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Article

In vitro biological activities of silver nanoparticles synthesized from *Scedosporium* sp. isolated from soil

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One of the important fields in nanotechnology is the development of an environment friendly method for the synthesis of nanoparticles. Many approaches show that microorganisms are the most reliable tools for biosynthesis of nanoparticles compared to physical and chemical methods. In our study, fungi have been exploited for extracellular production of metal nanoparticles. It was observed that in *Scedosporium*, silver ions are reduced to silver nanoparticles, which was confirmed by UV-visible spectrophotometry and AFM. Optimization studies showed that as the concentration of $AgNO_3$ used for synthesis increased, particles' size also increased. Size of the particles at different concentrations of $AgNO_3$ was observed to be 79-107 nm with particles being ellipsoidal to spherical in shape. Silver nanoparticles synthesized from 2.0 mM silver nitrate, showed maximum antimicrobial activity compared to all antibiotics tested including synergistic effects. *In vitro* cytotoxicity of silver nanoparticles against MCF 7 and PC 3 showed that as the concentration of silver nanoparticles increased, a decrease in the percentage cell viability was observed with IC_{50} values being 60.09 and 57.43 µg/ml respectively. Therefore, through this study, it could be said that extracellular synthesis of silver nanoparticles from *Scedosporium* was simple, ecofriendly, proving excellent antimicrobial and anticancer agents.

Keywords: Atomic force microscopy. *In vitro* cytotoxicity. *Scedosporium* sp. Silver nanoparticles. UV-visible spectroscopy.

INTRODUCTION

Nanoparticles are considered as fundamental building blocks of nanotechnology. Synthesis of nanoparticles is thus a major component of research in nanoscience (Vahabi, Mansoori, Karimi, 2011). Different types of nanoparticles are being synthesized in a number of ways, including the physical and chemical methods which pose a few disadvantages which are expensive, labor intensive, and are potentially hazardous to the environment and living organisms. Thereby, great interest is being created in researchers for the development of environmentally friendly method for the synthesis of nanoparticles. Nanoparticles synthesized from different fungal species have been reviewed by Siddiqi and Husen (2016). The antimicrobial potential of silver nanoparticles synthesized form different fungi has been explored by determining the size of the inhibition zone. A similar review was reported by Keat *et al.* (2015), which also dealt with the biosynthesis of silver nanoparticles. Several methods followed for the synthesis has been elaborated, determining its antimicrobial activity, followed by several applications of silver nanoparticles. Silver nanoparticles, a major type of nanoparticles are widely used as an antiseptic agent due to its low toxicity against mammalian tissues (Sunderamoothi *et al.*, 2009). These particles are known to be effective antimicrobial agents against a wide range of pathogenic microorganisms (Vigneshwaran *et al.,* 2007). They have also been used in anti bacterial clothing, as coating for medical devices, and for burn ointments due to their mutation resistant anti microbial activity (Mohammadian, Shojaosadati, Rezaee, 2007). Silver nanoparticles are widely used in the fields of biolabelling, biosensors, and filters (Bhainsa, D'Souza, 2006). Apart from these, they are used in spectrally selected coatings for solar energybsorption, as optical receptors, as intercalation agents for electrical batteries, and also as catalysts in chemical reactions (Kalimuthu *et al.,* 2008). The application of silver nanoparticles

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synthesized from *Candida albicans* isolated from dental caries has been reported by Saminathan (2015). The nanoparticles were observed to show potent antimicrobial activity against several multidrug resistant human pathogens. Biological syntheses have been developed as an alternative for physical and chemical synthesis, which are cost-effective and environmentally friendly (Sadowski *et al.,* 2008). Many organisms, unicellular and multicellular have been used for the production of nanoparticles either intracellularly or extracellularly (Varshney *et al.,* 2009). Organisms such as bacteria, algae, fungi and yeast have been exploited for the production of silver nanoparticles (Gajbhiye *et al.,* 2009). The use of fungi has said to be more advantageous than bacteria and yeast. Some of the advantages include tolerance towards metals, uptake of metals intracellularly and maximum binding capacity (Dias *et al.,* 2002). The production of fungi is also easy, thus producing large biomass for the synthesis of nanoparticles (Chen, Lin, Ma, 2003). Of the methods followed for the biosynthesis of nanoparticles, extracellular method is widely carried out. Intracellular synthesis allows the better control of size and shape, but harvesting the product and recovery are cumbersome and expensive. In case of extracellular synthesis, the enzyme nitrate reductase which is responsible for the production of silver nanoparticles is released into the medium where the Ag^+ ions are reduced to silver nanoparticles (Fayaz *et al.,* 2010). Thus, the extracellular approach is the most effectively adapted method for the biosynthesis of silver nanoparticles from fungi.

