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CHARACTERISATION AND ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED BY *ASPERGILLUS SP.*

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ABSTRACT

The use of microorganisms in the synthesis of nanoparticles emerges as an ecofriendly approach. The microorganisms such as bacteria, fungi and actinomycetes and plant materials are used in the biosynthesis of nanoparticles. The biological synthesis of silver nanoparticles has many advantages over physical and chemical methods. In the present study, the isolated fungal strain *Aspergillus sp.* was used for silver nanoparticle synthesis. Cell free filtrate of isolated *Aspergillus sp*. was incubated with 1mM silver nitrate on a rotary orbital shaker at 120 rpm. The nanoparticles synthesis was visually observed by brown color change. The formation of silver nanoparticles was monitored by UV-Visible spectroscopy and further characterized by SEM, EDAX, Particle size and Photoluminescence analysis. The nanoparticles exhibited a maximum absorbance at 422nm in UV-Visible spectroscopy corresponding to the plasmon resonance of silver nanoparticles. SEM analysis reveals the nanoparticles with the size range of 40-70nm. The antibacterial effect of silver nanoparticles reported herein reveal its importance in advantage over conventional antibiotic against *Pseudomonas aeruginosa.*

KEYWORDS: Silver nanoparticles, *Aspergillus sp*, Scanning Electron Microscope, Antibacterial activity

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INTRODUCTION

Nanotechnology is an emerging field of science which involves synthesis and development of various nanomaterials. At present, different types of metal nanomaterials are being produced using copper, zinc, titanium, magnesium, gold, alginate, and silver. Classically the nanoparticles are produced by physical and chemical method. However these methods are not only costly, and non-ecofriendly, but also lead to toxic effects. Scientists are looking forward to synthesize cost effective, nontoxic nanoparticles that also will be ecofriendly[1]. One of the most important criteria of nanotechnology is that of the development of clean, nontoxic and environmentally acceptable "green chemistry" procedures involving organisms ranging from bacteria to fungi and even plants[2,3]. A number of microorganisms have been found to be capable of synthesizing intra or extra cellular inorganic nanocomposites [4]. Biological production systems are of special interest due to their effectiveness and flexibility[5]. Silver nanoparticles are undoubtedly the most widely used nanomaterials among all. Silver nanoparticles are used in textile industries, water treatment, sunscreen lotions and as antimicrobial agents [1].Resistance of bacteria to bactericides and antibiotics has increased in recent years. Some antimicrobial agents are extremely irritant and toxic and there is much interest in finding ways to formulate a new type of safe and cost effective biocidal material [6]. Silver ions and silver based compounds are highly toxic to microorganisms[7]. Recent studies have demonstrated that specially formulated metal oxide nanoparticles have good antibacterial activity[8]. The highly reactive metal oxide nanoparticles exhibit excellent biocidal action against gram positive and gram negative bacteria[9]. In the present work, we have investigated the synthesis of silver nanoparticles by the fungal isolate *Aspergillus sp*. EV-4 that we have isolated and characterized the same by using UV-Visible spectrophotometer, SEM, EDAX. We have also studied the particle size and evaluated for its antimicrobial activity.

MATERIALS AND METHODS

SOURCE OF MICROORGANISMS

Fungal strain *Aspergillus sp*. EV-4 was isolated from soil sample, collected at metal cottage industry, Madurai. Enumeration of microbes present in the soil was done by serial dilution technique on Potato Dextrose Agar plates. Serial dilution of soil suspension was prepared upto 10^{-6} dilution. 1ml of suspension from each dilution was transferred to the potato dextrose agar medium in petriplates and incubated at 28°C for 3-4 days. The fungal colony was picked and maintained on PDA slants. The isolated fungal colony was stained using Lacto phenol cotton blue and confirmed based on colony characteristics and microscopic observation.

