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Biogenesis of silver nanoparticles using *Aspergillus terreus*, its cytotoxicity and potential as therapeutic against human pathogens

Pooja Bansal*[1], Pawan Kaur[1], Surekha[2], Anil Kumar[3] and Joginder Singh Duhan[1].

[1]Department of Biotechnology, Chaudhary Devi Lal University, Sirsa-1250055, India
[2]Department of Botany, Govt. National College, Sirsa- 125055, India
[3]Centre for Medical Biotechnology, Maharishi Dayanand University, Rohtak- 124001, India

ABSTRACT

In recent years, much research is being done towards greener synthesis methods of nanoparticles and their use as potential therapeutic agents. As compared to other nano-products silver nanoparticles have shown prominent biomedical applications. In present research work, fungi *Aspergillus terreus* is being used as nano-factory for synthesis of silver nanoparticles by reducing silver nitrate using a simple protocol. Synthesized nanoparticles were characterized for their size, stability and functional groups involved in their synthesis. Its cytotoxicity using sister chromatid exchange frequency in human lymphocytes has been evaluated and it was found that synthesized nanoparticles are bio friendly up to concentration of 100µg/ml. Antimicrobial activity against five opportunistic human pathogens has been studied and showed effective antimicrobial activity against fungal pathogens under *in vitro* conditions.

Keywords: Nanoparticles, therapeutic, cytotoxicity, sister chromatid exchange, opportunistic pathogens

Corresponding author
E-mail id: Poojabansal08@gmail.com

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INTRODUCTION

With increasing public concern towards adverse effects of antibiotics it become very important to switch to smarter antimicrobial agents as compared to conventional antibiotics. Silver nanoparticles are known for their antimicrobial potential for years. As compared with physical and chemical methods of synthesis, biogenesis of nanoparticles using microbes has additional benefits in terms of safety, compatibility and cleaner environment [1, 2, 3, 4]. Chemically synthesized nanoparticles are generally considered unsafe for human consumption due to involvement of toxic chemicals used as reducing and capping agents. Different researcher had earlier used biological entities including bacteria [5, 6], fungi [7, 8] plant extract [9, 10] and algae etc. for biogenesis of nanoparticles. Over past several years efficiency of nanoparticles as potential therapeutic agent and its cytotoxicity in humans is the area of concern for researchers. Due to one or more mechanism pathogens may develop resistance lead to development of multi drug resistant pathogens (MDR) [11]. Therefore, development of alternative strategies for treating diseases becomes very important. Looking nanoparticles as potential antimicrobial and therapeutic agent is a new and advanced strategy with more benefits and less harm as compared to conventional approaches [12]. When it comes to human application possible negative impact of nanoparticles and its cytotoxicity must be known.

In present research work silver nanoparticles had been synthesized using fungi *Aspergillus terreus* using simple and economical protocol. Synthesized silver nanoparticles had been characterized using different techniques including UV-visible spectroscopy, particle size analyzer, transmission electron microscopy and fourier transform infra red spectroscopy. Antimicrobial potential of synthesized nanoparticles was tested against human pathogens *Enterobacter* sp., *S. aureus, K. pneumoniae*, *P. florescence*, *Bacillus* sp.

MATERIALS AND METHODS

Chemicals and cultures

Silver nitrate ($AgNO_3$), nutrient agar (NA), potato dextrose agar (PDA), nutrient broth (NB), colchicines, sodium heparin, Hoechst 33258, 5-Bromo 2-deoxyuridine etc. were supplied by Sigma Aldrich. Antibiotics streptomycin and penicillin, Giemsa stain, L- glutathiame, phytohaemagglutin, fetal bovine serum etc were purchased from Hi-Media. All reagents used were of analytical grade. Human pathogenic strains *Enterobacter* (MTCC 5112), *Staphylococcus aureus* (MTCC 3160), *Pseudomonas florescence* (MTCC 2421) and *Klebsiella pneumoniae* (MTCC 4030) were procured from MTCC IMTECH, Chandigarh. Pathogenic *Bacillus* sp. was kindly provided by Vegetable department, HAU, Hisar.

