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GREEN SYNTHESIS OF SILVER NANOPARTICLES FROM *AZADIRACHTA INDICA* AND *OCIMUM SANCTUM* AND THEIR ANTIMICROBIAL EFFICACY

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ABSTRACT

Nanoparticles are used immensely due to its small size and physical properties which are reportedly shown to change the performance of any other material which is in contact with these tiny particles. These particles can be prepared easily by different chemical, physical and biological approaches. But the biological method is the most efficient approach for the preparation of nanoparticles because it is eco - friendly and less time consuming. The leaves extracts of *Azadirachta indica* and *Ocimum sanctum* acts as a capping agent, reducing agents, and posses antibacterial activities and healing properties. In this study, simple and rapid approach was applied for the green synthesis of silver nanoparticles from *Azadirachta indica(neem)* and *Ocimum sanctum (Tulsi)* leaves extracts. The biosynthesized silver nanoparticles were characterized using UV – Visible Spectroscopy, XRD, and SEM. The size of biosynthesized silver nanoparticles was found to be within the range of 20-60 nm. To identify the compounds for the reduction of silver ions, the functional groups present in plant extract were investigated by FTIR. The nanoparticles were tested for their antibacterial potential against drug – resistant bacteria, *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii* and against pathogenic fungus, *Rhizopus oryzae, and Aspergillus Niger.* It was found that the biosynthesized nanoparticles had an inhibitory effect towards these microbes. The toxicity analysis of silver nanoparticles was determined by treating the Moong seeds (*V. radiata*) with different concentrations of silver nanoparticles. The results showed that 25% of silver nanoparticles solution had the better effect towards the seed germination and oxidative stress enzyme activity.

KEYWORDS: **Silver nanoparticles, *Azadirachta indica*, *Ocimum sanctum*, antibacterial, antifungal, *V. radiata***

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INTRODUCTION

In today's era of development in research, nanoparticles are the key component which is being used widely. As the name suggests these are particles having the dimension of nano size, which in other words referred as one billionth or 10^{-9}. These particles are measured in nanometres scale with the size range of 1 - 100nm. Nanoparticles are more effective because of its novel characterization and exceptional features and also the surface area to volume ratio of nanoparticles, which makes them more reactive than bigger molecules.[1] There are basically three ways to synthesize nanoparticles. The approaches include chemical, physical and biological method. The chemical method includes photochemical,[2] chemical reduction,[3] ionization radiation method, and classical chemical method in which reducing agents such as hydrazine, hydrogen etc. were used.[1] These processes produce a huge amount of nanoparticles within a short period of time. But, it produces hazardous byproducts which may have the deleterious effect on the nanoparticle synthesis and may be toxic to human health.[4] The reducing and capping agents are added externally for the synthesis of metal nanoparticles[1]. The physical method includes milling, pyrolysis, condensation, and evaporation.[5-8] These processes are energy consuming and also time-consuming as it takes a lot of time to achieve thermal stability.[1] The laser ablation method depends on duration and wavelength of the laser.[1, 9,10] Moreover, nanoparticles size and shape gets modified when the laser is passed.[11] In the biological method, plants and microbes are used for biosynthesis of nanoparticles. When microbes are employed for the synthesis of metal nanoparticles, the rate of synthesis is very slow when compared to the plant-mediated synthesis, whereas the plant extracts acts as reducing and capping agent.[12] The biological method is referred as greener synthesis of nanoparticles as it is cost effective, eco – friendly and nontoxic.[13] Medicinal plants such as *Azadirachta indica* and *Ocimum sanctum* are known to have a wide range of application in pharmaceutical industry. The presence of phytochemicals such as alkaloids, flavonoids, and tannins etc. make them more effective against pathogens.[14,15] These plants and trees are commonly available in India and each of the plants and tree has been used as a household remedy against various human ailments from antiquitiy and for treatment against viral, bacterial and fungal infections. These leaves extracts have been tested against endodontic pathogens.[16] Silver nanoparticles (AgNPs) have unique properties such as antimicrobial, optical, and catalytic property.[1,17-19] It causes cell death through apoptosis and DNA fragmentation,[20] inactivation of proteins and DNA replication.[21] It exhibits strong antifungal activity against pathogenic *Candidasp*. It was found that AgNPs inhibit the growth of yeasts comparable to common antifungals.[22] Due to its antimicrobial capability, it is suitably employed in the household product, manufacturing industries, and medical services.[23] AgNPs have the positive effect on the seed germination and seedling growth.[24,25] It plays an important role in the suppression of stress thereby increasing the activities of superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidise.[26] Catalase is an enzyme present in living organisms which are exposed to oxygen. It plays an important role in protecting cells from oxidative stress caused by ROS (Reactive Oxygen Species). It catalyses the decomposition reaction of hydrogen peroxide, where it is broken down into water and oxygen. It was found that breakdown of hydrogen peroxide follow first order kinetics and increases linearly with catalase concentration.[27] In this present research work, AgNPs are biosynthesized from leaf extracts of two medicinal plants – *A. indica* (neem) and *O. sanctum* (tulsi). The synthesized nanoparticles are characterized. Disc diffusion method is employed to test the antimicrobial effects of leaf extracts, newly biosynthesized AgNPs and silver nitrate solution. Toxicity test of biosynthesized AgNPs was done on moong bean seeds (*Vigna radiata*). The seeds were treated with four concentrations of AgNPs of respective plants.

