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Synthesis Characterization and Evaluation of the Antimicrobial Activity of Neem Leaf Extract-Mediated Silver Nanoparticles

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ABSTRACT

Now a days Production and applications of green silver nanoparticles is been an attracting research activity. In this study we report the aqueous leaf extract of Azadirachta indica was used as the reducing agent for the synthesis of silver nanoparticles (AgNPs) from silver nitrate solution. AgNPs formation was confirmed by UV-VIS, FT-IR, XRD, DLS and TEM. UV–VIS spectrum of the aqueous medium containing silver nanoparticles exhibited absorption peak at around 405 nm. Fourier transform infrared spectroscopic analysis shows the absorption peaks at 580.90, 622.83, 1050.20, 1252.08, 1394.30, 1636.89, 1772.82, 2107.52, 2821.94, 2885.18, 2989.34 and 3,361.38 cm-1. X-ray diffraction analysis conformed the face centered cubic structure of the synthesized AgNPs. By dynamic light scattering technique hydrodynamic diameter (12.4nm) and zeta potential (-34.3mV) were measured. TEM analysis revealed AgNPs were spherical in shape and were poly-dispersed. The measured average size of AgNPs was 5-50nm. Antimicrobial efficacy of synthesized AgNPs was tested (in -vitro) by disc diffusion method against both bacteria and Fungi. A green synthesized silver nanoparticle has the potent antimicrobial activity. In addition, toxicity tests were conducted to analyze the toxicological effects of particle size on Brine shrimps. Less toxicity on brine shrimps were noted after exposure to aqueous extracts and synthesized AgNPs.

Key words: *Azadirachta indica, Antimicrobial efficacy, AgNPs, Nanoparticles*

INTRODUCTION

Now a days Production and applications of green silver nanoparticles is been an attracting research activity. Nanotechnology is emerging as a rapidly growing field with its applications in science and technology for the purpose of manufacturing new materials at the nano scale level.

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Nanoparticles are building blocks of nanotechnology, generally considered as particles with a size of up to 100 nm, exhibit improved properties when compared to the bulk material that they are composed of based on specific characteristics such as size, distribution, and morphology[1]. Among the different synthesis methods, plant-mediated nanoparticles synthesis is preferred as it is cost-effective, environmentally friendly and safe for human therapeutic use[2].

Biosynthesis of nanoparticles using microorganisms, enzymes, and plant extracts has been suggested as possible eco-friendly alternatives to chemical and physical methods[3]. Plant extract mediated nanoparticles synthesis can be advantageous over other biological synthesis because it eliminates the elaborate process of maintaining cell cultures and can also be suitably scaled up for large-scale nanoparticles synthesis[4].

Among the metal nanoparticles, silver nanoparticles have a special interest. Silver nanoparticles synthesized in chemical method that may have general toxicity. Advantages of synthesis of silver nanoparticles using plant extracts is an economical, energy efficient, cost effective, protecting human health and environment leading lesser waste and safe products[5,6]. *Azadirachta indica* commonly known as Neem belongs to Meliaceae family, and is well known distributed in India and its neighboring countries for more than 200 years and also most versatile medicinal plant having a wide spectrum of biological activity. Each and every part of the neem tree has been used as a traditional medicine for household remedy against various human ailments, from antiquity[7], also observed some anti bacterial[8] and anthelmentic properties of its extracts[9].

Azadirachta indica leaf extract has also been used for the synthesis of silver, gold and bimetallic (silver and gold) nanoparticles. The major advantage of using the neem leaves is that it is a commonly available medicinal plant and the antibacterial activity of the biosynthesized silver nanoparticles might have been enhanced as it was capped with the neem leaf extract[10]. Keeping in view of these activities, we synthesized and characterized the green Ag NPs using *Azadirachta indica*, for subsequent therapeutic applications in veterinary medicine.

MATERIALS AND METHODS

Materials

For nanoparticles synthesis Silver nitrate brought from Sigma Aldrich. Antimicrobial trails Potato dextrose broth, Potato dextrose agar, Nutrient broth, and Nutrient agar plates were purchased by Hi-media, India.