BIOSYNTHESIS OF SILVER NANOPARTICLES[1]

The isolated *Aspergillus sp*. EV-4 was selected for the production of silver nanoparticles. The *Aspergillus sp*. was inoculated in liquid media containing KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, yeast extract and glucose. The flasks were incubated at room temperature for 3 days in a rotary orbital shaker at 120 rpm. The biomass was harvested after 72 h of growth by sieving through a sieve (~100micron size) and washed with sterile double distilled water to remove any medium component. The biomass was mixed with 200 ml of sterile double distilled water in a 500 ml Erlenmeyer flask and agitated in the same condition for 72 h. After incubation, the cell free filtrate was obtained by passing it through Whatman filter paper no.1 and used further for nanoparticle synthesis. For the synthesis of silver nanoparticle, silver nitrate (1mM of final concentration) was mixed with cell free filtrate in an Erlenmeyer flask and agitated in dark. Control was also run along with the experimental flasks.

CHARACTERIZATION OF SYNTHESIZED SILVER NANOPARTICLES

(i) UV-VISIBLE SPECTROSCOPIC ANALYSIS[10]

The reduction of silver ions (Ag^+) by the cell free filtrate in the solution and formation of silver

nanoparticles were monitored by UV- Visible spectroscopy measuring the UV-Vis spectrum of the aqueous component. The UV-Vis spectra of these samples were measured at a resolution of 1nm from 200-800nm using Systronics Double beam UV-Vis spectrophotometer.

(ii) SCANNING ELECTRON MICROSCOPY AND EDAX[11,12]

After the synthesis of silver nanoparticles, the dried samples were analysed by JOEL –JSM 6290 Scanning Electron Microscopy (SEM) equipped with a Thermo Energy Dispersive X-ray spectroscopy (EDAX).

(iii) PARTICLE SIZE ANALYSIS[11, 12]

The particle size distribution of silver nanoparticles was evaluated using Dynamic Light Scattering (DLS) measurement conducted with a Malvern Zetazier Instrument. Measurements were taken in the range between 0.1 and 1000µm. Data obtained were analysed using Zetasizer software.

(iv)PHOTOLUMINESCENCE ANALYSIS[13,14]

Photoluminescence spectra of synthesized nanoparticles were recorded in Fluorolog 3 (HORIBA Jobin Yvon) using 90º illumination. Based on the excitation maximum 422 nm emission scan was carried out in the range of 400-750nm. The entire scanning was done at the speed at 150nm/second. The data were analysed using the FluoroEssence software.

(v) ANTIBACTERIAL ACTIVITY[15]

The ***Escherichia coli NCIM 2931, Pseudomonas aeruginosa NCIM 5029, Bacillus subtilis*** **NCIM 2920** ***and Klebsiella pneumonia*** **NCIM 2707** were obtained from NCIM, Pune . A loop of single colony of each test organism was grown in a tube overnight containing nutrient broth on a shaker (120 rpm) at 30ºC. The synthesized nanoparticles were tested for their antibacterial activity by agar diffusion method and commercial antibiotics were also used. Muller-Hinton agar plates were prepared. The inoculum suspension, adjusted by diluting to a 0.5 McFarland turbidity standard using spectrophotometer in 600 nm, (equal to 10^8 cells) was swabbed uniformly in different plates. Wells were made in each plate using a well-cutter and it was filled with different concentrations of silver nanoparticles (25µg, 50µg, 75µg, 100µg) and then incubated at 37ºC. The same method was used for evaluation of antibacterial activity using antibiotics with the same concentration as mentioned above. The antibiotics were chosen for the antibacterial activity based on the reference[16]. The formation of a clear zone around the well is an indication of antibacterial activity.

RESULTS AND DISCUSSION

There are several physical and chemical methods for synthesis of metallic nanoparticles. The development of simple and ecofriendly biological systems which will be of immense value in the synthesis and application of metallic nanoparticles [17]. The fungal isolate was characterized on the basis of colony characteristics and microscopic appearance. Silver nanoparticles were synthesized from silver nitrate solution containing Ag^+ ions by treating with the cell free filtrate of *Aspergillus sp*. The cell free filtrate of *Aspergillus sp*. changed the silver nitrate solution to brown colour during the reaction with Ag^+ ions. The appearance of brown colour in the conical flask suggested the formation of silver nanoparticles. This colour is primarily due to its Surface Plasmon Resonance (SPR) of deposited silver nanoparticles. The cell free filtrate incubated with deionized water (positive control) retained its original colour(Fig 1).

