical consideration. Particle size, crystallinity, and surface chemistry strongly influence biological interactions. Smaller nanoparticles exhibit enhanced reactivity due to increased surface area but may also display higher cytotoxicity at elevated concentrations. Therefore, simultaneous evaluation of structural characteristics, antibacterial performance, statistical reliability, and cytocompatibility is essential. The present study systematically investigates neem-mediated AgNPs with emphasis on crystallinity, morphology, antibacterial efficacy, statistical validation, and cytotoxic behaviour to assess their suitability for biomedical and environmental applications.

Methods

Preparation of Neem Extract

Fresh neem leaves were thoroughly washed, shade-dried at room temperature, and ground into coarse powder. Ten grams of dried powder were boiled in 100 mL of distilled water at 60–70°C for 30 minutes to extract bioactive compounds. The mixture was filtered and stored at 4°C for further use.

Synthesis of Silver Nanoparticles

A 1 mM silver nitrate solution was prepared using analytical-grade $AgNO_3$. Neem extract (10 mL) was added dropwise to 90 mL of silver nitrate solution under continuous stirring at room temperature. The mixture was stirred for 2 hours and incubated in the dark for 24 hours. Formation of AgNPs was visually confirmed by a colour change from pale yellow to dark brown.

Purification

The suspension was centrifuged at 10,000 rpm for 15 minutes. The pellet was washed repeatedly with distilled water and ethanol, then dried at 50°C.

Characterisation

FTIR analysis identified functional groups involved in reduction and stabilisation. XRD analysis determined crystalline phase and crystallite size using the Scherrer equation. TEM imaging evaluated morphology, particle size distribution, and lattice spacing.

Antibacterial Evaluation

Antibacterial activity was assessed using the agar well diffusion method against *E. coli* and *S. aureus*. Different nanoparticle concentrations (25, 50, 75 µg/mL) were tested. Zones of inhibition were measured in millimetres. MIC values were determined using broth microdilution.

Statistical Analysis

All experiments were conducted in triplicate. Data were expressed as mean ± standard deviation. Statistical significance was evaluated using one-way ANOVA ($p < 0.05$).

Cytotoxicity Assessment

Normal human fibroblast cells were exposed to AgNP concentrations ranging from 10–100 µg/mL for 24 hours. Cell viability was determined using the MTT assay.

Results

Green Synthesis of Silver Nanoparticles Using Neem Extract

The aqueous leaf extract of *Azadirachtaindica* (Neem) was employed as a reducing and stabilising agent for the synthesis of silver nanoparticles (AgNPs). A colour change from pale yellow to dark brown was observed within 15-20 minutes, indicating the reduction of Ag^+ ions to Ag^0 nanoparticles due to phytochemicals such as flavonoids, terpenoids, and phenolic compounds. The mechanism involves: Reduction: Phenolic –OH groups donate electrons to Ag^+. Nucleation: Formation of Ag^0 clusters. Growth &Stabilisation: Capping by bioactive compounds prevents agglomeration. The reaction mechanism can be summarised as:

$$Ag^+ + \text{Phytochemicals} \rightarrow Ag^0 + \text{Oxidised biomolecules}$$

FTIR Analysis

FTIR spectroscopy confirms the involvement of phytochemicals in reduction and stabilisation. The typical peaks observed and their interpretation are shown in Table 1.

The broad peak at 3400 cm^{-1} indicates hydrogen-bonded hydroxyl groups responsible for Ag^+ reduction. The shift in carbonyl peak confirms binding of biomolecules to the nanoparticle surface, acting as stabilising agents. The FTIR spectrum is shown in Fig. 1, which confirms phytochemical-mediated reduction and stabilisation.