Isolation and characterization of fungi

Based on $AgNO_3$ tolerance capacity, fungi colonies having tolerance upto 5 mM had been isolated from soil and characterized by IARI, Delhi.

Biosynthesis of nanoparticles

Revived fresh biomass of fungi was allowed to grow in PDB for 7 days. Biomass was filtered using Whatman filter paper no. 1 and washed with deionized water to remove unwanted traces of media. Obtained biomass was resuspended in 100 ml deionized water for studying intracellular synthesis. Supernatant obtained after filtration was further used for extracellular synthesis of nanoparticles. $AgNO_3$ was added to both supernatant and biomass. Final concentration of both supernatant and biomass was kept at 5 mM. Experiment was allowed to run for 5 days at 28°C and 180 rpm. Complete dark conditions were maintained throughout the experiment. Controls containing cultures without $AgNO_3$ was also kept at similar conditions.

Characterization of NPs

Synthesized silver nanoparticles were characterized for their size and functional groups using UV visible spectroscopy, PSA, TEM, FTIR.

Cytotoxicity assay

For culture set up five milliliter of venous blood was taken from healthy individuals in sodium heparin coated vacutainer tubes with Moorhead et al. protocol [13] with minor modifications. During culture set up, 500 µl of heparinized blood was added to a culture tube having 5 ml of RPMI-1640 culture medium with L glutamine (1%). Other supplement such as fetal bovine serum (20%), phytohaemagglutinin (2%) and antibiotic [penicillin (100UI/ml) and streptomycin (100µg/ml)] were also added.
The culture was treated with silver nano-particles (AgNPs) in varying concentrations (25 -150 µg/ml) and sister chromatid exchange (SCE) analysis was done. The cultures were then incubated for 72 h at 37 °C and ±5% CO_2. For differential staining of chromosomes, 5-bromo-2-deoxyuridine (1 mg/ml) was added after 24 h. To arrest the chromosomes at metaphase stage colchicine was added 45 min prior to the harvesting at final concentration of 0.2 µg/ml. The harvesting was performed using treatment with hypotonic solution (0.075 M KCl) kept at 37 °C and repeated washing of colchicine treated cells with fixative (methanol: acetic acid in 3: 1 ratio was done). The slides were prepared by dropping the cells on pre-cleaned slides and allow to air dried. Differential staining with Hoechst 33258 and 4% Giemsa stain was done. Then slide analysis was done to determine the frequency of SCE per cell, by analyzing well spread 25 metaphase plates/per slide.

***In vitro* antimicrobial potential**

Antimicrobial potential of synthesized silver nanoparticles against human pathogenic bacteria was studied using agar well diffusion assay. Twenty ml nutrient agar medium was poured in well autoclaved petri plates. One ml of fresh bacterial inoculum was spread on the solidified agar plates using a sterile glass swab. After 10 minutes settling time, with the help of sterile cork borer 5 wells of 6 mm diameter were made. Each well was filled with 60 µl of well sonicated nanoparticles solution made in different concentrations (10, 20, 40 60 µg/ml respectively) and one well was filled with antibiotic penicillin 60 µl (1mg/ml). All Plates were incubated at 30°C. After 24 hours of incubation zone of inhibition was measured using zone inhibition scale.

RESULTS AND DISCUSSION

Isolation and characterization of fungi- Isolated fungi (Figure 1) was characterized as *Aspergillus terreus*.

Visual observation

As shown (Figure 2) after 24 hours of reaction, color changed from yellow to brown indicating synthesis of silver nanoparticles. This change in color was observed due to excitation of surface Plasmon vibrations unique for AgNPs.

UV visible spectroscopy

A UV visible spectrum in the range 200-800 nm was recorded. Broad spectrum peak was observed at 430 nm (Figure 3). Past studies suggested that presence of peak 410- 450 nm indicate synthesis of small sized spherical silver nanoparticle synthesis. Duran et al. [14] suggested presence of peaks at lower wavelength is attributed to aromatic amino acids of proteins. It is well known that the absorption band around 250-300 nm arises due to electronic excitations in proteins due to amino acids such as tryptophan and tyrosine residues. To check for shelf life and stability of synthesized UV visible spectroscopy was repeated after one month of synthesis and no major shifting in peak was observed indicating no evidence of aggregation of nanoparticles.

