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Research Paper

Green Synthesis of Silver Nanoparticles from the Leaf Extract of *Persea americana*: Characterization and Antibacterial Activity Studies

Mallenahalli Puttashetty Somashekarappa[1*], Kinnigoli Raghava Thriveni[2]

[1]Department of Studies in Chemistry, GFG College, Kadur, Chikkamagaluru, Karnataka, India, Affiliated to Kuvempu University, Karnataka, India.

[2]Department of PG Studies in Chemistry, IDSG College, Chikkamagaluru, Karnataka, India, Affiliated to Kuvempu University, Karnataka, India.

*Corresponding author: mpsomashekar1@gmail.com

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ABSTRACT

Silver nanoparticles (AgNPs) were synthesized using the leaf extract of *Persea americana*. Characterization of the particles was done by UV-visible extinction spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and powder X-ray diffraction (PXRD) studies. The particles are spherical to quasi-spherical and the diffraction pattern in the PXRD spectrum and the selected area x-ray diffraction (SAED) confirms the silver being crystallized into a face-centered cubic (FCC) structure. The average particle size calculated through PXRD data is 23 nm. Antibacterial activity of the synthesized AgNPs against the spreading of gram-negative bacteria, *Escherichia coli* and gram-positive bacteria, *Staphylococcuus aureus,* were determined and the results were compared with that exhibited by ciprofloxacin, a commercial reference substance. The minimum inhibitory concentrations (MIC) of the AgNPs in their solid solutions in nutrient agar media were found to be 1.00×10^{-4} g/mL against *E. coli* and 7.50×10^{-5} g/mL against *S. aureus*. *The bactericidal efficacy exhibited against S. aureus* bacterium by AgNPs synthesized from *P. americana* is superior compared to that exhibited by the reference compound selected for the study.

Keywords: Silver nanoparticles, *Presea americana*, Antibacterial activity, *Escherichia coli, Staphylococcus aureus.*

INTRODUCTION

Size-dependent surface plasmon resonance [1] and the consequent interesting electronic and optical properties [2] of the silver nanoparticles (AgNPs) make them a very interesting and widely studied area among metal nanoparticles.[3] The established applications of the AgNPs include photocatalysis [4], optical sensors [5], nanosphere lithography [6], optoelectronics [7], solar energy conversion devices [8] and surface-enhanced Raman scattering (SERS) substrates [9]. Further, the inherent antimicrobial character [10], of the AgNPs makes them applicable in various biomedical uses.[11-13] Consequential demonstration of the antimicrobial properties of AgNPs is as follows. The AgNPs embedded polyurethane-based antibacterial water filter [14], blotting paper-based point-to-use antibacterial water filter [15-17], AgNP-embedded carbon-based antibacterial air filter [18] and AgNP tethered antibacterial textile fabrics.[19-22] AgNPs have also been investigated as carriers in drug delivery systems and the related precedents have been reviewed.[23] Chemical reduction methods of synthesis of AgNPs using harsh reducing agents, like $NaBH_4$, $LiAlH_4$, $R_4N^+(Et_3BH^-)$ or hydrazine [24] results in unstable AgNP solutions contaminated with reaction by-products like borides, metal borates [25], B_2H_6, $NaNO_3$ etc., Thus chemical reduction methods also involve use of stabilizing agents [26], such as certain polymers, and cationic polynorbornenes [27], making the synthesis expensive.

However, using extracts of plants, bacteria, fungi and biopolymers as reducing agents for the synthesis of AgNPs has proven to be a greener, cheaper and eco-friendly method in recent years.[28-30] The AgNPs synthesized using plant extracts were found to be a more sensitive substrate for biosensing of fungicides, exhibit excellent photocatalytic activity [31], and have extensive pharmacological applications in the treatment of cancer, malaria, microbial and cardiovascular diseases.[32] Therefore, exploring medicinal plants for their constituent medicinally important phytonutrients to be useful as both reducing and stabilizing agents for the synthesis of AgNPs is interesting. WHO has published four volumes of monographs consisting of detailed study, phytonutrient constituents and relevant references related to number of medicinal plants.[33] It has also been reported that the AgNPs synthesized using extracts of plants proved to inhibit the growth of HT-29 colon cancer cells and HepG2 liver cancer cells.[34] Thus, simple reduction chemistry could be done using natural plant resources to obtain sensitive materials for antimicrobial an pharmacological applications.

