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RESEARCH PAPER

Green Synthesis of Silver Nanoparticles Using the Mushroom Fungus *Schizophyllum commune* and Its Biomedical Applications

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Abstract Silver nanoparticles, which are small metallic colloidal particles, ranging 1 ~ 100 nm in size, have several nano biotechnological applications in medicine, manufacturing and engineering industries. Fungus-mediated synthesis of silver nanoparticles is an ecofriendly, green process. Further, extracellular enzymes and proteins elaborated by fungi are involved in the synthesis of the silver nanoparticle, which makes the downstream processing relatively simpler. In the present investigation, *Schizophyllum commune*, a mushroom fungus, was tested for its ability to synthesize extracellular as well as intracellular silver nanoparticles. When the fungus was challenged with 1 mM silver nitrate, a change in colour of the broth and the mycelium was observed, indicative of extracellular and intracellular synthesis of silver nanoparticles. The presence of silver nanoparticles was confirmed by studying its Surface Plasmon Resonance absorption band in the visible wavelength. FTIR spectrum analysis of the silver nanoparticles indicated the presence of biomolecules in association with the reduction of silver ions. Scanning Electron Microscopic analysis of the silver nanoparticles revealed the nanorange dimensions of both the extracellular and the intracellular silver nanoparticles. Analysis of biological activities of the silver nanoparticles disclosed their significant antibacterial activity against *Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae* and *Pseudomonas fluorescens*. Additionally, the silver nanoparticles inhibited the growth of the dermatophytic fungal pathogens *viz. Trichophyton simii*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* significantly. Anticancer activity of silver nanoparticles, assayed through MTT cytotoxicity assay, uncovered that 27.2 and 64% mortality could be obtained in Human Epidermoid Larynx Carcinoma (HEP -2) cell lines at concentrations between 10 and 100 μg/mL, respectively. The results obtained indicate that *Schizophyllum commune* is capable of synthesizing silver nanoparticles in shaken broth cultures (120 rpm) at 25 ± 2°C and pH 7.

Keywords: silver nanoparticles, *Schizophyllum commune*, antibacterial activity, anti-dermatophytic fungal activity, anti-cancer activity

1. Introduction

Nanotechnology is the application of science to control matter at the molecular level and it involves a wide variety of technologies such as material science to biotechnology where it is useful in the field of diagnostics and new delivery systems. It searches for particles in the dimension smaller than 100 nm and works on design, synthesis and manipulation of their structures and applications. The potential benefits of nanomaterials for human health and environment are reported by several researchers [1-4]. There is a search for nanoparticles produced by microorganisms and isolation, characterization, uptake and subcellular distribution of several nano materials have been brought to light. However, only a few nanoproducts are currently in use for medical purposes such as disease diagnosis and drug delivery. The development of better experimental procedures for the synthesis of nanoparticles of different chemical composition, sizes, shapes and controlled polydispersity is

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vital in this field [5-7].

Rapid and green synthetic method using extracts of living organisms have been designed for AgNPs synthesis. Microbe mediated synthesis of nanoparticles has been proved to be an efficient method in comparison to chemical methods due to the large amount of capital and energy involved in latter method. The use of hazardous chemicals such as hydrazine eliminates the method from being an eco-friendly one. Additionally, nano-crystalline silver colloids produced by such aqua-chemical routes exhibit aggregation with time, thereby compromising with the size factor upon storage. These de-merits necessitated the search for a novel "greener synthesis of nanoparticles", based on environment friendly methods involving the synthesis at biological pH [8-10]. Silver nanoparticles exhibit unique unusual physicochemical properties and biological activities that extend its application in antibacterial, antifungal, anti-viral, anti-inflammatory therapy as well as in cancer therapy [11-14].

Fungi are highly diverse and versatile organisms adapted to all kinds of environment. They are present in moist soil, compost, dead organic substances as saprophytes or in other plants and animals as parasites or as symbionts. Fungi are found to be the choice organisms for use in nanoparticle synthesis due to their mycelial nature of their plant body and their ability to grow on the surface of the liquid culture media. Saprophytic fungal species excrete large number of extracellular enzymes and proteins which act as the reducing and capping agents that mediate the extracellular nanoparticle synthesis making the downstream process of isolation and purification easier [15].

