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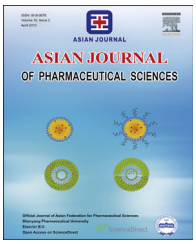

Original Research Paper

Mycosynthesis, characterization and antibacterial properties of AgNPs against multidrug resistant (MDR) bacterial pathogens of female infertility cases

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ABSTRACT

Recently, biosynthesis of silver nanoparticles using bacteria, fungus and plants has emerged as a simple and viable alternative to more complex physical and chemical synthetic procedures. The present investigation explains rapid and extracellular synthesis of silver nanoparticles using fungus *Fusarium oxysporum* NGD and characterization of the synthesized silver nanoparticles using UV-Vis spectroscopy, scanning electron microscopy, energy dispersive spectroscopy and X-ray diffraction analysis. The size range of the synthesized silver nanoparticles was around 16.3–70 nm. The FTIR studies showed major peaks of proteins involved in the synthesis of silver nanoparticles. Further, antibacterial effect of the silver nanoparticles against multidrug resistant pathogens *Enterobacter* sp. ANT 02 [HM803168], *Pseudomonas aeruginosa* ANT 04 [HM803170], *Klebsiella pneumoniae* ANT 03 [HM803169] and *Escherichia coli* ANT 01 [HM803167] was tested using turbidometric assay at 10, 20, 30, 40 μg AgNPs/ml alone and in combination with ampicillin using agar well diffusion assay. All the resistant bacteria were found to be susceptible to the antibiotic in the presence of the silver nanoparticles.

1. Introduction

Infectious agents can impair several essential human functions, including reproduction. Microbes can interfere with the reproductive function in both sexes. Especially, bacterial infections have long been recognized in association with female infertility [1,2]. Indeed, the treatment of bacterial infections is increasingly complicated because of their ability to develop

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resistance to antimicrobial agents. This resulted in severe debilitating and even life-threatening local and systemic infections, thus demanding the search for new antibacterial agents [3].

Presently, nanoscale materials have emerged as novel antimicrobial agents owing to their high surface area to volume ratio and their unique chemical and physical properties [4,5]. Metal nanoparticles such as copper, zinc, titanium [6], magnesium, gold [7], and silver [8,9] have proved to be effective against a range of bacteria, viruses and eukaryotic microorganisms [10]. Specifically in the case of silver, the broad spectrum antimicrobial activity encourages its use in biomedical application, water and air purification, food production, cosmetics, clothing and numerous household products [11].

Metal nanoparticles have been studied extensively because of their exclusive catalytic, optical, electronic, magnetic and antimicrobial properties [12,13]. Recent studies have shown that nanoparticles-based antimicrobial formulations could act as an effective bactericidal material [14]. Therefore a lot of research has been directed in finding out an inexpensive and environmental friendly method for nanoparticle synthesis. In accordance to the above, the present study is to synthesize silver nanoparticle using a soil fungus, and assess its antibacterial activity alone and in combination with ampicillin against multidrug resistant bacterial pathogens of female infertility cases.

2. Materials and methods

2.1. Collection of microbial strains

Multidrug resistant bacterial isolates of female infertility cases (*Enterobacter* sp. ANT 02 [HM803168], *Pseudomonas aeruginosa* ANT 04 [HM803170], *Klebsiella pneumoniae* ANT 03 [HM803169] and *Escherichia coli* ANT 01 [HM803167]) were obtained from the Germplasm, Department of Microbiology, Bharathidasan University, Tiruchirappalli, India and maintained on nutrient agar slants. The isolates were tested against various groups of antibiotics in clinical practice to determine their antibiotic resistance [3]. The fungus *Fusarium oxysporum* NGD was also obtained from the Germplasm.

2.2. Preliminary screening of soil fungi for nanoparticle synthesis

F. oxysporum NGD was cultured in 250 ml of sabouraud dextrose broth (SDB) at 28 ± 2 °C for 7 days at 200 rpm (pH-6.5). After incubation, the biomass was separated by filtration and washed in sterile distilled water to remove any medium components. The separated biomass (20 g) was then introduced into 200 ml of sterile distilled water and incubated for 3 days. After 3 days of incubation the clear cell free extract was collected by filtration and was used for nanoparticle synthesis. 50 ml of $AgNO_3$ solution (1 mM final concentration) was challenged with 50 ml of cell filtrate in a 250 ml Erlenmeyer conical flask and stirred constantly for 30 min. Control (without silver ions) was run along with the experimental flasks. The ability of the fungi to synthesize silver nanoparticles was determined visually based on change in colour of the reaction mixture [9,12].

