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Research Article

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ABSTRACT

The present study aimed at the biosynthesis of extracellular silver nanoparticles from *Pseudomonas* sp. and their characterization by means of UV-visible spectrophotometer. Moreover, the study focused on the bactericidal effects of the silver nanoparticles on human pathogenic bacteria. The nanoparticles demonstrated maximum absorbance at 410 nm corresponding to the surface plasmon resonance of nano-sized materials. However, the biosynthesis of metallic nanoparticles of silver by *Pseudomonas* sp. was carried out through the reduction of aqueous silver ions with culture supernatants. Pathogenic bacteria being resistant to antimicrobial agents have emerged as a major health problem in recent years. In this study, the silver nanoparticles were evaluated for their role as the antibacterial agents by well diffusion method against *Pseudomonas aeroginosa, Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Vibrio cholerae, and Staphylococcus aureus*. The silver nanoparticles showed satisfactory results against all six pathogenic bacteria. The extent of bactericidal effects of these nanoparticles was analyzed through the formation of clear zones around the wells in the plates containing bacterial culture. Thus, the synthesis, characterization and application of biologically synthesized silver nanoparticles could present an important concern for the development of antimicrobial agents by pharmaceutical companies to control infectious diseases caused by different pathogenic bacteria.

Keywords: Silver nanoparticles, *Pseudomonas* sp., biosynthesis, characterization, antibacterial activity.

List of Abbreviations: Me-NPs: Metal Nanoparticles, Ag-NPs: Silver Nanoparticles, LB: Luria–Bertani.

INTRODUCTION

Bacteria are the most abundant organisms in our biosphere. Living beings including human beings are often infected by bacteria in their living environment. Thus, the need for antibacterial material containing various natural and inorganic substances is of utmost importance (kim *et al*., 1998; Cho *et al*., 2005). Metal nanoparticles (Me-NPs), usually having a maximum size of 100nm, are thought to have antimicrobial properties. Among various Me-NPs, silver nanoparticles (Ag-NPs) have shown the best inhibitory and bactericidal effects (Kowshik *et al*., 2003; Khabat *et al*., 2011; Durán *et al*., 2005). Silver ions are highly toxic to bacteria and the antibacterial properties of these ions are highly applicable in biomedical experiments. Generally in Ag-NPs, a large surface area is available for the silver ions to enhance their antibacterial effects and therefore, the action of Ag-NPs on bacteria is directly proportional to the size and shape of the nanoparticles (Gurunathan *et al*., 2009; Catalina and Eric, 2010).

Nanoparticles of noble metals can be synthesized by several methods viz. hard templating, bio-reduction and solution phase analyses (Zhou *et al.,* 1999; Canizal *et al.,* 2001; Yu *et al.,* 1997). Synthesis of gold and silver (Mukherjee *et al.,* 2001) nanoparticles by eukaryotic cells such as fungi, synthesis of gold nanoparticles by *Shewanella* algae (Konishi *et al.,* 2006) and silver nanoparticles by fungus *Verticillium* (Mukherjee *et al*., 2001) have been reported (Anuradha *et al*., 2010). Also, several bacteria (for instance, *Pseudomonas stutzeri* AG259 bacterium) have been utilized to synthesize Ag-NPs intracellularly or extracellularly, containing nanocrystals of different compositions (Silambarasan and Jayanthi, 2012). Ag-NPs are synthesized by the reduction of silver salts mainly

$AgNO_3$ with agents such as glucose, citrate, ethylene glycol or sodium borohydride (Svitlana and Matthias, 2013; Leaper, 2006).

In the present study, *Pseudomonas* sp. was employed for extracellular biosynthesis of Ag-NPs. The synthesized Ag-NPs were characterized and examined for antibacterial activities against various human pathogenic bacteria such as *Pseudomonas aeruginosa, E. coli, Klebsiella pneumoniae, Serratia marcescens, Vibrio cholerae and Staphylococcus aureus*.

