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Mycobased Biosynthesis of Silver Nanoparticles and its Synergistic Antibacterial Activity Combined with Ofloxacin and Moxifloxacin

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The use of microbes in the synthesis of silver nanoparticles emerges as a cost effective and eco-friendly approach. In the present study, the biosynthesis of silver nanoparticles using *Aspergillus flavus* and its antibacterial properties were evaluated. The biologically synthesised silver particles are characterized by UV-Vis spectrophotometry. The silver nanoparticles showed the absorbance peak at 409nm in the UV-spectrophotometer. These silver nanoparticles were further characterized by Fourier Transform Infrared (FTIR) spectroscopy, Atomic force microscopy (AFM). The AFM study of silver nanoparticles showed the shape and size of individual nanoparticles as well as it focused a few aggregates having the size in the range of 55-70nm.The bactericidal action of biologically synthesised silver nanoparticles against gram positive and negative bacteria was found with high potentiality. When Ofloxacin (5 mcg ml^{-1}) and Moxifloxacin (5 mcg ml^{-1}) were combined with silver nanoparticles (20µg/ml^{-1}), it resulted in greater bactericidal efficiency on selected bacterial pathogens. The results confirmed that the solutions with more silver nanoparticles had better antimicrobial effects which would be the novel remedy substituent in the place of higher doses of antibiotics.

Key words: Silver nanoparticles, FTIR, AFM, Antibacterial activity, Ofloxacin, Moxifloxacin.

Green nanotechnology has emerged as significant division in the field of research from last decade for the synthesis and characterization of noble metals such as gold, silver, platinum, and palladium. In addition to this, physical and chemical methods are also used for producing controllable nanoparticles with well divined shapes and sizes[1]. Green synthesis of nanoparticles is not useful only due to its reduced environmental impact as compared with physio-chemical methods but can be used to produce in bulk quantities free from contamination with well defined size and morphology[2-5]. Among various noble metals, silver is the most effective agent used for the treatment of disease, preservation of food and keeping water safe and it is widely commercialised nanomaterials[6]. Silver nanoparticles having broad spectrum properties are being used as novel therapeutic agents with wide biomedical applications used in medical and consumer products such as antimicrobial, anti-inflammatory and anti-cancer agents including household antiseptic sprays, cosmetics, clothes and antimicrobial coatings for medical devices that sterilize air and surfaces[7-10]. While synthesised biologically, silver nanoparticles showed bactericidal properties as per our earlier reports[11-12]. The silver nanomaterials are among the most promising antimicrobial agents have wider effect against pathogenic organisms, which needs

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detail study. In this work, we have done the synthesis and characterisation of eco-friendly extracellular silver nanoparticles using an air fungal isolate, *Aspergillus flavus*. The antimicrobial activity of synthesized silver nanoparticles was carried out individually and also in combination with different antibiotics viz., Ofloxacin and Moxifloxacin against pathogenic bacteria using agar disc diffusion method.

MATERIALS AND METHODS

Isolation of *Aspergillus flavus*

The airborne fungi were isolated from indoors of the working environment of Sathyabama University by exposing Sabouraud's Dextrose agar media plates for 5 minutes based on gravitation method and incubated for 3-7 days in the Microbiology and Biomedical Research Laboratory, Sathyabama University, Chennai for their enumeration. *Aspergillus flavus* was isolated, pure cultured and stored in a refrigerator at 4°c for further studies.

Synthesis of silver nanoparticles

Silver nanoparticles were prepared for this study following the procedure described as per our earlier reports[13]. Seven day old culture of *Aspergillus flavus* was inoculated, grown aerobically in Erlenmeyer flasks and incubated on orbital shaker at 25°C and agitated at 140rpm. After 72 hours, the biomass was filtered using Whatman filter paper No.1 followed by three time wash with Milli-Q water in order to remove the residues. The fresh and clean biomass was taken into the Erlenmeyer flasks containing 100ml of Milli-Q deionised water and further it was incubated at 25°C in a shaker at 140 rpm for 72 hours. After the period of incubation the aqueous solution components were separated by filtration and the cell free extract was used with metal ion solution for the reduction of metals. For intracellular synthesis, the biomass was subjected to sonication for 10minutes followed by centrifugation at 12000rpm for 10 minutes. Both pellet and supernatant were separated. 1mM of $AgNO_3$ was poured into supernatant and pellets each and kept in dark condition. Fungal mycelium was simultaneously incubated in Milli-Q deionised water without adding silver nitrate as positive control.

