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Research Article

Medicinal Plant Extract Mediated Green Synthesis and Stabilization of Silver Nanoparticles using *Plumbago zeylanica* L: Characterization and Assessment of Bactericidal Activity

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ABSTRACT

Water extract of a medicinal plant, *Plumbago zeylanica* L, is used to synthesize and stabilize the silver nanoparticles (AgNPs). Particles were characterized using UV-visible extinction spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and powder X-ray diffraction (PXRD) studies. Phenolic compounds and flavonoids present in the extract, as confirmed by the systematic qualitative phytochemical analyses, reduce silver nitrate into metallic silver. The resulting 10 nm sized particles are spherical in shape and confined to a face-centered-cubic (FCC) structure. Bactericidal effect of AgNPs was determined against the spreading of gram-negative bacteria, *Escherichia coli (E. coli)* and gram-positive bacteria, *Staphylococcuus aureus (S. aureus)* and the results were compared with antibacterial effect exhibited by a commercial standard ciprofloxacin. The bactericidal efficacy was determined in terms of minimum inhibitory concentrations (MIC) of the AgNPs in the solid solution of nutrient agar media and was found to be 37.625 $\times 10^{-5}$ g/mL and 43.0 $\times$ 10-^{4} g/mL against *E. coli* and *S. aureus,* respectively.

Keywords: Silver nanoparticles, *Plumbago zeylanica* L, Antibacterial activity, *Escherichia coli, Staphylococcus aureus.*

INTRODUCTION

Silver nanoparticles (AgNPs) are one of the widely investigated classes among all the metal nanoparticles [1], because of their interesting electronic and optical properties [2] owing to their size-dependent surface plasmon resonance [3]. As a result, they have been explored as photocatalysts [4], optical sensors [5], in nanosphere lithography [6], optoelectronics [7], solar energy conversion devices [8] and surface-enhanced Raman scattering (SERS) substrates [9]. The antimicrobial properties possessed by AgNPs [10] made them useful for various biomedical applications [11-13], an AgNP-embedded polyurethane-based antibacterial water filter [14], a 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]. Further, AgNPs have also been developed as carriers for drug delivery systems [23].

The good old reduction methods of synthesis of AgNPs using reducing agents, like $NaBH_4$, $LiAlH_4$, $R_4N^+(Et_3BH^-)$ or hydrazine [24] results in unstable AgNP solutions along with reaction by-products like borides, metal borates [25], B_2H_6, $NaNO_3$ etc., The reduction methods also involve use of stabilizing agents [26], such as certain polymers, and cationic polynorbornenes [27], which adds to the cost of preparation..

However, greener, cheaper and eco-friendly methods are also being developed for the synthesis of AgNPs, using extracts of plants, bacteria, fungi and biopolymers as reducing agents [28-30]. As a value addition, 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]. According to the 1999 WHO report, "in many developing countries a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs" [33], and "there has been a tremendous increase in the use of herbal medicine" through the year 2009 [34]. Therefore, simple, mild and controlled reduction chemistry could be done using natural, plant resources to obtain sensitive materials for antimicrobial and pharmacological applications. Being interested and involved in exploring medicinal plants for their constituent medicinally important phytonutrients to be useful as both reducing and stabilizing agents for the synthesis of AgNPs, we selected a phytoconstituent-rich medicinal plant, *Plumbago zeylanica* L., for the study.

P. zeylanica L (chromosome number 2n = 24) is a multipurpose medicinal herb of the family Plumbaginaceae, most commonly used in the Indian traditional system of medicine [35]. *P. zeylanica* L is a versatile plant having a plethora of medicinal uses. This plant is a distinctive source of phytoconstituents and has tremendous therapeutic properties. Major constituents reported in the plant were flavonoids, alkaloids, glycosides, saponins, steroids, tannins, triterpenoids, coumarins and phenolic compounds and the identified

compounds are plumbagin, 3-chloroplumbagin, binaphthoquinone named as 3′, 6′-biplumbagin, 3,3′-biplumbagin, isozeylanone, zeylanone, elliptone, droserone, isoshinanolone and a new napthalenone (1, 2(3)-tetrahydro-3, 3′-biplumbagin) plumbagic acid such as glucosides; 3-O-beta-glucopyranosyl plumbagic acid and 3-o-beta-glucopyranosyl plumbagic acid, chitranone, maritinone, elliptone and isoshinanolone, seselin, methoxyseselin, suberosine, xanthyletin [35]. Literature indicates its huge utility towards numerous diseases, including cardiovascular disorders, ulcers, liver problems, diabetes, obesity, wound healing, cancer etc.[36].