Persea americana, popularly known as avocado, being cultivated in 75 countries, is known to contain various phenolic compounds such

as eugenol, syringic acid, vanillic acid, ferulic acid, epicatechin, epigallocatechin, apigenin, naringenin, and kaempferol.[35] Research related to the pharmacological potential of *P. americana* in novel drug discovery for the prevention and treatment of cancer, microbial, inflammatory, diabetes, and cardiovascular diseases was reviewed.[35,36]

This paper describes the synthesis of stable AgNPs using the water extract of the leaf sample of *P. americana*, the characterization of the AgNPs using Uv-visible extinction spectroscopy, powder X-ray diffraction, scanning electron microscopy and transmission electron microscopy analyses, and the study of their bactericidal effects against *E. coli* and *S. aureus* bacteria.

MATERIALS AND METHODS

Materials

The chemicals were of Merck and S. D. Fine chemicals. The nutrient agar was obtained from Himedia. Distilled water was used wherever required. The bacteria selected for the study were *E. coli* and *S. aureus*. A laboratory centrifuge, R-8C from Remi was used for the isolation of particles for SEM and powder XRD analyses. Systronics UV-visible spectrophotometer 119 was used for recording the UV-visible extinction spectra in the wavelength range of 300 to 700 nm. Powder XRD patterns were recorded on a Rigacu Smartlab X-ray diffractometer and the SEM and EDS were recorded on an Ultra 55 scanning electron microscope from GEMINI technology. TEM imaging of the drop-coated samples was done on Titan Themis 300kV from FEI.

Methods

Extraction

Freshly collected leaves of the selected plant were sliced and crushed into a paste with a small amount of warm distilled water using a mortar and pestle. The paste was transferred into a 250 mL beaker, suspended in 100 mL water, stirred on a magnetic stirrer for about 30 minutes at 45 to 50°C temperature, cooled to lab temperature and filtered through a pre-weighed piece of qualitative filter paper. The difference in weight method calculated the weight of the contents transferred to the extract. Qualitative phytochemical analysis of the extract was done following a routine method.[37]

Synthesis of silver nanoparticles

Fresh extract (50 mL) containing approximately 0.02 ± 0.005 g/mL of extracted substances was taken in a round-bottomed flask fitted with a pressure-equalizing dropping funnel. It was heated to 60°C while stirring and 20 mL of 0.002 M $AgNO_3$ solution was added dropwise. Temperature was maintained at 60 ± 5°C during the addition of $AgNO_3$ solution and for a further 1-hour time. Contents were cooled to lab temperature. The AgNP solution so obtained was centrifuged in order to isolate the material for powder XRD and SEM analyses. The solid was then dried in a vacuum over anhydrous phosphorous pentoxide and powdered.

Antibacterial activity studies

A known volume of the AgNP solution was evaporated on a pre-weighed watch glass, dried and weighed to determine the amount of AgNP material in its solution. A suspension of 28 g of nutrient agar in 1000 mL of distilled water was boiled and autoclaved. About 20 mL aliquots of the nutrient agar media were contaminated with various increased concentrations of AgNP solutions and transferred into sterilized petri dishes. When the media hardened, the surface of the media was applied with stains of selected bacteria using a cotton swab. The growth or spread of bacteria was followed for a period of 12 to 15 hours. The standard reference for analysis of the data was the results of the same experiments using ciprofloxacin conducted at the same condition.

