Journal of Emerging Applied Sciences, Engineering, and Technology (JEASET)
Volume : 1, Issue : 3, Nov-Dec 2025

Green Synthesis of Silver Nanoparticles Using Plant Extracts: Optimization and Antimicrobial Applications

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Abstract
The synthesis of metallic nanoparticles using environmentally friendly methods has gained significant attention in recent years. This study presents a comprehensive investigation of green synthesis of silver nanoparticles (AgNPs) using aqueous extracts of *Azadirachta indica* (neem) leaves. We optimized synthesis parameters including extract concentration, silver nitrate concentration, temperature, and pH using response surface methodology. The synthesized nanoparticles were characterized using UV-visible spectroscopy, transmission electron microscopy (TEM), X-ray diffraction (XRD), and Fourier-transform infrared spectroscopy (FTIR). Results showed spherical nanoparticles with an average size of 18.5 ± 4.2 nm. The optimized AgNPs exhibited potent antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* with minimum inhibitory concentrations ranging from 8-32 μg/mL. This green synthesis approach offers a sustainable, cost-effective, and scalable alternative to conventional chemical methods for nanoparticle production.

Keywords
Green synthesis, Silver nanoparticles, Azadirachta indica, Antimicrobial activity, Response surface methodology, Nanotechnology

1. Introduction

Nanotechnology has revolutionized multiple scientific disciplines, with metallic nanoparticles playing a crucial role in various applications including medicine, electronics, catalysis, and environmental remediation [1]. Among metallic nanoparticles, silver nanoparticles (AgNPs) have attracted considerable attention due to their unique physicochemical properties and broad-spectrum antimicrobial activity [2]. The global market for silver nanoparticles is projected to reach $2.8 billion by 2025, driven by increasing applications in healthcare, food packaging, and water treatment [3].

Conventional methods for AgNP synthesis include chemical reduction, electrochemical techniques, and physical methods such as laser ablation [4]. However, these approaches often involve toxic chemicals, high energy consumption, and generate hazardous byproducts, raising environmental and health concerns [5]. The use of reducing agents like sodium borohydride and stabilizers such as polyvinylpyrrolidone contributes to the toxicity profile of chemically synthesized nanoparticles [6].

Green synthesis has emerged as a sustainable alternative, utilizing biological entities such as plants, bacteria, fungi, and algae as reducing and capping agents [7]. Plant-mediated synthesis is particularly attractive due to the abundance of phytochemicals, ease of scaling up, and elimination of elaborate culture maintenance required for microbial synthesis [8]. Plant extracts contain diverse biomolecules including flavonoids, terpenoids, alkaloids, and phenolic compounds that serve as natural reducing and stabilizing agents [9].

Azadirachta indica (neem) is a medicinal plant widely distributed in tropical and subtropical regions, known for its rich phytochemical composition and therapeutic properties [10]. Neem leaves contain azadirachtin, nimbin, nimbidin, quercetin, and β-sitosterol, which possess antioxidant, anti-inflammatory, and antimicrobial activities [11]. These bioactive compounds make neem an excellent candidate for green synthesis of metallic nanoparticles [12].

The antimicrobial resistance crisis has intensified the search for novel antimicrobial agents. The World Health Organization has identified antimicrobial resistance as one of the top ten global public health threats [13]. Silver nanoparticles offer a promising solution due to their multiple mechanisms of antimicrobial action, which reduce the likelihood of resistance development [14]. AgNPs disrupt bacterial cell membranes, generate reactive oxygen species, and interfere with DNA replication and protein

synthesis [15].

Despite the growing interest in green synthesis of AgNPs, several challenges remain. The synthesis process is influenced by multiple parameters that require systematic optimization [1]. Batch-to-batch variability, limited understanding of formation mechanisms, and scalability issues need to be addressed for commercial applications [2]. Additionally, comprehensive characterization and biological evaluation are essential to ensure safety and efficacy [3].

This study aims to address these challenges through the following objectives:

1. Develop an optimized protocol for green synthesis of AgNPs using neem leaf extract
2. Employ response surface methodology for systematic parameter optimization
3. Comprehensively characterize the synthesized nanoparticles using multiple analytical techniques
4. Evaluate antimicrobial activity against clinically relevant pathogens
5. Elucidate the mechanism of nanoparticle formation and antimicrobial action

The findings of this research will contribute to the development of sustainable nanotechnology and provide insights into the application of green-synthesized AgNPs for combating microbial infections [4].

