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ORIGINAL RESEARCH PAPER

Antibacterial and Cytotoxicity Studies of Silver Nanoparticles Synthesized by Endophytic *Fusarium solani* Isolated from Withania *somnifera (L.)*

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ABSTRACT

The present study establish extracellular production of silver nanoparticles (AgNP) using *Fusarium solani,* from medicinal plant *Withania somnifera* (L.) (ashwagandha) and it's antibacterial and cytotoxicity effects. Biological- AgNP (Bio- AgNP) were synthesized by using fungal cell free extract and characterized by SEM, TEM, UV spectroscopy, XRD, FTIR and AFM analysis. Antibacterial properties were assayed by well diffusion and cytotoxicity by RBC lysis test and MTT assay respectively. X- ray diffraction and microscopic analysis revealed the well dispersed and crystalline nature of spherical nanoparticles with a calculated size ranging from 10 - 50 nm. The Bio-AgNP exhibited significant antibacterial properties in a range of 50-100 $\mu g ml^{-1}$ against the selected clinical pathogens *Escherichia coli*,*Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. The observed hemolysis of 3.906 % at 50 $\mu g\ ml^{-1}$ suggested the safe therapeutic application of Bio - AgNP. MTT assay revealed that at the suggeseted concentration 69 % of cells are viable. These outcomes are extremely encouraging to utilize Bio-AgNP as a medication. Exploiting the endophytic organisms from therapeutic plants for improvement of nanomaterial is a uninvestigated and relatively novel territory. This may improve the likelihood in future to push the limit ahead in nanomedicine.

KEYWORDS: *Endophytes; Fusarium solani; Withania somnifera; Bio-AgNP; RBC lysis; MTT assay*

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INTRODUCTION

In the area of nanotechnology evolution of dependable and green collar process for construction of nanoparticles is of great importance. Nanotechnology deals with the materials which exhibits remarkable physical, chemical and biological properties because of their nanoscaled size [1]. Nanoparticles are particles with a size range of 1-100 nm [2] . A wide variety of physical and chemical methods to synthesize nanoparticles are in practice but their inherent flaws that include contamination from precursor chemicals, use of toxic solvents and generation of hazardous by products makes their usage inappropriate in biological systems. These disadvantages insisted the use of novel and well refined methods that opened the doors to explore benign and green routes for synthesizing high – yielding, low cost, non toxic and environment friendly nanoparticles

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[3]. Microorganisms have been explored as potential bio factory for synthesis of nanoparticles such as cadmium sulphide, gold and silver [4]. Sastry *et a*l.[5] studied that fungus *Verticillium sp.* and *Fusarium oxysporum* when exposed to gold and silver ions, reduced the metal ion fairly into metal nanoparticles. Biological synthesis of silver nanoparticles using microorganisms has received profound interest because of their potential to synthesize nanoparticles of various size, shape and morphology either extracellularly or intracellularly [5; 6; 7]. Chauhan *et al*.,[8] reported the extracellular biosynthesis of silver and zinc oxide nanoparticles from extracellular components of *Pichia fermentans* JA2.Fungi are more versatile in growth and metal tolerance in contrast to bacterial population. Hence they have been used as bio factories for synthesis of nanoparticles and offer environment friendly solutions for different problems. Bio fabricated silver nanoparticles produced by various fungi via different approaches [9; 10; 11]. Intracellular synthesized nanoparticles require additional downstream processing steps to release the particles from cells. The extracellular synthesis is economical and downstream processing is simple [12].

Endophytes are one such organism that are under explored and can be exploited in the biogenic synthesis of silver nanoparticles. According to Baker *et al.* endophytes are microbes that colonize living internal tissue of plants without causing any immediate, overt negative effects. Owing to the fact that the endophytic fungi provide a broad variety of bioactive secondary metabolites with unique structures, they could be explored for their ability to biosynthesis of silver nanoparticles to develop an efficient environment friendly process. An attempt was made to investigate endophytic fungi associated with an ethno medicinal plant *Potentilla fulgens* L. for their ability to synthesize silver nanoparticles and the ultra structure and size distribution of the particles were studied using electron microscopy techniques [13]. Plant endophytic fungi are significant and unique source of natural bioactive composites with their possible applications in medicine, food and agriculture. Some of the endophytic fungi are associated with the production of anti-tumor drug taxol (paclitaxel) [14; 15; 16]. So endophytic fungi can be used as an encouraging optional unit in mycofabrication of nanoparticles with a progressive employment for potential upcoming drug [17].