RESULTS AND DISCUSSION

Phytochemical Screening

Water extract of the selected plant having a phytonutrient concentration of $\sim 25 \times 10^{-3}$ g/mL was subjected to qualitative phytochemical analyses adopting routine procedures [37] and the results are summarized in Table 1. The phytonutrients found in the water extract of *P. americana* include carbohydrates and glycosides, saponins, proteins and amino acids, oils and fats, phenolic compounds and flavonoids, gums and mucilages. Our phytochemical analytical data is in conformity with the earlier analytical reports.[35]

The important physicochemical parameters that are to be tuned for the AgNP dispersions to be useful for biomedical applications are size, shape, concentration, surface charge, colloidal state and agglomeration.[28,38] Synthesis of AgNPs using bioresources is highly diverse and eco-friendly, compared to their synthesis by physical and chemical methods, because of the natural origin of the reduction, dispersion and stabilizing medium. It has already been established that the presence of secondary metabolites or phytonutrients in the plant extract, such as phenolic compounds, flavones triterpenoids, exhibit free radical scavenging ability,[39-41] and hence makes the extract comfortably reducing in nature.

Table 1: The results of phytochemical analyses of the leaf extracts of *P. americana*

Phytonutrient	*Test*	*Inference*
Alkaloids	Mayers test	−
	Wagners test	−
	Hagers test	−
	Molish test	+
Carbohydrates and glycosides	Fehling's test	−
	Benedicts test	+
	Barfoed's test	−
Saponins	Foam test	+
	Millons's test	−
Proteins and amino acids	Biuret's test	+
	Ninhydrin test	−
Phytosterols	Liberman-Burchards test	−
Oils and fats	Spot test	+
	Saponification test	−
Phenolic compounds and flavonoids	Ferric chloride test	+
	Lead acetate test	+
	Alkaline test	−
Gums and mucilages	Gelatin test	+
		+

Synthesis

The procedure adopted for the synthesis of AgNPs in the present case, using the dilute leaf extract of *P. americana* is highly reproducible and does not involve any reducing agent and stabilizing molecules. The extract obtained at 45 to 50°C provides various phenolic compounds and flavonoids as identified by us (Table 1) and earlier workers as well [35] reduces the Ag^+ in the $AgNO_3$ into Ag^0 [30,39-41] that coalesce further to form nanosized particles. The fresh surfaces of the particles so formed get coated with a monomolecular layer of the oxidized secondary metabolites of the extract after reduction and other bioactive and functional molecules not involved in reduction.[26,32] All the AgNPs in the solution coated with a monomolecular layer of phytonutrient molecules possess the same surface properties. Hence, individual particles in their dispersing medium experience some degree of repulsion from the neighboring particles. This repulsion between the individual particles in the dispersion medium overpowers the possibility of agglomeration and, hence, stabilizes the particles.

Characterization

Turning off a pale green-colored plant extract into reddish brown color upon the addition of $AgNO_3$ solution at the reaction temperature is indicative of the formation of AgNPs. However, recording a surface plasmon resonance absorption band in the UV-visible region of electromagnetic radiation, characteristic of AgNPs [42, 43], confirms the presence of AgNPs.[44, 45] Recording an absorption spectrum between 300 to 700 nm, for dilute leaf extract of the selected plant does not show any absorption peak but only a baseline (Fig. 1 (a)). Fig. 1(b) shows the UV-visible absorption spectrum of the AgNP solution synthesized using leaf extract of *P. americana***.** The relatively bigger full width at half maximum (FWHM) of the absorption peak at 439 nm could be ascribed to the overlapping of several sharp absorption bands, qualitatively representing a wider particle size distribution. The present UV-visible absorption spectral data are consistent with that of AgNPs synthesized using hydrazine hydrate and sodium citrate as reducing agents.[46] Repetition of scanning the same AgNP solution for the same absorption band in the same region (300–700 nm) with regular intervals for a period of 12 weeks shows no decrease in intensity and no change in the λ_{max} of the absorption peak at 439 nm. This is an indication of good stability of the particles in the dispersion medium consisting of phytonutrient molecules.

Morphology of the AgNP material evolved when the reddish-brown colored solution was centrifuged to isolate the particles by recording SEM imaging and the data is presented in Figure 2.