Pseudomonas aeruginosa, mainly causing endocarditis, respiratory infections, septicemia, urinary tract infections, gastrointestinal infections and so on, has gained resistance to a large number of antibiotics. *Escherichia coli* causing cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), and traveler's diarrhea has shown resistance to multiple types of fluoroquinolone antibiotics such as ciprofloxacin and levofloxacin. *Klebsiella pneumoniae* which causes pneumonia, bronchopneumonia, bronchitis, thrombophlebitis, urinary tract infection (UTI), cholecystitis, and diarrhea is also resistant to many classes of antibiotics including aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and sulfamethoxazole. On the other hand, *Serratia marcescens* is resistant to antibiotics such as ampicillin, macrolides, and first-generation cephalosporins (such as cephalexin) and is the causative agent of conjunctivitis, keratitis, endophthalmitis, tear duct infections, white pox disease and flacherie disease. *Vibrio cholerae* which cause cholera, profuse and watery diarrhea has demonstrated resistance to tetracycline and other antimicrobial agents. *Staphylococcus aureus* causing furuncles, carbuncles, staphylococcal scalded skin syndrome (SSSS), septic arthritis, staphylococcal endocarditis, pneumonia has become resistant to most β-lactam antibiotics (Wright *et al*., 2009; Nathisuwan *et al*., 2001; Auwaerter, 2008; Weber *et al*., 1994; Capparelli *et al*., 2007).

The aim of the study was to test and use the biosynthesized Ag-NPs as the antibacterial agent against these human pathogenic bacteria and to provide a new hope to the medical field against the use of bacteria resistant antibiotics.

MATERIALS AND METHODS

Isolation of the bacteria

Some waste materials were collected from the dustbin of M.A.G. Osmani Medical College. They were serially diluted in sterile distilled water and plated on nutrient agar plates. The plates were incubated at 37°C for 48 h. The morphological and biochemical characterization of the bacterial strain was performed as methods described in Bergey's manual of determinative bacteriology (Holt *et al*., 2000).

Production of biomass

In the present study, LB (Luria–Bertani) media was used for the production of biomass by *Pseudomonas* sp. The flasks containing the bacterial culture were incubated on an orbital shaker at 37°C and agitated at 180 rpm. The growth profile of *Pseudomonas* sp. was studied here with 6 h interval. The culture was then centrifuged at 12000 rpm for 10 minutes to obtain biomass. The supernatant was collected for further reaction with silver salt ($AgNO_3$) to synthesize nanoparticles.

Biosynthesis of silver nanoparticles

As described by Kannan *et al.* (2010), the sample was added separately to the reaction vessel containing silver nitrate (Ag-NO_3) at a concentration of 10^{-3} (1% v/v) while the control contained only the biomass (without the silver nitrate). The reaction between the supernatant and Ag^+ ions was carried out in an orbital shaker under light at 37°C under 180 rpm and monitored after each 6 h interval (Kannan *et al*., 2010).

Characterization of silver nanoparticles

The formation of Ag-NPs was observed by the change in color of the silver nitrate solution incubated with the supernatant of *Pseudomonas* sp. culture. Usually the change of color of culture supernatant from light yellow to yellowish brown suggests the formation of Ag-NPs which is due to the bio-reduction of the Ag^+ ions in the solution. A sample of 2ml was withdrawn and the absorbance was measured within a resolution range of 350-450 nm using UV–visible spectrophotometer (model-T60 U, PG Instruments Ltd, England) to check the highest peak (Habeeb *et al*., 2013).

Antibacterial activity by well diffusion method

The Ag-NPs synthesized from *Pseudomonas* sp. was tested for their antibacterial activity by well diffusion method against human pathogenic bacteria namely *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Vibrio cholerae, and Staphylococcus aureus*. The organisms were obtained from three different diagnostic centres (Popular Diagnostic Centre, Medinova Diagnostic Centre and Jalalabad Diagnostic Centre) of Sylhet. The investigation was made by culturing these organisms in nutrient agar media and subsequently adding crude Ag-NPs into well made on plates. Each bacterium was swabbed uniformly onto the individual plates using sterile cotton swab and 20µl of crude Ag-NPs solution was poured into wells on all plates and incubated at 37°C for 20 h while for control 20µl of sterile distilled water was poured into the wells made on the same plates (Anima and Saravanan , 2009).

