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Phytofabrication of silver nanoparticles using Horse Gram (*Dolichos biflorus* L.) seed extract and assessment of its bactericidal and antioxidant activities

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Abstract

The silver nanoparticles synthesized by phytofabrication method receive bigger attention due to their fascinating properties. This study deals with the green mediated synthesis of silver nanoparticles using seed extract of horse gram (*Dolichos biflorus* L.) and assessment of their bactericidal and antioxidant activities. The green synthesized silver nanoparticles were characterized by UV–Vis absorption spectroscopy, FTIR, EDAX, XRD and SEM. In UV- vis spectroscopic analysis, a strong peak was observed at 442 nm. The XRD pattern showed that the silver nanoparticles were crystalline nature. The average estimated particle size of the sample was 29.09 nm. Scanning Electron Microscopy (SEM) revealed that the silver nanoparticles were cubic in shape. FTIR analysis showed the possible functional groups involved in the AgNPs formation. The EDAX result exhibited a large peak of silver which confirmed its presence in the suspension. Further, the silver nanoparticles were evaluated for their antibacterial efficacy against different human pathogens by standard well diffusion method. The highest antibacterial activity was observed against *Bacillus subtilis* followed by *Pseudomonas aeruginosa*, *Klebsiella* sp., *E. coli*, *Staphylococcus aureus* and no activity was noticed against *Proteus* sp. The percentage inhibition of free radicals increased with increase in concentration of substrates. The 60 µl concentration of nanoparticles showed 99% of scavenging activity. The present study suggests that plant mediated silver nanoparticle synthesis using horse gram seed extract is quick with potential bactericidal and anti-oxidant activities.

Keywords: Silver nanoparticles, *Dolichos biflorus*, seeds, Phytofabrication, Bactericidal activity, Antioxidant activity

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Introduction

Nanoparticles are particles lesser than 100 nm in diameter that exhibit new and increased size-dependent properties compared to their bulk material [1]. These are the fundamental units of nanotechnology. Living cells are acting as machines that operate at the nano-level and performing various jobs at terribly high potency [2]. In nanotechnology, nanoparticles research is an important aspect due to its huge applications. Among the various inorganic metal nanoparticles, silver nanoparticles have received additional attention due to their strong antimicrobial and anticancer, pesticidal, antioxidant and wounds and burns healing activities. They are used in paints, contraceptive agent, water disinfectant and room

spray [3, 4]. Therefore the research on silver nanoparticle synthesis and assessment of its activity is growing quicker.

There are two approaches of nanoparticle synthesis viz., top-down approach (Physical method) and bottom-up approach (Chemical and Biological methods). The physical and chemical strategies of nanoparticle synthesis are bound with various limitations like expensive, generation of hazardous toxic chemicals etc., which urged the researchers to develop environmentally safe alternative approaches to synthesize nanoparticles. The biological systems have been focused and exploited as a preferred source of green principle process for the synthesis of nanoparticles. There are amble evidences to indicate the efficiency of biological systems for the production of silver nanoparticles.

The biological method of nanoparticle synthesis employs the use of biological agents like bacterium, yeast, fungi, actinomycetes, algae and plants thereby providing an array of resources for the synthesis of nanoparticles [5]. Microbial mediated nanoparticle synthesis is environmentally benign and compatible with the use of the product for medical applications; but the production of microorganisms are more expensive and laborious than the production of plant extracts [4]. The advantage of using plants for the synthesis of nanoparticles is that they are readily and commonly available; most of them possess medicinal value, safe to handle and contain a broad variability of metabolites that may aid in reduction. Moreover, the plant mediated synthesis is an advancement of bioscience, high- yielding, low cost technology and non-toxic to vertebrate animals [6]. Therefore, plants are considered as nanofactories due to their ability to function as reducing agents and stabilizing agents in the synthesis of silver nanoparticles [7, 4]. The literature to date revealed that all the components of the plants like the extracts of leaf, flower, bark, fruit, root and seed [8-20] are used for silver nanoparticle synthesis [4].

Horse gram seeds are useful in lowering cholesterol level, controlling skin rashes and boils, and treatment of kidney stones. Further, these are good source of antioxidants [21]. Hitherto, there is no evidence for the phytofabrication of silver nanoparticles using horse gram (*Dolichos biflorus* L.) seeds. Hence the present study was undertaken to synthesize silver nanoparticle using these seeds and to evaluate its antibacterial and antioxidant activities.