We describe in this paper the synthesis and stabilization of AgNPs using the water extract of the leaf sample of *P. zeylanica* L, a very important medicinal plant, characterization of the AgNPs using UV-visible extinction spectroscopy, PXRD, SEM and TEM analyses, and the assessment of their antibacterial activity against *E. coli* and *S. aureus* bacteria.

MATERIALS AND METHODS

Materials

All the chemicals used for the study are either from Merck or from S. D. Fine Chemicals. The nutrient agar media is from Himedia. Distilled water was used wherever required. 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 Rigaku 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, *P. zeylanica* L were sliced, crushed in to paste with a little amount of distilled water warmed up to 45 to 50°C, using a mortar and pestle. The paste was transferred into a 250 mL beaker, suspended in 100 mL of water, stirred on a magnetic stirrer for about 30 minutes at 45 to 50°C, cooled to lab temperature and filtered through a pre-weighed piece of qualitative filter paper. The weight of the contents transferred to the extract was calculated by the difference in weight method. Qualitative phytochemical screening of the extract was done following a routine method [37].

Synthesis of silver nanoparticles

50 ml of the fresh extract containing approximately 0.03 ± 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 further 1 hour. 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 desiccator over anhydrous phosphorous pentoxide and powdered.

Antibacterial activity studies

A suspension of 28 grams of nutrient agar in 1000 mL of distilled water was boiled and autoclaved. 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 cotton swabs. 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 conditions.

RESULTS AND DISCUSSION

A water extract of *P. zeylanica* L with approximately 25 $\times 10^{-3}$ g/mL concentration of phytonutrients was subjected to qualitative phytochemical screening following routine procedures [37] and the results obtained are presented in Table 1. The plant's extract is found to contain carbohydrates and glycosides, saponines, phytosteroids, and phenolic compounds and flavonoids. The phytochemical analytical results are in good agreement with literature reports [35, 36].

For the synthesis of stable AgNP dispersions to be useful in biomedical applications, it is essential to tune their size, shape, concentration, surface charge, colloidal state and agglomeration [28, 38]. Using easily available bio resources for the synthesis of AgNPs is cheaper and eco-friendly, compared to their synthesis by

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

Phytonutrient	*Test*	*Inference*
Alkaloids	Mayers test	−
	Wagners test	−
	Hagers test	−
Carbohydrates and glycosides	Molish test	+
	Fehling's test	−
	Benedicts test	−
	Barfoed's test	−
Saponins	Foam test	+
Proteins and amino acids	Millon's test	−
	Biuret's test	−
	Ninhydrin test	−
Phytosteriods	Libeirman – Burchard's test	+
Oils and fats	Spot test	−
	Saponification test	−
Phenolic compounds and flavonides	Ferric chloride test	+
	Lead acetate test	−
	Alkaline test	+
	Gelatin test	−
Gums and mucilages		−

physical and chemical methods. Further, bio resources and medicinal plant extracts in particular containing secondary metabolites or the phytonutrients such as phenolic compounds, flavones, triterpenoids, etc., and possessing free radical scavenging ability [39-41], can play the roles of mild and controlled reducing medium, capping medium and the dispersion medium as well.

