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Green synthesis and characterization of *Azadirachta indica*-mediated silver nanoparticles and their *in vitro* antibacterial activity against *Xanthomonas axonopodis* pv. *Punicae* and *Ralstonia solanacearum* pv. *Lycopersici*

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Abstract

The development of eco-friendly antimicrobial nanomaterials has emerged as a promising strategy for the sustainable management of bacterial plant diseases. In the present investigation, silver nanoparticles (AgNPs) were biosynthesized using *Azadirachta indica* (neem) leaf extract as a green reducing and stabilizing agent, followed by physicochemical characterization and evaluation of their *in vitro* antibacterial efficacy against *Xanthomonas axonopodis* pv. *punicae* and *Ralstonia solanacearum* pv. *lycopersici*. Successful nanoparticle synthesis was confirmed by a distinct colour transition from light yellow to dark brown. UV-Visible spectroscopic analysis revealed a characteristic surface plasmon resonance peak at 424.58 nm (absorbance 1.761), while transmission electron microscopy demonstrated predominantly spherical AgNPs with particle sizes ranging from 17.42 to 18.80 nm. The antibacterial activity of the biosynthesized AgNPs was assessed by the paper disc diffusion method at concentrations of 10, 25, 50, 75 and 100 ppm. A clear concentration-dependent antibacterial response was observed against both phytopathogens. The highest inhibition zones were recorded at 100 ppm, measuring 18.50 mm against *X. axonopodis* pv. *punicae* and 21.75 mm against *R. solanacearum* pv. *lycopersici*, whereas no inhibition was observed in the untreated control. The enhanced antibacterial activity is attributed to the synergistic interaction between silver nanoparticles and neem-derived phytochemicals, which collectively disrupt bacterial cell integrity and physiological functions.

Keywords: *Azadirachta indica*, silver nanoparticles, green synthesis, antibacterial activity, *Xanthomonas axonopodis* pv. *Punicae*, *Ralstonia solanacearum* pv. *Lycopersici*, TEM, UV-Visible spectroscopy

1. Introduction

Silver nanoparticles have emerged as a focal point of research due to their exceptional physicochemical properties and broad-spectrum antimicrobial activity. Compared to bulk silver, AgNPs possess a high surface-area-to-volume ratio, enhancing their interaction with microbial cells. Their mechanisms of action are multifaceted, including disruption of cell membrane integrity, interference with enzymatic functions and ATP production, and interaction with sulfur- and phosphorus-containing cellular components like DNA and proteins.

Plant extracts contain a wide array of bioactive compounds like polyphenols, flavonoids, alkaloids, proteins, sugars and antioxidants, which collectively facilitate reduction of metal ions and stabilization of NPs. Efficiency of NPs synthesis varies among plant species and depends on the chemical composition and reducing potential of the extract (Jha *et al.*, 2009; Nair *et al.*, 2010)^[9, 17].

Azadirachta indica (neem), a medicinal plant belonging to the family Meliaceae, is widely recognized for its antimicrobial and therapeutic properties (Krishnan and Wong, 2015). Neem leaf extracts contain a rich diversity of phytochemicals that act as efficient reducing agents for Ag ions, resulting in the synthesis of biologically active AgNPs (Patel and Pratibha, 2014; Gupta *et al.*, 2017)^[8, 14, 18]. The antimicrobial efficacy of AgNPs

synthesized using neem extracts has been extensively documented against various plant pathogens (Roy *et al.*, 2017; Chinnasamy *et al.*, 2021) [5, 22].

Biosynthesis AgNPs was carried out by using *Azadirachta indica* (Neem) leaf extract and their characterization was done by using UV-Visible spectroscopy and Transmission Electron Microscopy (TEM) available at Eco-friendly Disease Management and Beneficial Microbes Research Laboratory, MPKV, Rahuri (M.S) India.

2. Materials and methods

2.1 Chemical

Silver nitrate (AgNO_3) used in the present investigation was procured from Hi-Media Laboratories Pvt. Ltd. The molecular weight of AgNO_3 is $169.87 \text{ g mol}^{-1}$.

