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Evaluation of spectroscopic and antimicrobial studies on silver nanoparticles synthesised from *AzadirachtaIndica*(neem) using green synthesis methods

Dhani Ram Deka[1], Pranjoli Das[2], Dhiman Chandra Paul[1], Minakshi Bhattacharjee[1], ManashPratim Sarma[1*]

1- Assam down town University, Panikhaiti, Guwahati (781027), Assam, India
2-Centre for Nanotechnology, IIT Guwahati (781039), Assam, India

Corresponding Author: Dr. Manash Pratim Sarma, PhD
Associate Professor and Dean, I/C Faculty of Science
Department of Biotechnology
Assam down town University
Assam
Phone: 91-8255075275
Email: sarmadrmanash@gmail.com

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Abstract:
Silver nanoparticles have been synthesised by green synthesis using aqueous leaf extracts of herbal medicinal plants *Azadirachta indica* (Neem) and silver nitrate solution. The synthesised silver nanoparticles (AgNPs) were characterised through UV-Visible Spectroscopy, FTIR (Fourier Transform Infrared Spectroscopy), and TEM (Transmission Electron Microscopy). Antibacterial activities of leaf extract and bio-reduced silver nanoparticles (AgNPs) were investigated using the nutrient agar well diffusion method against different gram-negative bacteria, *Escherichia coli* (NCIM 5346), and gram-positive bacteria, *Staphylococcus aureus (NCIM* 5345). A peak was seen in the UV-visible range between 420 and 450 nm. This means that surface plasmon resonance (SPR) excitation is making the silver ions change from Ag+ to Ag0. FTIR spectroscopy monitored the biomolecules, bio-organic compounds, and functional groups involved in the reduction, capping, and efficient stabilisation of biologically synthesised AgNPs. Transmission Electron Microscopy (TEM) imaging revealed the agglomeration of small grains, with particle sizes ranging from 2 to 10 nm. The selected area electron diffraction (SAED) analysis confirmed the presence of a face-centred cubic (FCC) crystal structure in the crystalline phase. The antimicrobial activity of the biologically synthesised silver nitrate solution against two bacterial strains (*E. coli and S. aureus*) was investigated and. The SAED and TEM analyses confirm the existence of a crystal structure with a diameter of about 0.5 mm and a size of 10 mm, respectively. Further, efficient antimicrobial activity proves the potential application of green synthesis methods in the area of Nano medicine.

Keywords: *AzadirachtaIndica*, Green synthesis, Silver nanoparticles, Antimicrobial activity and TEM.

Introduction:

Since the dawn of civilization, human society has been used herbal medicinal plants to combat diseases and certain bacterial illnesses. Due to the growing practical utility of the medicinal plants in treating several health care problems, the research interest on herbal plants has been increasing and gaining importance in international level. The herbal products or medicines are used to prepare from various parts of medicinal plants such as bulb, gel, leaves, roots, barks peels, etc. by using different methods such as extraction, fractionation, purification, concentration or physical or biological processes or methods [1-2].

Countries such as India and China have a wide knowledge in varieties of medicinal plants and their potential applications in health care. The pharmaceutical companies are interested to use this knowledge as a resource for research and development programs in the pursuit of discovering novel drugs. India is a varietal emporium of medicinal plants and it is one of the richest countries in the world as regards genetic resources of medicinal plants. The practice of using traditional medicine has become an integral part of the culture in most of the developing countries [3]. The silver nanoparticles synthesized from plant extracts prepared from leaves, flowers, seeds, fruits, roots and bark has several important applications in the field of nanotechnology and exhibit new physicochemical properties [4-6].

Azadirachta indica (Neem tree) is one of the most versatile medicinal plants and exhibit immuno-modulatory, anti-inflammatory, anti-hyperglycaemic, antiulcer, anti-malarial, antifungal, antibacterial, and antiviral, antioxidant, anti-mutagenic and anti-carcinogenic properties [7]. People in India consider it as a “Home Village Dispensary" and describe it as a "Reliever of Sickness". It belongs to Family- Meliaceae (mahogany family), Subfamily-Melioideae, Genus-Azadirachta, Species-indica, Order-Rutales, Suborder-Rutinae and Tribe-Melieae.

The medicinal value and beneficial effects of different parts of *Azadirachta indica* (Neem) are attributed to its biologically active principle 'Azadirachtin'. Natural sources of flavonoids, terpenoids, polyphenols, isoprenoids, sulphurous compounds, and polysaccharides play an important role in scavenging the free radical and subsequently arresting disease pathogenesis [8]. The phytochemicals found in Neem, specifically terpenoids and flavanones, which act as reducing as well as capping agent and helping in stabilizing the nanoparticles. The synthesized nanoparticles, which are capped with neem extract also exhibit and demonstrate enhanced antibacterial activity [9].