Synthesis

A host of various phenolic compounds and flavonoids present in the extract reduce Ag+ in the $AgNO_3$ solution added dropwise, into Ag^0 [30, 39-41]. Resulting in atomic silver aggregate into nanosized particles, followed by their coating with a monomolecular layer of the oxidized secondary metabolites of the extract after reduction and other molecules with electron-rich functionality, and not involved in reduction [26, 32]. The AgNPs coated with phytonutrient molecules will have the same surface charge and hence repel each other. This repulsion amongst the particles keeps them away from each other, and this gives them stability in their solution for a long time. The size of the AgNPs may be a function of the relative velocities of reduction to coating or formation of a monolayer, after reduction. This, in turn, may depend upon the concentrations of $AgNO_3$ and active phytonutrient molecules in the extract. By careful identification and isolation of active phenolic compounds and/or flavonoids, careful control over the concentration of such active phytonutrients and that of $AgNO_3$, and by careful control over the rate of mixing and reaction temperature, it may be possible to tune the size of the resulting AgNP particles. However, since the plant extracts contains enormous number of complex phytonutrient molecules and certain gums and mucilage of complex chemical nature, synthesized by nature, it is difficult to identify and isolate the required active molecules and use them for the purpose. Therefore, it is beyond the scope of this paper.

Characterization

Appearance of reddish brown colour upon stirring the dilute colourless solution of extract in the presence of $AgNO_3$ at 50°C for 30 minutes indicates the formation of AgNPs and it is confirmed by observing a characteristic surface plasmon resonance absorption band [42, 43] at 430 nm in the uv-visible spectrum recorded between 300 nm and 700 nm as shown in Fig. 1(b) [44, 45]. Fig. 1(a) shows a baseline in uv-visible spectrum recorded for a dilute leaf extract of the selected plant for the study. A wider full-width at half maximum (FWHM) of the absorption peak at 430 nm could be understood as a result of the overlapping of several sharp absorption bands. This broad surface plasmon resonance absorption band indicates a wider distribution of particle size. Appearance of the UV-visible spectrum is consistent with that of AgNPs synthesized using hydrazine hydrate and sodium citrate as reducing agents [46]. Repeated scanning of the AgNP solution kept in the laboratory, with regular intervals of one week for the spectrum shown in Fig. 1(b), between the same region of 300 to 700 nm, did not show any decrease in intensity at its λ_{max} or any change in the width of the band up to 12 weeks. This observation indicates the remarkable stability of the AgNPs synthesized using the extract of *P. zeylanica* L. The absorption edge observed towards 300 nm of the spectrum observed in Fig. 1 (both (a) and (b)) may be attributed to certain absorbing phytonutrient molecules.

Fig. 2 shows the SEM image recorded on the AgNP material isolated by centrifugation, in order to understand the morphology.

SEM image (Fig. 2) reveals that the spherical AgNPs have aggregated into micron-sized lumps, and cracks have developed during drying in a vacuum. Aggregation of the particles may be explained as follows. Forcing the organic monolayer-coated particles one over the other under the influence of centrifugal force at the bottom of the centrifuge tube results in interdigitation of the peripheral parts of the monolayer coated on the particles with those of the neighbouring particle. Aggregation is retained and the lumps develop when the material is dried in a vacuum.

Elemental composition of the AgNP material isolated by centrifugation is obtained by recording EDS and the data is presented in Fig. 3. The title material contains 79.82% by weight of silver, 6.77% carbon, 13.41% oxygen and 0 % nitrogen (inset in Fig. 3(b)). Indication of the presence of carbon and oxygen in the EDS data can be ascribed to the formation of a monomolecular layer of the phytonutrient molecules around the particles in order to stabilize them.

Powder XRD pattern recorded on isolated and dried powder sample of AgNPs prepared using the water extract of *P. zeylanica L is presented in Fig. 4.*

