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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *NELUMBO NUCIFERA* RHIZOME EXTRACT AND INVESTIGATION OF THEIR ANTI-OXIDANT AND ANTIBACTERIAL ACTIVITY

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ABSTRACT

The biosynthesis of nanoparticles has been proposed as a cost effective and environmentally benevolent alternative to chemical and physical methods. In the present study, green synthesis of silver nanoparticles (AgNPs) has been demonstrated using rhizome extract of *Nelumbo nucifera* reducing aqueous $AgNO_3$ solution. The synthesized nanoparticles have been characterized on the basis of fourier transform infrared spectroscopy (FT-IR), UV–Vis spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive X-ray (EDX) analysis. The presence of a characteristic surface plasmon resonance (SPR) absorption band at 435 nm in UV–Vis reveals the reduction of silver metal ions into silver nanoparticles. FT-IR analysis was carried out to probe the possible functional group involved in the synthesis of AgNPs. Further leaf extracts and AgNPs were evaluated for antiradical scavenging activity by DPPH assay and anti microbial activity against five bacteria and two fungi. The results proved that the synthesized silver nano particles found to inhibit the growth of the pathogenic microbes in the study and can serve as promising antiradical agents.

Key words: Silver nanoparticles, Rhizome extract, *Nelumbo nucifera*, Green synthesis, anti-oxidant activity, antibactrial activity

INTRODUCTION

One of the most important fields of research in nanotechnology is the synthesis of different nanoparticles such as silver, gold, iron, etc. [1, 2]. There are various chemical and physical methods for the synthesis of metallic nanoparticles for example, one can mention reduction of solutions, photochemical reactions in reverse micelles, and electrochemical reduction, heat evaporation and radiation assisted methods, among others. Physical and chemical methods have usually been successful in the synthesis of nanomaterials in large quantities in short periods of time, as well for specific size and shape. However, most of these methods are extremely expensive and they also involve the use of toxic, hazardous chemicals as the stabilizers which may pose potential environmental and biological risks [3]

In recent years, the use of biological methods for the synthesis of metallic nanoparticles has received considerable attention, because these are inexpensive and eco–friendly; also, they can be carried out in one step [4]. Green synthesis methods utilize miscellaneous biological natural substances such as microorganisms, whole plants, plant tissues and fruits, plant extracts, marine algae and micro–fluids for the reduction and stabilization of nanoparticles. Synthesis of nanoparticles using plant extracts has several advantages over other environmentally green synthesis methods, because plants are broadly distributed, readily scalable, easily available, safe to handle and less expensive [5]

Silver nanoparticles due to various properties such as catalysis, electrochemical conductivity and antimicrobial activity, can be used in different applications like biomedicine, agriculture, photo chemicals and food chemistry [6]. Since ancient times, the silver has been known to be efficient against a wide range of microorganisms [7]. Nowadays, the most important applications of silver nanoparticles in biotechnology science correspond to their antibacterial and antifungal activities.

Nelumbo nucifera is the national flower of India and deep religious meaning to Hindus and Buddhists, to whom the flower of *N nucifera* symbolizes beauty, purity and divinity. *Nelumbo nucifera* plant has traditional medicinal uses. The rhizomes or leaves are used with other herbs to treat sunstroke, fever, diarrhoea, dysentery, dizziness, vomiting of blood, haemorrhoids. The whole plant is used as an antidote to Mushroom Poisoning. The embryonic seeds for high fever, cholera (Chinese), nervous disorders and insomnia; the seeds to stop vomiting, relieve indigestion and diarrhoea or just as a tonic. Pounded petals for syphilis; for cosmetic unguents (Java); the flower stalk with other herbs to treat bleeding from the uterus. The pods contain alkaloids that stop bleeding [8].

In this research, the rhizome extract of *Nelumbo nucifera* was used for rapid, simple and biosynthetic synthesis of silver nanoparticles. Furthermore, green synthesis Ag nanoparticles were characterized by FT-IR, XRD, TEM, and SEM techniques. Finally, its antibacterial and anti-oxidant activity was investigated by the disk

diffusion and DPPH scavenging activity method respectively.

MATERIALS AND METHODS

Materials:

Silver nitrate of analytical grade was purchased from Indian Research Products, Chennai. Composition for the preparation of growth media for the growth of bacteria and fungi were purchased form Fisher Scientific Company, Mumbai. The standard antibiotic ciprofloxacin for antibiotic standard was obtained from Dr. Reddy's laboratories, Hyderabad. DPPH (2, 2-diphenyl-1-picrylhydrazyl) was purchased from Merck chemicals, Mumbai.