2. Experimental Materials and Methods:

The chemicals used for the experiments were Silver Nitrate from Sigma Aldrich Chemical Company Ltd and Nutrient broth from Himedia Lab. Ltd., Mumbai of high purity analytical grade. The nutrient media was used as a solid supportive medium for bacterial growth. The bacterial strains used to study antimicrobial studies were *E. coli* (NCIM 5346) and *S. aureus* (NCIM 5345).

Collection of Sample:

The fresh natural leaves of *Azadirachta Indica* collected from the North Guwahati, Kamrup, Assam, India were washed and cleaned thoroughly with running tap water as well as double distilled water to remove all the dust and debris and also unwanted other contaminated organic adherent particles. After that the leaves were allowed to dry at room temperature.

2.2. Preparation of *Azadirachta indica* (Neem) Leaf Extract:

The air dried leaves of *Azadirachta indica* were chopped into tiny pieces and kept at room temperature for a week and then in Hot Air Oven at 50°C for 24hr. After that these leaves were grinded into the fined powder using mortar and pestle and stored in air-tight container for the preparation of crude plant extract [10]. An aqueous extract of a plant sample was prepared using, with minor adjustments, the procedures of Dhanalakshmia, T., & Rajendran, S. (2012) [11]. *Azadirachta indica* leaf was extracted completely of its constituent compounds using water. Ten grammes of these finely ground *Azadirachta indica* leaf were weighed, then put into 250 millilitre beakers with 100 millilitres of Mili Q water and mixed thoroughly. After keeping it in a hot water bath at 60º C for four hours, it was filtered through Man No. 1 filter paper, freeze-dried, and stored at -20º C for future experiments.

2.3 Preparation of 1 M Silver Nitrate ($AgNO_3$) Stock Solution:

The concentration of 1 M silver nitrate ($AgNO_3$) was prepared by dissolving 1.698g of $AgNO_3$ in 10 ml of deionised water and stored in amber coloured bottle to prevent the self-oxidation of silver nitrate solution.

2.4 Biosynthesis silver nanoparticles (AgNPs) by green method:

In a conical flask 5 ml of aqueous *Azadirachta indica* leaf extract was taken and 45 ml of 1mM from 1M stock solution silver nitrate solutions were added. The flask was covered with

aluminium foil and incubated at room temperature in shaking condition in dark for 48 hours. After incubation the mixture of colour changed from orange brown to dark brown **(Figure 1)**. The colour change seen in the mixture is due to the synthesis of silver nanoparticles for the reaction between silver nitrate solutions and leaf extracts. The results were analysed by TEM, UV-Visible and IR spectroscopy as well as monitored by visual observation due to change in colour from orange brown to dark brown [12-14].

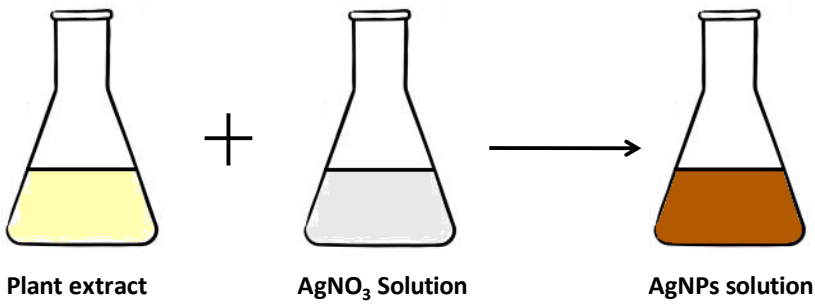

Figure 1: *Azadirachta indica* leaves extract mixed with aqueous Silver nitrate 1 mM solution and the change in colour

3. Characterization of synthesized silver nanoparticles by instrumentation:

3.1 UV-visible spectroscopy:

Multiskan Skyhigh Microplate UV-Visible Spectrophotometer (Thermo Scienctific, Cat No: A51119600DPC) with different wavelength (350, 400, 420, 450, 500, 550, 600 and 650 nm) was used for spectral analysis and to investigate the formation of Ag-NPs as well as the bio-reductive property of *Azadirachta indica* (Neem) leaf extracts. Optical Density (OD) was taken to confirm the reduction of silver nitrate. The absorbance spectra of the reaction were recorded after 24 hour using varying concentration of silver nitrate (1mM, 2mM, 3mM, 4mM, 5mM 6mM and 7mM keeping constant 100µL leaf extract) and leaf extracts solution (100- 700µL keeping constant 2 mM $AgNO_3$).