2.2. Preparation of *Azadirachta indica* leaf extract

Fresh leaves of *Azadirachta indica* (Neem) were collected and washed thoroughly with double distilled water and then air dried under shade for ten days. Air dried leaves were chopped into fine pieces and powdered. Out of this powder, 200 gm of powder was taken and equal amount of water i.e., 200 mL was mixed with plant extract powder. The mixture was boiled for 20 minutes and filtered through Whatman

No. 1 filter paper. The filtered extract was store in refrigerator for further studies.

2.3 Synthesis of silver nanoparticles from *Azadirachta indica* leaf extract

The 20 mL extract of *Azadirachta indica* (Neem) leaves was mixed with 180 mL of 5mM silver nitrate (AgNO_3) solution in 1:9 proportion, separately in conical flask under aseptic condition for bioreduction of Ag^+ ions in the solution. After mixing, final solution of 200 ml was poured in 250 mL flask and the flask was covered with the aluminum foil to avoid direct contact of light with the solution to avoid the photo activation of silver nitrate under static conditions. The covered flask was kept for overnight and observed for color changes of mixture. Synthesis of the nanoparticles was indicated with change in the color of solution i.e. light yellow to dark brown. Change in color indicated the formation of silver nanoparticles in the aqueous solution. The silver nanoparticles solution was centrifuged at 10000 rpm for 20 minutes to separate the AgNPs. The resultant pellet was kept in hot air oven overnight at 60°C to make a fine powder of nanoparticles. Photographs of Plate 1. illustrates the protocol adopted for the biosynthesis of silver nanoparticles from *Azadirachta indica* (Neem) leaf extract.



Plate 1: Photographs showing biosynthesis of silver nanoparticles from *Azadirachta indica* (Neem) leaf extract

2.3 Characterization of Biosynthesized Silver Nanoparticles: Silver nanoparticles synthesized by *Azadirachta indica* (Neem) leaf extract was analyzed using visual observation in color change and techniques such as UV-visible spectroscopy and Transmission Electron Microscopy (TEM).

Visual observation in color change

Visual observation in color change and color intensity during the incubation period of 24 hrs. was recorded.

UV-visible spectroscopy

UV-Visible spectroscopy analysis was done by using Evolution 201 UV-Visible Spectrophotometer available at Department of Soil science, NRC on Grape, Pune. The reduction of silver ions were monitored after 24 hrs. by recording the UV-Vis spectrum of the reaction medium after diluting a small aliquot of the sample into deionized water. The spectra of the surface plasmon resonance of AgNPs in the reaction mixture was recorded using UV-Vis spectrophotometer at wavelengths between 200 to 800 nm.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was utilized for the analysis of shape and size and used to image the synthesized AgNPs. TEM analysis of the sample was carried out at Eco-friendly Disease Management and Beneficial Microbes Research Laboratory, MPKV, Rahuri.

2.4 Culture Collection

For assessment of antibacterial activity, two phytopathogenic bacterial species were employed, namely *Xanthomonas axonopodis* pv. *punicae*, the causal agent of bacterial blight of pomegranate and *Ralstonia solanacearum* pv. *lycopersici*, which causes bacterial wilt of tomato. The pure cultures were obtained from Department of Plant Pathology and Agricultural Microbiology, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra.

2.5 In vitro antibacterial potential of *Azadirachta indica* mediated biosynthesized silver nanoparticles (AgNPs) against test bacterial plant pathogens

Silver nanoparticles synthesized by *Azadirachta indica* (Neem) extract was evaluated under *in vitro* condition against the test bacterial plant pathogens by inhibition zone method (Paper disc method) using NA as basal medium to observe the inhibitory effect of AgNPs on test bacterial plant pathogens.

The agar plate surface was inoculated by spreading a volume of the microbial inoculums over the entire nutrient agar surface. Then, a sterilized paper disc with a diameter of 5 mm was dipped in biosynthesized AgNPs solution at the concentrations of 10 ppm, 25 ppm, 50 ppm, 75 ppm and 100 ppm, then aseptically transferred it at the center of nutrient agar plate inoculated with test bacterial plant pathogens. In control plate sterilized paper disc dipped in distilled water. Then nutrient agar plates were incubated under suitable conditions depending upon the test bacterial plant pathogens. The nanoparticles diffuses in the agar medium and inhibits the growth of the test pathogen.

