Bioactive Silver Nanoparticles from Endophytic Fungus Fusarium sp. Isolated from an Ethanomedicinal Plant Withania somnifera (Ashwagandha) and its Antibacterial Activity

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Abstract
Nanotechnology has become a platform to alter and modify and develop the important properties of metal in the form of nanoparticles with its promising applications in various fields for the benefit of mankind. In the present study endophytic fungus Fusarium sp. was isolated from healthy leaves of Withania somnifera (Ashwagandha) and was subjected for the extracellular biosynthesis of silver nanoparticles (AgNps). The synthesized AgNps were characterized using visual observation, UV-Vis spectroscopy and Transmission Electron Microscopy (TEM). Further AgNps synthesized were tested for their efficacy against the bacterial pathogens like E.coli, S.typhi and S.aureus. The formation of AgNps was confirmed by visual observation by change in colour from pale white to brown and the Surface Plasmon Resonance was determined by UV-Vis spectra at 422 nm. TEM revealed the formation of small sized spherical shaped nanoparticles ranging 12-20 nm. Antibacterial activity of AgNps against E.coli, S.typhi and S.aureus showed encouraging results, showing maximum zone of inhibition of 26mm, 26mm and 28 mm respectively at 60µl concentration of AgNps against E.coli, S.typhi and S.aureus.

Key words: Silver nanoparticles, Endophytic fungi, Fusarium sp., TEM, Antibacterial activity.

1. Introduction
The field of nanotechnology is one among the foremost important and active areas of research in modern science and technology [1]. Nanotechnology mainly deals with the formulation of experimental process for the synthesis of nanomaterials using different systems with their wide applications. The use of metal nanoparticles has received extensive attention in present century due to their remarkable properties and wide range of the applications [2]. Use of toxic chemical synthesis greatly limits their biomedical applications particularly in clinical fields. Therefore, the focus for the green synthesis of nanoparticles is an emerging branch of nanotechnology with the help of biological resources like marine algae [1], plant extract [3], Bacteria [4], Fungi [5,6,7] actinomycetes [8], which offers numerous benefits of ecofriendliness and compatibility for pharmaceuticals and other biomedical applications. Amongst inorganic antimicrobial agents silver has been known to be a good antimicrobial agent since ancient times before Neolithic revolution, to fight against the infections. The researchers are trending towards nanoparticles, especially silver nanoparticles (AgNps) to solve the problem of emerging human pathogens which are highly resistant to conventionally used antibiotics. This has resulted in an inevitable and urgent need for the development of novel antimicrobial agents [9].

Owing to the fact that endophytic fungi provide a broad variety of bioactive secondary metabolite with unique structures they could be explored to their ability for biosynthesis of AgNps to develop an efficient environmental friendly process [10]. Very few reports showed the proficiency of AgNps using endophytic fungi like Penicillium sp isolated from curcuma longa (turmeric) [6], Alternaria solani (GS1) and Penicillium fumiculsum (GS 2) are the endophytic fungi isolated from ethanomedical plant Gloria superba [2].

In the present study, endophytic fungus Fusarium sp. isolated from the leaves of Withania somnifera (Ashwagandha) a medicinal plant, has been employed for the biosynthesis of AgNps and antibacterial efficacy was evaluated against resistant pathogenic bacteria which can explore the possibilities of exploiting this endophytic
fungus in pharmaceutical industries as a source of antimicrobial compounds.

2. Materials and Methods

2.1 Isolation of Endophytic Fungi:
Healthy leaves of Withania somnifera (Ashwagandha) were collected from Department of Botany, Gulbarga University Gulbarga. The leaves were gently washed several times with tap water to remove adherent dirt and then cut into small pieces. Surface sterilized by immersing into 70% ethanol (C2H5OH) for 30 sec, followed by 0.01% mercuric chloride (HgCl2) for 5 min and then soaked in 0.5% sodium hypochlorite (NaOCl) solution for 2-3 mins, then finally rinsed with sterile distilled water and blot dried with sterile filter paper, cut surface of the leaf was placed on petridish containing Potato Dextrose Agar (PDA) amended with streptomycin sulfate (250mg/ml) to suppress the bacterial growth and incubated at 28°C for 6-8 days for the growth of endophytic fungal colony from leaf segment. Fungi which grew out of the explant was isolated and pure cultured onto PDA plates and then the isolate was identified based on its morphological and reproductive characters using standard identification manual [11, 12].