MATERIALS AND METHODS

Preparation of Leaf Extract

Azadirachta indica (neem) and *Ocimum sanctum (tulsi)* leaves were used to prepared silver nanoparticles on the basis of ease of availability, cost effectiveness and their medicinal properties. Fresh leaves of *Azadirachta indica* and *Ocimum sanctum* were collected from the University campus. 20 grams of these collected leaves were washed several times with distilled water to remove any dust and dirt particles. Leaves were chopped into fine pieces and mixed into 100ml of distilled water. The mixture was maintained at 60°C in the water bath for 10 minutes for *A. indica* and 60 minutes for *O. sanctum*. The mixture was then filtered with Whatmann Filter Paper No.1. The extract was stored at 4°C for further studies.[4,25,28]

Synthesis of silver nanoparticles

0.1M silver nitrate ($AgNO_3$) was prepared and stored at 4°C for future work. 15 ml each of collected leaf extracts was added separately to 45 ml of the prepared solution of $AgNO_3$ at room temperature while stirring. It was further stirred for 5 – 10 minutes. The solution was then kept aside for 24 hours for complete bio – reduction and saturation of AgNPs. The experiment was repeated thrice. The formation of AgNPs was confirmed using spectrophotometric analysis.[4]

Characterization of silver nanoparticles

UV – visible spectroscopy

It was performed to observe the optical properties of biosynthesized AgNPs. After addition of extract to $AgNO_3$, the absorbance was taken at different time intervals up to 24 hours. The time intervals were taken as 15 minutes, 30 minutes, 60 minutes and 24 hours.[25] The final reading was taken at 48 hours from the initiation of the reaction. The wavelength range was taken as 350nm – 750nm.

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy(FTIR)analysis, of biosynthesized AgNPs, was performed to determine its chemical composition. The mixture was centrifuged at 15,000 rpm for 15 minutes to separate AgNPs from other compounds which may cause hindrance in the

analysis of protein–AgNPs interaction. The pellet thus obtained was dispersed in distilled water. The centrifugation and redispersion of pellet in distilled water was repeated thrice. Finally, KBr pellet method was carried out and the range was kept as 400 – 4000 cm^{-1}.[25,28,29]

XRD analysis

X – Ray Diffraction (XRD) was carried out for phase determination and crystal structure and orientation of biosynthesized AgNPs (thin film analysis of the solution). It was done at the voltage of 40 kV and current of 20 mA with Cu K (α) radiation of X – ray wavelength, that is, 1.54187 nm. The scanning was done at 0.02/min in the region of 2θ.[28] The crystal size of the prepared samples was determined by using Scherrer's equation – $D \approx 0.9\lambda / (\beta \cos \theta)$. Where, D is the crystal size, λ is the wavelength of X-ray, θ is the Braggs angle in radians and β is the full width at half maximum of the peak in radians.