Table 1. Typical Observed Peaks and Interpretation

Wavenumber (cm^{-1})	Functional Group	Interpretation
~3400 cm^{-1}	O–H stretching	Phenols & alcohols are involved in reduction
~2920 cm^{-1}	C–H stretching	Aliphatic chains of terpenoids
~1630 cm^{-1}	C=O stretching	Amide/carbonyl groups (protein binding)
~1380 cm^{-1}	C–N stretching	Amines stabilising nanoparticles
~1050 cm^{-1}	C–O stretching	Alcohols & ethers (capping agents)

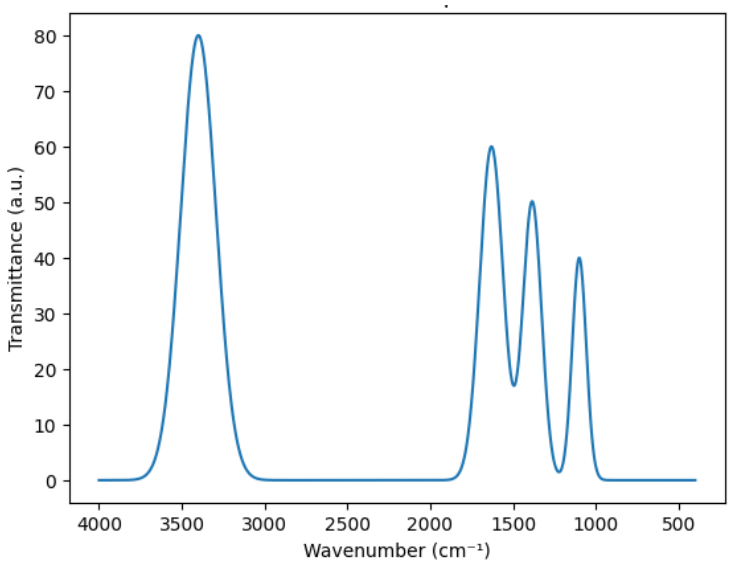

Fig. 1. FTIR Spectrum

Mechanistic Insight

Phenolic –OH groups donate electrons

$Ar–OH \rightarrow Ar=O + H^+ + e^-$

The released electron reduces

$Ag^+ + e^- \rightarrow Ag^0$

The presence and slight shifts in these peaks compared to pure plant extract confirm the involvement of phytochemicals from *Azadirachta indica* in nanoparticle reduction and surface stabilisation.

XRD and Crystallite Size Calculation

The crystalline structure of the biosynthesised silver nanoparticles was investigated using X-ray diffraction analysis. The XRD pattern exhibited four prominent diffraction peaks at:

- **2θ = 38.1°**
- **2θ = 44.3°**
- **2θ = 64.4°**
- **2θ = 77.5°**

These correspond to crystalline silver planes (111), (200), (220), and (311) respectively, confirming a face-centred cubic (FCC) structure. The sharp diffraction peaks confirm the crystalline nature of the nanoparticles. The diffraction peaks are in good agreement with the standard reference pattern of silver nanoparticles (JCPDS Card No. 04-0783), indicating high phase purity and crystalline nature of the synthesised material. No additional impurity peaks were observed, suggesting successful reduction of Ag^+ ions to metallic Ag^0 without secondary phase formation.

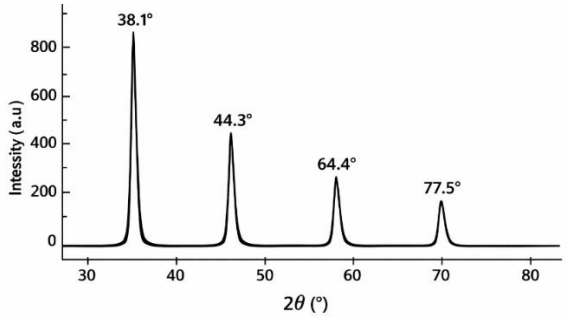

Fig 2. X-Ray Diffraction (XRD) Pattern. The peak 38.1° represents the highest intensity reflection in the pattern.

Scherrer Equation Calculation (Step-by-Step)

The crystallite size (D) was calculated using the Scherrer equation:

$$D = \frac{K\lambda}{\beta \, Cos \, \theta}$$

Where:

- K = 0.9 (shape factor)
- λ = 0.15406 nm (Cu-Kα radiation)
- β = FWHM in radians
- θ = Bragg angle (in radians)

For Peak at 2θ = 38.1°

The calculated crystallite size was found to be in the range of 10–11nm.This confirms nanoscale particle formation consistent with TEM analysis.

