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ARTICLE

Synthesis of Silver Nanoparticle Using Bioactive Phenolic Compound Extracts of *Leucas aspera* and *Leucas cephalotes* and Evaluation of its Antibacterial Activity

M. Salma Kausar, B.K. Manjunatha✉, C. Purvika and Mizba Farkana

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ABSTRACT

The present study was carried out to investigate the antibacterial activity of the bioactive phenolic extract from *Leucas aspera* and *Leucas cephalotes*. The phenolic compounds were extracted using water: ethanol (1:3, v/v) by hydroethanolic extraction method. The hydroethanolic extracts were subjected to qualitative and FTIR analysis as a confirmatory step for the presence of phenolics. Synthesis of silver nanoparticle from both plants was carried out by acid hydrolysis method and subjected to UV-visible spectrophotometry, SEM, TEM and XRD analysis, for confirmation of tagged bioactive compound to $AgNO_3$. The nanoparticle size distribution ranged between 50-94 nm in *L. aspera* and 40-67 nm in *L. cephalotes.* The antibacterial study was carried out using both crude phenolic extract and synthesized nanoparticles and tested against 5 pathogens namely *Escherichia coli* (ATCC® 8739™), *Pseudomonas aeruginosa* (ATCC® 25619™), *Staphylococcus aureus* (ATCC® 6538™), *Bacillus subtilis* (ATCC® 11774™) and *Klebsiella pneumonia* (ATCC® 13882™) for their antibacterial activity. From present study, the crude extract of *L. cephalotes* showed good antibacterial effect against test pathogen species wherein highest inhibition was observed in, *P. aeruginosa,* followed by *B. subtilis* and *S. aureus* with an average zone of inhibition of 23, 14 and 12 mm, *E. coli* and *K. pneumonia* measured 9 and 7 mm. The crude extract of *L. aspera* showed the highest inhibition in *P. aeruginosa* followed by *S. aureus* and *E. coli* with an average zone of inhibition of 12,11 and 10 mm *B. subtilis* and *K. pneumonia* measured 8 and 7 mm. Statistical analysis was calculated using One way ANOVA and was found to be statistically significant with $p < 0.05$.

KEYWORDS

Silver nanoparticle, *Leucas aspera*, *Leucas cephalotes*, Antibacterial activity.

Author affiliations:

Department of Biotechnology, The Oxford College of Engineering, Bengaluru-560068, India

✉To whom correspondence to be addressed:

E-mail: enghodbt@theoxford.edu; dr.manjunath.toce@gmail.com

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INTRODUCTION

Traditionally, the species of *Leucas* is used as a medicinal herb because of its potential for treating many infections and curing diseases. The plant is found to exhibit good antimicrobial, phytotoxic, anticarcinogenic, anti-inflammatory, antioxidant antimalarial, insecticidal and antidiabetic effects. The genus hence has enormous healing potential [1]. Maximum species diversity was reported from East Africa and India. Various phytochemicals were isolated and most of which belong to the classes of terpenes, fatty substances, glycosides, flavo-

noids, lignans and alkaloids [2]. About 43 species have been discovered in India, some of which have been proven to have therapeutic benefits which are *L. aspera*, *L. cephalotes*, *L. hirta*, *L. linifolia*, *L. lavendulaefolia*, *L. lanata*, *L. stelligra*, *L. ciliata*, *L. biflora*, *L. martinicenesis* [3]. The chemical components such as diterpenes, tannins, saponins, sterols, oleic acid, linoleic acid, palmitins, stearins, oleanols and alkaloids have also been isolated from this plant [4]. Plant phenols are structurally varied group of chemicals which give plants their organoleptic qualities. Simple phenolic acids or complex compounds linked to an oxygenated heterocyclic ring, such as benzoic acid derivatives, stilbenes, tannins, lignans, anthocyanins, flavonoids and coumarins, can all be found in plants [5]. Organic acids, such as methoxybenzyl benzoate, 4-hydroxybenzoic acid and uric acid, have been recovered from the chloroform fraction of a methanolic extract of the whole plant of *Leucas* species [6].