The aim of this study was to green synthesize (biological synthesis) silver nanoparticles and to assess its cytotoxic ability on cancer cells. Therefore, this study presents the biological synthesis of silver nanoparticles from the fungus, *Scedosporium*, and the formation of nanoparticles was monitored through spectroscopic and microscopic characterization, followed by *in vitro* cytotoxicity study.

MATERIAL AND METHODS

Biomass production

For the production of fungal biomass, the culturing was carried out in liquid broth composed of KH_2PO_4 (7.0g/L), K_2HPO_4 (2.0g/L), $MgSO_4$. $7H_2O$ (0.1g/L), $(NH_4)_2SO_4$ (1.0g/L) yeast extract, (0.6g/L) and glucose (10g/L) aerobically. The flask was incubated for about 11 days at a temperature of 29ºC. After incubation, the biomass produced was then harvested. This was then subjected by immense washing with Milli Q water.

Synthesis of silver nanoparticles

About 10 g of the biomass, after washing was inoculated into 100 mL Milli Q water. This was incubated for about 72 h at 29ºC. The biomass, after incubation was filtered using Whatmann No. 1 filter paper. The pH of the filtrate was observed to be 6.79. To 100 mL of filtrate, silver nitrate was added at a concentration of 1 mM. The reaction was carried out in dark. A control was maintained along with the test sample, where it contained only the cell filtrate without silver nitrate. The formation of silver nanoparticles was monitored at a time interval of 2 h using a UV-visible Spectrophotometer, where wavelength scan was carried out from 200-600 nm.

Optimization of synthesis of nanoparticles

The cell filtrate containing the silver nanoparticles were further studied. The influence of substrate on the formation of silver nanoparticles was studied by changing the substrate concentration (0.5, 1.0, 1.5, 2.0 mM) at different time intervals. The absorbance was measured at wavelength of 430 nm using a UV- visible spectrophotometer.

Characterization of silver nanoparticles

The cell filtrate having the silver nanoparticles were characterized using Atomic Force Microscopy (AFM) to determine the size and shape of nanoparticles synthesized at various concentrations of $AgNO_3$. The morphology of the synthesized nanoparticles was studied by AFM and was carried out to determine the particle height and volume. The sample analysis was carried out by preparing a smear with the pellet obtained from the filtrate containing the nanoparticles. This was then subjected to analysis through AFM.

Antimicrobial activity

The synergistic effect of antibiotics was studied using gram positive bacteria for the synthesized nanoparticles. Standard antibiotic discs were used. Standard antibiotics and antibiotics along with different concentrations of AgNPs (0.5, 1.0, 1.5 and 2.0 mM) were plated onto the Muller-Hinton agar plates. Overnight cultures of the test samples were used and incubated at 37ºC for about 24 hrs. The zone of inhibition was measured.

In vitro cytotoxicity studies

The *in vitro* cytotoxicity studies of AgNPs were

studied through MTT assay. Briefly, the cancer cells (MCF 7 and PC3, procured from National Center for Cell Science (NCCS), Pune, India) were cultured in DMEM and RPMI medium respectively containing 10% FBS in a culture flask at 37 ºC and 5% carbon dioxide. On reaching confluency, the cells were seeded in a 96 well plate at a cell concentration of approximately 5 x 10^3 cells/well and incubated for 24 h. After incubation, the medium was removed and washed with PBS. To the control plates, serum free media was added and to the experimental plates, different concentrations of AgNPs were added (12.5, 25, 50, 75 and 100 µg/mL). After 24 h of incubation, the medium was removed and the cells were washed twice with PBS to remove any traces of drug and fresh medium was added. To each well, 5 µL of MTT solution was added having a concentration of 10 mg/mL and further incubated for 4 h. The medium was removed and the cells were dissolved in 100 µL DMSO. The absorbance was measured at 570 nm in an ELISA micro plate reader. The data are shown as percentage viable cells of the test group in comparison with the control group.

