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Research article

Biogenic fabrication, characterization, and assessment of antibacterial activity of silver nanoparticles of a high altitude Himalayan lichen - *Cladonia rangiferina* (L.) Weber ex F.H. Wigg.

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Abstract: Silver nanoparticles synthesized using plant metabolites provide an edge over chemically synthesized compounds due to their comparatively efficient antimicrobial activity. In the present study, AgNPs was prepared by bioreduction of silver nitrate ($AgNO_3$) using the aqueous extract of *Cladonia rangiferina* collected ≥3500 m in Uttarkashi district of Uttarakhand, western Himalaya. The formation of Ag NPs was indicated by yellow-brown color after 72 h. The AgNPs were characterized by UV-Vis spectrophotometry, Fourier transformed infrared (FTIR) spectroscopy, scanning electron spectroscopy (SEM) analysis. Ag-NPs thus obtained were tested for antimicrobial activity against selected gram-negative (*i.e. Escherichia coli*, *Klebsiella pneuomoniae*, and *Pseudomonas aeruginosa*) and gram-positive (i.e. *Bacillus subtilis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*) bacterial strains employing Bauer-Kirby's disk diffusion assay using Gentamicin as positive control and distil water as negative control. The bioreduction of $AgNO_3$ yielded stable spherical and rod-shaped Ag-NPs showing characteristic UV-Vis spectral band peak with specific color change. The FTIR showed the role of many functional groups of different organic lichen secondary metabolites in AgNPs fabrication and stabilization. The synthesized AgNPs showed enhanced activity to positive control (*i.e.* Gentamicin). The study highlighted that lichen AgNPs can be used as better antibacterial material.

Keywords: Bactericidal - Cladonia - Gram-negative - Himalaya - Silver nanoparticles.

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INTRODUCTION

Nanoparticles are extensively used in optronics, textile industry, pharmacology, agriculture, environmental care, food processing, and as antimicrobial agents (Shekhawat *et al.* 2014, Roohizadeh *et al.* 2015, Hussain *et al.* 2015, Singh *et al.* 2017, Qayyum *et al.* 2019, Rodrigues *et al.* 2019, Yazdian-Robati *et al.* 2019, Tryfon *et al.* 2019, Singh & Singh 2019, Alvarez-Ordóñez *et al.* 2019, Dixit & Tripathi 2019). Among the various strategies of nanoparticle synthesis, the biogenic/ green synthesis of nanoparticles exploiting plant components or their extract in non-hazardous solvents (*i.e.* water) has emerged as an alternative approach, because of its easy methodology, cost-effectiveness, eco-friendliness and high-yields (Husen & Siddiqi 2014, Husen 2017). In the majority of biogenic synthesis of phytonanoparticles, organisms such as bacteria, fungi, algae, and flowering plants have been utilized (Korbekandi *et al.* 2009, Yadav *et al.* 2015), very rarely lichens are used to synthesize nanoparticles (Siddiqi *et al.* 2018). Out of about 800 known lichen secondary metabolites, 80–85% are unique to them and are known to possess antibacterial, antiviral, antioxidant, anticancer and antigenotoxic activities (Elix & Stocker-Wörgötter 2008, Ranković 2015).

Among the metal nanoparticles, silver nanoparticles are the most preferred nanoparticles (Mishra *et al.* 2019). The few reported studies on lichen/ lichen metabolite mediated fabrication of silver nanoparticles (AgNPs) have shown that lichens can be used for green synthesis of AgNPs which show antimicrobial activity

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against a broad spectrum of bacterial strains (Mie *et al.* 2012, Dasari *et al.* 2013, Yıldız *et al.* 2014).

The present study was carried out to fabricate the AgNPs from a terricolous lichen *Cladonia rangiferina* (L.) Weber ex F.H. Wigg. collected from alpine habitat (≥3500 m) of western Himalaya and assess the antibacterial properties of these AgNPs to bacterial strains.

