



# Synthesis and Characterization of Antimicrobial Silver Nanoparticles by an Endophytic Fungus Isolated from *Nyctanthes arbor-tristis*

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**Abstract** Biofabrication of metal nanoparticles is a cost-effective, one-step and ecofriendly technique. Cell filtrate of an endophytic fungus isolated from *Nyctanthes arbor-tristis* was challenged with 1 mM solution of AgNO<sub>3</sub> for the silver nanoparticles (AgNPs) synthesis. A reduction of silver ions into AgNPs was observed by surface plasmon resonance at absorption maxima 422 nm. The average size of AgNPs was 35.05 nm. The maximum inhibition zones by AgNPs were 14 mm each against *E. coli* and *Pseudomonas aeruginosa* compared to AgNO<sub>3</sub> solution used as control (10 mm and 9 mm). The fungus was identified as *Phomopsis helianthi* by the sequencing of ITS region of rDNA. This experiment demonstrates a single-step and ecofriendly method for biosynthesis of AgNPs and their usage as an antimicrobial agent.

**Keywords** Endophyte · Fungi · Nanoparticle · Antimicrobial · Plasmon resonance

**Significance statement** Green synthesis of nanoparticles using fungal biomass is an eco-friendly and cost-effective process. This study signifies that endophytic fungus *Phomopsis helianthi* could be a good agent for the synthesis of antimicrobial silver nanoparticles.

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## Introduction

Nanotechnology is a science of designing, characterization, production, and application of materials, devices, and systems by controlling the shape and size at the nanometer scale [1]. The exercise in nanotechnology is done for the transformation of materials in the form of nanoparticles, nanoclusters, nanodots, nanotubes, nanofibres, nanowires which reduce the cost and space for the application of those materials. The nanoparticles of gold are used in targeted drug delivery in cancer cells, whereas iron NPs are used for groundwater pollutants. Fabric treatment is done with AgNPs for the removal of microbial contaminations [2]. Interestingly, an excellent antiviral activity against HIV-1 was reported from silver nanoparticles. AgNPs bind to gp120 in a manner that prevents CD4-dependent virion binding, fusion, and infectivity, acting as an effective viricidal agent against the cell-free and cell-associated virus [3]. Despite other physical and chemical techniques, using a fungal endophyte *Phomopsis helianthi*, the authors report the one-step, ecofriendly, and cost-effective biosynthesis of AgNPs and their antibacterial potential. This endophytic fungus resides inside healthy plant tissues of *Nyctanthes arbor-tristis* without causing any symptoms to the host [4]. Interestingly, fungal endophytes are reported to produce a variety of bioactive and anticancer compounds [5].

## Material and Methods

### Isolation of Endophytic Fungus

The fungus was isolated from the healthy leaf of *Nyctanthes arbor-tristis* growing in the campus of Banaras Hindu

University, Varanasi (25.5°N, 82.9°E; elevation 85 m) according to the standard protocol [4].

### Biosynthesis of Silver Nanoparticles (AgNPs)

Biosynthesis of silver nanoparticles was done according to Singh et al. [6] with slight modifications. The fungus was grown in 1-l flasks containing 800 ml potato dextrose broth for three weeks on a rotary shaker at 200 rpm. Fungal biomass was filtered and resuspended in 100 ml deionized distilled water and agitated at 200 rpm for 24 h. The biomass was filtered with filter paper (Whatman No 1), and the filtrate was further centrifuged at 10,000 rpm to get the cell-free extract. The 50 ml cell-free extract was challenged with 50 ml of 0.1 mM ionic solution of AgNO<sub>3</sub>. A parallel control was kept using metal solution and distilled water in place of cell-free extract. The reaction mixture was left for 24 h on a shaker (200 rpm) at room temperature. The absorption spectra were recorded after 24 h from wavelength 250 nm to 700 nm on UV–visible spectrophotometer (Hitachi, UV-2910) at 1 nm resolution.

### Characterization of AgNPs by Transmission Electron Microscopy (TEM)

A drop of the suspension of nanoparticles was placed onto a copper grid coated with 2% collodion in amyl acetate. After one minute, the extra solution was removed using blotting paper and stained with 2% phosphotungstic acid, air-dried, and examined under a transmission electron microscope (Technai-12 G<sup>2</sup>, FEI, The Netherlands) equipped with SIS Mega View III CCD camera at 120 kV. Measurements were done using the AnalySIS software (SIS, Germany).

### Characterization of AgNPs by X-rays Diffraction (XRD) Analysis

The nanoparticles were further characterized by XRD spectrometry (Cu-K $\alpha$  radiation source) using a 12 kW rotating Cu anode-based Rigaku (Tokyo, Japan) powder diffractometer (PRINT 2000/PC series) operating in Bragg–Brentano geometry and fitted with curved crystal graphite monochromator in diffraction beam and a high-temperature attachment.

