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Phytofabrication of fluorescent silver nanoparticles from L*eucaena leucocephala* L. leaves and their biological activities

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ABSTRACT
The aim of this study was to expand an ecofriendly route for the fabrication of spherical shape silver nanoparticles (AgNPs) using an aqueous extract of *Leucaena leucocephala* L. leaves to act as stabilizing and reducing agent. Several biomolecules present in plant extract are accountable for single step reduction of metal ions into nanoparticles. The synthesized AgNPs were characterized by X-ray diffraction (XRD) profile, Fourier transform infrared (FTIR) spectroscopy, field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), Energy-dispersive X-ray spectroscopy (EDS) and Photoluminescence. Besides these, AgNPs evinced potent antibacterial, antimalarial and antimycobacterial activity against *Pseudomonas aeruginosa, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Salmonella typhi, Bacillus subtilis, Plasmodium falciparum* and *Mycobacterium tuberculosis*. The results suggest that the efficiently synthesized AgNPs can be used as potential candidates for various medicinal applications in bionanotechnology based industries.

Keywords: *AgNPs; Biological activity; Leucaena leucocephala L.; Nanotechnology*

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INTRODUCTION

Noble metal nanoparticles are amending enormous significance for the past decades due to their interesting and miraculous applications in the field of material science, biotechnology, bio-engineering, catalysis, optoelectronics, water treatment, chemistry and metal consumer products [1-2]. Amongst them, AgNPs evince novel properties depending upon their specific size, morphology and crystalline structure [3]. On account of their widespread utility, synthesis of AgNPs have been practiced via various methods such as electrochemical [4], microwave-assisted [5], radiation assisted [6], chemical reduction method [7], thermal decomposition, chemical and photochemical reactions [8], via micro-organisms [9] and plant extract. In an environmental standpoint, bio-inspired methods proffer ample benefits over chemical and physical methods as they are economically affordable, environmental benign, easy to scale up, non-mephitic and simple processes [10]. Hence, synthesis of AgNPs using plant extract has already been popular and well documented in the literature. Various plants such as *Artemisia annua* [11], *Ziziphora tenuior* [12], *Azadirechta indica* [13], *Eucalyptus chamaniana* [14], *Boerhaavia diffusa* [15], *Pistacia atlantica* [16], *Pomegranate peel* [17], *Mulberry leaves* [18], *Nelumbo nucifera* [19], *Cissus quadrangularis* [20],

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Eucalyptus hybrid [21], *Catharanthus roseus* [22], *Sesivium portulacastrum*[23], *Mangosteen* [22], *Acaltpha indica* [25], *Caralluma fimbriata* [26], *Ocimum sanctum* [27] and unripe fruit of *Annona reticulata* [28] are utilized for approachable biogenic synthesis of AgNPs. Moreover, the biological activity of AgNPs has long been recognized against a number of bacterial strains or microorganisms [29] and notably, AgNPs played an overriding role as a catalyst due to their interesting properties for several transformations [30]. The use of hazardous chemical methods have become the major concern for the scientific community and to get rid of it, to develop green protocols for industrial scale up and material synthesis is the sole option.

Leucaena leucocephala L. is a thornless long-lived shrub (miracle tree) which may grow up to heights of 5-20 m. Its origin is in Central America where its fodder value was recognized. Some common names of *Leucaena leucocephala* L. are found in various countries such as Leucaena in Australia, Lamtoro in Indonesia, Katin in Thailand, Yin ho huan in China, Subabul in India and Guaje in Mexico. This evergreen plant is deep-rooted. Leaves have a high nutritive value and live weight gain compared with feeding on pure grass pasture (Fig. 1). The leaves of this plant are highly productive; combines well with campanion (companion) grasses can be grazed with minimum losses from trampling and are also internationally marketed as animal feed. Moreover, brown and black dyes are extracted from the leaves and bark of *Leucaena leucocephala* L. The plant can provide timber, green manure, firewood, resin, reclamation, erosion control and shade. Other uses include the young leaves and seeds as vegetables for human consumption. In the present phase, *Leucaena leucocephala* L. has spread to most countries of the tropical world where it is used as a shade plant for plantation crops.

Fig. 1: Leaves of *Leucaena leucocephala* L.