Materials and Methods

Materials

Silver nitrate and Nutrient agar were purchased from Hi-Media, India. Seeds of *Dolichos biflorus* (Fig. 1) were collected from local market at Sivakasi, Tamil Nadu, India. The microbial strains such as *Pseudomonas aeruginosa*, *Klebsiella* sp., *Proteus* sp., *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* were obtained from Microbial type culture collection (MTCC), Pune, India.

Figure 1 *Dolichos biflorus* seeds used in the synthesis of silver nanoparticles

Preparation of seed broth

Fresh *Dolichos biflorus* seeds were thoroughly washed with distilled water and dried at 37 ºC. 10 gram of dried seeds was taken and added with 100 ml distilled water. The contents were boiled at 60 ºC for 15 minutes. The resulting crude extract was filtered through Whatmann filter paper no.1 and stored at -20 ºC for further study.

Green synthesis of silver nanoparticles

10 ml of *Dolichos biflorus* seed extract was added with 90 ml of 1 mM silver nitrate for reduction into Ag^+ ions and kept in magnetic stirrer for 5 hrs at room temperature. The colour of the solution turned from light brown to dark brown.

UV-visible spectroscopy

The sample was diluted 20 times with distilled water. The formation of silver nanoparticles by the reduction of silver ions was monitored by measuring the UV-Visible absorption of the solution. The spectra were recorded from 300 to 700 nm against distilled water as blank.

Recovery of silver nanoparticles

The silver nanoparticles solution was purified by centrifugation at 1000 rpm for 10 min. Then the pellet was washed with Millipore water. The same process was repeated thrice to obtain pure nanoparticles.

Characterization of silver nanoparticles

The purified silver nanoparticles were further characterized by SEM, XRD, EDAX and FTIR.

Bactericidal activity

Agar well diffusion method was followed to evaluate the bactericidal activity of the synthesized silver nanoparticles. A known volume of the seed extract, synthesized nanoparticles and $AgNO_3$ (1mM) were loaded in separate wells. After incubation at 37°C for 24 hours, the diameter of zone of growth inhibition around the well was measured and recorded.

Antioxidant assay

Free radical scavenging activity was measured by DPPH method [21]. Briefly, 4 mg of DPPH was dissolved in 100 ml ethanol. Then 0.5 ml of sample solution was added with 1 ml of DPPH solution and incubated for 1 hr at room temperature in dark condition. The absorbance was measured at 517 nm and the % inhibition of DPPH radical was calculated by the following formula:

DPPH scavenging (%) = Ac – As/Ac x 100

Where, Ac- is absorbance of the control and As- is for the test samples.

Results

The synthesis of Ag nanoparticles was confirmed by visible observation of the colour change in the reaction mixture. The colour of reaction mixture changed from light brown to dark brown within 15 mins of incubation. It indicates the production of silver nanoparticles in the reaction mixture.

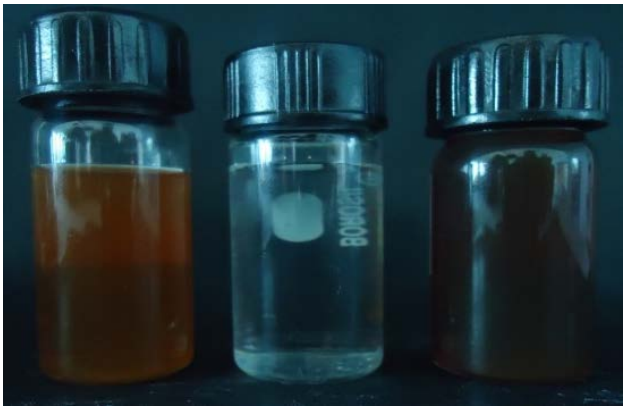

Figure 2 A. *Dolidhos biflorus* seed extract, B. 1mM $AgNO_3$ without seed extract and C. 1mM $AgNO_3$ with seed extract after 15 mins of incubation

The synthesis of silver nanoparticles was further confirmed by UV-visible spectroscopy. It is widely used for the verification of nanoparticle synthesis in reaction mixture. The observation of peak at 442 nm in the UV spectrum (Fig. 3) confirmed the formation of silver nanoparticles. This is characteristic to silver nanoparticles. The broadening of peak indicated that the synthesized naoparticles were polydispersed.

