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SHORT COMMUNICATION

A facile and rapid method for green synthesis of Silver Myco nanoparticles using endophytic fungi

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Abstract

The Myco silver nanoparticles (AgNPs) are synthesized through bio-reduction reaction of silver nitrate by cell-free filtrate of endophytic fungi, which act as both reducing and capping agent. The synthesis of silver nanoparticles (AgNPs) was confirmed through UV-VIS spectroscopy, Fourier Transform Infrared (FTIR), Transmission Electron Microscope (TEM), Scanning Electron Microscope (SEM). Energy Dispersive spectroscopy (EDAX) was used to study the structure, morphology, shape, and composition of synthesized nanoparticles. The efficacy of the silver nanoparticles (AgNPs) was tested against the pathogenic bacterial strains such as *K. pneumonia, A. Baumannii, P. mirabilis, S. Typhimurium, P. aeruginosa and E. Coli*. The myco silver nano particles treatment significantly reduced the growth of all the bacterial species tested in this study. The results suggested that myco nanoparticles can be utilized as an alternative to antibiotics or to break antimicrobial resistance.

Keywords: *Antimicrobial Effects; EDAX; Endophytic Fungi; SEM; Silver Nanoparticles;TEM.*

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INTRODUCTION

Endophytic fungi inhabit the inner healthy tissues of plants without causing any disease to the host plant [1]. They are the novel source of secondary metabolites and are considered as promising source of secondary bioactive compounds [2]. The myco synthesis of metal nanoparticles or Myconanotechnology is the utilization of fungi or fungal extract for the synthesis of nanoparticles [3]. The endophytic fungi can be grown to produce the commercially important metabolites and can be utilized for nano synthesis [4]. In recent years, much attention has been given to nanoparticles research due to the wide spectrum of applications in different fields [5, 6]. The green synthesis of nanoparticles is simple, environment-friendly, cost-effective and stable [7].

The application of medicinal plants for treatment of various ailments is very ancient traditional system of Indian medicine including Ayurveda and Siddha that use indigenous plants or herbs for conventional treatment methods [8]. *Solanum nigrum* or black berry or night shade or Makoi is a member of family Solanaceae. The plants possess various medicinal properties including anti-inflammatory, antioxidant, antipyretic, antitumor, antiulcerogenic, antinociceptive,cancerchemopreventive,immunomodulatory and hepatoprotective effects [9, 10].

Pharmacologically, different parts of the *S. nigrum* plants have been used for different treatments including anti-hyperglycaemic and hypolipidemic [11]. It has been reported that alkaloids and Solanine present in *S. nigrum* possess the antidiabetic activity [12] in albino rats [13] and prevented diabetic nephropathy in diabetes induced rats. [14] It has also been reported the larvicidal activity of leaf extract isolated from *S. nigrum* against the *Culex vishnui* and *Anopheles subpictus*. *S. nigrum* aqueous and methanolic extract also showed the antibacterial activity against the gram negative bacteria *Xanthomonas campestris* and *Aeromonas hydrophila* [15]. *S.*

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nigrum leaf extract significantly inhibited the growth of *Aspergillus niger* and *A. Flavus* [16, 17]. For the past few decades, silver metal has been used as anti-microbial agent to cure various diseases [18]. In the present study, a simple and rapid method was utilized for the synthesis of silver myco nanoparticles from endophytic fungi. The synthesized silver nanoparticles were characterized by various biophysical techniques to analyze its structure and stability. Furthermore, the efficacy of silver myco nanoparticles was assessed against different human pathogenic strains.

MATERIALS AND METHODS

Sample collection and Isolation of endophytic fungi

The healthy stem of *Solanum nigrum* was collected from B. S. Abdur Rahman Institute of Science & Technology, Vandalur, and Chennai. Samples were brought to the laboratory in sterile plastic bags for further processing. The stem was washed under tap water to remove the dust particles followed by sterile water and then surface sterilized [19]. The sterilized stems were then dried by placing over sterile blotting paper and dissected into pieces of around 1mm size.. Each piece was then inoculated on PDA medium. The plates were wrapped with parafilm and incubated in an incubator at 28ºC until the growth of endophytic fungi. The plates were observed every day for fungal growth. After 7 days mycelium was isolated and sub cultured in PDA medium.

