

# **Biosynthesis of Silver Nanoparticles Using Aqueous *Azadirachta Indica* (Neem) Flower Extract-Optimization, Characterization And Study of Antimicrobial and Anti-Oxidant Effects**

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**ABSTRACT:** Nanotechnology has recently attracted various branches of applications due to the dissimilar behavior of the bulk material when reduced to its nano-size. The use of plant extracts for the synthesis of nanoparticles plays a significant role in biomedical applications in nanotechnology field as it is simple, fast, environment friendly and no use of toxic chemicals. The present research work investigates the optimization of different experimental variables and conditions like reaction pH, silver nitrate concentration, time, temperature and mixing ratio of the reactants on the green synthesis of silver nanoparticles using aqueous extract of *Azadirachta indica* (Neem) flower. These biosynthesized nanoparticles were characterized by using UV-Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and High Resolution Tunneling Electron microscopy (HRTEM), which proved the spherical shape and average size of the particle to be 20 nm. The results showed that the flower extract of *Azadirachta indica* is a very good bioreductant for the synthesis of AgNPs. These silver nanoparticles were found to exhibit antibacterial activity against two human bacterial pathogens such as gram positive (*Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli*). Anti-oxidant behavior of these silver nanoparticles against DPPH is also observed. Green synthesis of AgNPs with effective antioxidant and antibacterial activity may help in the development of cost effective therapeutic agents.

**KEYWORDS:** Silver Nanoparticles, Neem flower extract, antimicrobial, antioxidant.

## **I. INTRODUCTION**

In recent years, noble metal nanoparticles are widely studied and various approaches have been employed for the synthesis of metal nanoparticles [1-6]. Out of all the noble metal nanoparticles, silver nanoparticles have received greater focus [7-13] for their advantage on significant studies such as photosensitive components [14], catalysts [15-17], surface-enhanced Raman spectroscopy [18-19], healthcare and biomedical applications [20-22], sensors [23], spectroscopy [24] and catalysis [25]. The green synthesis of nanoparticles is more advantageous over the conventional chemical and physical methods like thermal decomposition of silver compound [26], microwave assisted process [27], electrochemical method [28] as it is cost effective, does not involve the usage of hazardous chemicals and so environment friendly [29-31]. In the past few years, noble metal nanoparticles have been in the focus of research due

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to their unique optical, electronic, mechanical, magnetic and chemical properties that are importantly different from those of bulk materials. Although biosynthesis of silver nanoparticles by plants viz. papaya fruit [32], *Jatropacurcus* latex [33], *lantana camera* fruit [34], *Coriandrumsativum* [35], Cauliflower [36], *Portulacaoleracea* [37] and tea extract[38] have been reported, the efficacy of the plant as biological material for the synthesis of nanoparticles is yet to be fully explored. Silver nanoparticles are reported to possess anti- fungal[39], anti- inflammatory[40], anti-viral[41], anti-angiogenesis [41] and antiplatelet activities[42]

Neem (*Azadirachta indica*) tree belongs to the family of *Meliaceae*, is a large ever green tree growing up to 60 ft high in the dry tropical forests of India, Sri Lanka, Burma, Pakistan, tropical Australia and Africa. Neem twigs are used as tooth brushes in India from ancient times. Neem flowers are white in colour, generally 5 mm long and have a peculiar fragrance and produce nectar. Flowers are used in pharmaceutical, food and cosmetic industries. The effect of extracts of flowers of the neem tree on the levels of cytochrome P450, aniline hydroxylase (ANH) and aminopyrine-N-demethylase (AMD) as well as its capacity to activate the mutagenicity against aflatoxin B1 (AFB1) and benzo[a]pyrene (BaP), and to induce glutathione S-transferase (GST) in rat liver were assessed. It was found that feeding of the diets containing 12.5% neem flowers for 2 weeks strongly enhanced GST activity and extracts of neem flowers possessed chemo-preventive potential [43]. The neem flower powder could be a good source of lipid with polyunsaturated fatty acids such as oleic, linoleic, linolenic and few unconventional fatty acids. The volatile oil has characteristic flavour from caryophyllene [44]