Observations on inhibition zone (mm) of the test pathogen were recorded after 96 hrs of incubation and the inhibition zone was measured with measuring scale in mm.

3. Results and discussion

3.1 Characterization of Biosynthesized AgNPs from *Azadirachta indica* (Neem) leaf extract

Visual Observation (Color Change): The neem leaf extract-mediated reaction mixture exhibited a noticeable dark brown coloration after 24 hrs of incubation, with moderate color intensity (++), confirming AgNP formation. Color development occurred gradually and was less intense. The brown coloration became evident after prolonged incubation, indicating moderate reduction of Ag⁺ ions. Reaction mixture remained homogenous without visible precipitation.

The color change observed in neem-mediated synthesis is attributed to reduction of Ag⁺ ions by phytochemicals such as flavonoids, terpenoids, phenolics and reducing sugars found in extract of leaves. These compounds act as electron donors, facilitating conversion of Ag⁺ ions into metallic silver nanoparticles.

UV-Visible Spectroscopy: UV-Visible spectral analysis of neem-synthesized AgNPs depicted a prominent absorption peak at 424.58 nm, with an absorbance of 1.761, confirming nanoparticle formation as shown in graph Fig. 1. The absorbance intensity was moderate, suggesting a lower concentration of nanoparticles in the colloidal suspension. No absorption peak was observed in the control sample.

The SPR peak observed in neem-mediated synthesis confirms successful formation of silver nanoparticles. The broader absorption peak reflects variability in nanoparticle size, which is commonly associated with plant-based synthesis due to the heterogeneous nature of phytochemical constituents. Variations in concentration and composition of reducing agents can influence nanoparticle nucleation and growth, leading to polydispersity. Despite this, formation of stable NPs demonstrates the effectiveness of neem leaf extract as a biological reducing agent. UV-Visible spectroscopy thus validates neem-mediated biosynthesis as a viable green synthesis approach.

The peak position aligns well with earlier results on neem-mediated silver nanoparticle synthesis, which typically range between 400-450 nm reported by Sharma *et al.*, 2013; Ramteke *et al.* (2013); Silva *et al.* (2013); Bindhani and Panigrahi (2015); Kumar *et al.* (2015); Arumugam *et al.* (2017); Kuchekar *et al.* (2017); Shaikh *et al.* (2017); Khan and Javed (2021) and Regmi *et al.* (2021) [1, 4, 11, 15, 16, 20, 21, 24, 25, 27].

Transmission Electron Microscopy (TEM)

TEM analysis of neem-mediated silver nanoparticles revealed predominantly spherical particles ranged in size from 17.42 nm to 18.80 nm as shown in photographs of Plate.2. Nanoparticles were moderately dispersed, although occasional clustering was observed.

The spherical morphology observed in neem-derived nanoparticles is consistent with most plant-mediated nanoparticle synthesis reports. The comparatively larger particle size and occasional aggregation may be attributed to variable capping efficiency of phytochemicals present in the leaf extract.

These outcomes are in line with prior reports on neem-mediated AgNP synthesis, where spherical nanoparticles with sizes ranging from 5-50 nm have been documented by Ramteke *et al.* (2013); Bindhani and Panigrahi (2015); Kumar *et al.* (2015); Arumugam *et al.* (2017); Avinash *et al.* (2017); Khan and Javed (2021) and Regmi *et al.* (2021) [1, 2, 4, 11, 16, 20, 21].

