Green Synthesis and Phytochemical Analysis of Silver Nano Particles Using Root of *Nelumbo nucifera* and their Antibacterial Activity

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Abstract - *Nelumbo nucifera* root was found to be an excellent source of vitamins, minerals, iron and secondary metabolites. The preliminary phytochemical studies on *N. nucifera* extract show the presence of tannins, alkaloids and glycosides. The green synthesized silver nanoparticles were characterized by UV-Visible spectroscopy and Scanning Electron Microscope (SEM).The SEM analysis showed the average particle size of 60 nm with spherical shape. Further, the silver nanoparticles showed antibacterial activity against *Escherichia coli [gram negative], Bacillus subtilis [gram positive], Pseudomonas aeruginosa [gram negative], Aeromonas hydrophila* [gram negative] and *Staphylococcus aureus [gram positive]*. *N. nucifera* exhibited high activity against *E. coli*, moderate activity against *S. aureus*, and low activity in *Pseudomonas aeruginosa, Aeromonas hydrophila* and no more activity in *B.cereus.* This study revealed that silver nanoparticles synthesized with *N.nucifera* could be used as eco friendly and low cost biological reducing agents, which could produce metal nanostructures in aqueous solution at ambient temperature, avoiding the presence of hazardous and toxic solvents.

Key words: Silver nanoparticles, phytochemicals, antibacterial activity, *N. nucifera*.

I.INTRODUCTION

Green synthesis of nanoparticles is one of the aesthetic, ecofriendly, low cost, non toxic and upcoming technology, which shows very promising results in medicine and other fields. There are varied metals like, gold, silver, tungsten used as a source nanoparticles synthesis. Among various metals, silver is one of the most important metal which has the natural immune system. Silver is a naturally occurring precious metal most often as a mineral ore in association with other elements. It has been positioned as the 47^{th} element in the periodic table having a atomic weight of 107.8 u and 2 natural isotopes 106.90 Ag to 108 .90 Ag with abundance 52 – 48 per cent [1].

Recent years have shown notable progress in research and development of metal nanoparticles that takes expansion of their unique optical, magnetic, electronic, catalytic and other physicochemical properties in a wide range of practical and potential applications such as biomedical, chemical engineering, energy and environment [2].

As nanoparticles have great application in medical world like gene therapy, cancer therapy, drug delivery, etc., the medical world also easily accept the plant world for nanoparticle synthesis and welcome the angiosperms for their potentiality in synthesizing the non-polluted, environmentally acceptable, safety for human health nanoparticles [3]. Silver nanoparticles are of increasing interest since silver shows evidence of potent antibacterial properties with low toxicity for humans and animals by comparison with other heavy metals [4]. Silver has been proven to have a non toxic effect and has been found in applications ranging from traditional medicines to culinary items. Moreover, several salts of silver and their derivatives are commercially manufactured as antimicrobial agents [5].

The synthesis of metal nanoparticles using physical and chemical methods increases toxic effects to all living natures. Synthesis of silver nanoparticles has also been achieved through microbes and sea weeds. In addition there are several reports initiates the synthesis of silver nanoparticles using the plant parts of higher plants such as *Helianthus annus, Basella alba, Oryza sativa, Saccharum officinarum, Sorghum bicolor, Murraya koenigii, Citrullus colocynthis, Camellia sinensis, Carrica papaya, Chenopodium album, Enicostemma littorale, Cinnamomum camphora, Gliricidia sepium* and *Capsicum annuum* etc. Green synthesis of silver nanoparticles provides an alternative approach to chemical and physical methods as it is cost-effective and aesthetic manner. It does not involve use of high pressure, energy, temperature and toxic chemicals [6].

Due to the outbreak of the infectious diseases caused by pathogenic microbes and the development of antibiotic resistance, pharmaceutical companies and the researchers are searching for new antibacterial agents [7]. Thus, there is a immediate need to develop a green protocol using the leaves of higher plants for the synthesis of silver nanoparticles which does not produce any toxic substances and that can suitably scaled up for a large scale synthesis. The present study, report the synthesis of silver nanoparticles, reducing the silver ions present in the solution of silver nitrate by the cell free aqueous extract of *N. nucifera* root. Furthermore, these biologically synthesized nanoparticles and crude chloroform and aqueous extracts of *N. nucifera* were found to produce a high antibacterial activity. Hence, the present study is aimed to synthesize the silver nanoparticles in an eco-friendly manner using the roots of *N. nucifera* and characterize them in terms of their size, shape and distribution.