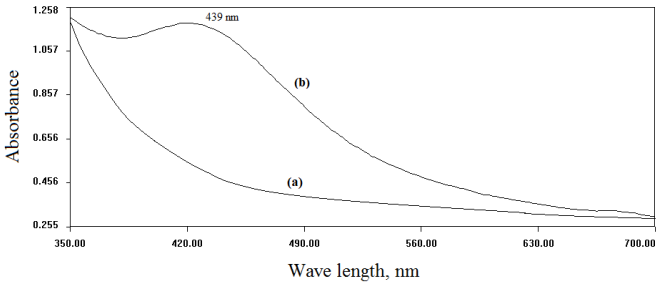

Fig. 1: UV-visible absorption spectrum of (a) extract of *P. americana* with phytonutrient concentration of 14.25×10^{-5} g/mL, and (b) AgNP solution prepared using leaf extract of *P. americana*

SEM image of the powder samples of AgNP material reveals that the spherical particles have aggregated into bigger, micron-sized lumps. Aggregation of the particles may be a result of forcing the organic monolayer-coated particles against each other under the influence of centrifugal force at the bottom of the centrifuge tube and drying of the same material as such in a vacuum. Fig. 3 shows the results of the elemental analysis of the AgNPs synthesized using an extract of *P. americana*, and the material consists of 47% by weight of silver, 25% carbon, 19% oxygen and 9% nitrogen (inset in Fig. 3(b)). The presence of carbon, nitrogen and oxygen in the EDS data can be accounted for the formation of a monomolecular layer of the phytonutrient molecules around the particles in order to stabilize them.

Fig. 4 shows the powder XRD pattern recorded on isolated and dried powder samples of AgNPs prepared using the water extract of *P. americana*.

The XRD pattern shows the sharp peak positions corresponding to the diffractions from (111), (200), (220), (222) and (311) crystallographic planes, and the characteristic positions are indicative of silver being formed by the reduction of silver nitrate, and getting crystalized to face-centered cubic (FCC) structure. The relatively wider full width at half maximum of the PXRD signals is characteristic of the material in the nanocrystalline form. The average particle size was calculated using Debye – Scherrer's formula

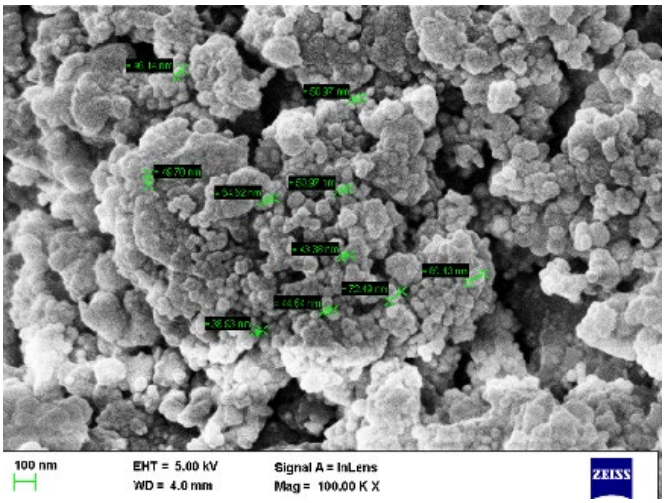

Fig. 2: Scanning electron micrograph of AgNPs synthesized from the aqueous leaf extract of *P. americana*

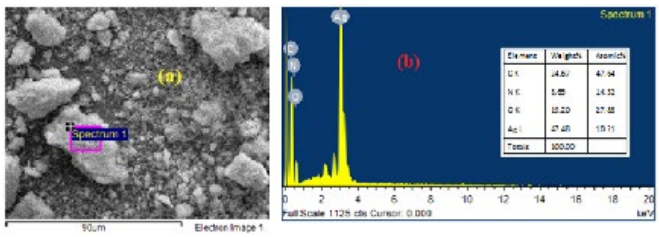

Fig. 3: (a) Micron-sized material lumps upon which the point EDS was recorded and (b) the EDS spectrum of the silver nanoparticles synthesized using the leaf extract of *P. americana*. Inset in (b), a table showing the elemental percentage of silver, carbon, nitrogen and oxygen in the material