Herein, we report the cost-effective, rapid and eco-friendly green synthesis of AgNPs using plant extracts of *Leucaena leucocephala* L. and their antibacterial, antimalarial and antimycobacterial activity against selected bacterial pathogens has been evaluated. Hence it is proposed that the as-synthesized AgNPs have biomedical applications in near future.

MATERIALS AND METHODS

Materials

Silver nitrate ($AgNO_3$, AR grade, 99.99%, Sigma-Aldrich), sodium bicarbonate ($NaHCO_3$, Analytical grade, 99.7%, Sigma-Aldrich) and dimethyl sulfoxide (DMSO, ACS reagent, 99.9%, Sigma-Aldrich) were used. All chemicals were used as such without any further purification. All the solutions were prepared using deionized water during the synthesis. The fresh leaves of *Leucaena leucocephala* L. were collected from Chandwad college campus, Maharashtra, India. The collected leaves were washed with deionized water and cut into small pieces. All glassware's are washed with HNO_3 and distilled water and dried in an oven.

Biosynthesis of AgNPs

10g small wizened pieces of *Leucaena leucocephala* L. leaves were transferred into 250 mL beaker containing 100 mL deionized water. The mixture was boiled at 80 °C for 15 minutes and cooled at room temperature followed by filtered through ordinary filter paper. Thereafter, resultant filtrate was again filtered through Whatmann No.1. The filtered extract was stored in a refrigerator at 4 °C and used for the synthesis of AgNPs. The solution of 2 mM silver nitrate ($AgNO_3$) was prepared in double distilled water, kept at room temperature and used for the synthesis of AgNPs. *Leucaena leucocephala* L. leaf extract was mixed to 2 mM aqueous $AgNO_3$ solution in 1:8 ratios in a 250 ml beaker and color of medium changed to brown within 1 min. After a period of time, the color of solution turns to dark brown. The solution was incubated at room temperature in dark for 24 hrs. The resultant solutions were centrifuged at 10000 rpm for 5 min. Then the residue was collected after discarding the supernatant liquid. The collected AgNPs were allowed to dry in a Petri dish.

Characterization of the Synthesized AgNPs

The completely dried powder of synthesized AgNPs was used for the Fourier transform Infra-red (FT-IR) (JASCO 4100) analysis. Transmission electron microscopic (TEM) images were taken on Philips CM 200 operated at accelerating voltages of 20 and 200 kV. X-ray diffraction (XRD) pattern of AgNPs was obtained using Bruker D8-Advanced Diffractometer (λ=1.54 Å) from which average crystal size of AgNPs was calculated. The surface morphology and elemental study of synthesized AgNPs were carried out by field emission scanning electron microscopy (FE-SEM) and Energy Dispersive Spectroscopy (EDS) (JEOL JSM-6701). Photoluminescence studies were evaluated by using fluorescence spectrophotometer (Jobin Yvon Flurolog-3-11, Spectrofluorimeter).

Phytochemical Screening-Qualitative Analysis

Fresh aqueous extract of *Leucaena leucocephala* L. leaves was used for phytochemical screening-Qualitative analysis. Phytochemical screening was carried out by a standard method [31].

Antibacterial Activity of Synthesized AgNPs

The antibacterial activity of synthesized AgNPs was determined by using disc diffusion method. This method was employed against selected human pathogens i.e. *Pseudomonas aeruginosa, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Salmonella typhi and Bacillus subtilis* obtained from Institute of Microbial Technology, Chandigarh, India. The nutrient agar medium (g/l) plates were prepared, well sterilized and solidified. After solidification, bacterial cultures spread evenly over the plate, and then the different concentration of AgNPs solution was poured into each plate. Thereafter, these plates were incubated in an incubator at 37 ^{0}C for 24 hrs and measured zone of inhibition of selected bacterial strains against standard reference drugs.