Fermentation and metabolite extraction

Endophytic fungus was cultured in 1000 mL Erlenmeyer flasks each containing 500 ml of PDB and incubated at 180 rpm, 25^{o}C on a rotary shaker for 20 days. After 20 days, fermented broth was filtered and extracted using ethyl acetate (1:1 ratio) for three times and the organic phase was dried by evaporation

Phytochemical analysis of endophytic fungal extract

The phytochemical analysis was carried out to detect the presence of secondary metabolites such as alkaloids, amino acids, carbohydrates, tannin, flavonoids, saponin, terpenes, lipids, and phenols [20].

Fungal DNA isolation and identification of endophytic fungi

DNA was isolated by cetyltrimethylammonium bromide (CTAB) method [21]. The Agarose gel electrophoresis was carried out to check the quality of the DNA, ITS region was amplified and sequenced by ITS1 (5′-3′) forward primer TCCGTAGGTGAACCTGCGG and reverse Primer TCCTCCGCTTATTGATATGC.

Construction of phylogenetic tree

The ITS sequence of the isolated fungal strain was compared with the fungal strains recovered from GeneBank using the Nucleotide Blast. The nucleotide sequence was used for the identification of the fungal isolate, isolated from *solanum nigrum*. MEGA version 4 (Molecular Evolutionary Genetics Analysis) software, was used for the construction of the phylogenetic tree [22].The phylogenetic tree was constructed using the closely related strains retrieved from NCBI Gene Bank.

Biosynthesis and characterization of silver nanoparticles

The biomass of endophytic fungi was grown aerobically in potato dextrose broth in Erylen Mayer flasks and kept in an orbital shaker at 150 rpm, 28^{o}C for 96 hrs. The biomass was harvested and filtered through whatman filter paper no. 1. The filtered broth was used for nanoparticle synthesis. The fungal broth was mixed with 1mM $AgNO_3$ and incubated at room temperature for 24 h for the synthesis of myco silver nanoparticles. The colour change was observed by visual observation. The reduction of pure silver ions was recorded by measuring the UV-Vis spectra of the suspension at room temperature at the range of 350-800 nm, followed by the IR analysis. The collected pellet was lyophilized with freeze dryer (Model: Alpha 2-4 LD plus) and was characterized by high resonance Transmission Electron Microscope (HR-TEM), Scanning Electron Microscope (SEM) and Energy Dispersive spectroscopy (EDAX).

Antibacterial assay by well diffusion method

Silver nanoparticle suspension was used for antibacterial activity against different human pathogens (*Escherichia. coli, Pseudomonas aeruginosa, Salmonella typhimurium, Proteus mirabilis, Acinetobacter baumannii and Klebsiella pneumoniae*) by well diffusion method using different concentrations of nanoparticles (1 mg/5 ml) and Ampicillin (25 µg/ml) was used as a positive control, crude (Fungal broth) as a negative control. Plates were observed subsequently for the formation of zone of inhibition [23].

RESULTS AND DISCUSSION

Endophytic fungus was isolated from the stem of *Solanum nigrum*. By 16S sequencing, it was identified as *Setosphaeria sp.* BAB-4662. The phylogenetic tree was constructed using the closely related strains retrieved from Gene Bank. (Figs. S1, S2).The fungus was initially white in colour however, after 4-5 days, colour was changed to grey to dark grey. The fungus was inoculated in PDA broth for 21 days to induce the production of the bioactive compounds. Fungal broth was mixed with equal volume of 1 mM of silver nitrate solution. The synthesis of silver nanoparticles from fungal extract was monitored by the colour change from pale white to dark brown colour which was observed after 24h, due to the reduction of silver ions and was determined by the UV-VIS spectra showing absorbance at 350 nm and 430 nm after 24 hours of incubation (Fig. 1a). The results of UV-VIS spectra suggested that the silver nanoparticles synthesized from fungal extract was very rapid and started within 15 minutes, which indicated that the silver nanoparticles were dispersed in the suspension and reaction was rapid. This is the first study where BAB-4662 sp. was used for biosynthesis of silver nanoparticles.