2.3. Characterization of AgNPs

Since rapid reduction of silver ions in *F. oxysporum* NGD cell filtrate was noticed visibly through gradual change in the colour of the filtrate, the reaction mixture was subjected to optical measurements using an UV-Vis spectrophotometer scanning range between 250 and 800 nm at the resolution of 1 nm. Scanning electron microscope (JEOL Model JSM-6390 LV) with secondary electron detectors at an operating voltage of 20 kV was used to record the images of synthesized AgNPs (dry powder). Energy-dispersive X-ray (EDX) spectroscopy analysis for the confirmation of elemental silver was carried out using Thermo Noran EDS attachment equipped with SEM, JEOL Model JSM-6390LV. The size and the morphology of the AgNPs were examined using atomic force microscope (di CP-II Veeco USA). The dried powder of silver nanoparticles was used for X-ray diffraction (XRD) analysis. XRD patterns were recorded on X'Pert Pro, PANaltyical, USA operating at 40 kV and a current of 30 mA with Cu Ka radiation ($1 = 1.54A^{\circ}$). The diffracted intensities were recorded from 30 to 75 2θ angles. FTIR spectrum of the synthesis mixture was recorded on a PerkinElmer 1600 instrument in the range 400–4000 cm^{-1} at a resolution of 4 cm^{-1}.

2.4. Antibacterial activity of AgNPs against multidrug resistant pathogens

2.4.1. Preparation of MDR bacterial cultures

The MDR *Enterobacter* sp., *P. aeruginosa*, *K. pneumoniae* and *E. coli* were inoculated in sterile Luria Bertani broth (LB) and the broth was incubated at 37 °C for 18 h.

2.4.2. Antibacterial activity of AgNPs

Effect of AgNPs on bacterial growth was determined in broth culture. To 50 ml LB broth, 100 μl of bacterial culture was added, followed by the addition of silver nanoparticles suspension ranging from 10 to 40 μg/ml for each test organism. All the flasks were incubated at 37 °C. Growth of *Enterobacter* sp., *P. aeruginosa*, *E. coli* and *K. pneumoniae* was indexed by measuring the optical density (OD) at $\lambda = 620$ nm at regular intervals in UV-Vis spectrophotometer. The control was treated in a similar fashion without silver nanoparticles. The average triplicate value of growth curve was plotted between optical density and time.

2.5. Effect of AgNP-ampicillin mixture on MDR bacteria

The antibacterial activity of AgNPs in combination with ampicillin (10 μg/ml) was investigated by agar well diffusion method. Muller Hinton agar plates were inoculated separately with bacterial cultures namely *Enterobacter* sp., *P. aeruginosa*, *E. coli* and *K. pneumoniae* at a concentration of 10^{-4} to 10^{-5} CFU/ml using a sterile cotton swab and three wells (6 mm dia) were punched in each plate using sterile cork borer. 100 μl each of ampicillin (10 μg/ml), silver nanoparticle suspension (20 μg/ml) and mixture of AgNPs and ampicillin were added to agar wells. The plates were incubated at 37 °C for 24 h and the

antibacterial activity was measured as zone of inhibition. This procedure was repeated thrice.

3. Results and discussion

3.1. *Synthesis and characterization of silver nanoparticles using F. oxysporum NGD*

The fungus *F. oxysporum* NGD showed rapid colour change in less than a minute and the colour intensity increased with time and became dark brown within 30 min. Appearance of brown colour is due to the surface plasmon resonance of silver nanoparticles. With the addition of $AgNO_3$ solution, the crude cell filtrate of *F. oxysporum* NGD changed from light yellow to brown within 3–7 min, whereas the control showed no colour change under the same experimental conditions (Fig. 1). Thus the colour change of the reaction mixture clearly indicates the formation of AgNPs. Further the colour intensity of the reaction sustained even after 24 h of incubation, indicating that the particles were well dispersed in the aqueous medium, and there was no obvious aggregation [9,12].