Instruments Used

The preliminary detection of AgNPs was carried out by visual observation based on colour change in the solution. Periodically, small aliquots (1ml) of the reaction solution of supernatant was withdrawn and the absorbance was measured in between the ranges of 350-700nm against culture suspension without silver nitrate as control after 24hrs. Observation peak was being measured continuously to check their stability through T-60 UV Vis-spectrophotometer (T-60, PG Instruments Ltd. *Lutterworh*, United Kingdom) after change in the solution colour. The synthesised nanoparticles were freeze dried into powder form and diluted with potassium bromide for Fourier transform infrared spectrophotometer (FTIR). The samples were scanned using infrared in the range of 4000-400cm^{-1} at a resolution of 4cm^{-1}using Fourier Transform Infrared Spectrometer (Thermo Nicolet Model-6700). The synthesised nanoparticles were further characterised through Atomic Force Microscope (AFM); NTMDT, Ireland, for topography, particle size and agglomeration of nanoparticles through three dimensional images. The sample used for AFM study was sonicated for 7minutes, centrifuged at 1000rmp for 5minutes and a small volume of sample was spreaded on well cleaned glass cover slip and dried at room temperature for analysis.

Determination of Bactericidal activity

The efficacy of metal ions (AgNPs) was determined by performing antimicrobial susceptibility test using disk diffusion method against clinical bacterial pathogens following NCCLS guidelines. Bacterial stains viz., *Bacillus cereus, Escherichia coli, Proteus vulgaris, Staphylococcus aureus* and *Vibrio chorea* were grown separately on nutrient agar medium (Qualigens, India). The agar plates (Hi-Media) was prepared using distilled water and sterilized in autoclave at 15lb/in^2 for 15 minutes as directed by the manufacturer. Inoculum was prepared from fresh culture of the microbial strain, kept for 18-24hrs at 37°C. Three to five colonies of microbial strain was inoculated into a tube containing 4- 5ml of freshly prepared Nutrient broth (Hi-Media) and incubated for 2-4hrs to standardize the culture to *0.5McFarland* standards CFU/ml. Inoculums density was compared with *Mac Farlands* standard solution of $BaSO_4$ (0.1 ml of 1% $BaCl_2$

+9.9 ml of 1% H_2SO_4). The inoculums suspension with the help of swab was inoculated on Petri plates by streaking over the entire sterile agar surface. Hi media; sterile disk (6mm) were kept on solid agar in the centre along with the antibiotics, Ofloxcian and Moxifloxacin. To determine the bactericidal effect, each strile disk was treated with biologically synthesised silver nanoparticle at 20¼g/disk. The zone of inhibition was measured and compared with the control. While performing the synergistic effect of antibiotics with extracellularly synthesised silver nanoparticles, each standard antibiotic disk was further impregnated with 20µg/disk of freshly prepared AgNPs along with standard antibiotics, Ofloxacin (5 mcg/ml) and Moxifloxacin (5 mcg/ ml) on agar plates and incubated at 37°C for 18-24hrs. The antibacterial assay was done in triplicate.

Statistical analysis

The assays were performed in triplicate and the representative data is presented in the research paper. For bactericidal assays, arithmetic mean values were taken into consideration for data analysis. t-test was performed for comparative analysis of unpaired data.

RESULTS AND DISCUSSION

The isolated fungal colonies observed by the author's expertise and laboratory manuals based on the colony morphology with respect to colour, shape, size and nature of colonies and was identified as *Aspergillus flavus* (Fig 1: a & b).

Synthesis of Nanoparticles

The interaction between fungal cultured filtrate containing extracellular component and metal ion was observed by colour change from chalky white before the addition of 1mM silver nitrate solution into brownish colour on completion of reaction with Ag+ ions after 24hrs (Fig 2: a & b). The appearance of yellow brown colour in the silver nitrate treated flask indicated the formation of silver nanoparticles due to the reduction of metal ions and plasmon resonance.