IR spectra of fungal silver nanoparticles synthesized from endophytic fungi revealed three bands at 1634.38 cm^{-1}, 1725.98 cm^{-1} and 3361.32cm^{-1}(Fig. 1 b) that correspond to the binding vibrations of amide I, amide II and alcohols and phenol. Small bands indicated that different molecules are present inside the silver nanoparticles. The IR spectra confirmed that bioactive compounds including proteins, Polyphenols, terpenoids, and flavonoids act as reducing as well as bio capping agent of these nanoparticles and increased the stability of the nanoparticles [24, 25]. Earlier studies [26, 27] also demonstrated the role of polyphenols and flavonoids in the reduction of a pure silver ion.

Scanning electron microscopy provided the

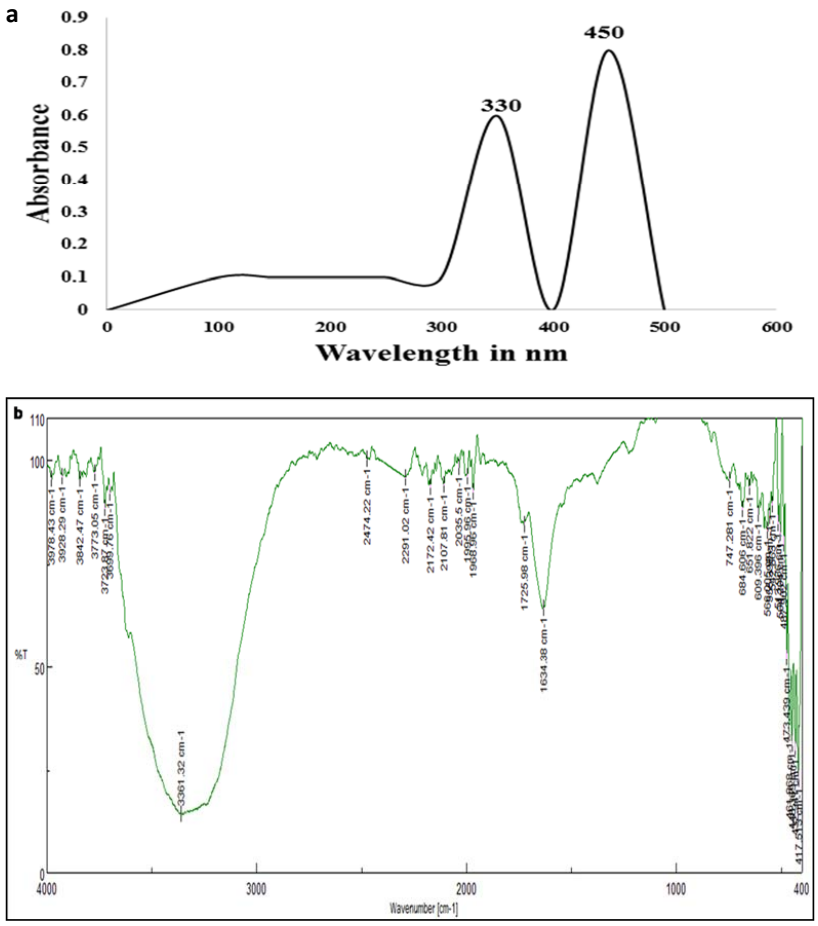

Fig.1: (a) UV-VIS. Spectra and (b) FTIR analysis of AgNPs synthesized from fungal broth.

