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Original Article

RAPID SYNTHESIS OF SILVER NANOPARTICLES USING AQUEOUS LEAF EXTRACT OF *ACHYRANTHES ASPERA* AND STUDY OF THEIR ANTIMICROBIAL AND FREE RADICAL SCAVENGING ACTIVITIES

PUSHPALATHA BOBBU[1], VASUDEVA REDDY NETALA[1], AISHWARYA S[1], INDIREDDY RAMA MANOHAR REDDY[1], VENKATA SUBBAAH KOTAKADI[2], VIJAYA TARTTE[3*]

[1]Deparment of Biotechnology, Sri Venkateswara University, Tirupati, A. P, India, [2]DST-PURSE Center, Sri Venkateswara University, Tirupati, A. P, India, [3]Deparment of Botany, Sri Venkateswara University, Tirupati, A. P, India
Email: tvijayasvu@yahoo.com

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ABSTRACT

Objective: To study the biosynthesis of silver nanoparticles (AgNPs) using the aqueous leaf extract of *Achyranthes aspera* and check the antimicrobial and free radical scavenging activity of the biosynthesized AgNPs

Methods: 20 ml of aqueous leaf extract of *A. aspera* was added to 80 ml of 2 mM silver nitrate and the reaction solution was heated at 55-60 °C for 20 min and incubated. Biosynthesized AgNPs were characterized by different spectroscopic measurements including UV-Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction pattern (XRD), Transmission electron microscopy (TEM) and Dynamic light scattering (DLS). Antimicrobial activity of AgNPs was checked against *Staphylococcus aureus, Bacillus subtilis* (Gram+ve bacteria), *Klebsiella pneumonia, Pseudomonas aeruginosa* (Gram-ve bacteria), *Aspergillus niger, Candida albicans* and *Candida nonalbicans* (Human pathogenic fungi) by employing disc diffusion method. The free radical scavenging activity of AgNPs was checked against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Hydrogen peroxide (H_2O_2) radicals.

Results: After 1 h of incubation, the light yellow color of the reaction solution was turned to dark brown. UV-Vis spectra showed the absorption peak at 445 nm and confirmed the synthesis of AgNPs. FTIR spectra revealed the functional groups plausibly involved in the biosynthesis and stabilization of AgNPs. XRD pattern revealed that the synthesized AgNPs were crystalline in nature with face-centered cubic (FCC) phase. TEM revealed that the synthesized AgNPs were spherical in shape with 20-40 nm in size. DLS analysis revealed that the average size of AgNPs was 24.5 nm. Biosynthesized AgNPs were highly stable due to their high negative zeta potential value of-28.1 mV. AgNPs showed effective antimicrobial activity against *S. aureus* (16.4 mm), *B. subtilis* (14.5 mm), *K. pneumonia* (13.2 mm), *P. aeruginosa* (12.4 mm), *A. niger* (12.2 mm), *C. albicans* (11.5 mm) and *C. nonalbicans* (11.8 mm). AgNPs showed effective free radical scavenging activity with IC_{50} values of 77.73 and 90.53 µg/ml respectively against DPPH and H_2O_2 radicals.

Conclusion: Successful and rapid synthesis of AgNPs was achieved using aqueous leaf extract of *A. aspera*. Biosynthesized AgNPs were proved to be excellent antimicrobial agents and free radical scavengers.

Keywords: *Achyranthes aspera*, Silver nanoparticles, TEM, DLS, Antimicrobial and Radical scavenging activity

INTRODUCTION

Metal nanoparticles attracted researchers, a considerable attention due to their unique physicochemical properties including surface plasmon resonance phenomenon, large surface area to volume ratio and reduced imperfections [1, 2]. Owing to their unique physicochemical properties, metal nanoparticles, particularly silver nanoparticles (AgNPs) found to possess wide variety of applications including optical receptors [3], intercalation materials in batteries [4], catalysts in chemical reactions [5], sensors [6], gene and drug delivery systems [7], diagnostic and biolabelling agents [8], cytotoxic [9], antioxidant [10] and antimicrobial agents [11-13].