Collection of Plant material:

The selected medicinal plant for the study *Nelumbo nucifera,* was collected in Botanical garden at Dr N R S Ayurvedic Medical College, Bandar Road, Vijayawada. Primarily the collected plant was washed and the cleaned herbal parts were dried with water absorbent paper (wet filter paper). Different parts of the plant like Leaf, Flower, Rhizome and Seeds were individually cut into small pieces, powdered using clean pestle and mortar. The plant powders were stored in an air tight container and were used for the extraction of plant components.

Microorganisms:

In this study five bacterial strains [*Bacillus Subtilis* (MTCC 10619), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 1688), *Staphylococcus aureus* (MTCC 3160), and *Bacillus cereus* (MTCC 1305)] and two fungal strain [*Aspergillus niger* (MTCC 282) and *Rhizopus oryzae* (MTCC 262)] was used for the determination of antimicrobial activity. All the pathogenic bacterial strains were obtained from Microbial Type Culture Collection and Gene Bank (*MTCC*), Chandigarh, India.

Preparation of the plant extract:

5grams of selected plant part powder and equal volume mixture of selected parts were dispensed in 100 ml of sterile distilled water and boiled for one hour at 80ºC. Then individual extracts were collected in separate volumetric flasks by standard filtration method. The final volume in the volumetric flasks was made up to 100ml mark. The obtained plant extracts were used for the synthesis of nano particles.

Synthesis of Silver nanoparticles:

The 10-2 M Silver nitrate solution was prepared and stored in brown bottles. 5ml of plant extracts was taken in volumetric flasks separately and to this 95 ml of AgNO3 solution was added. The same protocol

was followed for all prepared extracts. All the extracts were added individually and in combination of extracts in order study the impacts of various existing phytochemicals in different parts. The volumetric flasks were incubated at room temperature. The color change of the extracts from pale yellow to dark brown was checked periodically. The brown color formation indicates that the silver nano particles were synthesized from the herb and they were centrifuged at 5000 rpm for 15 minutes in order to obtain the pellet. The collected pellet was air dried and the dry synthesized nano particles were studied for further characterization and activity studies.

Characterization of silver nanoparticles:

The physical characterization of synthesized nanoparticles such as bulk density, moisture content, loss on ignition, ash content, PH, point of zero charge, iodine number and decolorizing power was studied.

UV–visible absorption analysis was carried out using a Teccomp UV-2301 double beam UV-Visible spectrophotometer. FT–IR spectra of silver nanoparticles were performed using a BRUKER VERTEX 80/80v FT-IR spectrometer with KBr pellet in the range of 4000–400 cm−1. The crystalline nature of silver nanoparticles was investigated by XRD analysis. X–ray diffraction data of AgNPs were obtained using a Bruker- D4 ENDEAVOR with Cu kα radiation (λ = 1.54 Å) in the 2θ range of 20° to 80°, and with a steps size of 0.02° at 40 kV and 30 mA. The morphology of the AgNPs was examined by TEM (Hitachi H7500). Furthermore, SEM (LEO 1420 VP Compact variable pressure Digital SEM) study was carried out to investigate the shape, size and the surface area of the AgNPs.

Antibacterial Activity:

Antibacterial activity of the synthesized nanoparticles was determined by using the Kirby-Bauer disc diffusion method [9] against different pathogenic bacteria and fungi. The anti fungal zone inhibition activity was studied against *Aspergillus niger* (MTCC 282) and *Rhizopus oryzae* (MTCC 262).The anti bacterial zone inhibition activity was studied against five human pathogenic bacteria namely, *Bacillus Subtilis* (MTCC 10619), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 1688), *Staphylococcus aureus* (MTCC 3160), and *Bacillus cereus* (MTCC 1305). Stock cultures were maintained at 4°C on agar slants of nutrient media. Prior to the experiment, pure cultures were sub-cultured in Muller Hinton broth and incubated overnight at 37°C. The inoculums suspensions were swabbed uniformly in different Petri plates. Filter paper discs saturated with nanoparticles were placed aseptically in the plates with the help of sterile forceps and incubated at 37°C. 1% dimethyl sulfoxide was taken as positive and negative control respectively. After 24 hours of incubation, the zone of inhibition was observed and measured.

Antioxidant activity by DPPH method:

The free radical scavenging activity of the synthesized AgNPs was determined by using DPPH method

described by Marsden S. Blois et al 1958 [10]. Briefly, DPPH solution of 0.1 mM was prepared in 95% methanol and 1 ml of this solution was added to 3.0 ml of synthesized AgNPs solution of 5–250µg/ml. The solution was incubated for 30min at dark conditions at room temperature and absorbance was measured at 517 nm using a UV–Vis spectrophotometer.