### Characterization of AgNPs by Fourier Transforms Infrared (FTIR) Spectroscopy

The nanoparticles solution was centrifuged at 14,000 rpm for 25 min. The pellet was washed three times with 20 ml of deionized water to get rid of the free proteins/ enzymes that were not capping the nanoparticles. The samples were

dried and ground with KBr pellets and analyzed on a Varian 1000 FTIR Scimitar series.

### Antimicrobial Assay of AgNPs

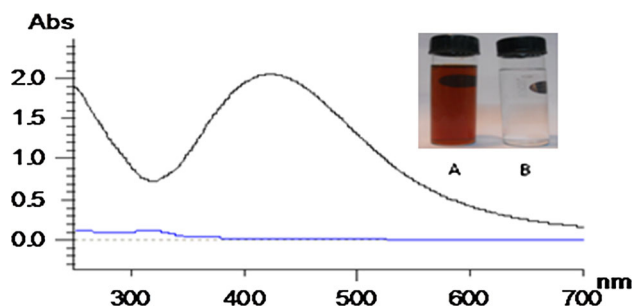
The antimicrobial activity of AgNPs was observed by disk diffusion assay against *Escherichia coli*, *Staphylococcus* sp., *Morganella morganii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and *Shigella boydii*. The sterile filter paper disks (5.0 mm dia.) were impregnated with 5  $\mu$ l suspension of AgNPs (0.5 mM) and placed onto agar plates pre-seeded with test bacterium. The filter paper disks impregnated with 5  $\mu$ l solution of silver nitrate with the same concentration used for nanoparticles were used as control. The diameter of the inhibition zone of bacteria around the disks was measured and compared with control.

### Identification of Endophytic Fungus

The genomic DNA of endophytic fungus was extracted and amplified following the protocol of Sim et al. [7]. The universal primers ITS1 and ITS4 (Metabion International, Martinsried, Germany) were used to amplify 5.8S rDNA and ITS2 regions flanked between the 18S and 28S rRNA genes. Amplified PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide (0.5  $\mu$ g/ml) for visual examination. PCR product was sent to First BASE Laboratories (Malaysia) for sequencing. Obtained ITS rDNA sequences were compared by BLAST search among mentioned sequences at the NCBI GenBank for the identification.

## Results and Discussion

The conversion of colorless silver nitrate solution to yellowish-brown after treatment with cell filtrate of *P. helianthi* was considered a positive signal of AgNPs biosynthesis and was confirmed by UV–visible absorption peak at 422 nm of surface plasmon resonance (SPR) (Fig. 1). SPR excitation is the absorption and scattering of a wavelength-selective photon which leads to a remarkable enhancement of the local electromagnetic field near the surface of the AgNPs [8]. Variable shapes from spherical to hexagonal were found in AgNPs suggesting the polydispersed conditions (Fig. 2a). Through TEM observation, the maximum size of AgNPs was recorded up to 40 nm. Only Saifuddin and colleagues reported a similar size range (5–60 nm) of AgNPs which corroborates this study [9]; however, the considerable numbers of AgNPs in this study were below 05 nm which is quite impressive. The histogram of nanoparticles showed that more than 50%



**Fig. 1** UV-visible spectrum of aqueous silver nitrate solution (Blue line) and AgNPs (black line), inset A—AgNPs, B—control ( $\text{AgNO}_3$ ). AgNPs solution is giving characteristic peak at 422 nm. After synthesis of AgNPs, the color of  $\text{AgNO}_3$  solution changed from white to yellowish-brown