Collection of Plant

Fresh (A*zadirachta indica*) neem leaves were collected from Regional Agricultural Research Station, Tirupati. Later these were identified by the Department of Botany, Sri Venkateswara University, Tirupati. The leaves were washed thrice in water to remove dust and air dried for 10 days under shade.

The aqueous leaf extract was prepared as described earlier[4]. Fifty grams of leaf powder was mixed with 500mL distilled water and boiled for about 30 min on a hot plate. The boiled solution was filtered using Whatman No.1 filter paper and clear aqueous extract was obtained. The extract was stored at 4°C for further experiment.

Synthesis of AgNPs by neem leaf extract

Silver nanoparticles (AgNPs) were synthesized as per the description[4]. 90mL of 2mM silver nitrate solution was mixed with the 10mL of neem leaf extract and maintained at 80^{o} C on a magnetic stirrer. After addition, the solution was kept at room temperature for 24 hours. Nanoparticles formation was confirmed by a distinct change of the hydrosol. Silver nanoparticles were collected by centrifuging at 10000 rpm for 4 min. of the hydrosol for further characterization.

Characterization

For characterization of the green silver nanoparticles, UV-Vis spectroscopy, Fourier Transformed infrared FT-IR, X-ray diffraction (XRD), dynamic light scattering, transmission electron microscopy (TEM) analyses were adopted.

UV–Vis spectroscopy

The localized surface plasma resonance of green silver nanoparticles were recorded UV–Vis spectrophotometer (UV-2450, SHIMADZU). A 5mL sonicated sample of the hydrosol was scanned from 200 to 800 nm to obtain peak absorption.

Fourier Transformed Infrared Analysis (FT-IR)

Fourier Transformed Infrared Analysis (FT-IR) analysis was used to identify the organic functional groups involved in the synthesis of silver nanoparticles. By using attenuated total reflectance (ATR) technique, FT-IR spectrum was taken in the mid IR region of 400–4000 cm^{-1} and recorded in transmittance mode. The dried sample was mixed with the potassium bromide crystal in the ratio of 1:200 (Tensor 27, BRUKER).

X-ray Diffraction (X-RD)

The X-ray diffraction (XRD) analysis determines the crystalline structure of silver nanoparticles . The XRD pattern was carried out on a green silver nanoparticles using computer controlled XRD-system in the range of 40 kV and 20 A. And finally identification of XRD peaks by using a software (Syn-master 7935) program.

Dynamic Light Scattering (DLS)

The aqueous suspension of the synthesized nanoparticles was filtered through a 0.22µm syringe-driven filter unit and the size and distribution of the nanoparticles and zeta potential was measured using Dynamic Light Scattering (DLS) technique (Nanopartica, HORIBA, SZ-100).

Transmission Electron Microscopy (TEM)

The morphology and size of the nanoparticles were studied by transmission electron microscopy (JEOL-JEM-1010 instrument) with an accelerating voltage of 80 kV. A drop of aqueous AgNPs was dried on carbon-coated-copper TEM grids under vacuum in a desiccator and loaded into the specimen holder. The particle size and surface morphology of nanoparticles were evaluated using Image J 1.45s software.

Antimicrobial activity

Green synthesized silver nanoparticles were examined for their antimicrobial activity by disc diffusion method against different pathogenic bacteria organisms, like *Staphylococcus aureus, Pseudomonas aeruginosa, Sphingobacterium thalpophilum* and *Escherichia coli*, and pathogenic fungal organisms like *Aspergillus niger*, *Aspergillus flavus*, *Sclerotium rolfsii* and *Rhizopus oligosporus* .

These pure culture isolates were collected from dept of microbiology RARS Tirupati. Silver nanoparticles were mixed with fungal suspension and bacterial suspensions to make a final volume of 1 ml also added deionized water used as a control.

A 10 µl subsample of the conidia and *A. indica* silver nanoparticles were taken at 50 ± 0.9, 100 ± 1.1 and 170 ± 1.4ppm after silver treatments and diluted 100-fold with the deionized water. Later spreading of 10 µl aliquot of the diluted spore suspension on PDA (Becton, Dickson and Company, Sparks, MD) medium.