In Vitro Antimalarial Screening of Synthesized AgNPs

In vitro antimalarial assay was carried out in 96 well microtitre plates according to the microassay protocol of antimalarial activity [32]. The cultures of *Plasmodium falciparum* strain was maintained in medium RPMI-1640 supplemented with 25 mM HEPES, 0.23% $NaHCO_3$, 1% D-glucose and 10% heat-inactivated human serum. The asynchronous parasites of *Plasmodium falciparum* were synchronized after 5% D-sorbitol treatment to get only the ring stage parasitized cells. For carrying out the assay, an initial ring stage parasitemia of 3% hematocrit in a total volume of 200 µl of medium RPMI-1640 was determined by Jaswant Singh Bhattacharya (JSB) staining [33] to evaluate the percent parasitemia and uniformly maintained with 50% RBCs (O^{+ve}). The culture plates were incubated at 37 °C in a candle jar. After 36 hrs incubation, thin blood smears from every cell was prepared and stained with JSB stain. Thereafter, slides were microscopically observed to note that maturation of the ring stage parasites into schizonts and trophozoites in the presence of different concentrations of the synthesized AgNPs. Therein, synthesized AgNPs concentration which inhibits the complete maturation into schizonts was recorded as the minimum inhibitory concentration (MIC). Chloroquine and Quinine were used as the reference drugs for experiments.

In Vitro Antimycobacterial Screening of Synthesized AgNPs

The antimycobacterial screening for green synthesized AgNPs was obtained for *Mycobacterium tuberculosis* $H_{37}RV$, by using L. J. (Lowenstein and Jensen) MIC method [34]. Stock solutions of primary 1000, 500, 250 and secondary 200, 100, 62.5, 50, 25, 12.5, 6.25, 3.25 µg/ml of AgNPs in DMSO were added in the liquid L. J. Medium and then media was sterilized. A culture of *Mycobacterium tuberculosis* $H_{37}RV$ growing on L. J. medium was harvested in 0.85% saline in bijou bottles. These tubes were then incubated at 37°C for 24 hrs followed by streaking of *Mycobacterium tuberculosis* $H_{37}RV$. The growth of bacilli was seen after 12 days, 22 days and finally 28 days of incubation respectively. Thereafter, AgNPs containing tubes were compared with control tubes where medium alone was incubated with *Mycobaterium tuberculosis* $H_{37}RV$. The concentration at which no development of colonies occurred or < 20 colonies was taken as MIC concentration of test compound. The standard strain *Mycobacterium tuberculosis* $H_{37}RV$ was tested against reference drug isoniazid [35].

RESULTS AND DISCUSSION

XRD analysis

The crystal structure and crystallite size of synthesized AgNPs were confirmed by using the

XRD pattern (Fig. 2) in the 2θ range of 20° to 80°. The prominent and intense peaks at 2θ=38.00°, 44.27°, 64.47° and 77.30° corresponding to the (111), (200), (220) and (311) Bragg's reflections of the face-centered cubic (FCC) crystal structure (JCPDS card no. 04-0783) of AgNPs, respectively. The average crystallite size (D) of AgNPs was calculated by using Scherrer's equation.

$$D = K\lambda / \beta \, COS\theta \qquad (1)$$

Where, D is the crystal size of synthesized AgNPs (nm), θ is Bragg angle (degrees), λ is the wavelength of the X-ray source used (1.54060 Å), β is the angular width at the half maximum of the diffraction peak (radians) and K is the constant of Scherrer's equation which is generally, for the spherically grown nanoparticles 0.94. Therefore, in our present investigation, we have used K= 0.94 to calculate the D value for synthesized material. The average crystal size of the synthesized AgNPs is estimated to be around 32-50 nm. The overall optostructural study indicated that synthesized material has a pure face-centered cubic crystal structure with nanocrystalline nature and was good agreement with TEM and FE-SEM results.

Phytochemical screening studies

Table 1 describes the qualitative pharmacognostic evaluation of aqueous leaf extract of *Leucaena leucocephala* L. highlighted the presence of tannins, saponins, coumarins, flavonoids, cardial glycosides, steroids, phenols, carbohydrates, amino acids, etc. The amino acids and phenols play a vital role in the bio-reduction process due to scavenging capabilities of their –OH groups. The antioxidant property of plant constituents is accountable, thus act as a reducing agent for stabilization of AgNPs. A various compound like Gallic acid, Mimosine, Caffiec acid, β-sitosterol, Chrysoenol, Kaempferol-3-o-rutinoside, Luteonil -7-o-glucoside, etc. (Fig. 3) [36-38] which can play a role in reduction and stabilization in the biosynthesis of nanoparticles.