3.2 FT-IR (Fourier Transform Infrared Spectroscopy) analysis:

FTIR spectra were recorded using THERMO NICOLET iS10 FTIR SPECTROMER (THERMO SCIENTIFIC) instrument of Guwahati Biotech Park Incubation Centre. The IR spectra of leaf extract were recorded using KBR pellet at a resolution of 400 - 4000cm^{-1} in % transmittance mode for the qualitative assessment of the functional groups present in the synthesized AgNPs.

3.3 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM), Make JOEL, Model 2100F TEM of Central Instruments Facility, Indian Institute of Technology, Guwahati, Assam was used to characterize the external morphology, crystalline nature as well as the size of the synthesized AgNPs using carbon-coated copper grids at an acceleration voltage of 200 kV. The AgNPs was analysed by

putting a drop of the sample on a copper grid and then allowed it to dry before loading the copper grid onto the specimen holder.

3.4 Antimicrobial studies of silver nanoparticles:

The study on the antimicrobial activity of the biologically synthesized silver nanoparticles (AgNPs) was done on Gram-negative bacteria *Escherichia coli* (NCIM 5346) and Gram-positive bacteria *Staphylococcus aureus* (NCIM 5345), by nutrient agar well diffusion method. As per the standard protocol the sterile nutrient broth inoculated with *E. coli* and *S. aureus* strains was kept overnight at 37^0C in an incubator. The nutrient agar media plates were prepared by pouring the nutrient agar on Petri plates and kept overnight. After it solidifies each freshly grown pathogenic strain (bacteria 100µL) were taken and spread evenly by a sterile glass spreader on the nutrient agar media plates in order to cultivate bacteria. 8-mm wells were created using sterile gel puncture borer and sealed with 50µl of soft agar to prevent leakage of the NPs from the bottom. The silver nanoparticles of different volumes (20, 40, 60, & 100µL) of and *Azadirachta indica* plant leaf extracts were loaded into the wells using a micropipette. All the plates were incubated at 37^0C for 24hrs and after incubation, the antimicrobial activity was studied based on the zone of inhibition around each well with plant extract and synthesized silver nanoparticle. For control, 100µL of 2mM silver nitrate ($AgNO_3$) was used.

4. Results and Discussions:

4.1 UV-Visible Absorption spectra of silver nanoparticles:

The formation of silver nanoparticles (AgNPs) from the reaction of leaf extract of *Azadirachta indica* (Neem) and Silver Nitrate solution (AgNO3) was confirmed by the absorbance peak in the range of 420-450 nm using UV-Visible spectrophotometer. The reaction was carried out with varying concentration of $AgNO_3$ (1-7mM) with constant leaf extracts (100µL) constant and vice-versa with varying the leaf extract solution (100-700µL) with constant $AgNO_3$ (2mM)

under dark condition to avoid photo-activation of silver nitrate at room temperature and monitored at different time intervals as shown in **Table 1** and **Figure 2**. It was observed that colour of the solution changed from light yellow to pale yellow and final dark brown colour after a period of time interval. The initial colour of the $AgNO_3$ solution on treatment with *Azadirachta indica* (Neem) leaf extracts was light yellow. After 24 hours the colour turned pale yellow, finally the colour changed to dark brown during a period of 120 hours which is due to the reduction of silver ions from Ag^+ to Ag^0(i.e. change in colour from pale yellow to brown) resulting in the formation of silver nanoparticles. The reduction of silver ions from Ag^+ to Ag^0 is due to excitation of Surface Plasmon Resonance (SPR). On the other hand, that there was no colour change in the control test of 2mM $AgNO_3$ solution**.**

Table 1: Absorbance recorded with varying concentration of $AgNO_3$ solution keeping *Azadirachta indica* (Neem) leaf extract concentration constant and with varying concentration of *Azadirachta indica* (Neem) leaf extracts keeping 2mM AgNO3 concentration constant.