**A** **B**  
**Fig.1: Azadirachta indica (Neem) flower- fresh (A) and dried sample(B)**

Neem flower extract was chosen for the present study, because of the aforesaid properties and since it has synergistic effects to amplify the antimicrobial properties of the synthesized silver nanoparticles. Here, the synthesis of silver nanoparticles is done by using aqueous flower extract of neem (*Azadirachta indica*). Furthermore biosynthesis of these nanoparticles were optimized by changing different factors like temperature, pH, silver nitrate concentration, leaf extract volume and reaction time, studied by UV-Vis spectroscopy and were characterized by, FT-IR, XRD and TEM. Further, these biologically synthesized nanoparticles were found to be toxic against multidrug resistant human pathogens. The investigation is also focused for the first time to outline the potential use of neem flower extract as reducing, stabilising and capping agent in the reaction.

## II. MATERIALS AND METHODS

All chemicals were of high level purity and obtained from Sigma Chemicals (Bangalore, India). The raw material *Azadirachta indica* (Neem) flower was collected from campus of Ramakrishna Mission Vivekananda College, Mylapore, Chennai, Tamil Nadu, India. Nutrient Agar medium was purchased from HiMedia, Mumbai, India. *Staphylococcus aureus*, *Escherichia coli* was isolated from soil for antibacterial assay.

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**2.1 Preparation of Azadirachta indica (Neem) flower extract:** The Azadirachta indica (Neem) flowers were washed, dried under sun shade for seven days and crushed to effectively extract the biomolecules. 2.5 g of the crushed powder is accurately weighed and transferred to a 250 ml Erlenmeyer flask with 100ml de-ionized water. After completely homogenizing the mixture, the whole content was heated on a hot water bath for 1 hour at 80°C. The solution was cooled and filtered first through a muslin cloth and then using Whatmann No.1 filter paper to obtain the aqueous extract filtrate. The filtrate is used immediately for the biosynthesis of AgNPs..

**2.2 Synthesis of silver nanoparticles:** The biosynthesis of silver nanoparticles was optimized by varying the concentration of AgNO<sub>3</sub> solution, contact time, volume of the extract, pH and temperature. The volume of AgNO<sub>3</sub> solution was fixed at 7ml. To this solution, at optimum pH and temperature, when optimum volume of the extract was added and allowed to react till, silver nanoparticles were synthesized. The reaction mixture was maintained in sun light till the mixture colour changed from golden yellow colour to dark yellowish brown colour. The change in colour of the mixture indicated the reduction of Ag<sup>+</sup> ions to silver nanoparticles (AgNPs). The silver nanoparticles were separated by lyophilisation. The absorbance of the resulting solutions were spectrophotometrically measured.

### 2.3 Fixation of parameters for Biosynthesis of Silver Nanoparticles

**2.3.1. Concentration of silver nitrate solution:** The above mentioned approach was repeated for optimization of silver nitrate concentration, where the reaction was monitored using different concentrations of silver nitrate (0.1, 0.2, 0.5, 1, 2 and 4.4 mM). The absorbance of the resulting solutions was measured spectrophotometrically at the end.

**2.3.2 Volume of the plant extract:** The procedure was carried out at different volumes of the neem flower extract ranging from 1ml to 10ml and the UV-Vis absorbance measurements were recorded.

**2.3.3. Temperature:** The above mentioned protocol was repeated for optimization of temperature, where the reaction mixture was maintained at room temperature (32°C), 50°C and 60°C, respectively, using water bath. The absorbance of the resulting solutions was measured spectrophotometrically

**2.3.4. pH:** The above described protocol was repeated for optimization of pH where the reaction pH was maintained at 6, 7, 8, 9 and 10 respectively. The pH was adjusted by using 0.1 N HCl solution and 0.1 N NaOH solution. The absorbance of the resulting solutions was measured using UV-Vis spectrophotometry

## III. CHARACTERIZATION

**3.1 UV-visible spectroscopic studies:** The biosynthesized AgNPs were characterized by UV-visible spectroscopy. The reduction of Ag<sup>+</sup> ions to AgNPs ions was confirmed by measuring the spectrum of the AgNPs sample after proper dilution using Elico 2010 Double Beam spectrophotometer operated within the range of 300 – 700nm.