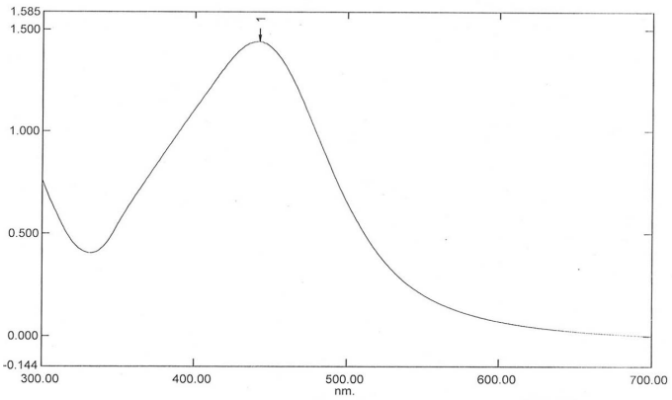

Figure 3 UV-Vis absorption spectrum of silver nanoparticles synthesized from seed extract of *Dolichos biflorus*

In XRD pattern, peaks with different intensity were observed.

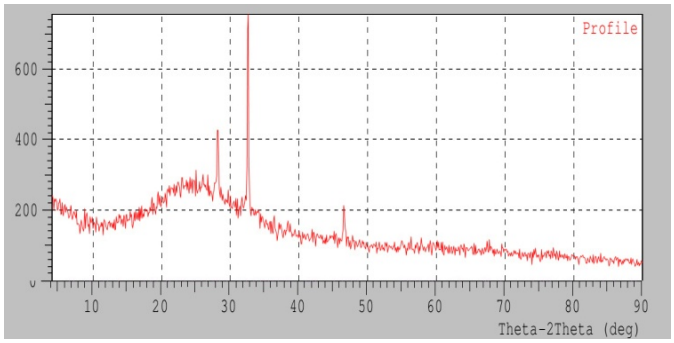

Figure 4 XRD patterns of synthesized silver nanoparticles

The 2θ values were noticed at 28.1º, 32.5º and 46.5º. The XRD pattern showed intense peaks in the whole spectrum of 2θ values ranging from 20 to 32. The average estimated particle size of the sample was 29.09 nm. SEM images revealed the shapes of the silver nanoparticles which were found to be cubic in nature.

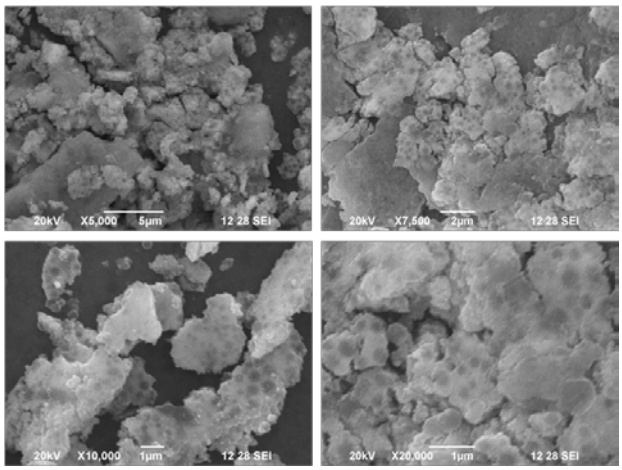

Figure 5 SEM image of synthesized silver nanoparticles

The EDAX analysis also confirmed the presence of silver nanoparticles synthesized using seed extract. The bactericidal assay was carried out by standard well diffusion method. The activity was shown to be higher with silver nanoparticles than the silver nitrate solution and seed extract (Fig. 8; Table 1). 50 µl concentration of silver nanoparticle was effective against the tested bacterial strains. The highest antibacterial activity was observed against *B. subtilis* which was followed by *P. aeruginosa*, *Klebsiella* sp., *E. coli*, *S. aureus* and no activity was detected against *Proteus* sp.