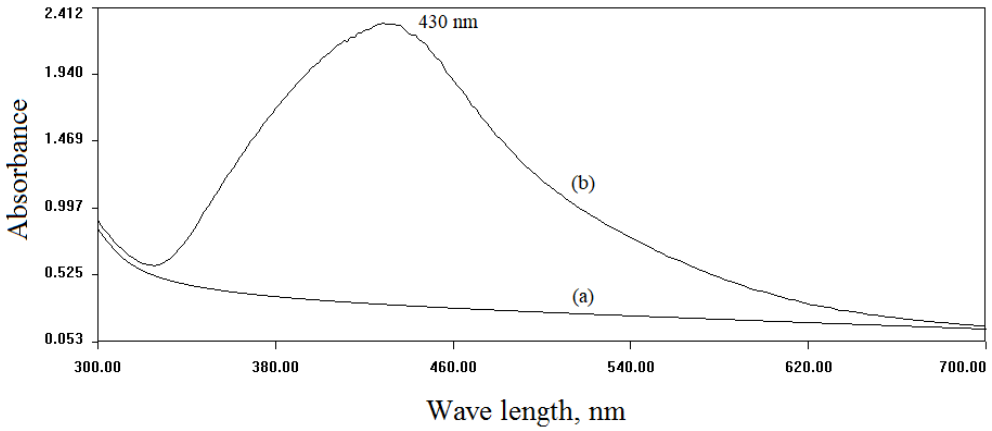

Fig. 1: UV-visible absorption spectrum of (a) extract of *P. zeylanica* L with phytonutrient concentration of 20 × 10^{-5} g/mL, and (b) AgNP solution prepared using leaf extract of *P. zeylanica* L

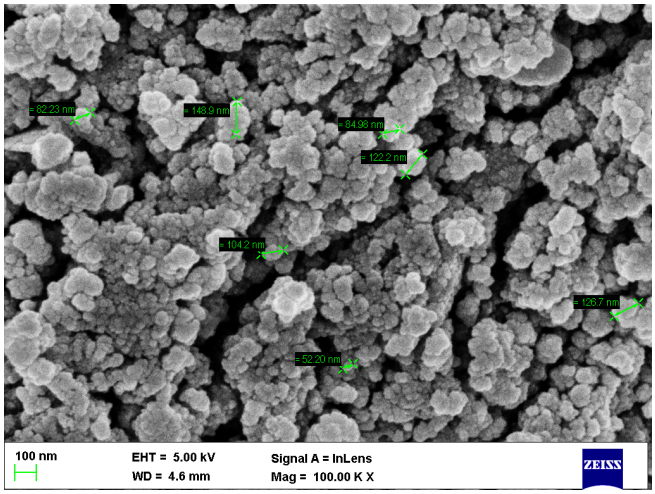

Fig. 2: Scanning electron micrograph of AgNPs synthesized from the aqueous leaf extract of *P. zeylanica* L

Fig. 4 shows some selective, relatively wider and sharp peak positions corresponding to the diffractions from (111), (200), (220), (222) and (311) crystallographic planes. The positions of these peaks are characteristic of silver being crystallized to face centred cubic (FCC) structure as and when it is formed by the reduction of silver nitrate by phytonutrient molecules. Relatively wider full-width at half maximum (FWHM) of the PXRD signals indicates that the material is in nanocrystalline form. The average particle size was calculated using Debye – Scherrer's formula $D = 0.94\ \lambda/\beta \cos\theta$, 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 10 nm.

The PXRD patterns observed for the title AgNPs are in conformity with those published earlier for AgNPs synthesized using 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], and* extract of *Sida cordifolia [49]. The other low intensity peaks,* observed at lower 2Θ values *in the PXRD pattern,* might be due to crystallization of phytonutrient molecules, which are not involved either in the reduction of $AgNO_3$ or capping of the particles, on or between particle aggregates.

Fig. 5 shows the TEM image recorded for a drop-cast sample of the AgNPs synthesized from *P. zeylanica* L. It is clear that the particles are spherical to quasi-spherical in shape, with most of the particles being spherical. The maximum of the particle size distribution histogram passes sharply through 10 nm (inset in Fig. 5) and this is in remarkable consistency with the particle size calculated using diffraction from the (111) crystallographic plane in the PXRD pattern.

