

Biogenic synthesis of silver nanoparticles using combined leaf extracts of *Azadirachta indica* and *Ocimum sanctum*: physicochemical characterization and antibacterial efficacy against drug-resistant pathogens including MRSA

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Abstract

Silver nanoparticles (AgNPs) synthesized by green routes represent a compelling solution to the global antimicrobial resistance (AMR) crisis. Here we report the first preparation of AgNPs using a binary 1:1 aqueous leaf extract combination of *Azadirachta indica* (Neem) and *Ocimum sanctum* (Tulsi), designated NL-AgNPs, exploiting the complementary phytochemical repertoires of both plants. Synthesis was optimized over a multi-parametric matrix (AgNO₃ concentration, temperature, pH, time) yielding NL-AgNPs confirmed by a surface plasmon resonance peak at 421 nm. Field emission scanning electron microscopy (FESEM) established a mean diameter of 22.8 ± 3.2 nm; X-ray diffraction (XRD) confirmed face-centred cubic (FCC) metallic silver; and dynamic light scattering (DLS) yielded a zeta potential of -31.7 ± 2.9 mV. FTIR spectroscopy identified eugenol, rosmarinic acid, quercetin, and gallic acid as primary reducing and capping agents. Antibacterial evaluation against six ATCC-certified strains including methicillin-resistant *Staphylococcus aureus* (MRSA ATCC 33591) demonstrated zones of inhibition of 15.8–22.6 mm and minimum inhibitory concentrations (MICs) of 3.12–25.0 µg/mL. NL-AgNPs showed activity against ampicillin-resistant MRSA (zone = 18.9 mm; MIC = 6.25 µg/mL), outperforming either individual plant extract and approaching fluoroquinolone efficacy. This hazardous-chemical-free, scalable approach offers a viable green route to clinically relevant antimicrobial nanomaterials.

Introduction

Antimicrobial resistance (AMR) stands among the most critical threats to global health, estimated to cause 1.27 million direct deaths annually and projected to exceed 10 million deaths per year by 2050¹. The WHO 2024 Bacterial Priority Pathogens List highlights methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Klebsiella pneumoniae*, and extended-spectrum beta-lactamase-producing *Escherichia coli* as organisms of critical concern². Silver nanoparticles (AgNPs) have attracted exceptional interest as alternative antimicrobial agents because their bactericidal action operates through multiple simultaneous pathways — membrane disruption, reactive oxygen species (ROS) generation, enzyme inhibition, and DNA intercalation — substantially reducing the likelihood of resistance development compared with single-target antibiotics^{3–5}.

Conventional physicochemical AgNP synthesis routes rely on hazardous reducing agents (sodium borohydride, hydrazine) and surfactant stabilizers (cetyl trimethylammonium bromide, polyvinylpyrrolidone), generating toxic by-products and requiring energy-intensive processing⁶. Green synthesis using plant extracts circumvents these drawbacks by exploiting phytochemicals — polyphenols, flavonoids, terpenoids, and organic acids — simultaneously as reductants and capping agents in a single-step aqueous reaction at moderate temperatures⁷. The resulting phytochemical corona additionally confers intrinsic biological activity to the nanoparticle surface, creating a synergistic material that exceeds the antimicrobial potency of bulk extract alone⁸.

Azadirachta indica (Neem; Meliaceae) leaves contain triterpenoids (nimbin, nimbidin), flavonoids (quercetin, rutin), and phenolic acids (gallic acid) bearing multiple hydroxyl groups capable of silver ion reduction⁹. *Ocimum sanctum* L. (Tulsi; Lamiaceae) provides a complementary phytochemical milieu comprising eugenol (70–85% of essential oil), rosmarinic acid, ursolic acid, and flavone glycosides (apigenin, orientin)¹⁰. Although both species have independently been used as AgNP synthesis platforms^{9–11}, no study has explored their binary combination. Here we address this gap, hypothesizing that complementary phytochemical pools will enhance both nucleation efficiency and surface capping diversity, yielding superior NL-AgNPs compared with either single-plant system.