2.2 Biological Synthesis of Silver Nanoparticles:
The endophytic fungus, Fusarium sp. was screened for the biosynthesis of AgNps. Fungus obtained was grown in 250 ml Erlynmyer flask containing 100 ml of MGYP (3g of Malt & Yeast Extract, 5g of Peptone per litre of distilled water) at 28°C in static position for 7 days of incubation, the fungal mycelia was separated by filtering through filter paper and then repeatedly washed with distilled water to remove the media components from the biomass. The biomass obtained was resuspended into the flasks containing sterilized double distilled water and the fungal filtrate was harvested by filtering through Whatman filter paper No 1, then fungal filtrate was treated with 1mM silver nitrate (AgNO3) solution and incubated at 28 °C for reduction (Ag+ to Ag0) [13].

2.3 Characterization of Silver Nanoparticles

2.3.1 UV-Vis Spectroscopy:
Change in colour of mycelium free filtrate incubated with 1mM silver nitrate solution was visually observed over a period of time which indicates the bioreduction of AgNps which was monitored by sampling aliquots of 1 ml with different time intervals. Absorption measurements were carried out on UV-Vis spectrophotometer at a resolution between 350 and 500 nm.

2.3.2 Transmission Electron Microscopy (TEM):
The morphology of the biosynthesized AgNps was studied using Transmission Electron Microscopy technique (Hitachi H-7500). A drop of biosynthesized AgNps solution was placed on a carbon coated copper grids and kept overnight under vaccum desiccation and dried. The sample was then loaded onto a specimen holder. Transmission electron micrographs of samples were taken. Particle size distribution and average silver core diameter were calculated.

2.3.3 Antibacterial Activity:
Antibacterial activity was analysed with synthesized AgNps by well diffusion method against Escherichia, coli Salmonella typhi and Staphylococcus aureus. These pathogenic microorganisms were inoculated in separate nutrient broth test tubes and incubated at 37°C for 6 h and with the help of cotton swabs the cultures were plated onto Muller Hinton Agar (MHA) plates. Wells were made on MHA plates with the help of sterilized cork borer [13] and loaded with varying concentrations (10-60 μl with a difference of 10 μl concentrations) of synthesized AgNps solution in individual wells and the AgNO3 solution was used as positive control and distilled water as negative control. The plates were incubated at 37°C for 24h, and observed for the zone of inhibition.

3. Results and Discussion

3.1 Isolation of Endophytic Fungi:
After 7 days of incubation on Potato Dextrose Agar (PDA) plate the endophytic fungus was grown out of the sterilized leaf segment of Withania somnifera (Ashwagandha) which was subcultured and identified as Fusarium sp. (Fig-1) based on the morphological and microscopic observations at Agharkar Research Institute, Pune.

Fig-1. Endophytic fungus on PDA medium after 7 days

The endophytic fungus Fusarium sp. isolated was used for the production of extracellular filtrate by treating it with 1mM AgNO3 solution and reduction was indicated by the change in color from pale white to brown which gives an indication for the production of AgNps (Fig-2), further confirmed by using UV-Vis spectrophotometric analysis.

Fig-2 a) Filterate of endophytic fungus Fusarium sp. b) Color change to reddish brown after treating with 1mM AgNO3

3.2 UV-Vis Spectroscopy:
UV-Vis spectrophotometric analysis was carried out for AgNps produced using endophytic fungus Fusarium sp. showed the maximum Surface Plasmon Resonance peak at 422 nm (Fig-3) scanned in the range between 350 and 500 nm which confirms the absorption of AgNps production.
Singh et al. 2013 reported the production of AgNPs using endophytic fungus *Penicillium* sp. with maximum Surface Plasmon Resonance peak at 420 nm [12], Devi et al. 2012 reported the confirmation of AgNPs production at 415 nm absorption peak by Surface Plasmon Resonance [2]. Similarly Shivaraj et al. 2013 revealed Surface Plasmon Resonance of AgNPs between 380 and 450 nm [13]. Gitanjali et al. 2014 reported 419 nm absorption peak by Surface Plasmon Resonance [7].

This indicates the AgNPs produced are highly stable and well dispersed, further the morphology and particle size of AgNPs was analysed by TEM.