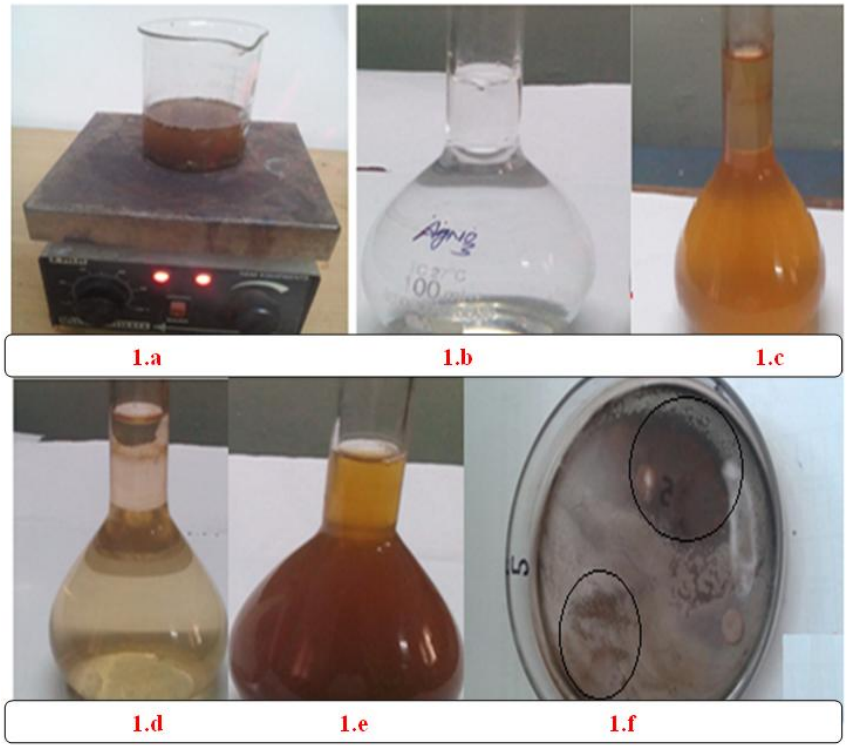
Ascorbic acid was used as a standard. The experiment was repeated triplicate and the DPPH scavenging activity was calculated by using the formula

$$\% \text{ Inhibition} = \frac{A0 - A1}{A0} \times 100$$

Where A_0 is the absorbance of the control and A1 the absorbance of the AgNPs solution

RESULTS AND DISCUSSION

The *Nelumbo nucifera* plant extract was employed for the green synthesis of AgNPs. After the addition of the plant extract to the silver nitrate solution, it was observed that the color of the reaction mixture was gradually changed from light yellow to dark brown, indicating the formation of silver nanoparticles. The color change of silver solution from light color to dark brown was observed on increasing the time up to 24Hrs. The preparation of plant extract, process of synthesis of silver nano particles and the nano particles obtained after the synthesis process was given in figure 1.



1.a: Process of Aqueous Rhizome extraction of *Nelumbo nucifera*, **1.b**: Silver Nitrate solution, **1.c:** Quantified

Plant extract solutions, **1.d:** Mixture of silver nitrate solution and plant extract immediately after mixing, **1.e**: Solution containing sysnthesised silver nano particles before centrifugation, **1.f:** Cluster of Silver Nanoparticles after centrifugation

Figure 1: Process of synthesis of Silver nanoparticles by using *Nelumbo nucifera*

Different extracts with individual and combination of plant parts were used for reduction silver. Among them addition of Rhizome extraction was exhibited the better color change at less time. The result obtained in this investigation is very interesting in terms of identification of potential plant i.e rhizome for synthesizing the silver nanoparticles. The yield of nanosilver material was also found high in rhizome extract, hence it is confirmed that rhizome extract was best choice for synthesis of nano silver with *Nelumbo nucifera* plant. The physical characterization of *Nelumbo nucifera* rhizome mediated silver nano-particles was given in table 1.

S. No	Character (Units)	Results
1	Bulk Density in gm/cc	5.12
2	Moisture Content in %	8.13
3	Loss on Ignition in %	79.64
4	Ash Content in %	5.6
5	pH	7.76
6	Point of zero charge	7.26
7	Water soluble Matter in %	5.311
8	Acid soluble Matter in %	38.84
9	Iodine Number mg/gms	411.33
10	Decolorizing Power in mg/gms	264
12	Carboxyl Group on Surface (%)	0.316
13	Lactone Group on Surface (%)	0.017
14	Phenol Group on Surface (%)	0.012
15	Total Basic Groups (%)	0.693

*results given in table are the average of three replicate experiments

Table 1: Physical characteristics of *Nelumbo nucifera* silver nano-particles

UV–Vis absorption spectroscopy is an important method to detect the formation and stability of metal NPs in the reaction mixture. Figure 2 shows the UV–Visible spectra recorded at different times of reaction. No change in absorbance was observed after 2H, indicating the complete conversion of Ag+ to Ag. In this work, the UV–vis spectra of silver nanoparticles synthesized displayed a strong broad peak around

435nm due to the formation of AgNPs. This peak corresponded to the surface plasmon resonance of the synthesized AgNPs [11].