Results

Visual observation and parametric optimization

Progressive colour change from pale yellowish-green to stable dark brown confirmed biogenic NL-AgNP formation, consistent with localized surface plasmon resonance (LSPR) excitation of metallic silver nanoparticles in the 400–450 nm range¹². Table 1 summarizes systematic parametric optimization. At 0.5 mM AgNO₃ no surface plasmon resonance (SPR) peak was detected; 1.0 mM gave the sharpest SPR peak (421 nm, A = 1.91 AU, FWHM = 42 nm). Concentrations above 1.0 mM produced red-shifted, broadened peaks indicating aggregation, consistent with LaMer nucleation theory at supersaturation¹³. Optimal temperature (70°C) gave narrow FWHM (42 nm); 90°C produced broadening (FWHM = 68 nm) attributable to Ostwald ripening. Mildly alkaline pH 7.5 maximized polyphenolic deprotonation and electron-donation capacity¹⁴.

Table 1
Parametric optimization of NL-AgNP synthesis.

Parameter	Batch A	Batch B	Batch C	Batch D	Optimized
AgNO ₃ conc. (mM)	0.5	1.0	2.0	3.0	1.0
Temperature (°C)	30	50	70	90	70
pH	5.0	6.0	7.5	9.0	7.5
Reaction time (min)	120	90	60	40	60
UV-Vis peak (nm)	No peak	432	421	418	421
Particle size – FESEM (nm)	–	31.2 ± 4.1	22.8 ± 3.2	28.6 ± 5.4	22.8 ± 3.2
Zeta potential (mV)	–	–21.3	–31.7	–26.4	–31.7
Visual colour	Pale yellow	Light brown	Dark brown	Dark brown	Dark brown

Values are mean $\pm$ SD ($n = 3$). (–) = no nanoparticles detected. SPR peak and particle parameters reported only for batches producing detectable nanoparticles.

UV-Visible spectroscopy

A single symmetric SPR absorption peak at 421 nm ($A = 1.91$ AU; FWHM = 42 nm) was observed in purified NL-AgNPs. The absence of secondary peaks above 500 nm confirms uniform spherical morphology without significant anisotropic nanostructures. Time-resolved spectra ($t = 10$ –60 min) showed monotonic absorbance increase at constant peak position, consistent with controlled nucleation-and-growth¹³. The 421 nm peak is intermediate between single-plant Neem-AgNPs (428–435 nm)⁹ and Tulsi-AgNPs (415–422 nm)^{10,11}, with narrower FWHM (42 nm vs. 50–65 nm in comparators), suggesting more uniform particle distribution from the binary extract platform.

FTIR spectroscopy

FTIR spectra of NL-extract and purified NL-AgNPs were recorded and compared. Key extract bands: O – H stretch (3418 cm^{-1}); C – H stretch (2924 cm^{-1}); C = O stretch (1714 cm^{-1}); aromatic C = C ($1618, 1516\text{ cm}^{-1}$); C – O–C (1241 cm^{-1}). In NL-AgNPs, systematic red-shifts were observed: O – H to 3396 cm^{-1} ($\Delta = -22\text{ cm}^{-1}$); C = O to 1698 cm^{-1} ($\Delta = -16\text{ cm}^{-1}$), confirming direct coordination of phenolic and carbonyl groups with the AgNP surface¹⁵. These interactions identify eugenol and rosmarinic acid (Tulsi) and quercetin with gallic acid (Neem) as the primary reducing and stabilizing phytochemicals.

FESEM, EDX, XRD, and DLS characterization

FESEM analysis (250 particles, ImageJ v1.53) revealed predominantly spherical NL-AgNPs: mean diameter 22.8 ± 3.2 nm, range 10–42 nm, coefficient of variation 14.0%. EDX confirmed elemental silver as the principal constituent: Ag L α peak at 2.98 keV (72.6 at.%), with C (18.4 at.%) and O (9.0 at.%) from the organic corona. XRD diffractogram displayed reflections at $2\theta = 38.2^\circ, 44.4^\circ, 64.5^\circ, 77.5^\circ$, corresponding to (111), (200), (220), (311) planes of face-centred cubic (FCC) metallic silver (JCPDS 04-0783). Scherrer analysis of the (111) reflection gave an average crystallite size of 20.4 ± 1.6 nm. DLS yielded z-average hydrodynamic diameter 36.8 ± 4.7 nm (PDI = 0.19) and zeta potential -31.7 ± 2.9 mV, confirming colloidal stability over 120 days at 4°C ($|\zeta| > 30$ mV threshold)¹⁶.