Statistical analysis

All experiments were carried out in triplicates and the results are expressed as mean±S.D, with $p=0.05$.

RESULTS

The extracellular synthesis of silver nanoparticles from the fungus, *Scedosporium*, which was followed in this study and the flasks containing the filtrate in the presence and absence of substrate that taken as a control were presented in the Figure 1. As observed visually, the color of the filtrate was changed remarkably with increasing concentration of $AgNO_3$, indicating the formation of silver nanoparticles. For further confirmation of this finding, UV- Visible spectral analysis was recorded between 200-600 nm at a regular time intervals, and an increase in spectra with a peak around 420-430 nm might be responsible for the production of silver nanoparticles due to its strong surface Plasmon resonance (Figure 2).

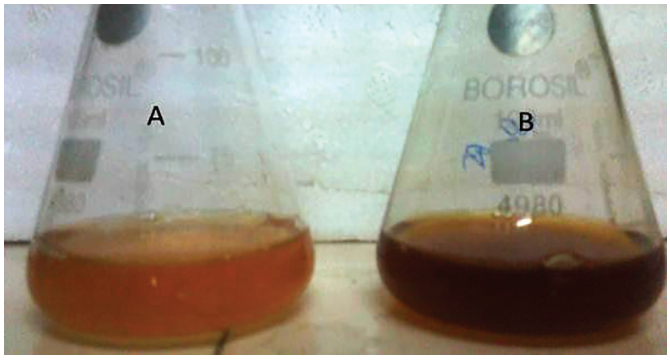

FIGURE 1 - Flasks showing only the cell filtrate (A) and cell filtrate with $AgNO_3$ (B). Change in color in flask B shows the formation of silver nanoparticles.

Remarkably, it was clearly demonstrated that the production of nano-particles increased with increasing concentration of $AgNO_3$, and the maximum amount was found to be produced at 2 mM concentration (Figures 3 and 4). The size of the produced silver nano-particles as determined by AFM and same as color intensity the highest size of the nanoparticles was observed to be 107 nm at 2 mM concentration, the maximum concentration used for analysis (Figure 5).

A study was carried out to determine the antibacterial activity of the silver nanoparticles synthesized extracellularly. The zone of inhibition of the antibiotics and the antibiotics along with the nanoparticles were measured. The results pertaining to the different concentrations of $AgNO_3$ with the standard antibiotics are tabulated as in

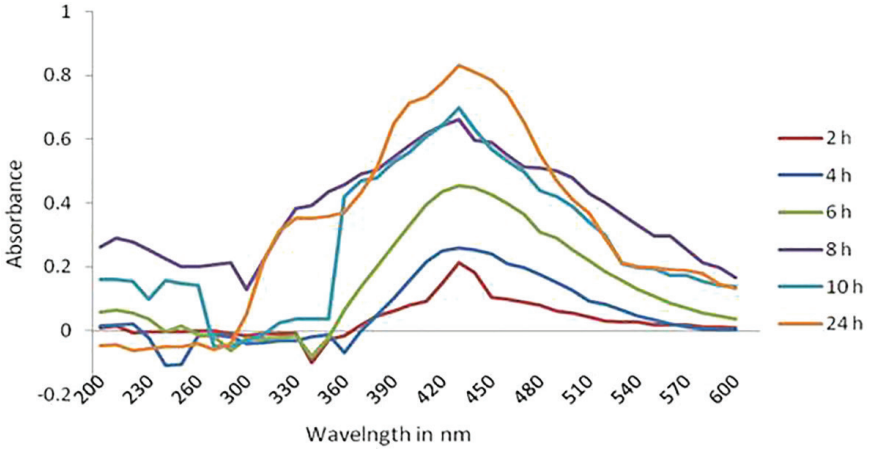

FIGURE 2 - UV spectrophotometric measurements at 2, 4, 6, 8, 10 and 24 h of incubation of cell filtrate with $AgNO_3$.