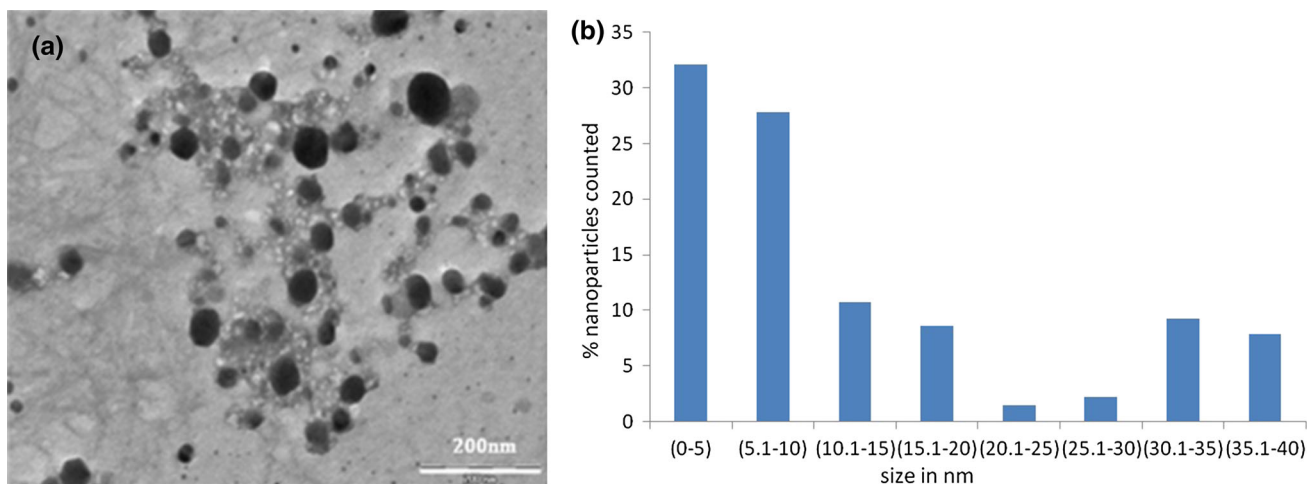
nanoparticles were below 10 nm (Fig. 2b). The smaller AgNPs were spherical, while larger were of pentagonal or hexagonal. Two fungal species *Aspergillus tubingensis* and *Bionectria ochroleuca* have been reported to biosynthesized silver nanoparticles in a spherical shape with a size of  $35 \pm 10$  nm [10]. The XRD indexing of AgNPs was in accordance with Bragg's reflection (JCPDS No: 03–0921) (Fig. 3) [11]. The lattice parameter was 4.074. The nanoparticles were crystallized in the face-centered cubic (FCC) structure with a space group of Fm3m (No. 225). In a similar study, Gajbhiye et al. [12] have reported the average size of spherical AgNPs as 32.5 nm synthesized by *Alternaria alternata*.

The FTIR analysis showed broad peaks around  $3400\text{ cm}^{-1}$  and  $1000\text{ cm}^{-1}$  (Fig. 4) due to the presence of a large number of hydroxyl, carboxyl and carbonyl groups, amides or amino-methyl stretching, and hydrogen bonding between them [13]. The peak around  $3400$  and  $2900\text{ cm}^{-1}$

may refer to N–H stretch vibration of primary and secondary amides of protein and O–H stretch of alcohols and phenols. The peaks around  $1300\text{ cm}^{-1}$  may refer to amine and amino-methyl stretching groups of protein [14]. These metabolites act as capping or stabilizing agents for AgNPs to protect them from agglomeration.

The bacterial inhibition was comparatively greater by AgNPs than  $\text{AgNO}_3$  (Table 1). AgNPs have displayed maximum inhibition zones (14 mm) against *Escherichia coli* and *Pseudomonas aeruginosa*. The combination of penicillin and AgNPs synthesized using *Citrus reticulata* juice showed an inhibition zone of 18 mm against *E. coli* [15]. AgNPs were active against both gram – ve and gram + ve bacteria. It differs from the antimicrobial activity of AgNPs synthesized by *Aspergillus clavatus* against *Pseudomonas fluorescens* and *E. coli* [16] where activity was only against gram-negative bacteria. The interaction between AgNPs and bacterial membrane causes structural changes and damage leading to death [17]. The biological synthesis of antimicrobial AgNPs was also characterized by an endophytic fungus *Alternaria* sp. isolated from the healthy leaves of *Raphanus sativus* [18].