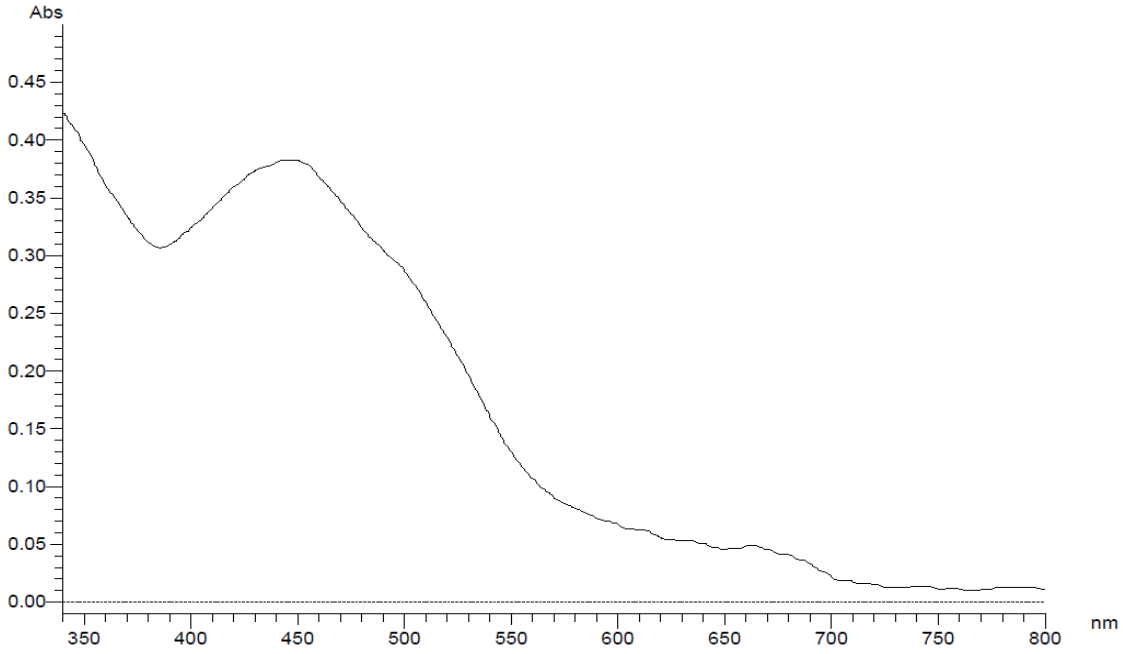
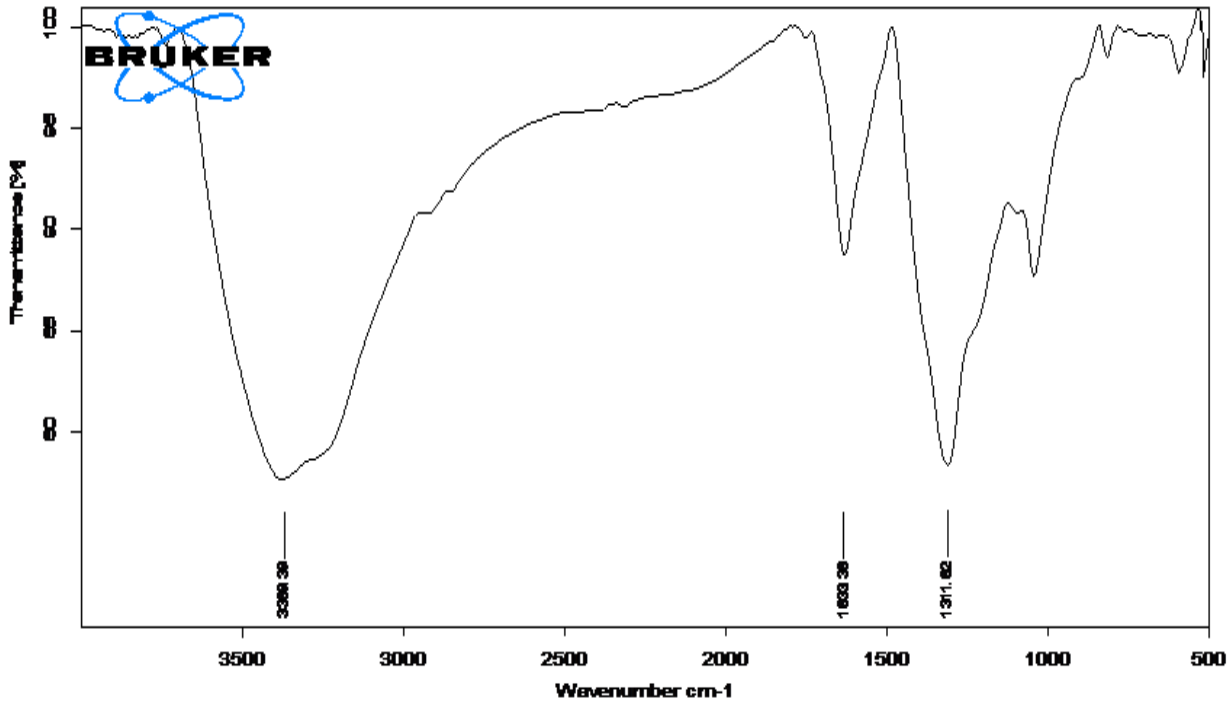