The geometric shape of the AgNPs obtained in the present case is very much comparable with those synthesized using the extract of *Terminalia bellirica* [50], the extract of apiin [51] as reducing agent, and those obtained by extracellular synthesis using Fungus, *Aspergillus niger, which* are spherical [52]. The appearance of 50 to 150 nm sized aggregates or lumps observed in the SEM image (Fig. 2) recorded centrifuged and vacuum-dried powder samples may be understood as follows. When silver nanoparticles of approximately 10 nm diameter and having a layer of phytonutrient molecules upon them are compressed against one another at the dip of the centrifuge tube under the influence of centrifugal force, interdigitation of the phytonutrient monolayer occurs and it results in aggregation of about 5 to 15 particles together. These bigger aggregates appear to be intact when centrifuged material is dried in a vacuum and subjected as such to scanning electron micrography. Due to con one understanding could be further looked into as a phenomenon in which the organic phytonutrient molecular layer anchored on the adjacent silver particles undergoes interdigitation under the influence of centrifugal force, and interdigitized aggregates stay intact when the sample was dried in a vacuum. Fig. 6(a) shows an HRTEM image captured on a drop-cast sample.

The selected area electron diffraction (SAED) pattern recorded on a drop-cast sample of AgNPs solution (Fig. 6(b)), synthesized by the leaf extract of *P. zeylanica* L., shows concentric circles with consecutively increasing radii, embedded with bright intermittent spots. The concentric rings with increasing radii represent the electron diffraction from 111, 200, 220, 222 and 311 planes of the FCC structure of the crystalline AgNPs. This result is an additional conformity with the PXRD pattern shown in Fig. 4 [46]. TEM and SAED data of the present AgNPs are in good agreement with those obtained for the AgNPs synthesized by a simple chemical reduction method and by using the extract of *Hibiscus rosasinensis [53].*

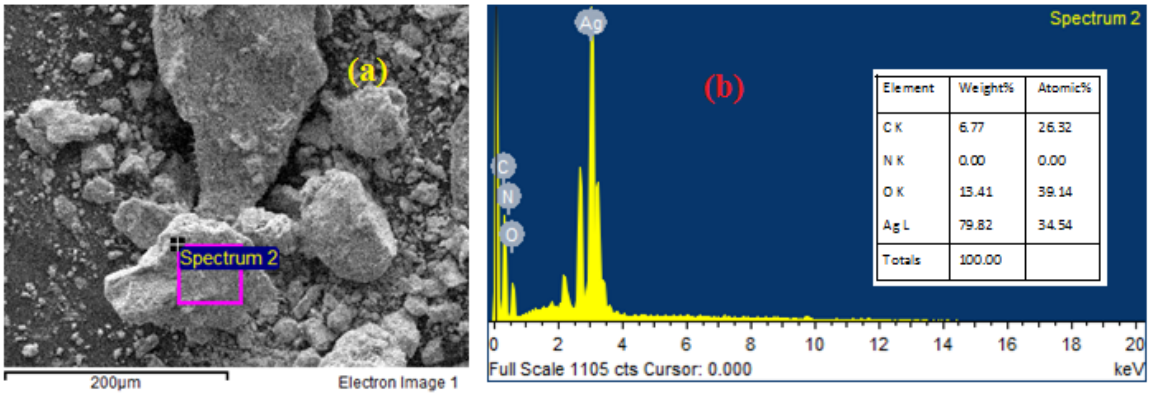

Fig. 3: (a) Micron-sized material lumps upon which the point EDS was recorded and (b) the EDS spectrum of the silver nanoparticles synthesized using the leaf extract of *P. zeylanica* L. Inset in (b), table showing the elemental percentage of silver, carbon, nitrogen and oxygen in the material

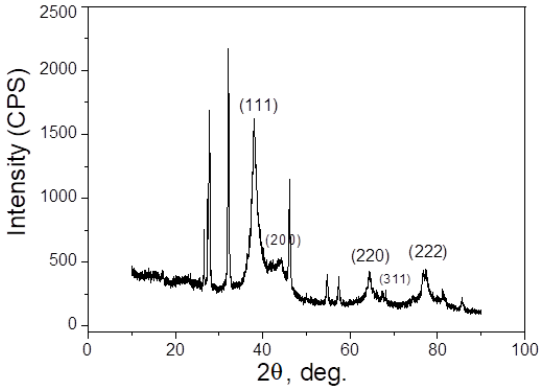

Fig. 4: Powder XRD pattern of AgNPs synthesized from the root extract of *P. zeylanica* L