(i) UV-VISIBLE SPECTRAL ANALYSIS

UV-Vis spectroscopy is one of the most widely used techniques for structural characterization of silver nanoparticles[4].The optical absorption spectrum of metal nanoparticles is dominated by the SPR which exhibits a shift towards the red end or blue end depending upon the particle size, shape, state of aggregation and the surrounding dielectric medium. The absorption band in the visible light region is typical for silver nanoparticles [18]. The silver nanoparticle synthesized by fungi *Aspergillus sp.* showed a strong silver plasmon absorption maxima was at 422nm in UV-Vis spectrophotometer(Fig 1) and this confirms the formation of silver nanoparticles synthesis.

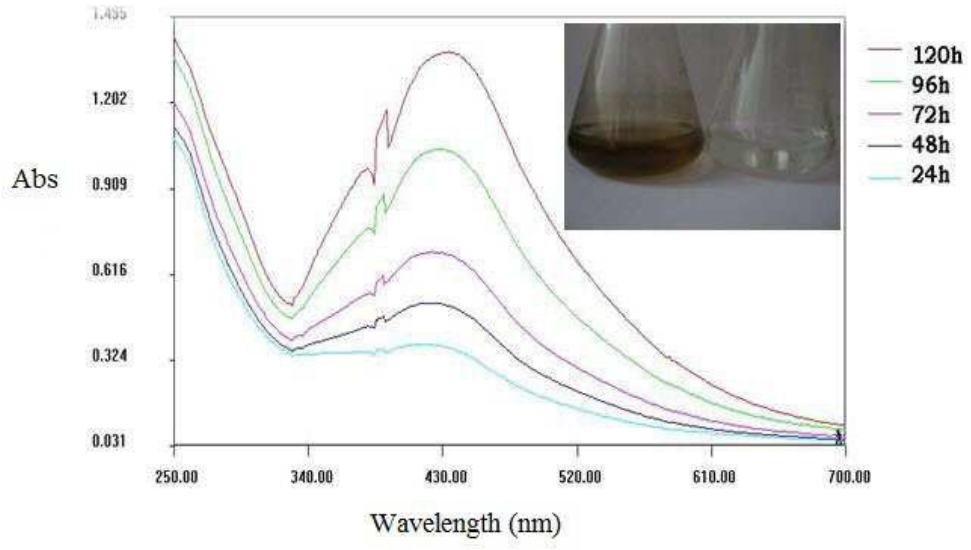

Figure 1
UV-Vis spectrum of silver nanoparticles synthesized by Aspergillus sp. EV-4. The inset shows the silver nanoparticle formation.

(ii) SCANNING ELECTRON MICROSCOPY

The silver nanoparticles synthesized by *Aspergillus sp*. EV- 4 were characterized by SEM. The results showed the particles in the range of 40-70nm. Similar results were reported that silver nanoparticle synthesized by *Trichosporon beigelii* NCIM 3326 showed the particle size of 50-100 nm [19].

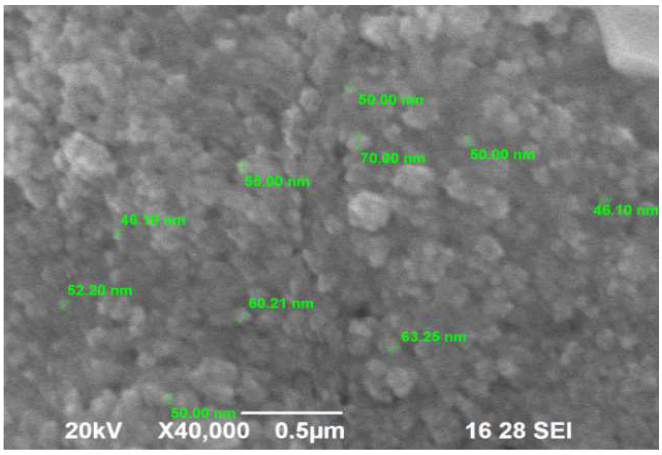

Figure 2
SEM image of silver nanoparticles synthesized from Aspergillus sp. EV-4.

(iii) EDAX ANALYSIS

EDAX analysis gives qualitative as well as quantitative status of elements that may be involved in formation of nanoparticles[14].EDAX spectrum recorded from silver nanoparticles synthesised by fungal isolate EV-4 (Fig.3) confirmed the presence of 70% elemental silver in the reaction mixture.