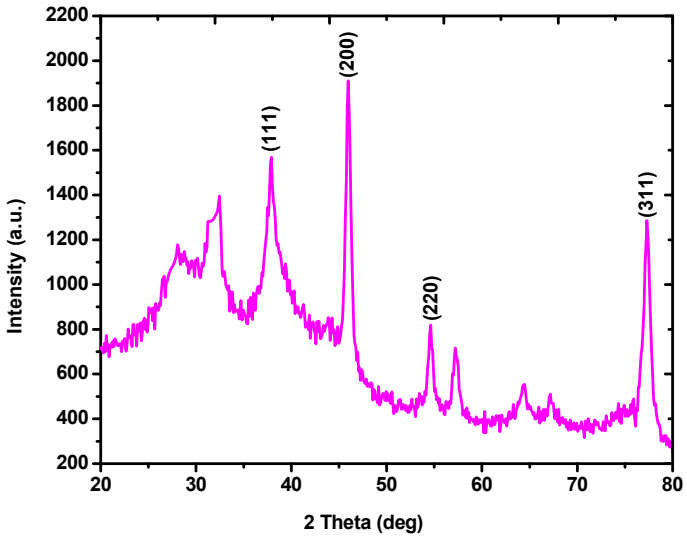

Fig. 2: X-ray diffraction profile of synthesized AgNPs

Table 1: Phytochemical Screening of aqueous leaves extract of *Leucaena leucocephala* L.

Phytochemical	Test	Phytochemical	Test
Tannin	+	Flavonoids	+
Coumarins	+	Emodins	-
Proteins	-	Saponins	+
Cardial Glycoside	+	Anthraquinone	-
Anthocyanosides	-	Phenols	+
Steroids	+	Amino acids	+
Carbohydrate	+		

FT-IR studies

FT-IR spectrum (Fig. 4) of AgNPs exhibited bands around 3630 cm^{-1} corresponds to O-H stretching vibration. The bands at 1739 cm^{-1} are observed as amide, ester, and acids arise due to carbonyl group stretching vibration. The band at 1417 cm^{-1} O-H bend of polyphenol, confirm the presence of the aromatic group. The peak at 1026 cm^{-1} corresponds to C-OH stretching frequency in phenolic compounds. The biomolecules in leaf extract of *Leucaena leucocephala* L. i.e. amino acids (Mimosine), phenols, flavonoids(Luteolin-7-o-glucoside) [38] and enzymes are responsible for the capping and stabilization of AgNPs as