surface morphology and size of the synthesized nanoparticles (Fig. 2a, 2b, and 2c). SEM images revealed plenty of dispersive nanoparticles with the average size of about 35 nm. As it is clear in the SEM image, the nanoparticles are relatively uniform. EDAX spectroscopic analysis confirmed the composition of silver nanoparticles and showed the presence of oxygen, copper and metallic silver (Fig. 3). Potassium was present as an impurity. TEM micrographs showed nano-sized dispersed silver nanoparticles of different sizes including spherical with size ranging from 20 nm-50 nm (Fig 4a, b, and c).

The efficacy of biosynthesized silver nanoparticles was tested against pathogenic bacteria and zone of inhibition was shown in Fig. 5 and 6. The wells were loaded with different concentrations (30 µl, 50 µl), of crude extract and silver nanoparticles respectively. Maximum zone of inhibition (45 mm) was observed in *P. aeruginosa* at 50 µl of AgNPs while *A. baumannii* showed no zone of inhibition in the above said concentration of AgNPs. At a minimum concentration of 30 µl amongst pathogenic bacteria, *P. aeruginosa* showed 40 mm zone of inhibition. *K. pneumoniae*, *P. mirabilis* showed 21 mm each and *E coli* showed 20 mm zone of inhibition while *A baumannii* showed no zone of inhibition. The results of the study suggested that effects of silver myco-nanoparticles are specific and inhibited the growth of *P. Aeruginosa, K. Pneumonia, E.coli* and *P. Mirabilis. However, no effect on growth of A*

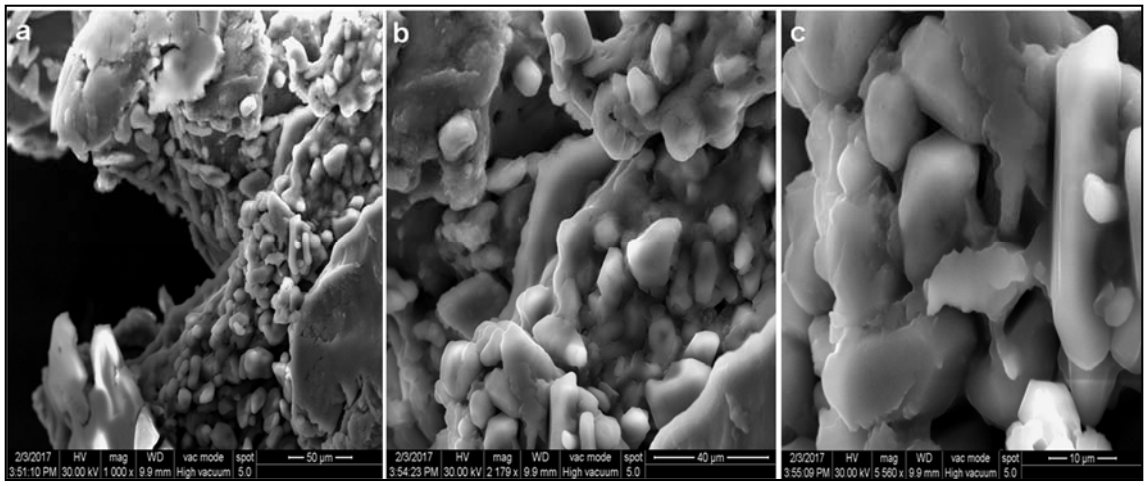

Fig.2: SEM (Fig. a, b, and c), images of synthesized silver nanoparticles synthesised from fungal broth.