SEM analysis

Scanning Electron Microscopy (SEM) is performed to determine the morphological features of the AgNPs. Few drops of solution were put on the small thin aluminium sheet. It was heated till the liquid phase evaporated. The samples were characterized by SEM at a voltage of 20 kV.

Antimicrobial analysis

Three strains of bacteria namely, *Staphylococcus aureus*, *Pseudomonas aeruginosa*and *Acinetobacter baumannii* (MCC 2366) were taken for studying antibacterial activities and two strains of fungi namely, *Aspergillus niger* and *Rhizopus oryzae* were taken for studying antifungal activities of AgNPs synthesised from the respective leaf extracts. The strains were subcultured by inoculating it in nutrient broth and incubating for 24 hours at 37°C.Under sterile conditions, nutrient agar media was poured into the petriplates which were intended to be used for bacteria. Potato dextrose agar was poured into the petriplates to be used for fungi. Swabbing of microbial culture was done when media was hardened. Sterile - discs were placed on the plates containing media and microbial culture. The discs were loaded with double distilled water, plant leaf extract, 0.1M $AgNO_3$ solution and synthesised AgNPs of 10µl each. The disc with double distilled water served as negative control and the disc with $AgNO_3$solution served as positive control. The plates were sealed with paraffin wax and were incubated at 37°C for 24 to 48 hours.[30] Then, the zone of inhibition were observed and measured. The plating was repeated thrice for each type of test bacteria. The mean and standard deviation were calculated for the obtained zone of inhibition.

Toxicity Assay

Germination Test

Moong seeds were selected for the germination test. Four concentrations of biosynthesized AgNPs from each plant leaf extract were prepared separately in distilled water. The concentrations prepared were 25%, 50%, 75% and 100%. The germination test was carried out in petriplates. The seeds were surface sterilized with 0.1% mercuric chloride solution and rinsed thoroughly with double distilled water thrice. An equal number of seeds were kept in labelled petriplates. The solution of each concentration was added to the labelled petriplates respectively to rehydrate the seeds. The petriplates were kept in dark for germination to take place. The length of root obtained following each 24, 48 and 72 hours after germination of seeds was observed.[25] The germination percentage was determined using following equation:[31]

$$\text{Germination Percentage (GP \%)} = (Gf/n) \times 100$$

Where, Gf is the total number of germinated seeds at the end of experiment and n is the total number of seed used in the test.

Catalase test

100mg of roots were collected from control and treated seeds. They were rinsed with distilled water and homogenized with cold phosphate buffer (0.1M, pH – 7). The obtained mixture was then centrifuged at 12,000rpm for 15minutes at 4°C. The pellet thus obtained was discarded and the supernatant was used further to test the oxidative stress. The substrate mixture consisting of 50mM of phosphate buffer and 15mM of H_2O_2 was added to the enzyme extract. The absorbance at 240nm was recorded. It was expressed as µmol H_2O_2 consumed $min^{-1}mg^{-1}$ protein.[25] The hydrogen peroxide consumption was calculated by the following equation:[32]

$$\mu\text{mol } H_2O_2 \text{ consumed } min^{-1} mg^{-1} \text{ protein} = \frac{\text{Difference in OD} \times V_t}{\text{Extinction coefficient of } H_2O_2 \times D \times V_s \times C_t \times 0.001)}$$

Where, V_t = Final Volume, Extinction coefficient of H_2O_2 (at 240 nm) is 34.9 (mMol min^{-1}), D = Path length (1 cm), V_s = Volume of sample (ml), C_t = Concentration of protein (mg/ml).

