

Fungus-Mediated Synthesis of Silver Nanoparticles and Their Activity against Gram Positive and Gram Negative Bacteria in Combination with Antibiotics

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Abstract

Silver nanoparticles were synthesized biologically from the white rot fungus, *Schizophyllum radiatum* and its synergistic antibacterial activity with ceftriaxone and streptomycin was studied. The nanoparticles were characterized using UV-Vis spectroscopy and Scanning Electron Microscopy (SEM). In the UV-Vis spectrum the peak at 420 nm confirmed the synthesis of silver nanoparticles and SEM studies revealed that the silver nanoparticles have different shapes and sizes. Particle size analysis revealed a range between 10 - 40 nm with a mean diameter of 14.5 nm, suggesting the production of different-sized nanoparticles. The antibacterial activity of the silver nanoparticles conjugated with antibiotics was assessed using agar well diffusion with 2 gram positive bacteria *Staphylococcus aureus*, *Bacillus subtilis*, and 2 gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. The antibacterial activity of ceftriaxone and streptomycin were evaluated with diameter of inhibition zones for the test bacteria. The antibacterial activity of ceftriaxone and streptomycin increased significantly in presence of Ag-NPs. The maximum antibacterial activity was observed when ceftriaxone and streptomycin conjugated with Ag-NPs. The applications of Ag-NPs and antibiotics together improved their efficiency, reduced side effects and also their cost.

Keywords: White rot fungi, silver nanoparticles, SEM, antibiotics, antimicrobial activity

Introduction

Silver nanoparticles have a wide range of advantages in the agriculture, healthcare sectors, staining pigments ceramics and environment. From past times, silver has been famous for its disinfectant property and has been used in various traditional medicines as well as antimicrobial drugs [1]. With the emergence of nanotechnology simultaneously there is an increase of microbial organisms resistant to multiple antibiotics. Microorganisms such as bacteria, yeasts, molds, and viruses, in the living environment are more frequently pathogenic and lead to intense infections in human beings. Nanotechnology provides a good path for having propitious advantages in diagnostics, antimicrobial agents and drug delivery systems [2]. Both unicellular and multicellular organisms readily produce inorganic materials either intra- or extracellularly [3]. Microorganisms such as bacteria, actinomyetes and fungi play a major role in remediation of toxic metals through reduction of metal ions and are considered as potential nanofactories [4]. Fungi may be considered nanofactories and are good sources for the synthesis of metal nanoparticles and ideal sources in the synthesis of metal nanoparticles, because of their capacity to secrete large amounts of enzymes [5]. Compared to other microorganisms in many ways fungal mycelial mesh can withstand flow pressure and agitation and other conditions in bioreactors or other chambers compared to

plant materials and bacteria [6]. Several fungi synthesize silver nanoparticles through extracellularly [7]. Silver and other metal nanoparticles are capable of facilitating microbial activities. Silver has been in use since ancient times in the form of metallic silver, silver nitrate, or silver sulfadiazine for the treatment of burns, wounds, and severe bacterial infections [8]. Nowadays antibiotics have proved to have many side effects but are used as a life saving agents. Silver nanocolloids usage is significant, as several pathogenic bacteria and fungi have developed resistance against various antibiotics. For example, *S. aureus* has exhibited resistance to methicillin, *E. coli* showed resistance to numerous antibiotics like, kanamycin, streptomycin, tetracycline and ampicillin [9,10]. The combination of antibiotics and metal nanoparticles could increase the antibiotics efficacy against resistant pathogens. Nanoparticle-antibiotic conjugates lower the amount of both agents in the concentration, which reduces harmfulness and increases antimicrobial properties. These conjugates were effective against resistant bacteria species due to this conjugation; the concentrations of antibiotics were increased at the place of antibiotic-microbe interaction and thus accelerate the binding between microbes and antibiotics [11]. For this reason the present research was carried out for biological synthesis of nanoparticles and their use in decreasing the concentration of ceftriaxone and streptomycin. The reduction of side effects and cost-effectiveness were also considered.