Sl.No.	Conc. of Leaf extract	Conc. of $AgNO_3$	Absorbance in nm							
			350	400	420	450	500	550	600	650
A. With varying (1mM-7mM) Conc. of AgNO3 keeping Leaf Extract (100µL) constant	100µL	1mM	0.3053	1.2137	1.5194	1.6108	0.8708	0.498	0.3378	0.2177
	100µL	2mM	0.4097	1.4707	1.8252	1.7233	0.937	0.5365	0.3332	0.2013
	100µL	3mM	0.5621	1.0062	1.1531	1.229	1.0104	0.7582	0.5671	0.4442
	100µL	4mM	0.2301	1.2822	1.6034	1.497	0.8221	0.4831	0.3019	0.1785
	100µL	5mM	0.2899	1.2735	1.63	1.5654	0.8763	0.4998	0.3256	0.2177
	100µL	6mM	0.0114	0.9321	1.2374	1.1778	0.647	0.3563	0.2211	0.1456
	100µL	7mM	0.0524	0.843	1.1416	1.1502	0.6407	0.3488	0.2191	0.1515
B. With varying Leaf Extract (100-700µL) keeping Conc. of AgNO3 (2mM) constant	100µL	2mM	0.1583	0.2210	0.2420	0.2406	0.1342	0.0536	0.0229	0.0124
	200µL	2mM	0.2787	0.4054	0.4487	0.4462	0.2226	0.0860	0.0350	0.0162
	300µL	2mM	0.4335	0.6181	0.6840	0.6805	0.3393	0.1478	0.0697	0.0391
	400µL	2mM	0.7226	1.1283	1.2488	1.2240	0.7101	0.3022	0.1391	0.0773
	500µL	2mM	0.732	0.9901	1.0987	1.1195	0.6051	0.2905	0.1500	0.0801
	600µL	2mM	0.8445	1.1849	1.3185	1.3121	0.6608	0.3312	0.1783	0.0966
	700µL	2mM	0.8356	1.1743	1.3060	1.3001	0.6554	0.3281	0.1780	0.0968

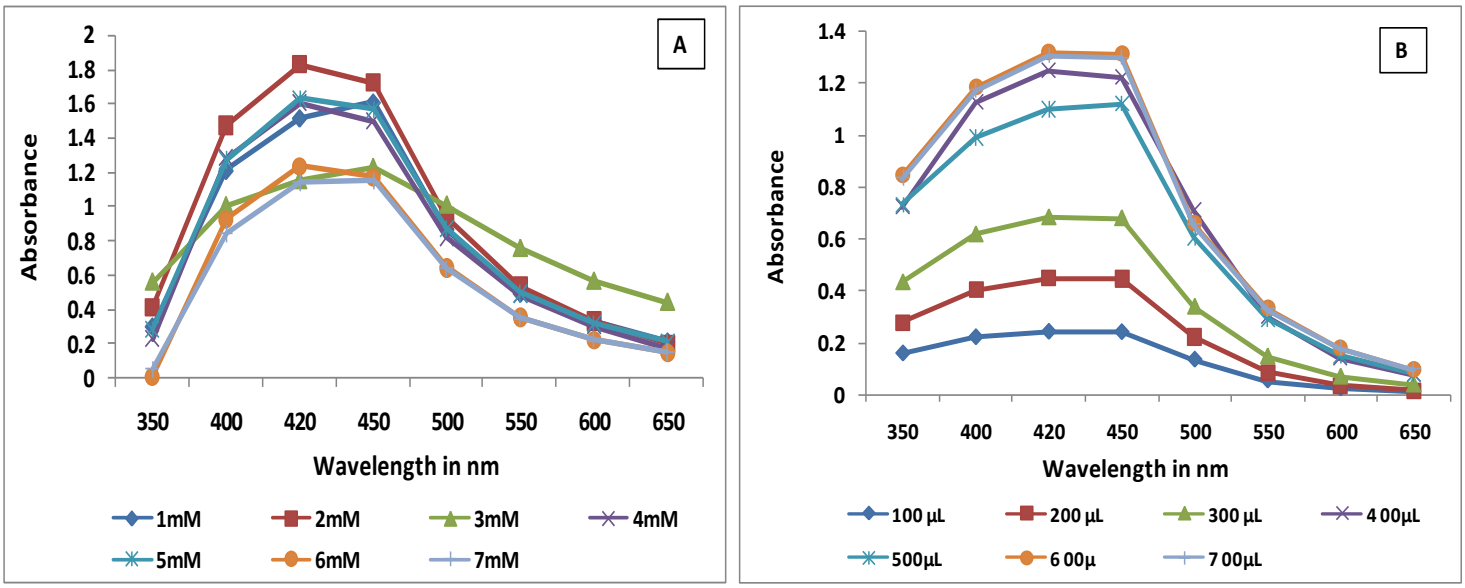

Figure 2: Absorbance spectra of silver nanoparticle formed from *Azadirachta indica* leaf extract [A] Varying concentration of $AgNO_3$ (1mM-7mM) keeping leaf extract concentration constant (100µL); [B] Varying concentration of leaf extract (100-700µL) keeping AgNO3 concentration constant (2mM).