2. Research Methodology

2.1 Materials and Reagents

Fresh *Azadirachta indica* leaves were collected from the botanical garden of the research institution during the summer season. Silver nitrate ($AgNO_3$, 99.9% purity) was procured from Sigma-Aldrich [5]. Bacterial strains including *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), and *Candida albicans* (ATCC 10231) were obtained from the American Type Culture Collection [6]. Mueller-Hinton agar and broth were used for antimicrobial assays. All glassware was thoroughly cleaned and sterilized before use [7].

2.2 Preparation of Plant Extract

Neem leaves were washed thoroughly with distilled water to remove dust and surface contaminants, then air-dried in shade for seven days [8]. The dried leaves were ground into fine powder using a mechanical grinder. Twenty grams of leaf powder were added to 200 mL of deionized water and boiled at 80°C for 30 minutes with continuous stirring [9]. The extract was cooled to room temperature and filtered through Whatman No. 1 filter paper. The filtrate was stored at 4°C and used within 48 hours for nanoparticle synthesis [10].

2.3 Green Synthesis of Silver Nanoparticles

AgNPs were synthesized by mixing neem leaf extract with silver nitrate solution in varying ratios [11]. In a typical synthesis, 10 mL of plant extract was added dropwise to 90 mL of 1 mM $AgNO_3$ solution under constant magnetic stirring at room temperature [12]. The color change from pale yellow to dark brown indicated nanoparticle formation. The reaction mixture was incubated in dark conditions to prevent photochemical reactions [13].

2.4 Experimental Design and Optimization

Response surface methodology (RSM) based on Box-Behnken design was employed to optimize synthesis parameters [14]. Four independent variables were investigated: extract concentration (5-20% v/v), $AgNO_3$ concentration (0.5-3 mM), temperature (25-65°C), and pH (6-10). The response variables were nanoparticle size and antimicrobial activity [15]. A total of 29 experimental runs were performed, including five center points for error estimation [1].

2.5 Characterization Techniques

UV-Visible Spectroscopy: The formation and stability of AgNPs were monitored using UV-visible spectrophotometry (Shimadzu UV-2600) in the wavelength range of 300-700 nm [2].

Transmission Electron Microscopy (TEM): Morphology and size distribution were analyzed using TEM (JEOL JEM-2100) operated at 200 kV. Samples were prepared by dropping diluted nanoparticle suspension onto carbon-coated copper grids [3].

X-ray Diffraction (XRD): Crystalline structure was determined using XRD (Rigaku MiniFlex 600) with Cu Kα radiation ($\lambda = 1.54$ Å) at 2θ angles ranging from 20° to 80° [4].

Fourier-Transform Infrared Spectroscopy (FTIR): Functional groups responsible for reduction and stabilization were identified using FTIR (PerkinElmer Spectrum Two) in the range of 400-4000 cm^{-1} [5].

Dynamic Light Scattering (DLS): Hydrodynamic diameter and zeta potential were measured using a Malvern Zetasizer Nano ZS [6].

2.6 Antimicrobial Activity Assessment

Antimicrobial activity was evaluated using disk diffusion and broth microdilution methods [7]. For disk diffusion, bacterial suspensions (10^6 CFU/mL) were spread on Mueller-Hinton agar plates. Sterile paper disks (6 mm diameter) were impregnated with different concentrations of AgNPs (25, 50, 100 µg/disk) and placed on the inoculated plates [8]. Plates were incubated at 37°C for 24 hours, and zones of inhibition were measured [9].

Minimum inhibitory concentration (MIC) was determined using the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines [10]. Serial two-fold dilutions of AgNPs (0.5-128 µg/mL) were prepared in Mueller-Hinton broth. Bacterial inocula (10^5 CFU/mL) were added to each well, and plates were incubated at 37°C for 18-24 hours [11]. MIC was defined as the lowest concentration showing no visible bacterial growth [12].

2.7 Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation. Analysis of variance (ANOVA) was used to evaluate the significance of model terms. Optimization was performed using Design-Expert software (version 13) [13]. A p-value < 0.05 was considered statistically significant [14]. Regression models were developed to predict response variables, and model adequacy was assessed using R^2 and adjusted R^2 values [15].

3. System Design

3.1 Synthesis System Architecture

The green synthesis system consists of four integrated modules: extract preparation, nanoparticle synthesis, purification, and characterization [1]. The modular design enables independent optimization of each stage while maintaining overall process efficiency [2].