Materials and methods

Collection, culturing and molecular identification of *Schizophyllum radiatum*

Fungi in the form of fruit bodies collected from the Eturnagaram forest of Warangal, Telangana, India, sterilized with disinfectants and approximately 3×3 mm were placed on an MEA agar medium in petri-dishes. When the mycelium had grown on the medium in the surrounding tissues, the culture was transferred to a fresh Malt agar media in tubes. This was regularly carried out until pure culture was obtained. Molecular-based characterization on ribotyping of 18S rRNA was carried at the Scigenome, Kochin, India and the sequence was deposited at the EMBL database for an accession number.

Bacterial cultures used

Staphylococcus aureus, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* were procured from Microbial Type Collection (MTCC) center, IMTECH, Chandigarh, India.

Antibiotics

Ceftriaxone, streptomycin were procured from National Chemical Laboratory (NCL), Pune, India.

Production of extra cellular silver nanoparticles

Basidiomycetous fungi *Schizophyllum radiatum* was grown in malt glucose broth containing glucose 10 g/L, malt extract 5 g/L. The final pH was adjusted to 6.0. The flasks were incubated in an orbital shaker at 200 rpm at 32 °C. After 5 days of incubation, the mycelium was separated by filtration and the supernatant was challenged with an equal amount of 1 mM silver nitrate solution (prepared in deionized water) and incubated in the shaker at 200 rpm in the dark at 32 °C. Simultaneously, a positive control of silver nitrate solution and deionized water and a negative control containing only silver nitrate solution were maintained under the same conditions.

Characterization of AgNPs

After 24 h of incubation the preliminary detection of Ag-NPs was carried out by visual observation (colour change of the filtrate). This cell filtrate was later subjected to optical measurements, which were carried out by using UV-visible spectra (ELICO SL-159 Spectrophotometer) in the range 350 - 470 nm. After freeze drying of the purified silver particles, the size and shape were analyzed by scanning electron microscopy (JOEL- Model 6390). FT-IR spectrum of the samples was recorded on a FT-IR instrument (Digital Excalibur 3000 series, Japan) in diffuse reflectance mode (DRS-800). All measurements were carried out in the range of 400 - 4,000 cm⁻¹ at a resolution of 4 cm⁻¹ [12]. The fungal mycelium embedded with the silver nanoparticles were freeze-dried, powdered and used for XRD analysis. The spectra were

recorded on a Philips® automatic X-ray Diffractometer with Philips® PW 1830 X-ray generator. The diffracted intensities were recorded from 30° to 90° 2θ angles.

Antibacterial activity

Biologically synthesized silver nanoparticles produced by the *Schizophyllum radiatum* were tested for antimicrobial activity using a method suggested by [13]. This involved using 2 Gram-positive and 2 Gram-negative bacteria by the agar well-diffusion method. Approximately 25 ml of nutrient agar medium was poured into sterilized petri dishes. The bacterial test organisms were grown in nutrient broth for 24 h. A 100 µl nutrient broth culture of each bacterial organism (1×10^5 CFU/ml) was used to prepare bacterial lawns. Agar wells of 8 mm diameter were prepared with the help of a sterilized stainless steel cork borer. The wells were loaded with 60 µl of Ag nanoparticles solution, 60 µl of ceftriaxone (10 mg in 100 ml of distilled water), 60 µl of ceftriaxone in combination with silver nanoparticle solution, 60 µl of streptomycin (10 mg in 100 ml of distilled water), and 60 µl of streptomycin in combination with the silver nanoparticle solution. The plates were incubated at 37 °C for 24 h and then were examined for the presence of zones of inhibition. The diameter of such zones of inhibition was measured and the mean value for each organism was recorded and expressed in millimeters.

Assessment of Increase in fold area

Increase in fold area was assessed by calculating the mean surface area of the inhibition zone generated by an antibiotic alone and in combination with silver nanoparticles [14]. The fold increase area was calculated by the equation, Fold increase (%) = $(b-a)/a \times 100$ where a and b refer to the zones of inhibition for antibiotic alone and antibiotic with silver nanoparticles.