Instrumental Analysis

The analysis of synthesised silver nanoparticles was initially performed by Uv-Vis spectroscopic analysis. The presence of absorption spectrum of silver nanoparticles prepared by biological reduction showed a surface plasmon absorption band with a maximum of about 409nm (Fig 3) is the characteristic of silver nanoparticles[14]. The observation of this peak was measured at regular intervals to determine their stability.

FTIR analysis of the freeze-dried samples of silver nanoparticles was carried out to identify the possible interactions between silver and bioactive molecules of fungi, which may be responsible for synthesis and stabilization of silver nanoparticles. Sanghi and Verma[15] suggested that the amide linkages between amino acid residues in proteins may give rise to well known signatures in the infrared region of the electromagnetic spectrum for its stability. In the FTIR spectrum analysis (Fig 4), different peaks were at 2921 (C-H stretch) and 1643cm^{-1}, (C=O symmetric stretch of amide), 1533 N-H bend of amide II, while their corresponding stretching vibrations were seen in 2921cm^{-1}(C-H stretch) the presence of signature peaks of amino acids supports the presence of proteins in cell-free filtrate as observed in UV-Vis spectra. Other peaks were at 1387 cm^{-1} (CH_3 bend of alkanes), 1083cm^{-1} (C-N stretch of aliphatic amines), 696cm^{-1} **(**acetylenes C-H bend of alkynes**)**, which suggests the binding of protein to nanoparticles either through free amine groups and cysteine residues or through electrostatic attraction of negatively charged carboxylate groups presents in cell wall of mycelia, which may be stabilizing the silver nanoparticles[16].

The topological appearance, porosity, roughness and fractal dimension of biologically synthesised silver nanoparticles was evaluated by analysing the AFM image analysis and the size of the biosynthesized silver nanoparticles was found in the range of 55-70 nm (Fig 5). During the analysis, we found that silver nanoparticles formed were predominantly spherical and poly dispersed. The image study was used to find average particles size analysis of the silver nanoparticles and to analyse the average roughness and particle in homogeneity (Fig 5), which was also confirmed by earlier workers[17].

The biologically synthesised nanoparticles were further analysed to determine the surface morphology by using FESEM and size of nanoparticles. The nanoparticles distributed uniformly showed that they were dispersed densely and having smooth surfaces and rough surfaces. The nanoparticles appearance showed

Table 1. Comparison of antibacterial activity of silver nanoparticles alone and in combination with antibiotics (Ofloxacin) and filtrate against pathogens

Pathogens	Zone of inhibition (mm)			
	Filtrate	AgNPs	Ofloxacin	Of. + AgNPs
Staphylococcus aureus	07±0.71	14±0.23	20±0.88	27±0.63
Bacillus cereus	07±0.91	12±0.32	18±0.12	24±0.54
Proteus vulgaris	08±0.23	15±0.26	19±0.72	26±0.13
Escherichia coli	07±0.39	16±0.44	20±1.0	25±0.54
Vibrio cholerae	08±0.12	15±0.51	21±0.36	26±0.38

Table 2. Comparison of antibacterial activity of silver nanoparticles alone and in combination with antibiotics (Moxifloxacin) and filtrate against pathogens

Pathogens	Zone of inhibition (mm)			
	Filtrate	AgNPs	Mox.	Mox. + AgNPs
Staphylococcus aureus	07±0.71	14±0.23	20±0.71	26±0.47
Bacillus cereus	06±0.39	16±0.44	21±0.78	27±0.76
Proteus vulgaris	07±0.23	15±0.26	20±0.31	24±0.24
Escherichia coli	08±0.91	12±0.32	15±0.34	21±0.23
Vibrio cholerae	06±0.12	15±0.51	13±0.12	20±1.0

that they were spherical to ovate in structure with having average dimensional size in the range of 50-70nm.