MATERIALS AND METHODS

The lichen was collected adjacent to Morinda lake (N 31º 09′57.5″ E 80º 25′39.48″) from the average elevation of 3798 m in Govind wildlife sanctuary situated in Uttarkashi district of Uttarakhand in western Himalaya (Fig. 1). The compound lichens found growing on soil over flat rock bed were collected for the experiment. The terricolous lichen was collected in paper bags in field with frequent air drying during the transit from field to laboratory. The lichen sample was air-dried and curated according to standard procedure (Rai *et al.* 2014a). The lichen sample was identified and authenticated up to species level using standard lichenological procedure of morpho-anatomical study, chemical spot tests, TLC profiling with reference to relevant keys and monographs at the lichenology laboratory of the CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh, India (Rai *et al.* 2014b).

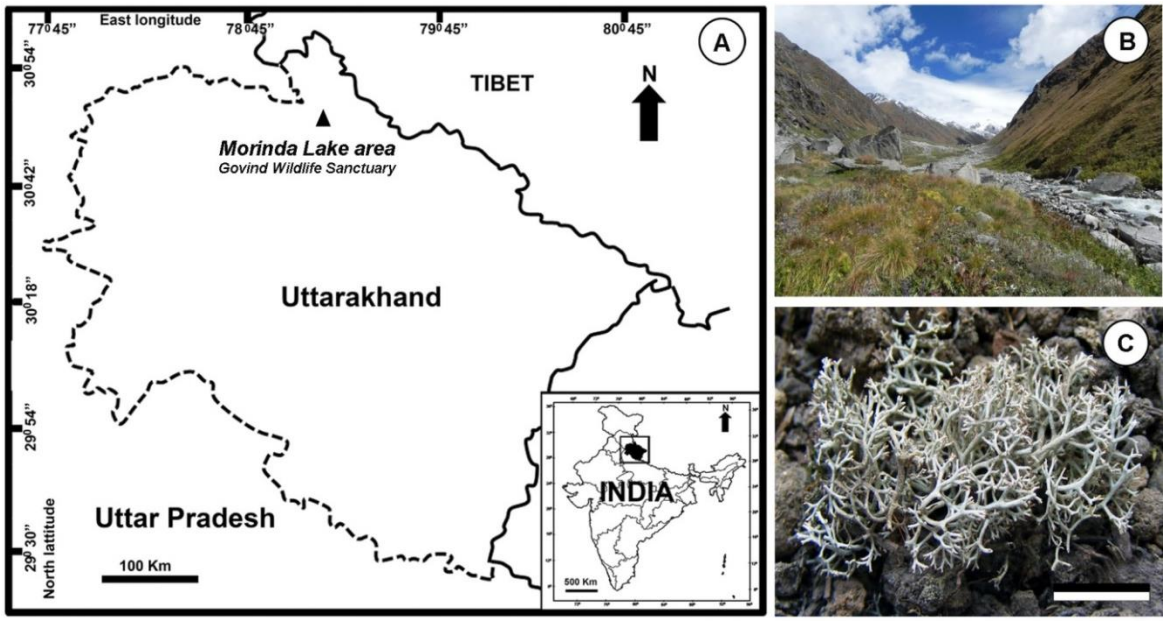

Figure 1. The site of lichen collection: **A**, The location map of the collection site; **B**, Morinda lake habitat; **C**, *Cladonia rangiferina* (L.) Weber ex F.H. Wigg. (LWG) habitus, (Scale = 2 cm).