For each combination used three plates and experiment conducted thrice. Filter paper discs were dipped in different concentrations and inserted on mediums (PDA). Later the plates were incubated at 37°C for 24 hrs. After 24 hrs of incubation zone of inhibition were calculated and compared with water control, zone of inhibition was determined[11].

Brine Shrimp Lethality Assay (BSLA)

Bioassay was performed with slight modifications of Lilybeth[12]. Brine shrimp eggs were purchased and artificial seawater was prepared (dissolving 38 g of sea salt in 1 liter of distilled water) for hatching eggs. Water was kept in a small plastic container (hatching chamber) with a separation for dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber while the lamp above the other side (light) will attract the hatched shrimp. After two days eggs were matured as larva (nauplii).

In each test tube add 4mL of artificial seawater and 10 brine shrimps were introduced

For each dilution triplicate should be done. 1 ml of each concentration of extract added finally volume was adjusted with artificial seawater up to 10mL per test tube. Different concentrations of 200ppm, 170ppm, 85ppm and 42.5 ppm of AgNPs of sample solution were used in this assay. After 24 hours counted the live shrimps. By probit analysis, the lethality concentration (LC_{50}) was assessed at 95% confidence intervals. LC_{50} of less than 100 ppm was considered as potent (active)[13]. LC_{50} value of less than 1000 µg/mL is toxic while LC_{50} value of greater than 1000 µg/mL is non-toxic according to Meyer[14].

The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

Statistical Analysis

For the antimicrobial analysis all of the data from three independent replicate trials were subjected to analysis using Statistical package for the Social Sciences (SPSS) version 16.0. The level of significance was set at $p<0.05$. The data obtained from brine shrimp assay was subjected to probit analysis to calculate lethal concentration (LC_{50} and LC_{99}) for respective compounds using Statistical Package for Social Sciences (SPSS 19.0 V IBM, Illinois, Chicago). The lethal concentrations were expressed by 95% fiducial limits. The level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

By addition of aqueous neem leaf extract to AgNO3 solution, the colour has been changed from colorless to brown. The change in color was noticed and is due to the localized surface Plasmon resonance phenomenon of the formed Ag NPs. The metal nanoparticles have free electrons, which give the Plasmon resonance peak absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave16.

UV–Vis spectroscopy

The UV-Vis spectroscopy could be used to examine the size and shape-controlled nanoparticles in aqueous suspensions[15]. The absorption spectrum was recorded for the sample in the range of 200-800 nm and the maximum absorbance was recorded at 405 nm. (Fig.1). The results reveal that the reduction of Ag+ into Ag NPs during the exposure to plant extracts was observed. Earlier research findings also explained that, the Ag NPs showed a surface Plasmon resonance peak in a range of 400-450 nm [21]. Based on the characteristic Ag NPs surface Plasmon resonance range, it was confirmed that, *Azadirachta indica* has huge potential to reduce Ag+ into Ag NPs. The present observation (405 nm) contrast to some of the previous results[8,18] who noticed a Plasmon peak at 351 nm and 370 nm, respectively. Factors such as concentration and combination of plant extract, pH, incubation temperature, reaction time and electrochemical properties of metal ion are influenced in this lower absorbance. The variation in the values of absorbance confirms the changes in the particle size[19].

FT-IR analysis

FT-IR spectrum was used identification of the functional groups which are responsible for the reduction and stabilization of silver nanoparticles.

FT-IR spectra peaks of neem leaf extract mediated silver nanoparticles were recorded as 580.90, 622.83, 1050.20, 1252.08, 1394.30, 1636.89, 1772.82, 2107.52, 2821.94, 2885.18, 2989.34 and 3,361.38 cm-1. (Fig.2). The peak at 3,361.38 reveals the presence of O–H stretch, indicating the alcohols and phenols. 2989.34 bend and 2,885.18cm-1 reveals the presence of C-H bend, indicating the alkenes. The band present at 2821.94 reveals the presence of H-C=O, C-H stretch indicates the aldehydes. 2107.52 shows the –C≡C stretch indicates the alkynes. 1772.82 shows the C=O stretch reveals the carbonyls. 1636.89 band peak shows the N-H stretch indicating the primary amine groups of protein. The band present at 1394.30 shows

the C–C stretching, likewise the bands at 1,252.08 shows the C-N stretch reveals the aromatic amine groups.1050.20 shows the C-N stretch reveals the aliphatic amines. Band at 622.83 indicates alkynes with C-H bend and band at 580.90 indicates the alkyl halides with C-Br stretch.