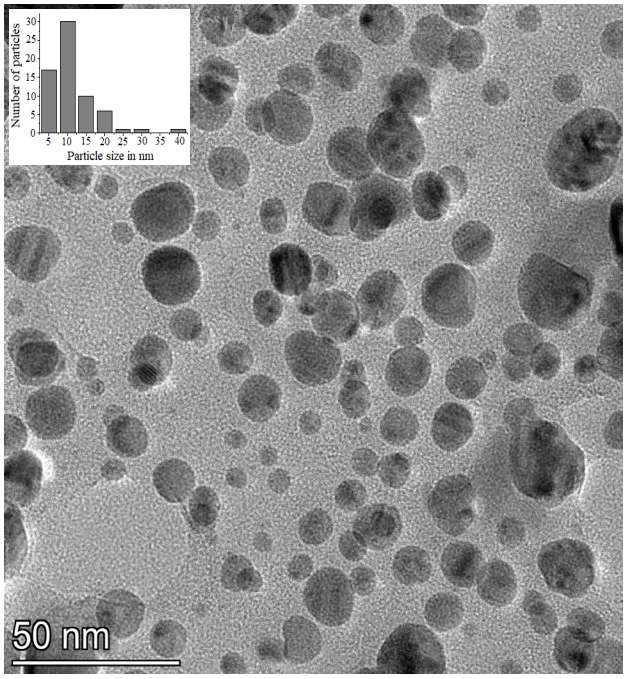

Fig. 5: Transmission electron micrographic image of the AgNPs synthesized from the leaf extract of *P. zeylanica* L. Inset: Histogram showing the particle size distribution worked out using TEM image

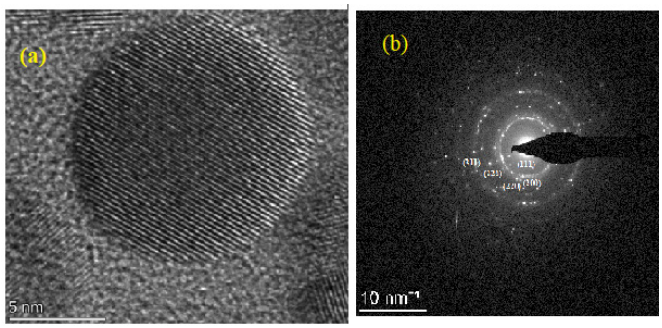

Fig.6: (a) High resolution TEM image of the single 11 nm sized silver nanoparticle synthesized from the leaf extract of *P. zeylanica* L (b) SAED pattern on the drop-cast layer of AgNP solution.

Antimicrobial Activity Studies

Use of metals such as silver, copper, iron, lead and tin, and some alloys of copper like brass and bronze are known to be used for therapeutic purposes for thousands of years in ancient Indian Ayurvedic medicinal practice [54]. Silver and the herbal extract-based phytonutrient formulations are understood to work as a bio-enhancer [55]. Antimicrobial activity is a well-known characteristic property of silver [56] and applications related to this property of the AgNPs synthesized from various possible methods have been elaborately reviewed [57, 58].

In the present paper, the relative assessment of bactericidal activity of AgNPs synthesized using the leaf extract of *P. zeylanica* L is made in terms of minimum inhibitory concentration (MIC) of AgNPs in their solid solution in nutrient agar media. MIC is the lowest possible concentration of AgNPs at and above which the bacterial growth does not take place. MICs are compared with those obtained using ciprofloxacin and are presented in Figs 7 and 8.