II.MATERIALS AND METHODS

Plant material and preparation of the extract:

The plant *N. nucifera* is belongs to the family Nymphaeaceae, collected from Siruvani in Tamil Nadu. The collected plants were carefully examined and identified in Botanical Survey of India, Coimbatore. Roots of *N. nucifera* were collected and kept for further study. The roots collected from wild and were shade dried and mechanically powdered after keeping them in an oven at 35º C for 24 hours. Aqueous dilution of the extract was prepared by fine crushing of the roots [2.85 gm with 50 ml sterile distilled water and boiled the extract between 55° - 65°C for 5 minutes. The extract was finally filtered by standard filtration method.

Qualitative phytochemical analysis:

The phytochemical tests was carried out using standard methods of analysis of carbohydrates, tannins, flavonoids, alkaloids, glycosides terpenoids, phenols, protein, amino acids and saponins.

a. Test for Carbohydrates

To 2ml of root extract, 1ml of Molisch's reagent and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates.

b. Test for Tannins

To 1ml of root extract, 2ml of 5% ferric chloride was added. Formation of dark blue or greenish black indicates the presence of tannins.

c. Test for Saponins

To 2ml of root extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15 minutes lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.

d. Test for Flavonoids

To 2ml of root extract, 1ml of 2N sodium hydroxide was added. Presence of yellow color indicates the presence of flavonoids.

e. Test for Alkaloids

To 2ml of root extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

f. Test for Glycosides

To 2ml of root extract, 3ml of chloroform and 10% ammonia solution was added. Formation of pink color indicates presence of glycosides.

g. Test for Terpenoids

To 0.5ml of root extract, 2ml of chloroform was added and few drops of concentrated sulphuric acid were added carefully. Formation of red brown color at the interface indicates presence of terpenoids.

h. Test for Phenols

To 1ml of the root extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols.

i. Test for proteins

The root extracts were dissolved in 10 ml of distilled water and filtered. The filtrate was mixed with biuret reagent. Appearance of peptide linkage of the molecule indicates presence of proteins.

j. Test for amino acids

Ninhydrin test: Test root extracts were dissolved in 10 ml of distilled water and filtered, 2ml of filtrate mixed with 2 drops of freshly prepared 0.2% of ninhydrin solution and heat. A characteristic purple color indicates the presence of amino acids.

Synthesis of Silver nanoparticles

$AgNO_3$ (10^{-3}M) solution was prepared by dissolving 0.016 gm of $AgNO_3$ into 100 ml distilled water and stored in dark conditions to avoid oxidation of Ag+ ions. 5:95 aqueous dilution of root extract in 10^{-3}M $AgNO_3$ was prepared by adding 95 ml of 10^{-3}M $AgNO_3$ to 5 ml of filtered root extract, into a standard flask with constant stirring and observed the color change. The standard flask was incubated at room temperature for 24 - 48 hours. The color change of the solution from yellow to dark brown indicated that the silver nanoparticles were synthesized.

UV-Vis spectra analysis

UV-Vis spectral analysis was done by using UV-Vis spectrophotometer, UV159 [Elico]. The reduction of pure silver ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 48 hours of incubation using small aliquot of the sample, along with the reference sample at 430 nm.

Effect of root extracts concentration on nanoparticle synthesis

To investigate the effect of root extract concentration for the synthesis of silver nanoparticles. The reduction mixtures with the ratios of 1:1,1:2,1:3,1:4,1:5,1:6,1:7,1:8, extract to 10^{-3} silver nitrate were prepared. The reduction mechanism of silver ions into nanoparticles was investigated through the UV-visible spectrophotometer analysis.

SEM Analysis of Ag Nanoparticle

Characterization of silver nanoparticle was done by using Scanning Electron Microscopy [SEM]. The sample was dried prior to SEM analysis. The 5:95 aqueous dilution of the root extract in 10^{-3}M $AgNO_3$ was centrifuged at 18,000 rpm for 25 minutes. The pellets were collected using petroleum ether and evaporated. After freeze-drying of the purified Ag nanoparticle, the structure, composition and average size of the synthesized Ag nanoparticles were analyzed by Hitachi S-4500 SEM machine.