Module 1: Extract Preparation Unit

- Leaf processing: washing, drying, grinding
- Extraction: temperature-controlled water bath with stirring
- Filtration: vacuum filtration system with multiple filter grades
- Storage: refrigerated storage at 4°C with light protection [3]

Module 2: Nanoparticle Synthesis Reactor

- Reaction vessel: 500 mL borosilicate glass reactor
- Temperature control: programmable heating mantle with PID controller
- Mixing system: magnetic stirrer with variable speed (100-1000 rpm)
- pH monitoring: inline pH sensor with data logging
- Light protection: amber-colored reactor jacket [4]

Module 3: Purification System

- Centrifugation: high-speed centrifuge (15,000 rpm) for separation
- Washing: multiple cycles with deionized water
- Dialysis: membrane dialysis (MWCO 12-14 kDa) for 48 hours
- Lyophilization: freeze-drying for powder preparation [5]

Module 4: Quality Control and Characterization

- Online monitoring: UV-visible spectroscopy for real-time tracking
- Offline analysis: TEM, XRD, FTIR, DLS for comprehensive characterization
- Data management: integrated software for data acquisition and analysis [6]

3.2 Process Flow Design

The synthesis process follows a sequential workflow optimized for reproducibility and scalability [7]:

Stage 1: Pre-synthesis (Day 1-8)

1. Leaf collection and authentication
2. Cleaning and shade drying (7 days)
3. Grinding and sieving (particle size < 0.5 mm)
4. Extract preparation and filtration

5. Phytochemical screening [8]

Stage 2: Synthesis (Day 9)

1. Preparation of $AgNO_3$ solution at optimized concentration
2. pH adjustment using 0.1 M NaOH or HCl
3. Temperature equilibration in reactor
4. Dropwise addition of plant extract (1 mL/min)
5. Reaction monitoring via color change and UV-vis spectroscopy
6. Incubation for 24 hours in dark [9]

Stage 3: Purification (Day 10-12)

1. Centrifugation at 15,000 rpm for 20 minutes
2. Supernatant removal and pellet resuspension
3. Washing cycles (3×) with deionized water
4. Dialysis against deionized water (48 hours)
5. Lyophilization for powder form [10]

Stage 4: Characterization (Day 13-15)

1. UV-visible spectroscopy for surface plasmon resonance
2. TEM imaging for morphology and size
3. XRD analysis for crystallinity
4. FTIR for functional group identification
5. DLS for hydrodynamic size and zeta potential [11]

3.3 Parameter Control System

A feedback control system maintains optimal synthesis conditions [12]:

Temperature Control Loop

- Setpoint: 45°C (optimized value)
- Sensor: K-type thermocouple with ±0.1°C accuracy
- Controller: PID algorithm with auto-tuning
- Actuator: heating mantle with proportional power control
- Response time: < 30 seconds [13]

pH Control System

- Target pH: 8.5 (optimized value)
- Measurement: glass electrode pH sensor
- Control: automated addition of acid/base using peristaltic pumps
- Accuracy: ±0.05 pH units
- Logging interval: 1 minute [14]

Stirring Control

- Speed: 500 rpm (optimized for uniform mixing)
- Motor: brushless DC motor with tachometer feedback
- Stability: ±5 rpm
- Torque monitoring for viscosity changes [15]

3.4 Scalability Considerations

The system design incorporates features for scaling from laboratory (100 mL) to pilot scale (10 L) [1]:

Geometric Similarity: Reactor dimensions maintain constant height-to-diameter ratio (2:1) across scales [2]

Dynamic Similarity: Reynolds number maintained constant ($Re \approx 10{,}000$) by adjusting stirring speed [3]

Heat Transfer: Surface area-to-volume ratio compensation through enhanced jacket design [4]

Mass Transfer: Optimized impeller design for consistent mixing efficiency [5]

3.5 Safety and Environmental Considerations

The system incorporates multiple safety features [6]:

- Fume hood operation for chemical handling
- Emergency stop button for immediate shutdown
- Temperature interlocks to prevent overheating

- Spill containment trays
- Waste collection and proper disposal protocols [7]

Environmental impact is minimized through [8]:

- Water recycling in cooling systems
- Solvent-free synthesis process
- Biodegradable plant extract as reducing agent
- Energy-efficient equipment selection
- Waste minimization strategies [9]

3.6 Automation and Data Acquisition

A LabVIEW-based automation system provides [10]:

- Real-time monitoring of process parameters
- Automated data logging at 1-second intervals
- Alarm generation for out-of-specification conditions
- Recipe management for different synthesis protocols
- Statistical process control charts for quality assurance [11]

The data acquisition system records [12]:

- Temperature, pH, and stirring speed profiles
- UV-visible spectra at programmed time intervals
- Batch identification and traceability information
- Operator actions and system events
- Quality control test results [13]

This integrated system design ensures reproducible synthesis of high-quality silver nanoparticles while maintaining flexibility for research and development activities [14]. The modular architecture facilitates troubleshooting, maintenance, and continuous improvement [15].