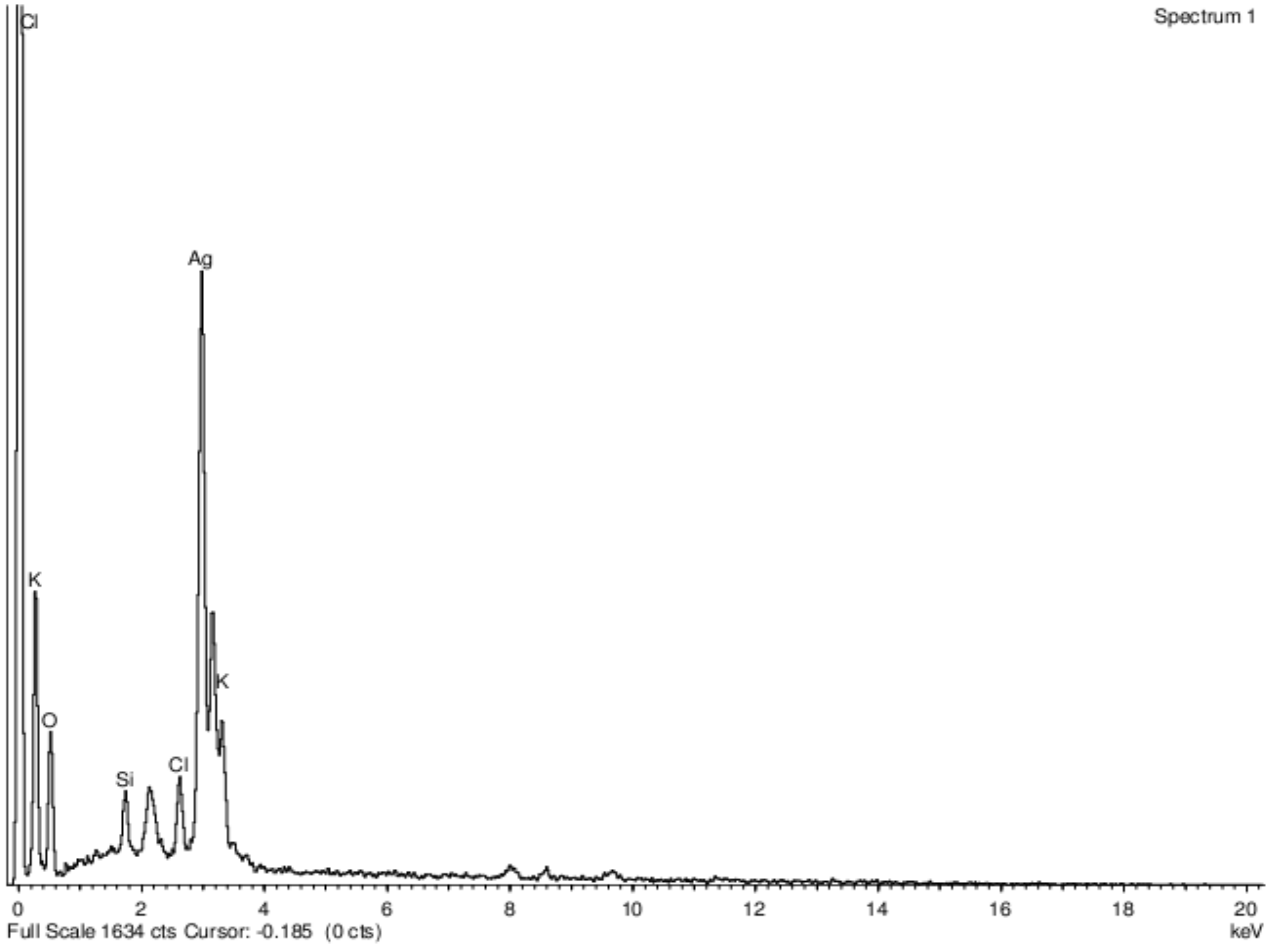

Figure 6 EDAX spectra of synthesized silver nanoparticles

The FTIR spectrum (Fig. 7) of synthesized silver nanoparticles revealed the presence of functional groups like 1043.49 cm^{-1} (C-O stretch), 437.84 cm^{-1}(C-I stretch), 451.34 cm^{-1} (C-I stretch) , 503.42 cm^{-1} (C-Br stretch) and 675.09 cm^{-1} (C-Br stretch) .