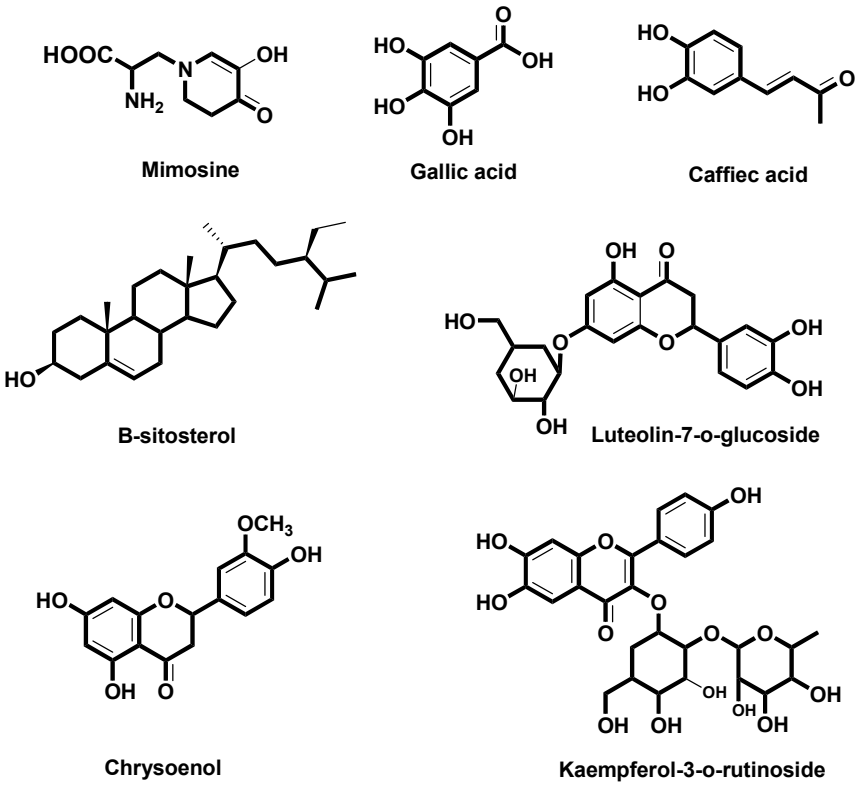

Fig. 3: Major bioactive compounds in the natural extract of *Leucaena leucocephala* L.

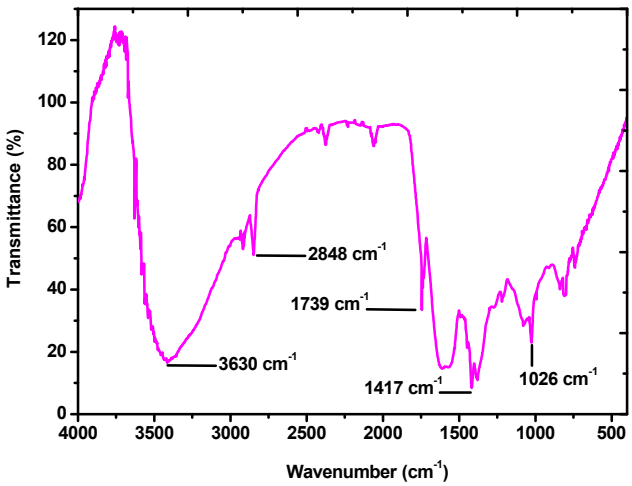

Fig. 4: FT-IR spectrum of green synthesized AgNPs

well as to interact with metal salts (Ag^{+}) via these functional group and mediated their reduction to metal nanoparticles (Ag^{o}). A plausible mechanism [39] for the biosynthesis of silver nanoparticles is indicated in Fig. 5. Mostly phenolic and flavonoid compounds are involved in the biosynthesis of nanoparticles, however, the exact mechanism by which type of nanoparticles are produced is an open area of research.

FE-SEM microphotographs

FE-SEM provides the acquaintance about surface morphology and grain size of the synthesized AgNPs. Fig. 6 shows FE-SEM images at different magnifications. It can be seen that the average crystal grain size of the quasi-spherical morphology AgNPs was mainly 35-47 nm except for slight agglomeration. This result exceeds the literature result which spherical shape of AgNPs was prepared by green synthesis method [16].

