

GREEN SYNTHESIS OF SILVER NANOPARTICLES (AgNPs) USING *HELVELLA LEUCOPUS* PERS. AND THEIR ANTIMYCOTIC ACTIVITY AGAINST FUNGI CAUSING FUNGAL ROT OF APPLE

MEHRAJUD DIN TALIE*, ABDUL HAMID WANI, NUSRAT AHMAD, MOHD YAQUB BHAT, JOHN MOHD WAR

Department of Botany, Section of Mycology and Plant Pathology, University of Kashmir, Srinagar, Jammu and Kashmir, India.
Email: danishmehraj143@gmail.com

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ABSTRACT

Objectives: The main objective of the present study was to synthesize silver nanoparticles (AgNPs) by green approach using *Helvella leucopus* and to evaluate the antimycotic activity of synthesized AgNPs against fungi causing fungal rot of apple.

Methods: During the present study for green synthesis of AgNPs using *H. leucopus*, equal volumes of both mushroom extract (100 ml) and silver nitrate solution (100 ml) were mixed and incubated at room temperature for the bioreduction process. These synthesized AgNPs were characterized by ultraviolet-visible spectroscopy, scanning electron microscopy, Fourier transmission infrared spectroscopy, and X-ray diffraction analysis. Furthermore, these synthesized AgNPs were evaluated for their antimycotic activity by spore germination method and agar well diffusion assay against different tested fungi.

Results: The results revealed that strong plasmon absorbance band was observed at 420 nm which confirms the synthesis of AgNPs using *H. leucopus*. The synthesized AgNPs were spherical in aggregated form with size ranging from 80 to 100 nm. Furthermore, different concentrations of synthesized AgNPs caused significant inhibition in spore germination and reduction in zone of inhibition of tested fungal pathogens. The highest inhibition in spore germination by AgNPs at highest concentrations was observed against *Penicillium chrysogenum* followed by *Aspergillus niger* and *Alternaria alternata*, respectively. Similarly, the synthesized AgNPs at highest concentrations showed maximum zone of inhibition against *P. chrysogenum* followed by *A. niger* and *A. alternata*, respectively.

Conclusion: It is concluded from the present study that synthesized AgNPs have good potential to be used as antifungal agents against many fungal plant pathogens. The synthesized AgNPs using mushroom fungi also have potential for the development of nanofungicides against fungal pathogens but after proper investigation.

Keywords: Silver nanoparticles, *H. leucopus*, Fungal rot pathogens, Characterization, Antimycotic activity.

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INTRODUCTION

Nanotechnology, a multidisciplinary and emerging field of science, covers diverse area of research and technology in physics, chemistry, and biology [1]. Physical synthesis of nanoparticle gives low yield [2] while as chemical methods are toxic to the environment. However, biological synthesis of nanoparticles is advantageous than physical and chemical methods because of rapid synthesis, better control over size and shape characteristics, less toxicity, cost-effectiveness, and eco-friendly approach [3,4]. Various plants, bacteria, fungi, algae, and viruses have been used for biological synthesis of nanoparticles [5]. However, the most widely accepted approach is using bacteria and fungi because they are easy to manipulate and handle [6].

Several methods have been applied for the management of fungal and bacterial pathogens but these have some limitations [7-10]. However, synthesized nanoparticles have great potential to be used as antifungal and antibacterial agents because they are considered as alternate, cost effective, and eco-friendly management strategy for the control of pathogenic microbes [4,11,12].

Various mushrooms, namely, *Volvariella volvacea*, *Pleurotus sajor-caju*, *Pleurotus florida*, *Ganoderma lucidum*, *Ganoderma applanatum*, *Agaricus bisporus*, *Fomes fomentarius*, *Helvella* sp., and *Microporus xanthopus* have been assessed for the synthesis of silver and other nanomaterials [13-17]. Silver nanoparticles (AgNPs) have become increasingly popular as antifungal, antibacterial, antioxidant, and anti-inflammatory agents [18,19].