RESULTS AND DISCUSSION

UV – visible spectrophotometer analysis

As soon as $AgNO_3$was added to each plant extracts colour change was observed from faint yellow to brown to reddish brown within 72 hours of incubation at room temperature. Similar colour changes were observed in other studies.[25,28,33] which confirmed the reduction of silver ions to AgNPs. Corresponding UV – Visible absorption spectrum of AgNPs was recorded at the interval of 15 minutes, 30 minutes, 60 minutes, 24 hours and 48 hours from the initiation of the reaction. The

absorption band was observed at around 380nm - 420nm for *A. indica* and *O. Sanctum.*[25,28,34] From the results obtained, it was found that the formation of AgNPs occurred rapidly within 15 minutes, representing rapid biosynthesis of AgNPs and the absorption peak increased as the time increased. After 24 hours of incubation, highest absorbance was observed and it remained constant after 48 hours of reaction. It has been reported that the biosynthesized AgNPs remained stable for four weeks due to capping material on the surface.[35,36] On comparing the two selected plant, *A. indica* and *O. sanctum*, according to the UV- Visible spectra recorded the most rapid bioreduction was achieved using *O. sanctum* leaf extract as reducing agent followed by *A. indica*. Similar peak at 420nm, 425nm and 438nm were reported for silver nanoparticles synthesized from the seed extract of *Psoralea corylifolia*[38] and thalli of *Anthoceros*[39] and from the leaf extract of *Acalypha indica*[40] respectively. This spectral characteristics of synthesized AgNPs interprets the NPs were polydispersed.

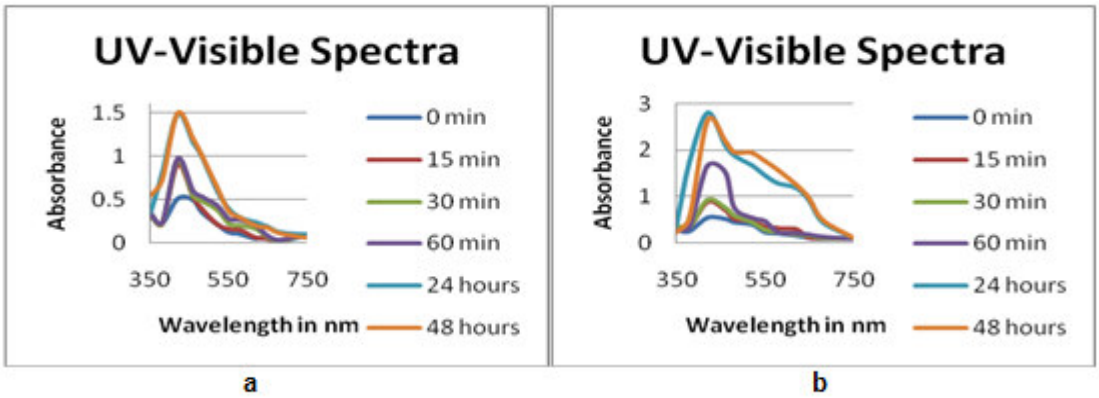
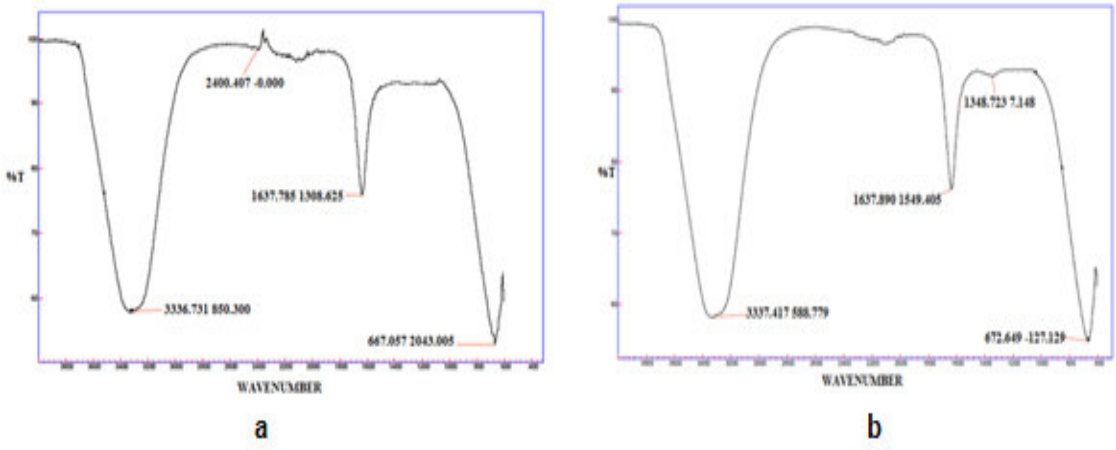