Withania somnifera(L.) Dunal (aswaganda) is regularly used in ayurvedic medicine. It holds anti-inflammatory, antitumor, antistress, antioxidant, immunomodulatory, hematopoietic, and rejuvenating characteristics. It also influences the endocrine, cardiovascular and pulmonary system. Introductory research shows that diverse components of aswaganda display a range of remedial effects. It nurture with large population of endophytes and they contributes to the medicament compounds bring out by the plant [18]. Hence in the current study we have adopted *Withania somnifera* for the isolation of endophytes and are then used for production of silver nanoparticles. The antimicrobial, preliminary cytotoxicity studies and MTT assay were carried out with the biogenic nanoparticles. The accurate component which silver nanoparticles utilize to bring about antimicrobial impact is not unmistakably known and is a wrangled about subject. There are however different speculations on the activity of silver nanoparticles on organisms to bring about the microbicidal impact. Silver nanoparticles can grapple to the bacterial cellwall and in this way enter it, consequently bringing about auxiliary changes in the cell membrane like the porousness of the cell layer and passing of the cell. There is development of "pits" on the cell surface, and there is gathering of the nanoparticles on the cell surface. The silver Nanoparticles also interact with respiratory enzymes.[19]

Principle and extreme objective of this work is to study in point of interest the procedure variables like concentration of silver nitrate , response pH, temperature and time of response on the bio mimetic amalgamation of silver Nanoparticles. In the current study we evaluated the antimicrobial and cytotoxicity patterns of Bio- AgNP. The reports are exceptionally encouraging.Further *in vitro* and *in vivo* studies are required to establish the medicinal exploitation of the particles.

MATERIALS AND METHODS

All chemicals used in the experiment were collected from sigma (Sigma, Bangalore, India) and Medias from Himedia (Himedia Labs, India).

Isolation of Endophytic Fungi

The healthy leaf samples of *Withania somnifera* (L.) (ashwaganda) were collected and identified with the snippets of Herbarium of Department of Botany at St. Berchmans college, Changanacherry (RHK 6350). The samples were then cleansed in the running tap water for 5- 8 min (4 – 5 times). Then leaf samples were cut in to small fragments of 1 cm length and surface sterilized with 70% ethanol for 2 minutes proceeded by soaking in Sodium hypochlorite (NaOCl 1-13%) for 3-10 minutes and washed in 70% alcohol for 1 minute. The segments were then washed 3 – 4 times in sterile distilled water and dried by using sterile blotting paper. The dried pieces (4-5) were placed aseptically on sterile Potato Dextrose Agar plate (PDA) plates augmented with streptomycin (100 µg ml^{-1}) and incubated at room temperature for 3 – 5 days [20]. The potency of surface sterilization was established by spread plating the water sample used for last wash on the PDA plates and offered as control. The fungi that rise out from the portions were regularly isolated and identified by dispensing the hyphal tips to fresh PDA plates without antibiotics.

Screening of Silver Nanoparticles Produced by Isolated Endophytic Fungi

The acquired fungal isolates were cultured in liquid Potato Dextrose medium and incubated at room temperature in rotary shaker for 5 days. The biomass obtained was accumulated by a sterile sieve and cleansed with sterile distilled water to remove components of medium. It was then centrifuged at 7000 g at 4°C for 10 minutes and the pellets were collected. Generally 20 g of biomass were carried in contact with 100 ml sterile double distilled water for 72 hours at room temperature in rotary shaker. Later the cell filtrate was obtained by filtering with sterile sieve and then centrifuged at 7000 g at 4° C for 10 minutes to remove cell debris. The collected supernatant was treated with 1mM silver nitrate ($AgNO_3$) and incubated at dark condition. It was then observed for colour change. $AgNO_3$without supernatant was served as control.

Identification of Fungi

The potential fungal isolates which showed colour change from colourless to yellowish brown were selected for further studies. The preliminary identification was carried out by Lacto Phenol Cotton Blue staining. Cultural characteristics were also studied. The Fungi was then identified by molecular characterization using 18 S rRNA amplification. Pure culture pellet was used for genomic DNA extraction. D1/D2 region was amplified by PCR from fungal genomic DNA using universal primers DF- 5'-ACCCGCTGAACTTAAGC-3' and DR - 5'-GGTCCGTGTTTCAAGACGG-3'.The amplified DNA was sequenced at Sci Genomics (Sci Genomics, Cochin). The sequences were submitted to NCBI gene bank for accession code.

Purification of Silver Nanoparticles

Suspension containing Bio- AgNP was centrifuged at 5000 g at 4° C for 10 minutes. The pellets were collected and resuspended in Milli Q water and again centrifuged at 5000 g at 4° C for 10 minutes. The cleansing step was replicated three to four times to remove any impurities present in it. Ultrasound sonication was carried out with the pellets and then again subjected to centrifugation. Finally the purified nanoparticles were weighed and put back into suspension with Milli Q water. This purified nanoparticles were stored at 4° C for further characterization [21].

Characterization of Biosynthetic Silver Nanoparticles

Formation of Bio- AgNP was proved by UV Vis spectrophotometer (Hitachi U5100) in the visible region from 350 – 800 nm. The morphology, size, shape and distribution of nanoparticles were studied by Scanning Electron Microscope (SEM). SEM analysis of dried samples was performed by mounting nanoparticles on specimen stubs with double adhesive tape and coated with platinum in a sputter coater and examined under JEOL 6390 SEM JSM at 10 kV. Transmission Electron Microscope (TEM) analysis of drop-coated solution of nanoparticles onto the carbon-coated copper grid was carried out for better evaluation of the size and shape of the nanoparticles using JOEL-JEM-2011. The possible bimolecules associated with the synthesis and stabilization of nanoparticles was listed using Fourier Transform Infrared (FTIR) Spectroscopy analysis (Nicolet, USA) in 400 to 4000 ranges. Atomic force microscopy (AFM (APER-A-100 SPM, Italy)) was carried out for the sample to know their size and three dimensional

Fig. 1. Biosynthesis of silver nanoparticles by *Fusarium solani* showing colour change from colourless to brown colour

Fig. 2. Cultivation of *Fusarium solani* on the PDA and Microscopic observation showing micro and macro conidia by Lacto phenol cotton blue staining.

arrangements. The purified powdered Bio- AgNP were analyzed using powder X-Ray Diffractometer (BRUKER, Germany) in a range of 20° -80° 2 Θ angles.