Bioreduction of silver ions into nanoparticles was further confirmed by spectrophotometric analysis. The UV absorption spectra of silver nanoparticles at different time intervals are presented in Fig. 2A. A surface plasmon absorption band with a maximum at 425 nm, indicate the presence of spherical or roughly spherical AgNPs. The position and shape of the plasmon absorption depends on the particles, size and shape, and dielectric constant of the surrounding medium [15]. Absorption band at 425 nm rapidly increased initially in 15 min time interval, later reaching a plateau phase after 30 min (Fig. 2B), which indicated that the synthesis was completed within 30 min of incubation [16].

Extracellular synthesis of silver nanoparticles by cell free filtrates of *Aspergillus terreus* [17], *F. oxysporum* [18], *Fusarium acuminatum* [13], *Penicillium fellatum* [19], *Phytophthora infestans* [20], *Alternaria alternata* [21], *Agaricus bisporus* [22] and *Biopolaris nodulosa* [23] has been demonstrated. Though the actual mechanism involved in AgNP synthesis has not been worked out in this study, according to the earlier reports several proteins, free amino acids and NADH-dependent reductases [18] secreted by the fungus is responsible for the synthesis of nanoparticles. It could be noticed that in all the previous reports the reaction mixtures required 24 h–48 h for the reduction of the silver ions in the aqueous solution into nanoparticles. In the present study, the synthesis process initiated in few min and completed within 30 min. Therefore, this is the first report on shortest time required to generate silver nanoparticles using fungi.

The SEM micrographs of silver nanoparticles (5 mg) (Fig. 3) were roughly spherical to oval in nature with a little aggregation. The aggregation of AgNPs occurred during drying process (lyophilization) which is required for XRD analysis as well as for antibacterial studies. Ultra sonication technique was employed to separate the aggregated particles prior to antibacterial studies. EDS analysis provided additional evidence for the reduction of silver ions to nanoparticles. The optical absorption peak (Fig. 4) approximately at 3 keV is typical for the metallic silver nanocrystals [24]. Throughout the scanning range of binding energies, no peak belonging to impurity was detected. The results indicated that the reaction product was composed of highly pure Ag nanoparticles. The spectrum shows strong signal for Ag along with weak oxygen and carbon peak, which may be originated from the biomolecules bound to the surface of silver nanoparticles. The topological images of the AgNPs are shown in Fig. 5. The depth image of atomic force microscopy (AFM) (Fig. 5B) shows the spherical arrangement of silver nanoparticles within the diameter range of 6.3–12.67 nm. The XRD pattern of silver nanoparticles were compared and interpreted with standard data of International Centre of Diffraction Data (ICDD). Three characteristic peaks for silver nanoparticles appeared at 38.1°, 44.3° and 64.4° 2θ angle (Fig. 6) which corresponds to energetically distinct sites based on atom density. It suggests that the prepared silver nanoparticles are biphasic in nature. The size range of the nanoparticles was calculated using Scherrer formula ($D = 0.94\ \lambda/\beta\ \mathrm{Cos}\ \theta$) which was found to be 16.3–70 nm. The variation in the nanoparticles size range in AFM and XRD studies may due to the aggregation of particles in lyophilisation process.

The functional groups mainly involved in the reduction of Ag^+ ions were predicted using FTIR spectroscopy (Fig. 7). The absorption peaks of *F. oxysporum* cell filtrate (Fig. 7B) located at 1092, 1164, 1240, 1412, 1630, 2464, 2572, 2676 and 2778 cm^{-1} in