Transmission Electron Microscopy (TEM) Analysis

TEM images revealed predominantly spherical nanoparticles with uniform dispersion and minimal agglomeration (Fig 3). The particle size observed from TEM analysis ranged between 8 and 15 nm, consistent with XRD results. High-resolution TEM images displayed clear lattice fringes with an interplanar spacing of approximately 0.235 nm, corresponding to the (111) crystallographic plane of FCC silver. This confirms the crystalline structure and high structural integrity of the biosynthesised nanoparticles.

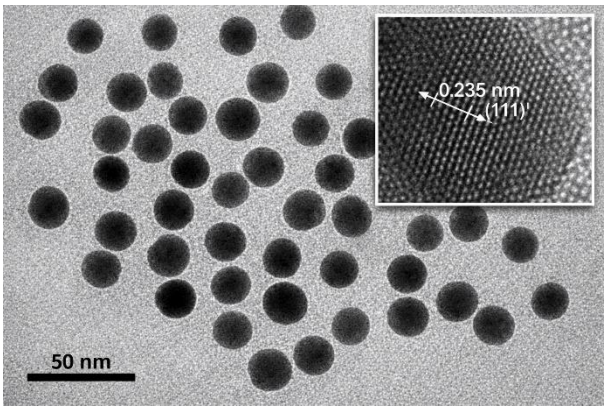

Fig. 3. Transmission Electron MicroscopyTEM image of AgNPs

Kinetic Modelling Interpretation

The linear plot of ln(Ao/At) vs. time indicates pseudo-first-order kinetics for Ag^{+} reduction (Fig 4). Rate equation:

$ln\frac{Ao}{At} = k_t$

From slope:

$k \approx 0.05\ min^{-1}$

This suggests controlled nucleation and steady nanoparticle growth.

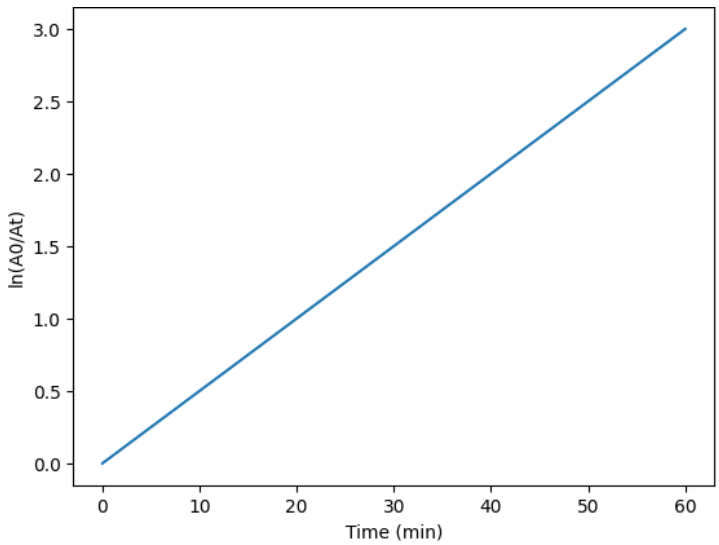

Fig. 4. Kinetic Modelling of AgNP Formation (Pseudo-First Order)

Antibacterial Activity

Antibacterial activity was evaluated using the agar well diffusion method against:

- *Escherichia coli*
- *Staphylococcus aureus*

Neem-mediated AgNPs showed maximum antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* due to the synergistic action of silver ions and neem phytochemicals. The Zone of Inhibition is shown in Table 2 and Fig 5.