### 3.3 Transmission Electron Microscopy (TEM):

TEM measurements were used to determine the size and shape of synthesized nanoparticles using endophytic fungus *Fusarium* sp. from the leaves segment of *Withania somnifera* (Ashwagandha) found to be well dispersed small in size and spherical in shape ranging from 12-20 nm (Fig-4). Our report correlates with other researchers, Singh et al. 2013 reported well dispersed spherical shaped nanoparticles with size range of 25-30 nm using extracellular filtrate of endophytic fungus *Penicillium* sp. [12] Shivaraj et al. 2014 reported characterization of AgNPs by TEM and found to be spherical with size range of 20-55 nm using *Aspergillus niger*. [5] Similarly, Banu et al. 2011 reported characterization of AgNPs by TEM size ranging between 3-20 nm which are nano sized and well dispersed. [14].

#### 3.3.3 Antibacterial activity:

Antimicrobial efficacy of AgNPs was studied against test bacterial strains of *E.coli*, *S.typhi* and *S.aureus* using agar well diffusion method. AgNO₃ solution was taken as positive control (1mM AgNO₃ Solution) and negative control (Distilled water). Bacterial lawn was prepared and wells were loaded with different concentrations 10, 20, 30, 40, 50 and 60µl of AgNPs respectively. The results were encouraging. AgNPs exerted good antibacterial activity (Table-1). The highest zone of inhibition was observed against *S.aureus* i.e 28mm with 60µl AgNPs and the lowest zone was 20mm with 10µl AgNPs. The antibacterial efficacy of AgNPs against *E.coli* positive control shows 25mm zone of inhibition whereas the highest 26mm zone of inhibition with 60µl of AgNPs and efficacy of AgNPs against *S.aureus* was found to be 16mm zone of inhibition with positive control and no zone of inhibition with negative control i.e distilled water, the highest zone of inhibition 28mm with 60µl AgNPs and antibacterial studies against *S.typhi* revealed 23mm zone of inhibition for positive control and no zone of inhibition to negative control while the highest zone of inhibition 26mm with 60µl of AgNPs was observed (Fig-5). Sinha et al., 2014 reported the antibacterial property of AgNPs produced by *Pithophora oedogonia* which revealed the zone of inhibition of 16.8mm in case of *E.coli* (MTCC 443) using 20µl of AgNPs [1], similarly Sharanabasava. et al. 2012 reported the antibacterial activity of AgNPs produced by *P.diversum* indicating maximum zone of inhibition against *S.typhi* to be 16mm by using 20µl of AgNPs [9]. The antimicrobial studies of our results reveal the increase in the concentration of AgNPs increases the zone of inhibition. Compared to the results of antibacterial studies against pathogenic clinical isolates of Singh et al. 2014 reported synthesis of AgNPs from endophytic fungus *Penicillium* sp. showed the maximum zone of inhibition against MDR *E.coli* and *S.aureus* as 17mm and 16 mm respectively with 80µl of AgNPs [12], Shivaraj et al. 2013, reported the biosynthesis of AgNPs from *Aspergillus flavus* and showed the maximum zone of inhibition against MDR *E.coli* of 16mm at 80 µl AgNPs concentration [13]. The AgNPs produced by the endophytic fungi appears to show highest
zone of inhibition with minimum concentration of AgNps produced by *Fusarium* sp. isolated from an ethanomedicinal plant *Withania somnifera* (Ashwagandha) which can potentially eliminate the problem of chemical agents, which may have adverse effects in its application, thus making AgNps more biocompatible.

4. **Conclusion**

In our study we have reported the isolation and screening of endophytic fungus, *Fusarium* sp. from an ethanomedicinal plant *Withania somnifera* (Ashwagandha) and screened for their efficacy to reduce AgNO$_3$ to AgNps. Now a day’s fungal synthesis of AgNps have got significance as they contain larger cell wall protein which leads higher production of AgNps. The synthesized AgNps were characterized using UV-Vis spectrum and TEM. All over the world the bacterial strains are getting resistance to conventional antibiotics because of indiscriminate use of antibiotics to modify the existing antimicrobials or search for new antibiotics in the form of AgNps is burgeoning topic of research in Pharma and healthcare companies which are concentrating more on AgNps to control on pathogenic microorganisms.

**References**


**Fig-5.** Antibacterial activity of AgNps against different pathogenic bacteria.

**Table-1.** Zone of Inhibition against different pathogenic bacteria

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<th>Sl No.</th>
<th>Pathogenic Bacteria</th>
<th>Zone of Inhibition (mm)</th>
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<tr>
<td></td>
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<td>Positive Control</td>
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<tr>
<td>1.</td>
<td><em>E.coli</em></td>
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<td>2.</td>
<td><em>S.typhi</em></td>
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<td>3.</td>
<td><em>S.aureus</em></td>
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