Figure 1
UV-Visible spectra at different time intervals
a. A. Indica with 0.1M $AgNO_3$
b. O. Sanctum with 0.1M $AgNO_3$

Fourier Transform Infrared Analysis

FTIR analysis was carried out to identify the functional groups that act as capping agents and stabilizing agents for biosynthesized AgNPs and are responsible for the reduction of silver ions.[12] As observed in the graph, the broad band with strong intensity obtained in the region 3330 – 3340 cm^{-1} is due to O – H stretching vibrational frequency. It could have occurred due to the hydroxyl group of phenol and alcohols. The weak absorption intensity peak at 1637 – 1638 cm^{-1} may be obtained due to C=C stretching of aromatic ring which is present in terpenoids. These terpenoids play a major role in the reduction of metal ions. The peak can also be obtained due to C=O which is present in flavonoids. Also, it may be due to carbonyl stretch of proteins which may be assigned to amide I group. The peak at around 2400 cm^{-1} is due to N – H bond. Peaks at 1130, 1139 and 1349 cm^{-1} are due to binding of the molecule to the surface of AgNPs through COOH carboxylate group. The peak at around 670 cm^{-1} is due to the C – H bond.[29,34,37]



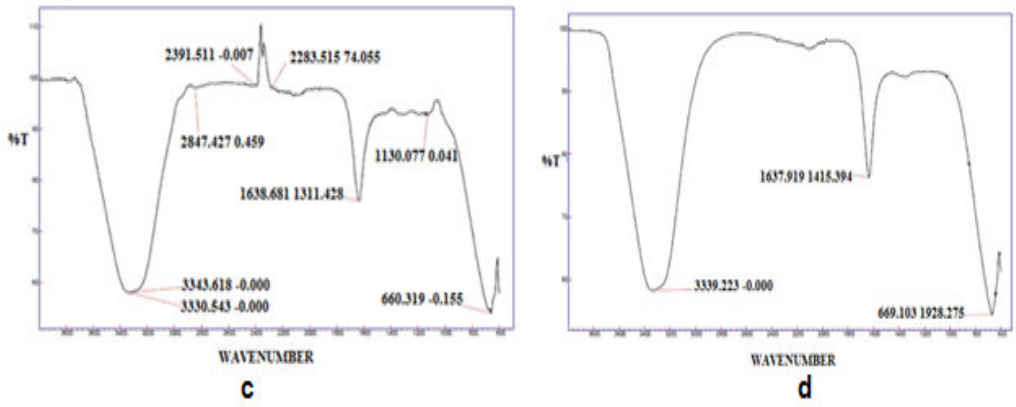


Figure 2
FTIR analysis
a. A. indica leaf extract
b. A. indica with 0.1M $AgNO_3$
c. O. sanctum leaf extract
d. O. sanctum with 0.1M $AgNO_3$