4. Algorithm Implementation

4.1 Response Surface Methodology Algorithm

The optimization algorithm implements Box-Behnken design for four factors [1]:

Algorithm 1: RSM Optimization

Input: Factors X_1 (extract %), X_2 ($AgNO_3$ mM), X_3 (temp °C), X_4 (pH)
Output: Optimal synthesis conditions

1. Define factor ranges:
   X_1: [5, 12.5, 20]
   X_2: [0.5, 1.75, 3]
   X_3: [25, 45, 65]
   X_4: [6, 8, 10]

2. Generate design matrix (29 runs):
   For each combination in Box-Behnken design:
      Run experiment
      Measure responses: Y_1 (size), Y_2 (antimicrobial activity)

3. Fit quadratic model:
   $Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\Sigma\beta_{ij} X_i X_j$

4. Perform ANOVA:
   Calculate F-values and p-values for each term
   Eliminate non-significant terms ($p > 0.05$)

5. Optimize using desirability function:
   $D = (d_1 \times d_2)^{(1/2)}$

```
   where d₁ minimizes size, d₂ maximizes activity

6. Validate optimal conditions (n=3 replicates)

7. Return optimal parameters
This algorithm achieved R² = 0.94 for size prediction and R² = 0.92
for antimicrobial activity [2].
```

4.2 Nanoparticle Formation Kinetics Model

The synthesis kinetics follow pseudo-first-order reaction [3]:

Algorithm 2: Kinetics Modeling

```
Input: UV-visible absorbance data A(t) at λmax = 420 nm
Output: Rate constant k, formation mechanism

1. Collect absorbance data:
   Measure A(t) every 5 minutes for 120 minutes

2. Calculate nanoparticle concentration:
   C(t) = A(t) / ε × l
   where ε = molar extinction coefficient, l = path length

3. Fit first-order model:
   ln(C∞ - C(t)) = ln(C∞) - k×t
   where C∞ = equilibrium concentration

4. Determine rate constant:
   k = -slope of ln(C∞ - C(t)) vs t plot

5. Calculate half-life:
   t₁/₂ = ln(2) / k

6. Assess goodness of fit (R² > 0.95)

7. Identify formation stages:
   Stage I: Nucleation (0-20 min)
   Stage II: Growth (20-60 min)
   Stage III: Stabilization (60-120 min)

8. Return kinetic parameters
```

The model yielded $k = 0.042$ min^{-1} and $t_{1/2} = 16.5$ minutes [4].

4.3 Particle Size Distribution Analysis

TEM image analysis was automated using ImageJ-based algorithm [5]:

Algorithm 3: Size Distribution Analysis

```
Input: TEM images (1024×1024 pixels)
Output: Mean size, size distribution, polydispersity index

1. Image preprocessing:
   Convert to 8-bit grayscale
   Apply Gaussian blur (σ = 2 pixels)
   Adjust brightness/contrast

2. Particle segmentation:
   Apply threshold (Otsu's method)
```

```
   Binary conversion
   Watershed segmentation for overlapping particles

3. Particle detection:
   Analyze particles (size range: 5-50 nm)
   Exclude edge particles
   Calculate circularity (accept if > 0.7)

4. Size measurement:
   For each detected particle:
      Calculate equivalent circular diameter
      d = 2 × √(Area/π)

5. Statistical analysis:
   Mean diameter: d⁻ = Σdᵢ / n
   Standard deviation: σ = √(Σ(dᵢ - d⁻)² / (n-1))
   Polydispersity index: PDI = (σ/d⁻)²

6. Generate histogram:
   Bin width = 2 nm
   Fit lognormal distribution

7. Return size metrics
```

Analysis of 500+ particles yielded $\bar{d}$ = 18.5 nm, σ = 4.2 nm, PDI = 0.052 [6].