4.2 FTIR (Fourier Transform Infrared Spectroscopy) analysis:

The bio-molecules, bio-organic compounds and functional groups involved in reduction, capping and efficient stabilization of biologically synthesized AgNPs was analyzed by FTIR spectroscopy [15-17]. The characteristic IR spectrum band for AgNPs as shown in Figure 4 were observed at 3262.94cm-1, 2119.05cm-1, 1634.95cm-1, and 508.75cm-1 and a small shoulder peak at 2119.05cm-1. The IR bands at 3262.94cm-1, 2119.05cm-1 and 1634.95cm-1 for AgNPs formed from *Azadirachta indica* (Neem) leaf extract is due to the presence of -OH, amide C=O as well as small C-O, and C=C bond stretching. The amide C=O stretching vibrations may be due to the carbonyl stretching in the amide group of proteins. Similarly, the OH, C-O, C=C stretching vibrations will be due to the presence of phytochemicals in leaf extract such as alkaloids, terpenoids, flavonoids, phenolics and so on. On the other hand, the leaf extract also exhibited a wide and strong peak, reaching its maximum intensity at 508.75

cm-1. The presence of these functional groups allows *Azadirachta indica* (Neem) leaf extract to act as both reducing as well as capping agent.

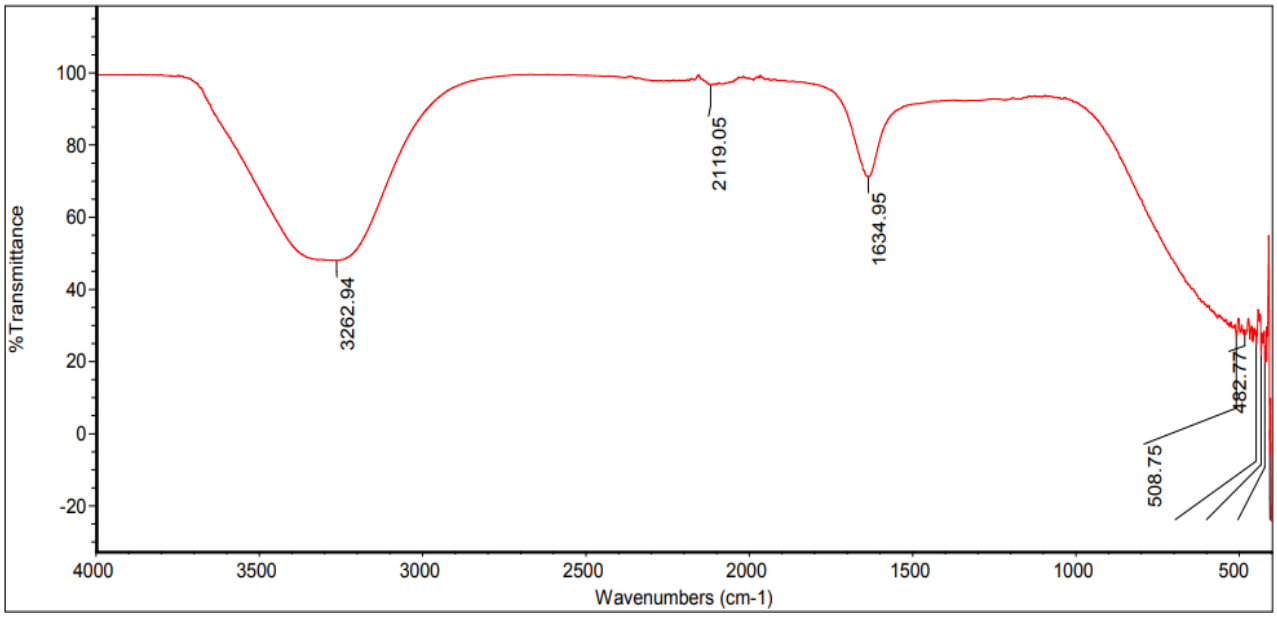

Figure 4: FTIR Analysis of Silver Nanoparticles from *Azadirachta indica*.