Silver nanoparticles have a wide range of physical, chemical and biological properties, making them appealing for use in a variety of sectors [7]. Biosorption, particle aggregation and cellular absorption are all examples of interactions between nanoparticles and bacteria. The target organism suffers from membrane damage and toxicity as a result of this, the target organism is less likely to develop resistance to AgNPs and cell death is most likely caused by DNA replication inhibition or protein inactivation caused by the release of K^+ ions. The antibacterial efficiency of nanomaterials is influenced by their size and surface modification [8,9]. As a result, nanoparticles are an important technique in drug delivery. Therefore, an alternate treatment is necessary that has a low risk of causing the bacteria to grow resistant. Because *L. aspera, L. cephalotes* have antifungal, antioxidant, antimicrobial and cytotoxic properties, it was chosen to make silver nanoparticles (AgNPs) as antimicrobial agent [10].

EXPERIMENTAL

Bacterial strain collection: 5 Test pathogens namely *Escherichia coli* (ATCC® 8739™), *Pseudomonas aeruginosa* (ATCC® 25619™), *Staphylococcus aureus* (ATCC® 6538™), *Bacillus subtilis* (ATCC® 11774™) and *Klebsiella pneumonia* (ATCC® 13882™) were procured from American type culture center (ATCC) for the study.

Plant sample collection: The plant of *L. aspera* and *L. cephalotes* were collected from Western ghats and Turahalli forest from Karnataka, India.

Plant extract preparation: The whole plants of *L. aspera* and *L. cephalotes* were washed with distilled water for removal of dust particles and shade dried at room temperature for 8-10 days and were pulverized using blender. 100 g of powdered plant samples were mixed with 250 mL of distilled water and 750 mL of ethanol and the content was allowed to stand over a period of 3 days with intermittent stirring. The suspension was vacuum filtered and excess solvent was evaporated to concentrate the extract to dryness under a rotary flash evaporator. The extracts were stored at 4 ºC and used for *in vitro* screening of antibacterial activity [11].

Green synthesis of silver nanoparticles using plant extracts: 10 mL of plant extracts were mixed with 90 mL of 1 mM $AgNO_3$ aqueous solution and the reaction mixture was heated to 80 ºC at different time interval. The reaction mixture were cooled to room temperature and the absorbance was measured at regular intervals using a UV spectrometer (200-600 nm). The solution was later subjected to centrifugation at 13000 rpm for 15 min. The pellet was dried at 55 ºC in the oven, scraped and used for antibacterial study [12].

FTIR analysis: The FTIR measurements were performed using FTIR spectrometer (Bruker Tensor 127) in the wavenumbers region 4000-400 cm^{-1}. Spectral resolution was set at 4 cm^{-1}.

UV-visible analysis: A UV-visible spectrophotometer was used to do a preliminary evaluation of the tagged nanoparticle. The silver nanoparticle synthesis was monitored at regular interval by observing the reduction of silver ions until no further colour change in the sample was found, the absorbance spectra monitored was in the range of 200-600 nm.

Scanning electron microscopy (SEM) analysis: For validation, the produced nanoparticle tagged with crude bioactive chemical extract in powdered form was exposed to SEM using an FEI Quanta scanning electron microscope at an accelerating voltage of 20 kV.

Transmission electron microscopy (TEM) analysis: Aqueous sample of tagged AgNP was placed on TEM grid and preparation of grid was carried out. The sample was air dried before the imaging process was initiated. Separate photos were captured at 200 kV and magnification was between 20,000x to 100,000x.