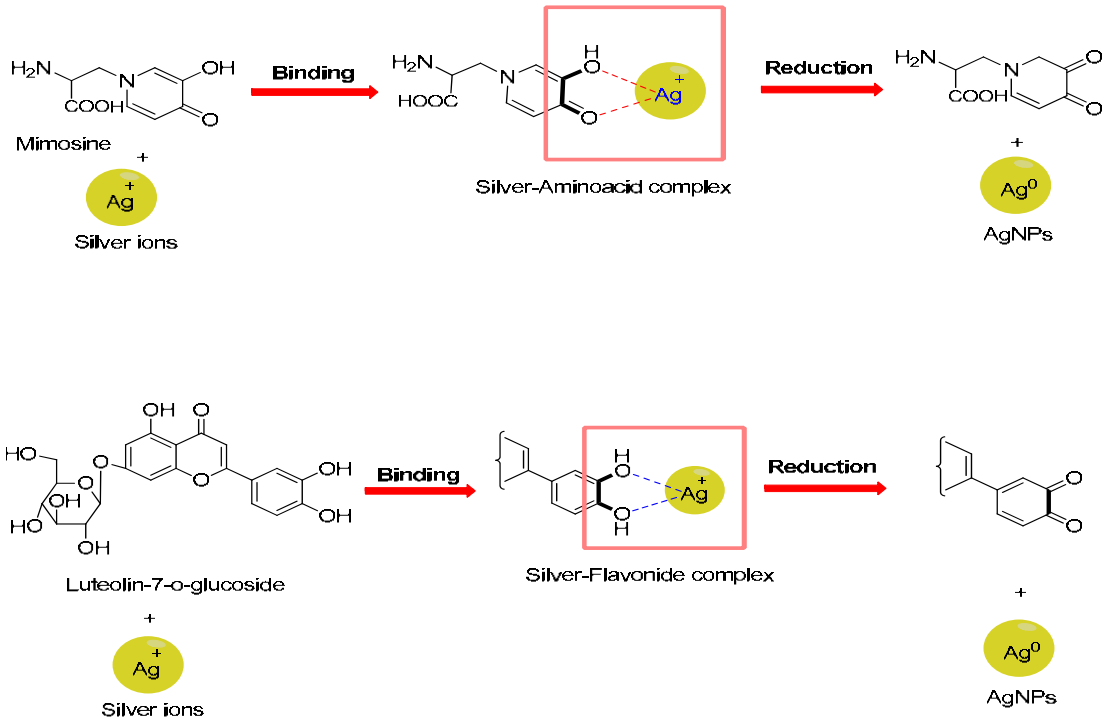

Fig. 5: Plausible reaction mechanism for generation of AgNPs using biomolecules of *Leucaena leucocephala L.*

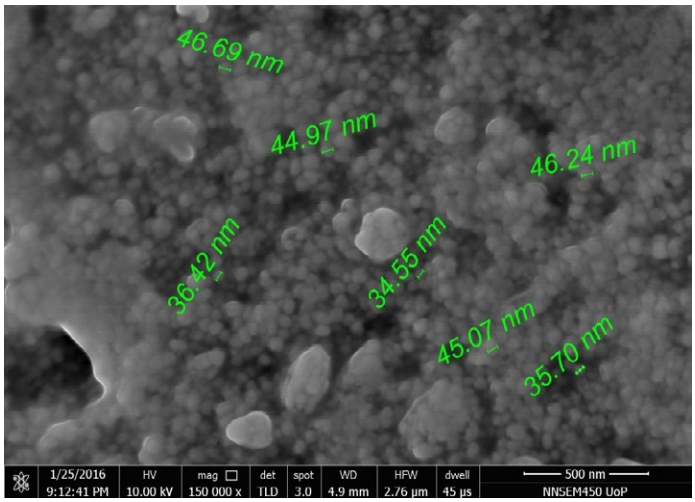

Fig. 6: FE-SEM microphotographs of AgNPs deposited on a carbon strip

TEM images

The TEM images of biosynthesized AgNPs recorded after completion of reaction are depicted in Fig. 7. The AgNPs formed were quasi-spherical in shape in the range of 25-50 nm size having medium surface area. The particles were monodisperse, with only a few particles of different size. However, TEM results were good in agreement XRD results also.

EDS analysis

Fig. 8 highlights EDS spectrum of synthesized AgNPs. EDS spectrum confirmed the presence of Ag, along with C and O in the synthesized material. The peaks for C are from the grid used while peak O corresponds to the phenols and enzyme capping over the synthesized AgNPs. The biosynthesized metallic silver nanoparticles exhibit typical optical absorption peak in the range of 3 to 4 keV.

Photoluminescence study

Biosynthesized AgNPs are reported to exhibit visible photoluminescence and their fluorescence spectra are shown in Fig. 9 (a and b). The optimized AgNPs were found to be luminescent with four emissions at 284, 399, 508 and 559 nm for an excitation at 250 nm. When AgNPs were excited at 300 nm, it showed excitation at 605 nm, the excitation of 300 nm is of high intensity in comparison to another one. The luminescence at 250 and 300 nm may be due to the presence of phytoconstituents or antioxidants present in the plant extract. The AgNPs synthesized using *Azadirechta indica* leaf extract are also reported to be luminescent with emission band at 561 and 600 nm [13].