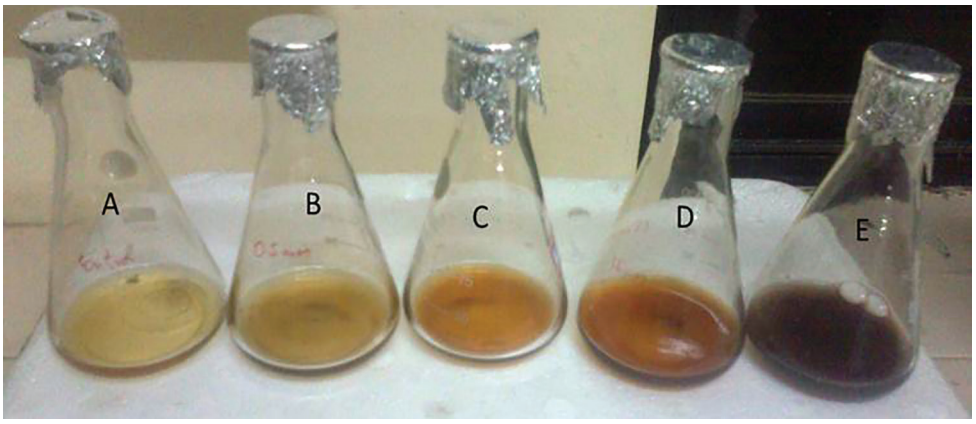

FIGURE 3 - Flasks showing cell filtrate with varying substrate concentration with control (A), 0.5 mM $AgNO_3$ (B), 1 mM $AgNO_3$ (C), 1.5 mM $AgNO_3$ (D) and 2 mM $AgNO_3$ (E).

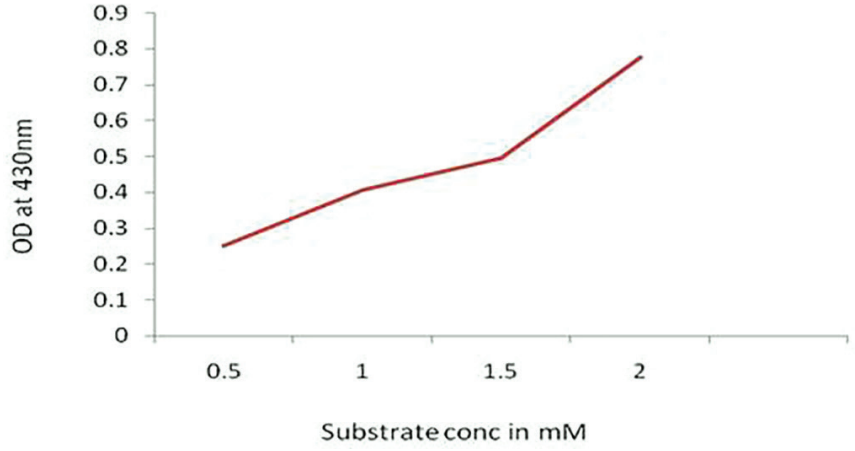

FIGURE 4 - Effect of substrate concentration on $AgNO_3$ measured at 430 nm in a UV-vis sepectrophotometer.