Bacterial strain preparation: Bacterial strain *E. coli* (ATCC® 8739™), *P. aeruginosa* (ATCC® 25619™), *S. aureus* (ATCC® 6538™), *B. subtilis* (ATCC® 11774™) and *K. pneumonia* (ATCC® 13882™) were procured from American type culture center and were cultured in appropriate media at 37 ºC for 24 h, with 250 rpm agitation. The strains were plated on Muller Hinton Agar (MHA)

Antibacterial activity studies: Antimicrobial property of the extract was evaluated by well diffusion technique [13]. A 6-8 mm diameter hole was aseptically punched using a cork borer over the solidified agar plate. Then, 20-50 µL sample of desired concentration was loaded and incubated at 37 ºC for 24 h on MHA platted bacterial strains. Then, the zone of inhibition was measured and recorded in mm diameter. Both positive and negative control were maintained alongside the experimental strains. The study was carried out in triplicates and one-way ANOVA was calculated to assess the statistical significance with $p < 0.05$.

Determination of minimum bactericidal concentration (MBC): The MBC of the crude extract and tagged nanoparticle was tested against the test organism by macro broth dilution assay method [14]. The test organisms were grown in the media containing crude extract and bioactive compound tagged nanoparticles and a group without tagged nanoparticles was taken as control. The overnight grown culture of the test organism was later streaked on agar plates from each concentration and MBC was determined.

RESULTS AND DISCUSSION

FTIR studies: The presence of polyphenolic bioactive compounds as part of the phytoconstituents present in extract

tagged to AgNP was detected using FTIR analysis. IR (KBr, ν_{max}, cm^{-1}): 3397 (OH), 2091 (CH *str.*), 1620 (CO *str.*), 1328 (CH *str.*) in *L. cephalotes*. IR (KBr, ν_{max}, cm^{-1}): 3400 (OH), 2926 (CH *str.*), 1632 (CO *str.*) in *L. aspera* was observed. Fig. 1a-b obtained spectral evidence, confirmed that the isolated extract contains OH, CH and CO regions, which corresponds to phenol, aromatic and cyclic groups, respectively. The phyto-compounds and proteins/enzymes found in plant extracts have been shown to behave as a capping agent [15,16].

UV-vis analysis: The preliminary characterization of AgNP tagged with the bioactive compound of both extracts was carried out using UV-vis spectroscopy. The addition of $AgNO_3$ to the extract resulted in the colour change to dark brown when incubated up to 4 h, thus indicating the formation of nano-particles, this is due to the reducing ability of the extract. Fig. 2a-b shows a remarkable peak in the range of 220-240 nm and an SPR band was observed at 228 nm indicating the synthesis of AgNPs, which showed saturation at the end of 4th h.

SEM analysis: A detailed investigation information of nanoparticles was validated using SEM. Fig. 3a-b reveals the image of the bioactive compound attached to the nanoparticle at 100 µm magnification, this conclusion was drawn with the observation of the image which shows the largest magnification of the nanoparticle. An observation showed the spheroidal shape of nanoparticles and also the formation of small aggregates was observed.

TEM analysis: The absorption spectra reveal only the formation of nanoparticles; Hence TEM analysis was carried out to assess the size of the synthesized AgNPs. It reveals that the smallest size of the AgNP as observed on a scale of 500 nm was 50 nm in *L. cephalotes* and *L. aspera* showed the smallest size of 40 nm on a scale of 400 nm (Fig. 4). The nanoparticle showed spheroidal morphology and few were found as aggregate assumed as a result of improper capping or aggregation.

Antibacterial assay: The effect of the AgNPs and crude extract of both the species *L. aspera* and *L. cephalotes* on pathogenic bacterial strains of *E. coli*, *B. subtilis*, *P. aeroginosa, K. pneumonia* and *S. aureus* by well diffusion method was investigated. In the present study, clear zones more than 10 mm were considered as highly susceptible, 7-10 mm was considered as moderate and less than 7 mm was considered resistant. From the results obtained against the control drug azithromycin (30 µg/mL), it was visible that the AgNPs tagged extracts showed better antibacterial effect against the pathogenic strains as compared to the crude extract as indicated in Fig. 5a-b. Further, One way ANOVA results of *L. aspera* showed the *p*-value of 0.010576 and *L. cephalotes* showed a *p* value of 0.008907. Hence, the experimental work can be considered statistically significant.

