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Biogenic silver nanoparticles of resistant *Aspergillus flavus* AUMC 9834 against some pathogenic microorganisms and its synergistic effect with the antifungal fluconazole

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Abstract: The aim of this study was the biosynthesis of silver nanoparticles (AgNPs) by *Aspergillus flavus* AUMC 9834 isolated from oral suspension of β-lactam antibiotic Cefpodoxime proxetil. Antimicrobial activity and the synergistic effect of AgNPs in combination with the antibiotic fluconazole against resistant fungus were investigated. The synthesized AgNPs from cell-free filtrate were characterized by using UV-VS spectrophotometer analysis, Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD) and Transmission Electron Microscopy (TEM). UV-VS spectrophotometer analysis showed a peak at 420 nm corresponding to the plasmon absorbance of silver nanoparticles and FTIR analysis showed the potential biomolecule responsible for the reduction of silver. The structural properties of silver nanoparticles were confirmed using XRD technique, while TEM micrographs revealed that the silver nanoparticles are dispersed and aggregated, and mostly having spherical shape within the size range between 5 and 19 nm. The synthesized silver nanoparticles plus fungal filtrate exhibited a varied growth inhibition activity against the tested pathogenic bacteria. A significant increase in the area of growth inhibition was observed when a combination of silver nanoparticles and fluconazole was applied. The current results revealed that the synthesized silver nanoparticles produced by *A. flavus* strain are promising for the use in medical therapy, due to their broad spectrum against some pathogenic bacteria and resistant tested fungus.

Key words: *A. flavus* strain, biosynthesis, silver nitrate nanoparticles, synergism with fluconazole, antimicrobial activity.

Introduction

Green nanotechnology is the utilization of microbial resources for the fabrication of nanoparticles. Nanoparticles have been synthesized using various fungi (Ahmad *et al*. 2003, Bhainsa and D'souza 2006), bacteria (Nanda and Saravanan 2009) and plants (Kumar and Yadav 2009). When comparing with chemical and physical methods, biological synthesis of nanoparticles has proved to be free of any limitations associated with production of hazardous by-products, and it is simple and cost effective (Singh *et al*. 2011). Microbiological synthesis can take place either intracellularly (Seshadri *et al*. 2012) or extracellularly (Bhambure *et al*. 2009) .The mechanism underlying the synthesis of silver nanoparticles (AgNPs) by fungi can also be predicted. Biosynthesis is basically associated with the reducing mechanism of the cellular components (Bhattacharyya *et al*. 2009). The specific position of reductase enzymes was typically cited (Mukherjee *et al*. 2001, Bhainsa and D'souza 2006). The silver ions were subsequently reduced to AgNPs by enzymes present at the fungal cell surfaces (Bhainsa and D'souza 2006, Jain *et al*. 2011, Mukherjee *et al*. 2001). The reduction process was also facilitated by certain extracellular enzymes like naphthoquinones and anthraquinones (Ahmad *et al*. 2003). Metal nanoparticles of silver, copper, and gold have been found to be active against certain pathogenic bacteria and fungi (Kim *et al*. 2008, Rai *et al*. 2012). Comparatively, AgNPs have been intensely studied owing to their distinct properties such as, chemical stability, nonlinear optical behaviour, and bactericidal activity (Vaidyanathan *et al*. 2009, Kollef *et al*. 2011, Rai *et al*. 2012). These properties make them suitable for use as a microbial disinfectant. The production of AgNPs is relatively inexpensive, and the addition of these particles into goods such as plastics, clothing, creams, and soaps increase their market value (Allahverdiyev *et al*. 2011). New classes of compounds that include nanoparticle–antibiotic conjugates are undergoing clinical evaluations (Li *et al*. 2005, Fayaz *et al*. 2010, Sekhon 2010, Kollef *et al*. 2011, Pissuwan *et al*. 2011, Rai *et al*. 2012). The combination of antibiotics and metal nanoparticles could increase the antibiotics' efficacy against resistant pathogens (Li *et al*. 2005, Naqvi. *et al*. 2013). Moreover, nanoparticle antibiotic conjugates lower the amount of both agents in the dosage, which reduces noxiousness and increases antimicrobial properties. These conjugates are effective

against resistant bacteria. Additionally, due to this conjugation, the concentrations of antibiotics are increased at the place of antibiotic–microbe contact (Fayaz *et al*. 2010, Li *et al*. 2005).