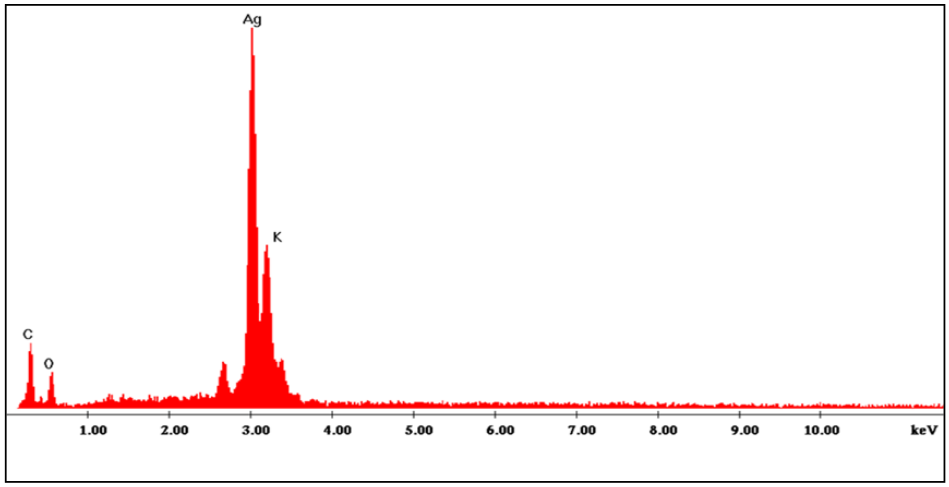

Fig. 3: EDAX image of mycosilver nanoparticles.

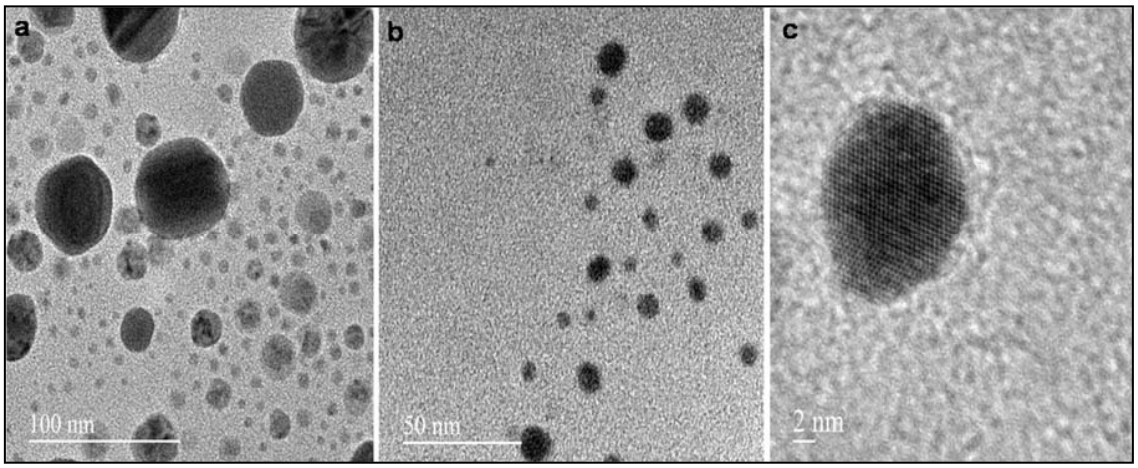

Fig. 4: (a, b, and c) HR-TEM, images of Mycosilver nanoparticles.