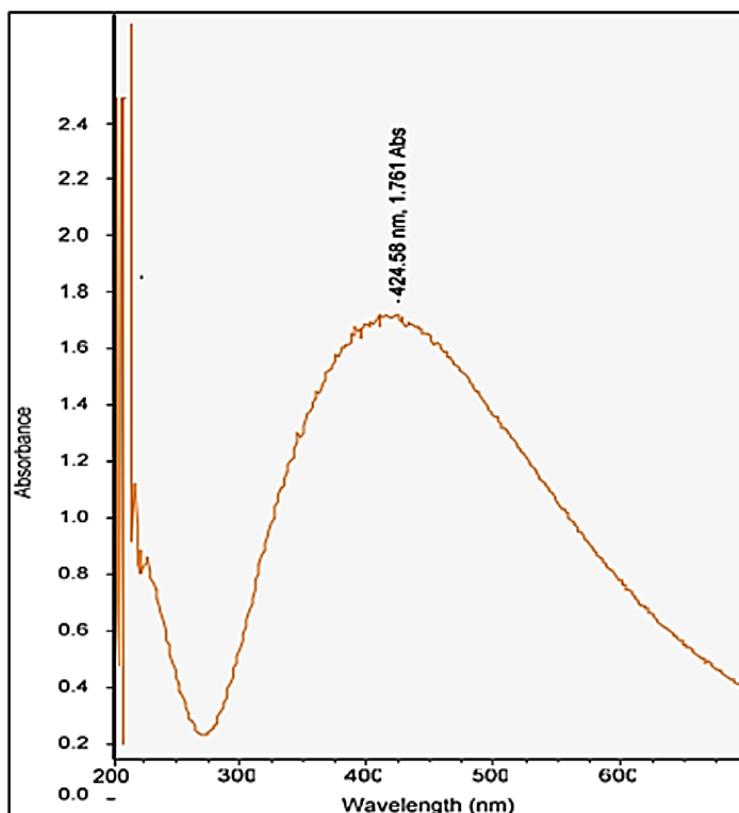


Fig 1: Graphical presentation of UV-visible spectroscopy characterization of biosynthesized silver nanoparticles from *Azadirachta indica* (Neem) leaf extract

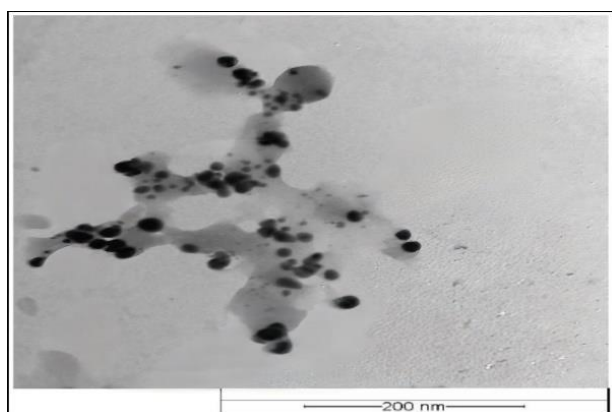


Plate 2: TEM micrograph showing the relatively spherical shape silver nanoparticle with range of 17.42 To 18.80 nm, synthesized by using *Azadirachta indica* (Neem) leaf extract

inhibition zone of 18.50 mm was documented at 100 ppm that was substantially more than all other treatments. The control treatment showed no inhibition.

Table 1: *In vitro* evaluation of antibacterial potential of *Azadirachta indica* (neem) mediated biosynthesized AgNPs against *Xanthomonas axonopodis* pv. *punicae*

Tr. No.	Treatment details	Mean inhibition zone (mm)
T ₁	AgNPs @ 10 ppm	7.75
T ₂	AgNPs @ 25 ppm	10.25
T ₃	AgNPs @ 50 ppm	12.50
T ₄	AgNPs @ 75 ppm	16.25
T ₅	AgNPs @ 100 ppm	18.50
T ₆	Control	0.00
	SE ₊	0.3385
	CD at 1%	1.3779

3.2 *In vitro* evaluation of antibacterial potential of *Azadirachta indica* (neem) mediated biosynthesized silver nanoparticles (AgNPs) against *Xanthomonas axonopodis* pv. *Punicae*

Neem-mediated silver nanoparticles demonstrated significant bactericidal efficacy against *Xanthomonas axonopodis* pv. *punicae*, with inhibition zone diameter increasing consistently across tested concentrations. The observed results have been substantiated by pictorial proof in Plate 3. and tabulated in Table 1.