4.3 Transmission electron microscopy (TEM) analysis:

The morphological characterization such as shape and size of synthesized AgNPs was analysed by Transmission Electron Microscopic (TEM) technique as shown in Figure 5. The typical bright-field TEM image micrograph silver nanoparticles represented in Figure 5A appeared to be uniform, well distributed and spherical in size. However, agglomeration of nanoparticles in some regions would be due to the surface forces, van der Waal forces, capillary forces, and electrostatic forces [18]. The diameter of the nanoparticles ranges from 0.5nm to 60nm but a maximum number of AgNPs had an average size of 10nm. It is also observed that the nanoparticles obtained from plant sources were comparatively larger than microbial sources because the AgNPs synthesized from leaf extract interacted with several other phytochemical

constituents present in the leaf extract and hence diameter of the nanoparticle increases. The Selected Area Electron Diffraction (SAED**)** pattern of synthesized AgNPs as shown in Figure5B had resulted in characteristics ring pattern from inner to outer which could be indexed as (111), (200),(220) and (311) reflection of pure face centered cubic (FCC) silver structure [19]. Selected area electron diffraction (SAED) is a crystallographic experimental technique associated with TEM and basically useful for the phase identification [20].

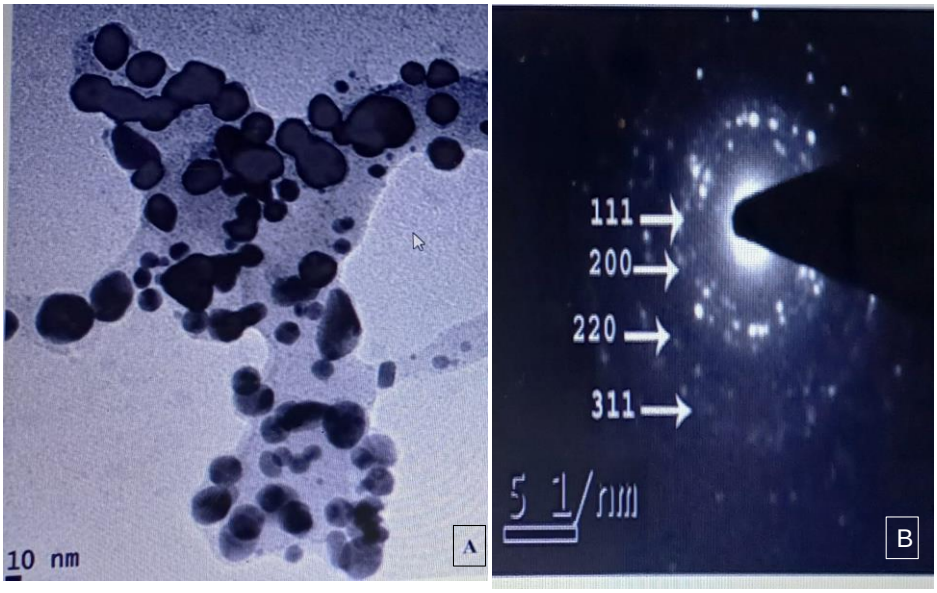

Figure 5: [A] Bright-field TEM image micrograph of AgNPs; [B] Selected Area Electron Diffraction (SAED) pattern of AgNPs synthesized from *Azadirachta indica* leaf extract by green method.

4.4 Evaluation of Antibacterial activity:

The antimicrobial activities of *Azadirachta indica* leaf extract and bio-reduced silver nanoparticles (AgNPs) were investigated using the agar well diffusion method against two bacterial strains Gram-negative bacteria *Escherichia coli* (NCIM 5346) and Gram-positive bacteria *Staphylococcus aureus* (NCIM 5345) species. All the plates were incubated at 37^0 C for 24 hrs and then the antimicrobial activities were determined by measuring the zone of inhibition against each bacterial strains at different concentration of leaf extract and synthesized Ag-NPs such as 20 µl, 40 µl, 60 µl, & 100µl respectively as shown in Table 2 and Figure 6.