XRD analysis

It was performed to analyze the particle size of the biosynthesized AgNPs. It was determined by Scherrer's equation:$D_p = 0.94\lambda/(\beta \cos\theta)$Where λ = 1.5402 Å, β = Peak width (Degrees), 2θ = Peak position (Degrees).For the neem AgNPs, peaks were obtained at 7°, 11° and 15° position and the particle size was calculated to be 27.7 nm, 27.8nm, and 41.8 nm respectively. For the tulsi AgNPs, peaks were obtained at 10° and 28° position and the particle size was calculated to be 41.6 nm, and 42.7 nm respectively.

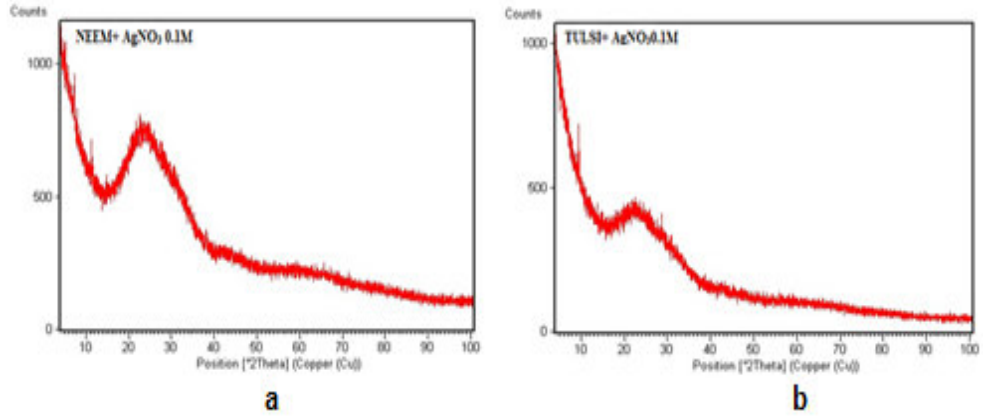


Figure 3
XRD analysis of AgNPs biosynthesized from
a. A. indica with 0.1M $AgNO_3$ b. O. sanctum with 0.1M $AgNO_3$

SEM analysis

The morphology and size of the AgNPs were obtained from the SEM. The diameters of biosynthesized nanoparticles were found to be of several nm. The size of prepared nanoparticles was found to be within the actual size range of nanoparticles, which is 1 - 100nm. It is evident from the image of SEM that the AgNP is spherical or roughly spherical shaped as seen in Fig 4. The average size of AgNPs biosynthesized from neem leaf extract and tulsi leaf extract was found to be upto 60nm respectively.

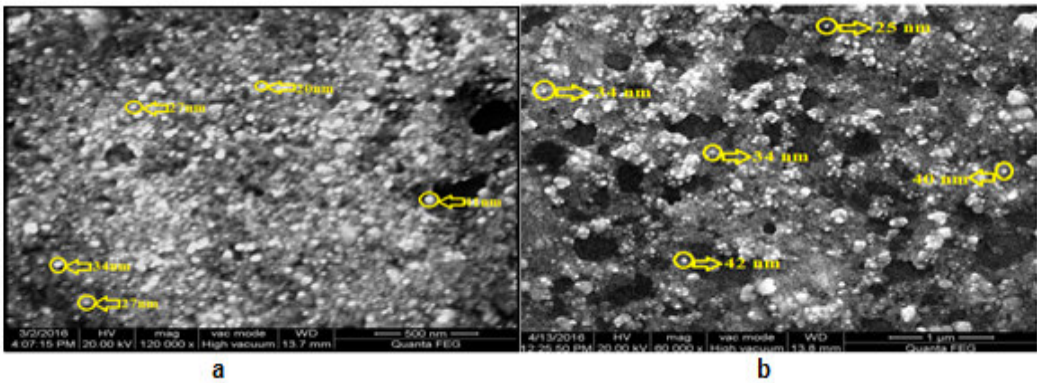


Figure 4
SEM analysis of AgNPs biosynthesized from
a. A. Indica with 0.1M $AgNO_3$
b.O. Sanctum with 0.1M $AgNO_3$