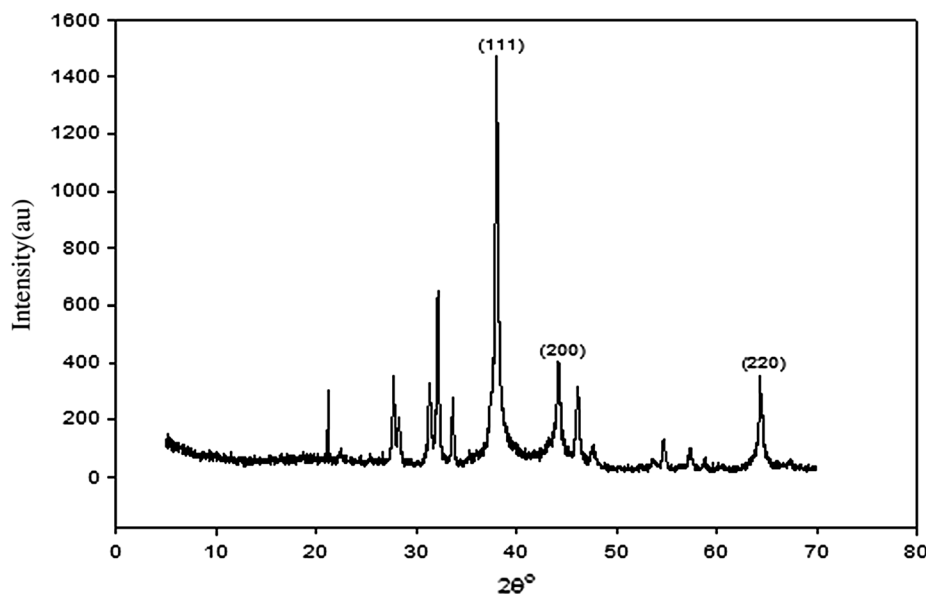
Based on molecular sequence similarity (96%), the fungus was identified as *Phomopsis helianthi* with NCBI GenBank accession No. JN163858. *Phomopsis* is a very common filamentous endophytic species found in tropical and temperate medicinal plants as well [19]. Endophytic *Phomopsis* species is reported to produce antimicrobial compounds such as phomol and antimalarial phomoxanthones [20]. It is hypothesized that there may be NADH-dependent reductase which plays a role in the reduction of silver ions into AgNPs in cell filtrate of *Fusarium oxysporum* [21].



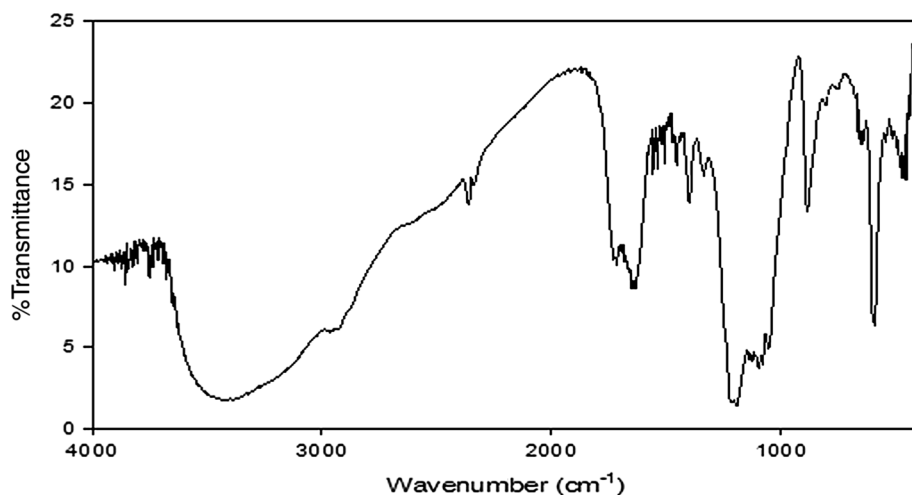
**Fig. 2** a TEM images of AgNPs. The NPs were different in size and shape. The NPs were surrounded by the organic layer stabilizing them, b particle size distribution histogram of AgNPs. Most of the

nanoparticles were in the size range below 5 nm. The maximum size of NPs was below 40 nm

**Fig. 3** X-rays diffraction pattern of AgNPs synthesized by *Phomopsis helianthi*. Three characteristic peaks were observed at  $2\theta = 38.07^\circ$ ,  $44.17^\circ$ , and  $64.52^\circ$  indexing to (111), (200), and (220) planes of AgNPs, respectively



**Fig. 4** FTIR spectrum of AgNPs synthesized by *Phomopsis helianthi*



**Table 1** Antimicrobial activity of AgNPs synthesized by *Phomopsis helianthi*

	Inhibition zone (mm) from of 0.5 mM						
	<i>E. coli</i>	<i>Staphylococcus</i> sp.	<i>Morganella morganii</i>	<i>Proteus vulgaris</i>	<i>Pseudomonas aeruginosa</i>	<i>Salmonella enteritidis</i>	<i>Shigella boydii</i>
AgNO <sub>3</sub>	10	10	07	06	09	08	07
AgNPs	14	13	09	08	14	12	09

## Conclusion

The authors report the extracellular, single-step and safe biosynthesis of AgNPs by endophytic fungus *P. helianthi* which gives advantage over intracellular nanoparticles synthesis because that requires an additional effort to purify from the adhering biomass and exploiting them as an antibacterial agent. This method is ecofriendly using a

biological agent for metal nanoparticle synthesis which does not affect our environment. Further research is needed for the investigation of the mechanism involved in nanosynthesis by biological molecules.

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### Compliance With Ethical Standards

**Conflict of interest** The authors declare no conflict of interest to publish this manuscript.

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