**3.2 FT-IR spectroscopic studies:** The biosynthesized AgNPs were mixed with KBr to make pellet and the FT-IR spectrum was recorded using PerkinElmer FT-IR spectrometer. FTIR spectroscopy analysis was carried out to identify the biomolecules responsible for the reduction of Ag<sup>+</sup> ions and capping of the reduced silver nanoparticles synthesized by using plant extract in the region 1000–7000cm<sup>-1</sup>.

**3.3 X-Ray Diffraction (XRD):** XRD studies of neem flower reduced nanoparticles was measured by uniformly coating the dried AgNPs on XRD grid and the recording the spectrum by using Philips PW 1830 X-Ray generator operated at a voltage of 40 kV and a current of 30 mA with Cu K<sub>α</sub> 1 radiation.

**3.4 HR – TEM Analysis:** A drop of biosynthesized AgNPs was placed on copper grid and vacuum evaporated to dry it. After drying the AgNPs nanoparticles were photographed using Philips CM20 Transmission Electron Microscope equipped with selected area electron diffraction pattern (SAED).

**3.5 Antimicrobial activity of silver (Ag) nanoparticles :** The silver nanoparticles synthesized from Azadirachta indica were tested for their antimicrobial activity by well diffusion method against pathogenic organisms like bacteria gram positive (Staphylococcus aureus) and gram negative bacteria (Escherichia coli), Nutrient agar (NA) was prepared and poured in the sterile Petri dishes and allowed to solidify. 24 h growing bacterial and fungal cultures, Staphylococcus aureus and Escherichia coli was swabbed on NA plates. Then, 5 wells (8mm diameter) were made by using a sterile cork borer. The different concentrations (250 µg, 500 µg, 750 µg and 1000 µg) of the test sample were

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loaded in the wells. Tetracyclin was used as the standard antibiotic. The plates were then incubated at 37°C for 24-48h. After incubation the inhibition diameter was measured.

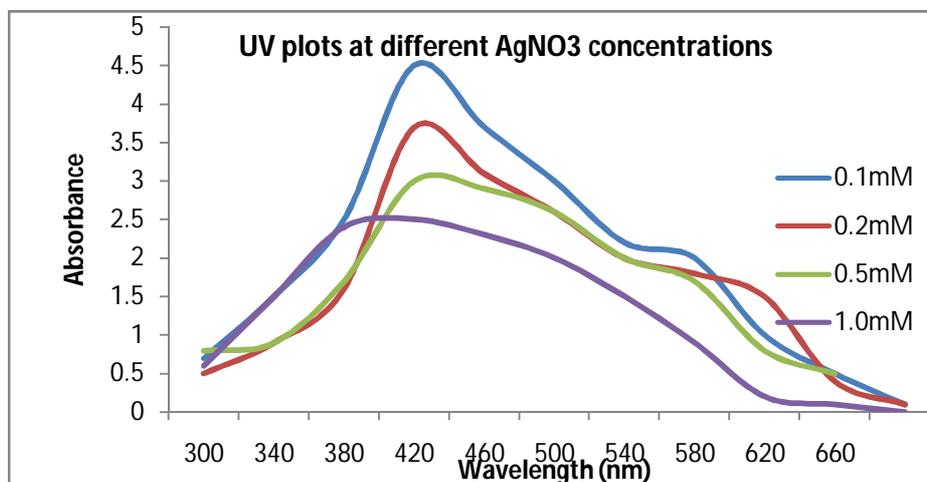
**3.6 Antioxidant activity assays of silver (Ag) nanoparticles :** The silver nanoparticles synthesized from neem flowers were tested for their radical scavenging activity by using DPPH(2, 2-diphenyl-1-picrylhydrazyl) assay. The decrease in the absorption at 517nm of the DPPH solution after the addition of the antioxidant was measured in a cuvette containing 2.960µl of 0.1mM ethanolic DPPH solution mixed with 20 to 100µg/ml of sample and vortexed thoroughly. The setup was left at dark at ambient (room) temperature and the absorption was monitored after 20min. BHT (Butylated hydroxytoluene) was used as standard control. The ability of the sample extract to scavenge DPPH radical was calculated by the following equation:

$$\% \text{ of DPPH Radical Scavenging Activity (\% RSA)} = \frac{\text{Abs.control} - \text{Abs.sample} \times 100}{\text{Abs.control}}$$