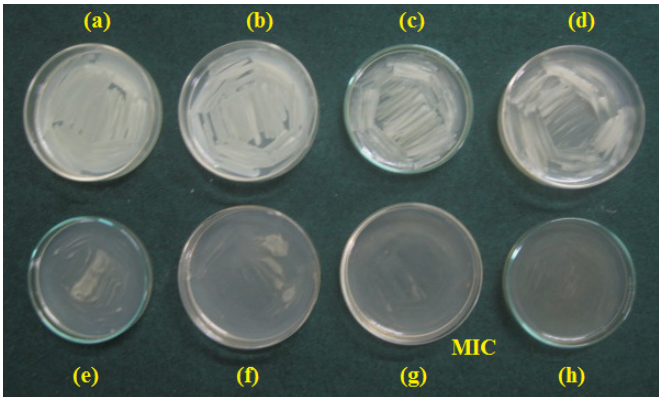

Fig. 7: Growth of the E. coli bacteria in 20 mL nutrient agar media contaminated with (a) 0.00 g/ml. (b) 1.075×10^{-4} g/mL, (c) 1.6125×10^{-4} g/mL, (d) 2.15×10^{-4} g/mL (e) 2.6875×10^{-4} g/mL, (f) 3.225×10^{-4} g/mL, (g) 3.7625×10^{-4} g/mL, (h) 4.30×10^{-4} g/mL of silver nanoparticles prepared from the extract of P. zeylanica L.

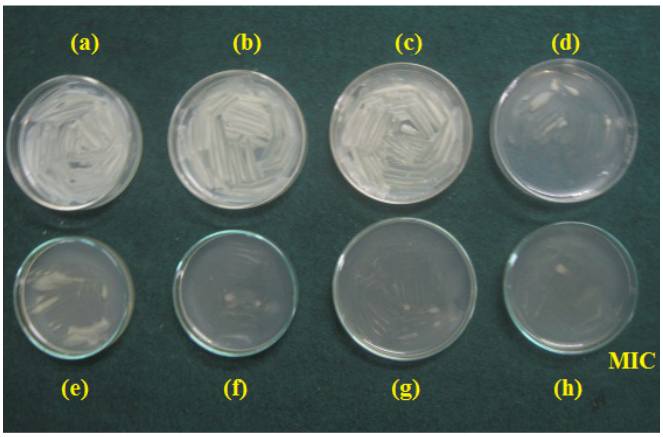

Fig. 8: Growth of the *Staphylococcus aureus* bacteria in 20 mL nutrient agar media contaminated with (a) 0.00 g/ml. (b) 1.075×10^{-4} g/mL, (c) 1.6125×10^{-4} g/mL, (d) 2.15×10^{-4} g/mL (e) 2.6875×10^{-4} g/mL, (f) 3.225×10^{-4} g/mL, (g) 3.7625×10^{-4} g/mL, (h) 4.30×10^{-4} g/mL of silver nanoparticles prepared from the extract of *P. zeylanica* L

It is clear from Figs 7 and 8 that an increase in concentration of AgNPs suppresses the growth of both the bacteria selected for the study. The determined MICs of the AgNPs synthesized from the leaf extract of *P. zeylanica* L are 37.625 $\times 10^{-5}$ g/mL and 43.0 $\times$ 10^-^{4} g/mL against *E. coli* and *S. aureus,* respectively. The experimentally determined MICs of ciprofloxacin are 20.00 $\times$ 10^{-5} g/mL against *E. coli* and 24.00 $\times$ 10^{-5} g/mL against *S. aureus* bacteria, cultured under the same experimental conditions.

CONCLUSION

The water extract of P. zeylanica L shows the presence of carbohydrates and glycosides, saponines, phytosteroids, and phenolic compounds and flavonoids. The phenolic compounds and flavonoids present in the extract reduce silver nitrate into metallic silver nanoparticles. The individual AgNPs thus formed are capped instantaneously with a monomolecular layer of phytonutrients. The high stability of the resulting AgNP solutions is due to repulsion between the individual particles possessing the same surface charge. The title AgNPs are spherical and the maximum of the particle size distribution curve appears at 10 nm. This determination is very much consistent with the particle size calculated using the PXRD peak corresponding to the (111) crystallographic plane. The metallic silver is found to crystallize in to FCC structure in the AgNPs, as evidenced by characteristic diffractions from 111, 200, 220, 222, and 311 crystallographic planes with respect to both PXRD and SAED analyses. *The AgNPs synthesized using the water extract of P. zeylanica L inhibit the growth of E. coli and S. aureus bacteria, and the magnitude of antibacterial nature is measured in MIC, which are* 37.625 $\times 10^{-5}$ g/mL and 43.0 $\times$ 10-^{4} g/mL against *E coli* and *S. aureus,* respectively. *However, the determined bactericidal effect is not superior to that shown by the commercial chemical compound ciprofloxacin.*

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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