Figure 2: UV scanning spectra for the synthesized nano particles using *Nelumbo nucifera*

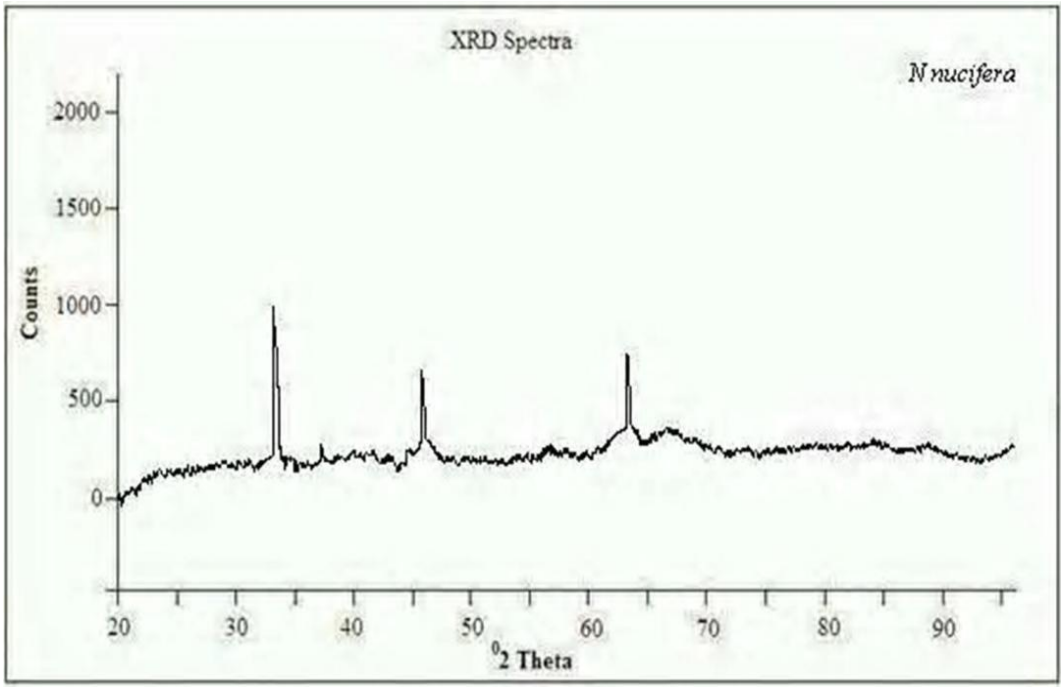
To determine the possible biomolecules and functional groups involved in reduction, capping and efficient stabilization of newly synthesized Ag nanoparticles, FTIR spectroscopy was employed. The FTIR spectrum of stabilized silver nanoparticles is depicted in Fig. 3. The FTIR spectrum of the *Nelumbo nucifera* rhizome plant extract, wherein some pronounced absorbance was recorded in the area between 4000 and 500 cm^{-1}. They include a 1311.62 (nitro compounds - N–O symmetric stretch, aromatic amines - C–N stretch and alcohols, carboxylic acids, esters, ethers - C–O stretch), 1633.36 (1° amines - N–H bend), 3369.39 (1°, 2° amines, amides - N–H stretch, alcohols, phenols - O–H stretch, H–bonded).



Figures 3: FT-IR Spectrum for the synthesized nano-particles using *Nelumbo nucifera*

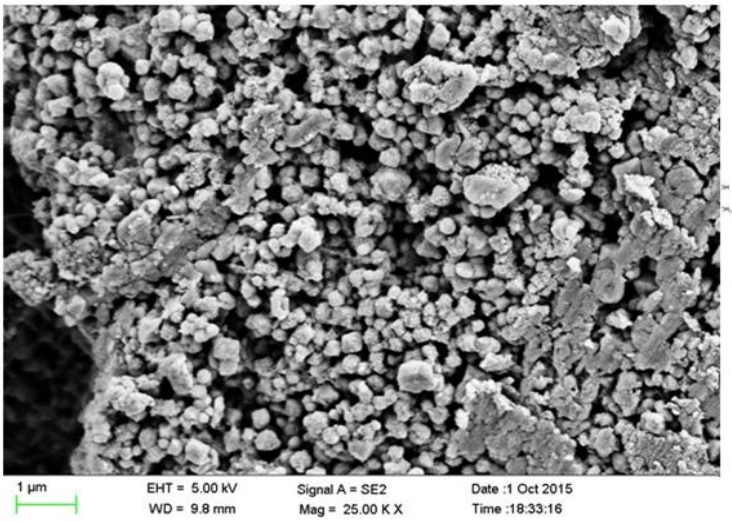
The precipitation stage during the reduction process usually involves fast chemical reactions and nucleation kinetics, thus the type of reducer a significant factor in the particle size distribution of the product [12]. The results previously reported indicated that the *Nelumbo nucifera* extract containing flavonoids, phenolic compounds and terpenoids ect and so may be held responsible for efficient capping and stabilization of obtained AgNPs.