The present study reports the extracellular and the intracellular production of silver nanoparticles by *Schizophyllum commune,* a mushroom fungus when grown in the presence of silver nitrate in the culture medium. The antibacterial, the antidermatophytic, and the cell line inhibition activity of extracellular silver nanoparticles have been assessed in the bacteria *Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae* and *Pseudomonas sp*, the fungi *Trichophyton simii*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* and cell lines Human Epidermoid Larynx Carcinoma (HEP -2) Cell Line respectively.

2. Materials and Methods

2.1. Culture maintenance

The pure culture of *Schizophyllum commune* was procured from Department of Plant Sciences in the University of Madras, Chennai, India. The culture was maintained on malt agar slants (malt extract 30 g/L, glucose 10 g/L, agar 20 g/L and pH 7), stored at 4ºC and sub-cultured every month.

2.2. Biosynthesis of silver nanoparticles

All the chemicals used were of analytical grade. The media components like glucose, malt extract, agar and silver nitrate were obtained from Hi-Media chemicals in Mumbai (India). *Schizophyllum commune* was grown aerobically in Erlenmayer flasks (250 mL) in liquid media (5 g/L malt extract and 10 g/L glucose) inoculated with a single mycelial agar plug from 6 days old culture of *Schizophyllum commune*. The flasks were maintained as shake cultures for 120 h on a rotary shaker at 120 rpm at 25 ± 2°C. Silver nitrate was added to a set of flasks for final concentration of 1 mM. The flasks were completely sealed with black paper and kept on a rotatory shaker and the same conditions were maintained. 1 mM silver nitrate was added to 20 mL of cell-free culture filtrate of 6 days-old *Schizophyllum commune* in Erlenmeyer flasks (100 mL), for testing the extracellular synthesis of silver nanoparticles. The flasks were completely sealed with black paper and maintained on a rotary shaker at 120 rpm. The control set of flasks had *Schizophyllum commune* grown in the absence of silver nitrate. The change in colour of the mycelium and the cell-free culture filtrate was noted after twelve hours [15].

For microscopic observation, a small tuft of mycelium grown in the medium containing silver nitrate was aseptically transferred to a clean glass slide; spread with sterile tweezers, and observed under 400× magnification of a compound microscope (Labomed, India).

2.3. Isolation of extracellular silver nanoparticles

The cell free filtrate containing silver nanoparticles was filtered through a 0.22 μm Millipore filter to remove residual cellular debris. The samples were centrifuged at 16,000 g for 30 min at 20 ± 2°C to separate the silver nanoparticles [10].

2.4. SEM analysis

Scanning Electron Microscopic (SEM) analysis was done using Hitachi S-4500 SEM machine. Thin films of the silver nanoparticles from cell free filtrate were prepared on a carbon coated copper grid by spotting a drop of the silver nanoparticle suspension on the grid. Excess solution was removed gently, using a blotting paper and then the film on the SEM grid was allowed to dry by keeping it under a mercury lamp for 5 min. SEM analysis of mycelium was performed by mounting the mycelium on specimen stubs with double adhesive tape and coated with platinum in a sputter coater and examined under Hitachi S-4500 SEM machine, 10 KV at 10,000 × and subsequently at 30,000 × magnification.

2.5. Characterization of silver nanoparticles

2.5.1. UV-VIS spectrum

The change in colour of the broth was observed as an indicator for silver nanoparticle synthesis. The reduction of silver ions was monitored by measuring the UV-VIS spectrum of 1 mL of the sample from the culture filtrate at 24 h time interval and the absorbance was recorded, using a UV-VIS spectrophotometer.

2.6. FTIR analysis

The isolated silver nanoparticles were dried, ground with infrared grade KBr (1: 10) and pressed into disks under vacuum using a Spectra Lab Pelletiser for FTIR spectrum. The spectrum (4,000 ~ 400/cm) at a resolution of 4/cm was recorded in a Burker 72 FTIR spectrophotometer [9].