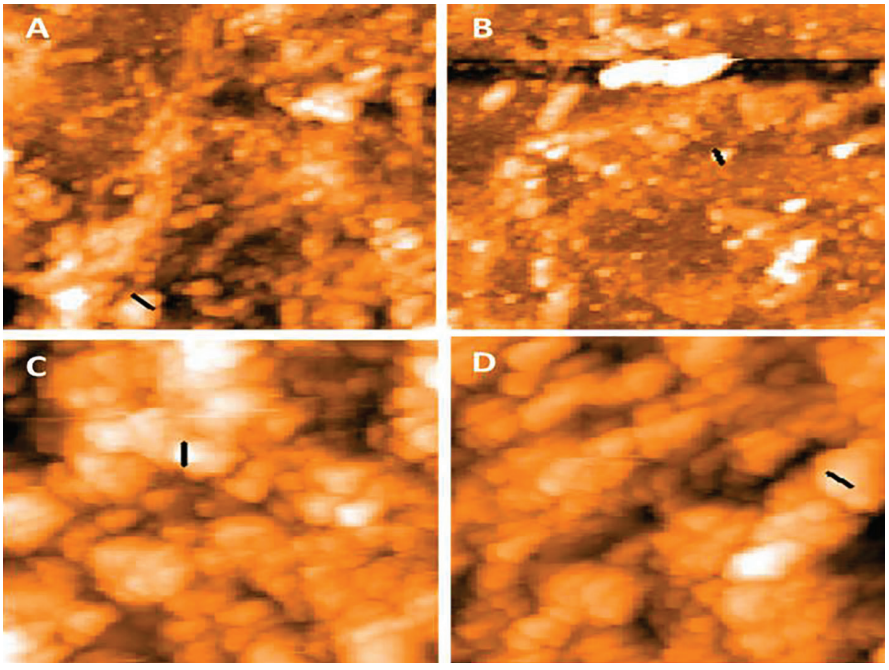

FIGURE 5 - Size of nanoparticles at different concentrations of $AgNO_3$. AgNPs of size 79 nm at a concentration of 0.5 mM (A), 88 nm at a concentration of 1 mM (B), 102 nm at a concentration of 1.5 mM (C) and 107 nm at a concentration of 2 mM (D).

Table I (Figure 6). The zone of inhibition for different antibiotics at varying concentrations of $AgNO_3$ against the gram positive bacteria was determined. Bacitracin was shown to have the maximum zone of inhibition against the test organism at all concentrations of $AgNO_3$, followed by activity of vancomycin. The percentage of fold increase of antibiotics and the synergistic effect of antibiotics with AgNPs were calculated and plotted as in Figure 7.

In vitro cytotoxicity studies of different concentrations of AgNPs against MCF 7 and PC 3 showed a concentration dependent decrease in the cell viability. It was observed that as the concentration of AgNPs increased, a decrease in the percentage viable cells was observed (Figure 8). Similar results were observed for both, MCF 7 and PC 3. The IC_{50} values were observed to be 60.09 and 57.43 µg/ml for MCF 7 and PC 3 respectively.

DISCUSSION

The extracellular synthesis of silver nanoparticle from the fungus, *Scedosporium* was carried out in this study. Figure 1 shows the two flasks containing the cell filtrate of *Scedosporium* with $AgNO_3$ and without the silver ions. It was observed visually that there was a change in colour from pale yellow to dark yellowish brown after about 24 hrs of incubation. The formation of yellowish brown colour serves as an indication for the formation of colloidal nanoparticles. The colour development was observed due to the excitation of surface plasmon vibrations in the silver nanoparticles (Ahmad *et al.,* 2003; Duran *et al.,* 2005; Kholoud *et al.,* 2010). Thus, the production of silver nanoparticles is characterized by intense brown colour. It was also observed that as the

TABLE I - Zone of inhibition for different antibiotics (ampicillin, vancomycin, and bacitracin) against Gram positive bacteria (*Staphylococcus aureus*)

	Ampicillin (10 µg/disc)				
	Amp (zone in mm)	**Amp +0.5 mM $AgNO_3$**	**Amp +1.0 mM $AgNO_3$**	**Amp +1.5 mM $AgNO_3$**	**Amp +2.0 mM $AgNO_3$**
S. aureaus	15±0.56	17±1.25	18±0.98	19±0.12	19±0.98
	Vancomycin (10 µg/disc)				
	Van (zone in mm)	**Van +0.5 mM $AgNO_3$**	**Van +1.0 mM $AgNO_3$**	**Van +1.5 mM $AgNO_3$**	**Van+2.0 mM $AgNO_3$**
S. aureaus	13±0.24	17±0.76	15±1.54	14±2.05	18±1.30
	Bacitracin (8 units/disc)				
	Bac (zone in mm)	**Bac +0.5 mM $AgNO_3$**	**Bac +1.0 mM $AgNO_3$**	**Bac +1.5 mM $AgNO_3$**	**Bac +2.0 mM $AgNO_3$**
S. aureaus	19±0.85	20±0.17	21±1.12	21±0.76	22±0.89