The present research was carried out in response to the significance of biological synthesis of nanoparticles and the implications of their use in controlling pathogenic microbes. Biogenic silver nanoparticles of fluconazole-resistant *Aspergillus flavus* AUMC 9834 isolated from oral suspension of beta-lactam antibiotic Cefpodoxime proxetil was evaluated against some pathogenic microorganisms as well as its synergistic effect with the antifungal fluconazole towards the resistant fungus.

Materials and Methods

Microorganism

The strain of *A. flavus* isolated from oral suspension finished product (cepodem 40mg/5 ml dry mix) was used for the synthesis of nanoparticles. Cepodem contains the active ingredient Cefpodoxime proxetil which is a semi-synthetic beta-lactam antibiotic belonging to the third generation of oral cephalosporin group produced by T3A pharmaceutical company, Assiut, Egypt. The fungus was isolated on Sabouraud Dextrose Agar medium (SDA), characterized and identified according to Raper and Fennell (1965) and Moubasher (1993) and was maintained at 4°C at Assiut University Mycological Center (AUMC 9834).

Assay for the synthesis of nanoparticles

The mycogenesis of AgNPs was performed using *A. flavus* AUMC 9834. The fungal biomass was cultivated aerobically in a broth medium of the following composition (g / l): KH_2PO_4 7; K_2HPO_4 2; $MgSO_4.7H_2O$ 0.1; $(NH_2)SO_4$ 1; yeast extract 0.6; glucose 10; pH 5.8 (Fayaz *et al*. 2010) and incubated at 22°C for 72 h in an orbital shaker at 200 rpm. At the end of incubation period, the fungal biomass was filtered using filter paper Whatman no 1. The cultural filtrate was later used for nanoparticle synthesis. About 200 ml of mycelium-free cultural filtrate containing 0.1 M precursor salt AgNO3 were disposed in a 500 ml Erlenmeyer flask. The flask was incubated in dark condition at 28°C on a shaker at 150 rpm for 72 hours (Bhainsa and D'souza 2006).

Characterization of silver nanoparticles
Visual observations

The formation of AgNPs was followed by visual observation of colour change into reddish color that indicates the reduction reaction (Mukherjee *et al*. 2001).

UV-Vs spectral analysis:

The reduction of pure Ag+ was further confirmed by the sharp peak given by scanning the AgNPs of the reacting solution using Perkin-Elmer Lamda-45 spectrophotometer, in a 1 cm path quartz cell at a resolution of 1 nm from 300 to 800 nm (Vahabi *et al*. 2011).

Fourier transforms infrared spectroscopy (FTIR)

FTIR spectra of dried synthesized AgNPs were recorded using FTIR Nicolet Avatar 660 FTIR spectrometer (Jeevan *et al*. 2012).

X-ray diffraction (XRD) analysis:

The synthesized AgNPs were dried and the powder form of the sample was subjected for XRD analysis using X-ray diffractometer (Model PW 1710 control unit Philips, Anode material Cu, 40 KV, 30 M.A, optics: automatic divergence slit) with Cu Kα radiation λ=1.540562 °A (Prema and Raju 2009).

Transmission electron microscopy (TEM) analysis

The morphology of the AgNPs was investigated by TEM using JEOL- JEM-100 CXII instrument in the electron microscope unit in Assiut University, by drying a drop of the washed colloidal dispersion onto a copper grid covered with a conductive polymer (Li *et al*. 2011).

Antimicrobial activity of fungal filtrate and AgNPs by agar well-diffusion method

Silver nanoparticles (AgNPs) synthesized from *A. flavus* AUMC 9834 and mixed with fungal filtrate were tested for their antimicrobial activity by the well-diffusion method against various pathogenic microorganisms. The tested microorganisms were used, namely gram-negative bacteria (*Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027) and gram-positive bacterium (*Staphylococcus aureus* ATCC 6538P), and the fungal strains of *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. Each strain was spread over the surface of nutrient agar using sterile cotton swab and wells of size 6 mm diameter were made with the help of a sterilized cork borer. Different volumes (50, 25, 15 µl) of the AgNPs and the fungal filtrate mixture were loaded separately into wells and the plates were incubated at 35 C° for 24 h in case of bacterial strains and at 28º C for 72 h for fungal strains. The zones of inhibition were measured and the lowest concentration of AgNPs and fungal filtrate mixture that inhibited the growth of each of the test microorganisms was determined as the minimum inhibitory concentration (MIC) (Kareem *et al*. 2008; Silambarasan and Jayanthi 2013).