Antibacterial activity

Table 2 presents the complete antibacterial dataset. NL-AgNPs (25 $\mu\text{g}/\text{disc}$) produced statistically significant zones of inhibition (ZOI) against all six strains ($p < 0.01$, one-way ANOVA, Tukey post-hoc), ranging from 15.8 ± 0.8 mm (*P. aeruginosa*) to 22.6 ± 0.9 mm (*S. aureus*). These values significantly exceeded crude NL-extract ZOIs (6.9–11.1 mm) and Ampicillin activity across all strains. Notably, NL-

AgNPs retained potent activity against MRSA ATCC 33591 (ZOI = 18.9 ± 0.6 mm; MIC = 6.25 µg/mL) despite complete Ampicillin resistance, demonstrating multi-mechanistic killing independent of beta-lactam resistance pathways. MICs ranged from 3.12 µg/mL (*S. aureus*, *B. subtilis*) to 25.0 µg/mL (*P. aeruginosa*).

Table 2
Antibacterial activity of NL-AgNPs against ATCC-certified bacterial strains.

Bacterial Strain	Zone of Inhibition (mm)		Antibiotic Controls (mm)			MIC (µg/mL)
	NL-AgNPs (25 µg)	Crude Extract	Ampicillin (10 µg)	Streptomycin (10 µg)	Ciprofloxacin (5 µg)	
<i>E. coli</i> ATCC 25922	19.2 ± 0.7	8.6 ± 0.5	13.5 ± 0.6	17.8 ± 0.5	25.1 ± 0.8	6.25
<i>S. aureus</i> ATCC 25923	22.6 ± 0.9	10.3 ± 0.6	14.2 ± 0.7	18.6 ± 0.9	27.3 ± 0.7	3.12
<i>K. pneumoniae</i> ATCC 700603	17.4 ± 0.6	7.8 ± 0.4	11.9 ± 0.5	15.4 ± 0.6	23.7 ± 0.6	12.5
<i>P. aeruginosa</i> ATCC 27853	15.8 ± 0.8	6.9 ± 0.3	10.6 ± 0.4	14.2 ± 0.7	21.4 ± 0.9	25.0
<i>B. subtilis</i> ATCC 6633	20.4 ± 0.7	11.1 ± 0.5	16.8 ± 0.6	20.3 ± 0.8	26.8 ± 0.7	3.12
MRSA ATCC 33591	18.9 ± 0.6	9.4 ± 0.4	Resistant	17.1 ± 0.6	22.6 ± 0.8	6.25

Data expressed as mean ± SD (n = 3). 'Resistant' = no zone of inhibition. MIC determined by resazurin-based broth microdilution (CLSI M07-A10). Superscript * denotes significant difference from crude extract (p < 0.01).

Discussion

The binary NL-extract strategy produced NL-AgNPs with narrower size distribution (CVsize = 14.0%) and stronger colloidal stability (ζ = -31.7 mV) than single-plant Neem-AgNPs (mean 28–35 nm, ζ = -20 to -25 mV)⁹ and Tulsi-AgNPs (mean 25–32 nm, ζ = -22 to -28 mV)¹⁰. We attribute this to complementary mechanisms: the high eugenol content of Tulsi provides rapid initial nucleation through fast electron donation, while gallic acid and ellagic acid polyphenols from Neem provide secondary surface passivation limiting Ostwald ripening. This cooperative action is reflected in the narrower SPR FWHM (42 nm versus 50–65 nm in comparators).

The multi-mechanistic antibacterial action of NL-AgNPs encompasses: membrane disruption via Ag⁺-mediated phospholipid oxidation; intracellular enzyme inactivation through thiol reactivity (NADH

dehydrogenase, succinate dehydrogenase); ROS cascade ($O_2^{\bullet-}$, $\bullet OH$, H_2O_2) causing oxidative damage to DNA, lipids, and ribosomes; and synergistic bactericidal activity from surface-adsorbed phytochemicals (eugenol, quercetin, nimbin)^{3-5,15}. Against MRSA – resistant to Ampicillin via *mecA*-encoded PBP2a – all four pathways remain operative since none targets the beta-lactam resistance mechanism, explaining the potent MRSA inhibition observed. The Gram-differential susceptibility pattern (*S. aureus* ZOI 22.6 mm > *P. aeruginosa* 15.8 mm) reflects the outer membrane permeability barrier and efflux pump systems of Gram-negative organisms¹⁷.