Results and discussions

The isolate yielded 1,112 base pairs, based on 18S rRNA gene sequencing and an NCBI BLAST search conducted based on the topology of phylogenetic analysis revealed that the obtained sequence was 99 % related with *S. radiatum*. Then the sequence was submitted to the EMBL database with the Accession number HE 863742.1. The cell culture filtrate was challenged with 1mM of AgNO₃ and incubated in shaker (160 rpm) at 30 °C in the dark. The colour of the solution turned yellow in 24 h and attained maximum intensity after 48 h with a dark brown colour. The reduction of silver was monitored by using UV-vis spectral analysis. The brown color appearance of the medium was caused due to the excitation of surface Plasmon vibrations which is the specific characteristic of silver nanoparticles [15]. In our experiment the maximum peak was noticed at 420 nm designating that the bioreduction of silver nitrate had taken place following incubation of silver nitrate solution in the presence of the culture filtrate extract (**Figure 1**). FT-IR measurements of the dried and powdered samples of Ag-NPs spectral data revealed two types of vibrations (i.e. stretching and bending) in the wavelength range of 4,000 to 500 cm⁻¹. The presence of an amine vibration band at 3,400 cm⁻¹ representing a primary amine (N-H) stretching, and amide (N-H) bending vibration bands at 1,650 and 1,644 cm⁻¹, see **Figure 2**. Furthermore, the FT-IR spectra of biologically synthesized silver nanoparticles also revealed peaks at 2,026 and 2,116 cm⁻¹ stretching vibrations of aliphatic C-H bonds. A band at 1,412 cm⁻¹ can be assigned to CH₂-scissoring stretching vibration at the planar region. Several C-N stretching vibration peaks at 1,258, 1,143, 1,102, 1,027 and 908 cm⁻¹ were also observed in the spectral range of 1,230 to 900 cm⁻¹. In addition, the presence of bands at 1,356 and 1,250 cm⁻¹ in the FT-IR spectra suggested the capping agent of biologically synthesized nanoparticles possesses an aromatic amine groups with specific identities of amide linkages between amino acid residues in the proteins in the infrared region of the electromagnetic spectrum [16]. This type of FT-IR spectra indicates the presence of a protein type of compound on the surface of the biologically synthesized nanoparticles, confirming that metabolically produced proteins acted as capping agents during production and prevented the reduced silver particles agglomeration.

In the present study, for the conformation of Ag-NPs, XRD spectroscopy analysis was performed and the diffractogram is shown in **Figure 3** revealing the phase purity of the material. The diffraction

peaks are above 37° , XRD patterns were recorded the 4 prominent 111, 200, 220 and 311 reflections at $2\theta = 38.2, 44.4, 64.5$ and 77.7 indicating the face centered cubic (FCC) structure of silver nanoparticles.

Scanning electron microscopy analysis was carried out to study the morphology and size of the produced nanoparticles. The biologically synthesized silver nanoparticles are different in size and shape and are mostly observed as individual particles and aggregates. SEM mediated characterization of biologically synthesized nanomaterials has been performed by several investigators. Particle size analysis revealed that the silver nanoparticles are in the size range of 10 - 40 nm with a mean diameter of 14.5 nm, suggesting production of different-sized nanoparticles (**Figure 4**). A range of 20 - 50 nm particles by *Lactobacillus* sp. [17], 35 - 46 nm silver nanoparticles by *Pseudomonas stutzeri* [18] have been reported.