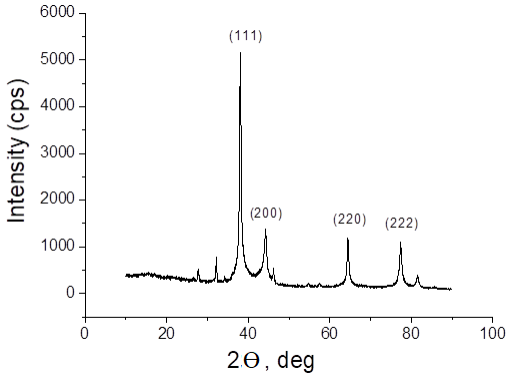

Fig. 4: Powder XRD pattern of AgNPs synthesized from the leaf extract of *P. americana*

$D = 0.94 \lambda/\beta \cos \theta$, where D is the average crystalline size, λ is the wavelength of X-ray, β is FWHM, θ is the angle of diffraction, with respect to (111) crystallographic plane, and is 23 nm.

The PXRD patterns obtained for the AgNPs in the present study are comparable and consistent with those documented earlier for AgNPs synthesized using an aqueous extract of *Ocimum sanctum and* quercetin (a flavonoid from the same plant) [38], root hair extract of *Phoenix dactylifera [47],* extracts of garlic, green tea and turmeric [48], extract of *Sida cordifolia.[49]* The other low-intensity peaks, around lower 2Θ values in the PXRD pattern, might be a result of the probable crystallization of phytochemicals, which are not involved either in the reduction of $AgNO_3$ or the capping of the particles on or between particle aggregates.

TEM image recorded upon drop casted sample of the AgNPs synthesized from *P. americana*, is presented in Fig. 5. The shapes of the title particles vary from spherical to quasi-spherical and the maximum of the particle size distribution worked and presented as the histogram passes through 15 nm (inset in Fig. 5).

Results related to the shapes of the particles obtained in the present study are comparable with the other reports documented earlier. The AgNPs synthesized using an extract of *Terminalia bellirica,*[50] and those obtained by extracellular synthesis using fungus *Aspergillus niger* are spherical.[51] In contrast, the particles synthesized using an extract of apiin as a reducing agent are quasi-spherical in shape.[52] The relatively bigger aggregation shown in the SEM image (Fig. 2) and the related understanding could be further looked closely into as a phenomenon in which the organic phytonutrient molecular layer anchored on the adjacent silver particles undergo interdigitation under the influence of centrifugal force and inter digitized aggregates stay intact when a sample was dried in vacuum. A high-resolution TEM image of a single 21 nm particle is presented in Fig. 6.

The FCC structure inferred using PXRD data for the AgNPs synthesized using the leaf extract of *P. americana* (Fig. 4) was further confirmed by recording selected area electron diffraction (SAED) pattern on a drop casted thin layer of corresponding AgNPs solution. The SAED pattern is shown in Fig. 6(b), which consists of the concentric rings embedded with bright intermittent spots, and the radii of the concentric rings increase. The consecutive increase in radii of these concentric rings is the result of electron diffraction from 111, 200, 220, 222 and 311 planes of FCC structure of the crystalline metallic silver in AgNPs. This is the confirmative inference for the reduction of $AgNO_3$ by phytonutrients in the extract into atomic silver and their consequent crystallization into FCC structure.[46] Results of the TEM analysis of the present AgNPs are in fair agreement with that obtained for the AgNPs synthesized by chemical reduction method and by using extract of *Hibiscus rosa-sinensis* [53] as well.