Particle size analyzer (PSA)

Size was confirmed by using zetasizer (Nanoseries Nano ZS90, Malvern). Well dispersed NPs solution was used for readings. The average size of NPs comes out to be 45.5 nm which is also indicated by UV visible spectra. Single and sharp peak with PDI value 0.7 was observed (Figure 4). Zomorodian et al. [15] observed similar results for biosynthesized AgNPs using *Aspergillus fumigates*.

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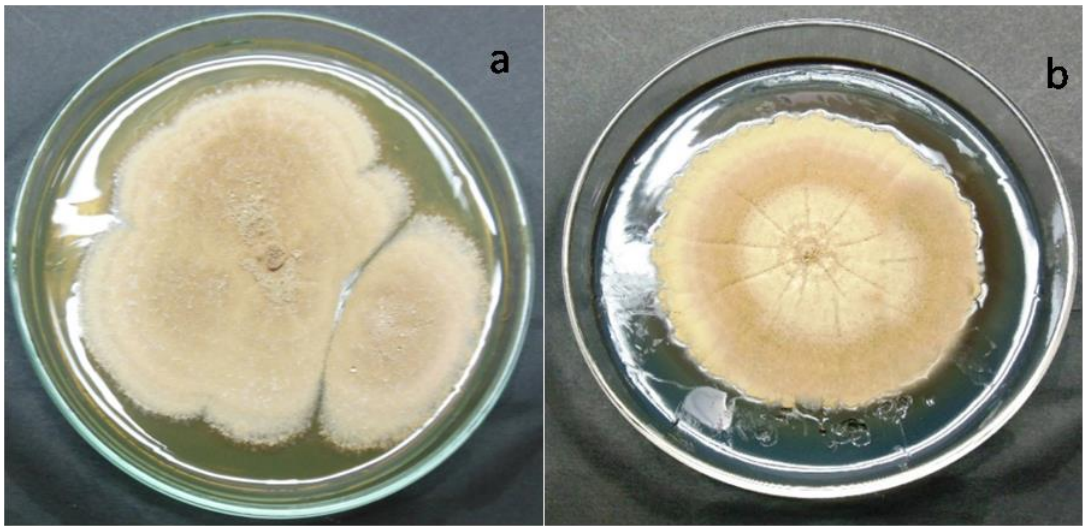

Fig. 1 *Aspergillus terreus* on PDA (a) and PDA embedded with 5mM AgNO3 (b)