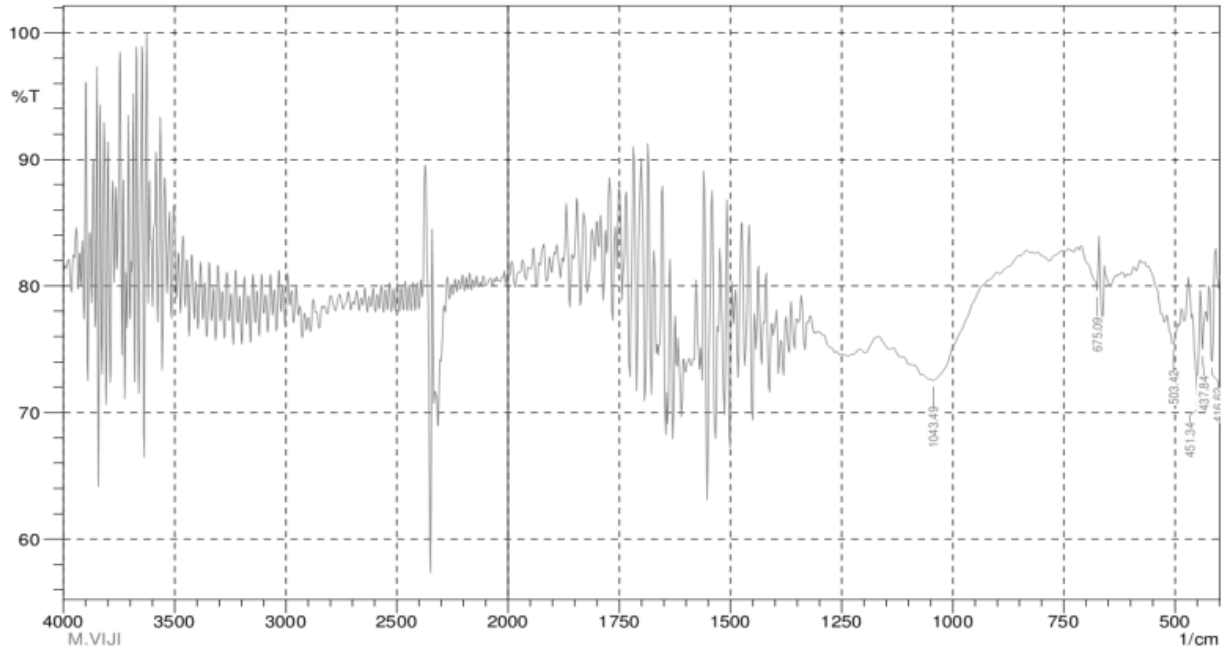

Figure 7 FTIR spectrum of silver nanoparticles synthesized using *Dolichos biflorus* seed extract

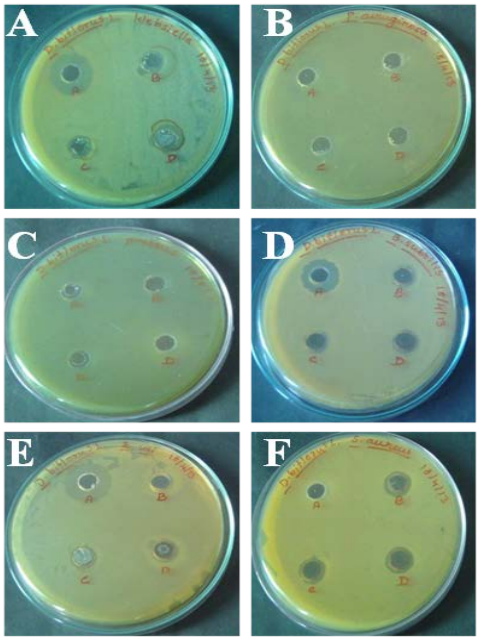

Figure 8 Bactericidal activity of different concentrations of synthesized silver nanoparticles against following human pathogens: A) *Klebsiella* sp., B) *Pseudomonas aeruginosa*, C) *Proteus* sp., D) *Bacillus subtilis*, E) *Escherichia coli* F) *S. aureus* (Inside the plate A- 50 µl Silver nanoparticle solution; B-10 µl Silver nanoparticle solution; 50 µl of Silver nitrate; 50 µl of seed extract)

Table 1 Bactericidal activity of synthesized silver nanoparticles using seed extract of *Dolichos biflorus* against pathogens

Bacterial strains	Silver nitrate (mm)	Seed Extract (mm)	Nanoparticle (50µl) (mm)
B. subtilis	11.66±0.30^{ab}	12.41±0.43^{ab}	18.75±0.34^{a}
Klebsiella sp.	5.50±0.28^{c}	13.16±0.25^{b}	15.25±0.40^{a}
S. aureus	14.16±0.64^{ab}	11.49±0.17^{b}	15.20±0.45^{a}
P. aeruginosa	13.69±0.27^{b}	13.41±0.31^{b}	17.37±0.19^{a}
E. coli	12.87±0.25^{b}	12.55±0.14^{b}	15.62±0.8^{a}
Proteus sp.	0.00±0.00	0.00±0.00	0.00±0.00

Values are expressed in Mean ± SE. Means in the same vertical column that are not marked with the same superscript (alphabets) letters are significantly different at a = 0.5 level (Duncan's test).

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay showed potent inhibitory capacity of synthesized silver nanoparticles when compared with ascorbic acid at higher concentrations (Table 2).