The X-ray diffraction analyses were carried out to determine the known phase of the silver nanoparticles. Figure 4 demonstrates the XRD pattern of the dried synthesized Ag nanoparticles by the *Nelumbo nucifera* rhizome extract. The spectrum exhibited three distinct separate peaks at 2θ of 33.8, 46.1 and 64.6 that could be indexed to (111) (200), (220), (311) and (222) planes, respectively. The diffraction peaks data obtained were in accordance with the reports of FCC structure. The mean grain crystalline size of green synthesized AgNPs was calculated by employing Debye–Scherrer formula. The average grain crystalline size of AgNPs was estimated to be approximately 70nm. A small number of unassigned peaks were also recorded that might be due to the crystallization of bioorganic phases present in *Nelumbo nucifera* rhizome extract on the surface of the silver nanoparticles. Similar results were also obtained for AgNPs synthesized using the beetroot extract [13] and *Ixora coccinea* leaves extract [14].

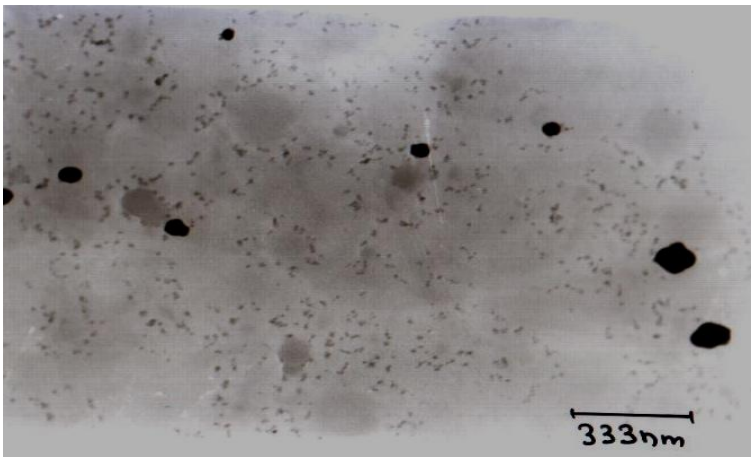


Figures 4: XRD Spectra for the synthesized nano-particles using *Nelumbo nucifera*

SEM technique was employed to determine the surface morphology and the topography of synthesized silver nanoparticles. Figure 5 shows the size of silver nanoparticles from 15 to 315 nm, with an average size 70nm. SEM image exhibited that the biosynthesized silver nanoparticles were mostly cubicin shape. The circular particles were found to be less in size than the cubic particles. The shape and size of the biosynthesized AgNPs were further analyzed by TEM. TEM image (Fig. 6) demonstrated that the most AgNPs were obviously cubic in shape and well dispersed, with an average size around 70nm. The obtained results from TEM image were in a good agreement with the SEM data.