(a) (b)

Fig. 1. *Aspergillus flavus* a) Macroscopic view b) Microscopic view

(a) Before addition of $AgNo_3$ (b) After addition of $AgNo_3$

Fig. 2. Synthesis of silver nanoparticles from *Aspergillus flavus*

Bactericidal activity

The bactericidal activity of silver nanoparticles[18] and its comparative analysis in a synergistic mode with Ofloxacin and Moxifloxacin was studied against gram positive and gram negative bacteria and was found valuable as nanomedicine. The antibacterial activities of Ofloxacin and Moxifloxacin have increased in the presence of silver nanoparticles against the bacterial strains (Fig 6). The synergistic effect of silver nanoparticles represented the highest percentage of increase in inhibition Table 1 & 2. Ofloxacin showed good activity against *Staphylococcus aureus* (27±0.60) followed by *Proteus vulgaris* (26±0.30), *Vibrio cholerae* (26±0.381), *Escherichia coli* (25±0.54) and *Bacillus cereus (24±0.54)* (Table 1 and Fig 7), while Moxifloxacin showed good activity against *Bacillus cereus* (27±0.76) and *Staphylococcus aureus* (26±0.47) followed by *Proteus vulgaris* (24±0.24), *Escherichia coli* (21±0.23), *Vibrio cholerae* (20±1.0) (Table 2 and Fig 8). Ofloxacin along with silver nanoparticles showed equally effect on both gram positive bacteria and gram negative bacteria while Moxifloxacin showed enhanced effect on gram positive bacteria instead of gram negative bacteria. The bonding reaction between the antibiotic and silver nanoparticles enhanced the bactericidal effect against the test

organisms. The antibacterial efficacy of silver nanoparticles alone and in combined form with antibiotic showed an increase in zone diameter to ensure the contribution of biologically synthesized silver nanoparticles to nanomedicine. The AgNPs impregnated with antibiotics was found more effective against gram positive and gram negative bacteria. Three possible mechanisms have been proposed for bactericidal activity of AgNPs either attached directly with cell membrane and its binding interaction with surface area with a smaller particle size, a large surface area will have a stronger bactericidal effect[19, 20]. The other possible mechanism suggests that AgNPs are able to penetrate the bacteria cell possibly by interacting with sulphur and phosphorus-containing

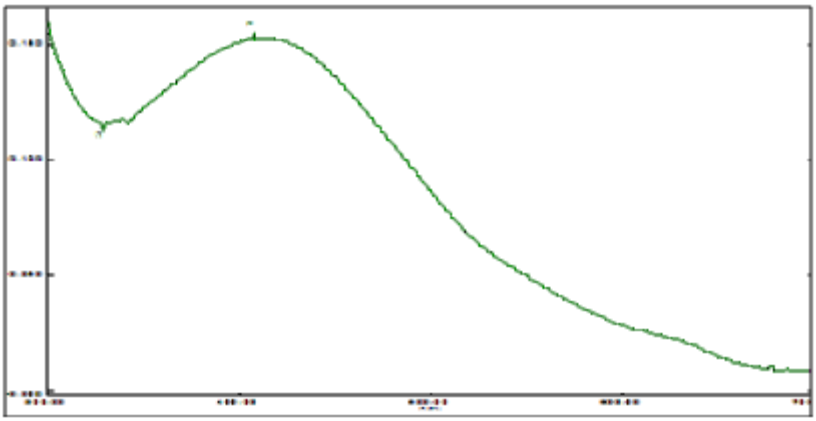


Fig. 3. Confirmation of AgNPs by UV-Spectrophotometry from *Aspergillus flavus*

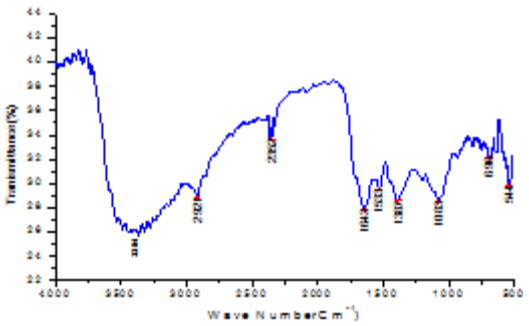


Fig. 4. FTIR analysis of Silver nanoparticles synthesized from *Aspergillus flavus*