Different physical and chemical methods including UV, γ-radiation assisted thermal decomposition, laser ablations, lithographic, polyaniline, polyal, photochemical, sonochemical and electrochemical methods have been reported for the synthesis of AgNPs. Most of these methods involve hazardous processes and toxic chemicals as well as very expensive [14]. Nanobiotechnology involves the application of biological entities derived from small prokaryotes to higher eukaryotes for the synthesis of AgNPs. Isoprenes to terpenoids, Peptides to proteins, polyphenols, and many other secondary metabolites involve in the bioreduction and stabilization of AgNPs. Whereas, plant assisted approaches involve simple, low cost, eco-friendly and rapid procedures. Plants containing different proteins and secondary metabolites such as alkaloids, quinines, flavonoids, terpenoids and saponins [15, 16] that are involved in the synthesis and stabilization of AgNPs. Different plant extracts including *Cassia alata* [17], *Catharanthus roseus* [18], *Centella asiatica* [11], *Gymnema sylvestre* [12] and *Melia dubia* [13] have been reported for the biosynthesis of AgNPs. The present study reports the rapid synthesis of AgNPs using aqueous leaf extract of *A. aspera*. Characterization of AgNPs was done using UV-Vis, FTIR, XRD, TEM and DLS techniques. The biological importance of the AgNPs was proved by antimicrobial and free radical scavenging assays.

MATERIALS AND METHODS

Biosynthesis of AgNPs

Leaves of the *A. aspera* were collected from Sri Venkateswara University Campus and authenticated with Taxonomist and voucher specimen (SVUBHBPL-0116) was deposited in the Herbarium, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. Leaves were washed 2-3 times with sterile double distilled water (SDDW). 5 g of wet leaves were cut into fine pieces, taken into 50 ml of SDDW and boiled at 55-60 °C for 20 min, cooled and filtered. The filtrate collected was used for the biosynthesis of AgNPs. 20 ml of leaf filtrate was added to 80 ml of 2 mM silver nitrate ($AgNO_3$) and incubated in the dark at room temperature. After 1 h incubation of reaction solution, biosynthesis of AgNPs was observed visually.

Characterization of AgNPs

UV-Vis spectra (Analytical technologies ltd, India) was recorded between 200-800 nm to confirm the biosynthesis of AgNPs. FTIR

spectra (ALPHA interferometer; Make: Bruker, Germany) was carried out between 500-4000 cm^{-1} to reveal the plant metabolites that plausibly involved in the synthesis and stabilization of AgNPs. XRD pattern (Rigaku, Tokyo operated at 40 kV and Cu Kα as a radiation source) was recorded to determine the crystalline nature of AgNPs. TEM analysis (FEI Tecnai F12, Philips, and Holland) was performed to determine the size and shape of the AgNPs synthesized. Particle size distribution and Zeta potential measurements were carried out using DLS (Horiba, Nanopartica) technique.

Antimicrobial activity

Antibacterial activity of AgNPs was carried out against *S. aureus, B. subtilis* (Gram+ve) and *K. pneumonia, P. aeruginosa* (Gram-ve) by using disc diffusion method [19]. 200 µl of bacterial inoculum was swabbed on the surface of nutrient agar (NA) plates. Each NA plate consists of three sterile paper discs. One disc containing 50 µl aqueous leaf extract, the second disc containing 50 µl solutions of AgNPs. Third disc containing standard antibiotic (Streptomycin). Plates were incubated for 24 h at 38 °C to observe the inhibition zones. The diameter of inhibition zones was measured.