The aqueous extract of lichen was prepared by boiling about 3 g of air-dried coarsely grounded lichen thallus in 45 ml of Mili-Q water at 80ºC for 20 min. The mixture war filter through Whatman No. 1 (pore size 125 mm) to discard any thallus debris. The filtrate was stored at 4ºC for further procedure. About 15 ml of lichen aqueous extract was added into 45 ml of 1 mM silver nitrate solution (prepared in Mili-Q water). The mixture was alkalized by 2–3 drops of 0.1 M sodium hydroxide and the solution mix was kept for 72 hrs at room temperature (25ºC) to facilitate bioreduction of silver ions. The reduction of silver ions to AgNPs in aqueous extract was monitored by measuring the UV-Vis spectrum of solution using after diluting a small aliquot of the sample into distilled water in UV-Vis Spectrophotometer (Thermo Fisher 160 UV-VIS). The shape and size of the nanoparticles were analyzed using JEOL-JEM 2100F Field emission gun-transmission electron microscope (FEG-TEM). For TEM the colloidal solution was first sonicated for 15 min, then a drop of it was loaded on a carbon-coated copper grid, allowing the solvent to evaporate under fume hood for 30 min. The functional groups in lichen extract and their possible role in synthesis of AgNPs was studied by Perkin Elmer Spectrum one: fourier transform infrared spectrometer (FTIR) with scan range 450–4000 cm^{-1}.

The antibacterial activity of synthesized AgNPs was studied employing Bauer-Kirby's disk diffusion assay (Bauer 1959, 1966) against gram-positive bacterial strains *Bacillus subtilis* (MTCC-2390), *Staphylococcus aureus* (MTCC-6908), *Staphylococcus epidermidis* (MTCC-6810) and gram-negative bacterial strains *Escherichia coli* (MTCC-595), *Klebsiella pneuomoniae* (MTCC-4030), and *Pseudomonas aeruginosa* (MTCC-4727). Gentamicin (10 µg discs, HIMEDIA-SD016) was used as positive control whereas distill water was used as negative control. 10 µl of nanoparticle solution was loaded (using sterile micropipette-10 µl) on sterile discs (HIMEDIA-SD067) which were placed together with Gentamicin discs on nutrient agar plates. The plates were

incubated at 37ºC for 24 h and the zone of inhibition was measured. Experiments were carried in triplicate and mean values were recorded. Antibacterial activity was assessed by measuring the inhibition zone diameter (IZD) around the discs. Percentage relative inhibition zone diameter (% RIZD) for different bacterial strains was calculated as:

$$\% \text{ RIZD} = \frac{\text{IZD of the AgNPs} - \text{IZD of negative control}}{\text{IZD of Gentamicin}} \times 100$$

RESULTS AND DISCUSSION

The reduction of silver ions into AgNPs by phytochemicals is often detected by a change in color of the reaction mixture which is (Mishra *et al.* 2019). The colour of the reaction mixture changed to yellowish-brown (Mie *et al.* 2012, Dasari *et al.* 2013, Mie *et al.* 2014a, Din *et al.* 2015, Paul *et al.* 2015, Leela & AnchanaDevi 2017, Khandel *et al.* 2018, Siddiqi *et al.* 2018). The UV-Vis spectra peaked at 402 nm (Fig. 2) due to surface plasmon absorption (SPA) which is within the range of characteristic SPA band range of 400–450 nm usually observed in AgNPs synthesized by lichen/ purified lichen secondary metabolites (Mie *et al.* 2012, Dasari *et al.* 2013, Mie *et al.* 2014a,b, Yildiz *et al.* 2014, Din *et al.* 2015, Paul *et al.* 2015, Çıplak *et al.* 2017, Leela & AnchanaDevi 2017, Khandel *et al.* 2018, Siddiqi *et al.* 2018).