### IV. RESULT AND DISCUSSION

#### 4.1 Optimization

**4.1.1 Optimization of silver nitrate concentration:**Effect of AgNO<sub>3</sub> concentration was studied by varying the concentration of AgNO<sub>3</sub> solution from 0.1 mM to 1mM.Maximum absorbance was recorded at 420nm for 0.1mM AgNO<sub>3</sub> concentration. At higher concentrations either a peak shift or decrease in absorbance was observed.



**Fig .2: UV spectra-optimization of AgNO<sub>3</sub> concentration**

**4.1.2 Optimization of volume of the plant extract:**The reducing ability of neem flower extract has been discussed in the literature [40]. Optimum extract volume was chosen by varying the volume of the extract from 5ml to 15 ml. The UV-Vis spectra for the same are shown in fig.3. It was observed that 9ml plant extract showed maximum absorbance.It was reported in literature [45] that optimum extract concentration is necessary for symmetrical preparation of nano particles. Lesser and greater volumes of extract showed lower absorbance indicating incomplete synthesis of silver nano particles.

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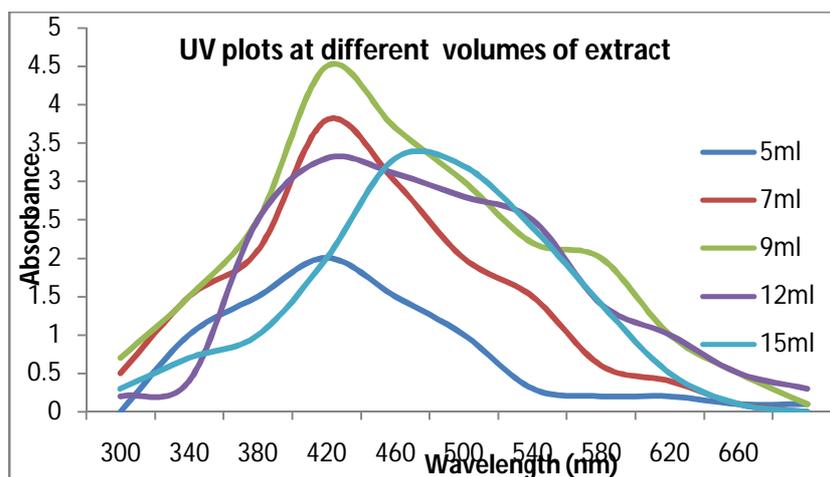
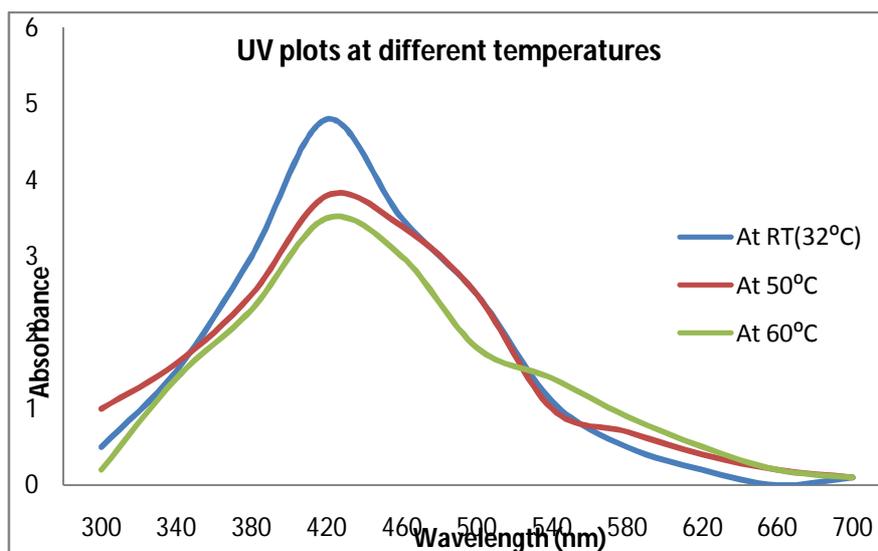