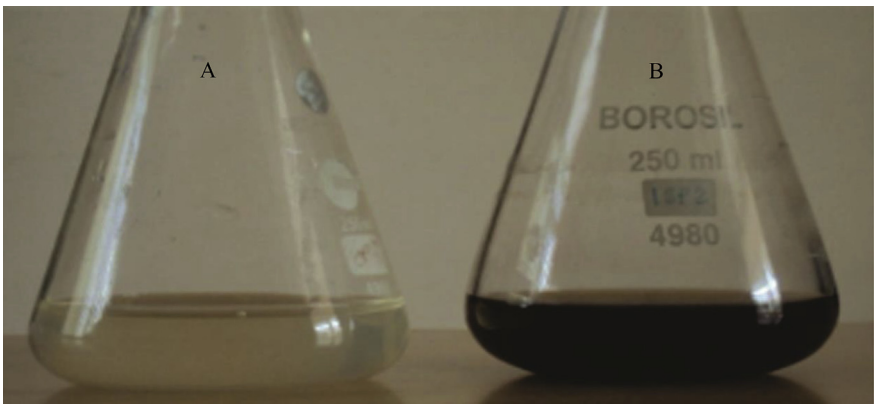

Fig. 1 – Silver nanoparticle synthesis from fungal extract. A) Cell free extract B) Synthesis mixture.

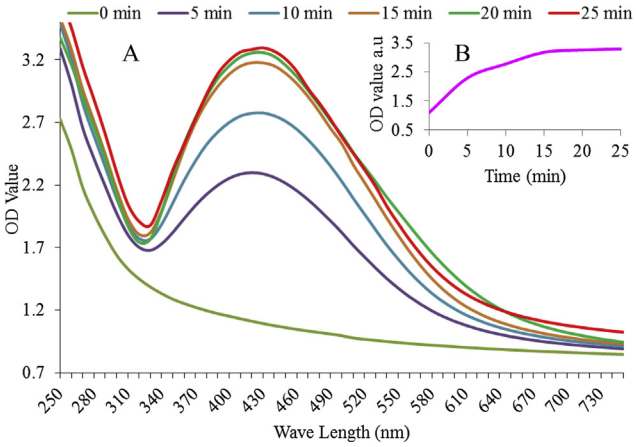

Fig. 2 – (A) UV-Vis spectrum of synthesis mixture at different time intervals. (B) Respective plot of absorbance at 425 nm as a function of time.

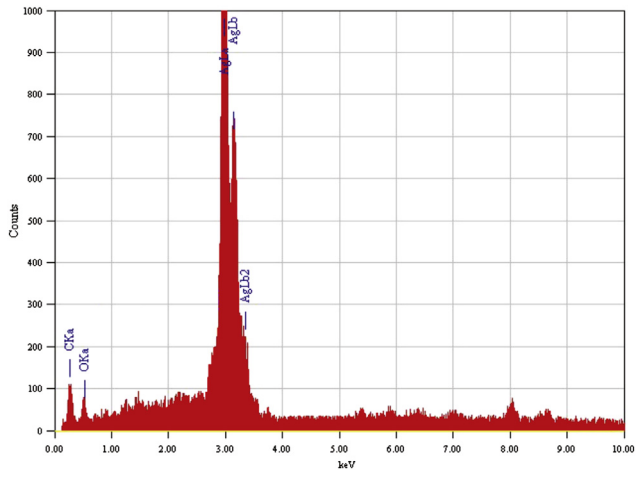

Fig. 4 – Energy dispersive spectroscopy analysis of AgNps.

the region of 500–3000 cm^{-1} are either red shifted or increased or diminished in the spectrum of the synthesis mixture containing cell filtrate and $AgNO_3$ (Fig. 7C). The distinct band at 1630 cm^{-1} is assigned to the $-NH_2$ group [25] and the band intensity increased and shifted to 1636 cm^{-1}, which indicates the formation of $-NH_3^+$ groups due to the complexation of amino groups and carboxylic groups [26]. During the silver nanoparticle synthesis, the relative intensity of peaks at 1092, 1164, 1240, 1412, and 2464 cm^{-1} of cell filtrate are either diminished or disappeared. The bands at 1092 and 1164 cm^{-1} are attributed to the vibration of C–O and C–OH stretch respectively [27,28]. The peaks observed at 2572 and 2778 cm^{-1} are assigned to the symmetric stretching of $N–H/N^+H_2$ groups and the bands at 2464 and 1412 cm^{-1} corresponds to the characteristic C–H and $C–H_2$ symmetric scissoring [29]. The absorption peaks at 1240 and 2676 cm^{-1} are due to the C–H stretch and O–H bending respectively [29]. In Fig. 7C, two new peaks appeared at 1111 and 2340 cm^{-1}, which is assigned to rocking of amino acids NH_3 structure [30,31] and physisorbtion of CO_2 generated due to the decomposition of oxygen containing functional groups [32,33] respectively. The changes in the peak intensity and disappearance of peaks in spectrum of synthesis mixture is mainly due to the involvement of the biomolecules and their functional groups for the AgNPs synthesis and all these functional groups are recognized as the carbonyl groups and amide I band of proteins. On the whole, it is observed that the protein molecules of the fungus are mainly responsible for the synthesis of AgNPs. Earlier FTIR reports [34,35] prove the involvement of carbonyl groups present in the amino acid residues and peptides of proteins for the synthesis of AgNPs. These protein molecules may also play a significant role in the prevention of agglomeration and stabilization of the AgNPs in the aqueous medium by binding on to their surface and forming a protein coat [36].