Conclusion

The silver nanoparticles synthesis from the hydroethanolic extract of *L. aspera* and *L. cephalotes* were developed which showed the significant antibacterial activity. Characterization of this nanoparticle showed the expression of spheroidal shape and its aggregation properties. Further, one way ANOVA disproves the null hypothesis as the results were statistically significant indicating α value to be < 0.05 and suggest that the study conducted has an encouraging *in vitro* potential for the bioactive compound to be used as an effective drug candidate. Hence, it is suggested that the extracted phenolic can be further

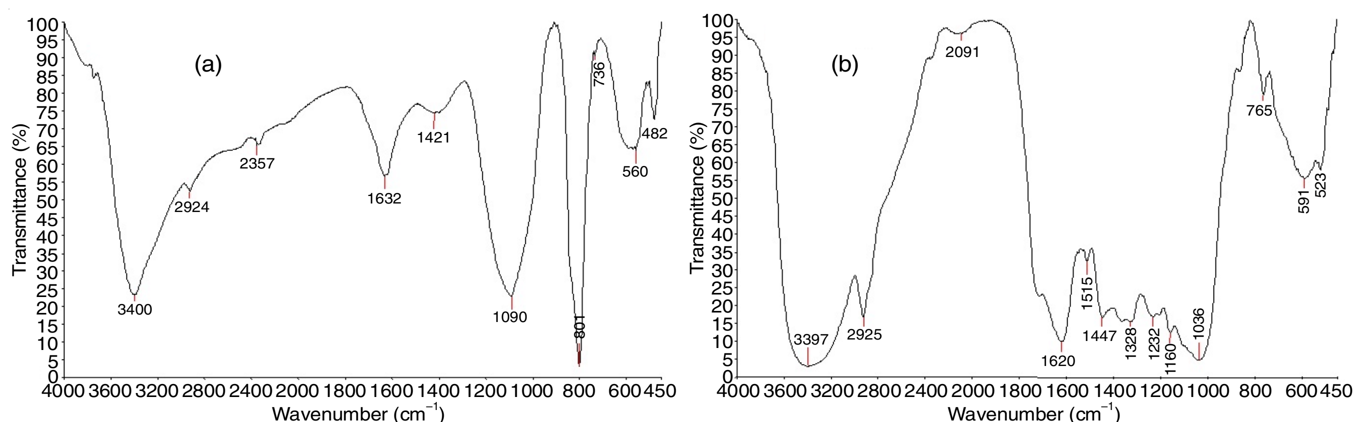

Fig. 1. FT-IR result for (a) *L. cephalotes* and (b) *L. aspera*

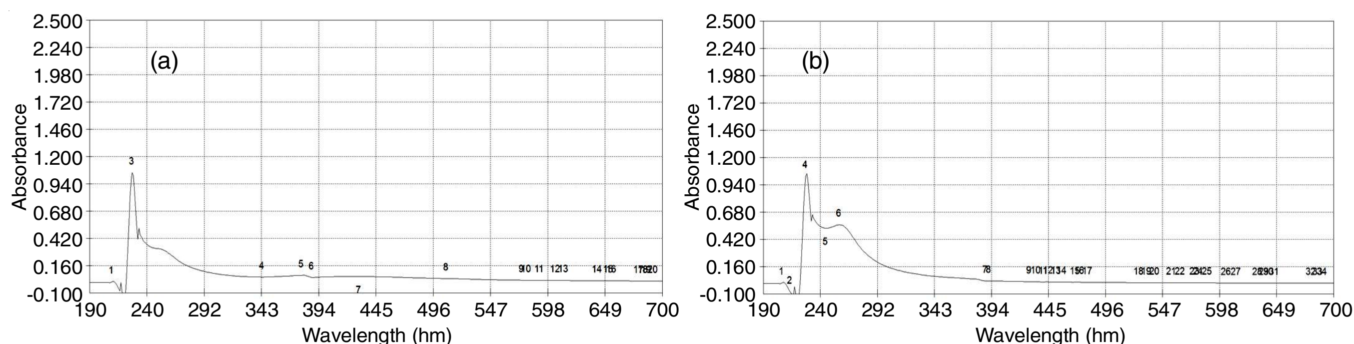

Fig. 2. UV-visible spectrum for (a) *L. aspera* and (b) *L. cephalotes*

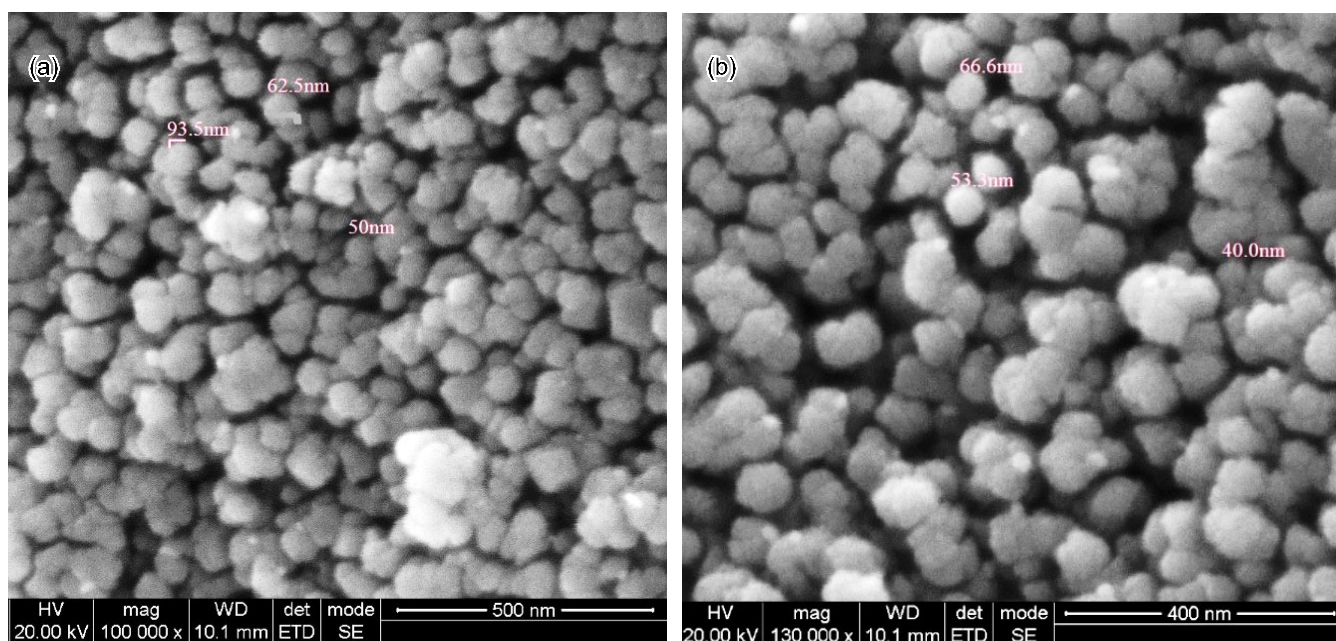

Fig. 3. SEM analysis of bioactive compound tagged AgNP (a) *L. aspera* and (b) *L. cephalotes*