The FT-IR analysis the green synthesized Ag NPs and confirmed that, the bio-reduction of Ag+ to Ag NPs. This is due to the reduction of capping material of plant extract. In the present study, the FT-IR analysis also showed the involvement of amines, phenols, alcohols, alkanes, alkenes, aromatics, aldehydes, alkyl halides, and carbonyls in the synthesis of Ag NPs which is similar to other reports[17]. Further, the FT-IR studies confirmed that, the alcoholic and phenolic groups of the amino acid residues and proteins has the strong ability to bind the metals indicating that, the proteins could play a vital role in capping of Ag NPs and preventing agglomeration and thereby stabilizes the nanoparticles. This strongly suggests that, the biological molecules could possibly perform the dual functions of formation and stabilization of Ag NPs. The presence of reducing sugars in solution could be responsible for the reduction and the formation of nanoparticles. These issues can be addressed once the various fractions of neem leaf extracts are separated, identified and assayed individually for the reduction of metal ions[20].

XRD analysis

The X-RD pattern of neem mediated Ag NPs showed the peaks corresponding to the whole spectra of 2θ values ranging from 10-80. The peaks at 2θ values corresponding to the Bragg's reflections of planes (111), (200), (220), (311) and (222) which confirrms the FCC crystalline structure of silver. The relatively higher intensity of planes (111, 222) in FCC crystalline structure supports the stability of the green synthesized Ag NPs. The lattice constant calculated from this pattern was 4.0860 A°, a value in agreement with the published literature value of 4.0855 A° (JSPCDS file No. 893722). This clearly indicates that, the Ag NPs formed by the reduction of Ag+ by the neem extract are crystalline in nature. The calculated crystalline size of the neem mediated Ag NPs was 50 nm (Fig. 3).

The X-RD pattern of neem leaf extract findings recorded in the present work was in corroborating with the previous observation[21]. The sharp Bragg's reflections might be due to the crystalline nanoparticles and the intense peaks suggest that, the strong X-ray scattering centers in the crystalline phase. Independent crystallization of the capping agents was ruled out due to the process of centrifugation and re-dispersion of the pellet in Millipore water after nanoparticles formation as a part of the purification process[16].

Dynamic light scattering analysis

Particle size and zeta potential values were measured using Nanopartica SZ-100 (HORBIA). The particle size distribution spectra for the silver nanoparticles were recorded as diameter (nm) versus frequency (%/nm) spectra with diameter (nm) on x-axis and frequency (%/nm) on y-axis. The zeta potential spectra for the silver nanoparticles were recorded zeta potential verses intensity spectra with zeta potential (mV) on x-axis and intensity (a.u) on y-axis. The HDD of the neem mediated AgNPs it was 12.4 nm (Fig. 4a). The recorded value of zeta potential was -34.3 mV for neem mediated (Fig. 4b).

The size measurements using the DLS technique for neem aqueous extract and other synthesized AgNPs were comparable to other findings[8,21,26]. The size measurements and zeta potentials of AgNPs indicates the good stability of the synthesized AgNPs.

TEM analysis

The transmission electron microscopy (TEM) technique was used to visualize the particles and to study the surface morphology, size and shape. From the TEM images, (Fig. 5) it is evident that AgNPs were spherical in shape and were poly-dispersed. The measured average size of AgNPs was 5-50nm. Occasional agglomeration of the AgNPs has been observed. From the TEM images, it is evident that, most of the neem mediated

AgNPs were spherical or pencil head in shape. In other studies, the shape was recorded as spherical[7,17,21,22] or pentagons[20], however the particle size ranges were within the limits. The variations in shape may be due to the variations in concentration, pH of the reaction mixture, incubation temperature, reaction time, concentration and electrochemical potential of a metal ion[19].