Fig .3: UV spectra-optimization of neem flower extract volume

**4.1.3 Optimization of temperature and pH:** Temperature of the reaction mixture and pH influences the synthesis of AgNPs significantly. Reaction temperature plays a major role in the nucleation process of nano particle configuration. Studies show that low pH (acidic) decreases the synthesis of AgNPs and results in larger sized AgNPs, while high pH (basic) increases the formation of AgNPs and results in the formation of highly dispersed and small nanoparticles. In the present study the synthesis of AgNPs was carried out at room temperature (32°C), 50°C and 60°C and varying the pH from 6 to 11. The pH was adjusted using 0.1N NaOH and 0.1N HCl. Fig.4 shows the UV-Vis spectra for these variations. It was found that the absorbance was maximum at room temperature and at pH 10.



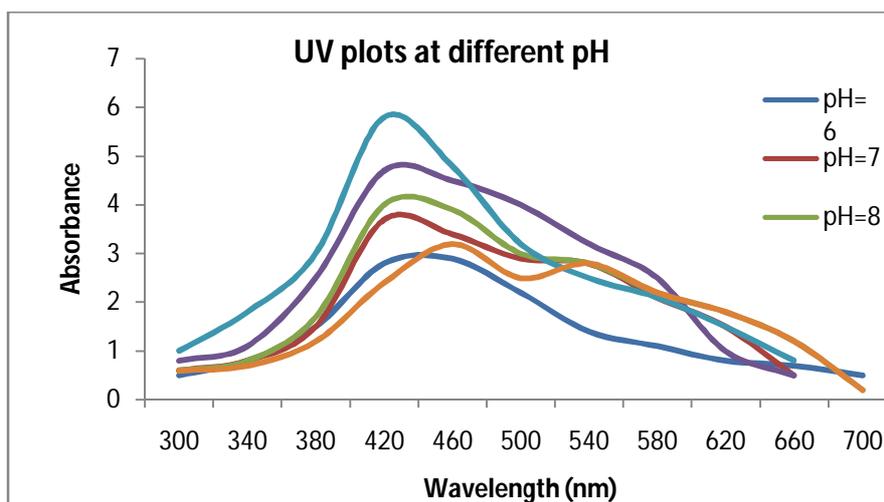


Fig.4: UV spectra-optimization of pH and temperature



Fig. 5 Photo showing the formation of silver nano particles in yellowish brown colour and the neem flower extract in yellow colour

**4.2: FT-IR spectroscopic studies:** FTIR measurements were carried out to identify the biomolecules for capping and efficient stabilization of the metal nanoparticles synthesized. The FTIR spectrum of silver nanoparticles (Fig 6) showed the band between  $3522\text{ cm}^{-1}$  corresponds to O-H stretching H-bonded alcohols and phenols. The peak found around  $1500\text{ cm}^{-1}$  showed a stretch for C-H bond, peak around  $1450\text{-}1500\text{ cm}^{-1}$  showed the bond stretch for N-H. Where therefore the synthesized nanoparticles were surrounded by proteins and metabolites such as terpenoids possessing functional groups. From the FTIR spectral study we confirm that the carbonyl groups from the amino acid residues and proteins has the stronger ability to bind metal indicating that the proteins could possibly form the metal nanoparticles (i.e.; capping of silver nanoparticles) to prevent agglomeration and thereby stabilize the medium. This suggests that the biological molecules could possibly perform dual functions of formation and stabilization of silver nanoparticles in the aqueous medium. Carbonyl groups proved that flavanones or terpenoids adsorbed on the surface of metal nanoparticles. Flavanones or terpenoids might have adsorbed on the surface of metal nanoparticles, possibly by interaction through carbonyl functional groups or  $\pi$ -electrons in the absence of other strong ligating agents in sufficient concentration. It is also possible that the terpenoids play a role in reduction of metal ions by oxidation of aldehydic groups in the molecules to carboxylic acids. These issues can be addressed once the various fractions of the neem flower extract are separated, identified and individually assayed for reduction of the metal ions. This rather elaborate study is currently