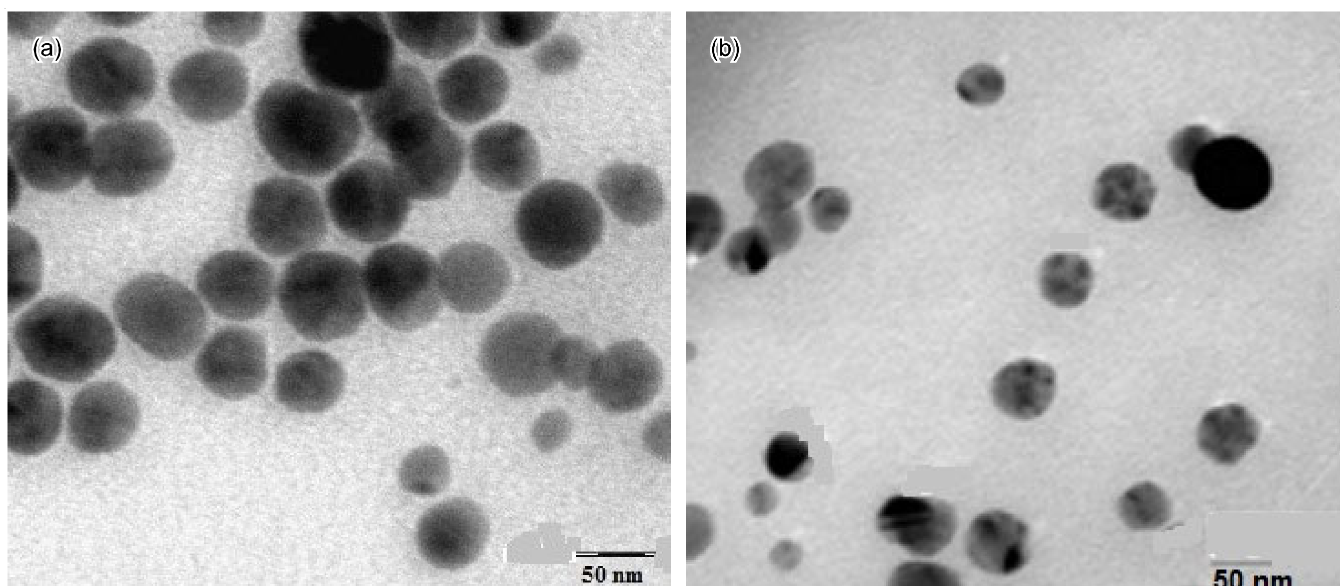

Fig. 4. TEM micrograph of AgNP tagged with bioactive compound (a) *L. cephalotes* and (b) *L. aspera*

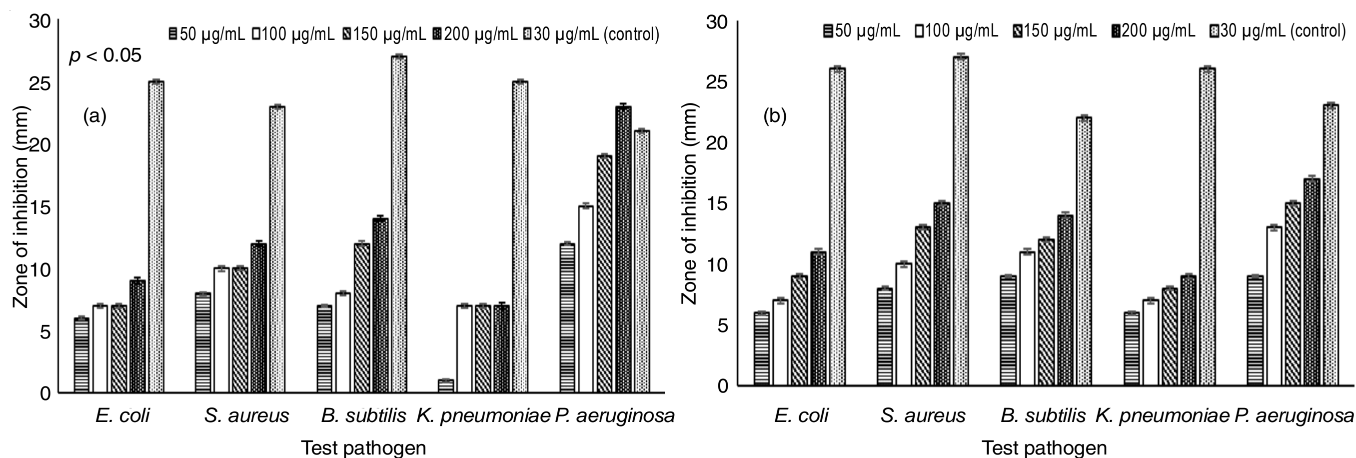

Fig. 5. (a) *L. aspera* and (b) *L. cephalotes*. Antibacterial activity of crude extract and tagged nanoparticle. Bars are the zone of inhibition (mm) against test pathogen present on the x-axis

purified as a single bioactive compound and used as a prospective drug candidate.

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