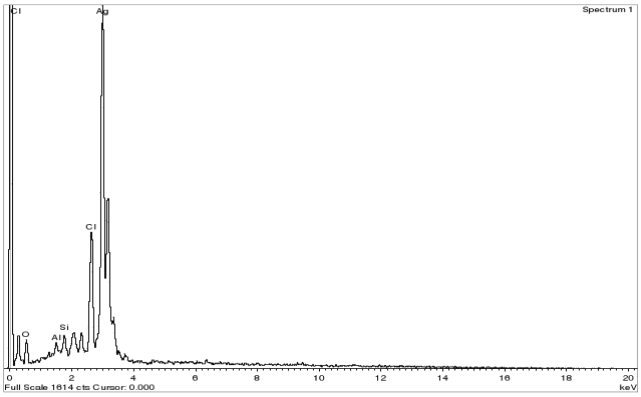

Figure 3
EDAX spectrum recorded from Aspergillus sp. EV-4 after formation of silver nanoparticles.

(iv)PARTICLE SIZE ANALYSIS

The particle size determination of the formulated nanoparticles was shown based on intensity(Fig.4).Laser diffraction revealed that the particle obtained were polydispersed mixture. The first and second peaks, corresponding to the average diameter of the particles were found to be 8.134nm(9.5%,1.504 width) and 73.03nm(90.5%,21.59 width) respectively.

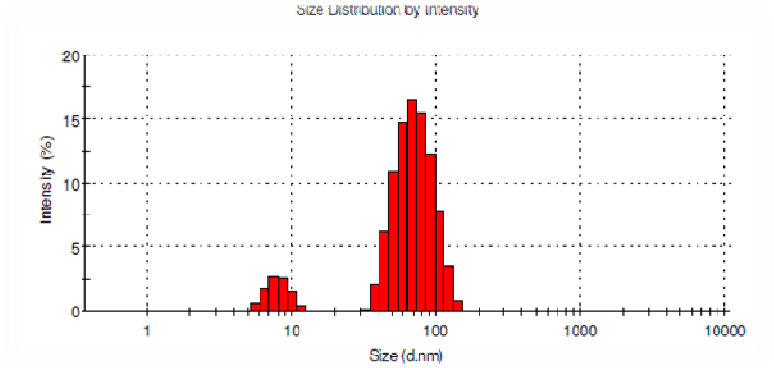

Figure 4
Particle size distribution for silver nanoparticles using Aspergillus sp. EV-4

(v) PHOTOLUMINESCENCE ANALYSIS

Photoluminescence spectra of synthesized silver nanoparticles were observed to evaluate its optical property[14]. Fig. 5 shows the typical PL spectra of silver nanoparticles synthesized using cell free filtrate of EV- 4. The emission peak of synthesized silver nanoparticles was observed at 533 nm when excited at 422 nm. The photoemission wavelength is independent of the particle size while the intensity increases sharply with decrease of particle size.

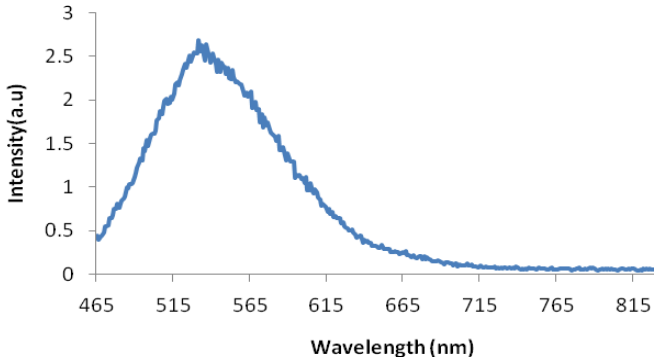

Figure 5
Photoluminescence spectra of silver nanoparticle synthesized by Aspergillus sp. EV-4.