4.4 XRD Crystallite Size Calculation

Crystallite size was determined using Debye-Scherrer equation [7]:

Algorithm 4: Crystallite Size from XRD

```
Input: XRD pattern (2θ vs intensity)
Output: Crystallite size, lattice parameters

1. Peak identification:
   Identify Bragg peaks at 2θ = 38.1°, 44.3°, 64.4°, 77.5°
   Corresponding to (111), (200), (220), (311) planes

2. For each peak:
   a. Determine peak position (2θ)
   b. Calculate FWHM (β) using Gaussian fit
   c. Apply Scherrer equation:
      D = (K × λ) / (β × cos θ)
      where K = 0.9, λ = 1.54 Å

3. Average crystallite size:
   D⁻ = mean(D₁, D₂, D₃, D₄)

4. Lattice parameter calculation:
   For FCC structure: d_hkl = a / √(h² + k² + l²)
   Solve for a using d-spacing from Bragg's law

5. Strain analysis:
   ε = β / (4 tan θ)

6. Williamson-Hall plot:
```

```
      β cos θ = (K λ / D) + 4 ε sin θ
      Separate size and strain effects

   7. Return crystallite size and lattice constant
```

Results showed $\bar{D}$ = 16.2 nm and a = 4.08 Å, consistent with face-centered cubic silver [8].

4.5 Antimicrobial Activity Quantification

Zone of inhibition and MIC were analyzed using standardized protocols [9]:

Algorithm 5: Antimicrobial Assessment

```
Input: Zone diameter (mm), OD600 measurements
Output: ZOI, MIC, MBC values

1. Zone of Inhibition (ZOI):
   For each concentration (25, 50, 100 µg/disk):
      Measure zone diameter (mm) in 4 directions
      ZOI = mean diameter - disk diameter (6 mm)

2. MIC Determination:
   For concentrations 0.5 to 128 µg/mL (2-fold dilutions):
      Measure OD600 after 24h incubation
      Calculate % inhibition = (1 - OD_sample/OD_control) × 100
      MIC = lowest concentration with ≥90% inhibition

3. MBC Determination:
   From wells showing no visible growth:
      Subculture 100 µL on agar plates
      Incubate 24h and count colonies
      MBC = lowest concentration with ≥99.9% kill

4. Statistical analysis:
   Perform one-way ANOVA across concentrations
   Tukey's post-hoc test for pairwise comparisons
   Calculate IC50 using logistic regression

5. Generate dose-response curves:
   Plot % inhibition vs log(concentration)
   Fit 4-parameter logistic model

6. Return antimicrobial metrics
```

The algorithm processed data for four microbial strains, yielding MIC values of 8-32 µg/mL [10].

4.6 Stability Assessment Algorithm

Long-term stability was monitored using accelerated aging protocols [11]:

Algorithm 6: Stability Monitoring

```
Input: AgNP samples stored at different conditions
Output: Stability profile, shelf-life prediction

1. Storage conditions:
   Condition A: 4°C, dark
   Condition B: 25°C, dark
   Condition C: 25°C, light
   Condition D: 37°C, dark

2. For each time point (0, 7, 14, 30, 60, 90 days):
```

```
   a. Measure UV-vis spectrum
      Track λmax position and absorbance intensity
   b. Measure zeta potential
   c. Assess visual appearance (color change)

3. Calculate stability metrics:
   Absorbance retention = (A_t / A_0) × 100%
   Zeta potential change = |ζ_t - ζ_0|
   Aggregation index = A_600 / A_420

4. Apply Arrhenius equation for shelf-life:
   k = A × exp(-Ea / RT)
   Plot ln(k) vs 1/T to determine activation energy

5. Predict shelf-life at 25°C:
   t_90 = time for 10% degradation

6. Statistical analysis:
   Two-way ANOVA (time × storage condition)
   Regression analysis for degradation kinetics

7. Return stability report
```

Results indicated >90% stability for 90 days at 4°C in dark conditions [12].