Evaluation of synergistic effects of AgNPs combined with Fluconazol antibiotic

The resistant strain *A. flavus* AUMC 9834 was also used for studying the synergism between synthesized AgNPs and fluconazole. Well-diffusion method on nutrient agar plates was used to assess the synergistic effect. Different concentrations of the tested antifungal (200, 150, 100, 50, 25 and 12.5 µg /ml) were prepared to determine the MIC. A twenty five µl of freshly prepared AgNPs and fungal filtrate mixture, and 25 µl of each concentration of the antifungal were loaded into each well as the final content of 50 µl of AgNPs and antifungal per well (Silambarasan and Jayanthi 2013). After incubation at 28°C for 24 hours, the inhibition zones were measured; the assays were performed in triplicates.

Statistical analysis

Results were compared statistically using Graphpad Prism v5.00 statistical software. The results are presented as mean ± standard deviation (S.D.). Statistical significance was set up at $p < 0.05$.

Results

Characterization of silver nanoparticles

Visual observation

The cultural filtrate of the filamentous fungus *Aspergillus flavus* AUMC 9834 was used for the biosynthesis of silver nanoparticles using 0.1 M silver nitrate salt. The colour changes to reddish after 24 h of incubation in the dark indicating the nanoparticles biosynthesis, comparable to the control (fungal filtrate without silver nitrate) which shows no color change (Fig. 1).

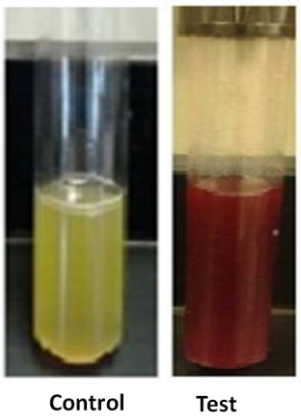

Fig 1: Biosynthesis of AgNPs by *A. flavus* AUMC 9834: Colour change test.

UV-VS spectral analysis

Bioreduction of silver nitrate ions was confirmed by the sharp peak given by the AgNPs in the visible region from UV–VS spectrum of the reaction solution at 420 nm (Fig 2). The spectrum showed a strong surface Plasmon absorption band indicating the presence of AgNPs**.**

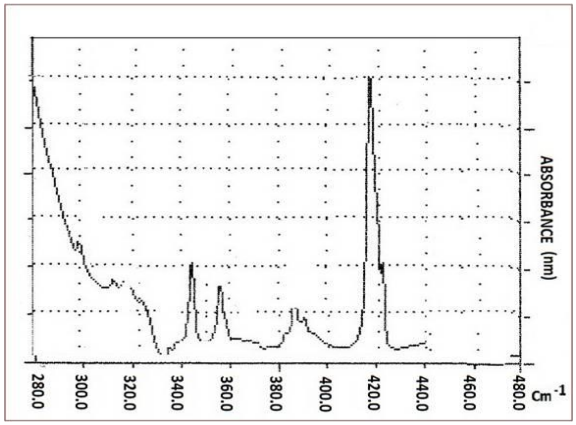

Fig 2: UV-VIS spectral analysis of AgNPs absorption band around 420 nm.

FTIR analysis of Ag NPs

The nature of the biomolecules involved in the reduction and formation of AgNPs was studied by FTIR (Fig 3). The FTIR signals of AgNPs were observed at 1072, 1365, 1648, 2145, 2350, 2933 and 3260 cm^{-1}. FTIR measurement was carried out to identify the potential biomolecule in enzyme filtrate responsible for the reduction of silver ions and capping agent responsible for the stability of the bioreduced silver nanoparticles**.** The FTIR spectra in 1400-1700 cm^{-1} region provide information about the presence of -C=O and -N=H groups which is responsible for the reduction of $AgNO_3$ to Ag. The bands in the region between 3260 and 2145 cm^{-1} were assigned to O-H stretching of alcohols and phenol compounds, and aldehyde -C-H-stretching of alkanes. The peaks in the region 1072, 1365 and 1648 cm^{-1} correspond to N-H of primary and secondary amides and –C-N-stretching vibrations of amines or –C-O-stretching of alcohols, ethers, carboxylic acids and anhydrides (Ninganagouda *et al.* 2013).