Optimization of Silver Nanoparticle Production

Prominent yield of nanoparticles were obtained by optimizing the conditions of production. Optimization studies were carried out for the crucial parameters such as temperature, pH, concentration of silver nitrate, and days of incubation. At first concentration of silver nitrate was optimized and that value was used in addition for optimization of pH. Then both optimized values were used for temperature optimization and then respectively for days of incubation. Optical density was measured at 430 nm in UV – spectral analysis for evaluating the production rate.

Antimicrobial Activities of Silver Nanoparticles

To analyse the antibacterial activity of the synthesized Bio- AgNP, antimicrobial sensitivity assay was performed by agar well diffusion in Muller Hinton Agar (MHA) plates[22]. The pathogens such as *Escherichia coli* (MTCC723) *Pseudomonas sp* (MTCC 121), *salmonella typhi* (MTCC 734), *Klebsiella pneumoniae* (MTCC 109) and *Staphylococcus aureus* (MTCC 734) were used for the assay. MTCC strains of bacterial pathogens were grown in Muller Hinton broth at 37 ^{0}C for 18–24 h. A confluent lawn of bacterial growth was prepared with the pathogens and wells were cut using sterile cork borer. In to the wells AgNP was applied at concentration of 50, 100, 150 µg/ml and incubated at 37 ° C. The plates were examined for appearance of zone of inhibition and the diameter of zones were measured in millimetres and compared with standard antibiotic Ciprofloxacin (Hi Media, India).

Cytotoxicity Assay-RBC Lysis Test for AgNp

In vitro erythrocyte lysis test was performed as a rudimentary cytotoxicity assay. The analysis was by quantifying the release of haemoglobin due to membrane disruption by the AgNP. Fresh blood drawn from a healthy donor was collected in anticoagulant solution (EDTA) and was centrifuged at 1000g for 10 minutes at 4° C. The washed RBC was diluted (50%) with 20mM PBS buffer. To study haemolysis RBC suspension was mixed with diverse concentrations of Bio-AgNP (50 to 150µg/ml) and incubated at 37 ° C for 1 hour [23]. It was again centrifuged at 1500 g after incubation and supernatant was interpreted by measuring the absorbance at 576 nm in UV – visible spectroscopy. The percentage haemolysis was calculated by equation (1);

$$\text{Percentage of hemolysis}(\%) = \left(\frac{Abs(T) - Abs(C)}{Abs(100) - Abs(c)}\right) \times 100 \quad (1)$$

Where Abs (T) is the absorbance of test and Abs(c) is absorbance for control (normal saline). Complete lysis in 1% triton X 100 act as Abs (100%).

Determination of in vitro Cytotoxic Effect of Bio-AgNp on Cultured L929 Cellsl929 (Fibroblast Cells)

Cell lines were purchased from NCCS Pune was maintained in Dulbecco's modified eagles media (Hi Media) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37^{0}C in 5 % CO_2 in a humidified atmosphere in a CO_2

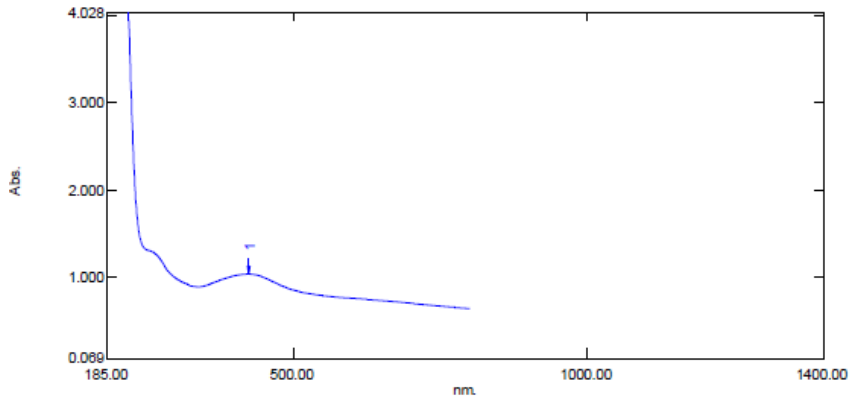


Fig. 3. UV – Vis spectroscopy of biosynthetic silver nanoparticles showing peak at 424 nm

incubator (NBS, EPPENDORF, GERMANY). The cells were trypsinized (500µl of 0.025% Trypsin in PBS/0.5 mM EDTA solution (Himedia)) for 2 minutes and passaged to T flasks in complete aseptic conditions. Extract was added to grown cells at a final concentration of 6.25, 12.5,25,50 and 100 µg/ml from a stock of 1mg/ml and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation.

MTT Assay [24]

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent Dimethyl sulfoxide (Himedia) and the released, solubilised formazan product was measured at 540nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells as in Equation (2).