3.2. *Antibacterial activity of synthesized silver nanoparticles*

The effectiveness of AgNPs on MDR bacterial strains were assessed by comparing bacterial growth rate under normal (control) and AgNPs treated (test) conditions. The lag, log, stationary and death phases were clearly represented in the control group but under treatment with various concentration of AgNPs (10, 20, 30 and 40 μg/ml), gradual decrease in the growth and lengthy log phase were recorded (Fig. 8). This clearly states the concentration dependent inhibitory effect of AgNPs on MDR bacteria. Particularly, in Fig. 8A and D there were no increase in the log phase of *Enterobacter* sp. and *E. coli* upto 12 h of incubation with 40 μg/ml of AgNPs, indicating complete inhibition of bacteria. Similarly, Fig. 8B and C show diminution in the growth rate of *P. aeruginosa* and *K.*

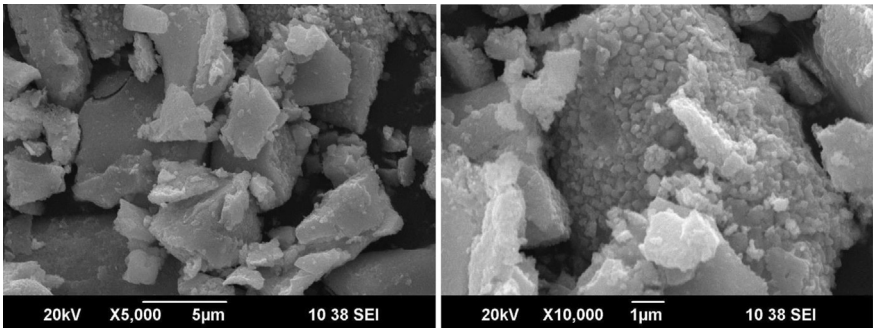

Fig. 3 – SEM image of silver nanoparticles synthesized using fungal extract.

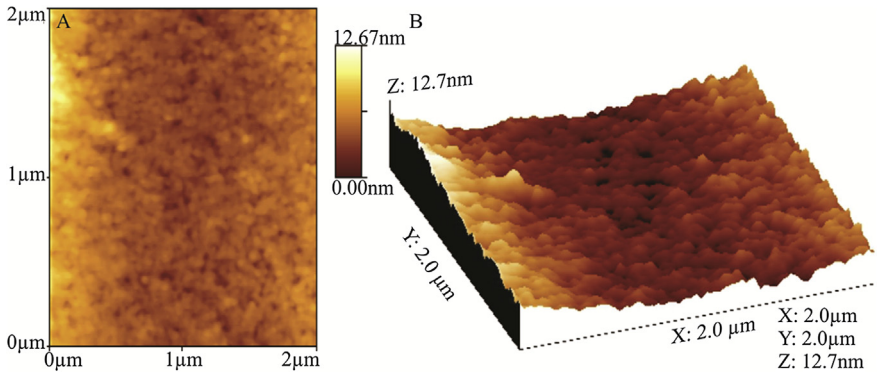

Fig. 5 – Atomic force microscopy images of AgNps (A) 2D view (B) 3D view.