Considering the present environmental scenario and other safety measures, there is widespread interest in developing cost effective, benign, and eco-friendly approach for the synthesis of nanoparticles [20]. Therefore, the present study entitled "Green synthesis of AgNPs using *Helvella leucopus* Pers. and their antimycotic activity against fungi causing fungal rot of Apple" was carried out 1st time from Kashmir, India. These synthesized AgNPs were characterized and evaluated for their antifungal activity against fungal rot pathogens such as *Penicillium chrysogenum*, *Aspergillus niger*, and *Alternaria alternata* with respect to spore germination and reduction in zone of inhibition.

METHODS**Sample collection**

During the present study, *H. leucopus* was collected from different localities of Northern Kashmir such as Sopore, Pattan, Tangmarg, and Sumbal Sonawari. The mushroom was then identified based on morphological, reproductive, and other characteristics [21,22]. This mushroom was cleaned and then dried at room temperature for the synthesis of AgNPs. The identified mushroom was found positive for AgNPs production and gives positive peak while performing spectrophotometric analysis.

Biosynthesis of AgNPs

About 5–20 g of dried mushroom specimen were taken and washed thoroughly with distilled water to free from the mud and dust adhering. The cleaned mushroom after complete redrying was ground to fine powder with the help of grinder. The powdered sample was suspended

in 150 ml of deionized water and heated for 5–10 min. The extract was cooled at room temperature, then filtered 2 times and stored at 4°C for further use. During the present study, equal volumes of both mushroom extract (100 ml) and silver nitrate (AgNO_3) solution (100 ml) were mixed and incubated at room temperature for the bioreduction process. Simultaneously, *H. leucopus* extract was taken as positive control while as AgNO_3 solution acts as negative control [23].

Characterization of AgNPs

Following techniques were employed for the characterization of synthesized AgNPs.

Ultraviolet (UV)–visible spectroscopy

UV–visible spectroscopic analysis was performed on UV–visible absorption spectrophotometer (UV-119 Systronics) between the wavelengths of 300 and 700 nm. The process of reaction between AgNO_3 ions and *H. leucopus* extract was assessed by UV–visible absorption spectra of AgNPs in aqueous solution.

Scanning electron microscopy (SEM)

SEM analysis was used to measure the size of AgNPs. For SEM, the AgNPs synthesized using *H. leucopus* were allowed to complete dryness and grounded well to superior quality powder.

Fourier transmission infrared (FTIR) spectroscopy

The resultant suspension of AgNPs synthesized using *H. leucopus* was initially centrifuged at 3000 rpm for 15 min, to remove the unwanted impurities. The supernatant was centrifuged 3 times up to 10,000 rpm for 15 min. Pellets obtained were washed with distilled water to get the pure AgNPs and were completely air-dried at room temperature. The collected powdered material of synthesized AgNPs was taken for FTIR spectroscopy analysis using broker (Alpha 200486) instrument in the range of 450–4500 cm^{-1} .

X-ray diffraction (XRD) analysis

After the reduction process, the suspension obtained was cleaned and purified by centrifugation at 5000–10,000 rpm for 20 min. After air-drying of the purified AgNPs, the structure as well as composition were examined by XRD analysis in the range of 20–90. However, to verify the UV–visible spectral results, the powdered sample of synthesized AgNPs was analyzed by XRD pattern to confirm its crystalline nature.

Antifungal assay

Test organisms

The pathogenic fungi such as *P. chrysogenum* Thom (1910), *A. niger* Van Tieghem (1867), and *A. alternata* (Fr.) Keissl. (1912) used during the present study were obtained from Plant Pathology and Mycology, Department of Botany, University of Kashmir, Hazratbal, Srinagar (190006).