Antimicrobial efficacy of neem extract mediated silver nanoparticles

Antimicrobial efficacy of green synthesized Silver nanoparticles were observed in (Plates 1 and 2). These pure culture isolates were collected from dept of microbiology RARS Tirupati. Three concentrations of AgNPs (170, 100, 50 ppm) were prepared and examined against different fungal and bacterial species. Highest antimicrobial efficacy observed at higher concentration at 170 ppm. (Tables 1 and 2).

Antimicrobial efficacy of silver nanoparticles due to the loss of replication ability of microbial DNA and also in activation of proteins and enzymes which are essential to ATP synthesis[16].

When compared to bulk materials highest efficacy observed in nanoparticles because nanoparticles having the large surface area to volume ratio and the surface activity[23-25]. Also nanoparticles are more penetration power compared to bulk material it causes the much mechanical cell membrane damage and improved the fungal efficacy[26].

For the clear understanding of mechanism of silver nanoparticles more detailed therapeutic investigations and toxicological assessment are required.

Brine shrimp Assay

This assay has been noted as a useful tool for the isolation of bioactive compounds from plant extracts[12] Brine shrimp (*Artemia salina*) lethality bioassay was carried out to check the cytotoxic activity of the plant extract and synthesized silver nanoparticles. The extract of *A. indica* and AgNPs were evaluated for brine shrimp lethality in different concentrations the assay was done with some modifications[12]. The possible toxic effects of exposure to different extracts and synthesized Ag NPs using the aqueous leaf extract of *A. indica* were tested against brine shrimps. As mentioned by Meyer[14] LC_{50} value of less than 1000 µg/mL is toxic while LC_{50} value of greater than 1000 µg/mL is non-toxic.

All experiments were done in triplicate and the mean result was noted. The lethal concentration LC_{50} of the test samples after 24 hrs was obtained by a plot of percentage of the dead nauplii against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis, Brine shrimp lethality activity of the plant extracts of neem and AgNPs were shown in (Table. 3). For AgNPs LC_{50} and LC_{99} values were 331.08 and 2796.63 ppm respectively. Crude neem extracts resulting in LC_{50} and LC_{99}values were 695.68 and 6949.34 ppm respectively.

The result on the lethality of *Azadirachta indica* on brine shrimps is lower than the previous studies[28] where its LC_{50} values are 1000 ppm. The result on the lethality of *Azadirachta indica* on brine shrimps is differ to the previous studies[29] where LC_{50} value of the leaf extract was determined and it was 37.15 mg/ml here it may be due to the methanolic extract have more potent than the crude extract. For a comparison of cytotoxic activity of neem mediated silver nanoparticles these observations, no published reports were available.

These green synthesized silver nanoparticles from crude extract of *Bergenia ciliata* nanoparticles showed the cytotoxic effects against brine shrimp (Artemia salina) nauplii with a value of 33.92 mg/ml LD_{50} [29]. It is concluded from our results that green synthesis of AgNPs was carried successfully by using *A. indica* extract. Incorporation of active phyto constituents such as flavonoid compounds is added advantage of synthesized nanoparticles. Further, these nanoparticles were evaluated for their activities and showed antimicrobial and cytotoxic activities compared to extract. The significances of this study demonstrate a broad range of applications of synthesized nanoparticles.