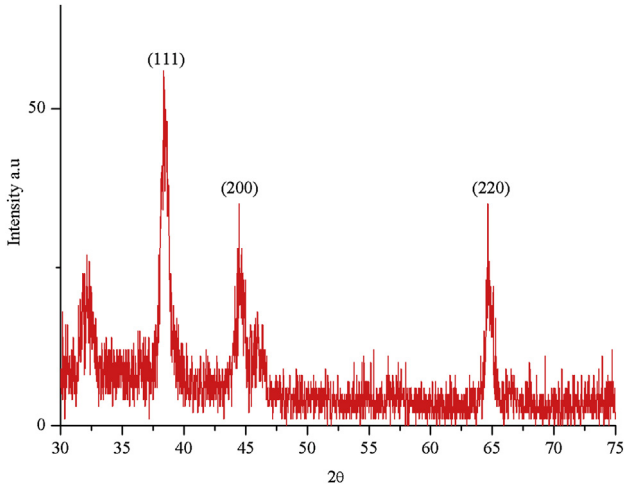

Fig. 6 – XRD pattern of synthesized silver nanoparticles.

pneumoniae when increasing the AgNPs concentration. Though silver nanoparticles are widely used in many antibacterial applications, the exact action of this metal on microbes is not clearly understood and is a debated topic. Quite a few mechanisms have been hypothesized that the AgNPs can cause cell lysis or growth inhibition. The proposed mechanisms are, (i) Silver nanoparticles have the ability to adhere to the bacterial cell wall and produces cracks and pits, through which the internal cell contents are released out [37], ultimately leading to structural loss and cell death [38]. (ii) Silver ions released by the nanoparticles react with thiol groups (–SH) of bacterial enzymes and other cell membrane components to produce a stable S–Ag bonds or disulfide bonds (R–S–S–R) [39,40]. It has been proposed that the catalytic oxidation reaction of Ag^{+} with the thiol group leads to inactivation or down regulation of 30 S ribosomal subunit protein, succinyl coenzyme A synthetase, maltose transporter (MalK), fructose bisphosphate adolase, transmembrane energy generation and ion transport, etc. This could possibly change the shape of cellular enzymes thereby affecting the cells function. (iii) Soft acid nature of silver eventually reacts with the cellular soft base components namely sulphur and phosphorus. During this reaction, the sulphur and phosphorus content of genomic DNA possibly gets affected by silver nanoparticles which would definitely block DNA replication [41,42] and thus kills the bacteria. (iv) It has also been found that the nanoparticles can inhibit the signal transduction in bacteria by dephosphorylating the peptide substrates on tyrosine residues. All the above said actions of the nanoparticles could cause the cell death. However, the actual and most reliable mechanism is not fully understood or cannot be generalized.

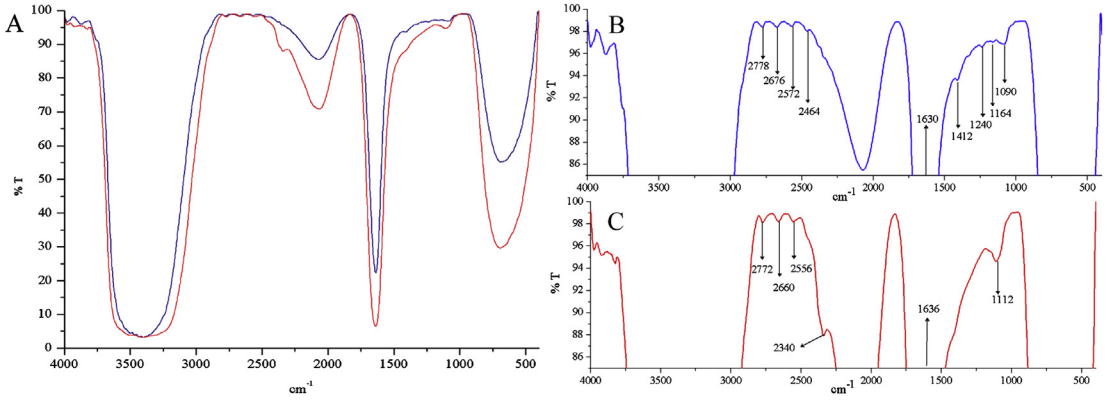

Fig. 7 – FTIR spectra of (A) *F. oxysporum* filtrate (blue) and synthesis mixture (red) during the synthesis of AgNPs. The enlarged and separate view at 85–100% transmittance of (B) *F. oxysporum* filtrate and (C) synthesis mixture.