Spore germination method

The evaluation of antimycotic activity of synthesized AgNPs was assessed by spore germination method against different test fungi. Different concentrations, namely, 10 mg/ml, 15 mg/ml, and 20 mg/ml were prepared from the dried and purified material of synthesized AgNPs. Furthermore, spore suspension of each test fungus was prepared in sterilized distilled water containing 1×10^3 conidia/ml. A drop of about 0.01 ml from a particular test fungus was placed on cavity slide. Equal volume of the respective concentration of synthesized AgNPs was also placed on the same cavity slide and was incubated for $25 \pm 2^\circ\text{C}$ in a soaked blotting chamber to maintain required humidity. For control, a drop of spore suspension as well as respective test fungi was taken on cavity slide. Each treatment was maintained in the replicates of three including standard. The slides after incubation were then examined on stereoscopic microscope. The percentage of germination of spores was calculated by the formula [24].

$$\text{Percent spore germination (\%)} = \frac{\text{No. of spores germinated}}{\text{Total No. of spores examined}} \times 100$$

$$\text{Inhibition of spore germination (\%)} = \frac{\text{Gc} - \text{Gt}}{\text{Gc}} \times 100$$

Where, Gc and Gt represent the average number of germinated conidia in control and treated plates, respectively.

Agar well diffusion method

The antimycotic activity of synthesized AgNPs was evaluated against rot causing fungi by agar well diffusion assay as adopted by Wiegand *et al.* [25]. Different concentrations, namely, 10 mg/ml, 15 mg/ml, and 20 mg/ml were prepared in sterilized distilled water. Nystatin 50 ul/disc was used as standard while as distilled water was taken as negative control. About 0.02 ml spore suspension of each test fungus was inoculated in 20 ml of molten Sabouraud dextrose agar medium in culture tubes. The culture tubes after proper homogenization were poured into 90 mm Petri plates. These cultures were allowed to solidify under aseptic conditions in laminar airflow chamber. With the help of standard, 5 mm cork borer wells were prepared in solidified media. During the present study, the efficacy of different concentrations of synthesized AgNPs against different test fungi was assessed and compared with the positive control. The culture plates were sealed with cello tape and incubated at $25 \pm 2^\circ\text{C}$ for 48 h. The antimycotic activity was determined by measuring the zone of inhibition [26].

RESULTS

Characterization of synthesized AgNPs using *H. leucopus*

Synthesized AgNPs using *H. leucopus* were characterized by the following techniques given below:

UV–visible spectroscopy

The preliminary indication of the synthesis of AgNPs using *H. leucopus* extract was confirmed by the color change from yellow to dark brown within 24 h (Fig. 1).

The reaction between AgNO_3 solution and mushroom extract was assessed by UV–visible spectroscopic analysis between the wavelengths of 300 and 700 nm. The strong plasmon absorbance band was observed at 420–450 nm (Fig. 2) which confirms the synthesis of AgNPs using *H. leucopus*.

SEM analysis

It was revealed from the results (Fig. 3) that synthesized AgNPs were spherical in aggregated form. The size of synthesized AgNPs ranges from 80 to 100 nm.

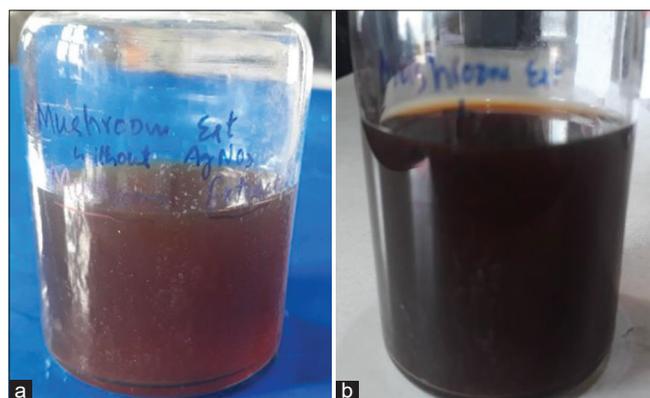


Fig. 1: Mushroom extract before and after the addition of silver nitrate (AgNO_3) solution (a) Mushroom extract before the addition of AgNO_3 , (b) mushroom extract after the addition of AgNO_3