The cells was washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT -5mg/ml dissolved in PBS). It was then incubated at 37^{0}C for 3 hours. MTT was removed by washing and centrifuged at top speed for 2 minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a microplate reader (ELISA SCAN, ERBA).

$$\%\ of\ viability = \left(\frac{OD\ of\ test}{OD\ of\ control}\right) \times 100 \qquad (2)$$

RESULTS

Primary Screening for Biosynthesis of Silver Nanoparticles

From the sterilized leaf segments of *Withania somnifera* fungal colonies were grown out after 5 days of incubation. The fungal colonies were then subcultured on PDA without antibiotics and maintained as pure cultures. The fungal strains were screened for their efficiency for the extracelllar production of Bio- AgNP. Cell free supernatant of one strain showed production of silver nanoparticles with a visible colour change from colour less to dark yellowish brown when exposed to silver nitrate solution Fig. 1. The potential isolate was selected and used for further studies.

Identification of Fungal Isolate

The isolate grown on PDA showed white cottony smooth appearance and on reverse it showed a pigmentation of dull white colour to pale yellow [25]. Hyaline crescent shaped macro conidia and small micro conidia were observed on Lacto Phenol Cotton Blue staining. Hence the isolate was preliminarily identified as *Fusarium sp.* Fig. 2 and was later identified by molecular characterization with 18S r RNA. Amplification of 18 S r RNA genes was carried out and sequenced. The accession code: KJ 193849 was obtained for the sequence from NCBI gene bank and the organism was identified as *Fusarium solani*.

Characterization of Biosynthetic Silver Nanoparticles

The colour change from colourless to brownish yellow perceived was later established by UV – Vis spectroscopy analysis. To analyse the particles they

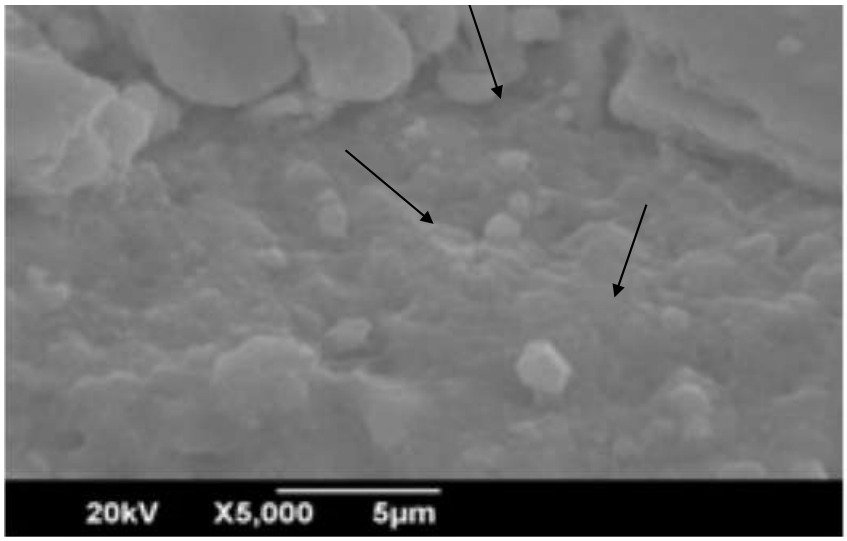


Fig. 4. SEM micrograph showing well dispersed spherical Silver Nanoparticles

Table 1 The particle size of Bio – Ag NP calculated by Debye – Scherrer formula

2Θ of intense peak	hkl (planes of silver)	Full width at half maximum [FWHM(β)] of intense peaks radians	Size of the particles (nm)	d spacing
38.2527	(111)	0.48	16.98	2.350
46.3376	(200)	0.384	51	1.957

were purified and were stored at 4° C for further studies. The purified nanoparticles were weighed and the total yield was calculated as 62 mg/100ml.

The initial characterization was carried out with UV –Vis spectroscopy analysis. The formation of distinguishing brown colour is due to Surface Plasmon Resonance (SPR). A distinctive strong absorption peak was observed at 424 nm Fig. 3. It indicates the reduction of silver nitrate to silver nanoparticles. In the blank solution there was no characteristic absorption spectral response.

SEM analysis of Bio - AgNP on drop coated films was recorded (Fig. 4). It confirmed the formation of AgNP. SEM micrographs showed well dispersed spherical nanoparticles with size ranging from10 to 50 nm. SEM images indicate the uniform size, distribution and spherical shape of stabilized nonagglomerated nanoparticles.

