Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(2):727-731

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Comparative antibiogram analysis of AgNPs synthesized from two *Alternaria* Spp. with amoxicillin antibiotics

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ABSTRACT

From long time ago, the use of silver nanoparticles to control the growth and prevalence of disease causing bacteria is well known. In recent times, bacteria make themselves resistant to varied antibiotics based on their genes present in the plasmids. During the present study, an effective approach was performed to synthesize potent silver nanoparticles (AgNPs) from two mould fungi; Alternaria sp. and Alternaria alternata. The appearance of yellowish brown color in the conical flask suggested the formation of AgNPs. The extract of the fungus culture changed the solution into brownish color during the reaction. The characterization of silver nanoparticles was confirmed by Uv-Vis spectroscopy, Field emission scanning electron microscopy (FESEM). Size of the nanoparticles measured between 20nm to 30nm by FESEM. The synthesized silver nanoparticles were subjected to their characterization by X-ray diffraction (XRD) technique to determine the metallic nature of nanoparticles. The XRD analysis confirmed that silver nanoparticles have been formed resulting in the diffraction peaks at 38, 45, 64 and 77 respectively showing the metallic nature of nanoparticles and peak were specific for the silver nanoparticles. Silver nanoparticles synthesized from both the fungi showed good antimicrobial activity against the selected bacterial pathogens like, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris, E. coli and Vibrio cholerae and the nanoparticles were found more effective against the test pathogens in combined with the available drugs viz., Amoxicillin (30mcg). The efficacy of the drugs was increased at high level of potency.

Key words: AgNPs, *Alternaria* spp., Antibiogram, Amoxicillin, Uv-Vis Spectrophotometer

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INTRODUCTION

Combined form of silver nanoparticles with various antibiotics show antibacterial activity [1]. Silver nanoparticle was used in medicine for its antimicrobial properties from the early of this century and more recently has been used in wound dressings and catheters. It is less reactive in comparison to silver ions, which makes it more suited to medical applications. It has also been shown to induce programmed cell death. Although the antimicrobial properties of silver have been known for long time, but we have recently understand the mechanisms by which silver inhibits the bacterial growth. The current challenge is to measure its activity, to understand the molecular level and then to determine the effective concentrations for therapeutic use. As per current report, bacteria are becoming more resistant to antibiotics, mostly superbugs like methicillin resistant *Staphylococcus aureus* in hospital communities. Alternate approach for therapies are being researched for antibacterial properties of AgNPs. Nanoparticles have put a great attention in recent days by their promising interdisciplinary fields of science which offers valuable nanomaterials of wide application in the range of areas, including catalysis, optics, mechanics and biomedical sciences [1]. In comparison to physical and chemical process, biological process has an interest because of its cost

effective and non-toxic synthesis techniques [2]. Biological synthesis of nanoparticles by bacteria, fungi, yeast and several plant extracts have been investigated due to their ability to reduce metal ions very easy way [1]. Silver nanoparticles (AgNPs) have drawn special attention owing to its immense potential as antimicrobials in health care applications. Since they are the attractive option because they are nontoxic to the human body at low concentrations and they have broad spectrum antibacterial efficacy [3]. Silver ions are very sensitive and are known to bind to the vital cell components, inducing cell death [1]. Fungal strains have the ability to resist environmental stresses and have the capability of growing in presence of high metal concentrations [4]. In the present study, biosynthesis of silver nanoparticles by extracellular method from two mould fungi; *Alternaria* sp. and *Alternaria alternata* was made and their characterization was also done with the evaluation of antibiogram against bacterial pathogens like, *Staphylococcus aureus*, *Bacillus cereus*, *Proteus vulgaris*, *E. coli* and *Vibrio cholerae* with their AgNPs and with one antibiotics. To explore and evaluate the potential antibacterial properties of silver particles, experiments were also carried out comparing the effects of nano-silver alone and in combination with traditionally used antibiotics, amoxicillin.