Table 2: Zone of inhibition (in mm) of *Azadirachta indica* leaf extract and silver nanoparticles against *E. Coli* and *S. aureus* at different concentration (in µL)

Bacterial Strain	Zone of inhibition along with well diameter 8mm								
	Control 100 µL 2mM $AgNO_3$	20µL		40µL		60µL		100µL	
		leaf extract	AgNPs solution	leaf extract	AgNPs solution	leaf extract	AgNPs solution	leaf extract	AgNPs solution
E.Coli	0	8	20	10	23	12	27	16	34
S.aureus	0	6	13	8	18	9	21	14	30

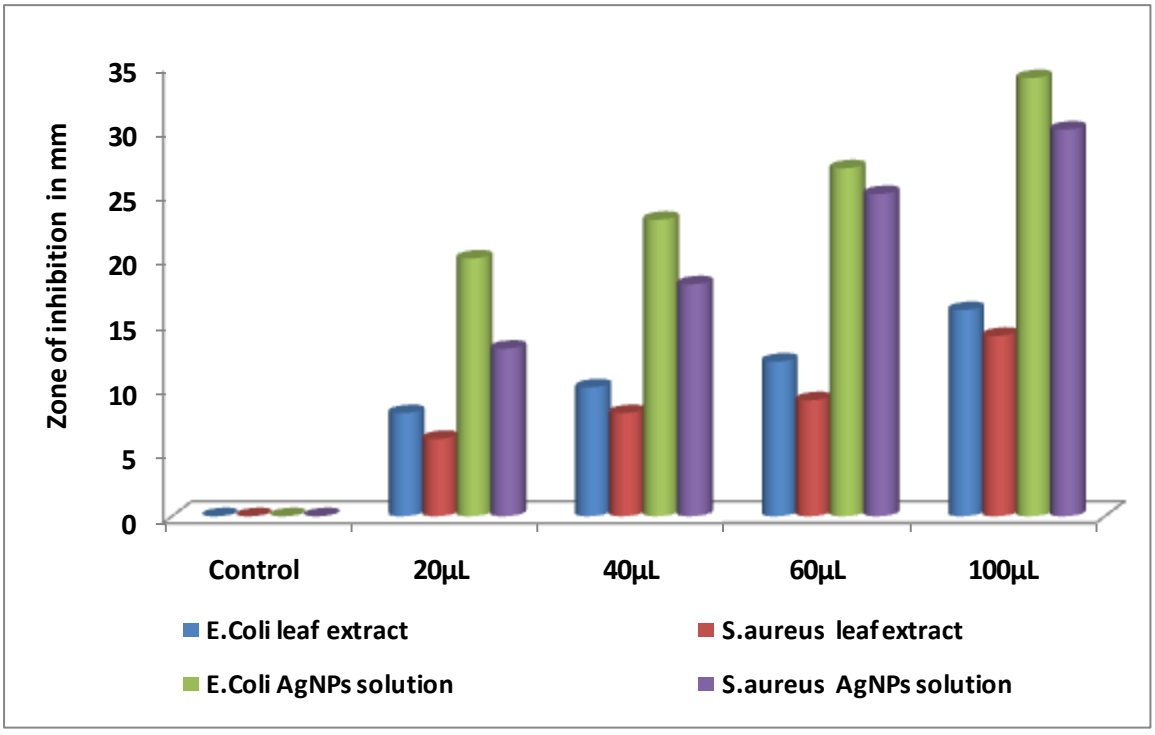

Figure 6: Bar diagram showing Zone of inhibition (in mm) of *Azadirachta indica* leaf extract and silver nanoparticles against *E. Coli* and *S. aureus* sp.

The phytochemical compounds present in *Azadirachta indica* leaf extract and the silver ions of AgNPs solution inhibits the growth of micro-organisms by disrupting the cell membrane, cellular proteins and DNA strands, hereby affecting the permeability and respiratory functions of the pathogens [21]. The significant antibacterial effect showing the growth of inhibition against each bacterial strains *Escherichia coli* and *Staphylococcus aureus* species is represented in Figure 7(A) and 7(C) for *Azadirachta indica* leaf extracts and Figure 7(B) and 7(D) for the synthesized AgNPs by green method.