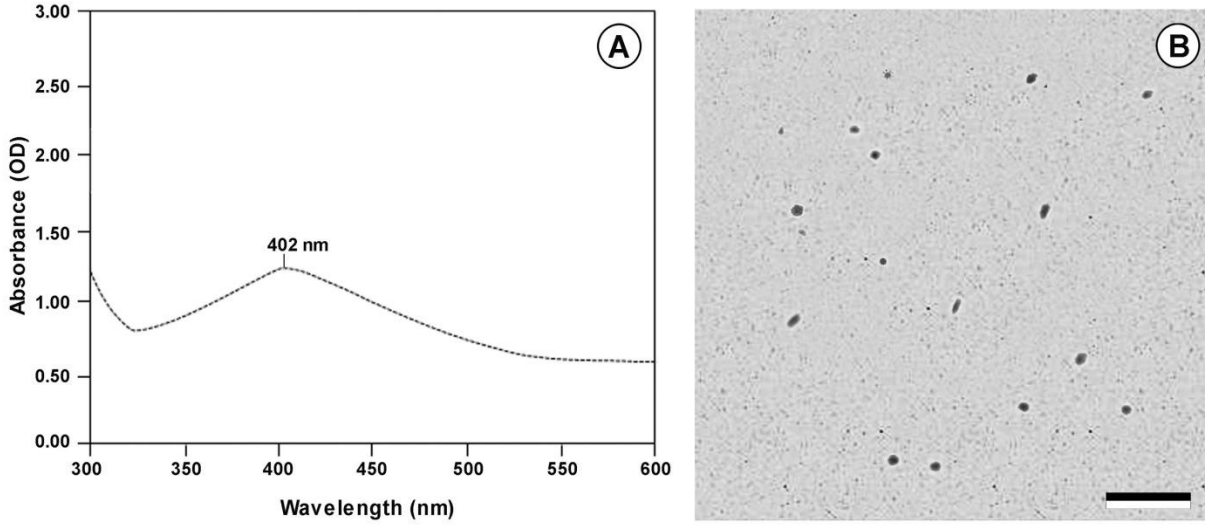

Figure 2. A, UV-Vis absorption spectrum of AgNPs synthesized using the aqueous extract of *Cladonia rangiferina* (L.) Weber ex F.H. Wigg.; **B**, TEM image of *Cladonia rangiferina*-AgNPs obtained after 72 h of the reduction reaction, (Scale = 1 μm).

The morphology and size distribution of the synthesized AgNPs determined by TEM analysis showed that the particles were both spherical (Mie *et al.* 2012, Çıplak *et al.* 2017, Leela & Anchana Devi 2017, Khandel *et al.* 2018, Siddiqi *et al.* 2018) and of rod shape (Paul *et al.* 2015) (Fig. 2 C). The particle size of the AgNPs ranged in size from 5 nm to 40 nm with an average diameter of 20 nm.

The FTIR analysis of lichen AgNPs showed peaks in the range of 1000–4000 cm^{-1} which corresponds to many functional groups found in two major secondary metabolites Atranorin and fumarprotocetraric acid of *Cladonia rangiferina*. The FTIR spectra of AgNPs synthesized from *Cladonia rangiferina* (L.) Weber ex F.H. Wigg., showed presence of O-H (3400 cm^{-1}), C-H (2853 cm^{-1}), C=O (1742 cm^{-1}), C=O (1691 cm^{-1}), C=O aldehyde (1651 cm^{-1}), C=C vibration (1573 cm^{-1}), CH_2, CH_3 (1443 cm^{-1}) and C-O (1273 cm^{-1}) suggesting the involvement of polyphenols and other functional groups of multiple organic lichen metabolites in reduction and stabilization of AgNPs (Dasari *et al.* 2013, Çıplak *et al.* 2017, Leela & AnchanaDevi 2017, Khandel *et al.* 2018, Siddiqi *et al.* 2018).

The antibacterial activity showed considerable efficiency of *Cladonia rangiferina* AgNPs against positive control Gentamicin (Fig. 3) (Din *et al.* 2015, Leela & AnchanaDevi 2017, Khandel *et al.* 2018, Siddiqi *et al.* 2018). The AgNPs showed much greater bactericidal activity against gram-negative bacterial strains than the gram-positive ones (Fig. 3) (Mie 2014a).

CONCLUSION

The study highlighted the probability of fabricating stable AgNPs from aqueous extracts of lichens such as *Cladonia rangiferina* (L.) Weber ex F.H. Wigg. from harsh high elevational alpine habitats. The effectiveness of lichen AgNPs was further established in the study with comparatively higher efficiency towards gram-negative

bacterial strains. The study concluded that the unique secondary metabolites of lichens make them an appropriate reducing and stabilizing agents for synthesis of AgNPs with higher antibacterial efficiency.