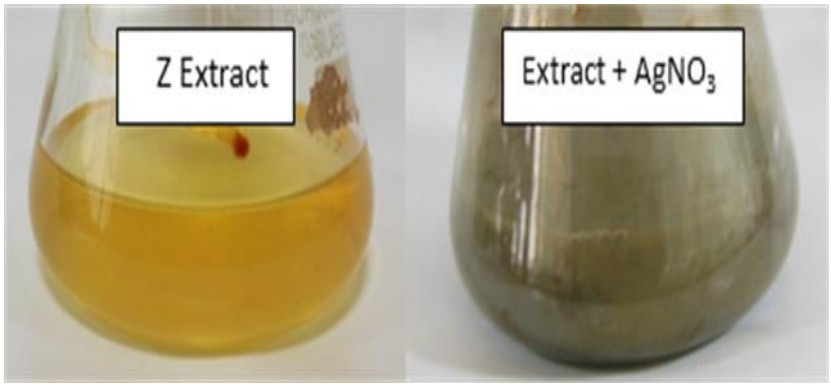

Fig. 2 Change in color of fungus extract after 24 h by addition of 5mM $AgNO_3$

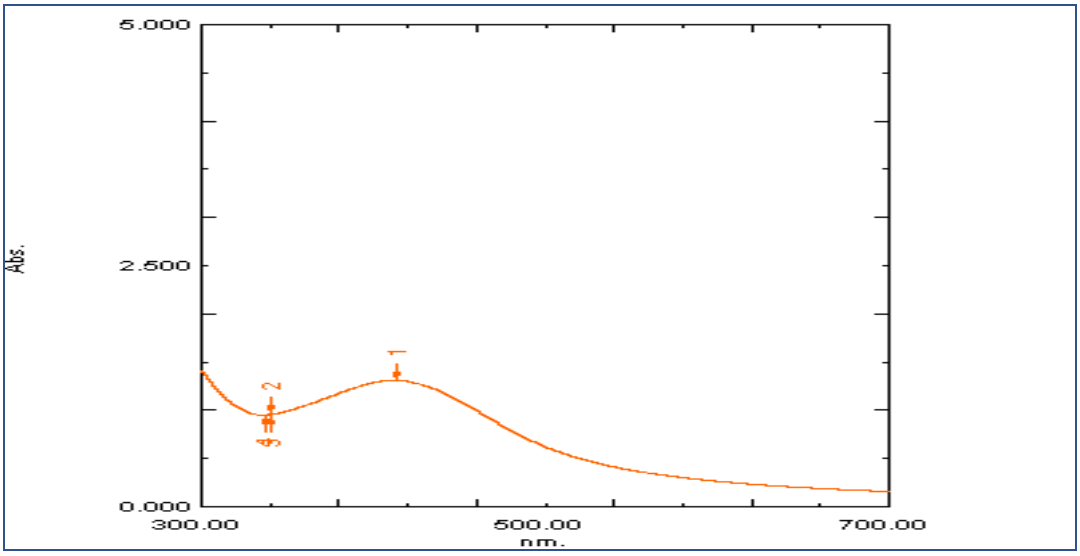

Fig. 3. UV Visible spectra of synthesized sliver nanoparticles

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		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 73.4	Peak 1:	45.5	100.0	9.93
PdI: 0.720	Peak 2:	0.000	0.0	0.000
Intercept: 0.941	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report