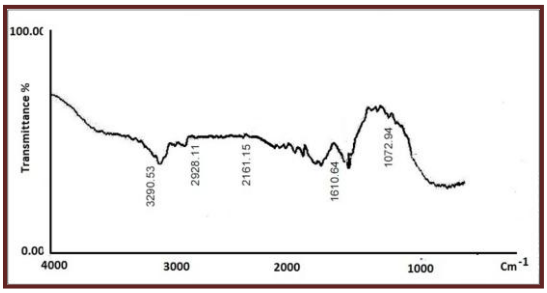

Fig. 3: FTIR spectra of synthesized AgNPs from *A. flavus* AUMC 9834.

X-ray diffraction (XRD) analysis:
The crystalline nature of the particles was confirmed using XRD. Figure 4 shows x-ray powder diffraction patterns of the synthesized AgNPs at 80º C. The line positions are consistent with metallic silver (Mc Clune 1993). The peaks assigned in the AgNPs sample were 108, 132, 206, 222 and 316 Aº, number of Bragg reflections with 2Ɵ values of 27.76°, 32.18°, 46.17°, 38.08°, 46.17°, 54.82°, 57.41°, 67.48°, 74.56° and 76.75Aº (ASTM No. 04-014-6889).

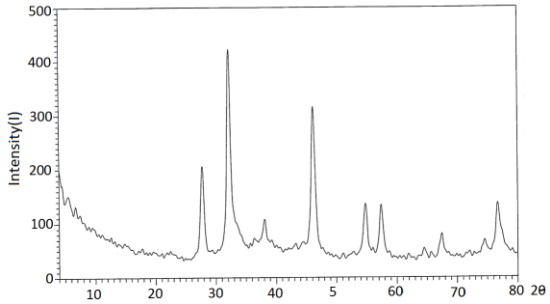

Fig 4: X-ray diffraction (XRD) pattern show silver nanoparticles synthesized from *A. flavus* AUMC 9834.

Transmission Electron Microscopy (TEM) analysis
The TEM image of AgNPs (Fig 5) shows that the particles were spherical in shape and the size of the AgNPs was found to be in the range from 5 to 19 nm.

Fig 5: Transmission Electron Microscopy (TEM) of AgNPs synthesized from *A. flavus* AUMC 9834.

Antimicrobial activity and Synergistic effect of AgNPs in combination with fluconazole

The silver nanoparticles mixed with fungal filtrate exhibited good antibacterial activity against Gram-negative bacteria, the inhibition zone in case of *E. coli* ATCC 8739 was 13.33±2.30 mm, while in *P. aeruginosa* ATCC 9027 was 15.00 ±2.00 mm. In case of Gram-positive bacterium, *S. aureus* ATCC 6538P, they showed inhibition zone of 12.66 ±1.15 mm. They also showed antifungal activity against *C. albicans* ATCC 10231 of 18.33 mm±0.57 and *A. niger* ATCC 16404 of 19.33 ±1.52 mm. The inhibition activity of fungal filtrate combined with silver nanoparticles against test strains were significantly promoted by the fold increase ranging from 25 - 66.2 %. On the other hand, biosynthesized AgNPs induced significantly higher antifungal activity when combined with fluconazole antifungal against resistant *A. flavus* AUMC 9834 than that of the fungal filtrate or fluconazole antifungal alone (Table 1).

Discussion

The scope of the present study was based upon exploring the capability of *A. flavus* AUMC 9834 to synthesize AgNPs and its possible biomedical application in controlling infection. The data showed a great deal of capability of synthesizing AgNPs. In addition, these nanoparticles alone and in conjugation with fluconazole proved to be effective in inhibiting the growth of the resistant fungus and some pathogenic bacteria. Fluconazole inhibits cytochrome P_{450}-dependent enzymes (particularly C-14- demethylase) involved in the biosynthesis of ergosterol, by altering the cell membrane function and permeability, resulting in cell dysfunction (Al-Badriyeh *et al.* 2009).