At 10 ppm, a distinct inhibition zone of 7.75 mm was recorded, indicating initial antibacterial action. When concentration was risen to 25 ppm, the inhibition zone expanded to 10.25 mm. At 50 ppm, the inhibition zone further increased to 12.50 mm, reflecting enhanced antibacterial efficacy. A notable increase was observed at 75 ppm, with an inhibition zone of 16.25 mm. The largest

The enhanced antibacterial performance of neem-based AgNPs suggests that botanical extracts provide a more effective surface functionalization of nanoparticles, thus enhancing their engagement with bacterial cells. The pronounced inhibitory effect observed against *X. axonopodis* pv. *punicae*, a Gram-negative bacterium, supports earlier reports indicating that AgNPs demonstrate an increased level of toxicity towards Gram-negative bacteria owing to their thinner peptidoglycan layer and negatively charged outer membrane.

Support for the present findings was reported by Divya (2013) [7], who investigated the *in vitro* bactericidal effect of silver and silica nanoparticles against *X. axonopodis* pv. *punicae*. Their research revealed that AgNPs were markedly more effective, producing a maximum inhibition zone of 23.3 mm at 100 ppm, while the lowest inhibition (12.30 mm) was observed at 20 ppm. These results closely align

with the inhibition trend recorded in the current investigation.

Additional corroboration was provided by Rajesh *et al.* (2012) ^[19], who documented significant antibacterial activity of green-synthesized silver nanoparticles against *Xanthomonas campestris* pv. *malvacearum*, recording inhibition zones of 14.00 ± 0.58 mm. Similarly, Sahayaraja *et al.* (2012) ^[23] reported that plant-mediated AgNPs suppressed growth of *X. campestris* pv. *malvacearum*, producing an inhibition zone of 10.33 ± 0.33 mm, further validating bactericidal potential of botanical silver nanoparticles.

Moreover, Sherkhane *et al.* (2018) ^[26] compared AgNPs

produced utilizing various plant extracts and reported that Tulsi-based AgNPs exhibited the highest antibacterial activity against *X. axonopodis* pv. *punicae* (22 mm), followed by Tridax (20 mm), Neem (19 mm) and Drumstick (18 mm). Their findings reinforce the present results, where neem-mediated AgNPs demonstrated strong antibacterial efficacy.

The superior bactericidal action of neem-mediated silver nanoparticles might be due to synergistic action of silver ions and neem-derived phytochemicals such as azadirachtin, nimbin and phenolic compounds, which enhance nanoparticle stability and facilitate effective interaction with bacterial cell membranes.