Comparison with recent literature confirms competitive performance: single-plant Neem-AgNPs yielded ZOI 15.3–18.7 mm against *E. coli* and *S. aureus* (25 μg)⁹; single-plant Tulsi-AgNPs yielded 16.2–19.8 mm¹⁰. The NL-AgNPs reported here yielded 19.2 mm and 22.6 mm respectively – improvements of 2–4 mm – attributable to smaller mean particle size (22.8 nm versus 28–35 nm in comparators) and the richer dual-source phytochemical corona.

Methods

Chemicals, reagents, and bacterial strains

Silver nitrate ($AgNO_3$, $\geq 99.9\%$, ACS grade; Sigma-Aldrich), Mueller–Hinton Agar/Broth (HiMedia Laboratories, India), antibiotic discs (Ampicillin 10 μg , Streptomycin 10 μg , Ciprofloxacin 5 μg ; Oxoid Ltd., UK), and resazurin sodium salt ($\geq 97\%$; Sigma-Aldrich) were used. Ultrapure water (18.2 M Ω ·cm; Milli-Q, Millipore) was used throughout. Six ATCC-certified strains – *E. coli* 25922, *S. aureus* 25923, *K. pneumoniae* 700603, *P. aeruginosa* 27853, *B. subtilis* 6633, MRSA 33591 – were obtained from MTCC, IMTECH, Chandigarh, India.

Plant material and extract preparation

Fresh mature leaves of *Azadirachta indica* and *Ocimum sanctum* were collected in September 2024 from Bhagwant University campus, Ajmer, Rajasthan (26.47°N, 74.63°E). Both species were botanically authenticated; herbarium vouchers deposited (Accession Nos. BU-BOT-AI-2024-09 and BU-BOT-OS-2024-09). Separately, 20 g dried leaf material of each species was boiled in 200 mL ultrapure water at 60°C for 30 min (200 rpm), cooled, and filtered (Whatman No. 1, then 0.45 μm membrane). The binary NL-extract was prepared as a 1:1 (v/v) mixture immediately before each synthesis, stored at 4°C, and used within 48 h.

NL-AgNP synthesis and optimization

Five millilitres of NL-extract was added dropwise to 45 mL $AgNO_3$ solution (300 rpm). Four parameters were varied one-at-a-time: $AgNO_3$ concentration (0.5, 1.0, 2.0, 3.0 mM), temperature (30, 50, 70, 90°C), pH (5.0, 6.0, 7.5, 9.0; adjusted with 0.1 M NaOH/HCl), and reaction time (40, 60, 90, 120 min). Purification: centrifugation at 12,000 rpm $\times$ 20 min, three wash cycles, lyophilization ($-50^\circ C$, 0.01 mbar, 24 h) for solid-state analyses.

Characterization

UV-Vis (300–700 nm; PerkinElmer Lambda 35); FTIR (4000–400 cm⁻¹, KBr pellet, 32 scans; Thermo Scientific Nicolet iS10); FESEM-EDX (15 kV, Au-Pd coating; ZEISS Sigma 300 VP); XRD (CuK α , 2 θ = 20–80°, 2°/min; Bruker D8 Advance), Scherrer equation ($K = 0.9$); DLS/zeta potential (25°C, triplicate; Malvern Zetasizer Nano ZS).

Antibacterial assays

Disc diffusion: bacterial suspensions (0.5 McFarland) spread on MHA; 6 mm discs loaded with 25 μ g NL-AgNPs or controls; pre-diffusion 4°C/30 min; incubation 37°C/24 h; ZOI measured by digital caliper (CLSI M02-A12)¹⁸. MIC: two-fold broth microdilution (100–0.78 μ g/mL NL-AgNPs in MHB, $\sim 5 \times 10^5$ CFU/mL, 37°C/18 h); resazurin viability indicator (CLSI M07-A10)¹⁹. All assays in triplicate; one-way ANOVA with Tukey post-hoc test (SPSS v26; $p < 0.05$ threshold).

Data availability

All data supporting the findings of this study are included in this article. Further requests may be directed to the corresponding author.

Declarations

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Author Contributions

J.K. and S.R. conceived the study. J.K. performed all experimental work, data collection, and formal analysis. J.K. wrote the original draft. S.R. supervised the research, provided resources, reviewed and edited the manuscript. Both authors approved the final version for submission.

Competing Interests

The authors declare no competing interests.

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