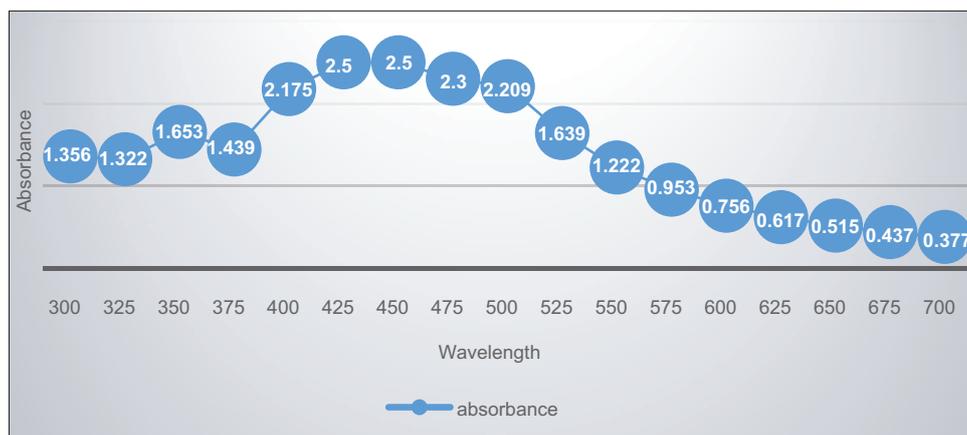


Fig. 2: Ultraviolet-visible spectroscopy of synthesized silver nanoparticles from *Helvella leucopus*

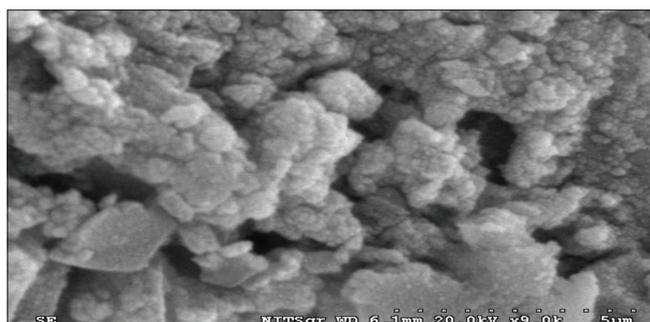


Fig. 3: Scanning electron microscopic micrograph of silver nanoparticles biosynthesized from *H. leucopus*

FTIR spectroscopy

It was observed from the FTIR spectroscopy measurements (Fig. 4) that synthesized AgNPs absorb strongly at 3452.06, 2937.59, 2851.96, 2370.87, 2345.48, 1637.25, 1384.41, 1105.42, and 407–496 cm^{-1} . However, the absorption band of 3421.14 is associated to N-H amine stretch and 2861.95–2937.69 absorption bands correspond to -OH of carboxylic acid. Likewise, the absorption band observed at 1384.41 cm^{-1} is in accordance with the C-N stretching vibrations of the aromatic amines while as 1637.25 absorption band is associated with unsaturated nitrogen compounds O-NO₂, nitrate, respectively.

Furthermore, the IR spectra show the bands which clarify the presence of N-H amine linkages, O-H of carboxylic acid, C-N linkages of aromatic amines, and O-NO₂ unsaturated nitrogen compounds that may be present in the AgNPs as stabilizing caps along with the proteins and amino acid residues.

XRD analysis

It was revealed from the XRD measurements (Fig. 5) that synthesized AgNPs showed peaks in the whole spectrum ranging from 20 to 90. The data obtained during the present study were matched with inorganic crystal structure database and international center for diffraction data using PDXL-2 software. While comparing the results of XRD spectrum obtained during present study with the standard, it was proved that these synthesized AgNPs were in the form of nanocrystal.

Antifungal assay

Effect of different concentrations of AgNPs on the spore germination of some test fungi

It was observed from the results (Table 1) that significant spore germination inhibition of all the test fungi was brought about at different concentrations of AgNPs. The highest concentrations of synthesized AgNPs caused maximum spore germination inhibition

against *P. chrysogenum* (83.21%) followed by *A. niger* (77.32%) and *A. alternata* (69.10%), respectively. However, lower concentrations of synthesized AgNPs also caused inhibition in spore germination against all the test fungal pathogens but to lesser extent.