Antibacterial activity of AgNPs

Scrutiny of the literature reveals that AgNPs

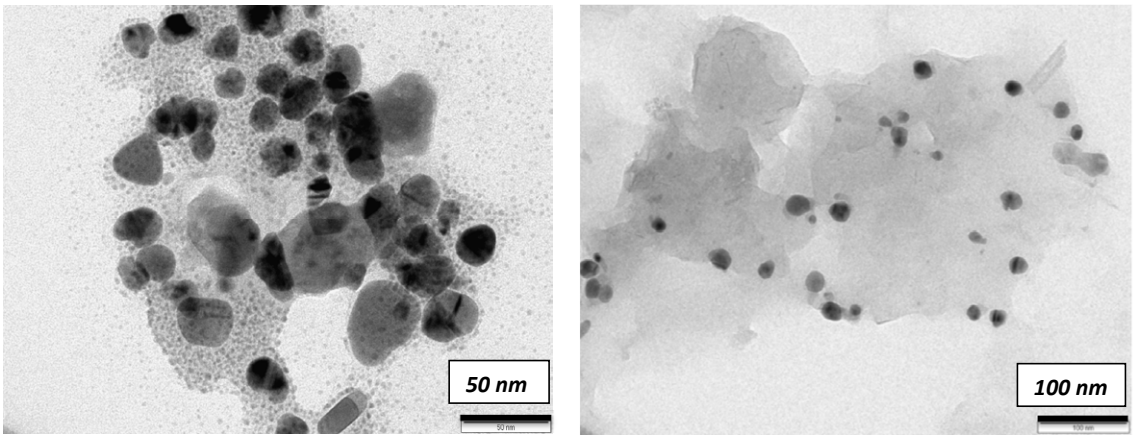

Fig. 7: TEM images indicating the presence of spherical AgNPs recorded at various magnifications.