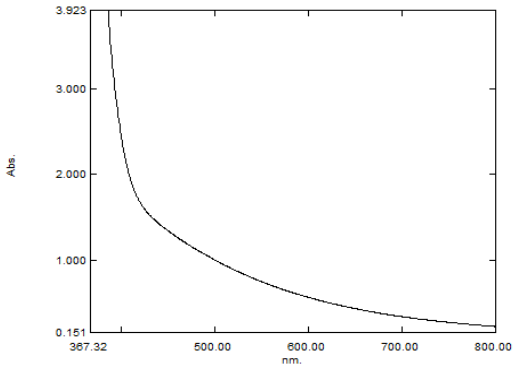


Fig. 1: UV–Visible absorbance spectra of neem mediated silver nanoparticles

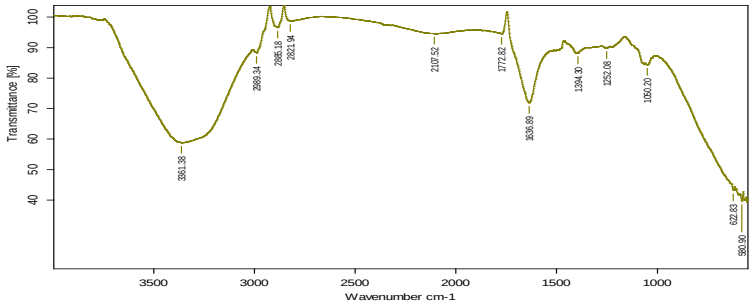


Fig. 2: FT-IR spectra indicates the functional groups in the neem mediated silver nanoparticles

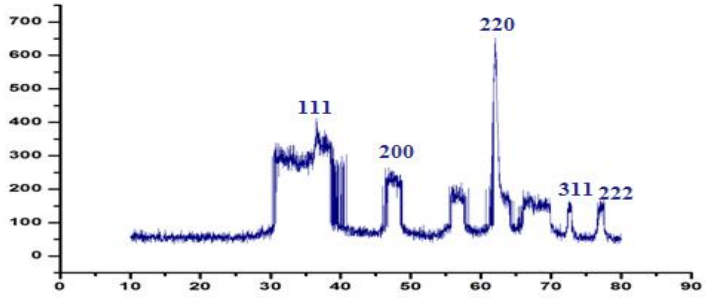
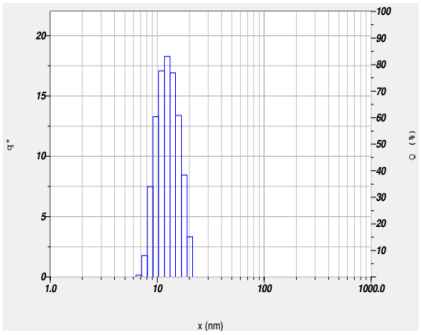
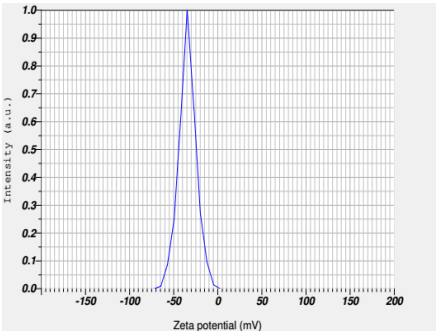


Fig. 3: X-ray diffraction analysis of the neem mediated silver nanoparticles





Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	12.9 nm	3.0 nm	12.4 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	12.9 nm	3.0 nm	12.4 nm

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-34.3 mV	-0.000266 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : -34.3 mV

Fig. 4: Dynamic light scattering analysis of neem mediated silver nanoparticles Size (a) and its zeta potential (b)