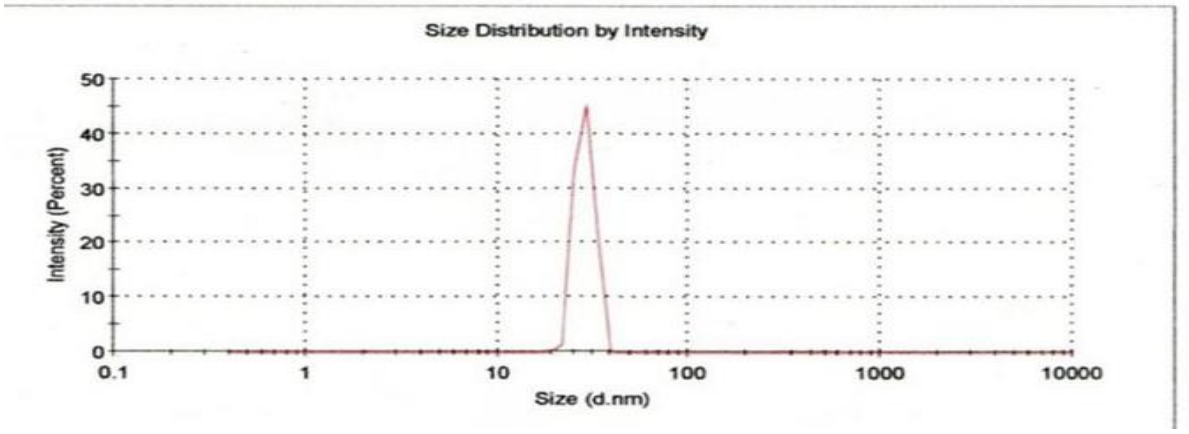

Fig.4. PSA spectra showing monodispersed nanoparticles

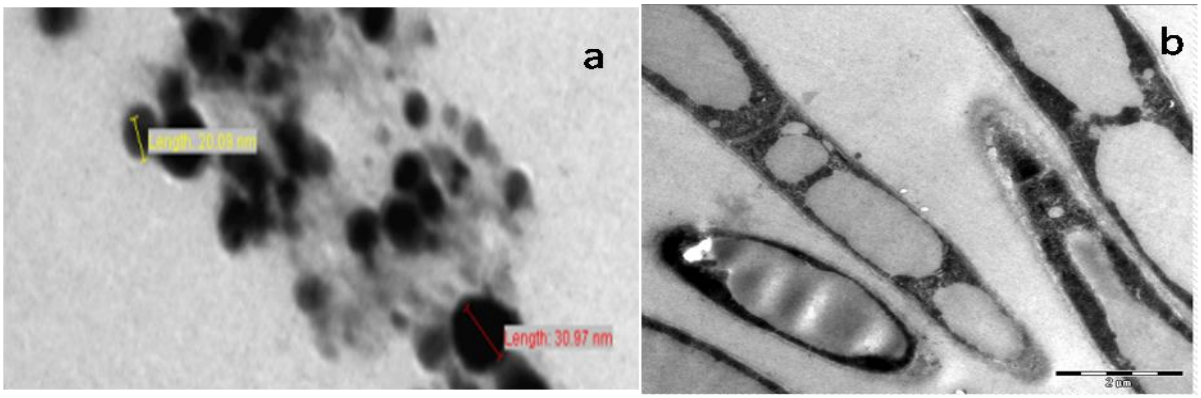

Fig. 5. TEM images of extra and intracellular synthesized silver nanoparticles

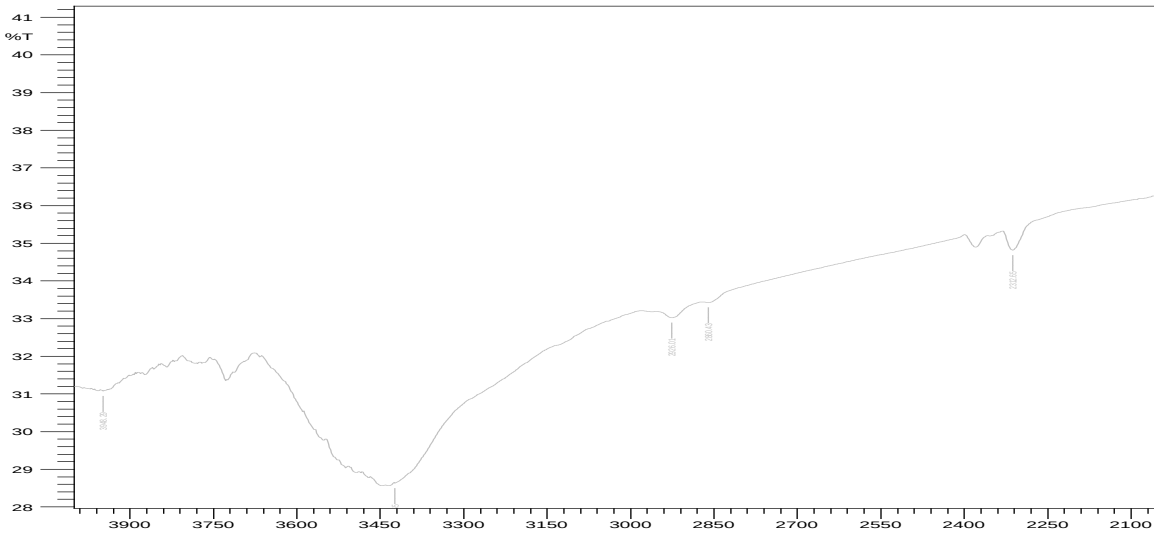

Fig. 6. FTIR spectra of silver nanoparticles recorded in the range of 2300-4000 cm^{-1}