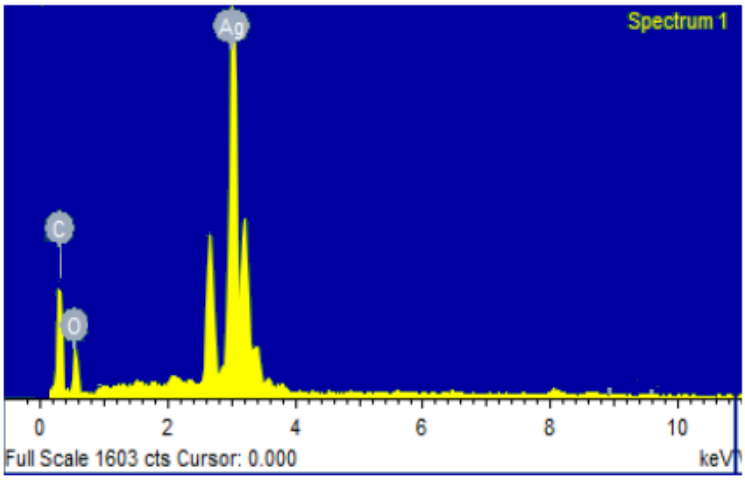

Fig. 8: EDS spectrum of synthesized AgNPs

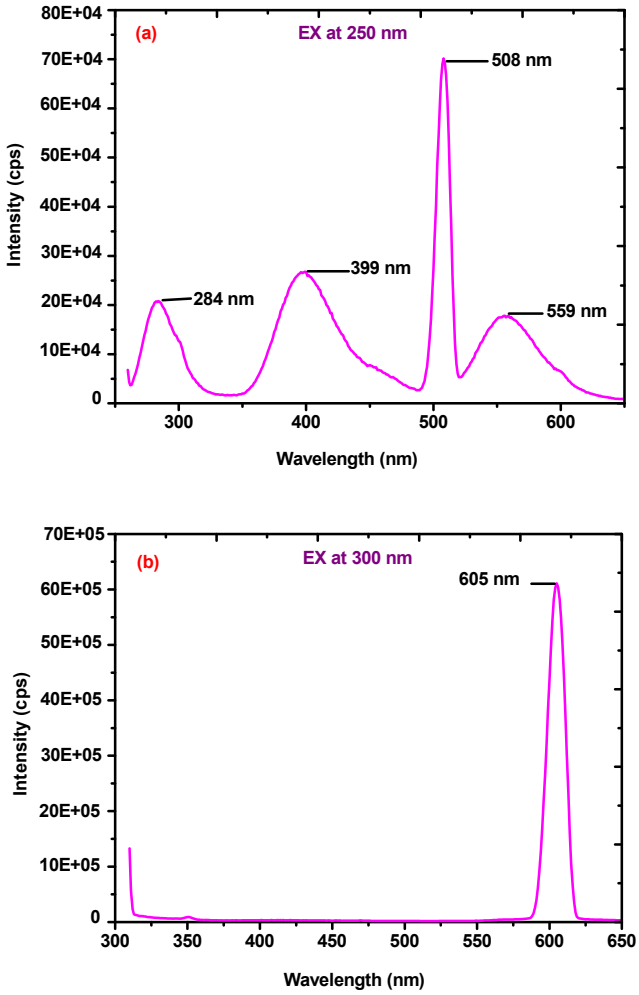

Fig. 9: Fluorescence spectra of green synthesized AgNPs formed with excitation at (a) 250 nm and (b) 300 nm

are highly poisonous to most of the human pathogens [29]. In this context, we decided to investigate antibacterial activity of green AgNPs against selected human pathogens *viz Pseudomonas aeruginosa, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Salmonella typhi, Bacillus subtilis*. These bacterial and fungal strains namely *P. aeruginosa* MTCC 1688, *S. pyogenus* MTCC 442, *S. aureus* MTCC 96, *E. coli* MTCC 443, *S. typhi* MTCC 98 and *B. subtilis* MTCC 441 were added on a nutrient agar plate and spread evenly over the plate with the help of glass spreader. The various concentrations of synthesized AgNPs (25, 50, 100, 250, 500 µg/ml.) were tested for antibacterial activity against these pathogens with ampicillin has a positive control. The plates were then kept at 4-5 °C for 1 hr. followed by incubated in an incubator at 37 °C for 24 hrs. Thereafter, 24 hrs, exact zone of inhibition was measured with respect to reference drugs (Table 2). Gratifyingly, it was observed that biosynthesized AgNPs exhibited potent antibacterial activity against the selected strains [40]. These results are in agreement with previous studies that examined the antibacterial activity of AgNPs [41].

Antimalarial activity

The synthesized AgNPs were screened for their in vitro antimalarial activity against *Plasmodium falciparum* by measuring the significant minimum inhibitory concentration (µg/mL) against standard reference drugs like Quinine and Chloroquine, as shown in Table 3.

Table 2: Zone of inhibition (mm) of biosynthesized AgNPs against selected human pathogens

Test pathogens	Inhibition zone (mm) of AgNPs (µg/ ml)					Control
	25	50	100	250	500	
P. aeruginosa	14	15	16	17	20	18
S. pyogenus	12	15	16	19	21	14
S. aureus	13	16	19	20	23	16
E. coli	12	14	17	18	22	19
S. typhi	14	16	17	19	20	24
B. subtilis	13	15	16	17	19	20

Table 3: Minimum inhibition concentration (MIC) of biosynthesized AgNPs against *Plasmodium falciparum*

Sr. No	Compound Name	Mean IC_{50} values
1)	AgNPs	0.96 µg/ ml
2)	Chloroquine (Standard)	0.020 µg/ ml
3)	Quinine (Standard)	0.268 µg/ ml

Table 4: Minimum inhibition concentration (MIC) of biosynthesized AgNPs against *Mycobacterium tuberculosis*

Sr. No	Compound Name	MIC (µg/ ml)
1)	AgNPs	125 µg/ ml
2)	Isoniazide (Standard)	0.20 µg/ ml

Antimycobacterial study

The antimycobacterial screening of biosynthesized AgNPs was performed using Lowenstein-Jensen MIC method (Table 4) and it is noted that AgNPs was the only displaying inhibition of *Mycobacterium tuberculosis* $H_{37}RV$ completely (99%) at the MIC of 125 µg/ml.

CONCLUSIONS

We have demonstrated an environmentally benign, robust and ecofriendly synthesis of stable and spherical AgNPs using an aqueous extract of *Leucaena leucocephala* L. The biosynthesized AgNPs are found good antibacterial, antimalarial and antimycobacterial agents and thus can be used as potential candidates for various biomedical applications and will play a vital role in medical devices in near future. The plant extract is ascribed to the relative levels of steroids, phenols, carbohydrates, flavonoids and amino acids which act as reducing as well as capping agents AgNPs. Moreover, phyto constituents provide stability of AgNPs as explicit from FT-IR and photoluminescence studies.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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