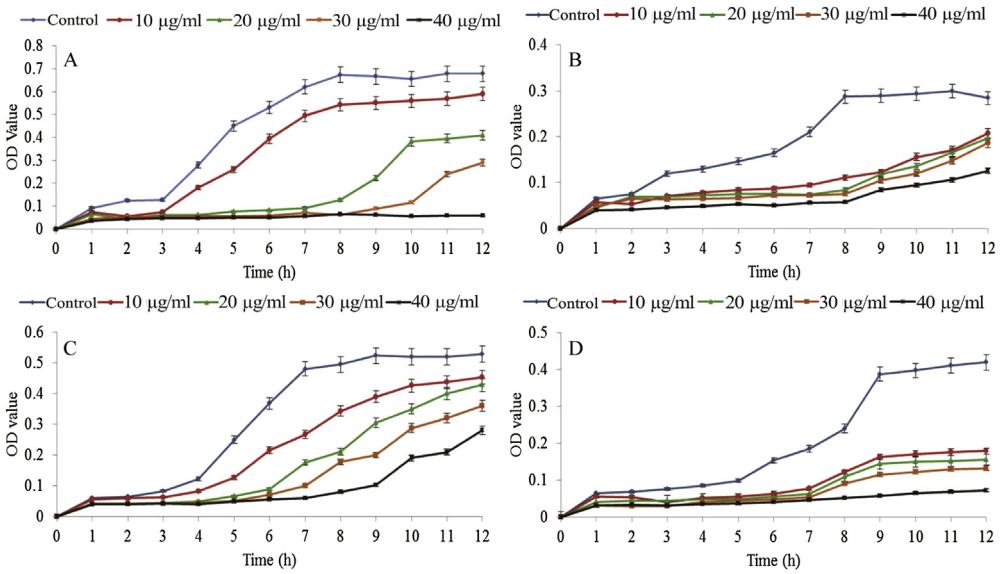

Fig. 8 – Turbidometric assay for the inhibitory effect of AgNps at 10, 20, 30, 40 μg/ml concentration against (A) *Enterobacter* sp (B) *P. aeruginosa* (C) *K. pneumoniae* (D) *E. coli*.

In the present study, we observed varying inhibitory effects of the AgNPs against *P. aeruginosa*, *K. pneumoniae*, *Enterobacter* sp. and *E. coli*. According to the reports, the inhibitory effect of AgNPs fluctuates in different bacteria due to the variation in their capsular and cell wall composition, thickness of the S-layer or a combination of these. Likewise, the bacterial growth rate and its byproducts may also influence the inhibitory activity of AgNPs. Thus we conclude that, an increasing concentration of AgNPs is required to achieve a higher inhibitory effect.