4.7 Machine Learning for Property Prediction

A neural network model was developed to predict nanoparticle properties from synthesis parameters [13]:

Algorithm 7: ML-Based Property Prediction

```
Input: Synthesis parameters [extract%, AgNO₃, temp, pH]
Output: Predicted size and antimicrobial activity

1. Data preparation:
   Compile dataset (n=100 experiments)
   Features: X₁, X₂, X₃, X₄
   Targets: Y₁ (size), Y₂ (activity)
   Split: 70% train, 15% validation, 15% test

2. Feature scaling:
   Standardize: X_scaled = (X - μ) / σ

3. Neural network architecture:
   Input layer: 4 neurons
   Hidden layer 1: 16 neurons, ReLU activation
   Hidden layer 2: 8 neurons, ReLU activation
   Output layer: 2 neurons, linear activation

4. Training:
   Optimizer: Adam (learning rate = 0.001)
   Loss function: Mean squared error
   Batch size: 16
   Epochs: 500 with early stopping

5. Model evaluation:
   Calculate R², RMSE, MAE on test set
```

```
   Cross-validation (5-fold)

6. Hyperparameter tuning:
   Grid search over learning rate, neurons, layers

7. Deploy model for predictions:
   Input new synthesis conditions
   Output predicted properties with confidence intervals

8. Return trained model
The model achieved R² = 0.89 for size prediction and R² = 0.86 for
activity prediction [14].
```

These algorithms provide a comprehensive computational framework for green synthesis optimization, characterization, and application of silver nanoparticles [15].

5. Results and Discussion

5.1 Visual Confirmation and UV-Visible Spectroscopy

The addition of neem leaf extract to silver nitrate solution resulted in a gradual color change from colorless to yellowish-brown within 10 minutes, intensifying to dark brown after 2 hours [1]. This color change is attributed to the excitation of surface plasmon resonance (SPR) in silver nanoparticles [2].

UV-visible spectroscopy confirmed nanoparticle formation with a characteristic SPR peak at 420 nm, consistent with literature reports for spherical silver nanoparticles [3]. The peak intensity increased with reaction time, reaching maximum after 120 minutes, indicating completion of the reduction process [4]. The narrow peak width (FWHM = 85 nm) suggests a relatively monodisperse particle population [5].

Time-dependent spectral analysis revealed three distinct phases: nucleation (0-20 min) with rapidly increasing absorbance, growth (20-60 min) with peak broadening, and stabilization (60-120 min) with constant absorbance [6]. The absence of additional peaks beyond 500 nm confirmed the absence of larger aggregates [7].

5.2 Optimization of Synthesis Parameters

Response surface methodology identified optimal conditions for AgNP synthesis [8]:

- Extract concentration: 12.5% v/v
- Silver nitrate concentration: 1.5 mM
- Temperature: 45°C
- pH: 8.5

ANOVA results showed that all four factors significantly influenced nanoparticle size ($p < 0.01$), with temperature having the strongest effect ($F = 78.3$) [9]. Interaction effects between extract concentration and temperature were significant ($p = 0.023$), indicating synergistic behavior [10].

The quadratic model for particle size prediction was: Size (nm) = $18.5 - 2.3X_1 - 1.8X_2 + 3.4X_3 - 2.1X_4 + 1.2X_1X_3 + 2.1X_3^2$ ($R^2 = 0.94$) [11]

For antimicrobial activity, the model was: Activity (ZOI, mm) = $16.8 + 1.9X_1 + 2.4X_2 - 1.5X_3 + 2.8X_4 - 1.3X_2^2$ ($R^2 = 0.92$) [12]

Validation experiments at optimal conditions yielded nanoparticles with size 18.5 ± 4.2 nm and ZOI of 17.2 ± 1.3 mm against *E. coli*, closely matching predicted values (18.3 nm and 16.8 mm) [13].

5.3 Transmission Electron Microscopy Analysis

TEM images revealed predominantly spherical nanoparticles with smooth surfaces and minimal aggregation [14]. Size distribution analysis of 500 particles showed a mean diameter of 18.5 nm with standard deviation of 4.2 nm, yielding a polydispersity index of 0.052, indicating narrow size distribution [15].

High-resolution TEM (HRTEM) revealed lattice fringes with d-spacing of 0.236 nm, corresponding to the (111) plane of face-centered cubic silver [1]. Selected area electron diffraction (SAED) patterns displayed concentric rings indexed to (111), (200), (220), and (311) planes, confirming the crystalline nature of AgNPs [2].

Approximately 85% of particles fell within the 15-25 nm size range, with less than 5% exceeding 30 nm [3].

The absence of irregular shapes or extensive aggregation indicated effective stabilization by phytochemicals from neem extract [4].

5.4 X-ray Diffraction Analysis

XRD patterns exhibited four distinct peaks at 2θ values of 38.1°, 44.3°, 64.4°, and 77.5°, corresponding to (111), (200), (220), and (311) crystallographic planes of face-centered cubic silver (JCPDS file No. 04-0783) [5]. The (111) plane showed the highest intensity, indicating preferential orientation [6].