2.7. PAGE analysis of proteins associated with silver nanoparticles

Analysis of silver nanoparticles bound proteins was performed by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). Isolated silver nanoparticles were washed thoroughly with deionised water and redissolved in minimum volume of deionised water. Next, the solution of nanoparticles was sonicated for a brief period, followed by heating at 60ºC for five minutes. The resulting solution of silver nanoparticles was then centrifuged at 20,000 rpm (37,118 × *g*) for 15 min. The supernatant was collected and stored at -20ºC for further experiments. 20 µL of supernatant was loaded on 12% gel for protein analysis. To another well, the proteins isolated from cell free filtrate of 6 days old *S. commune* culture was loaded [12].

2.8. Antimicrobial activity

Antimicrobial activity of the extracellular silver nanoparticles, synthesized by *S. commune*, was tested by agar well-diffusion method. Bacteria including *Pseudomonas fluorescens (P. fluorescens)*, *Klebsiella pneumoniae (K. pneumoniae)*, *Bacillus subtilis (B. subtilis)* and *Escherichia coli (E. coli)* and pathogenic dermatophytic fungi *Trichophyton simii*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* were used as hosts to test the antimicrobial activity. Pure cultures of bacterial and fungal pathogens were subcultured on nutrient agar and potato dextrose agar (PDA), respectively. Wells of 10 mm diameter were made on nutrient agar and PDA plates by gel puncture. Overnight cultures of each bacterial strain were swabbed uniformly onto individual plates, using sterile cotton swabs. Using a micropipette, different concentrations of the nanoparticle suspension were spotted into each well on all plates. After incubation at 37°C for 24 h, the zone of inhibition on the bacterial lawn was measured. To test the antifungal activity, PDA plates were inoculated with 6-days old fungal spore suspension and the plates were treated with silver nanoparticles of various concentrations. The zone of inhibition was measured upon incubation at room temperature for 48 h.

2.9. Anticancer activity of silver nanoparticles on human epidermoid larynx carcinoma (HEP -2) cell line

2.9.1. Cell culture

Human Epidermoid Larynx Carcinoma cell line was obtained from the Department of Biotechnology in Pondicherry University, Pondicherry. Cancerous cells were seeded in T - flask with Dulbecco's Modified Eagle Medium (DMEM). The medium used was adjusted to contain 5 ~ 10 % fetal bovine serum (FBS). The cells were incubated at 37°C in a 5% CO_2 atmosphere. After 24 ~ 48 h incubation period, surface adhered cells were trypsinized for 3 ~ 5 min and centrifugated at 1,400 rpm for 5 min. The cells were counted and distributed in 96 well ELISA plate with 10,000 cells in each well. The plate was incubated for 24 ~ 48 h at 37°C in a 5% CO_2 atmosphere to allow the cells attached to the bottom of the well.

2.10. MTT assay

Different concentrations of silver nanoparticle suspension (10, 25, 50 and 100 µg/mL) in dimethoxy sulphoxide solvent (DMSO) were added to each well in 96 well Elisa plates and the effect of silver nano particles on cell growth was analyzed by optical microscopy after 24 h. Cell viability was evaluated by the MTT colorimetric technique. 100 µL of the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) solution (5 mg/ml in PBS) was added to each well in the 96 well Elisa plate. The plates were incubated for 3 ~ 4 h at 37°C. Metabolically active cells reduce MTT by the action of dehydrogenase enzymes, and generate reducing equivalents such as NADH and NADPH resulting in purple formozan product. 100 µL of isopropanol (95%) was added to the wells for solubilization of the MTT crystals. The plates were placed on a shaker for 15 min for complete solubilization of crystals and then the optical density of the solutions in each well was determined. The quantity of formazan product, as measured by its absorbance at 545 nm, is directly proportional to the number of living cells in culture. Experiments were done in duplicate. The relative cell viability (%) over control wells, containing cell culture medium without silver nanoparticles was calculated by

$$\text{Cell Viablity } \% = \frac{[A]Test}{[A]Control} \times 100$$

Where [A] test is the absorbance of the test sample and [A] control is the absorbance of control sample [16].