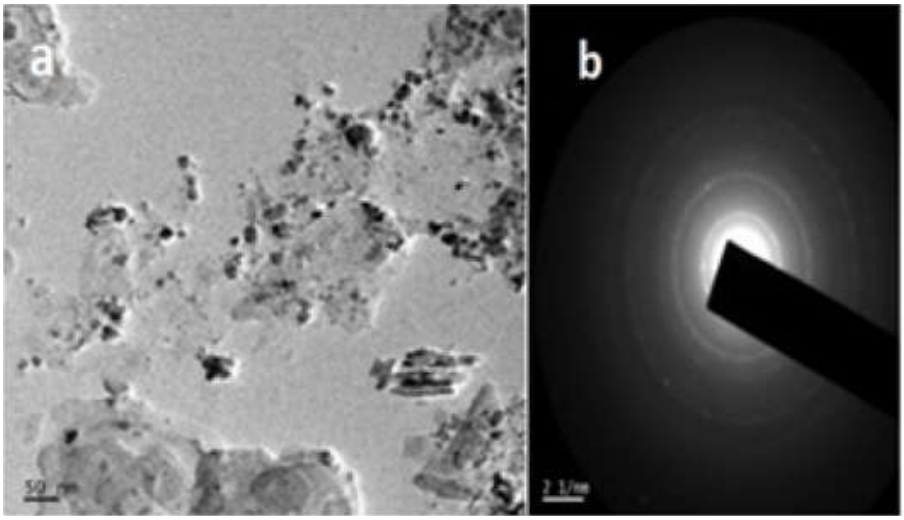
TEM micrographs Figs. 5(a) and 5(b) showed that the Bio - AgNP are spherical in nature with measurements ranging from 10 nm to 50 nm. The crystalline nature of AgNP was also analyzed and confirmed by TEM using Selected Area Electron Diffraction analysis (SAED). The SAED pattern showed well defined diffraction lattice in the form of rings in the silver region which confirmed the nanocrystalline nature of the particles.

AFM surface topology analysis of bio fabricated silver nanoparticles thin film on mica sheet showed more insight into the size, shape and distribution of nanoparticles. Spherical particles of varying size below 50 nm Fig. 6 were observed on AFM analysis. It also showed uniform distribution without agglomeration.

Additional studies were carried out using XRD to validate the crystalline nature of nanoparticles. The pattern displayed the powerful peaks in a 2Θ value ranging from 20 to 80. X- Ray diffractometer showed characteristic peaks at 32.367^0, 38.252^0 and 46.337^0 Fig. 7.

These values corresponds with the plane of silver 111(38.252^0) and 200 (46.337^0) when compared with the standard powder diffraction card of Joint Committee on Powder Diffraction Standards (JCPDS), silver file No. 04–0783. Two strong Bragg reflections were found which correspond to pure silver metal. The particles size can be calculated by using the Equation (3):

$$d = \left[\frac{k\lambda}{\beta cos\theta}\right] \quad (3)$$



Figs. 5(a) and 5(b) TEM micrograph showing (a) dispersed spherical particles (b) SAED pattern with well defined diffraction lattice in the form of rings in the silver region

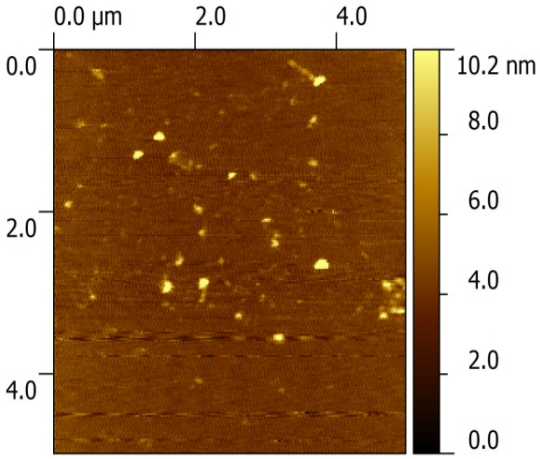


Fig. 6. Atomic force microscopy images of Bio - AgNP synthesized by *Fusarium solani* showing spherical nanoparticles without agglomeration

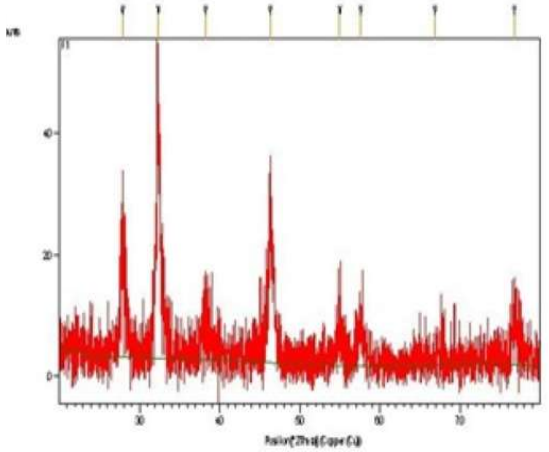


Fig. 7. XRD patterns of biosynthetic silver nanoparticles at 2 Θ showing prominent peak at 38 and 46

The calculated particle sizes (Table 1) for the particles are 16.98 and 51 nm respectively.

FTIR analysis of silver nanoparticle samples was carried out in order to obtain information about functional groups involved in reduction of silver nitrate Fig. 8. Compared to silver nitrate certain new prominent peaks at band width 2918.30, 1340.55, 1195.87, 1159.22, 1134.14, 943.19 and 916.19 were observed in treated sample, which represents C-H stretch of alkenes, N-O symmetric stretch of nitro compounds, C-H wag of alkyl halides, C-N stretch of aliphatic amines and O-H bend of carboxylic acid respectively. These representative peaks contributed by the supernatant manifest the possible role of biomolecules mainly proteins in reduction of silver nitrate to nanoparticles.