The exact mechanism through which silver nanoparticles employ to cause antimicrobial effect is not clearly known. There are however various explanations on the action of silver nanoparticles on microorganisms to cause the microbial effect. Silver nanoparticles have the capability to adhere to the bacterial cell wall and simultaneously penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. This leads to the formation of small pits on the cell surface, and there is accumulation of the nanoparticles on the cell surface [19]. The probable mechanism on enhancement with the addition of silver nanoparticles is partially known. Some studies have reported that the positive charge on the Ag ion plays a major role in its antimicrobial activity through the electrostatic attraction between negative charged cell membrane of the microorganism and positive charged nanoparticles [20-22]. The antibacterial activity of antibiotics increased in the presence of Ag-NPs against the bacterial strains. An increase in the synergistic effect may be due to the bonding reaction between the antibiotics and the silver nanoparticles. Further the nanoparticles have large surface area which allows them to closely interact with antibiotics. The antibiotic molecules contain active groups like hydroxyl and amido groups, which can easily react with Ag-NPs by chelation [23]. In another report by Dhar *et al.* the Ag-NPs along with streptomycin demonstrated enhanced antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Candida albicans* [24]. Fayaz *et al.* demonstrated that the silver nanoparticles in a mixture with ampicillin showed higher antibacterial against gram positive and gram negative bacteria they proposed a mode of activity; the dynamic groups of ampicillin connect with the Ag-NPs. The ampicillin - Ag-NPs complex interacts with the cell wall and represses the arrangement of cross connections in the peptidoglycan layer prompting cell wall lysis. Thus increases the penetration of the complex into the bacterium. The Ag-NPs - ampicillin complex responds with DNA and keeps the loosening up of DNA, which brings about genuine harm to bacterial cells [25].

The antibacterial activity of ceftriaxone and streptomycin were evaluated with diameter of inhibition zones for the test bacteria (*Bacillus subtilis*, *S. aureus*, *E. coli*, *Proteus vulgaris*). The antibacterial activity of ceftriaxone and streptomycin increased significantly in the presence of Ag-NPs, **Figure 5**. Silver Nanoparticles showed good antimicrobial activity alone. It was found that the silver nanoparticles produced from *S. radiatum* enhanced the reaction rates of the antibiotics in a synergistic mode as well as in its own way on these pathogens. **Figure 6** and **7** shows the graphical representation of the combination effect of ceftriaxone and streptomycin with Ag-NPs against the test bacteria. The highest fold increase percentage was found in case of ceftriaxone+Ag-NPs against *P. vulgaris*, *B. subtilis* followed by *S. aureus* and *E. coli*. In the case of streptomycin+Ag-NPs the order was *P. vulgaris*, *E. coli*, *B. subtilis* and *S. aureus* **Table 1**.

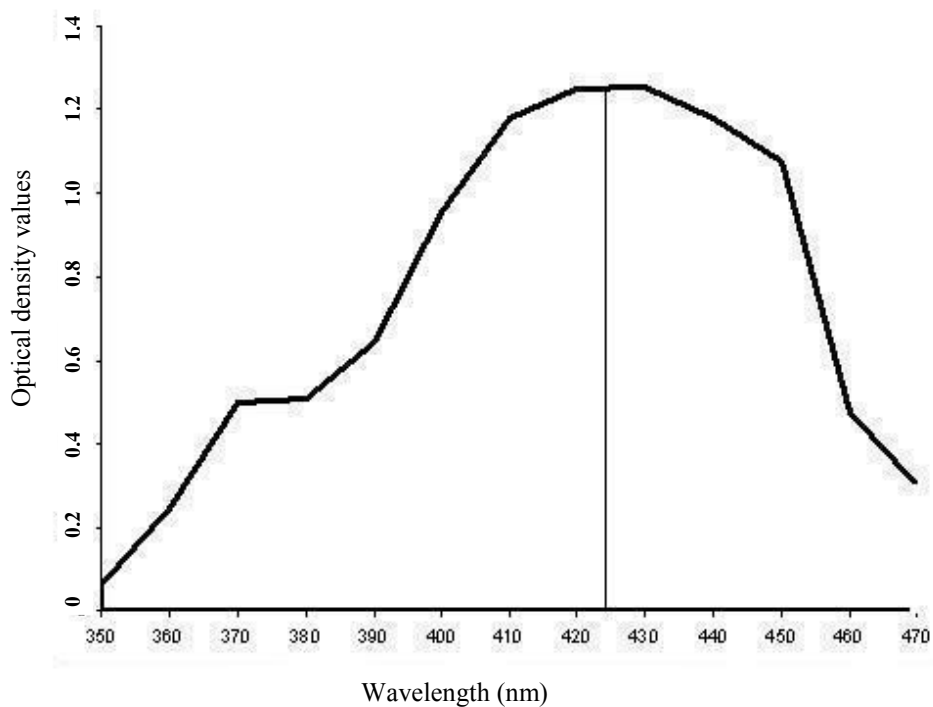


Figure 1 UV-Visible absorption spectra of silver nanoparticles after 24 h of incubation.