Antifungal activity of AgNPs was checked against *A. niger, C. albicans* and *C. nonalbicans* by employing disc diffusion method. 200 µl of fungal inoculum was spreaded on the surface of Potato dextrose agar (PDA) plates. Each PDA plate comprises of three sterile paper discs. One disc containing 50 µl aqueous leaf extract, the second disc containing 50 µl of AgNPs solution. Third disc containing standard fungicide (Voriconazole). Plates were incubated for 72 h at 24 °C to observe the inhibition zones.

Free radical scavenging activity

Free radical scavenging activity (RSA) of AgNPs was checked against both DPPH and H_2O_2 radicals. DPPH scavenging assay was performed according to the method of Aluru *et al.* [20]. 1 ml of a methanolic solution containing test samples of different concentrations (10, 20, 40, 60, 80 and 100 µg/ml) was added to 2 ml of a methanolic solution containing DPPH radical. The reaction solution was incubated at room temperature for 45 min. After incubation, the absorption of the reaction solution was measured at 517 nm. To further confirm the RSA of the AgNPs, H_2O_2 radical scavenging assay was performed according to the method of patel *et al.* [21]. Ascorbic acid was used as positive control in both assays. RSA was calculated using % RSA = [(Ac-As)/Ac] x 100. Where Ac is the absorbance of the control and As is the absorbance of the sample. IC_{50} values are determined using linear regression curves.

RESULTS AND DISCUSSION

Biosynthesis of AgNPs

After incubation, the reaction solution containing $AgNO_3$ and aqueous leaf extract turned from light yellow to dark brown color (fig. 1). The color change detected the formation of AgNPs in the solution called colloidal solution of AgNPs.

Fig. 1: Light yellow color of the reaction solution containing 2 mM $AgNO_3$ and aqueous leaf extract of *A. aspera* changes to dark brown color after 1 h incubation

UV-Vis analysis

UV-Vis analysis of the colloidal solution of AgNPs was carried out to confirm further the biosynthesis of AgNPs by aqueous leaf extract. UV-Vis spectra showed the characteristic absorption peak of AgNPs at 445 nm (fig. 2). The peak is due to surface plasmon resonance (SPR) excited in the visible region [1, 2]. SPR peak is responsible for the spherical shape of AgNPs synthesized in this study.

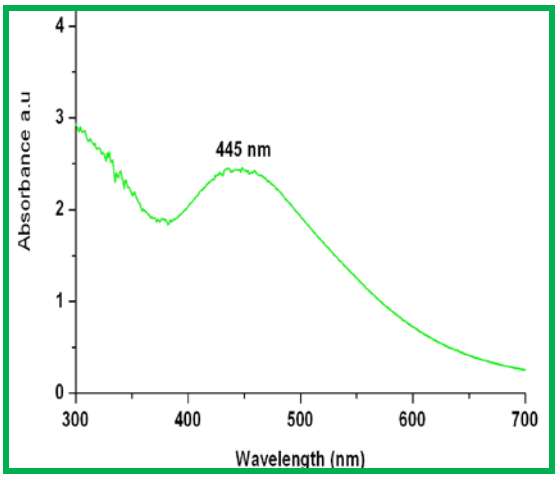

Fig. 2: UV-Vis spectrum of AgNPs synthesized using aqueous leaf extract of *A. aspera*

FTIR analysis of AgNPs

FTIR analysis was carried out to confirm the plant biomolecules that plausibly involved in the biosynthesis and capping of AgNPs synthesized. FTIR spectrum (fig. 3) showed the peaks at 3271, 1734, 1328, 1098 and 820 cm^{-1}. The peak at 3271 cm^{-1} is responsible for O–H stretching of the phenolic groups and the peak at 1734 cm^{-1} could be indexed to C=O stretching of the proteins. The peak at 1098 is responsible for C=O stretching of carboxylic acids [22]. The peak at 1328 is responsible for the N-H stretching of amide II of the proteins. The peak at 820 $cm^{-1}$is attributed to aromatic groups. FTIR peaks clearly revealed the presence proteins, terpenoids and flavonoids in the aqueous extract of *A. aspera*. Flavonoids and terpenoids might have involved in the synthesis of AgNPs while proteins could play an important role in the stabilization of AgNPs by coating around them.