Antibacterial activity of synthesized Ag nanoparticles

Aliquots of the chloroform, methanol and water extract [0.1 ml & 0.3 ml] were used to screen the antibacterial activity by the standard method. The antibacterial assay of Ag nanoparticles from root extract of *N. nucifera* was performed against various pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginos* and *Aeromonas hydrophila* by disc diffusion method on nutrient agar medium. Sterile filter paper discs [Whatmann No.1] were impregnated with different concentrations of the root extract. Gentamycin (2µg/disc) was used as the standard antibiotic. All the plates were then incubated for 24 hours at 37°C to determine the zone of inhibition by silver nanoparticles (mm).

III. RESULTS AND DISCUSSION

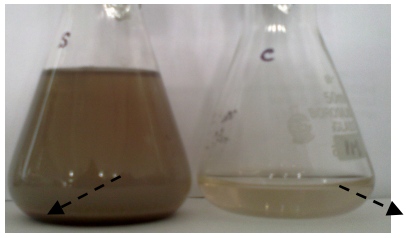
The result of phytochemical screening in *N. nucifera* reveals those alkaloids, tannins, glycosides, carbohydrates were present. Alkaloids were present in chloroform & water extracts of root and were absent in all other extracts. Tannins were present in methanol extracts of root. Carbohydrates were present in chloroform, methanol, and water extracts. Terpenoids and saponins were absent in chloroform, methanol, water extracts of root. Glycosides were present in chloroform & methanol extracts of root. Oil and fats were absent in all extracts of root. Hence the results showed the minimum number of secondary metabolites is present in the extracts of root.

Table 1 Phytochemical Analysis of bioactive compound in different solvent extracts of *N. nucifera*:

S.No	Solvents	Chloroform	Methanol	Water
1	Alkaloids	+	-	+
2	Tannins	-	+	-
3	Terpenoids	-	-	-
4	Glycosides	+	+	-
5	Phenols	-	-	-
6	Flavonoids	-	-	-
7	Saponins	-	-	-
8	Proteins	-	-	-
9	Carbohydrates	+	+	+
10	Fats & Oil	-	-	-

(+) positive, (-) negative

The preliminary analysis [Table 1] shows that the presence of secondary metabolites is much valuable, similarly [8] has pointed out the tannins are used as a medicine in the treatment of cough, asthma and fever. The presence of terpenoids exposed that the plants can act as a growth inhibitor and possesses considerable toxicity toward insects [9]. It should be noted that steroidal compounds are plays significant role in pharmaceutical industries due to their relationship with such compounds as sex hormones. The presence of steroidal compounds in plants is an indication that the plants can be used expectant mothers or breast feeding mother to ensure their hormonal balance, since steroidal structure could serve as potent starting material in synthesis of these hormones [10].



(b) (a)

Figure 1 Silver nanoparticle synthesis from *N. nucifera* root
(a) Before colour formation (b) After colour formation

From the formation peaks shows [Fig 3] silver nanoparticles present in the methanol extract the reaction media has absorbance peak at 450-550nm as like Papaya fruit extract have the peak at 450nm [11]. Similarly the silver nanoparticles from unexploited weed *Trianthema decandra* also have the absorbance peak between 420-450nm [12]. There was no peak is observed in the case of blank solutions which indicates, the absence of silver nanoparticles. Further the experiment was made twice for its concomitant value, it is observed that the silver nanoparticles are may be present only in the methanol extracts, due to their colour change [Fig 1] and readings of spectra analysis within the boundary of 450-650nm. [(Fig 2 and Fig 3] showed the photographs of the reaction mixture of *N.nucifera* root extract and silver nitrate solution as a function of time. Within 15 min reaction time, a visible color change from transparent to yellow indicates the formation of silver nanoparticles which was confirmed by UV-visible analysis. Further the color change to dark yellow was due to increased concentration as well as growth of silver nanoparticles. After 90 minutes there was no significant colour change, which is evidence for the completion of reduction reaction. Fig 3 shows the results of the UV- visible spectrophotometer scan at various time intervals. The peak occurs at 400nm - 420nm (λ max) which corresponds to the absorbance of silver nanoparticles [13]. The intensity of the peak at 415nm was increased with time until the reduction completes. The observed results at time frequency indicates an increased rate of silver nanoparticles formation and a higher final concentration of nanoparticles achieved after 3 hours.