Optimization of Silver Nanoparticle Production

Intensification of AgNP production can be obtained by optimizing the parameters such as pH, temperature, days of incubation and concentration of silver nitrate for the reaction Fig. 9. Optimized production of silver nanoparticles was obtained at pH 6, temperature of 45 ^{0} C, concentration of silver nitrate 3mM and five days of incubation. In the current study there was no colour change or nanoparticle production at acidic pH.

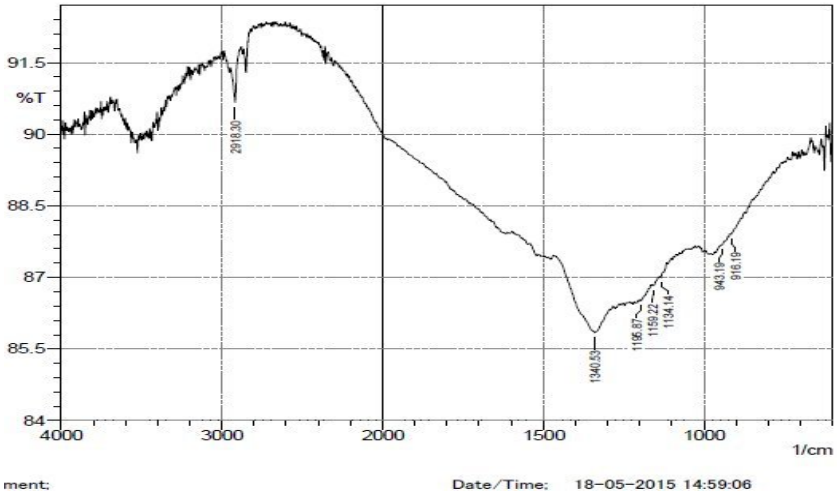


Fig. 8. FTIR patterns of Bio-AgNP

Table 2 Dose dependent antibacterial activity of Bio AgNP

Concentration of AgNp (µg/ml)	Zone of inhibition in mm(± SD)*				
	Escherichia coli	*Klebsiella pneumoniae*	*Salmonella typhi*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
50	18.1 ±0.1	9.1 ±0.05	13.0 ±0.10	18.2 ±0.10	13.0 ±0.12
100	18.6 ±0.05	15.2 ±0.25	18.3 ±0.58	18.3 ±0.58	16.6 ±0.04
150	20.1 ±0.10	18.0 ±0.05	20.5 ±0.50	18.5 ±0.50	20.3 ±0.58
Ciprofloxacin	20.1 ±0.10	15.0 ±0.05	18.5 ±0.50	18.1 ±0.50	20.0 ±0.58

* Values are represented as mean ± SD values.

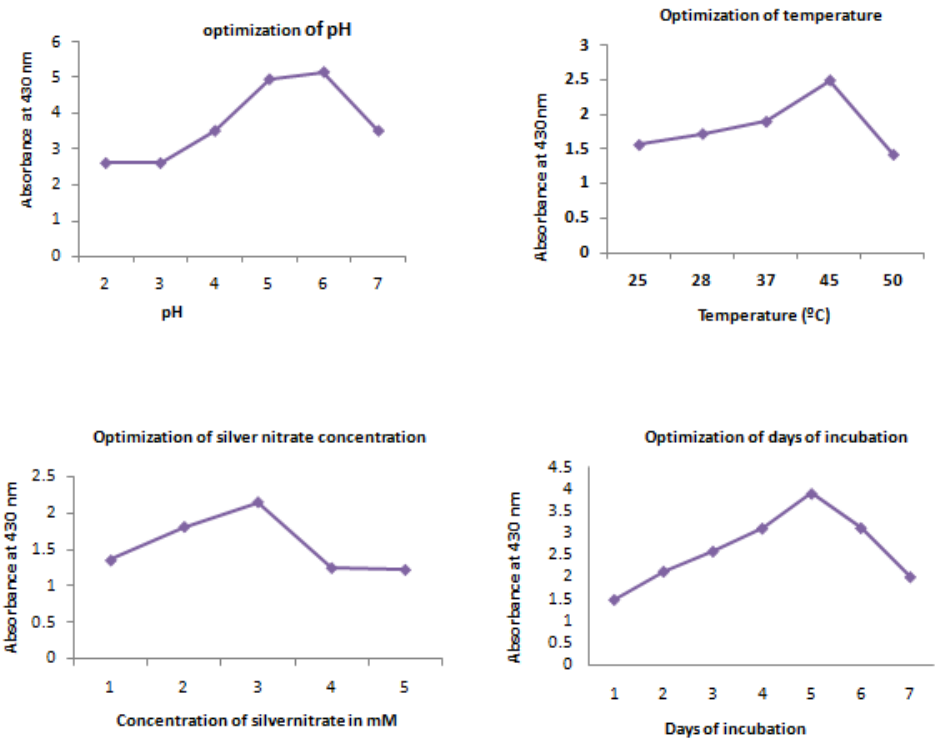


Fig. 9. Optimization of physical parameters pH, temperature, Concentration of silver nitrate and days of incubation for biosynthesis of silver nanoparticles

Table 3. Dose dependent RBC lysis assay of Bio – AgNP

Concentration of Bio - AgNP (µg/ml)	Absorbance at 576 nm ± SD*	% of hemolysis
25	0.135±0.001	3.794
50	0.139±0.0005	3.906
75	0.143±0.0005	4.019
100	0.178±0.0015	5.002
125	0.459±0.0011	12.92
150	1.139±0.001	32.012

* Values are represented as mean ± SD values.