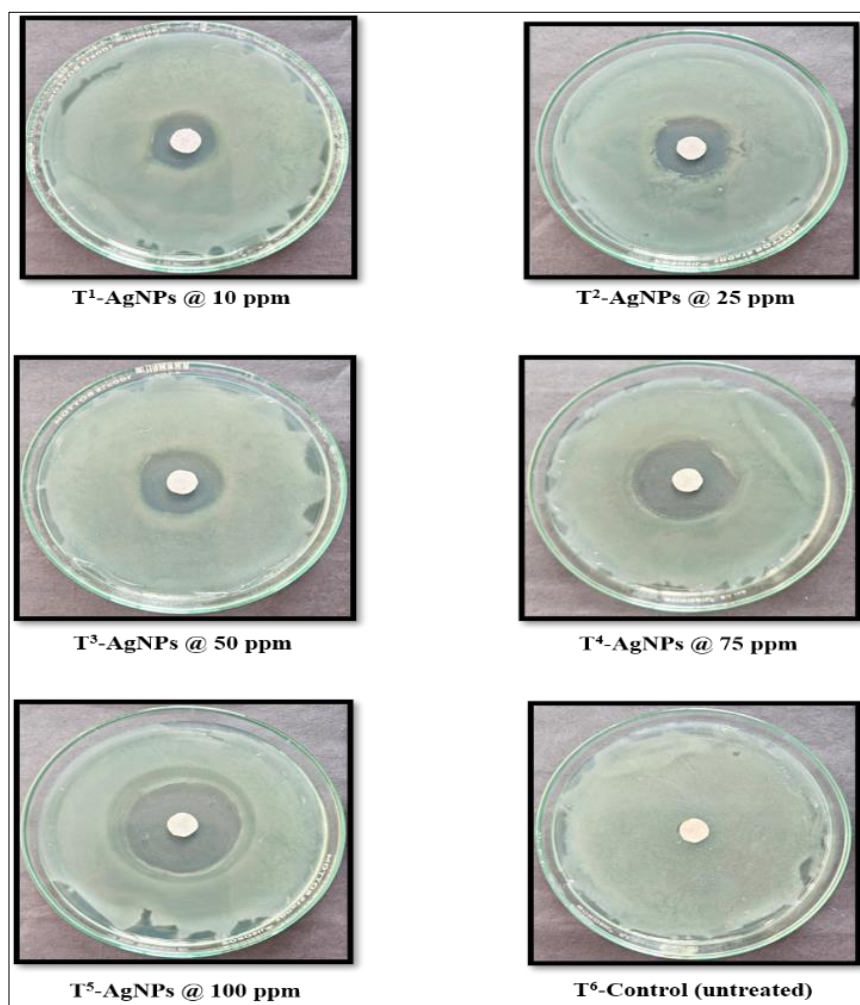


Plate 3: Photograph showing *in vitro* evaluation of antibacterial potential of *Azadirachta indica* (neem) mediated biosynthesized silver nanoparticles (AgNPs) against *Xanthomonas axonopodis* pv. *punicae*

3.3 *In vitro* evaluation of antibacterial potential of *Azadirachta indica* (neem) mediated biosynthesized AgNPs against *Ralstonia solanacearum* pv. *lycopersici*

The results demonstrated a wide range of inhibitory effects, with mean inhibition zone diameters varying from 9.00 to 21.75 mm across the tested AgNPs concentrations. The findings of research are captured in photographs of Plate 4 and systematically depicted in Table 2.

Among the treatments evaluated, AgNPs applied at 100 ppm (T_5) exhibited the highest antibacterial activity, producing a maximum inhibition zone of 21.75 mm that was substantially better than all other treatments as well as the untreated control. The treatment T_4 (75 ppm) ranked second in efficacy, recording an inhibition zone of 18.25 mm. An

observable antibacterial effect of moderate intensity was noted with T_3 (50 ppm), which resulted in an inhibitory zone of 14.50 mm.

Lower concentrations displayed comparatively reduced antibacterial activity. The treatment T_2 (25 ppm) produced an inhibition zone of 11.75 mm, while T_1 (10 ppm) recorded the least inhibitory zone of 9.00 mm. No inhibitory zone was detected in the untreated control, confirming lack of antibacterial activity without neem-mediated AgNPs. These findings distinctly suggest that the antibacterial efficacy of neem-synthesized silver nanoparticles increased proportionally with concentration.

The present findings demonstrate that neem-mediated silver nanoparticles exhibit significant antibacterial capabilities

towards *Ralstonia solanacearum* pv. *lycopersici*, a destructive soil-borne vascular pathogen. The progressive enhancement in the diameter of the inhibitory zone with an increase in nanoparticle concentration suggests a clear dose-response relationship, highlighting the importance of adequate AgNP concentration for effective bacterial suppression.

The pronounced antibacterial activity observed at higher concentrations can be ascribed to the collaborative interplay amid AgNPs and neem-derived phytochemicals, which act as natural capping as well as reducing agents in nanoparticle synthesis. These bioactive chemicals may augment nanoparticle stability and promote closer contact with cell membranes of bacteria, resulting in membrane disruption, enzyme inactivation and impaired cellular metabolism.

Table 2: *In vitro* evaluation of antibacterial potential of *Azadirachta indica* (neem) mediated biosynthesized silver nanoparticles (AgNPs) against *Ralstonia solanacearum* pv. *Lycopersici*

Tr. No.	Treatment details	Mean inhibition zone (mm)
T ₁	AgNPs @ 10 ppm	9.00
T ₂	AgNPs @ 25 ppm	11.75
T ₃	AgNPs @ 50 ppm	14.50
T ₄	AgNPs @ 75 ppm	18.25
T ₅	AgNPs @ 100 ppm	21.75
T ₆	Control	0.00
	SE ₊	0.4290
	CD at 1%	1.7463

Current findings are backed by earlier investigations. Khan *et al.* (2019) reported substantial inhibition of *R. solanacearum* at 100 $\mu\text{g mL}^{-1}$ concentration of biosynthesized AgNPs. Similarly, Khan *et al.* (2021) exhibited significant bactericidal efficacy against *R. solanacearum* when AgNPs synthesized from strawberry waste were applied at 100 $\mu\text{g mL}^{-1}$, producing distinct inhibition zones around treated discs. Bibi *et al.* (2023) [3] further validated the antimicrobial effectiveness of plant-mediated silver nanoparticles synthesized from *Hedera nepalensis*, where notable inhibitory zones were

documented against *R. solanacearum* at higher nanoparticle concentrations.