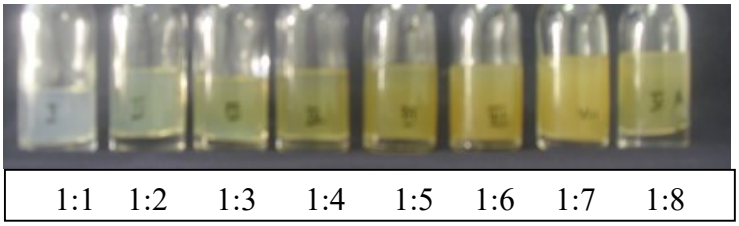


Figure 2 Effect of root extracts concentration on nanoparticle synthesis

The green synthesized silver nanoparticles by employing *N.nucifera* root extract as reducing agent was further demonstrated and confirmed by the structural view under the Scanning Electron Microscope (SEM).The formation of silver nanoparicles as well as their morphological dimensions in the Scanning Electron Microscope study demonstrated that the average size of silver nanoparticles synthesized in methanol extract was around 0.2 to 1 µm size (Fig 4). The shapes of nanoparticles were relatively spherical and cubic. In *Carica papaya* and *Trianthema decandra* Scanning Electron Microscope images shows the nanoparticles present in 10nm – 50nm and the shapes of nanoparticles are relatively spherical, cubic, hexagonal respectively [14,15]. Many reports are well documented on the biosynthesis of silver nanoparticles using several plant extracts. It is reported that silver nanoparticles were synthesized using stem bark of *Boswellia ovalifoliolata* [16]*; Aloe vera* [17]*;* leaf extract of *phyllanthus embica* [18] *Cinnamomum camphora* [19]*; Parthenium* [20]*; Acalypha* [21] *and Sesbania grandiflora* [22].

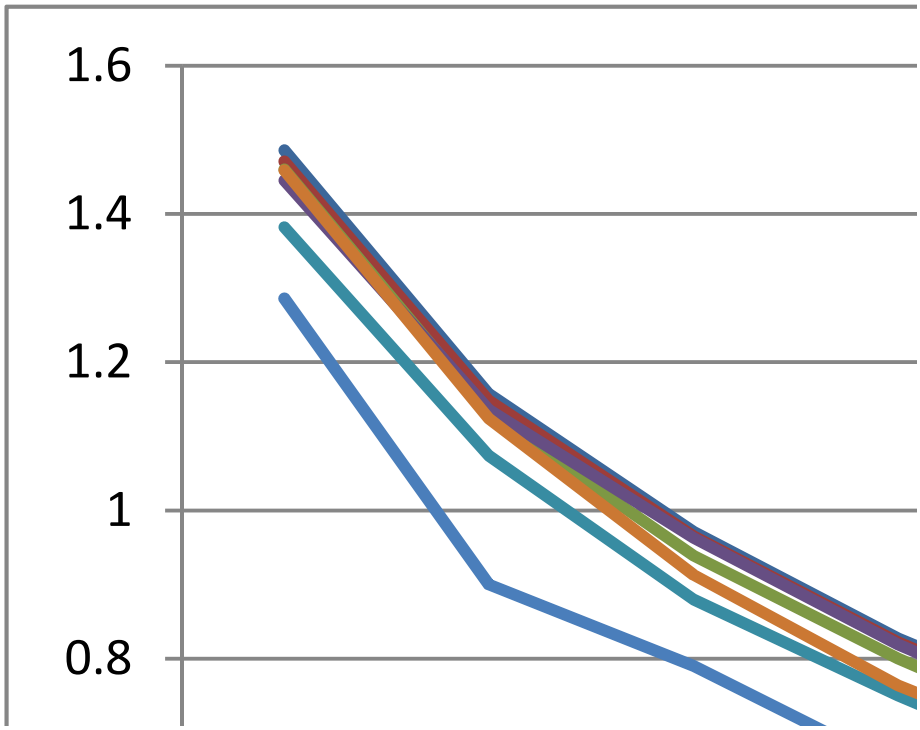


Figure 3 UV- visible spectrophotometer scan at various time intervals for the effect of root extract concentration on nanoparticles synthesis