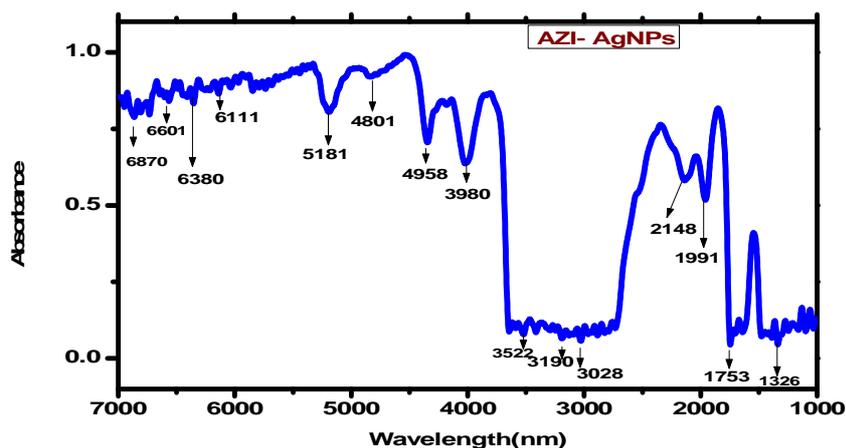


Fig.6: FT-IR spectrum of silver nanoparticles

#### 4.3: X-Ray Diffraction (XRD) study:

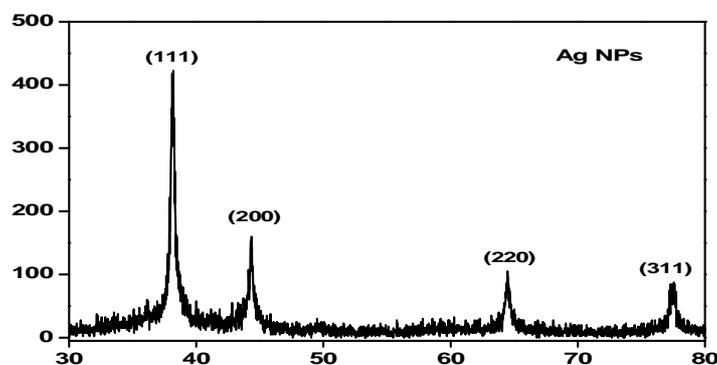


Fig.7: X-Ray Diffraction (XRD ) pattern: Intensity(Y axis) vs 2θ degrees(X axis)

The XRD pattern of silver nanoparticles( Fig.7) showed diffraction peaks at  $38.91^{\circ}$ ,  $44.27^{\circ}$ ,  $64.39^{\circ}$ , and  $77.54^{\circ}$ , can be assigned to face-centered cubic (FCC) silver. Can be observed together with a highly crystalline potassium chloride phase where as any peaks originating from potential silver oxides cannot be observed. These sharp Bragg peaks is an implication of the extract acting as a capping agent to stabilize the nanoparticle. Intense Bragg reflections suggest that strong X-ray scattering centres in the crystalline phase and could be due to capping agents. Therefore, XRD results suggested the crystallization of the bio-organic phase occurs on the surface of the silver nanoparticles or vice versa. Generally, the broadening of peaks in the XRD patterns of solids is attributed to particle size effects. Broader peaks signify smaller particle size and reflect the effects due to experimental conditions on the nucleation and growth of the crystal nuclei.