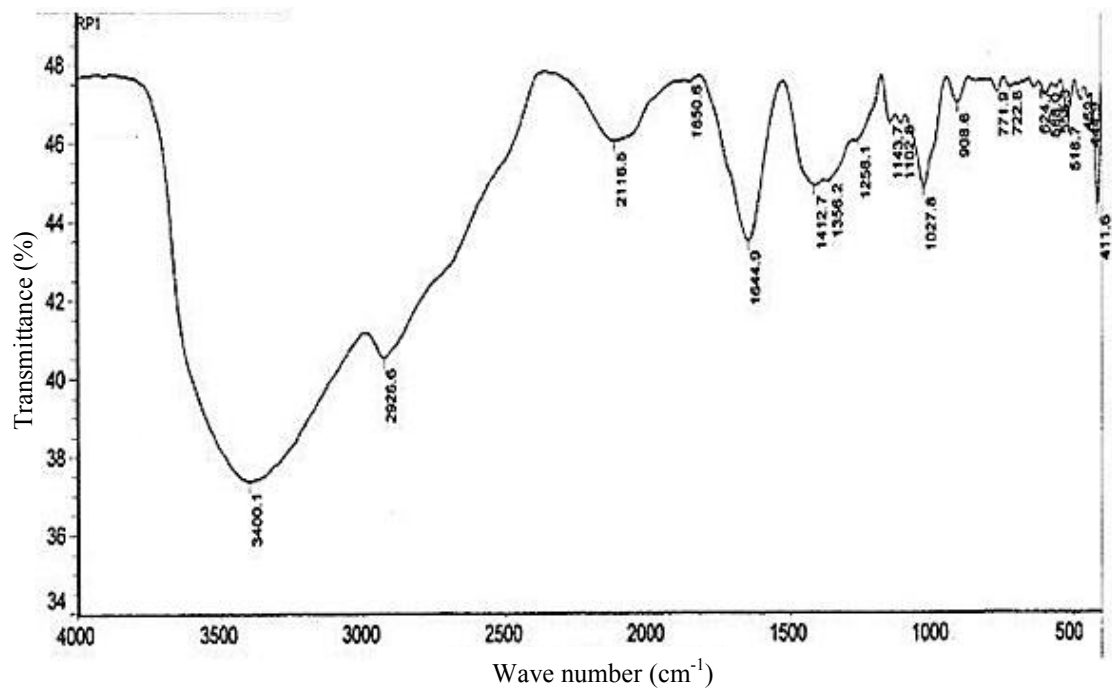


Figure 2 FT-IR spectrum of the synthesized silver nanoparticles.