Agar well diffusion method

The evaluation of antimycotic activity of different concentrations of synthesized AgNPs against *P. chrysogenum*, *Aspergillus niger*, and *A. alternata* was determined by measuring the zone of inhibition. It was revealed from the results (Table 2 and Fig. 6) that all the concentrations of synthesized AgNPs showed significant zone of inhibition. However, the highest concentrations of synthesized AgNPs brought about maximum zone of inhibition against *P. chrysogenum* (24.00±1.00 mm) followed by *Aspergillus niger* (20.33±0.57 mm) and *A. alternata* (19.33±1.54 mm), respectively. Furthermore, the lowest concentrations of synthesized AgNPs also bring about non-specific reduction in zone of inhibition against all the test fungal strains.

DISCUSSION

In the present study, AgNPs were biosynthesized using mushroom fungi, *H. leucopus* and such studies have been carried out for the 1st time in Kashmir, India. Likewise, in the present study, a strong plasmon absorbance band was observed at 420–450 nm which is in accordance with the work of Narasimha *et al.* [23]. Similar plasmon resonance peak was observed at 420 nm in case of AgNPs biosynthesized from *A. bisporus* by Haq *et al.* and Sudhakar *et al.* [17,27]. It has also been found in different studies that green synthesis of nanoparticles offers a simple, clean, non-toxic, and environmental-friendly approach of synthesizing nanoparticles with a wide range of morphological and physiochemical properties [3,28].

After characterization of synthesized AgNPs using different techniques revealed that synthesized AgNPs were spherical in aggregated form with their size ranging from 80 to 100 nm. Similar techniques were also used for characterization of other biosynthesized nanoparticles by different workers [28-31]. Furthermore, it was revealed from the results that synthesized AgNPs at different concentrations brought about significant spore germination inhibition and reduction in zone of inhibition against all the tested fungal rot pathogens indicating their antimycotic activity. In a similar study, Rajeshkumar *et al.* [32] reported antifungal activity of synthesized AgNPs against *A. niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Fusarium sp.*, and *Candida albicans*. Pulit *et al.* [33] also studied the antimycotic activity of synthesized AgNPs against *A. niger* and *Cladosporium cladosporioides* and also reported that these nanoparticles have potent biocidal activities even at lower concentrations as were found in the present study. The antifungal and antimicrobial activities of AgNPs synthesized from *A. bisporus* and *Tricholoma crassum* were also reported to be maximum at the highest concentrations followed by lower concentrations as were confirmed in the present investigation [17,34].

Table 1: Effect of AgNPs on spore germination % and spore inhibition % against different fungi

Fungal pathogens	Spore germination %			
	10 mg/ml	15 mg/ml	20 mg/ml	Standard
<i>Penicillium chrysogenum</i>	27.40±0.57 (57.38)	18.52±0.57 (69.00)	9.04±0.58 (83.21)	55.25±1.00
<i>Aspergillus niger</i>	57.68±0.52 (38.13)	41.1±0.57 (61.86)	21.56±1.54 (77.32)	74.39±1.00
<i>Alternaria alternata</i>	60.00±1.00 (19.81)	42.83±0.58 (39.94)	25.00±2.00 (69.10)	76.90±1.54

Values are represented as mean±SD. Figures in parenthesis indicate the inhibition in spore germination (%). AgNPs: Silver nanoparticles

Table 2: Zone of inhibition by AgNPs against different test fungi

Fungal pathogens	Zone of inhibition			
	10 mg/ml	15 mg/ml	20 mg/ml	Standard
<i>Penicillium chrysogenum</i>	16.33±1.52	21.00±1.00	24.00±1.00	27.33±2.51
<i>Aspergillus niger</i>	13.00±1.00	16.67±1.52	20.33±0.57	23.00±2.00
<i>Alternaria alternata</i>	13.33±0.57	15.67±0.57	19.33±1.54	24.67±0.57

