Green Synthesis Characterization with Antimicrobial Potential of Silver Nanoparticles using some Macrolichens

THESIS

SUBMITTED TO

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SUMMARY OF THE THESIS

In this chapter, an attempt has been made to summarize background and aim of the study. Including the most prominent results and findings reported in experiments and also to draw valid conclusions based on the significant findings of the present investigation entitled **"Green Synthesis Characterization with Antimicrobial Potential of Silver Nanoparticles using some Macrolichens".** The experimental study was conducted in the Lichenology Laboratory of CSIR-NBRI, Lucknow, and the Rhizosphere Laboratory, Department of Environmental Microbiology, BBA University, Lucknow.

Drug resistance among human pathogenic bacteria is a growing global concern, posing a significant threat to public health. The overuse and misuse of antibiotics in healthcare, agriculture, and livestock have accelerated the development of resistant strains. The consequences of drug resistance are profound, leading to prolonged illnesses, increased healthcare costs, and higher mortality rates. Moreover, the limited development of new antibiotics exacerbates the problem, creating a pressing need for innovative solutions. Nanotechnology, a burgeoning field in modern science, particularly in biotechnology emerging a unique way for development of novel antimicrobial agents. Silver nanoparticles are the most significant commercialized nanoparticles among nanostructured noble metals that have been used as antimicrobial agent. Nanoparticles are characterized by their synthesis process. Synthesis by using physical and chemical methods is quite costly and produces toxic byproducts. Green synthesis of nanoparticles is a simple and reliable method that creates nanoparticle with good stability and appropriate dimensions.

Lichens represent a symbiotic relationship between Photobiont (constituting 10% of the consortium) and Mycobiont (making up the remaining 90%). They serve as notable illustrations of emergent metabolic strategies and cohesive morphologies resulting from this synergistic association. Lichen cells, recognized for containing a diverse array of natural metabolites and bioactive molecules, have become a focal point of attention in industrial, pharmaceutical, biotechnological, medical, and cosmetic applications. Numerous studies underscore the potential of various lichen species to yield distinct natural compounds with diverse physicochemical and biological activities. The historical use of lichens in traditional medicine, documented across different pharmacopeia by native Americans, Indians, Chinese, and Europeans, further highlights their significance in treating various ailments. Lichens employ their metabolites in the stabilization of silver ions through the application of green nanotechnology, resulting in the formation of lichen silver nanoparticles. This approach is particularly favoured for its capacity to generate nanoparticles at minimal expenses, its environmentally sustainable attributes, and its safety for potential human therapeutic applications.

This study aims to contribute to the development of an innovative antimicrobial agent to address contemporary challenges posed by microbial drug resistance. By identifying four distinct macrolichen species, we harnessed their exclusive secondary metabolites for the green synthesis of nano antimicrobial agents. Subsequently, the bioactivity of these agents was evaluated against six human pathogenic microorganisms, successfully achieving the study's outlined objectives.

➢ Objectives:

- Collection and identification of lichen sample.
- Green synthesis silver nanoparticles of macrolichens sample.

- Characterization of lichen silver nanoparticles by (UV -UV-visible spectroscopy, FTIR, SEM, TEM).
- To screen the therapeutic potential of lichen silver nanoparticles for antimicrobial activity.

➢ Collection and identification of lichen sample:

The lichen samples were collected in different zones in India. Dolichousnea, Everniastrum, Stereocaulon from Uttarakhand. Roccella from Odisha.

- **Identification and characterization of selected lichen**

Categorized by their growth form, certain lichens exhibit two distinct patterns: **Foliose:** Referred to as leafy lichens, this category is characterized by a thallus loosely attached to the substrate, affording free borders.

Fruticose: Identified as fruticose lichens, this type features a thallus that is only partially connected to the overall plant structure. These lichens grow in an erect manner, resembling a dangling fruit.

Chemical approaches: Thin layer chromatography (TLC) and color spot tests served as pivotal techniques for the identification of specific lichen species metabolites. In the case of *Everniastrum cirrhatum* (E. cirrhatum), the presence of salazanic acid and dibaric acid was confirmed through the Color spot tests (P and K tests, producing a red or brown color). Additionally, TLC results demonstrated the occurrence of Salazanic acid and Protolichesterinic acid. *Roccella montagnei* exhibited the isolation of Erythrin metabolite, identified through TLC and color spot tests. The Color spot test (C test) specifically revealed a red coloration upon the presence of Erythrin. *Dolichousnea longissima* showcased the presence of evernic acid, diffractaic acid, and barbatic, as evidenced by the TLC results. The identification of these metabolites was integral to understanding the lichen's chemical composition. In the case of

Stereocaulon, lobaric acid and Atranorin metabolites were identified through color spot and TLC results. In summary, the utilization of Thin Layer Chromatography and colour spot tests facilitated the identification of distinct metabolites in specific lichen species, shedding light on the unique chemical compositions within each specimen.