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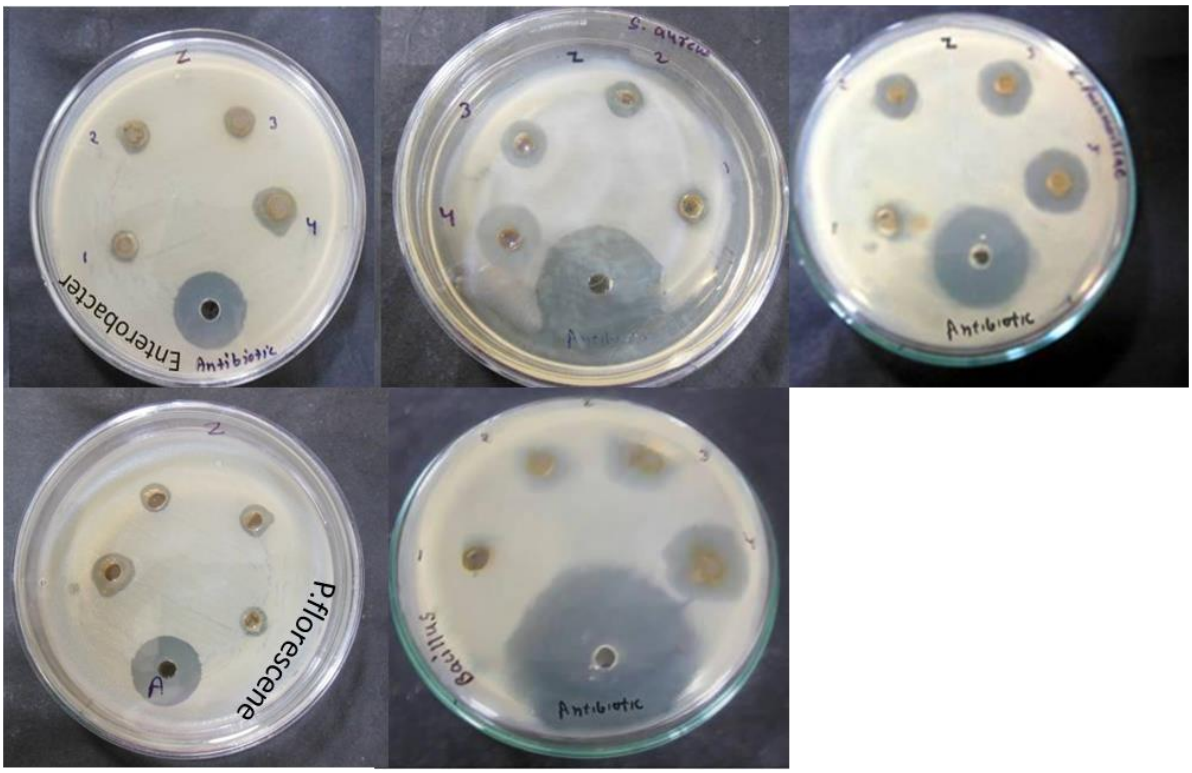

Fig. 7 Zone of inhibition of nanoparticles and standard antibiotic against 1) *Enterobacter* sp. 2) *S. aureus* 3) *K. pneumoniae* 4) *P. florescence* 5) *Bacillus* sp.

Transmission electron microscopy (TEM)

TEM images showed the presence of small sized NPs both intra as well as extracellular. Small sized well dispersed nanoparticles with size approx. 30 nm have been seen in case of extracellular synthesis (Figure 5). A bit agglomerated NPs in large quantity were observed in case of intra cellular synthesis. Nanoparticles were seen well dispersed indicating stabilization by capping agent. Results obtained are in accordance with previous techniques. Elbeshely et al. [16] reported synthesis of spherical AgNPs biosynthesized using Bacillus. In other report similar TEM results for gold and silver nanoparticles were observed [17].

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was done in order to find out the chemical functional groups in the sample responsible for reduction of $AgNO_3$ [18]. An FTIR spectrum was recorded in the range of 2200–4000 cm^{-1} (Figure 6). Presence of peaks at 2400-3200 and 3408, 3410and 3419 can be assigned to free ammonium ions and 1˚ amines which may be responsible for synthesis of silver nanoparticles by reducing $AgNO_3$ [19].