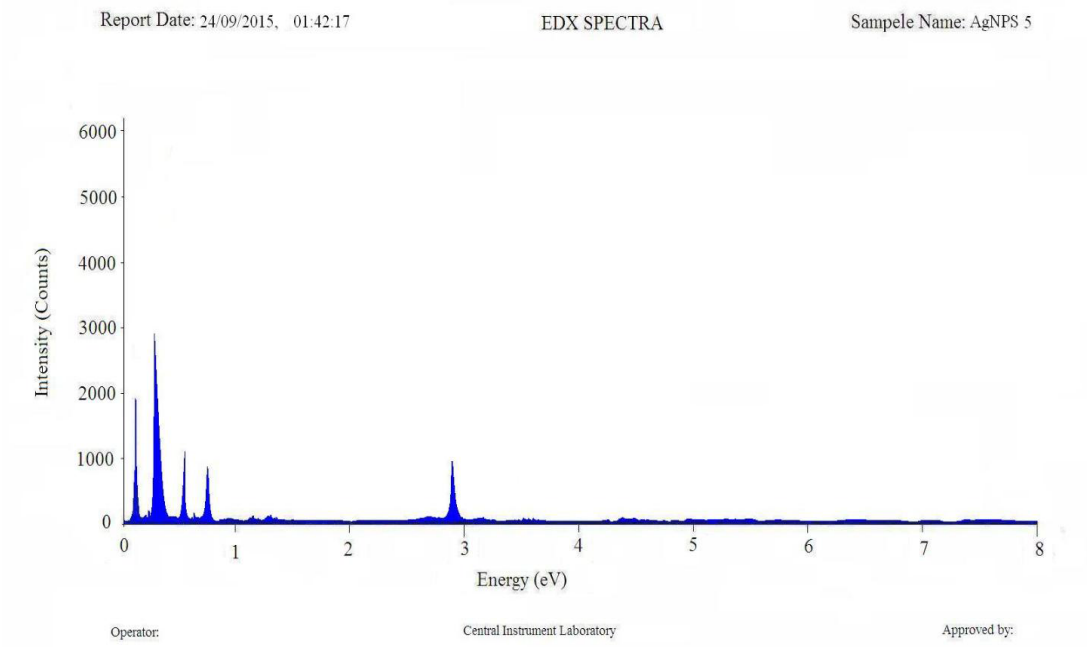


Figures 5: SEM images for the synthesized nano-particles using *Nelumbo nucifera*



Figures 6: TEM images for the synthesized nano-particles using *Nelumbo nucifera*

The EDX profile (Fig 7) shows a strong silver signal at 2.93eV. The graph also shows the presence of carbon (C), sulfur (S) and Nitrogen (N) are present in the EDX picture of silver nanoparticles. It has been reported that nanoparticles synthesized using plant extracts are surrounded by a thin layer of some capping organic material from the plant extract and are, thus, stable in solution up to 4 weeks after synthesis. This is another advantage of nanoparticles synthesized using plant extracts over those synthesized using chemical methods.



Figures 7: EDX images for the synthesized nano-particles using *Nelumbo nucifera*

Anti microbial Activity of synthesized silver nano particles:

The antibacterial activity of biosynthesized silver nanoparticles synthesized using *Nelumbo nucifera* rhizome extract was studied against five bacterial *B Subtilis*, *E coli*, *P aeruginosa*, *S aureus*, and *B cereus* and two fungal strain *A niger* and *R oryzae* using the agar well diffusion assay, and the zone of inhibition was tabulated as shown in Table 2 and 3. The synthesized AgNPs displayed efficient antibacterial activity against both Gram-negative and Gram-positive bacteria. The silver nanoparticles synthesized by *N nucifera* rhizome extract showed the maximum zone of inhibition around 19.4 mm for *S aureus*, which were followed by *P aeruginosa* (15.6mm), *B cereus* (14.7mm), *B Subtilis* (14.6mm) and *E coli* (12.1mm) at a synthesized nano particle concentration of 1000µg/ml. The two fungi studied high zone of inhibition was observed for *A niger* (13.5mm). On the other hand, the negative control (distilled water) did not exhibit any zone of inhibition and the rhizome extract shows very less zone of inhibition.

S No	Name of the Organism	Zone inhibition observed for concentration (µg/ml) of AgNPs					
		1000	500	250	100	10	1
1	*Bacillus Subtilis*	14.6	12.5	9.9	8.4	3.2	---
2	*Escherichia coli*	12.1	10.8	8.6	7.1	---	---
3	*Pseudomonas aeruginosa*	15.6	13.1	9.4	6.6	---	---
4	*Staphylococcus aureus*	19.4	15.5	13.2	10.6	5.4	---
5	*Bacillus cereus*	14.7	12.8	10.7	8.4	3.1	---

Table 2: Results of studies of anti bacterial activity of synthesized nano particles

S No	Name of the Organism	Zone inhibition observed for concentration (µg/ml) of AgNPs					
		1000	500	250	100	10	1
1	*Aspergillus niger*	13.5	10.5	8.9	5.6	---	---
2	*Rhizopus oryzae*	12.4	11.6	9.4	7.5	2.9	---

Table 3: Results of studies of anti fungal activity of synthesized nano particles