When the reaction mixture was incubated under dark conditions, the colour of the liquid mixture changed to reddish after 24 hours of reaction as the first indication of AgNPs biosynthesis. The change in color intensity (absorbance) was also monitored through ultraviolet-visible spectroscopy; peaks of the reaction mixture were obtained around 420 nm (Fig 2). It was reported that the reduction of Ag^+ to atomic silver Ag^o corresponds to absorption at 420 nm (Li *et al.* 2005, Fayaz *et al.* 2010). Various reports have provided evidence of extracellular generation of AgNPs by XRD and TEM images (Prema and Raju 2009, Narasimha *et al.* 2011). Also, several studies have proposed that AgNPs may attach to the surface of the cell membrane disturbing permeability and respiration functions of the microbial cell **(**Singh and Raja 2012, Rai *et al.* 2014). Smaller AgNPs having the large surface area available for interaction would give more bactericidal effect than the larger AgNPs **(**Singh and Raja 2012, Rai *et al.* 2014**).**

The mechanism of action of AgNPs is still not well defined. Many proposed mechanisms about antibacterial effect of silver ions have been presented by several researchers (Davies and Etris 1997, Li *et al.* 2005, Fayaz *et al.* 2010, Kollef *et al.* 2011, Rai *et al.* 2012). Being positively charged, they attack negative charges of transmembrane proteins and could destroy the cell membrane and block the transport channels (Davies and Etris 1997). It might be possible

that they penetrate inside the microbial cell and disrupt cellular activities like transportation, protein synthesis, and nucleic acid functioning (Senapati *et al.* 2005). It has also been proposed that silver ions penetrate the cell, intercalate themselves between pyrimidine and purine, and denature the DNA molecule (Rai *et al.* 2012).

When the AgNPs, have large surface area, were combined with the antifungal antibiotic fluconazole, the fungicidal effect is enhanced by interaction between active groups like hydroxyl and amino groups present in this antifungal with AgNPs by chelation (Batarseh 2004). As a result, antifungal–AgNP conjugate is formed in which an AgNPs core is surrounded by antibiotic molecules. Thus, the antimicrobial concentration is increased at the focal site, which leads to increased disruption of peptidoglycan of bacteria (Fayaz *et al.* 2010, Li *et al.* 2005). Likewise, fluconazole in combination with positively charged AgNPs both inhibited and disrupted cell-wall synthesis (Sekhon 2010, Herman and Herman 2014). More studies are needed to find out the exact mechanism of action to develop a novel potent antimicrobial drug against resistant fungus.

Table 1: Antimicrobial activity of the fungal filtrate, fungal AgNPs, fluconazole and fluconazole + AgNPs against some microorganisms (expressed as zone of inhibition in mm ± SD).

Test microorganisms	Fungal filtrate only[a]	Fungal filtrate AgNPs Only[b]	Fluconazole only (MIC)[c]	[Fluconazole (MIC)+filtrate AgNPs][d]	Fold increase%*	P value
	(50 µl)	(50 µl)	(50 µl)	25 µl + 25 µl		
E. coli ATCC 8739	8.00±1.00	13.33±2.30	-	-	66.25	p < 0.05
P. aeruginosa ATCC 9027	10.33±0.75	15.00±2.00	-	-	45.63	
S. aureus ATCC 6538P	9.33±1.52	12.66±1.15	-	-	35.69	
C.Albicans ATCC 10231	14.66±0.57	18.33±0.57	-	-	25.03	
A.niger ATCC 16404	12.66±1.56	19.33±1.52	-	-	52.68	
A.flavus AUMC 9834 (Resistant strain)	6.3±1.52	8.61±0.57	-	-	36.66	
			8±1.00	11.6±1.52	45	

MIC of Fluconazole = 200 µg/ml; *Percentage fold increases of antimicrobial activities were calculated using the formula ((b − a)/a) × 100 or (d − c)/d) × 100 (Fayaz *et al*, 2010).

Conclusion: Antifungal-resistant fungi have been continuously increasing over the past decade; hence, there is a need to detect another method to overcome this problem. In the present scenario, AgNPs have appeared as a promising antimicrobial candidate in the medical field. Hence it could be potentially applied in the fabrication of silver impregnated antimicrobial materials for biomedical applications. The present results demonstrated the synthesis of silver nanoparticles by *A. flavus* AUMC 9834 at room temperature. Within 24 hours of time, spherical-shaped nanoparticles below 20 nm size were formed. The method described is highly efficient and cost effective to produce silver nanoparticle. The activity of antifungal antibiotic fluconazole against resistant *Aspergillus flavus* AUMC 9834 was augmented when impregnated with AgNPs.

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