Cytotoxicity

In this study cytotoxicity of silver nanoparticle was determined by monitoring sister chromatin exchange frequency of human lymphocytes at different concentrations of AgNPs. All treatments were carried out in duplicates. One way ANOVA was applied for comparison of SCE frequency level among control and treated groups using SPSS 16.0. The level of significance was set at $P < 0.05$ and the results were expressed as Mean ± S.D. Silver nanoparticles induced genotoxicity was determined using SCE frequency as an indicator of genotoxicity. On treatment with different concentrations of AgNPs (25- 125 µg/ml), the cultured peripheral blood lymphocytes showed a very slight increase in SCE frequency but results were observed non-significant ($p > 0.05$) compared to control (Table1) up to concentration of 100µg/ml. However, the higher concentrations of AgNPs (125 µg/ml) induced increase in mean SCE frequency. Some previous studies showing dose dependent cytotoxicity of silver NPs on different cancer cell lines [20, 21, 22, 23] but less research has been done on cytotoxicity assays for finding biocompatible concentration of silver nanoparticle.

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Table 1: Frequency of SCE in peripheral blood lymphocytes *in vitro* treated with different concentrations of AgNPs.

AgNP treatments (µg/ml)	SCE/Cell (Mean±S.D.)
Control (untreated)	3.192±.034
25	3.192±.034
50	3.196±.039
75	3.201±.041
100	3.211±.046
125	3.512±.048

ANOVA with was applied for comparison between different treatment groups
F (4, 46) = .408, [P (.802) > 0.05)]

Antimicrobial activity against human pathogens

In present research, antimicrobial activity of synthesized silver NPs was tested against five human opportunistic pathogenic bacteria *Enterobacter* sp, *S. aureus*, *K. pneumonia, P. florescence* and *Bacillus* sp. by agar well zone diffusion method (Table 2, Figure 7). Maximum zone of inhibition was obtained against *Bacillus sp.* i.e. 28 mm. Antibiotic resistance developed in case of *Pseudomonas florescence* and *Enterobacter* bacteria after 72 h incubation period but no bacterial growth in ZOI was seen by zone developed by silver nanoparticles. Antibacterial activity was concentration dependent. Synthesized silver nanoparticles showed fairly good antimicrobial activity against both gram positive and gram negative bacteria. Some research work on antifungal effect of silver nanoparticles has already been done. Zafar et al. [24] reported antimicrobial activity of biosynthesized silver nanoparticle using zone diffusion assay against different human pathogens. In another research work Pandian & Chidambaram [25] had reported strong inhibitory effect of biosynthesized AgNPs against human pathogenic bacteria and fungi.

Table 2 Zone of inhibition (mm) obtained by using different concentrations of silver nanoparticles (4, 3, 2, 1) and standard antibiotic against human pathogens

Pathogen	Zone of inhibition (mm)	Conc. of NPs				Antibiotic (60µg)
		4 (60µg/ml)	3 (40µg/ml)	2 (20µg/ml)	1(10µg/ml)	
Enterobacter sp.		14.0± 1.5	10.0± 2.0	8.0± 1.2	7.0± 1.4	20.0±0.2
S. aureus		16.0±1.0	11.0±0.3	9.0±0.8	7.0±0.4	35.0±0.8
K. pneumoniae		18.0±2.0	15.0±1.0	11.0±1.3	8.0±1.0	26.0±0.4
P. florescence		14.0±0.2	10.0±0.8	8.0±0.2	7.0±0.9	20.0±0.7
Bacillus sp.		28.0±0.8	15.0±0.4	10.0±0.4	7 .0± 0.8	47.0±0.6

CONCLUSION

Biosynthesis of small sized, stable silver nanoparticles using Aspergillus terreus as bio-factory have been reported both intra as well as extracellularly. This synthesis method does not involve any toxic chemicals and hence a greener approach which can scaled at larger level for production of nanoparticles. Biocidal efficiency of synthesized AgNPs had been observed against all tested pathogens projecting AgNPs as potential therapeutic agent. After 72 h incubation period presence of microbial colonies in ZOI of antibiotics showed development of antibiotic resistant colonies in *Pseudomonas florescence* and *Entrobacter* but no such resistance was found against silver nanoparticles. Therefore, it clearly indicated theadvantage of using AgNPs as compared to conventional antibiotic.

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