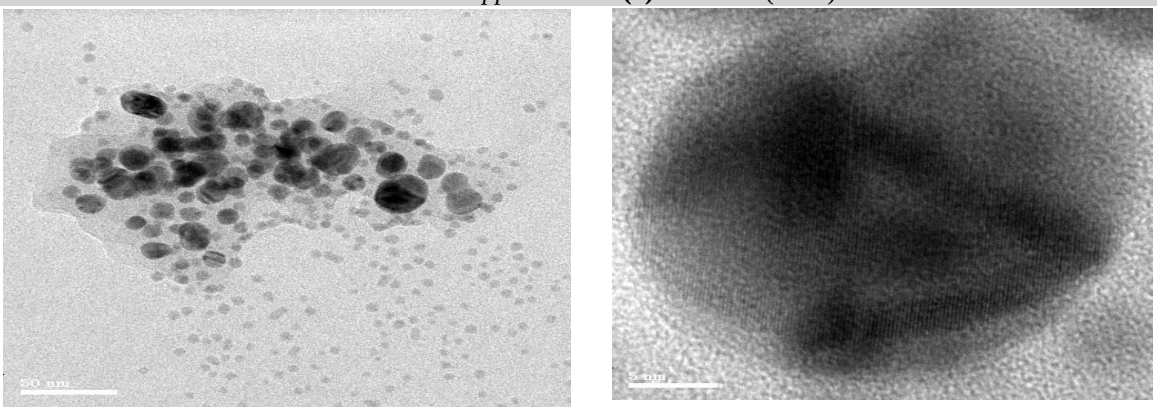
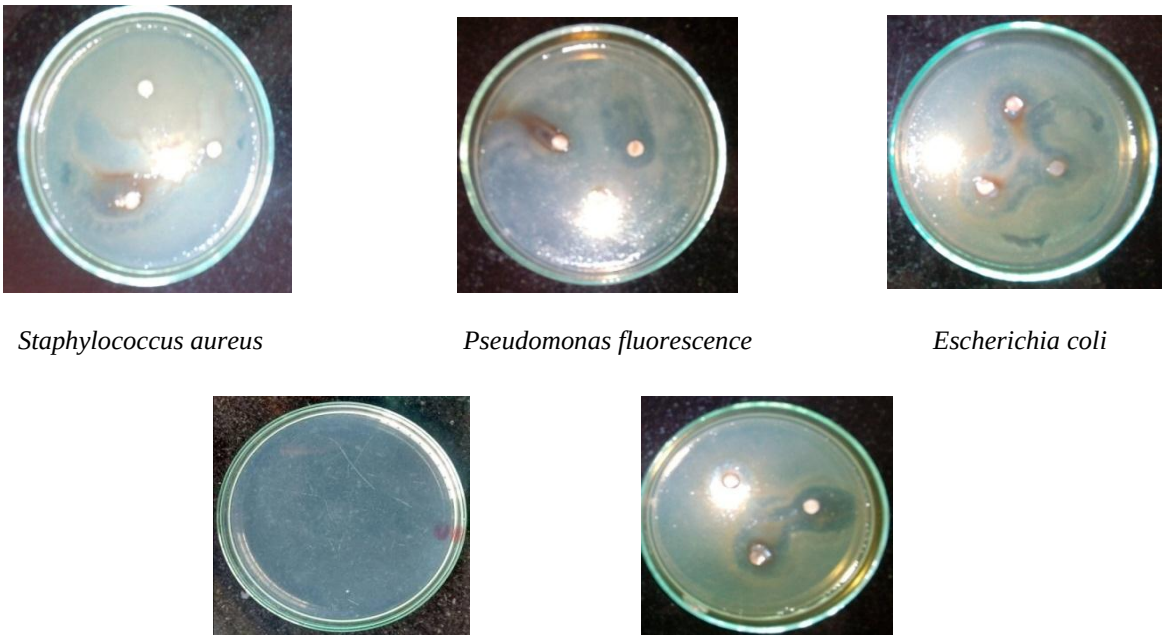


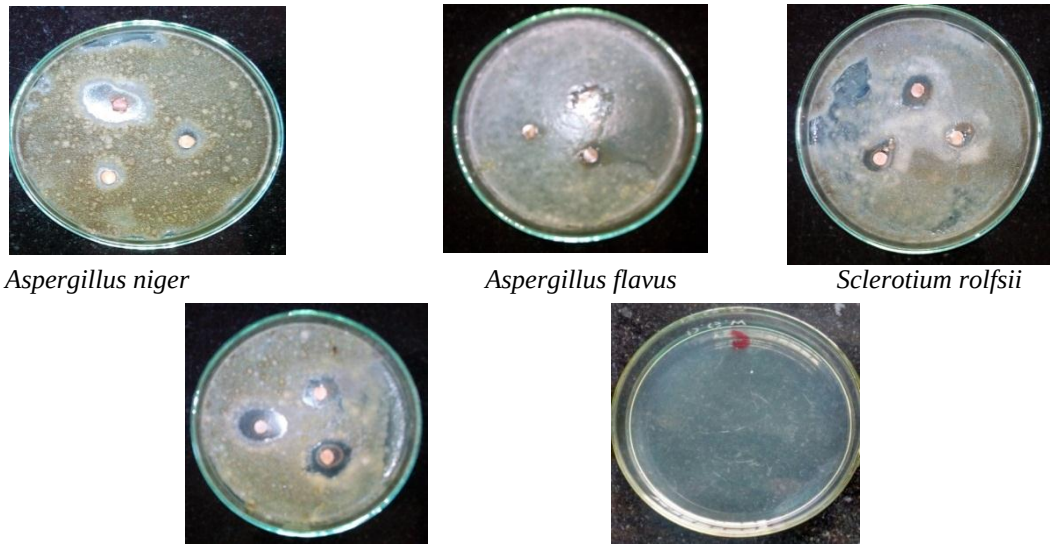
Fig. 5: TEM images of neem mediated silver nanoparticles