Table 2 Percentage of antioxidant activity of ascorbic acid and silver nanoparticles

Concentration of synthesized nanoparticles (µl)	Scavenging activity for ascorbic acid (%)	Scavenging activity for synthesized nanoparticles (%)
10	45.84	91.72
20	46.28	92.64
30	47.20	93.80
40	47.44	95.36
50	47.92	97.08
60	48.16	99.04

The percentage of inhibition of free radicals increased with increase in concentration of silver nanoparticles.

Discussion

Biomediated synthesis of silver nanoparticles overcomes the demerits of chemical and physical mediated approaches. Among the biological methods, plant mediated synthesis is ideal due to their easy availability, medicinal properties, safe to handle, existence and variability of biomolecules for early reduction [6]. The present study was carried out to synthesize silver nanoparticles using seed extract of horse gram (*Dolichos biflorus* L.). The appearance of brown colour in the reaction mixture was the primary confirmation for the synthesis of silver nanoparticles.

UV-spectroscopy is very helpful for further confirmation of silver nanoparticle synthesis in the reaction mixture. The wavelength in the 300-800 nm range is typically used for the characterization of various metal nanoparticles within the size range of 2 to 100 nm [22]. In the present study, the peak was observed at 442 nm. It indicates the successful reduction of silver nanoparticles which may be attributed to the biomolecules present in the horse gram seeds.

SEM is used for the morphological characterization of molecules at the nanometer to micrometer scale [23]. In this study, the SEM results revealed the shape of silver nanoparticles as cubic in nature. It is evident from earlier reports that the silver nanoparticles produced using papaya fruit extract [24] and *Cassia auriculata* flower extract [13] were found to be cubes.

XRD analysis was employed for the phase identification and characterization of the crystal structure of the nanoparticles [25]. XRD results showed that the average size of the silver nanoparticle was 29.09 nm. Chauhan *et al.* (2011) [26] also obtained similar results in which the XRD pattern revealed two intensive peaks in the spectrum of 2θ value ranging from 10-50.

The FTIR spectroscopic analysis is useful in the characterization of the surface chemistry of synthesized silver nanoparticles [27]. It is also very effective in the

identification of possible biomolecules responsible for capping and efficient stabilization of silver nanoparticles by plant extract [28]. In the present study, the identified functional groups might be responsible for capping and stabilization of synthesized silver nanoparticles.

As another phase of the present study, the bactericidal activity of synthesized silver nanoparticles was also evaluated. The highest bactericidal activity was noticed against *B. subtilis*. It is presumed that the selectivity of microorganisms differs owing to the presence of capping agents and stabilizers employed in the synthesis of silver nanoparticles [29]. In the present study, five functional groups were identified in the silver nanoparticles which may be responsible for bactericidal activity. The precise antimicrobial action of silver nanoparticles remains unknown. It is believed that bactericidal activity could also be owing to interaction of nanoparticles with bacterial plasma membrane, bacterial DNA, bacterial proteins and inflicting cellular injury [11, 30]. Inside a bacterium, nanoparticle will interact with DNA thereby losing its ability to replicate which can cause the cell death [31]. It is also believed that silver nanoparticles could amend the membrane structure of microbes which results in the increased membrane permeability of the bacteria leading to cell death [12, 32]. This study also confirms the bactericidal activity of nanoparticles against pathogenic bacteria.

The synthesized nanoparticles exhibited antioxidant effect also. The concentrations above 60 µl of synthesized nanoparticles may serve as potent antioxidants. If antioxidants are present in the medium, the purple colour disappeared. This is due to the antioxidant ability of silver nanoparticles. It is well documented that antioxidant molecules can quench DPPH free radicals and convert them to a colourless product. It gains support from the antioxidant properties of silver nanoparticles synthesized using seed of *Syzygium cumini* [33]. Therefore, the nanoparticle synthesized from seeds of *Dolichos biflorus* extract can be used as a strong bactericidal agent and potential free radical scavengers.

Conclusion

For the first time, the present study demonstrates the phytofabrication of silver nanoparticles using seed extracts of *Dolichos biflorus*. The characterization studies have proved the silver nanoparticle synthesis. The silver nanoparticles exhibited bactericidal activity against various human pathogens. Further, nanoparticles exhibited *in vitro* free radical scavenging activity which may be helpful to protect the cells from oxidative stress due to the free radicals. The detailed study would pave the way to fabricate new pharmaceutical products from these silver nanoparticles.

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