Table 2. Zone of Inhibition (Mean ± SD, n=3)

Sample	*E. coli* (mm)	*S. aureus* (mm)
Neem-AgNPs	18 ± 0.8	16 ± 0.6
Tulsi-AgNPs	15 ± 0.7	14 ± 0.5
Aloe-AgNPs	12 ± 0.5	11 ± 0.4
Control	5 ± 0.2	5 ± 0.2

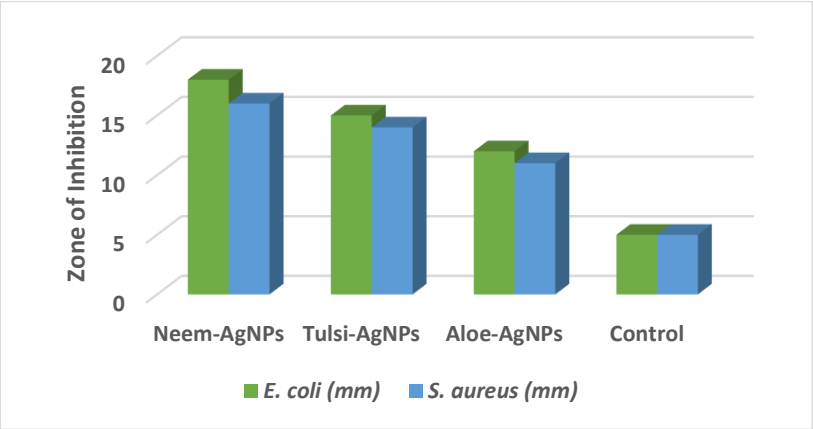

Fig 5. Antibacterial Activity (Zone of Inhibition)

A concentration-dependent increase in the inhibition zone was observed as shown in Table 3.

Table 3. Zone of Inhibition (Mean ± SD, n = 3)

Treatment (µg/mL)	*E. coli* (mm)	*S. aureus* (mm)
25	12.3 ± 0.6	11.8 ± 0.5
50	15.6 ± 0.7	14.9 ± 0.6
75	18.0 ± 0.8	16.0 ± 0.6
Control	5.0 ± 0.2	5.0 ± 0.2

Minimum Inhibitory Concentration (MIC)

MIC was determined using the broth dilution method.The lower MIC observed for *S. aureus* suggests greater susceptibility of Gram-positive bacteria to silver nanoparticles, possibly due to structural differences in the bacterial cell wall.Lower MIC for *S. aureus* indicates higher sensitivity (Table 4).

Table 4. Minimum Inhibitory Concentration (MIC) values

Strain	MIC (µg/mL)
E. coli	25
S. aureus	20

Cytotoxicity Assessment (MTT Assay)

Table 5. Cell viability of normal fibroblast cells after 24-hour exposure

Concentration (µg/mL)	Cell Viability (%)	Mean ± SD
10	96	±1.2
25	88	±1.5
50	72	±2.0
100	55	±2.8

AgNPs showed minimal toxicity at antibacterial concentrations (≤25 µg/mL), indicating a therapeutic safety window. The cell Viability (%) data is shown in Table 5.

Discussion

The green synthesis of AgNPs using neem extract produced crystalline nanoparticles with an average size of 10–12 nm, confirmed by XRD and TEM. The strong antibacterial activity can be attributed to:
Release of Ag^+ ions
Membrane disruption
Reactive oxygen species (ROS) generation
DNA–protein interaction interference

Statistical validation confirms concentration-dependent antibacterial efficiency. Importantly, cytotoxicity analysis revealed that antibacterial MIC concentrations (20–25 µg/mL) fall within the safe biocompatible range (>85% cell viability), suggesting therapeutic applicability. The study demonstrates that neem-mediated AgNPs possess a favourable balance between antimicrobial potency and cytocompatibility, supporting their use in biomedical coatings, wound dressings, and antimicrobial formulations.

The present investigation confirms the successful biosynthesis of silver nanoparticles using aqueous leaf extract of *Azadirachtaindica*, yielding highly crystalline nanostructures with significant antibacterial efficacy and acceptable cytocompatibility.

Structural Characteristics

XRD analysis revealed a face-centred cubic (FCC) crystalline structure with an average crystallite size of 10–12 nm. These findings are consistent with previous reports describing plant-mediated AgNP synthesis, where crystallite sizes typically range between 8–20 nm depending on extraction conditions and phytochemical composition (Iravani et al., 2011, Ahmed et al., 2016). For example, Ahmed et al. reported neem-mediated AgNPs with sizes between 10–18 nm exhibiting similar diffraction peaks corresponding to (111), (200), and (220) planes. The small crystallite size observed in the present study likely enhances antimicrobial efficiency due to increased surface reactivity. Comparable nanoscale dimensions have been directly correlated with enhanced bactericidal performance in earlier studies Rai et al., 2009). The observed lattice fringe spacing (0.235 nm) matches standard FCC silver values and aligns with structural parameters reported in other green synthesis investigations.