Table 4. MTT assay of Bio – AgNP on cultured L929 fibroblast cells

Concentration of Bio - AgNP (µg/ml)	% of cell viability
25	89.524 ± 0.005
50	69.244 ± 0.001
100	68.136 ± 0.002
200	53.191 ± 0.001
300	42.052 ± 0.0011

* Values are represented as mean ± SD values.

Antibacterial Activity of Biogenic Silver Nanoparticles

Antibacterial effects of Bio - AgNP were carried out in opposition to certain pathogens such as *Escherichia coli* (MTCC 723), *Pseudomonas aeruginosa* (MTCC 121), *Staphylococcus aureus* (MTCC 96), *Salmonella typhi* (MTCC 734) and *Klebsiella pneumoniae* (MTCC 109) against standard antibiotic Ciprofloxacin. Table 2 shows the antimicrobial activity of AgNP against *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae, Staphylococcus aureus* and *Escherichia coli*.

RBC Lysis Test for Preliminary Toxicity Study

By RBC lysis test the inherent toxicity of synthesized AgNP was measured Table 3. The trial particles persuade a smaller amount of toxicity compared to positive control (1% triton X 100).The percentage of haemolysis increases with increase in concentration. The percentage of toxicity is 3.794, 3.906, 4.014, 5.002, 12.92 and 32.012 for 25, 50, 75, 100, 125 and 150 µg ml^{-1} respectively. This toxicity study uncovers that Bio – AgNP are exceptionally sheltered to use as remedial operators in nanomedicine.

MTT Assay

The result of cytotoxicity tests revealed dose dependent cytoxicity at all the concentrations tested. The Trypan blue dye exclusion method revealed a dose dependent decrease in viability of cells Table 4. Bio – AgNp showed antimicrobial activity at a concentration of 50 µg ml^{-1} and at the concentration MTT assay showed only 69.244 % cytotoxicity. This result clearly emphasized that the Bio- AgNP are very secure to use as a healing factor in the cancer treatment and it is very bright as the issue of antibiotic resistance is a major problem today.

DISCUSSION

In the present study extracellular production of silver nanoparticles were carried out victoriously. This offers a rapid synthesis of silver nanoparticles in an eco-friendly way and make them as a successful alternative for more popular chemical and physical methods of synthesis. The produced particles showed the characteristic sharp peaks at 424 nm in UV- Vis spectroscopy analysis due to its Surface Plasmon Resonance. It was reported that for ellipsoidal particles there are two peaks and for spherical particle the peak is between 420 and 425nm [26]. This confirms that the obtained particles are spherical in shape. Previous studies[27] reported the production of silver nanoparticles using endophytic *Pencillium sp.* and the absorbance peak for spherical nanoparticles was at 425 nm. In their studies Birla *et al.* denoted that the shape and size of silver nanoparticles are also related to their absorbance peak [28]. The test sample also showed certain peaks at 250 and 280 nm in UV- Vis spectroscopy analysis which emphasise the presence of certain proteins in the culture supernatant and can be suggested as the mechanism behind the synthesis of nanoparticles[29].

SEM is a widely used method for evaluation of morphology and size of nanoparticles [30]. Korbekandi *et al.* [31]studied that silver nanoparticles were almost spherical in shape and appear as single (25- 50 nm) or in aggregates (100 nm). The well dispersed pattern of spherical nanoparticles shows the stabilization of biosynthetic nanoparticles without any external source of capping agents and stabilizing agents as in the chemical process of synthesis. These studies are in agreement with the findings of Selvi and Sivakumar [32]. TEM analysis confirmed the spherical shape and well dispersed pattern of nanoparticles. It also emphasized that the size of particles are between 10 nm and 50 nm. Rahi and Parmar [33] in their studies revealed the highly variable nature and size of nanoparticles by TEM analysis. Parashar *et al.* [34] reported the exploitation of SAED for the analysis of AgNP to reveal its crystalline nature. SAED lattice of silver nanoparticles verified the crystalline nature of biosynthetic nanoparticles. AFM is a very good technique for measuring surface morphology and fine structure of nanoparticles [35]. Sangappa and Thiagarajan [9] observed that AFM topology is very helpful in revealing the exact size and shape of silver nanoparticles. An AFM study of biosynthetic nanoparticles supports the data obtained by SEM and TEM analysis. All these studies revealed that the present nanoparticles are stable and spherical in shape without any agglomeration. The stability of nanoparticles is due to the bioactive components present in the fungal filtrates.

X-Ray Diffraction analysis of synthesized Bio - AgNP also states the crystalline nature [36]. The size of nanoparticles was calculated using full width at half maximum (FWHM) values with the Debye - Scherer equation and it is evidenced that the particles have size ranging from 16.5 nm to 51 nm. The obtained nanoparticles are very promising because of the fine size that in turn increases the catalytic activity[37; 38]. These results are compared with the standard values and were confirmed as silver nanoparticles. These peaks coincide with the values of Joint Committee on Powder Diffraction standards (file no.04-0783) and are showing reflections of pure silver. Thus XRD studies substantiate the resultant particles as silver nanoparticles.