3. Results and Discussion

3.1. Biosynthesis of Ag nanoparticles

The cell free culture filtrate *S. commune,* when challenged with 1 mM silver nitrate resulted in the change in colour from pale yellow to brown (Fig. 1A), indicating its potential to synthesize extracellular silver nanoparticles. Colour change was not observed in control broth (without inoculum) when supplemented with 1 mM silver nitrate. Similarly, the mycelium turned brown indicating its potential to reduce silver ions to metallic silver in the cell (Fig. 1B). To clearly observe the color change, mycelium of *S. commune*, grown in the presence or absence of silver nitrate, was observed through light microscopy. Indeed, a brown colored mycelium was observed in cultures supplemented with silver nitrate and not in the control sample (Fig. 2). Together, these observations indicate that silver nanoparticles are potentially synthesized by *S. commune*, both the in the cell and extracellularly when challenged with silver nitrate. Synthesis of silver nanoparticles has been reported in several mold fungal species including *Aspergillus niger, Trichoderma asperellum, Fusarium oxysporum, Fusarium semitectum, Aspergillus fumigatus, Aspergillus flavus, Cladosporium cladosporioides etc*, [12] but studies using mushroom fungi are meagre. Fungi with their "Silver Resistance Machinery" can synthesize silver nanoparticles provided the concentration of the silver ions that does not cross the "threshold limit". The resistance mechanism differs with organisms. Extracts from living organisms have been observed to act both as reducing and capping agents in silver nanoparticles synthesis. Silver nitrate upon being added to the broth dissociates as silver ions. It is followed by reduction of silver ions to metallic silver which later aggregates as silver nanoparticle. This process mediated by a combination of biomolecules found in these extracts such as enzymes/ proteins, amino acids, polysaccharides and vitamins provides the basics for a green synthetic approach, yet it is a chemically complex process [16].

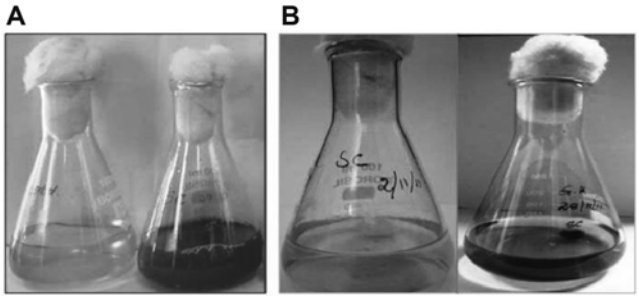

Fig. 1. Production of silver nanoparticles using *Schizophyllum commune.* (A) Extracellular production, (B) mycelium.

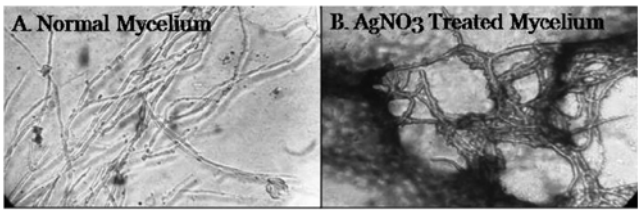

Fig. 2. Microscopic observation of *S. commune* mycelium. (A) Normal mycelium, (B) mycelium grown in the presence of silver nitrate.

3.2. Characterization of *S. coummune* synthesized silver nanoparticles

SEM analysis of silver nanoparticles synthesized using cell free filtrate showed the particles raging from size 51 ~ 93 nm (Fig. 3). SEM analysis of mycelium clearly shows the presence of hyphal cell wall-associated silver nanoparticles. These silver nanoparticles appeared to be synthesized intracellular and are associated with the cell wall. Further, analysis showed that the hyphal wall-associated silver nanoparticles are spherical in shape, with size ranging from 54 to 99 nm (Fig. 4). Together these results indicate that silver nanoparticles are synthesized both extracellularly and intracellularly.