EXPERIMENTAL SECTION

Isolation of *Alternaria* spp

Different fungi were collected from indoor air of a working environment by exposing Sabouraud Dextrose agar for 5 minutes on media plates based on gravitation method and the plates were incubated in BOD incubator at 25±3^{o}c for 3-7 days in the Microbiology Laboratory, Sathyabama University, Chennai for their enumeration and identification. *Alternaria* sp. and *Alternaria alternata* were isolated and identified from the mixed culture of airborne fungi [5, 6], grown on pure culture and stored in refrigerator at 4°c for further experiments.

Synthesis of silver nanoparticles

Both the *Alternaria* spp were subjected to biosynthesis of silver nanoparticles. Fungal biomass was grown aerobically in a specific liquid medium containing (g/L): 2.0 K_2HPO_4; KH_2PO_4 7.0; $MgSO_4$. $7H_2O$ 0.1; $(NH_4)_2SO_4$ 1.0; glucose 10.0; yeast extract 0.6 at 25±3 ^{o}c and incubated at 25 ^{o}c in a shaker at 140 rpm for 72 hours. Just after incubation, the biomass was filtered using whatman filter paper No.1 and washed extensively with distilled water to remove all residual components. The fresh and clean biomass was taken into the Erlenmeyer flasks containing 100ml deionized Milli Q water. The flask was again incubated at 25 ^{o}c in shaker incubator at 140 rpm for 72 hours. The biomass was filtered with whatman filter paper No.1 again and the cell free extract was used for the following experiments. 1mM $AgNo_3$ was prepared and 50ml was added to the cell free extract and kept in dark condition for 48 hrs.

Characterization of silver nanoparticles

The solution in the flask was observed for change in color and maximum absorbance was analyzed using UV-spectrophotometer. After 24hours, 1ml of sample supernatant was taken and absorbance was measured by UV-visible spectrophotometer at 300-600nm. FESEM was used to determine the surface morphology and particle size of the silver nanoparticles. The sample of AgNPs were sonicated and centrifuged later at 15000 rpm for 20 minutes. Before FESEM analysis, the samples were sonicated further to get the uniformity and better observation. Later the supernatant were discarded and pellet was washed with the Milli Q water for minimum four times. Later on, samples were transferred into the Petriplate and dried for about two hours at 50^{o}c, after that the sample were subjected to FESEM analysis. XRD analysis was used to observe the crystalline and metallic nature and also face centered cubic structure of silver nanoparticles. The sample was prepared by centrifugation of the silver nanoparticle solution at 15000 rpm for 20 minutes for XRD analysis. The supernatant was discarded and the pellet was washed with Milli Q water four times and dried in petriplates. The sample in the powder form was used for XRD analysis at International Research Centre, Sathyabama University, Chennai-600119, Tamilnadu, India.

Antibacterial study of AgNPs

The silver nanoparticles were checked for its antibacterial activity by disc diffusion method [7]. The antimicrobial activity of the prepared silver nanoparticles from both *Alternaria* spp was tested against the pathogenic bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Proteus vulgaris*, *Escherichia coli* and *Vibrio cholerae*. The amoxicillin (Amox.30mcg) were taken separately as control parallel to the AgNPs to find a comparative assessment of the antibiotic efficacy over the pathogenic bacteria. The combined effects of AgNPs with antibiotics were used to find out combine effect against all bacterial pathogens.

RESULTS AND DISCUSSION

In the present study, the fungal isolates, *Alternaria* spp used in this study were isolated from indoor air of the working environment and employed for the biosynthesis of silver nanoparticles. AgNPs were synthesized by the reaction of Ag+ ions from $AgNo_3$ with the supernatants under dark conditions. After 48 h incubation, appearance of yellowish brown color in the conical flasks indicated the formation of AgNPs [8]. The supernatants of the *Alternaria* spp cultures changed the solution to a brownish color upon completion of the 24 h reaction with Ag+.