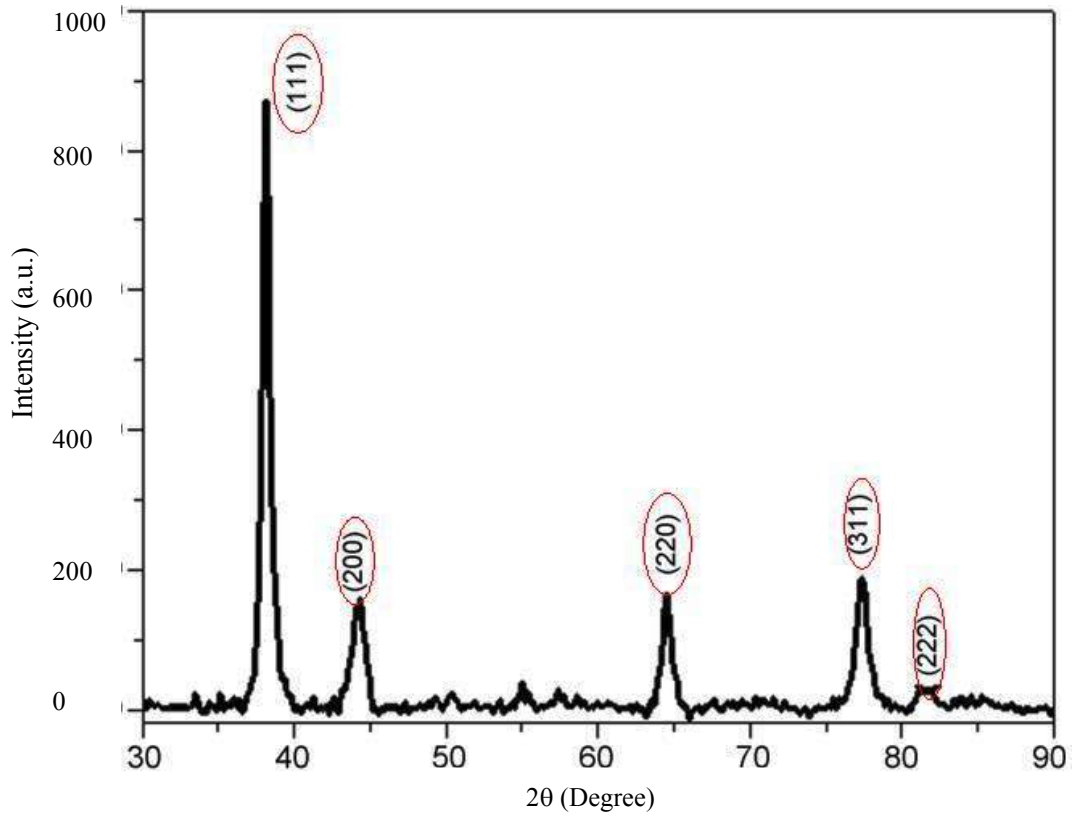


Figure 3 XRD pattern of the silver nanoparticles.

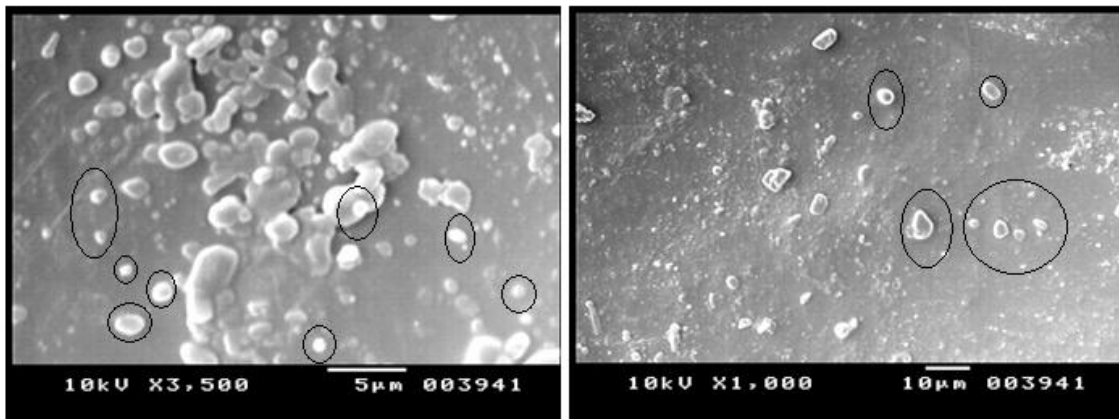


Figure 4 SEM Micrographs of the silver nanoparticles synthesized from fungal extracts.