Antimicrobial Activity Studies

Silver and other metals such as copper, iron, lead and tin, and some alloys of copper like brass and bronze are known and have been used for thousands of years for therapeutic purposes in ancient Indian Ayurvedic medicinal practice.[54] The traditional form of silver used with herbal-based phytonutrient-formulated potions is understood to work as a bio-enhancer.[55] Thus antimicrobial ability of silver is its well-known characteristic property.[56] Results in the documented literature related to the antimicrobial activity of silver nanoparticles synthesized from all possible methods have been elaborately reviewed.[57,58]

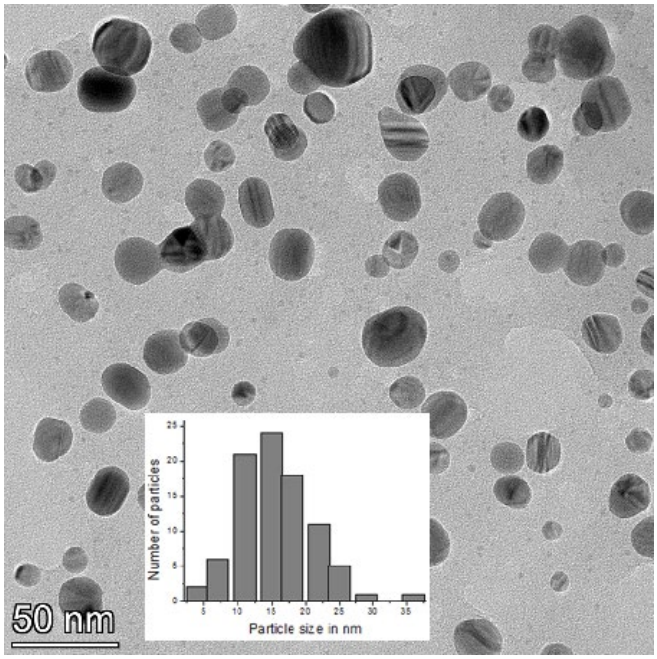

Fig. 5: Transmission electron micrographic image of the AgNPs synthesized from the leaf extract of *P. americana***.** Inset: Histogram showing the particle size distribution worked out using TEM image

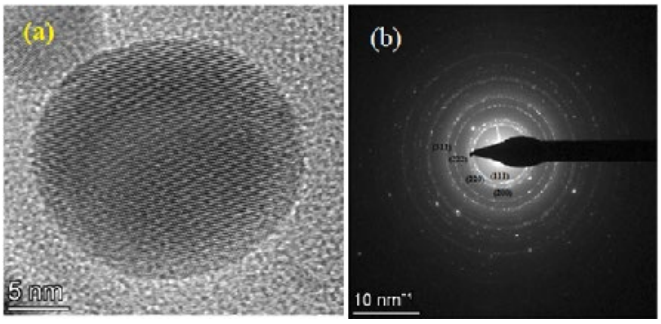

Fig. 6: (a) High-resolution TEM image of the single 21 nm sized silver nanoparticle synthesized from the leaf extract of *P. americana***.** (b) SAED pattern on the drop casted layer of AgNP solution

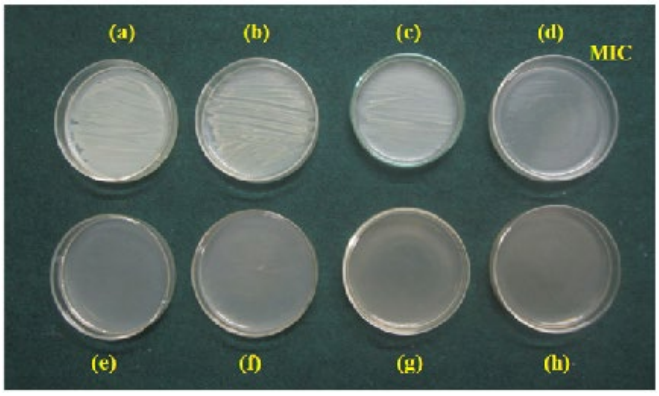

Fig. 7: Growth of the ***E-coli*** bacteria in 20 ml nutrient agar media contaminated with (a) 0.00 g/ml. (b) 5.00×10^{-5} g/mL, (c) 7.50×10^{-5} g/mL, (d) 1.00×10^{-4} g/mL (e) 1.25×10^{-4} g/mL, (f) 1.50×10^{-4} g/mL (g) 1.75×10^{-4} g/mL, (h) 2.00×10^{-4} g/mL of silver nanoparticles prepared from extract of *P. americana*.