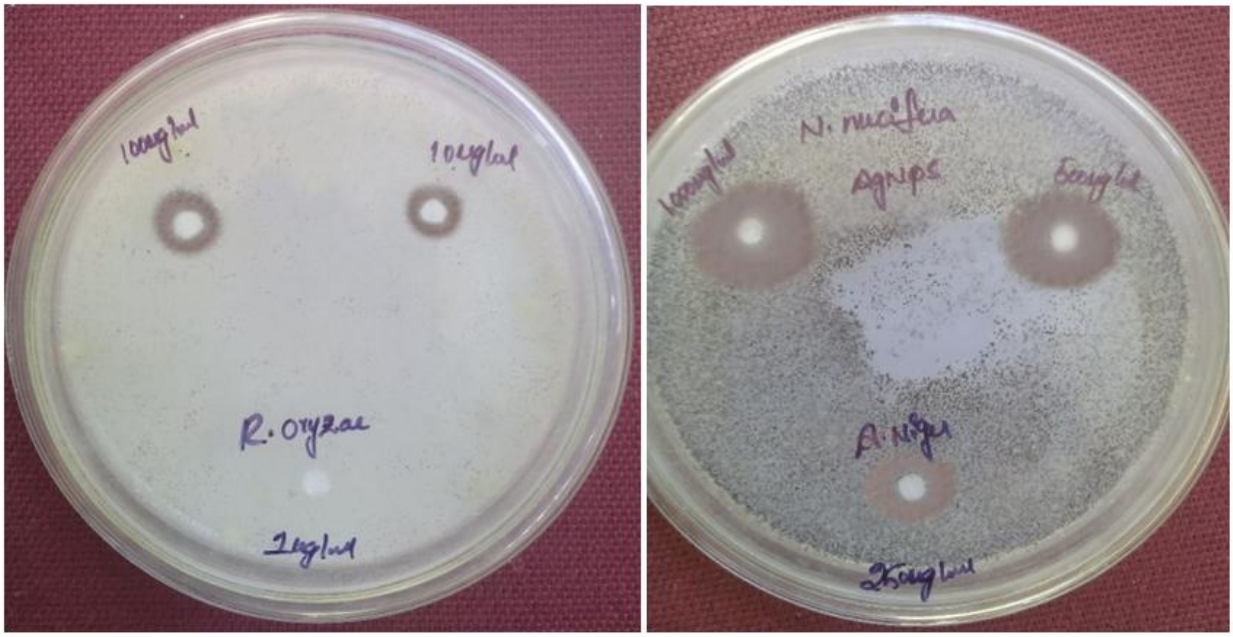


Figure 8: Results of studies of anti fungal activity of synthesized nano particles

The mechanisms of efficient antibacterial activity by silver nanoparticles against various pathogenic bacteria, are still unclear and required further investigation [15]. AgNPs might have been attached to the surface of the cell membrane of microorganisms, leading to the disturbance of its functions like permeability and respiration. It is obvious, therefore, that the binding of particles to the microorganism depends on the surface area available for intraction. In general, small nanoparticles have a larger surface area for intraction with bacteria, as compared to that of bigger particles, due to greater antibacterial activity [16].

Anti-Oxidant activity by DPPH method:

The antioxidant activity of the aqueous extract, Ascorbic acid and bio-conjugated AgNPs was evaluated using DPPH scavenging assay. The IC_{50} values of extract, standard and synthesized AgNPs are reported in Table 4. It can be inferred from the data that synthesized AgNPs displayed better antioxidant activity in comparison to crude extract. As can be seen from Table 1, there was a dose dependent increase in the percentage inhibition (% inhibition) of extracts and synthesized silver nanoparticles. DPPH free radical scavenging assay of the synthesized silver nanoparticle (AgNPs) when compared with the standard ascorbic acid showed promising activity. It was found that at a concentration of 25-30µg/mL, the bio-conjugated AgNPs exhibited better free radical scavenging activity with reference to drug ascorbic acid. These results suggest that at concentrations of 30µg/mL, the synthesized AgNPs may serve as potent radical scavengers. All these results suggest that intercalation of bio extracts with silver nanoparticles can serve as promising antiradical agents.

S No	Concentration in µg/ml	% DPPH inhibition		
		Ascorbic acid	Crude Extract	AgNPs
1	5	13.26203	0.962567	1.818182
2	10	21.39037	3.101604	8.770053
3	15	36.25668	8.877005	14.33155
4	20	45.34759	14.65241	21.71123
5	25	52.72727	22.56684	33.26203
6	30	60.53476	27.91444	51.01604
7	50	69.94652	42.6738	65.13369
8	100	86.63102	52.40642	74.75936
9	150	91.3369	66.31016	87.91444
10	200	93.79679	76.14973	93.79679
11	250	97.54011	84.27807	94.54545

Table 4: Quantitative screening of antioxidant activity of plant extract and AgNPs by DPPH assay and comparison with standard Ascorbic acid

CONCLUSION

The present work reports the facile, convenient and eco-friendly, *Nelumbo nucifera* rhizome extract mediated green synthesis of bio-conjugated silver nanoparticles using microwave. The characterization with UV–Vis spectroscopy, FT-IR, SEM, TEM and EDX analysis authenticate the formation of target silver nanoparticles. TEM analysis depicts the stabilization of synthesized nanoparticles by the capping organic materials thereby preventing their agglomeration. The synthesized silver nanoparticles exhibited promising ability to diffuse the toxic free radicals and can be used as a possible food additive or in nutraceutical and biopharmaceutical industries. It could be concluded that *Nelumbo nucifera* rhizome mediated silver nano particles can be used efficiently in the production of potential antioxidant and antibacterial AgNPs for commercial application

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