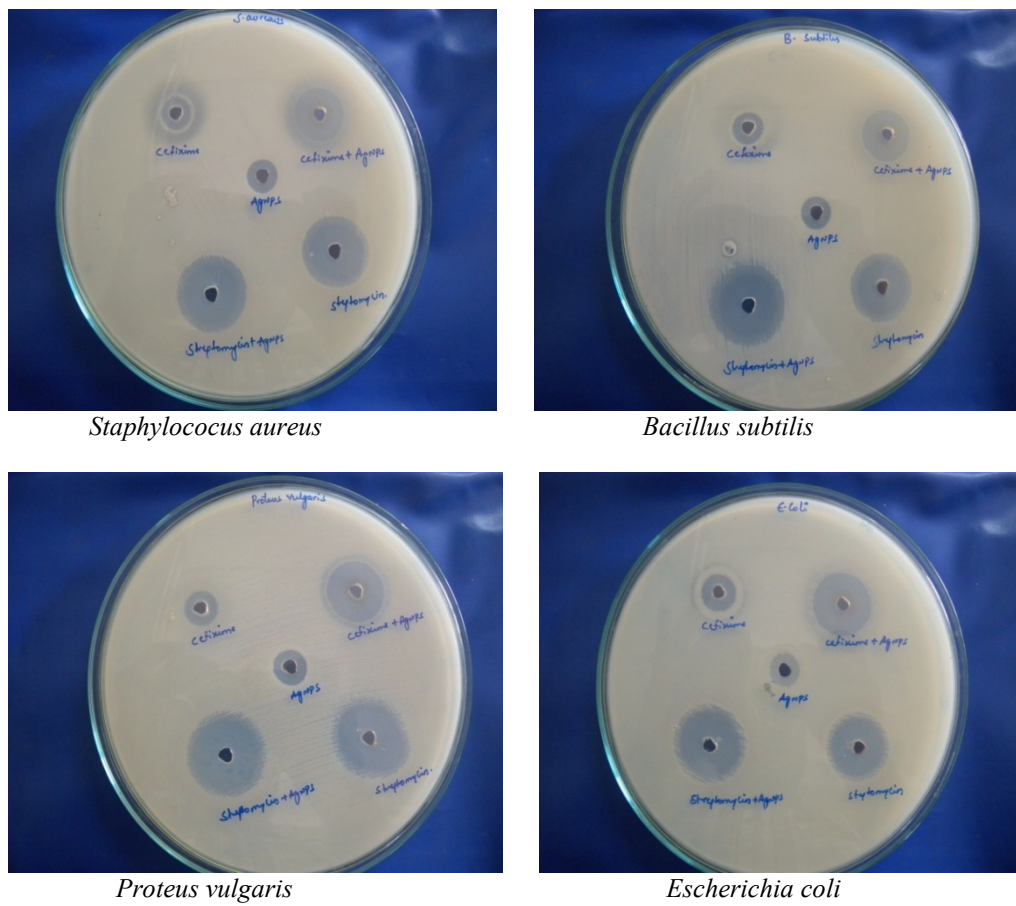


Figure 5 Antibacterial activity of silver nanoparticles produced by *Schizophyllum radiatum* conjugated with antibiotics against bacterial species.