N. nucifera root extracts were used to investigate antimicrobial activity of five bacterial isolates namely *Staphylococcus aureus*, *Bacillus cereus, Pseudomonas aeruginosa, Aeromonas hydrophila* and *Escherichia coli. N. nucifera* exhibited high activity against all the tested micro- organisms except *B.cereus*. The result showed *N. nucifera* exhibited high activity against *E. coli*, moderate activity against *S. aureus*, and low activity in *Pseudomonas aeruginosa, Aeromonas hydrophila* and no more activity in *B.cereus* (Fig 6).The inhibitory effects of root extracts were very close and identical in magnitude and are comparable with the standard antibiotics. Likewise the synthesized silver nanoparticles are generally found to be effective as antimicrobial agents against some important human pathogens. It's isolated from rhizome extract of *Discorea oppositifolio* shows highest zones of inhibition is observed against *S.typhi, E.coli, Bacillus cereus, Enterococus faecolis* [23]. The results shows that it was concordance with the findings of [24]. Similar results were obtained from stem bark extract of *Adansonia digitata* shows highest inhibiting effect on *E. coli, P. aeruginosa, B. subtilis* and *S. aureus* as confirmed by the diameter of zone of inhibition [25]. [26] Produced highly stabilized silver nanoparticles (25–40 nm) using a leaf extract of *O. tenuiflorum*. The nanoparticles shows antimicrobial activity against human pathogens such as B. cereus, *S. aureus, A. hydrophilla, S. typhimurium*. Gram positive bacterium *S. aureus* shows highest activity compared to Gram negative bacterium *Aeromonas hydrophila* in silver nanoparticles synthesized by *P. guajava* leaf extract [27].

Table 2 Antibacterial activity of silver nanoparticles *N. nucifera* root extract

S.No	Conc (µg)	Zone of inhibition(mm)				
		S.a	B.c	E.c	P.a	A.h
1	500	-	-	-	-	-
2	1000	10.0 ± 0.0	-	10.0 ± 0.0	10.0 ± 0.0	-
3	1500	10.0 ± 0.0	-	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0

4	2000	10.0 ± 0.0	-	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0

Values are expressed in Mean ± SD (n=3)

Note: ***S. a –*** *Staphylococcus aureus,* ***B. c*** *– Bacillus cereus,* ***E. c*** *– Escherichia coli,* ***P. a*** *– Pseudomonas aeruginosa,* ***A. h*** *– Aeromonas hydrophila*

They found to have maximum zone of inhibition against *E.coli, Pseudomonas.* They also concludes *cassia tora* plant extract with aqueous and synthesis of silver nanoparticles can be used as antimicrobial agent and also used as herbal medicine for curing of disease in the form of pellets of paste. The present study emphasizes the use of plants synthesis of silver nanoparticles with potent antimicrobial activity.

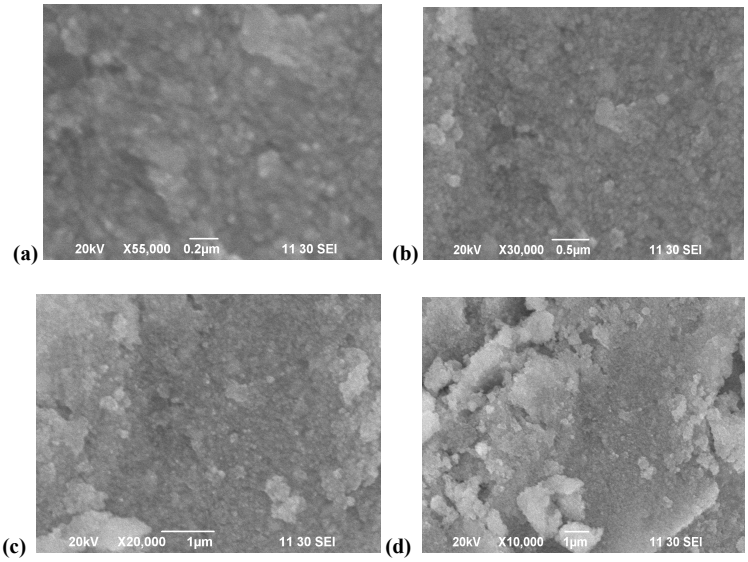


Figure 4 Scanning Electron Microscope view of silver nanoparticles from methanol extract
(a). 0.2 µm in size at X 55,000., (b). 0.5 µm in size at X 30,000., (c). 1.0 µm in size at X 20,000., (d). 1.0 µm in size at X 10,000.