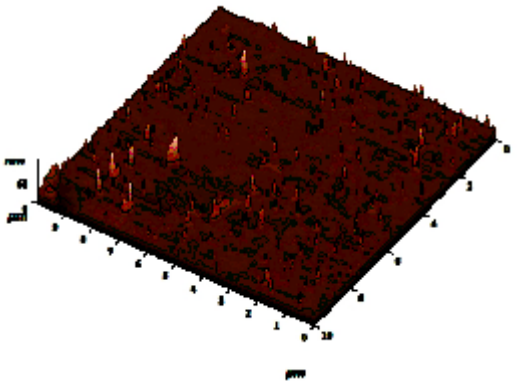
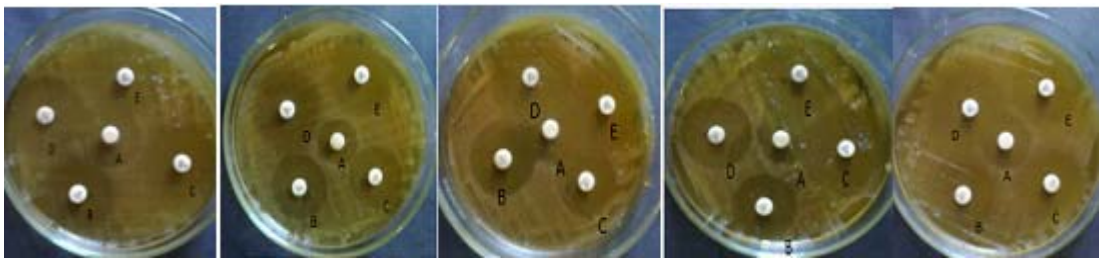


Fig. 5. AFM image of nanoparticles shows the topography and particle thickness



B. cereus A. AgNPs | *S. aureus* B. Ofloxacin | *P. vulgaris* C. Moxifloxacin | *E. coli* D. Of.+ AgNPs | *V. cholerae* E. Mox.+ AgNPs

Fig. 6. Antibacterial assay of AgNPs and its synergistic effect with antibiotics

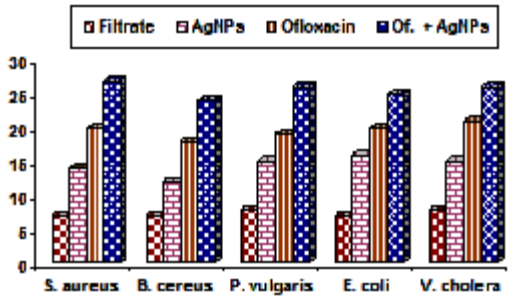


Fig. 7. Synergistic effect of Ofloxacin and AgNPs against pathogens

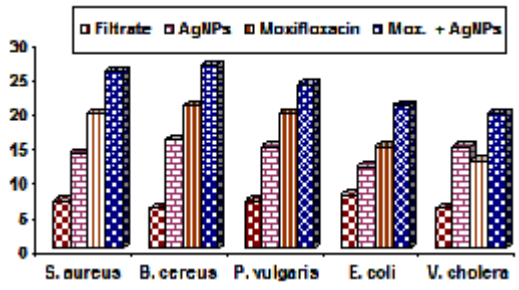


Fig 8: Synergistic effect of Moxifloxacin and AgNPs against pathogens

compounds such as DNA and cause further damage, while another mechanism suggests that the silver nanoparticles release silver ions, which may have bactericidal effect[21]

CONCLUSION

Antibacterial results had shown that the synthesised AgNPs posses discrete antibacterial activity against clinically isolated pathogens along with Ofloxacin and Moxifloxacin. But the mechanism behind the bactericidal effect of the silver nanoparticles against bacteria is not well known. We can conclude that the silver nanoparticles enhance the antibacterial activity in general but in our present finding its efficacy was further enhanced when pooled with two antibiotics viz., Ofloxacin and Moxifloxacin. Nanomaterials are considered as the leading requirements in the field biomedical research, but further studies are necessary to understand the cellular mechanism behind the biosynthesis of nanoparticles and their mode of action on pathogens.