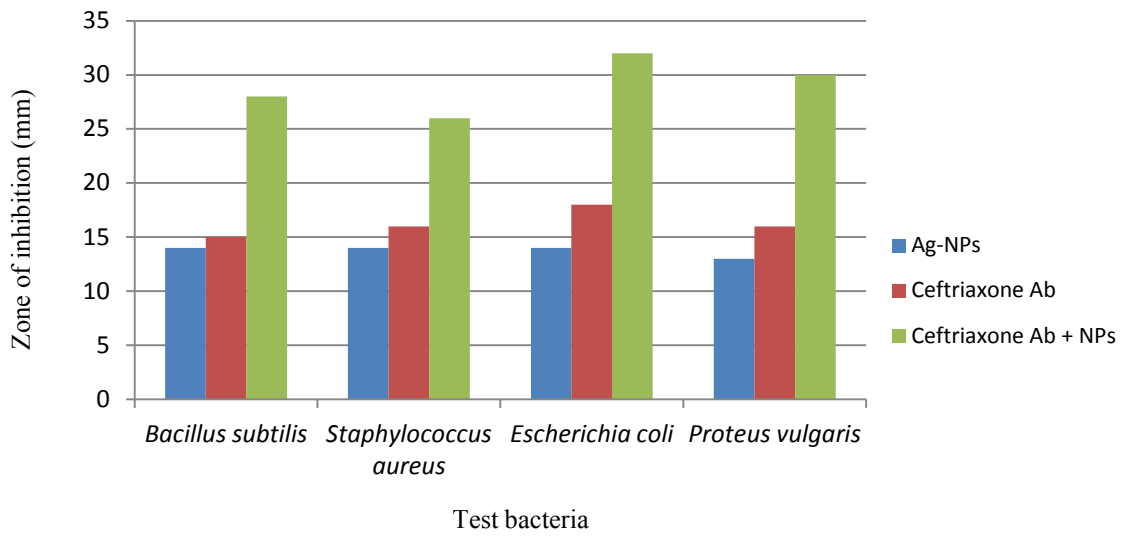


Figure 6 Graphical representation of the combination ceftriaxone with Ag-NPs against the Test bacteria.

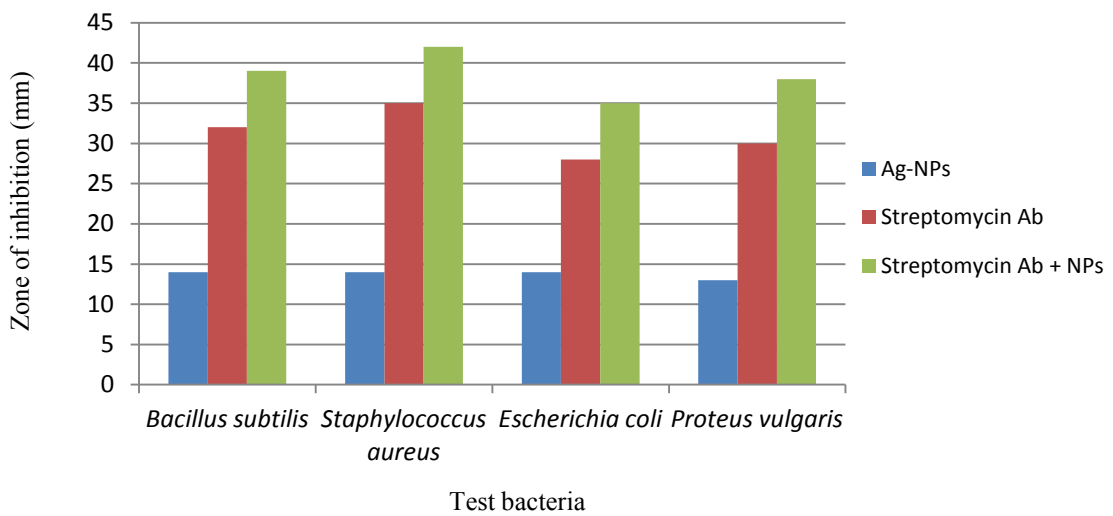


Figure 7 Graphical representation of the combination streptomycin with Ag-NPs against the Test bacteria.

Table 1 Synergistic effect of different antibiotics with and without extracellularly biosynthesized Ag-NPs against pathogens. F.I-Fold Increase $F = ((b-a)/a)*100$.

Organism	Ag-NPs	Ceftriaxone		FI %	Streptomycin		FI %
		Ab	Ab + NPs		Ab	Ab + NPs	
<i>Bacillus subtilis</i>	14	15	28	86.6	32	39	21.87
<i>Staphylococcus aureus</i>	14	16	26	62.5	35	42	20
<i>Escherichia coli</i>	14	18	32	77.7	28	35	25
<i>Proteus vulgaris</i>	13	16	30	87.5	30	38	26.6

Conclusions

Silver nanoparticles play a crucial role in enhancing the antibacterial activity of ceftriaxone and streptomycin. When nanoparticles are conjugated with ceftriaxone and streptomycin in lower concentrations it was also found to be effective. Moreover, the combination effect of standard antibiotics with Ag-NPs against pathogenic bacteria is similarly a new finding. Further, it can be concluded that the applications of Ag-NPs and antibiotics together improved their efficiency, reduced side effects and also their cost.

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