Additional evidence was provided by Dilbar *et al.* (2023), who evaluated AgNPs produced from *Salvia nubicola* and reported more than 90 per cent bacterial inhibition at higher concentrations against *R. solanacearum*. More recently, Khan *et al.* (2024) demonstrated complete or near-complete inhibition of *R. solanacearum* using *Rubus fruticosus*-derived AgNPs at elevated concentrations, reinforcing the strong antibacterial potential of plant-based silver nanoparticles [6, 11].

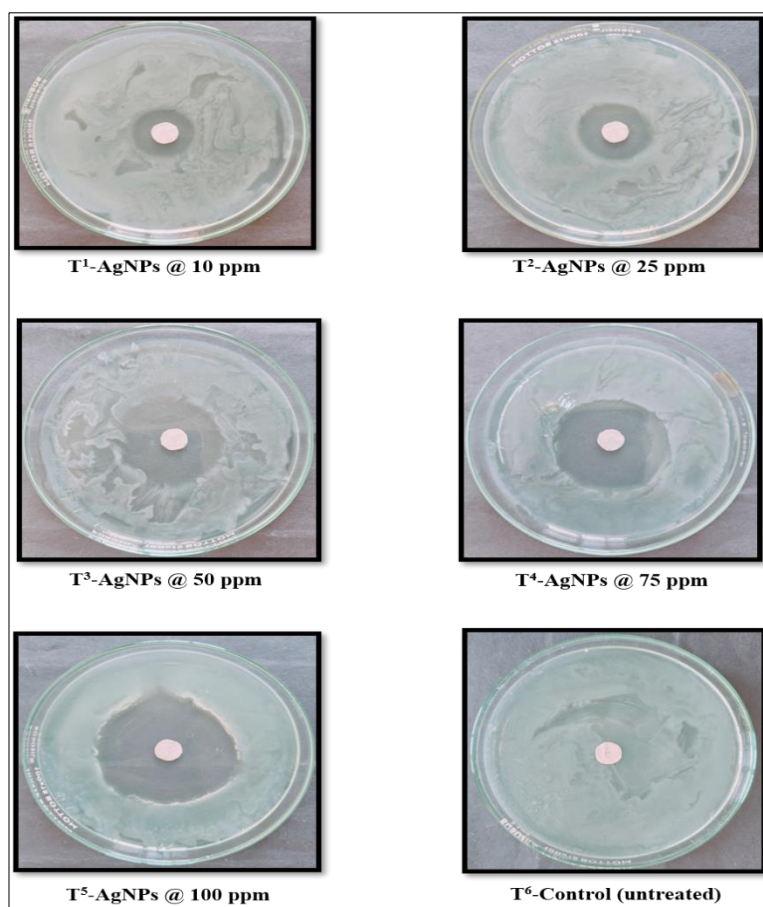


Plate 4: Photograph showing *in vitro* evaluation of antibacterial potential of *Azadirachta indica* (neem) mediated biosynthesized silver nanoparticles (AgNPs) against *Ralstonia solanacearum* pv. *Lycopersici*

Conclusions: Successful biosynthesis of AgNPs was attained utilizing *Azadirachta indica* (neem) leaf extract through an eco-friendly green synthesis approach.

UV-Visible spectroscopy and TEM analysis proved formation, stability and nanoscale size of synthesized AgNPs, validating the effectiveness of biological reducing and capping agents.

Biosynthesized AgNPs from *Azadirachta indica* (neem) demonstrated significant antibacterial activity against *Xanthomonas axonopodis* pv. *punicae* and *Ralstonia solanacearum* pv. *lycopersici*.

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