To further characterize the silver nanoparticles, UV-Visible spectrum of the silver nanoparticles synthesized using cell free filtrates of *S. commune* was studied. Shankar *et al.* [9] suggested that the absorption peak at 440 nm was due to the excitation of longitudinal plasmon vibrations of silver

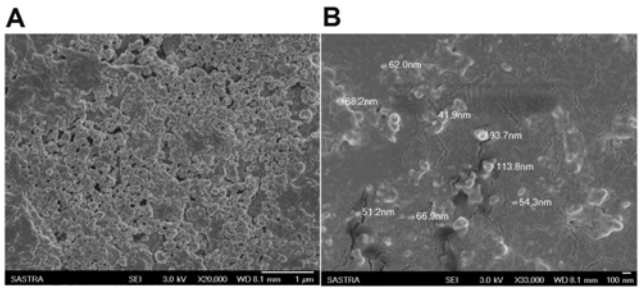

Fig. 3. SEM images of extra cellular silver nanoparticles produced by *S. commune.*

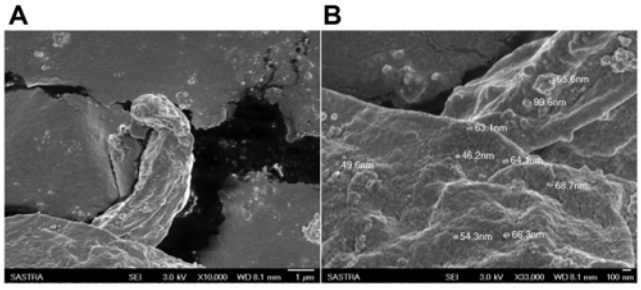

Fig. 4. SEM Images of mycelia bound silver nanoparticles. (A) Mycelium at 10,000 × magnification, (B) 33,000 × magnification.

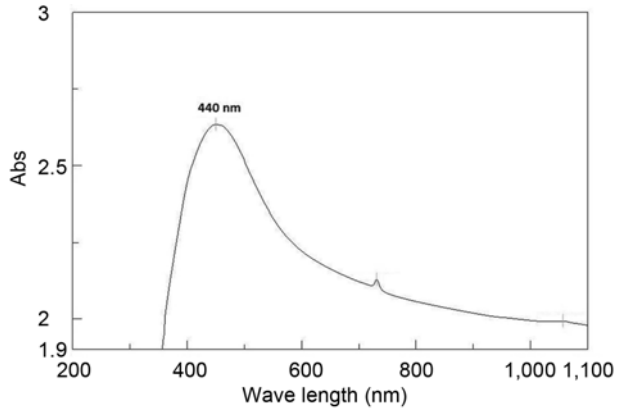

Fig. 5. UV visible spectrum of silver nanoparticles.

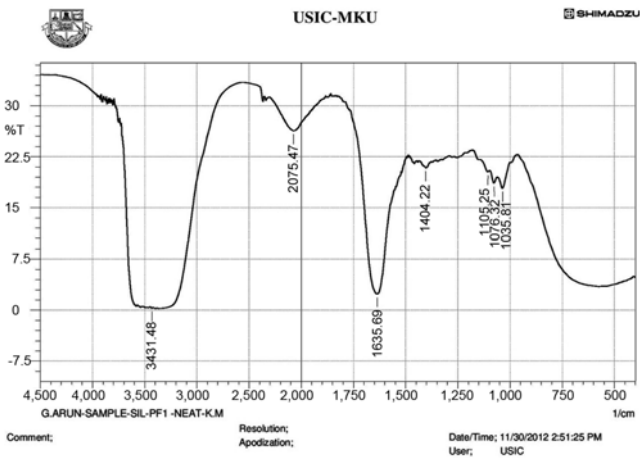

Fig. 6. FTIR spectrum of silver nanoparticles.

nanoparticles. Furthermore, according to Mie's theory [17], only a single SPR band is expected in the absorption spectra of spherical nanoparticles, whereas anisotropic particles could give rise to two or more SPR bands, depending on the shape of the particles. In the present study, a single SPR band at 440 nm in the culture filtrate containing silver nanoparticles (Fig. 5) was observed a characteristic of the silver nanoparticles. The presence of single SPR band at 440 nm is in accordance with the SEM results, indicating that silver nanoparticles are spherical in shape.