XRD pattern of AgNPs

XRD pattern (fig. 4) showed three diffraction peaks at 38.11 °, 44.51 ° and 77.54 ° that are corresponding to planes (111) (200) and (311) planes of the face centered cubic crystalline lattice respectively. XRD pattern revealed the crystal structure of the AgNPs with face centered cubic (FCC) phase. XRD pattern consisted with standard JCPDS data File no. 03-0921. The results of the XRD pattern are in line with earlier reports [11-14].

TEM analysis of AgNPs

TEM analysis was carried out to confirm the size and shape of biosynthesized AgNPs. TEM micrographs (fig. 5a-b) showed that the synthesized AgNPs were 20-40 nm in size with spherical in shape. The size of the AgNPs was further confirmed by particle size distribution by DLS analysis. TEM micrograph determined that the synthesized AgNPs were monodispersed without any aggregation which might be due to coating of the AgNPs by plant proteins. TEM results are consistent with many earlier reports [11-14].

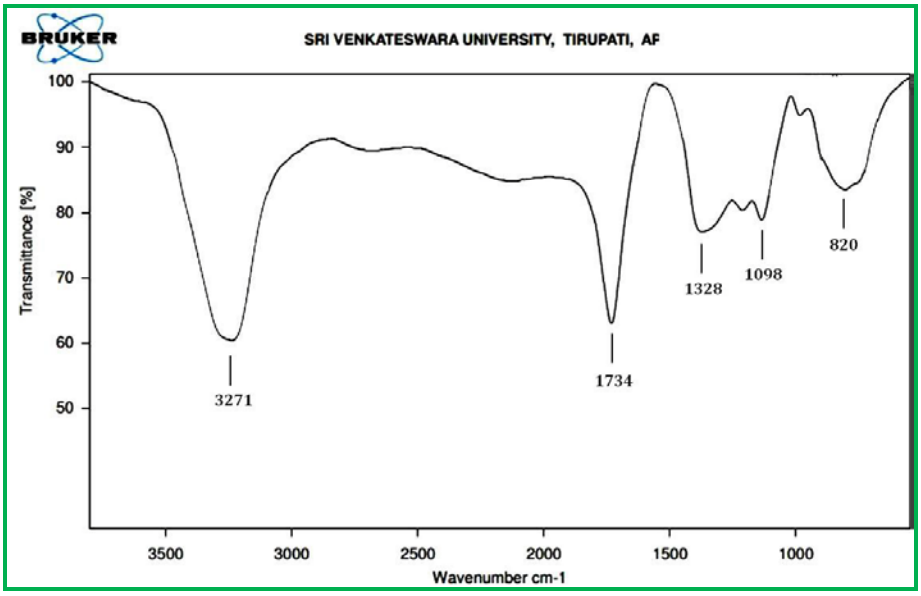

Fig. 3: FTIR spectrum of AgNPs synthesized using aqueous leaf extract of *A. aspera*