Antimicrobial analysis

The antibacterial properties of biosynthesized AgNPs were studied on three strains of bacteria, namely *Staphylococcus aureus, Pseudomonas aeruginosa* and *Acinetobacter baumannii*. These bacteria were cultured on nutrient media agar separately. The antifungal properties of biosynthesized AgNPs were studied on two strains of fungi, namely *Aspergillus niger* and *Rhizopus oryzae*. These fungi were cultured on potato dextrose agar separately. The zone of inhibition obtained is shown in table – 1 (For antibacterial analysis) and table – 2 (For antifungal analysis).

TABLE 1
Anti - bacterial effect of biosynthesized AgNPs

	SAMPLE	ZONE OF INHIBITION (Measured in mm)		
		Staphylococcus aureus **(OP-5517-VL)**	***Pseudomonas aeruginosa*** **(OP-5515-VL)**	***Acinetobacter baumannii*** **(MCC 2366)**
	$AgNO_3$	9.33±1.15	9.3±0.5	12.33±2.51
A.Indica	Leaf extract	8.33±0	8±0	No Inhibition
	AgNPs	11±0.57	12.3±1.52	14.6±1.5
	AgNPs - $AgNO_3$	1.33±1.52	1.33±0.57	2.33±2.08
O.Sanctum	Leaf extract	8.3±1.15	9±1	No Inhibition
	AgNPs	11±1	10.6±0.5	14.6±1.1
	AgNPs - $AgNO_3$	1.67±0.57	1.33±1.15	2.33±1.52

TABLE 2
Anti – Fungal Effect Of Biosynthesized AgNPs

	SAMPLE	ZONE OF INHIBITION(Measured in mm)	
		Aspergillus niger (OP-5601-VL)	*Rhizopus oryzae* (OP-5606-VL)
A.Indica	$AgNO_3$	39.3±3.78	41.3±3.05
	Leaf extract	No Inhibition	No Inhibition
	AgNPs	43.3±3.78	41.3±3.05
	AgNPs- $AgNO_3$	2.33±1.52	1±1
O.*Sanctum*	Leaf extract	No Inhibition	No Inhibition
	AgNPs	44±3.0	40.6±8.3
	AgNPs- $AgNO_3$	4.66±1.52	4±3

It was observed that the biosynthesized AgNPs possessed antimicrobial properties. On comparing its effectiveness among the selected three strains of bacteria and two strains of fungi, the highest zone of inhibition was obtained against *Acinetobacter baumannii* and *Aspergillus niger*. Among the two selected plants, tulsi showed higher antimicrobial activity. It is known that *A.Indica* and *O. Sanctum* leaf extracts have antimicrobial property, but very few or no inhibition zone was observed which may be due to its lower concentration.[12]

Toxicity analysis of silver nanoparticles

The toxic effect of AgNPs was analysed on Moong beans seeds (*V. radiata*). The germinated seeds were observed for its emergence of radicles. Maximum percentage of germination was found after 24 hours. It was observed that there was some variation in the growth of the seeds with the treatment. It indicated that seeds treated with neem AgNPs solution showed better growth than seeds treated with the tulsi AgNPs solution as seen in Figure 5.