Characterization of AgNPs

The AgNPs were characterized by Uv-vis spectrophotometer and was proved to be very useful for the analysis of nanoparticles [2]. Uv-Vis spectra, a strong surface plasmon resonance were centered at approximately 430nm and 420nm indicating the presence of silver nanoparticles. It may be possible that when the silver ions come in contact with the fungal biomass, the nitrate reductase enzyme from fungus causes the reduction of silver ions into silver nanoparticles [9]. Field emission scanning electron microscopy (FESEM) was used to understand the surface topology and the size of silver nanoparticles. Analysis of AgNPs by FESEM showed spherical shaped silver nanoparticles which were well dispersed within the diameter ranges of 34 nm and 47 nm. The synthesized silver nanoparticles were further characterized by X-ray diffraction (XRD) technique to determine the metallic nature of nanoparticles. The XRD pattern clearly showed that silver nanoparticles have been formed resulting in the diffraction peaks at 38, 45, 64 and 77 respectively confirming the metallic nature of nanoparticles and peak was specific for the silver nanoparticles. The results obtained were similar to the earlier studies made by the following workers [10, 11, 12]. Abeer et al [13] reported the synthesis of silver nanoparticles from *Aspergillus terreus* strain KC46206. These biologically synthesized nanoparticles were characterized by UV-Vis spectrophotometric analysis which showed the absorption peak at 420nm. These nanoparticles were further characterized by FTIR, XRD, SEM and TEM, which showed that the particle size was in the range 5 to 30nm, spherical and well dispersed.

Comparative analysis of antimicrobial efficacy of AgNPs

Comparative antimicrobial efficacy of synthesized silver nanoparticles by disc diffusion method against five clinically isolated pathogens, *Staphylococcus aureus*, *Bacillus cereus*, *Proteus vulgaris*, *Escherichia coli* and *Vibrio cholerae* was given in Table 1. Synthesized silver nanoparticle synthesized from *Alternaria* sp. was found to be the effective one in order to prevent the bacterial growth in comparison to *Alternaria alternata*. During the study, Amoxicillin (30mcg) on its own didn't show any impressive result over the test pathogens, but the combined formulations of AgNPs with amoxicillin showed remarkable results against all the pathogens (Table 1). *Bacillus cereus* was found to be more susceptible followed by *Vibrio cholerae* in the combined formulation of amoxicillin and AgNPs (Fig 1). The antimicrobial studies showed that combined formulation of amoxicillin and AgNPs were significantly effective [1,9]. The studies confirmed that the biologically synthesized AgNPs from *Alternaria* spp amplified the antibacterial property of commercial antibiotics when used in combination. Some of the previous workers narrated their finding in the same field like us like Feng et al [13], who conducted a study to observe the effects of silver ions on gram positive and negative bacteria, *Staphylococcus aureus* and *Escherichia coli* respectively. They treated cells with $AgNO_3$, which is a source of Ag^+ in aqueous environments and looked at the structural and morphological effects of these silver ions on the cells. During the study, they exposed cells to $AgNO_3$ for 4 to12 hours before being prepared for microscopy and then fixed and sliced with an ultramicrotome to produce ultrathin sections for transmission electron microscopy (TEM). They observed that cells exposed to the Ag^+ ions seemed to have activated a stress response that led to the condensation of DNA in the center of the cell. They also observed that the cell membrane detachment from the cell wall damage and electron dense granules outside and in inside the cell. It was found that condensation of DNA occurred as a protective measure in order to protect the genetic information of the cell [14], however condensation of DNA could also prevent cell replication by preventing the DNA from being accessed by transcriptional enzymes such as DNA polymerase. In their work, electron dense granules which formed inside and outside the cell were extracted and subjected to X-ray microanalysis to determine their composition. It was found that the granules were in part composed of silver and sulfur. This finding supports our idea that silver inactivates proteins by binding to sulfur-containing compounds [15]. It was also recorded that when treated with Ag^+, *E. coli*, a gram negative bacterium, sustained more structural damages than the gram positive *S. aureus* [14,18]. It was also been shown that treating cells with silver leads to cell shrinkage and dehydration leads to the death of the bacteria [16]. Feng et al [14] confirmed that the cells that sustained extensive damage eventually ended up with cell membrane damage, which would lead to the leaking of cytoplasm from the cell and result in dehydrated and shrunken cells agreed with Guggenbichler et al [16, 19]. Klueh et al [15] opined that silver forms stable S-Ag bonds with thiol-containing compounds in the cell