Values are represented as mean±SD. AgNPs: Silver nanoparticles

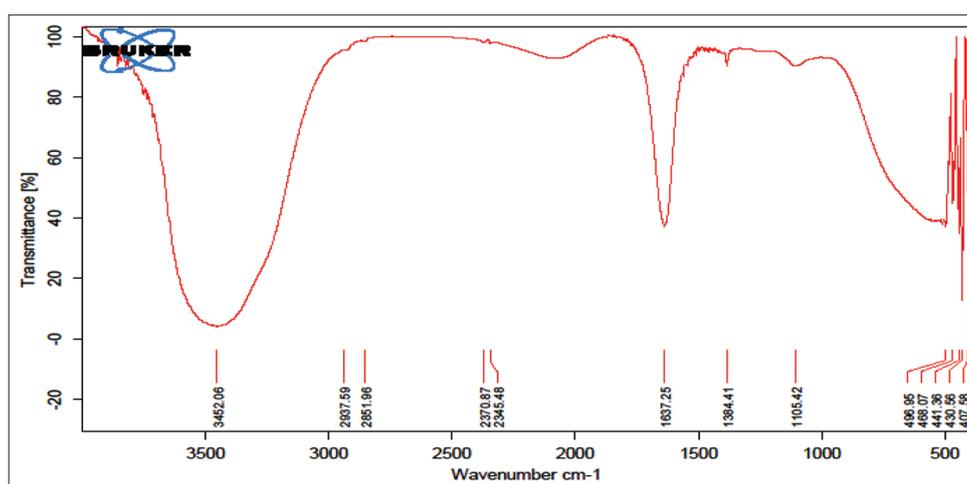


Fig. 4: Fourier transmission infrared spectra of silver nanoparticles biosynthesized from *Helvella leucopus*

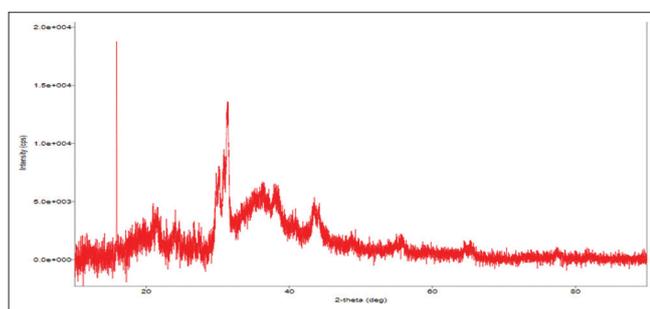


Fig. 5: X-ray diffraction spectrum of silver nanoparticles biosynthesized from *H. leucopus*

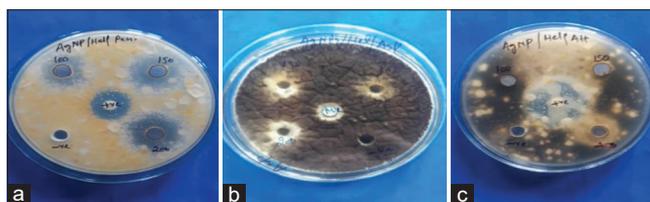


Fig. 6: Zone of inhibition by different concentrations of silver nanoparticles against (a) *Penicillium chrysogenum*, (b) *Aspergillus niger*, (c) *Alternaria alternata*

CONCLUSION

It is concluded from the present study that AgNPs have good potential to be used as antifungal agents against many fungal plant pathogens and controlling plant diseases caused by different fungal pathogens. The successful synthesis of AgNPs by active reduction of silver ions using *H. leucopus* extract as bioreductants can be developed an imperative new green technology solution, where mushrooms could be used actively for metal nanoparticles synthesis. The potential antimycotic activity against rot causing fungi will be boon for the apple growers and food industry to use such metal nanoparticles to reduce the contamination of fruits, vegetables, and food stuffs and also for their long time storage and preservation. The major complication of apple growers and food industry such as rotting, food contamination, and spoilage by fungi now can be resolved with continuous effort of using nanoparticles after proper investigation of their antifungal activity.

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AUTHORS' CONTRIBUTIONS

The first and second authors conducted the experimental work while as the third and fourth authors helped in writing of manuscript. The last author helped in conducting antimycotic assays.

CONFLICTS OF INTEREST

On behalf of all authors, the corresponding author states that there are no conflicts of interest with regard to the preparation or publication of this manuscript.

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