➢ Synthesis silver nanoparticles using selected macrolichens samples extract:

- **Extraction of metabolites from macrolichens:**

The lichen samples were cleansed using tap water and tween 80 detergent to remove debris. Following this, the purified samples were spread on sterile filter paper and left to air-dry at room temperature. After drying, the lichen sample underwent freezing with liquid nitrogen, followed by crushing. The extraction of metabolites from the chosen macrolichens was conducted using the cold extraction method with some modifications.

- **Green synthesis of silver nanoparticles:**

Macrolichen-extracted metabolite solution was added to an aqueous $AgNO_3$ solution to reduce Ag^+ ions. Reaction temperature was maintained at 50-80 ^{0}C. The color change of the reaction mixture dark brown indicates the formation of macrolichens silver nanoparticles (AgNPs).

➢ Characterization of green synthesized silver nanoparticles:

To confirm NPs formation, the characteristic absorption behaviour of NPs was measured using a UV-vis spectrophotometer. A constricted surface plasmon absorption peak formed at the wavelength of 410-440 nm (AgNPs) indicates the presence of nanometer-sized particles. Metabolites of macrolichens encapsulation on NPs was confirmed by Fourier-transformed infrared (FTIR) analysis measured at a

resolution of 4000-400 cm^{-1} in the percent transmittance mode. The morphological shape and size of the particles were determined by scanning electron microscope analysis. Biofabricated AgNPs are found to be irregular to spherical shaped. Further size distribution of NPs analysed through Transmission electron microscopy and results showed that the average size of AgNPs falls in the range of 10-50 nm. The Crystal lattice structure and phase identification of green synthesized nanocomposites observed by using an X-ray diffraction (XRD) system showed a face-centered cubic lattice of AgNPs. The stability of the nanoparticle has been confirmed by analyzing its zeta potential. The zeta-potential values of particles Dl@AgNPs, Ec@AgNPs, Rm@AgNPs, and Sf@AgNPs were found to be -25.3 mV, -30.4 mV, -11.6 mV and -25.5 mV. Green Ag nanoparticles exhibit a high negative charge due to phytochemical encapsulation, indicating particle stability. (Ramasamy et al., 2017). The size distribution histogram of zeta sizer of nanoparticles Dl@AgNPs, Ec@AgNPs, Rm@AgNPs, and Sf@AgNPs. The nanoparticle's diameter with an average size of 80.64 nm, 163.75 nm, 122.6 nm and 78 nm with a polydispersity index of 26.2 %, 24.3 %, 25.3 % and 22 % respectively. The GC-MS analysis reveals the presence of metabolites potentially participating in the synthesis of AgNPs. The peak area suggests the involvement of multiple compounds in both the bio-reduction and stabilization of silver ions into metallic silver nanoparticles.

- **Assessment of therapeutic potential of biosynthesized silver nanoparticles for antimicrobial activity:**

  - **Determination of Antimicrobial activity (well diffusion assay)**

Green synthesis AgNPs displayed remarkable antimicrobial efficacy against selected human pathogenic microorganisms. Their ability to combat microbes was

demonstrated through agar well diffusion assay, measuring Zone of Inhibition (ZOI). Significant antimicrobial activity was shown by *Everniastrum cirrhatum Dolichousnea longissima, Roccella montagnei, Stereocaulon foliolosum* macrolichens silver nanoparticles *(*Ec@NPs, Dl@NPs, Rm@NPs and Sf@NPs) against bacteria *B. cereus, S. aureus, C. albicans* (yeast) and *S.mutans* at the lowest concentration range (10µg/mL).

- **Determination of Minimum Inhibitory Concentration**

The Minimum Inhibitory Concentration (MIC) value was notably minimal with Dl@NPs, measuring ≤04 µg/mL against both *E. coli* and *B. cereus*. Furthermore, Ec@NPs and Rm@NPs exhibited a MIC that significantly inhibited the growth of *C. albicans* (≤08 µg/mL). In the case of Sf@NPs, the MIC against *B. cereus, E. coli*, and *S. mutans* was determined at ≤08 µg/mL. These findings underscore the considerable antibacterial efficacy of Dl@NPs against both gram-positive and gram-negative bacteria.