FTIR spectrum of the silver nanoparticles showed three absorption peaks, located at about 1020, 1538 and 1638/cm, in the region 1000-1750/cm (Fig. 6). The absorption peak at 1020 cm^1 can be ascribed to the absorption peaks of -C-O-C- or –C-O- bonds [9]. Moreover, the wide absorption spectra, at 1638/cm, may result from stretching of -C=C- bonds [8]. The peak at around 1640/cm indicates the presence of amide I bonds of proteins [10]. The bonds or the functional groups such as -C-O-C-, –C-O- and -C=C- may belong to heterocyclic compounds and the amide I bond may be derived from the proteins which are present

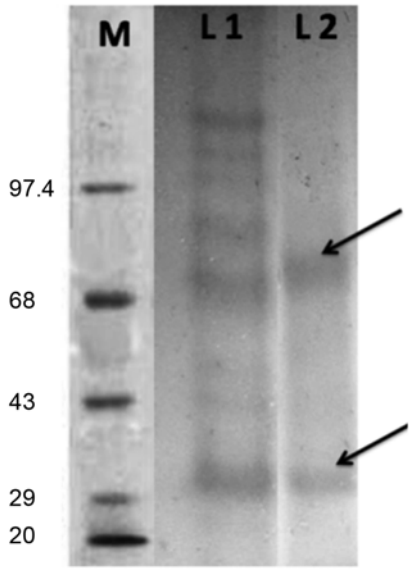

Fig. 7. PAGE analysis of proteins associated with silver nanoparticles. Lane 1: extracellular proteins in cell free culture filtrate of *S. commune.* Lane 2: proteins associated with silver nanoparticles.

in the mycelial extract [9]. This FTIR spectrum supports the presence of proteins and heterocyclic compounds in the culture filtrate, which might have aided in the synthesis of silver nanoparticles.

3.3. PAGE analysis of proteins associated with silver nanoparticles

The PAGE gel Fig. 7, Lane 1 represents the proteins isolated from the cell free filtrate of *Schizophyllum commune* and in the lane 2, the arrow points to two proteins of 30 and 70 KDa which may be associated with the synthesized silver nanoparticles. Several reports suggested the possible role for 95, 57, and 30 kDa proteins in the capping and stabilization of gold nanoparticles and 64, 52, and 29 kDa in the green synthesis of silver nanoparticles using bacterium *Actinobacter spp* [12].

3.4. Antibacterial activity of *S. commune* synthesized silver nanoparticles

S. commune-synthesized silver nanoparticles exhibited antibacterial activity against all the bacteria tested. Among the tested bacteria, the zone of inhibition was the highest for *K. pneumoniae* (3.1 cm). *B. subtilis*, *E. coli* and *P. fluorescens* showed a zone of inhibition of 3, 2.5, and 2.3 cm, respectively. In addition, the inhibition rate was concentration-dependent in the case of *E. coli* and *P. fluorescens,* whereas *K. pneumoniae* and *B. subtilis* were inhibited in a concentration in an independent manner (Fig. 8, Table 1). Together these results indicated that *S. commune* synthesized silver nanoparticles showed a significant antibacterial activity and it is effective against both Gram negative and Gram positive organisms.