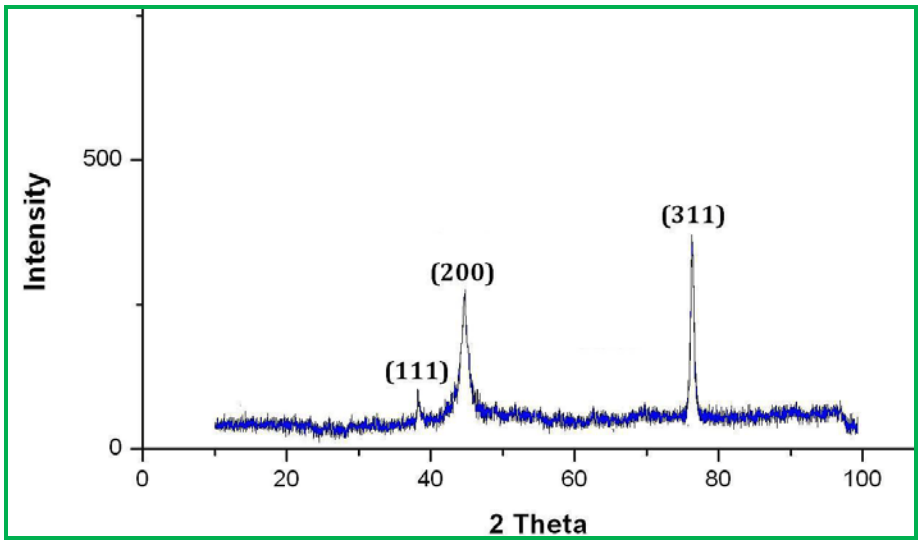

Fig. 4: XRD pattern of AgNPs synthesized using aqueous leaf extract of *A. aspera*

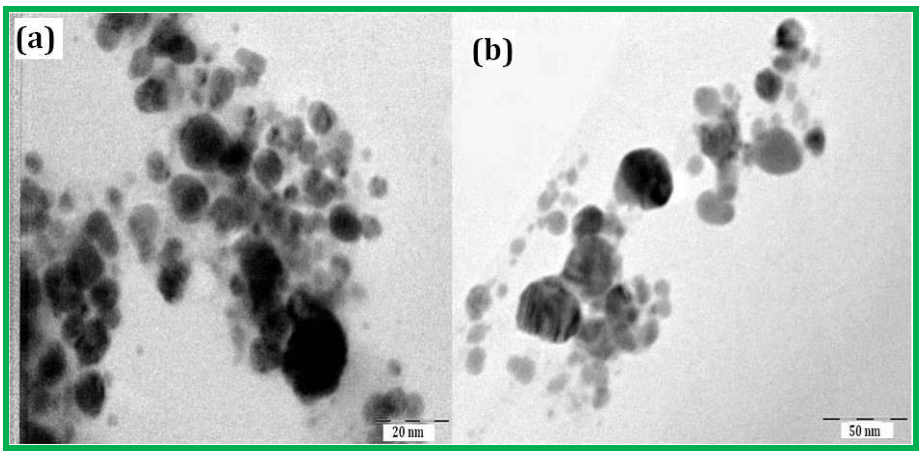

Fig. 5: TEM micrographs of biosynthesized AgNPs at (a) 20 nm and (b) 50 nm

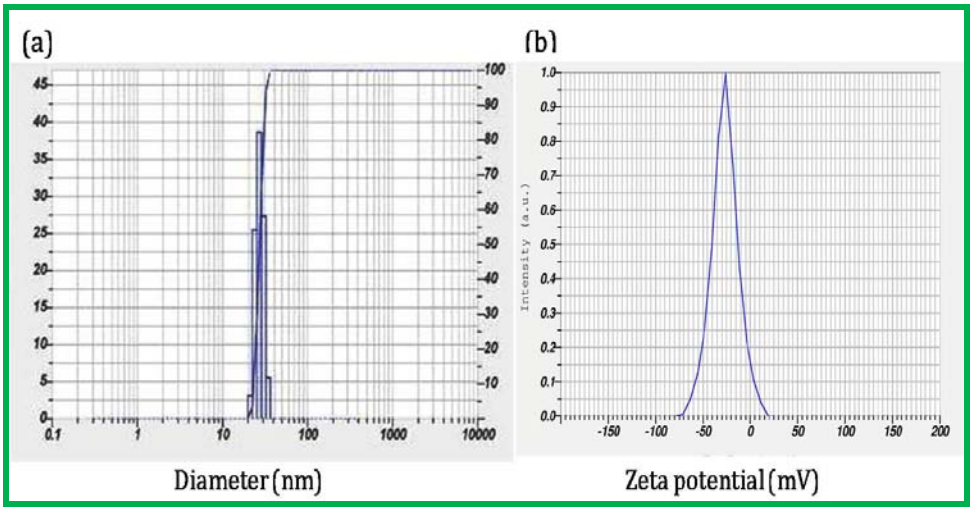

Fig. 6: (a) Particle size distribution and (b) Zeta potential measurement of AgNPs synthesized using aqueous leaf extract of *A. aspera*