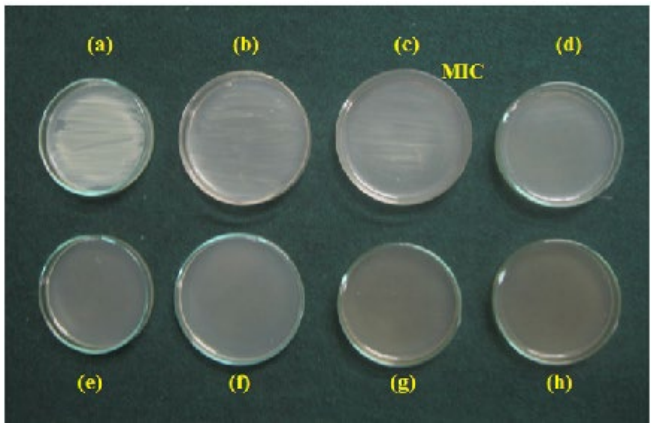

Fig. 8: Growth of the ***Staphylococcus aureus*** bacteria in 20 ml nutrient agar media contaminated with (a) 0.00 g/ml. (b) 5.00×10^{-5} g/mL, (c) 7.50×10^{-5} g/mL, (d) 1.00×10^{-4} g/mL (e) 1.25×10^{-4} g/mL, (f) 1.50×10^{-4} g/mL (g) 1.75×10^{-4} g/mL, (h) 2.00×10^{-4} g/mL of silver nanoparticles prepared from extract of *P. americana*.

For the present work, a relative assessment of the antibacterial activity of the AgNPs synthesized from the leaf extract of *P. americana* was determined against two selected bacteria, *E. coli* and *S. aureus*. The antibacterial effect was obtained in terms of the minimum inhibitory concentration (MIC) of AgNPs in their solid solution in nutrient agar media and compared with that exhibited by a reference substance, ciprofloxacin. Figs 7 and 8, respectively, show the MICs of the AgNPs determined against the growth of *E. coli* and *S. aureus*.

As the concentration of AgNPs in the solidified nutrient agar media increases, the growth of both the bacteria selected for the study decreases. The lowest possible concentration of AgNPs, at and above which the bacterial growth is completely diminished, is understood as MIC of the AgNPs. The MIC of the AgNPs synthesized from the extract of *P. americana* is 1.00×10^{-4} g/mL against *E. coli* and 7.50×10^{-5} g/mL against *S. aureus*. The experimentally determined MICs of the ciprofloxacin are 20×10^{-5} g/mL against *E. coli* and 24×10^{-5} g/mL against *S. aureus* bacteria in the same experimental conditions.

CONCLUSION

The qualitative phytochemical analyses of the water extract of *P. americana* indicate the presence of carbohydrates and glycosides, saponins, proteins and amino acids, oils and fats, phenolic compounds and flavonoids, gums and mucilages, among which phenolic compounds and flavonoids bring about a reduction of silver nitrate into metallic silver nanoparticles. The bare surfaces of each individual AgNP thus formed are capped instantaneously with a monomolecular layer of phytonutrients. The resulting AgNP solutions are highly stable and the stability could be ascribed to some degree of repulsion between the individual particles possessing the same surface properties because of the same molecular characteristics of the monomolecular layer around them. The TEM analysis of the title AgNPs reveals that the particles are spherical to quasi-spherical shaped, and the particle size distribution passes across 15 nm. The PXRD patterns recorded upon the AgNPs show the characteristic diffractions from 111, 200, 220, 222 and 311 crystallographic planes corresponding to FCC structure. The PXRD pattern and the FCC crystal structure ascertained are consistent with that concluded by the SAED pattern recorded upon drop casted sample of particles. The particle size calculated with respect to the (111) crystallographic plane in the PXRD pattern is 23 nm. The MICs of AgNPs synthesized using the water extract of *P. americana* are 1.00×10^{-4} g/mL against *E. coli* and 7.50×10^{-5} g/mL against *S. aureus*. The bactericidal efficacy exhibited against *S. aureus* bacterium, by the title AgNPs is superior compared to that exhibited by the reference compound selected for the study.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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