- **Microbial growth kinetic assay**

The microbial growth kinetics demonstrated changes in different growth phases of bacterial culture when treated with biosynthesized AgNPs. The study reported that treatment with nanoparticles effectively suppressed or delayed the lag phase in all test pathogens and in some cases eradicates the entire initial microbial population in inoculum. The excellent result represented by Sf@NPs, it was effectively inhibited the growth of *S. aureus* then after *S.mutans, B. cereus P. aeruginosa* and *C. albicans* as reported in growth kinetics analysis.

- **Determination of microbial metabolic activity**

The findings from the metabolic activity assay of biosynthesized AgNPs can effectively suppress the cellular metabolism of microbial cells at specific concentrations. According to experiments Ec@NPs, Dl@NPs, Rm@NPs and Sf@NPs, significantly suppress the cellular metabolism of *B. cereus, E. coli, C. albicans, S.mutans.* The membrane integrity test suggests that AgNPs efficiently cause membrane damage, leading to the release of intracellular metabolites due to permeabilized microbial cell membranes.

- **Membrane permeability test**

The outcomes of the membrane permeability test for biosynthesized silver nanoparticles (AgNPs) were employed to assess their impact on various microbial cultures, thereby elucidating one of the mechanisms underlying the antimicrobial activity of NPs. The results revealed that *Bacillus cereus* exhibited the most significant cell membrane damage upon treatment with Ec@AgNPs, as indicated by the red fluorescence observed in propidium iodide (PI) staining. *Escherichia coli* treated with Dl@AgNPs demonstrated the highest membrane permeability due to cell membrane damage. Subsequently, Sf@AgNPs induced notable damage to the bacterial cell membrane in the cases of *Streptococcus mutans, Staphylococcus aureus, and Pseudomonas aeruginosa*, the application of Sf@AgNPs resulted in maximum red fluorescence, suggesting increased cell membrane permeability. Conversely, in *Candida albicans,* treatment with Rm@AgNPs exhibited the most substantial cell membrane damage. These findings indicate a varied impact of distinct green synthesized macrolichen silver NP treatments on diverse microbial strains. The observed differences are likely attributed to the encapsulation of different metabolites in each of the four biosynthesized NPs. In essence, the cell permeability assay

suggests that membrane damage is a primary mode of action for biosynthesized NPs, correlating with their antimicrobial activity.

- **Determination of cell cytotoxicity**

The results of the cytotoxicity evaluation revealed that the viability of fibroblast cells was consistently maintained at levels surpassing 80% when subjected to macrolichen silver nanoparticles (Ml@AgNPs) at concentrations of 20 µg/mL. Moreover, even at an elevated concentration of 40 µg/mL in the treatment with Ml@AgNPs, viability levels remained above 75%. This suggests that the biosynthesized nanoparticles are non-toxic to human cells, indicating their potential suitability for applications in antimicrobial therapy.

➢ Conclusions:

The investigation concludes that macro-lichens exhibited several unique secondary metabolites possessing notable biological activities, effectively undermining the defence mechanisms of microbes and mitigating drug resistance observed in human pathogenic microbes. The application of various analytical techniques such as Thin Layer Chromatography (TLC), color spot tests, Fourier-Transform Infrared Spectroscopy (FTIR), and Gas Chromatography (GC) analysis reveals the active involvement of diverse macrolichen biomolecules in the reduction and encapsulation of silver nanoparticles (AgNPs). The utilization of green nanotechnology emerges as a strategic approach to enhance the bioactive properties of natural remedies, providing a potential avenue to augment the effectiveness of existing medications, counteract multidrug resistance in microbial pathogens, and facilitate the development of innovative antimicrobial agents.

Moreover, Macrolichens AgNPs contribute to the conservation of valuable macrolichen species, as the synthesis of nanoparticles requires significantly smaller quantities of lichen extracts compared to the traditional preparation of crude extract-based herbal drugs. Subsequent research endeavors should focus on evaluating the in vivo antimicrobial activity of bio-fabricated nanoparticles and determining sub-lethal doses in animal models. Such investigations are crucial for yielding valuable insights into the development of antimicrobial drugs with enhanced efficacy.