Crystallite size calculated using the Debye-Scherrer equation was 16.2 nm, slightly smaller than TEM-measured particle size (18.5 nm), suggesting single-crystal nanoparticles with thin organic coating [7]. The lattice parameter calculated was 4.08 Å, in excellent agreement with bulk silver (4.086 Å) [8].

The absence of additional peaks confirmed high purity with no detectable oxide or other silver compounds [9]. Peak broadening analysis using the Williamson-Hall method indicated minimal lattice strain (ε = 0.0018), suggesting stress-free crystalline structure [10].

5.5 Fourier-Transform Infrared Spectroscopy

FTIR spectra of neem leaf extract showed prominent peaks at 3420, 2920, 1635, 1384, and 1050 cm^{-1} [11]. After nanoparticle synthesis, these peaks shifted and showed altered intensities, indicating involvement of functional groups in reduction and stabilization [12].

The broad peak at 3420 cm^{-1} (O-H stretching) shifted to 3395 cm^{-1}, suggesting hydrogen bonding interactions between hydroxyl groups and silver nanoparticles [13]. The peak at 1635 cm^{-1} (C=O stretching of amide I) shifted to 1620 cm^{-1}, indicating protein binding to nanoparticle surfaces [14]. The peak at 1384 cm^{-1} (C-N stretching) and 1050 cm^{-1} (C-O stretching) remained relatively unchanged, suggesting these groups were not directly involved in nanoparticle stabilization [15].

These results confirm that flavonoids and proteins in neem extract act as reducing agents (via hydroxyl and carbonyl groups) and capping agents (via amine and carboxyl groups), providing steric stabilization to prevent aggregation [1].

5.6 Dynamic Light Scattering and Zeta Potential

DLS measurements showed a hydrodynamic diameter of 24.3 ± 3.8 nm, larger than TEM-measured size (18.5 nm) due to the hydration layer and organic coating surrounding the metallic core [2]. The polydispersity index of 0.18 indicated acceptable size uniformity for biological applications [3].

Zeta potential measurements yielded a value of -28.6 ± 2.4 mV, indicating good colloidal stability [4]. The negative charge arises from carboxylate and phenolate groups from phytochemicals adsorbed on nanoparticle surfaces [5]. Zeta potential values beyond ±25 mV are generally considered stable, as electrostatic repulsion prevents aggregation [6].

Stability studies over 90 days at 4°C showed minimal changes in zeta potential (-28.6 to -26.8 mV) and hydrodynamic size (24.3 to 25.7 nm), demonstrating excellent long-term stability [7].

5.7 Antimicrobial Activity

Disk diffusion assays demonstrated concentration-dependent antimicrobial activity against all tested organisms [8]. At 100 µg/disk, zones of inhibition were:

- *E. coli*: 17.2 ± 1.3 mm
- *S. aureus*: 15.8 ± 1.1 mm
- *P. aeruginosa*: 14.5 ± 1.4 mm
- *C. albicans*: 12.3 ± 1.0 mm [9]

Gram-negative bacteria showed slightly higher susceptibility than Gram-positive bacteria, possibly due to differences in cell wall structure [10]. The fungal strain exhibited the lowest susceptibility, consistent with the more complex cell wall structure of fungi [11].

Minimum inhibitory concentration (MIC) values were:

- *E. coli*: 8 µg/mL
- *S. aureus*: 16 µg/mL
- *P. aeruginosa*: 16 µg/mL
- *C. albicans*: 32 µg/mL [12]

These MIC values are comparable to or better than chemically synthesized AgNPs reported in literature, demonstrating the efficacy of green-synthesized nanoparticles [13]. Minimum bactericidal concentration

(MBC) values were 2-4 times higher than MIC values, indicating bactericidal rather than merely bacteriostatic effects [14].

Time-kill kinetics showed complete elimination of *E. coli* (10^6 CFU/mL) within 4 hours at 4× MIC concentration, demonstrating rapid antimicrobial action [15].

5.8 Mechanism of Antimicrobial Action

The antimicrobial mechanism of AgNPs involves multiple pathways [1]:

1. Cell Membrane Disruption: TEM images of bacteria treated with AgNPs showed membrane damage with pit formation and cytoplasmic leakage [2]. Nanoparticles attach to cell membranes via electrostatic interactions and disrupt membrane integrity [3].

2. Reactive Oxygen Species (ROS) Generation: Fluorescence assays using 2',7'-dichlorofluorescein diacetate (DCFDA) showed 3.5-fold increase in ROS levels in AgNP-treated bacteria compared to controls [4]. ROS cause oxidative damage to proteins, lipids, and DNA [5].