(vi) ANTIBACTERIAL ACTIVITY

Silver nitrate has long been considered as a powerful and natural antibiotic and antibacterial agent. Silver nanoparticles exhibited antibacterial properties against bacterial pathogens. The antimicrobial activity of the silver nanoparticles synthesized by *Aspergillus sp*.EV-4 was evaluated against *Escherichia coli* NCIM 2931*, Pseudomonas aeruginosa* NCIM 5029*, Bacillus subtilis* NCIM 2920 *and Klebsiella pneumonia* NCIM 2707 which showed different inhibition zone (Fig 6-9). The antibacterial activity was evaluated using various antibiotics and compared with synthesized silver nanoparticle using different concentrations(25, 50, 75 100µg/ml). The silver nanoparticles synthesized by *Aspergillus sp*. EV-4 showed effective inhibitory action against *Pseudomonas aeruginosa* when compared to *Klebsiella pneumonia*, *Escherichia coli* and *Bacillus subtilis (*Table.1*)*. The mechanism of inhibitory action of silver ions on microorganism shows that upon Ag^{+} treatment, DNA loses its replication ability and expression of ribosomal subunit proteins, as well as other cellular proteins and enzymes essential to ATP production, becomes inactivated[20]. The positive charge on Ag^{+} is an important factor for its antibacterial nature, through electrostatic interaction between the negatively charged cell membrane of the microorganisms and positively charged nanoparticles. It is proposed that the electrostatic force might be an additional cause for the interaction of the nanoparticles with the bacteria[21]. Similar results were reported that the silver nanoparticles might attach to the surface of the cell membrane, disturbing permeability and respiration functions of the cell. It is also possible that silver nanoparticles not only interact with the surface of the membrane, but also penetrate inside the bacteria[22]. The electrostatic attraction between negatively charged bacterial cells and positively charged nanoparticles is crucial for the activity of nanoparticles as bactericidal materials[9,23]. Similar results were reported that silver nanoparticles synthesized by *Aspergillus flavus* are known to have inhibitory and bactericidal effects against MDR strains of *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia*[24]*.*

Table 1
Antibacterial activity of silver nanoparticles synthesized by Aspergillus sp. EV-4 compared with antibiotics

Organisms	AgNp & Antibiotics	Concentration of antibiotics & AgNp			
		Zone of inhibition (mm)			
		25µg/ml	50µg/ml	75µg/ml	100µg/ml
Bacillus subtilis **NCIM 2920**	AgNp	10.06±0.58	12.6±0.58	12.6±0.58	13.3±0.58
	Chl	-	-	-	-
	Cip	-	17.4±0.51	21.4±0.36	23.9±0.61
	Cef	18.5±0.50	22.6±1.0	25.5±0.50	29.4±0.35
Escherichia coli **NCIM 2931**	AgNp	12.6±0.56	15.6±1.53	14.6±2.08	16.3±2.08
	Amk	21.4±0.40	23.3±0.41	25.6±0.30	27.5±0.45
	Amp	-	-	-	-
	Cef	20.5±0.45	23.5±0.15	28.1±0.1	30.1±0.23
Pseudomonas aeruginosa **NCIM 5029**	AgNp	19.0±2.21	23.0±1.00	20.0±1.15	21.6±1.05
	Amk	15.3±0.26	18.1±0.15	19.2±0.34	21.0±0.11
	Amp	-	-	-	-
	Chl	-	-	-	-
Klebsiella pneumonia **NCIM 2707**	AgNp	11.6±0.57	13.3±1.15	13.6±1.00	14.6±0.58
	Tet	-	-	-	-
	Amp	-	-	-	-
	Cef	-	-	-	-
AgNp - Silver nanoparticle; Chl- Chloramphenicol; Cip - ciprofloxacin ; Cef - Cefotoxamine;					
Amk - Amikacin ; Amp - Ampicilin ; Tet - Tetracycline ; - not detected					

Values are the Mean ± SD of triplicate experiments.

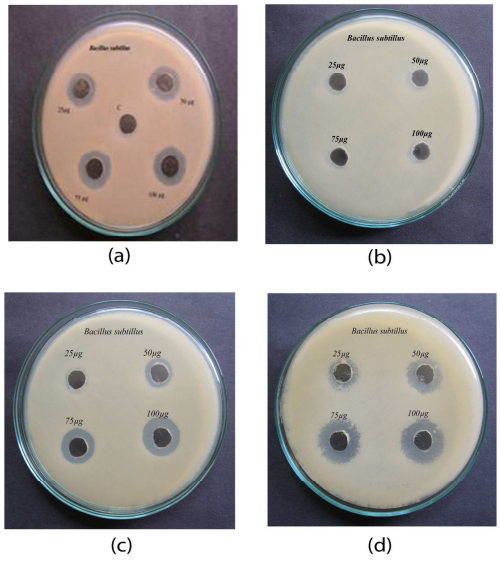

Figure 6
Antibacterial activity for *B. subtilis* using (a) Silver nanoparticle, (b) chloramphenicol, (c) Ciprofloxicin, (d) Cefotoxime.