All experiments were performed in triplicates and the results are expressed as mean ± S.D. (n=3)

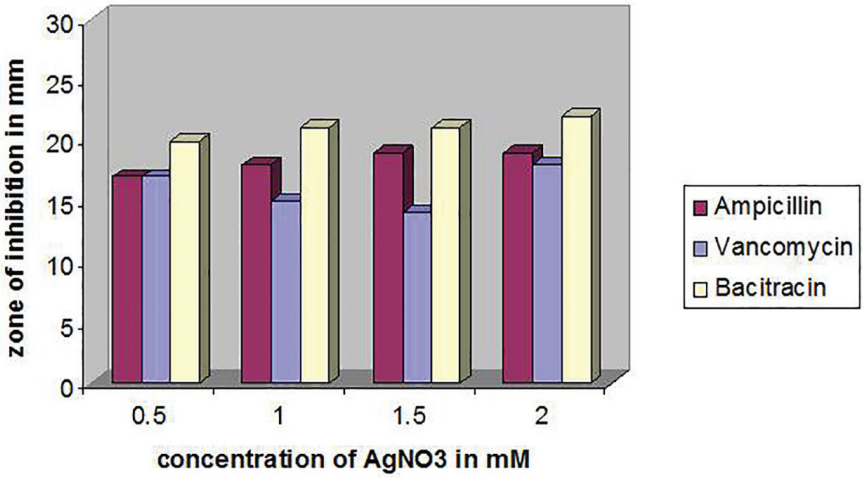

FIGURE 6 - Zone of inhibition (in mm) for different antibiotics (ampicillin, vancomycin and bacitracin) at different concentrations of $AgNO_3$ (0.5, 1, 1.5 and 2 mM).

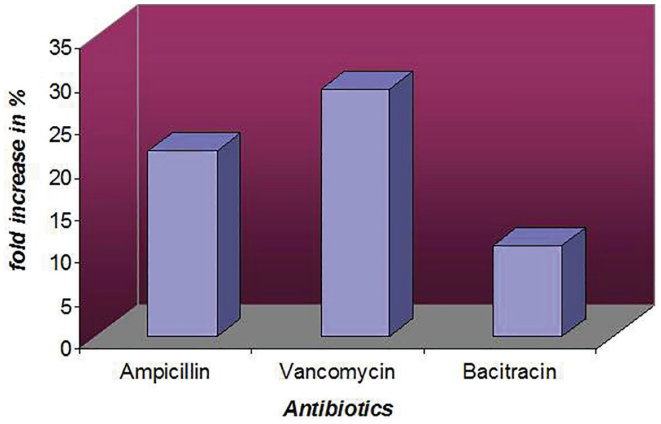

FIGURE 7 - Percentage fold increase in antibacterial activity of antibiotics and antibiotics with AgNPs.

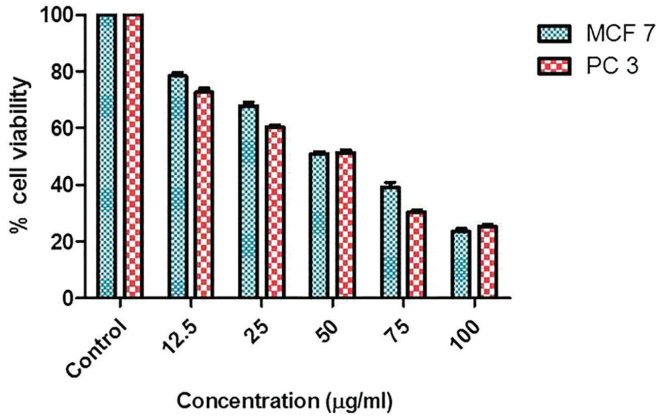

FIGURE 8 - In vitro cytotoxicity assay of different concentrations of AgNPs against MCF 7 and PC 3 cells, expressed in percentage viable cells.