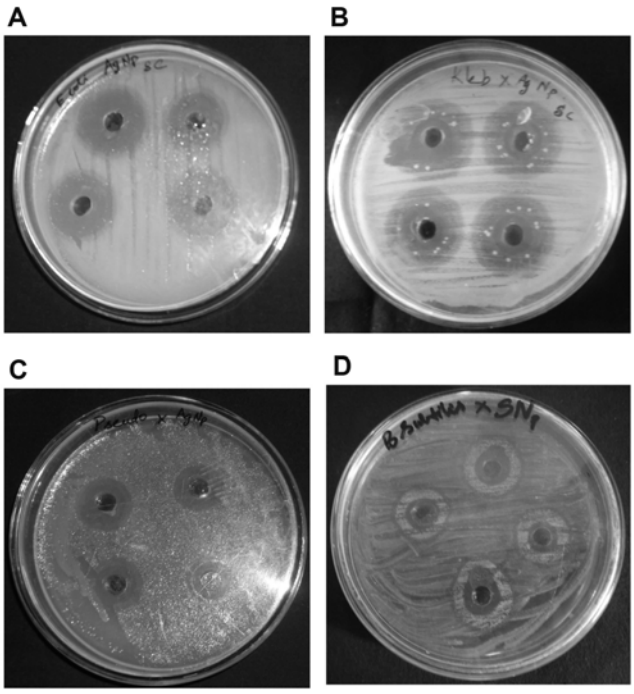

Fig. 8. Antibacterial activity of silver nanoparticles produced by *S. commune* against. (A) *E. coli*, (B) *Klebsiella pneumoniae*, (C) *Pseudomonas fluorescens*, (D) *Bacillus subtilis.*

3.5. Anti-fungal activity of *S. commune* synthesized silver nanoparticles

Similar to its antibacterial activity, the silver nanoparticles also exhibited antifungal activity against all the three tested pathogenic fungi. However, unlike the antibacterial activity, the antifungal activity was found to be concentration-dependent for all the tested fungi. Thus, when the concentration of silver nanoparticles was increased from 25 to 100 µg, the zone of inhibition increased from 12 to 17 mm for *Trichophyton simii,* 13 to 17 mm for *Trichophyton mentagrophytes* and 17 ~ 21 mm for *Trichophyton rubrum*. Commercially available antifungal antibiotic, fluconozole was used as the positive control for the experiment (Fig. 9, Table 2).

Table 1. Antibacterial activity of silver nanoparticles produced by *S. commune*

S. no	Test bacterium	1 mM Ag Np of *S. Commune* Zone of inhibition in cm			
		50 µL	100 µL	150 µL	200 µL
1	*E.coli*	2	2.2	2.2	2.5
2	*Klebsiella pneumonia*	2.5	2.5	3	3.1
3	*Pseudomonas fluorescence*	1.8	1.8	2.0	2.3
4	*Bacillus subtilis*	2.5	2.7	2.9	3

3.6. Cell line inhibition assay

Results of cell line inhibition assay showed that *S. commune* mediated synthesized silver nanoparticles exhibited significant anticancer activity. The inhibitory effect against HEP – 2 cell line showed a direct dose-response relationship with increased inhibition at higher concentrations. Silver nanoparticles demonstrated significant anti-cell proliferation

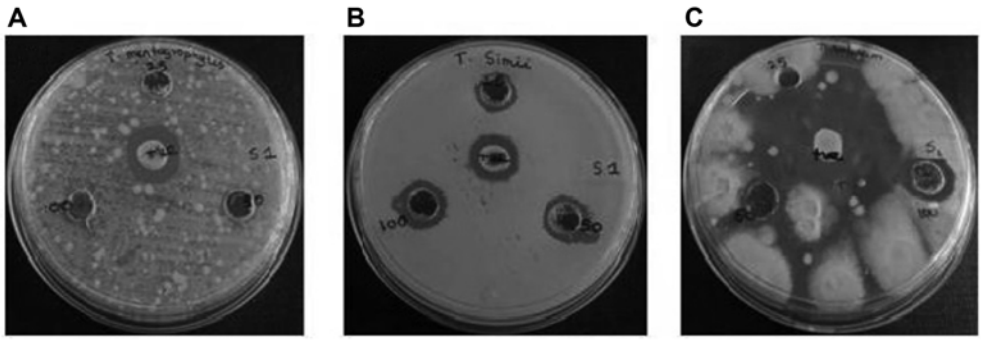

Fig. 9. Anti fungal activity of silver nanoparticles produced by *S. commune* against. (A) *Trichophyton mentagrophytes*, (B) *Trichophyton simii*, (C) *Trichophyton rubrum.*