membrane that may be involved in trans-membrane energy generation and ion transport. It is also believed that silver can take part in catalytic oxidation reactions that result in the formation of disulfide bonds (R-S-S-R) [19]. Silver does this by catalyzing the reaction between oxygen molecules in the cell and hydrogen atoms of thiol groups: water is released as a product and two thiol groups become covalently bonded to one another through a disulfide bond [17]. The silver-catalyzed formation of disulfide bonds could possibly change the shape of cellular enzymes and subsequently affect their function leading to inactivate protein synthesis, which later inhibits the bacterial growth and also death. It was further proposed by Klueh et al [15], that the mechanisms of the antimicrobial activity of silver may be like the followings, that Ag^+ enters the cell and intercalates between the purine and pyrimidine base pairs disrupting the hydrogen bonding between the two anti-parallel strands and denaturing the DNA molecule [15]. Whether this has yet to been proved, but it has been shown that silver ions do associate with DNA once they enter the cell according to Fox and Modak [18, 21]. Further investigation is required to study its cytotoxicity *in vivo* for accessing its biocompatibility before administrating as antimicrobial drugs for human welfare.

Fig 1: Comparative antibacterial susceptibility assay of AgNPs and amoxicillin against pathogenic bacteria

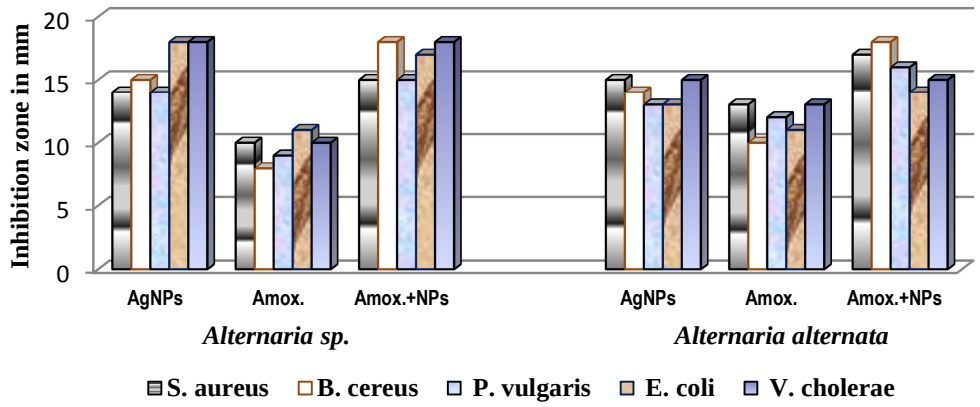

Table 1: Zone of inhibition (mm), Antibacterial activity of AgNPs against bacterial pathogens

Pathogens	*Alternaria sp.* (Zone of inhibition in mm)			*Alternaria alternata* (Zone of inhibition in mm)		
	AgNPs	AgNPs+Amox	Amox	AgNPs	AgNPs+Amox	Amox
S. aureus	14	15	10	15	17	13
B. cereus	15	18	08	14	18	10
P. vulgaris	13	15	09	13	16	12
E. coli	18	17	11	13	14	11
V. cholerae	18	18	10	15	15	13

S. aureus: Staphylococcus aureus, B. cereus: Bacillus cereus: P. vulgaris: Proteus vulgaris, E. coli: Escherichia coli, V. cholerae: Vibrio cholerae, Amox: Amoxicillin

CONCLUSION

Synthesis of different nanoparticles by biological path way is getting prominence in the recent scenario since it has varied characters. Nanoparticles especially silver nanoparticles have unique physical and chemical properties, which are clusters of silver atoms and have strong antimicrobial properties against pathogenic bacteria of resistant strains. In our present study, good antimicrobial activity was shown against the pathogenic bacteria by extracellular biosynthesized silver nanoparticles from two *Alternaria* spp and its activity were further enhanced in combination with amoxicillin in vitro condition, which may conclude that combined formulation of available drugs with silver nanoparticles would be an alternate approach in order to treat the multi drug resistant pathogenic bacteria and also to minimize the antibiotic doses to cure different dysfunctions made by these bacteria.

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