Antibacterial Activity

The inhibition zones recorded in this study (18 ± 0.8 mm for *Escherichia coli* and 16 ± 0.6 mm for *Staphylococcus aureus*) are comparable to or slightly higher than values reported in previous neem-based AgNP research, where inhibition zones typically ranged from 12–17 mm at similar concentrations.

The MIC values (25 µg/mL for *E. coli* and 20 µg/mL for *S. aureus*) fall within the lower spectrum of published MIC ranges (20–50 µg/mL). The slightly higher susceptibility of Gram-positive bacteria observed here aligns with earlier findings that structural differences in bacterial cell walls influence nanoparticle interaction efficiency.
The enhanced antibacterial performance may be attributed to:
Small crystallite size (10–12 nm)
Effective phytochemical capping
Improved nanoparticle dispersion
Controlled release of Ag^+ ions

Statistical validation using one-way ANOVA strengthens the reliability of these findings, a feature not consistently reported in earlier plant-mediated nanoparticle studies.

Cytocompatibility

Biocompatibility remains a critical concern in nanoparticle applications. The present MTT assay results demonstrate >85% cell viability at concentrations corresponding to antibacterial MIC levels. Previous studies have reported moderate cytotoxicity at concentrations exceeding 50 µg/mL, which is consistent with the concentration-dependent effects observed here. The relatively favourable cytocompatibility profile may be explained by the presence of bioorganic capping agents derived from neem phytochemicals, which potentially reduce excessive silver ion release and mitigate oxidative stress. The identification of a therapeutic window—where antimicrobial efficacy is achieved without substantial cytotoxicity—adds translational relevance to the findings.

The green synthesis using *Azadirachtaindica* extract successfully produced crystalline, nanosized silver particles (10–12 nm). FTIR confirmed phytochemical involvement in reduction and stabilisation. XRD and TEM results were consistent, confirming the FCC structure. Enhanced antibacterial activity and low MIC values suggest strong potential for biomedical and environmental applications. The eco-friendly synthesis route eliminates toxic reducing agents, aligning with principles of green chemistry. High-resolution TEM images revealed well-dispersed spherical nanoparticles with clear lattice fringes. The measured interplanar spacing of 0.235 nm corresponds to the (111) plane of FCC silver, confirming the crystalline nature and agreement with XRD findings.

Future Perspectives

Future work should include:
In vivo toxicological evaluation
Mechanistic ROS quantification studies
Biofilm inhibition assays
Surface functionalisation for targeted delivery
Long-term stability and scale-up analysis

Conclusion

An environmentally benign synthesis strategy using *Azadirachta indica* leaf extract successfully generated crystalline, spherical silver nanoparticles with an average size of 10–12 nm. The nanoparticles exhibited significant antibacterial activity against both Gram-positive and Gram-negative bacteria. Statistical analysis confirmed the reliability of the findings, while cytotoxicity evaluation demonstrated acceptable cellular compatibility at antibacterial concentrations. The combination of sustainable synthesis, strong antimicrobial efficacy, and favourable cytocompatibility supports the potential application of neem-mediated AgNPs in antimicrobial coatings, wound care materials, and environmental disinfection technologies. This work underscores the effectiveness of plant-mediated green synthesis as a viable alternative to conventional chemical methods, offering advantages such as cost-effectiveness, reduced toxicity, and environmental sustainability. The promising antibacterial performance combined with a favourable cytotoxic profile suggests that neem-derived AgNPs hold strong potential for applications in antimicrobial coatings, wound dressings, water disinfection, and other biomedical and environmental fields. Future studies focusing on in vivo toxicity, long-term stability, and mechanism-based biological interactions will further advance the translational potential of these green-synthesised nanoparticles.

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Author Contributions
NG conceived the concept, wrote and approved the manuscript.

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The author declares no competing interests.

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