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REFERENCES

1. Yuqing, M, Sun, K, Qiu, J, Liu, J., Preparation and characterization of gold nanoparticles using ascorbic acid as reducing agent in reverse micelles. *J. Mater. Sci.*, 2009; **44**: 754-758.
2. Dahl JA, Maddux BLS, Hutchison JE., Toward greener nanosynthesis. *Chem Rev*., 2007; **107**: 2228-69.
3. Shankar SS, Rai A, Ahmad A, Sastry M., Rapid synthesis of Au, Ag, and bimetallic Au core–Ag shell nanoparticles using Neem (Azadirachta indica) leaf broth. *J Colloid Interface Sci*., 2004; **275**: 496-502.
4. Hutchison JE., Greener nanoscience: a proactive approach to advancing applications and reducing implications of nanotechnology. *ACS Nano.*, 2008; **2**: 395-402.
5. Ravindra S, Mohan YM, Reddy NN, Raju KM. Fabrication of antibacterial cotton fibres loaded with silver nanoparticles via “green approach”. *Colloids Surf A.*, 2010; **367**: 31-40.
6. Silver S, Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. *FEMS Microbiol. Rev*., 2003; **27**:341–353.
7. Rai M., A. Yadav and A. Gade., Silver nanoparticles as a new generation of antimicrobials, *Biotechnol. Adv*. 2009; **27**:76–83.
8. Marambio-Jones C. and E. Hoek, J., A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment *Nanopart. Res*.2010; **12**: 1531–1551.
9. Chen, X.; Schluesener, H. J. Nanosilver, A nanoproduct in medical applications, *Toxicol. Lett*., 2008; **176**(1): 1-12.
10. Faunce, T.; Watal, A., Nanosilver and global public health: *international regulatory issues, Nanomedicine (London, U.K.)*, 2010; **4**: 617-632.
11. Saravanan M and Nanda A., Biosynthesis of Silver nanoparticles from Aspergillus clavatus

and its Antimicrobial Activity against MRSA and MRSE. *Colloids and Surfaces B,* 2010; **77**: 214-218.

12. Nanda A. and Saravanan M., Biosynthesis of silver nanoparticles from Staphylococcus aureus and its antimicrobial activity against MRSA and MRSE; *Journal of Nanomedicine: Nanotechnology, biology and medicine* 2009; **5**: 452–456.
13. Nayak B. K., Amin Bhat M, and Nanda Anima, Implementation of *Penicillium* sp. as raw material for synthesizing metal nanoparticles for antibiosis, *Materials Science Forum,* 2013; **760**: 33-38.
14. Mulvaney P., Surface Plasmon Spectrocopy of Nanosized Metal particles. *Langmuir.* 1996; **12**: 788-800.
15. Sanghi R, and Verma P., Biomimetic synthesis and characterisation of protein capped silver nanoparticles. *Bioresource Technol* 2009; **100**: 501-4.
16. Gole A, Dash C, Ramakrishnan V, Sainkar SR, Mandale AB, Rao M, et al. Pepsin-gold colloid conjugates: preparation, characterization, and enzymatic. *Langmuir* 2001; **17**: 1674-9.
17. Gopinath V., D. Mubarak Ali, Priyadarshini S., Meera N., Thajuddin N., Velusamy P., Biosynthesis of silver nanoparticles from Tribulus terrestris and its antimicrobial activity: a novel biological approach, *Colloids Surf. B.*, 2012; **96**: 69–74.
18. Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO. A mechanistic study of the antibacterial effect of silver ions on E. coli and Staphylococcus aureus. *J Biomed Mater Res*; 2000; **52**: 662–668.
19. Sadhasivam S, Shanmugam P, Yun KS., Biosynthesis of silver nanoparticles by Streptomyces hygroscopicus and antimicrobial activity against medically important pathogenic microorganisms. *Colloids Surf B Biointerfaces*. 2010; **81**: 358–362.
20. Chamakura, K., Perez-Ballestero, R., Luo, Z.P.; Bashir, S., Liu, J. Comparison of bactericidal activities of silver nanoparticles with common chemical disinfectants. *Colloids Surf. B,* 2011; **84**: 88–96.
21. Gibbins B, Warner L. The role of antimicrobial silver nanotechnology, *Med Device Diagnostic Indust Mag.*, 2005; **1**: 1–2.