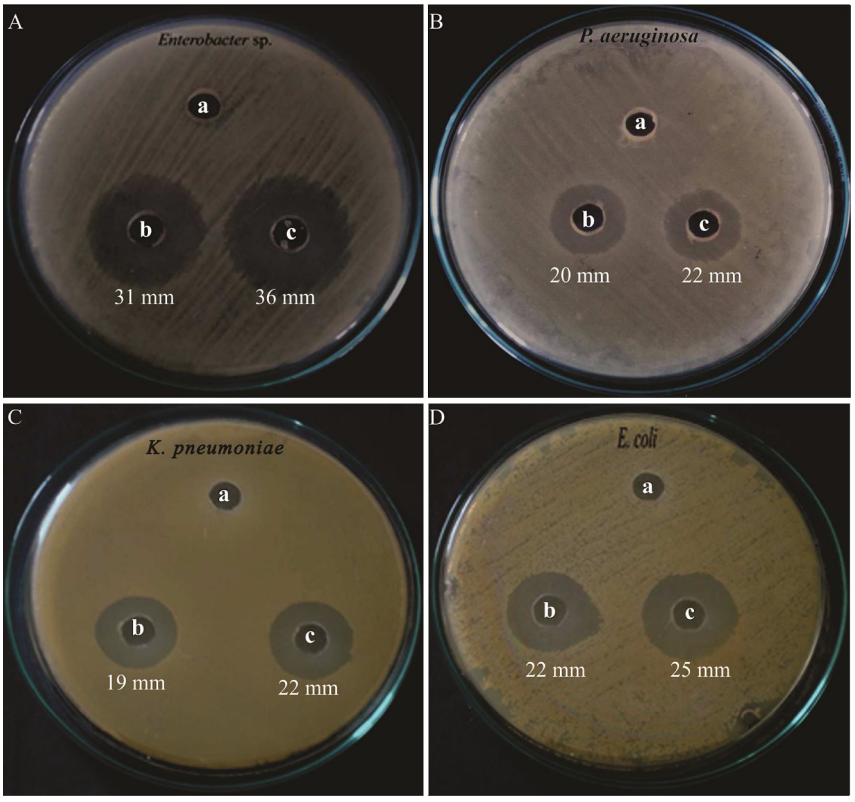

Fig. 9 – Antibacterial activity of AgNps-ampicillin complex against (A) *Enterobacter* sp (B) *P. aeruginosa* (C) *K. pneumoniae* (D) *E. coli* (a. ampicillin b. AgNps c. AgNps-amp complex). Diameter of zone of inhibition in mm excluding well size.

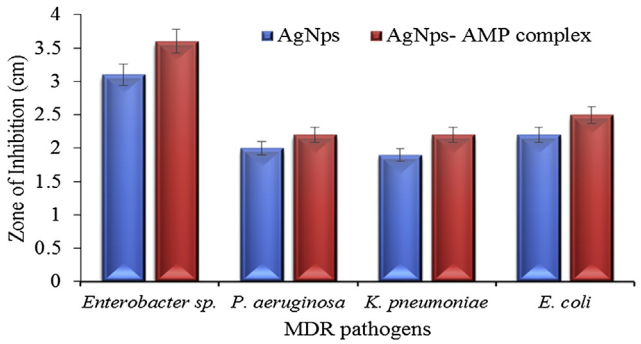

Fig. 10 – Zone of Inhibition of AgNps and AgNps-Amp complex against MDR bacteria pathogens.

3.3. *Antibacterial effect of AgNPs and ampicillin (AMP) complex*

Development of ampicillin resistance among a variety of bacteria is due to hyper production of classical β-lactamases, synthesis of inhibitor-resistant TEM (IRT) β-lactamases, hyperproduction of chromosomal AmpC β-lactamase, OXA β-lactamases and changes in membrane permeability. Resistance to antibiotics makes bacteria more difficult and expensive to eradicate. Thus treatment with a combination of drugs is necessary to clear infections of resistant bacteria.

Ampicillin molecule couples on the surface of silver nanoparticles due to synergistic effect producing AgNPs and ampicillin complex [43]. This complex greaten the inhibitory effect against all the ampicillin resistant bacteria when ampicillin alone had no/little inhibitory effect on the test organisms and therefore the bacteria are considered to be resistant to the antibiotic. However, all the bacteria were found to be sensitive to AgNPs to varying extent upto 20 μg/ml dose. Addition of ampicillin in the presence of AgNPs slightly enhanced the inhibitory effect towards all the tested bacteria by an increase of 2–3 mm inhibition zone (Figs. 9 and 10). Among the tested organisms *Enterobacter* sp. (Fig. 9A) was found to be highly sensitive with larger inhibition zone when compared to *P. aeruginosa* (Fig. 9B), *K. pneumoniae* (Fig. 9C) and *E. coli* (Fig. 9D).

As pointed above, all the tested clinical pathogens showed resistance towards ampicillin either by producing β-lactamases or changes in their membrane permeability. While treating these MRD isolates with AgNPs – ampicillin complex, the AgNPs lyse the cell wall and causes the leakage of internal cellular material leading to death of the pathogen. On the other hand, ampicillin enters the cell through damages caused by AgNPs and results in irreversible inhibition of the enzyme transpeptidase which ultimately stops their cell wall synthesis. Thus the results clearly represents that the sensitivity of the MDR bacterial pathogens increased with AgNPs – ampicillin complex in comparison with AgNPs.

4. Conclusion

Silver nanoparticles have been synthesized using fungal extract and positively complexed with ampicillin in order to destroy the ampicillin resistant bacterial pathogens. Though AgNPs and ampicillin complex undoubtedly combat antibiotic resistance pathogens, further researches are required to find out the exact mechanism of AgNPs and ampicillin complex formation to improve their inhibitory activity. Further investigations are required in this direction for the complete elimination of multidrug resistant pathogens using AgNPs as an adjuvant.

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