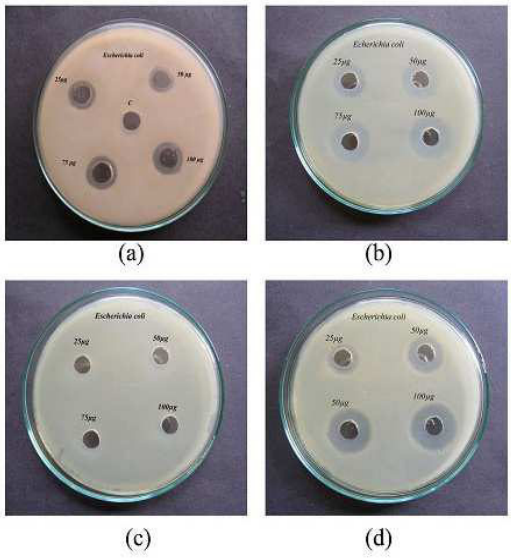

Figure 7
Antibacterial activity for *E.coli* using (a) Silver nanoparticle, (b) Amikacin, (c) Ampicilin,(d) Cefotoxime.

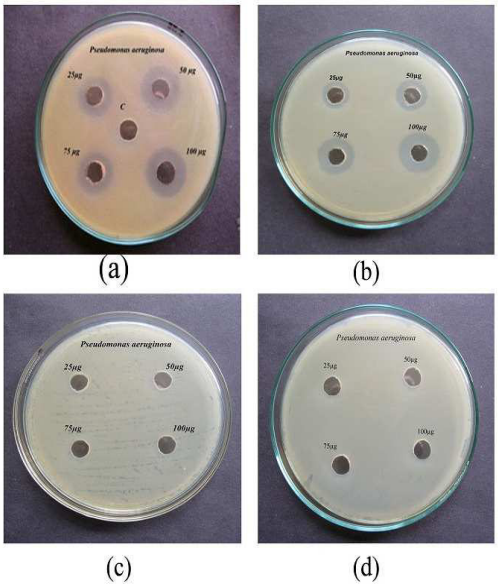

Figure 8
Antibacterial activity for *P.aeruginosa* using (a) Silver nanoparticle,(b)Amikacin, (c) Ampicilin, (d) Chloramphenicol.

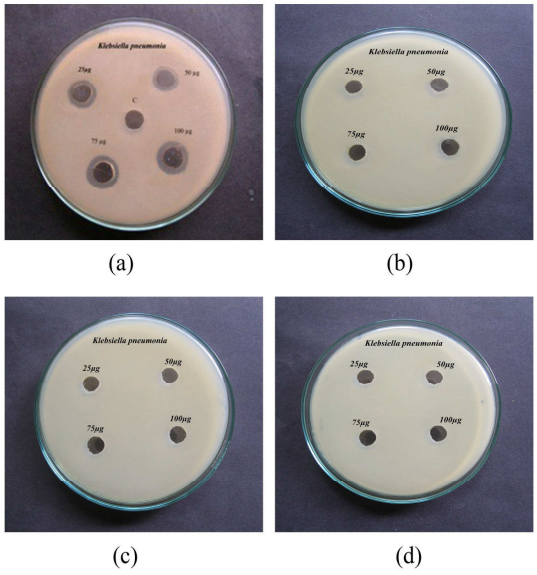

Figure 9
Antibacterial activity for *K.pneumonia* using (a)Silver nanoparticle, (b) Teracycline, (c) Ampicilin, (d) Cefotoxamine.

CONCLUSION

The microbial mediated synthesis of metal nanoparticles may replace some of the physical and chemical methods in use for nanoparticle production[25]. The present study revealed the antibacterial ability of silver nanoparticles synthesized by fungal strain *Aspergillus sp*. EV-4 is effective against bacterial species tested. Here it is concluded that the antibacterial efficiency of silver nanoparticles was more than commercial antibiotic against *Pseudomonas aeruginosa* NCIM 5029.

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