Particle size distribution and zeta potential measurement

Particle size distribution by DLS analysis showed that the AgNPs synthesized were 20-40 nm in size with average size or hydrodynamic radii of 24.5 nm (fig. 6a). Particle size analysis and size determination by TEM are inconsistent with each other. Zeta potential measurement showed that AgNPs possess a surface negative charge of–28.1 mV (fig. 6b). The high negative surface charge or zeta potential value indicates that the AgNPs synthesized in this study were long-term stable. Long term stability is very important for the biomedical applications of AgNPs.

Antimicrobial activity

Antimicrobial activity of the AgNPs was checked by employing disc diffusion method. AgNPs showed effective inhibitory activity against Gram+ve bacteria compared to Gram-ve bacteria. AgNPs formed maximum inhibition zone against *S. aureus* (16.4 mm) followed by *B. subtilis* (14.5 mm), *K. pneumonia* (13.2 mm) and *P. aeruginosa* (12.4 mm). Fig. 7 showed a comparison of the antibacterial activity of the AgNPs with aqueous leaf extract of *A. aspera* and streptomycin. The antibacterial activity of the AgNPs is 3-4 times effective compared to aqueous leaf extract. The effective antimicrobial activity is due to a spherical shape and small size of AgNPs. The small size confers large surface to volume ratio that increases the contact area between AgNPs and microbe which in turn increases the inhibitory activity.

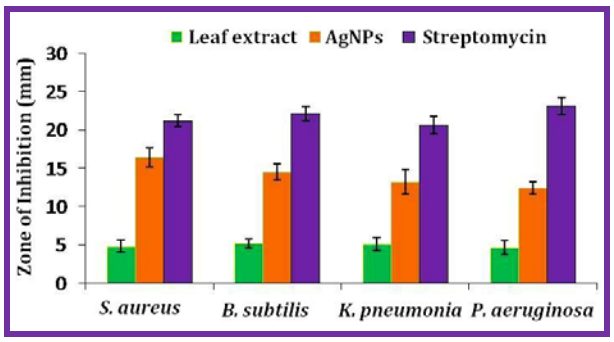

Fig. 7: Antibacterial activity of the biosynthesized AgNPs compared with aqueous leaf extract of *A. aspera* and Streptomycin

AgNPs also assessed for their antifungal activity against human pathogenic fungi. Diameters of inhibition zones were shown in fig. 8. AgNPs showed effective antifungal activity and the highest zone of inhibition was noticed against *A. niger* (12.2 mm) followed by *C. albicans* (11.5 mm) and *C. nonalbicans* (11.8 mm).

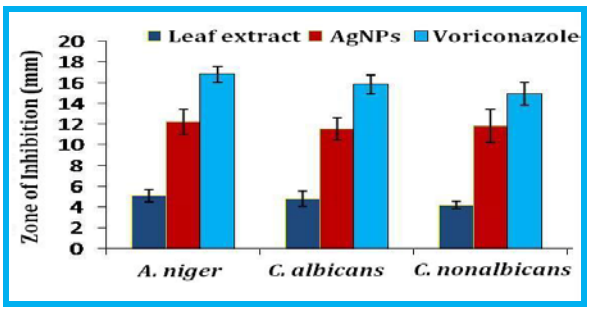

Fig. 8: Antifungal activity of the biosynthesized AgNPs compared with aqueous leaf extract of *A. aspera* and voriconazole

The plausible mechanistic approach behind the antimicrobial activity of AgNPs was also explained in this study. AgNPs form the pores or pits around the cell membrane and enter inside the cell and binds to thiol groups of many important proteins or enzymes that are essential for replication or division [23, 24]. AgNPs also caused the destructive fragmentation of DNA. Thus, AgNPs inhibits bacterial replication which in turn leads to bacterial death [11-14, 24]. The effective antimicrobial activity of the biosynthesized AgNPs in this study provides the capability of AgNPs as future topical ointments for various infections without any side effects.