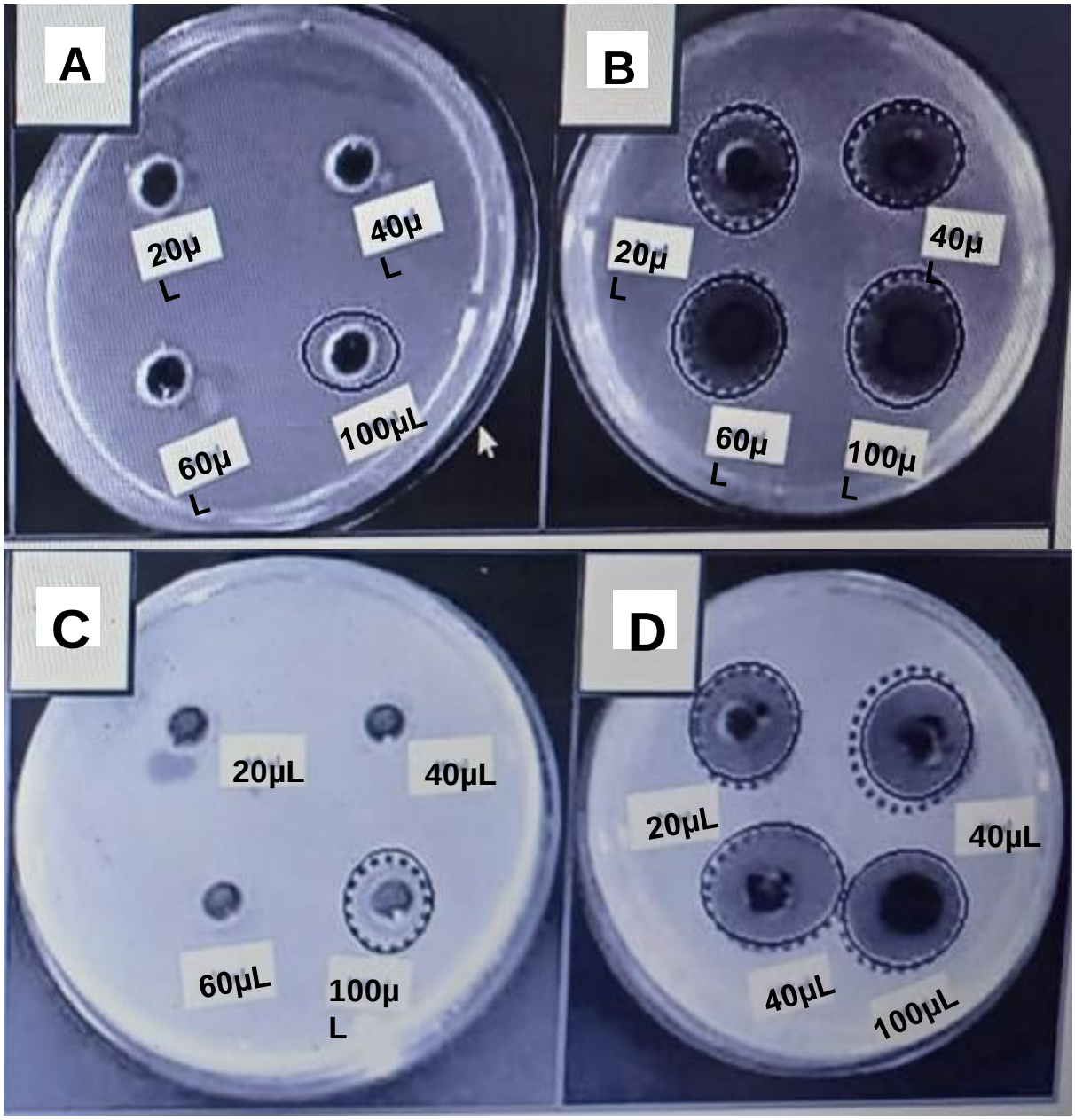

Figure 7: Zone of inhibition against bacterial strains: [A] *E.coli* after 24 hours using *Azadirachta indica* leaf extract; [B] *E.coli;* after 24 hours using silver nanoparticle; [C] *S. aureus* species after 24 hours using *Azadirachta indica* leaf extract; [D*] S.aureus* species after 24 hours using silver nanoparticle.

5. Conclusion:

The green synthesis of silver nanoparticles using *Azadirachta indica* leaf extract is an eco-friendly, simple and efficient method. It is evident from the experimental observation that the substantial amount of AgNPs synthesized using *Azadirachta indica* leaf extract acts as both reducing and capping agents. UV-Visible spectra display surface plasmon resonance absorption

peak at 420 nm. TEM analysis shows that the particles are between 2 to 10 nm in size, and the SAED analysis verifies and reveals with confirmation that the AgNPs have a face-centered cubic (FCC) crystalline structure. It has been shown that AgNPs can effectively kill both Gram-negative bacteria *Escherichia coli* (NCIM 5346) and Gram-positive bacteria *Staphylococcus aureus* (NCIM 5345) species. The synthesized AgNPs showed enhanced antibacterial property against Gram-negative bacteria (*E-Coli*), which suggests possible bio-medical applications. Also it adds scientific merit to long term use of *Azadirachta indica*as traditional herbal medicines.

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Conflict of interest:

None

Financial disclosure:

None to disclose

Code - Data Availability

Code and data sharing is not applicable to this article as no new code was created. All data in support of the findings of this paper are available within the article

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