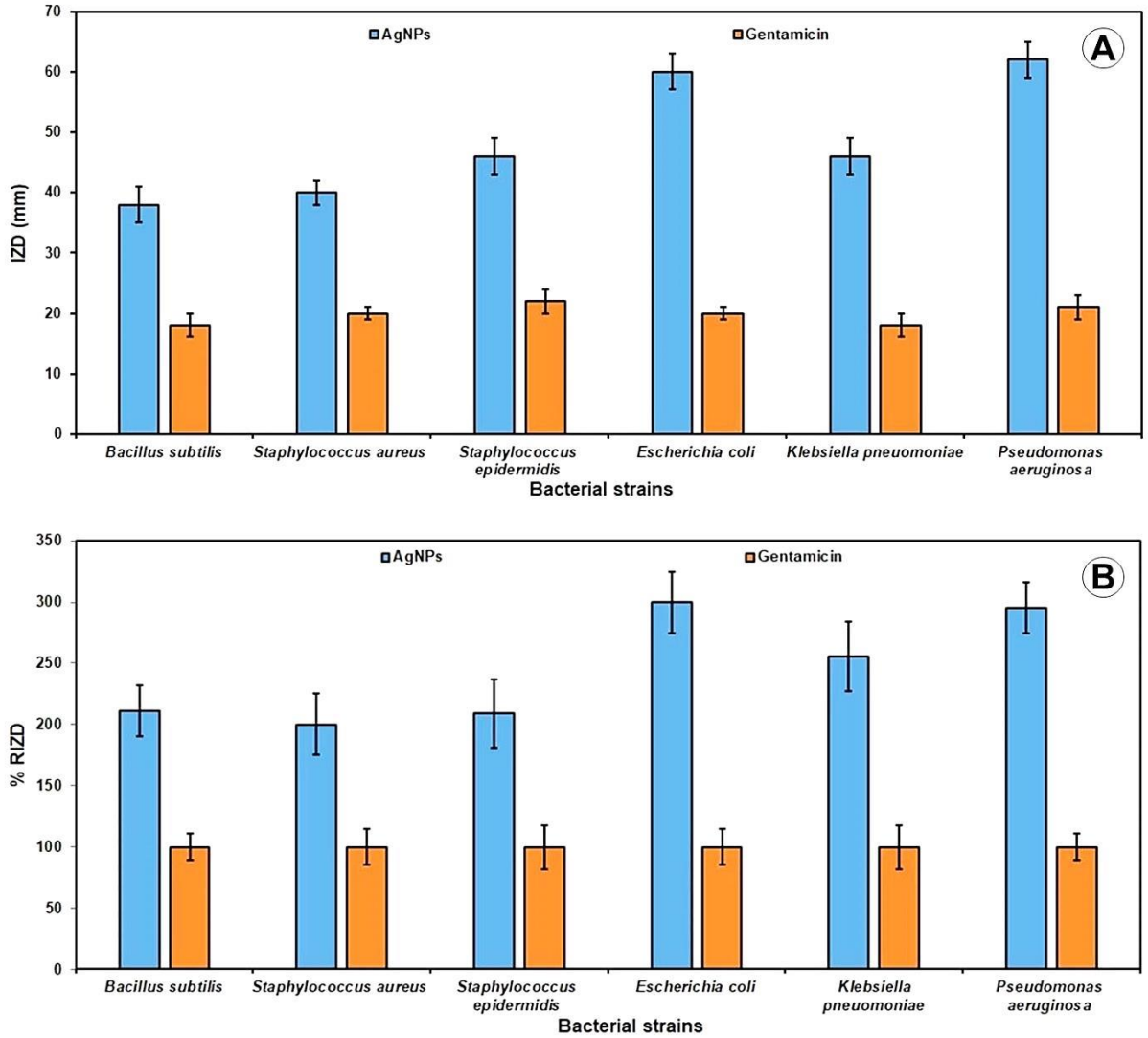

Figure 3. Antibacterial activity of *Cladonia rangiferina* (L.) Weber ex F.H. Wigg. AgNPs as A, IZD, and B, %RIZD on selected gram-negative and gram-positive bacterial strains.

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