Free radical scavenging activity

Radical scavenging activity of the AgNPs was checked against DPPH and H_2O_2 radicals. AgNPs showed dosage dependant RSA against both DPPH and H_2O_2 radicals. Increase in concentration of AgNPs showed an increase in % inhibition of radicals (fig. 9a-b). Biosynthesized AgNPs showed maximum RSA of 57.53 and 52.49 % respectively against DPPH and H_2O_2 radicals at the highest concentration used in this study (100 µg/ml). IC_{50} (inhibitory concentration at which half maximal radical are scavenged) values are determined using linear regression coefficient (**R^2=0.9**). IC_{50} values of AgNPs against DPPH and H_2O_2 radicals were found to be 77.73 and 90.53 µg/ml respectively. The RSA of the AgNPs might be due to the presence of polyphenols, saponins and secondary metabolites that are present in the colloidal solution of AgNPs [10]. Free radicals such as superoxides, peroxides, hydroxyl radicals and singlet oxygen cause oxidative stress which is linked inflammation,

obesity, diabetes and many neurodegenerative disorders. Free radical scavenging activity of the AgNPs synthesized in this study showed their biomedical importance against many diseases linked to oxidative stress.

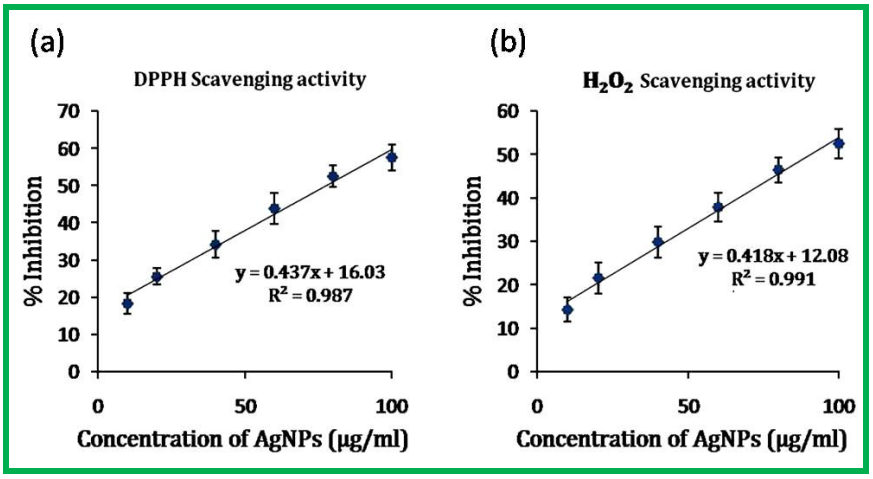

Fig. 9: Free radical scavenging activity of biosynthesized AgNPs against a) DPPH radicals and b) H_2O_2 radicals

CONCLUSION

The present study reports the rapid and successful synthesis of AgNPs using aqueous leaf extract of *A. aspera* as a bio-reducing agent. The synthesized AgNPs in this study were very small, monodispersed, spherical in shape, crystalline in nature with FCC phase and long term stable due to high negative surface charge. Secondary metabolites present in the aqueous leaf extract of *A. aspera* such as Polyphenols, terpenoids and flavonoids could play an important role in this bioreduction process while proteins could play as capping agents around AgNPs and provided stability to the synthesized AgNPs. Biosynthesized AgNPs showed effective antimicrobial and free radical scavenging activities and were proved their biomedical importance.

ACKNOWLEDGEMENT

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CONFLICT OF INTERESTS

All authors declare that there is no conflict of interest

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