**4.4: HR – TEM Analysis:**TEM analysis observed is characteristic of almost predominantly spherical silver nanoparticles. The typical TEM images obtained is shown in Fig. 8. The size of nanoparticles ranges from 20 to 50 nm. The Spherical like morphology is observed. The ability of neem flower extract to modulate the shape of nanoparticles is observed in this study which opens up exciting possibility of developing further synthetic routes using biological

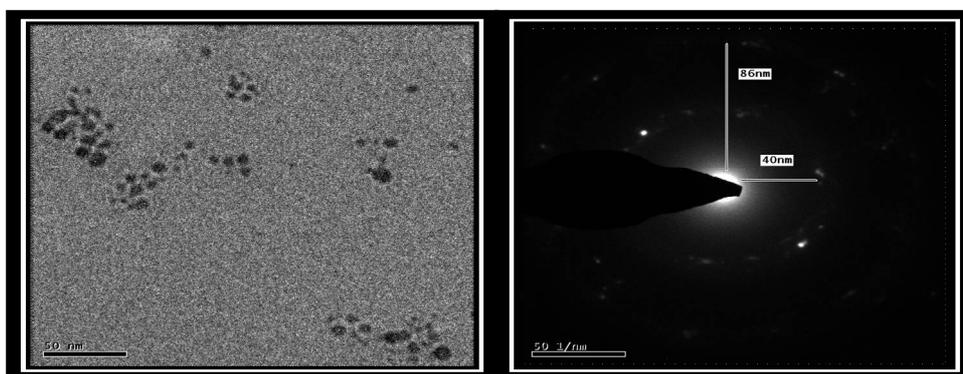
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sources. The inset of Figure 8 show selected area electron diffraction pattern (SAED) of AgNPs acquired to present in the clear rings confirms that the synthesized silver nanoparticles are highly crystalline nature is confirmed.

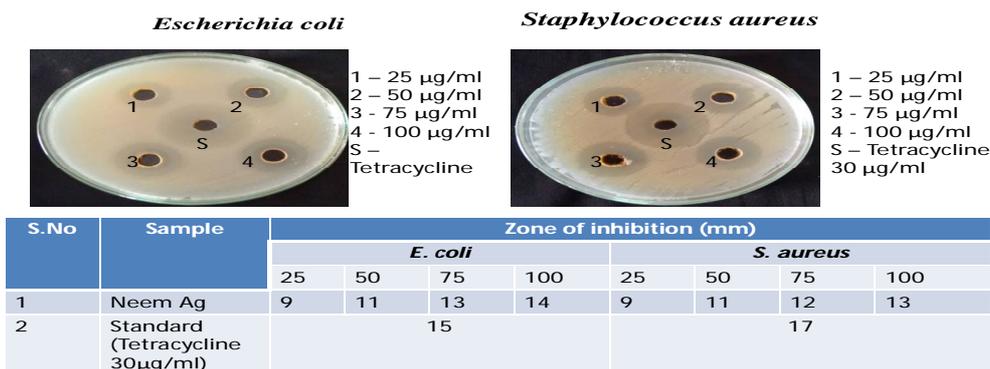
### HR – TEM with SAED pattern- Analysis