The FTIR spectroscopy analysis grants the idea about capping and stabilizing agents present in the fungal filtrate. There are several reports in the literature [39] that has located absorption peaks at about 3843.68 cm-1 (–NH group of amines), 3597.73 cm-1 (-OH group of phenols), 2080.65 cm-1 (aromatic –CH stretching), 1631.66 cm-1 (-NHCO of amide) and 767.16 cm-1 (C-Cl) in nanoparticles contributed by *Pencillium sp.* cell filtrate and explains the role of proteins in silver nanoparticle synthesis. Ranjitham *et al.* (2013) has also reported the presence of certain proteins in the synthesis of silver nanoparticles. In the present study also there are certain characteristic peaks which emphasis the role of bio molecules in reduction of silver nitrate to silver nanoparticles.

In order to expand the production of silver nanoparticles we studied the effect of various factors such as pH, temperature, concentration of silver nitrate and days of incubation. Chandrashekhar *et al.* studied the effect of various physico chemical factors on production of nanoparticles. In their study the optimum pH is alkaline for highest yield of particle and at low or acidic pH no production was observed. Sangappa and Thiagarajan (2012) reported the relation of temperature and concentration of silver nitrate in production of silver nanoparticles. Highest temperature gives maximum production but as the temperature rises above 50° C there may be chances for agglomeration of particles. The concentration of silver nitrate is a crucial factor for AgNP synthesis. Korbekandi *et al.* (2013) studied the effect of concentration of silver nitrate on nanoparticles production by *Fusarium oxysporum* and the results are coinciding with our data [31].

Antimicrobial activities against the tested pathogens are promising and these results are well documented in previous studies[40]. Oves *et al.* (2013) in his studies suggest that gram negative and gram positive bacterial strains were most susceptible to Ag NP**.** A high antimicrobial activity was exhibited by the AgNP of their small size which is below 50 nm. The AgNP contributed by *Fusarium solani* isolated from the medicinal plant *Withania somnifera* is very much stabilized because of the bioactive compounds produced by the fungi which act as capping agent. These findings are in connection with the FTIR analysis. Baker and Satish [41] suggest that functional metabolites of endophytes get tailored with the nanoparticles during biosynthesis and it act as potent antibacterial

complex. Endophytes are reported to secrete a large variety of antimicrobial bioactive compounds and so when they are employed for synthesis of nanoparticles, these compounds may get capped with the nanoparticles and can be more effective compared to bioactive compound or nanoparticle alone.

The results of RBC lysis are in agreement with Oves *et al.* (2013) and in his studies it is revealed that these toxic levels are safe in use as drugs. Hence the findings of the present study are reliable. The leading problem while using the silver nanoparticle for treatment is their toxicity but here the preliminary toxicity patterns were promising because they do not impart inherent toxicity. When observed together the bactericidal patterns and cytotoxicity patterns are more reliable in the current scenario of treatment. Further animal studies are required for the well establishment of the result. Compared to other methods of nanoparticle synthesis this method is more dependable as the synthesised particles are very stable with consistently good antimicrobial activity and flat cytotoxicity.

The cytotoxicity pattern of Bio- AgNP on L929 cells was evaluated by MTT assay and it showed differential cytotoxicity according to concentration of Bio- AgNP. It showed promising results with low toxicity towards the L929 cells. These results are in agreement with Oves *et al.* (2013). In the studies, it is reveled the cytotoxicity of Bio- AgNP against splenocytes. The Low inherent toxicity pattern is very promising in the area of anticancer and antimicrobial therapeutics.

Silver nanoparticles produced by endophytic fungus *Fusarium solani* with an intermediate size range of 10 to 50 nm offer guaranteed horizon as the fungal manufacturers for their green- collar and imperishable production. The antimicrobial activity gives assurance to therapeutic field as antibiotic resistance is an on-going problem. The particles are easily dispersible in water and are biocompatible in nature. The output is extremely controlled and reproducible in nature. Hence the biosynthesis is more advantageous compared to chemical or physical methods.

The data of the present study guides to the determination that the endophytic fungi isolated from the medicinal plant *Withania somnifera* reduces silver nitrate to silver nanoparticles. The formation of nanoparticles was identified initially by UV - Vis spectroscopy with the peak at 424 nm. The particles are again characterized by XRD, SEM, TEM and AFM analysis. These results showed the size, crystalline nature and spherical shape of the molecules. From XRD the size was calculated as 19.5 nm. The FTIR analysis showed certain prominent peak, which shows the presence of amines and carboxylic acids in the test sample. The production of silver nanoparticles was intensified with optimized parameters. The toxicity study by RBC lysis and MTT assay showed that AgNP are safe to utilize as a drug. In the safe concentration itself, they showed antibacterial effect against *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Escherichia coli*. The reduced toxicity of AgNP in the current survey is due to the biosynthesis of AgNP by endophyte isolated from the medicinal plant *Withania somnifera*. This is really promising and displayed prospect of synthesizing remedial AgNP from endophytic fungi *Fusarium solani* with a manageable and reasonable way. Further subjects are needed to establish the cytotoxicity studies, antitumor activities and the preparation of bio conjugates for drug delivery.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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