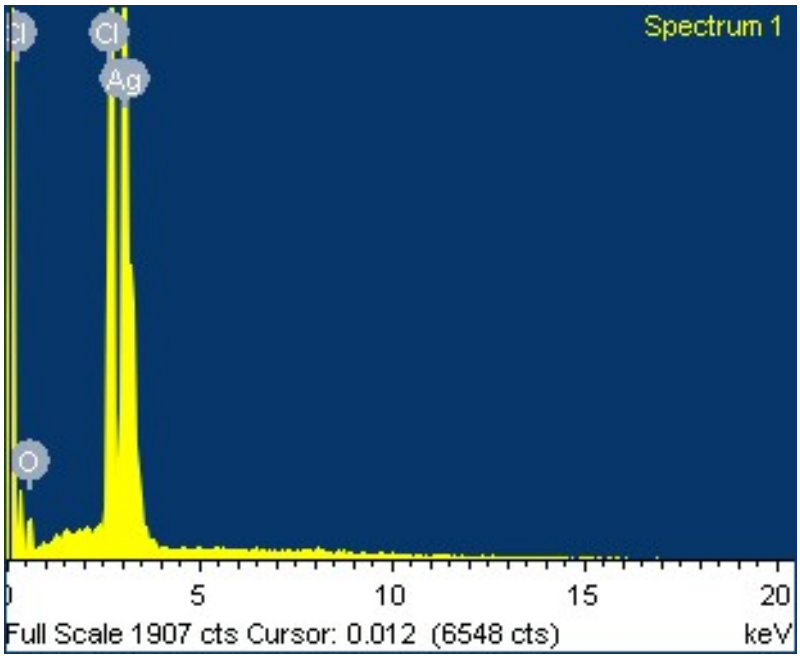


Figure 5 Elementary analysis of silver nanoparticles synthesized from methanol extract.

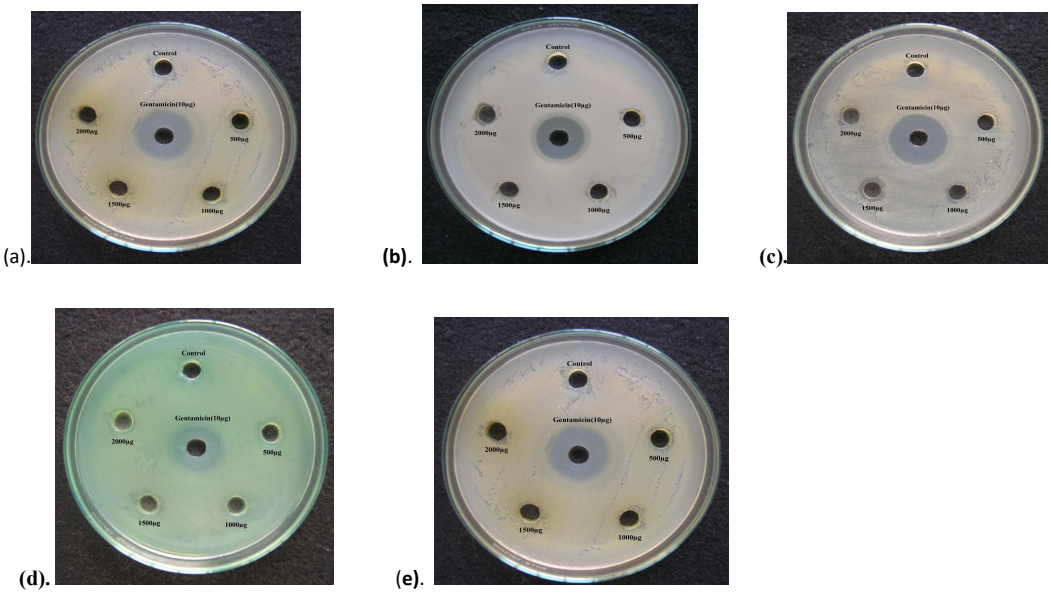


Fig 6 Antibacterial activity of silver nanoparticles *N.nucifera* root extract

(a). Staphylococcus aureus, (b). Bacillus cereus, (c). Escherichia coli,
(d). Pseudomonas aeruginosa, (e). Aeromonas hydrophila

IV. CONCLUSION

The present investigation reveals a simple biological and low-cost approach for preparation of stable silver nanoparticles by reduction of silver nitrate solution with a bio reduction method. Silver nanoparticles synthesized using *N.nucifera* possess an effective antibacterial property against *E. coli* and *B.subtilis* and low activity in *Pseudomonas aeruginosa, Aeromonas hydrophila* and no more activity in *B.cereus.* Hence, the present study emphasizes the use of medicinal plants for the synthesis of silver nanoparticles with potent antibacterial effect, against both gram negative and gram positive bacteria. These results shed the light on the ability of the root extract to use them as a potential source for production of antibacterial drug with a broad spectrum of activity

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