incubation time increases, the intensity of color in flask containing cell filtrate and $AgNO_3$ also increased. As shown in previous literatures, these observations indicate that the enzyme responsible for the formation of silver nanoparticles is being released into the cell filtrate (Das *et al.,* 2014; Kushwaha *et al*., 2015). Therefore, on addition of the substrate, the enzyme brings about the reduction of silver ions to silver nanoparticles, thereby achieving extracellular synthesis of nanoparticles. The production of silver nanoparticles in the medium was monitored through UV-visible spectroscopy.

UV-visible spectroscopy is one of the conformational techniques used to determine the formation of silver nanoparticles. Through the literatures it has been observed that an increase in the absorbance at 420-430 nm indicates the formation of silver nanoparticles due to the surface plasmon resonance of the metal (Basavaraja *et al.,* 2008). In our study, the UV recordings were analysed and the formation of silver nanoparticles were determined by a peak at the same wavelength. The silver nanoparticles produced were optimized by varying the substrate concentrations from 0.5-2.0 mM. From this study it was inferred that the maximum production of nanoparticles was observed at a concentration of 2.0 mM. Figure 3 indicates the gradual increase in the intensity of colour from pale yellow to dark brown. The morphology of the synthesized nanoparticles was studied by AFM. AFM studies were carried out for samples with varying substrate concentrations. It was concluded that as the substrate concentration, *i.e.,* $AgNO_3$ concentration increased, the size of the particles also increased which were observed to range from 79-107 nm for the substrate concentrations from 0.5-2.0 mM. The results obtained from this study showed that the fungus, *Scedosporium* is capable of producing silver nanoparticles of different sizes depending upon the concentration of silver nitrate. One of the applications of silver nanoparticles is that, these have been widely used as an effective antimicrobial agent against a wide range of pathogens.

The antimicrobial potential of silver nanoparticles synthesized from endophytic fungi was studied against *S. aureus*. The results obtained showed an inhibition zone of 14.0 mm, which was reported to show a potent antimicrobial activity (Netala *et al.,* 2015). A similar study was carried out to determine the antimicrobial property of silver nanoparticles synthesized from *Trichoderma viride*. This study also showed an excellent antimicrobial activity of silver nanoparticles synthesized from the fungi (Elgorban *et al.,* 2016). Our study was carried out to determine the antimicrobial activity of the silver nanoparticles synthesized. This was performed by using different standard antibiotics. The nanoparticles were used along with the antibiotics to determine the synergistic effects of AgNps and the antibiotics and were tested against a Gram positive bacterium. Promising results were obtained where the nanoparticles showed activity against the test organism. Maximum activity was observed when the nanoparticles were used along with the antibiotic, Bacitracin, showing its synergistic effect. The activity was tested for nanoparticles at different substrate concentrations against the same test organism and was observed that as the concentration of substrate increased, the antimicrobial activity also increased in case of nanoparticles used with antibiotics bacitracin and ampicillin. Therefore, from our study it was concluded that as the size of the nanoparticles increased from 0.5-2.0 mM, the antimicrobial activity also was observed to increase against the test organism, also showing a high synergistic effect with bacitracin and ampicillin. Nanoparticles with bacitracin showed to have maximum activity at all concentrations of the substrate.

AgNPs, along with their potent antibacterial activity are also known to possess anticancer activity. In our study, the anticancer activity of AgNPs against breast cancer cells (MCF 7) and prostate cancer cells (PC 3) were studied. It was observed that the AgNPs synthesized from *Sceidosporium* sps. isolated from soil showed good anticancer activity. As the concentration of AgNPs increased, a decrease in the cell viability was observed. This showed that AgNPs along with its antimicrobial nature could also be used as a potent anticancer agent for targeting breast and prostate cancers. Therefore, through this study, it could be said that extracellular synthesis of silver nanoparticles from *Scedosporium* was simple, ecofriendly, proving excellent antimicrobial and anticancer agents.

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