Staphylococcus aureus *Pseudomonas fluorescence* *Escherichia coli*

Sphingobacterium thalpophilum Control

Plate 1: *In-vitro* antibacterial activity of *Azadirachta. indica* mediated silver nanoparticles



Aspergillus niger *Aspergillus flavus* *Sclerotium rolfsii*

Rhizopus oligosporus Control

Plate 2: *In-vitro* Antifungal activity of different concentrations of *Azadirachta indica* leaf extract mediated silver nanoparticles

Table 1: *In-vitro* antibacterial studies of *Azadirachta indica* mediated silver nanoparticles (different concentrations)

Bacteria	AgNPs zone of inhibition (mm)		
	170 ± 1.4ppm	100 ± 1.1ppm	50 ±0.9ppm
Staphylococcus aureus	1.7 ±0.79	0.8 ± 0.92	0.3 ± 0.40
Pseudomonas fluorescence	2.0 ± 0.21	1.7± 0.16	0.8 ± 0.38
Escherichia coli	3.0 ± 0.42	2.5± 0.20	2.0± 0.20
Sphingobacterium thalpohilum	2.7 ± 0.20	2.2 ±0.27	1.1± 0.48

Table 2: *In-vitro* anti fungal studies of *Azadirachta indica* mediated silver nanoparticles (different concentrations)

Fungi	AgNPs Zone of inhibition (mm)		
	170 ± 1.4ppm	100 ± 1.1ppm	50 ± 0.9 ppm
Aspergillus niger	1.7± 0.73	1.5 ± 0.45	1.2 ± 0.29
Aspergillus flavus	2.1 ±0.49	1.9 ± 0.08	1.2 ±0.33
Sclerotium rolfsii	2.1 ±0.31	1.8 ± 0.24	1.6 ±0.17
Rhizopus oligosporus	1.9± 1.37	1.6± 1.05	1.4± 1.04

Table 3: Lethal concentrations of phytogenic silver nanoparticles and neem extract in Brine Shrimp Assay

S.No.	Compound	LC_{50}	LC_{75}	LC_{90}	LC_{99}	Coefficient	Intercept
1.	CNE AGNP	331.08 (282.94 – 414.63)	614.64 (477.96 – 890.67)	1072.61 (762.79 – 1780.30)	2796.63 (1700.28 – 5880.89)	2.510	-6.326
2	Neem extract	695.68 (554.26 – 1021.12)	1355.86 (943.48 – 2563.12)	2471.96 (1513.24– 5905.44)	6949.34 (3397.6 – 24943.62)	2.327	-5.915

Values are estimate of lethal concentration (ppm) with 95% Fiducial limits

Probit regression analysis with Log_{10} transformation of dose using Statistical Package for Social Sciences (IBM SPSS 19.0V)

Estimates with different super scripts are significantly different based on absence of overlapping of fiducial limits ($P<0.05$)

CONCLUSION

In conclusion, flavanon and terpinoid constituents of neem leaf broth are the surface active molecules which stabilize the Ag NPs. The X-RD results suggest that, crystallization of the bio-organic phase occurs on the surface of the Ag NPs or vice- versa. As observed from FT-IR studies and X-RD analysis, bio-organic components from neem leaf broth acted as a probable stabilizer for the Ag NPs. Whereas the TEM results shown partial agglomeration of the nanoparticles. Stability of cluster distribution was enhanced decreasing tendency for aggregation of the particles. Green synthesized silver nanoparticles has the potent antibacterial and anti fungal activity. Less toxicity on brine shrimps were noted after exposure to aqueous extracts and synthesized Ag NPs. In future studies, the green synthesized Ag NPs in the present study are to be evaluated in-vivo for their antimicrobial activity

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