**Fig.8: HR – TEM with SAED pattern – Analysis**

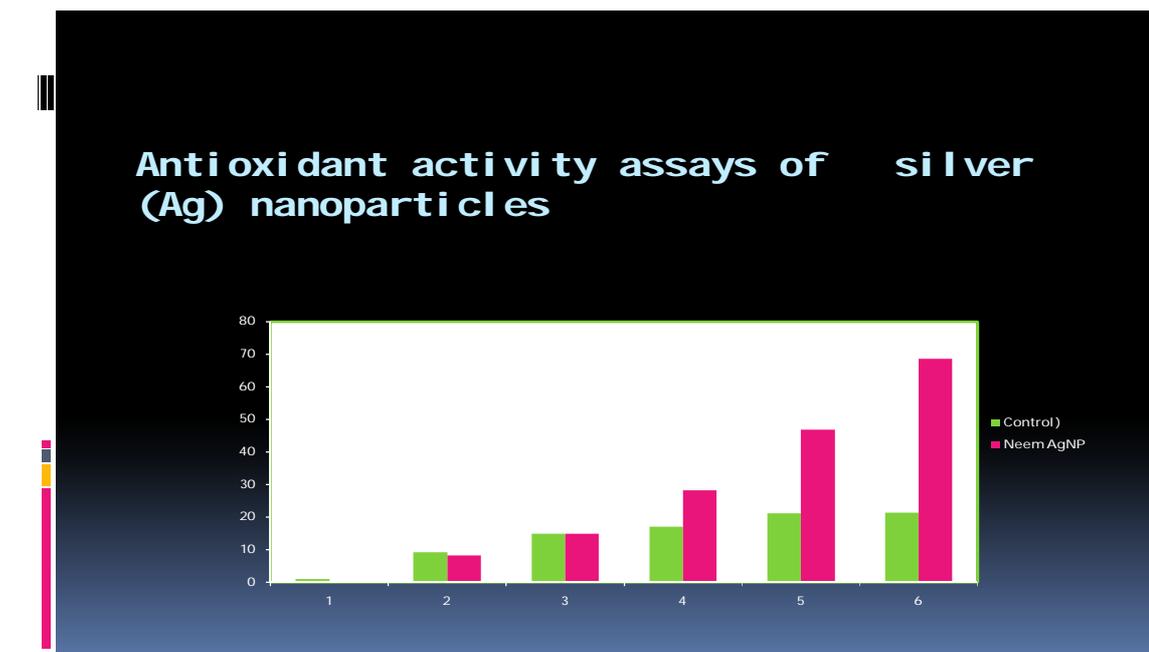
**4.5: Antimicrobial activity of silver (Ag) nanoparticles :** Biosynthesis of silver nanoparticles was studied for antimicrobial activity against pathogenic bacteria (clinical isolate) by using standard zone of inhibition (ZOI) microbiology assay with a well size of 14 mm, 13 mm, diameter and 25  $\mu$ l to 100  $\mu$ l concentrations were used as control for antimicrobial agent. The silver nanoparticles synthesized showed measurable antibacterial activity against the studied two bacterias. Maximum zone of inhibition was found to be 14 mm in Escherichia coli and minimum of 09 mm in Staphylococcus aureus bacteria as shown in fig. 9.

### Antimicrobial activity of silver (Ag) nanoparticles



**Fig.9: Antimicrobial activity of silver nanoparticles**

**4.6: Antioxidant activity assays of silver nanoparticles :** There has been a manifold interest in the study of antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body and to prevent the degradation of fats and other constituents in foodstuffs. In both cases, there is a preferential need for antioxidants from natural rather than from synthetic sources. Hence there is a steep and parallel increase in the use of methods for estimating the efficiency of such substances as antioxidants. The DPPH radical scavenging activity (Fig 10) of the aqueous extract of neem flower was relatively lower than that of synthesized AgNPs. The proton donating capacity of was found in the synthesized AgNPs. The absorbance decreased as a result of a color change from purple to yellow as radical was scavenged by antioxidants through donation of hydrogen to form the stable DPPH radical.



**Fig.10: Antioxidant activity assays of silver (Ag) nanoparticles**

#### IV. CONCLUSION

The development of reliable, cost effective and eco-friendly process for the synthesis of metallic nanoparticles is of great significance in the field of nanotechnology. In the present study, we have reported a simple, reproducible, reliable, environment friendly and cost efficient approach for the preparation of efficient and stable silver nanoparticles by using aqueous extract of the flowers *Azadirachta Indica* as the reducing, stabilizing and capping agent. The biosynthesized nanoparticles have been characterized by ultraviolet and visible spectroscopy, FT-IR spectroscopy, XRD and TEM. The AgNPs are crystalline in nature and the size of silver nanoparticles is in the range 20nm-50nm. The AgNPs have antibacterial activity and anti-oxidant effects. The biosynthesized silver nanoparticles proved to be potential candidates for medical applications where antioxidant and antimicrobial are highly essential. Hence the synthesized nanoparticles are more efficient in the drug delivery process. This neem flower extract can be extended to the synthesis of other metal and metal oxide nanoparticles which would have numerous applications.

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