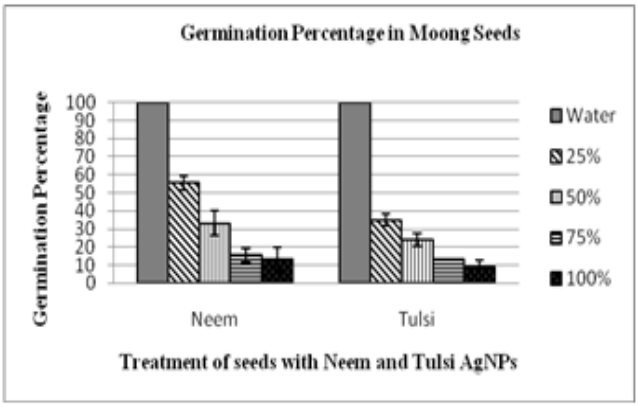


Figure 5
Effect of AgNPs on seed germination

Analysis of oxidative stress
Catalase Test

When the cells are exposed to hydrogen peroxide, stress is induced which is overcome by catalase enzyme. A decline in absorbance was observed at 240nm. This was due to the toxicity of the AgNPs which hindered the hydrogen peroxide consumption. The H_2O_2 consumption was found to be dependent on time and concentration of AgNPs.[25] It was observed that the seeds treated with 25% of AgNPs showed more activity towards the stress induced by addition of H_2O_2. $AgNO_3$ showed the similar effect as that of the AgNPs. On comparing the two plants, which was shown in figure 6 and 7, neem showed more activity towards H_2O_2 consumption than tulsi. Hence, the toxicity of AgNPs is higher in 100% concentration which had decreased H_2O_2 consumption.

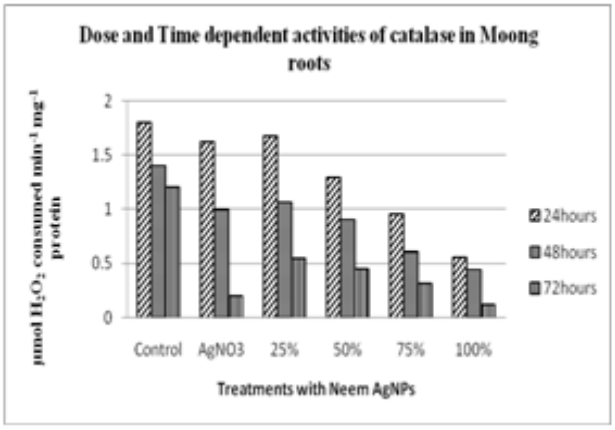


Figure 6
Catalase test with Azadirachta indica AgNPs

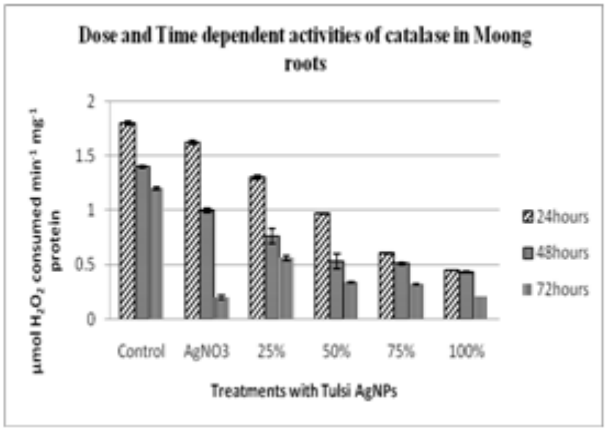


Figure 7
Catalase test with Ocimum sanctum AgNPs

CONCLUSION

The AgNPs were biosynthesized from neem and tulsi leaves by bioreduction of $AgNO_3$. Owing to varying properties of these two plant species, AgNPs obtained from them also varied in size, the smallest yield being the neem leaves. Synthesized AgNPs have been appropriately characterized using UV-vis spectroscopy, SEM, XRD and FTIR. Hence, it can be concluded that the biosynthesized AgNPs have potential medical application because of its antimicrobial properties and agricultural application due to its positive effect on seed germination.

CONFLICT OF INTEREST

Conflict of interest declared none.

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