Table 2. Anti dermatophytic fungal activity of silver nanoparticles

S. no	Test organism	Zone of inhibition in mm silver nanoparticle			Positive control
		25 µg	50 µg	100 µg	Fluconazole 100 µg / well
1	*Trichophyton simii*	12	21	27	22
2	*Trichophyton mentagrophytes*	13	20	26	21
3	*Trichophyton rubrum*	-	17	21	29

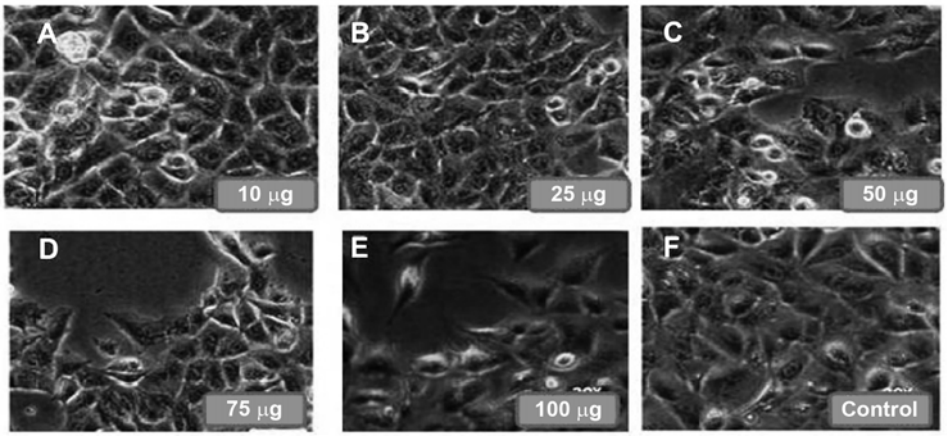

Fig. 10. Anti-cell proliferation activity of silver nanoparticles against Human Epidermoid Larynx Carcinoma (HEP -2) cell line. (A–E) HEP 2 cell line treated with 10, 25, 50, 75, and 100 μg of Ag NP respectively. F control untreated cell line.

Table 3. Anticell proliferation activity of silver nanoparticles against HEP-G2 cell line

S. no	Sample	Concentration (μg)	Cell viability (%)
1	Ag NP	10	70.8
		20	73.6
		50	46.8
		100	35.6
2	Control	-	100

effect on HEP - 2 cell line at concentrations ranging from 50 to 100 μg. The $IC_{50\%}$ concentration of silver nanoparticles, necessary to elicit 50% cell death, was found to be 53 μg/mL. As shown in Fig. 9, at the lowest concentration tested (10 μg/mL), silver nanoparticles were able to inhibit the cell line's growth by less than 30%. In contrast, silver nanoparticles at a concentration 100 μg/mL significantly decreased cell viability by 65% (Fig. 10, Table 3). Similar inhibitory effect of AgNps on cell inhibition had been reported by Moaddab [16] and Foldbjerg [18] in osteoblast cancer and lung cancer cell line, respectively.

4. Conclusion

This study demonstrates that silver nanoparticles can be synthesized in an ecofriendly manner by the mushroom fungus *S. commune*, when grown in the presence of silver nitrate. Silver nanoparticles are synthesized extracellularly through the action of cell-free filtrate as well as intracellularly, where the nanoparticles remained adhered to the hyphal cell wall. A significant antibacterial and antidermatophytic fugal activity of *S. commune*-synthesized silver nanoparticles was observed. Furthermore, the silver nanoparticles also showed significant anti-cell proliferation activity against HEP 2 carcinoma cell line. Taken together, our study emphasizes 'green synthesis' as a novel eco-friendly strategy for the production of nanoparticle with potential biotechnological and biomedical applications using the mushroom fungus *S. commune.*

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