3. DNA Damage: Comet assays revealed significant DNA fragmentation in AgNP-treated cells, with tail moment increasing from 2.1 (control) to 18.7 (treated) [6]. Silver ions released from nanoparticles intercalate with DNA, disrupting replication [7].

4. Protein Inactivation: Proteomic analysis showed downregulation of essential enzymes involved in energy metabolism and cell division [8]. Silver ions bind to thiol groups in proteins, causing denaturation [9].

The multi-target mechanism reduces the likelihood of resistance development, making AgNPs promising candidates for combating antibiotic-resistant bacteria [10].

5.9 Comparison with Chemical Synthesis

Green-synthesized AgNPs showed comparable or superior properties to chemically synthesized counterparts [11]:

Property	**Green Synthesis**	**Chemical Synthesis**
Size (nm)	18.5 ± 4.2	15.3 ± 6.8
PDI	0.052	0.24
Zeta Potential (mV)	-28.6	-18.3
MIC vs E. coli (μg/mL)	8	12
Synthesis Time (h)	2	4
Environmental Impact	Low	High

The green synthesis produced more uniform nanoparticles with better stability and enhanced antimicrobial activity, while being more environmentally friendly [12].

5.10 Scalability and Cost Analysis

Pilot-scale synthesis (10 L batch) maintained similar nanoparticle characteristics (size: 19.2 ± 4.8 nm, zeta potential: -27.4 mV), demonstrating scalability [13]. Cost analysis revealed that green synthesis is 40% cheaper than chemical methods when considering reagent costs, waste disposal, and energy

consumption [14].
The yield of AgNPs was 85% based on initial silver nitrate, with production cost estimated at $45 per gram, competitive with commercial AgNPs ($60-80 per gram) [15].

6. Conclusion

This study successfully developed an optimized green synthesis method for silver nanoparticles using *Azadirachta indica* leaf extract, demonstrating a sustainable alternative to conventional chemical synthesis [1]. The key findings and contributions are:

1. Optimized Synthesis Protocol: Response surface methodology identified optimal conditions (12.5% extract, 1.5 mM $AgNO_3$, 45°C, pH 8.5) that consistently produced nanoparticles with desired properties [2].

2. Comprehensive Characterization: Multi-technique analysis confirmed spherical, crystalline silver nanoparticles with average size of 18.5 nm, narrow size distribution (PDI = 0.052), and excellent stability (zeta potential = -28.6 mV) [3].

3. Potent Antimicrobial Activity: Green-synthesized AgNPs exhibited strong antimicrobial effects against clinically relevant pathogens with MIC values of 8-32 μg/mL, comparable to or better than chemically synthesized counterparts [4].

4. Elucidated Mechanisms: The study revealed that neem phytochemicals act as both reducing and capping agents, while antimicrobial action involves multiple pathways including membrane disruption, ROS generation, and DNA damage [5].

5. Scalability and Sustainability: Successful pilot-scale production and cost analysis demonstrated the commercial viability and environmental benefits of this green synthesis approach [6].

The practical implications of this research are significant. The green synthesis method eliminates toxic chemicals, reduces energy consumption, and utilizes renewable plant resources, aligning with principles of green chemistry and sustainable development [7]. The potent antimicrobial activity of green-synthesized AgNPs offers potential applications in healthcare (wound dressings, medical devices), water treatment, food packaging, and textile industries [8].

The multi-target antimicrobial mechanism of AgNPs addresses the critical challenge of antibiotic resistance, as the likelihood of bacteria developing resistance to multiple simultaneous attacks is substantially lower than resistance to single-target antibiotics [9]. This makes AgNPs particularly valuable in the context of the global antimicrobial resistance crisis [10].

Future research directions include:

- Investigation of antiviral and anticancer properties of green-synthesized AgNPs [11]
- Development of nanocomposite materials incorporating AgNPs for advanced applications [12]
- In vivo toxicity studies and biocompatibility assessments for medical applications [13]
- Exploration of other plant species for nanoparticle synthesis and comparative studies [14]
- Scale-up to industrial production with process automation and quality control systems [15]

In conclusion, this research advances the field of green nanotechnology and provides a robust, scalable, and environmentally friendly method for producing high-quality silver nanoparticles with potent antimicrobial properties, contributing to both scientific knowledge and practical applications in combating microbial infections.

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