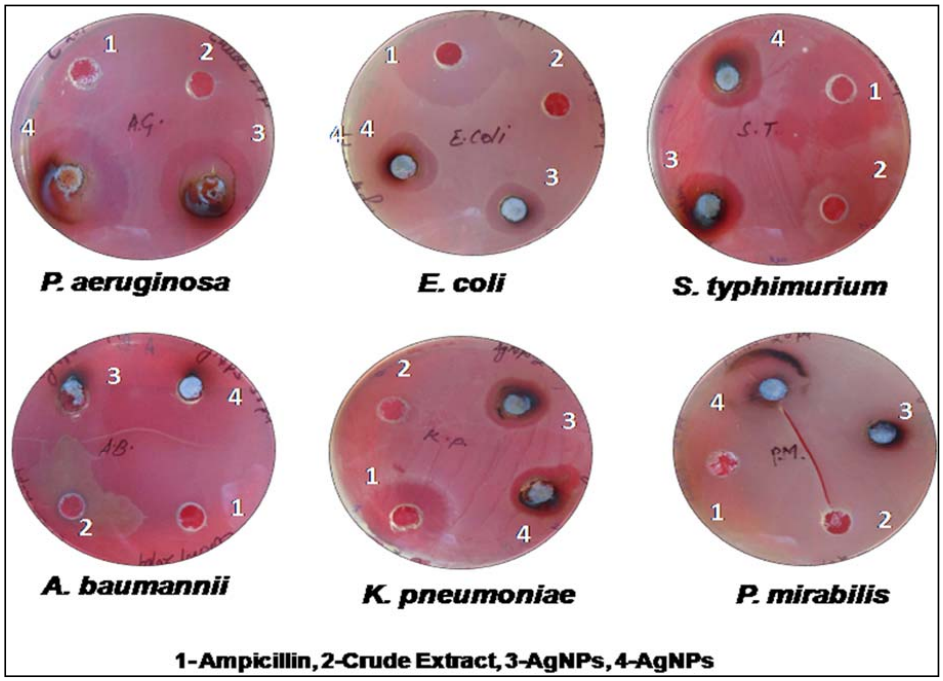

Fig. 5: Antibacterial activity of silver nanoparticles against *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Proteus mirabilis* by well plate method.

baumannii was observed. This suggests that myco nanoparticles specifically interact with the targets or virulence factors and hence it can be utilized as an alternative to antibiotics to break antibiotic resistance.

CONCLUSION

The current study suggests that endophytes could be the potential source of antimicrobial agents because they produce bioactive metabolites, and has the potential to synthesize the silver nanoparticles. The green synthesis of silver nanoparticles from *Setosphaeria sp* BAB-4662 sp. was synthesized for the first time. The characterization of nanoparticles was performed by UV-Vis spectroscopy followed by FTIR analysis which revealed the functional groups present in the biosynthesized fungal silver nanoparticles. Scanning electron microscopy (SEM), High Resolution Transmission electron microscopy (HR-

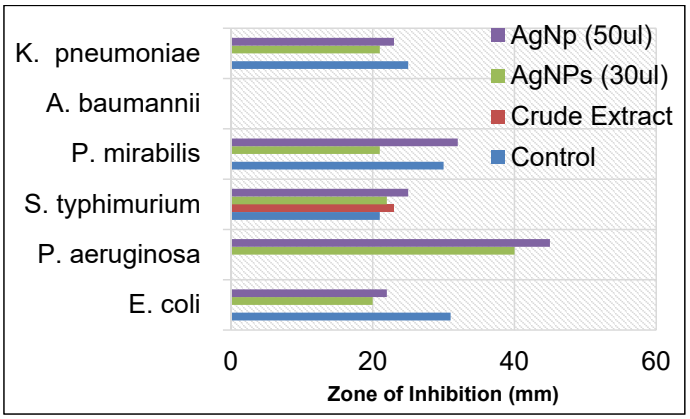

Fig. 6: Graphical representation of antibacterial activity of silver nanoparticles.

TEM), energy dispersive X-ray analysis (EDX or EDAX) techniques confirmed the synthesis of silver nanoparticles of different shapes, sizes and gave the confirmation of metallic silver present in fungal silver nanoparticles. The antibacterial potential of AgNPs revealed maximum efficacy of both gram positive and gram negative pathogenic strains. Furthermore, the endophytic fungi can be utilized to control broad-spectrum human pathogenic strains, which may be exploited further for the control of various bacterial diseases and targeted drug delivery. The synthesized myco nanoparticles have the potential to control both gram positive and gram negative bacteria and can be utilized to break the antibiotic resistance.

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CONFLICT OF INTEREST

The Authors declare that they have no conflict of interest.

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