RESULTS

Pseudomonas sp. was isolated, identified and cultured for the production of biomass. The bacterial isolate of *Pseudomonas* sp. was inoculated in LB media as the production culture and inoculated at 37 ^{0}C in orbital shaker at 180 rpm. The growth profile was then studied at 600 nm with an interval of 6 h, starting from the time of inoculation. The maximum growth kinetics was observed after 24 h of incubation as shown in figure 1.

Figure 1: Growth profile of *Pseudomonas* sp. in the LB media.

The bacterial culture was then subjected to centrifugation at 12000 rpm for 10 minutes to obtain the biomass. The supernatant thus obtained was mixed with 1 mm $AgNO_3$ solution at a concentration of 10^{-3} (1% v/v) in a culture flask. On the other hand, the flask containing only supernatant (without $AgNO_3$ solution) was used as a control. The experimental mixtures were incubated overnight for subsequent reaction. It is a well-known fact that the conformation of nanoparticle synthesis is characterized by the change in color of reaction mixture from light yellow to yellowish brown (Kannan *et al*., 2010; Sadowski *et al.,* 2008). Upon overnight incubation, the supernatant of *Pseudomonas* sp. culture with $AgNO_3$ solution changed its color from light yellow to brown after completion of reaction with silver ions. In contrast, the color of the supernatant used as control remained unchanged. Therefore, the appearance of yellowish brown color in the reaction vessel suggested the formation of Ag-NPs, which is similar to results described by Kannan *et al*., 2010; Sadowski *et al*., 2008; Ahmad *et al.,* 2003, as shown in figure 2.

Figure 2: Conical flasks having culture supernatant with silver nitrate (1 mM) (left) and culture supernatant without silver nitrate (right).

For the characterization of Ag-NPs, UV-Vis (model-T60 U, PG Instruments Ltd, England) spectroscopy was used. The formation of Ag-NPs was characterized by observing the changes in UV-Vis absorption. The supernatant containing $AgNO_3$ was exposed to UV-Vis spectroscopy at different wavelengths ranging from 350 to 450 nm, whereas this broad resonance suggests the accumulation of Ag-NPs in the solution. According to Shankar *et al.,* (2004), the spectrum with bands within this range corresponds to the surface plasmon resonance of nano-sized silver metal, thus, confirming the presence of the Ag-NPs in the given solution after exposure to UV light. The sample containing $AgNO_3$ in it was when exposed to UV-Vis spectroscopy showed a wide spectrum range between 400 and 430 nm, while the highest peak of surface plasmon resonance was observed at approximately 410 nm as illustrated in figure 3.

Figure 3: UV–vis absorption spectra of silver nanoparticles synthesized by *Pseudomonas* sp. culture.

The absorption spectrum of silver nanoparticles exhibited a strong broad peak at 410 nm and observation of such a band is assigned to surface Plasmon resonance of the particles. These results were almost consistent with the reports of Syed *et al*. (2013), Basavaraja *et al. (2008)*, Bhainsa *and* D'Souza (2006), who had reported strong broad peak at 420 nm. Similarly, Gaikwad *and* Bhosale (2012), reported that the reduction of Ag+ to atomic silver AgO corresponds to absorption at 440 nm.

The Ag-NPs synthesized by *Pseudomonas* sp. was investigated for their antibacterial properties against various pathogenic organisms such as *Pseudomonas aeruginosa*, *Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Vibrio cholerae, and Staphylococcus aureus* which are resistant to several antibiotics. The study was carried out by culturing these organisms in nutrient agar media and subsequently adding synthesized Ag - NPs

solution into well made on plates. For the purpose, each bacterium was swabbed uniformly onto the individual plates using sterile cotton swab and 20µl of crude Ag-NPs solution was poured into wells on all plates and incubated at 37℃ for 20 h while for control 20µl of sterile distilled water was poured into the wells made on the same plates. All six bacteria used in the present study showed susceptibility to Ag-NPs and formed clear zones around the wells in the plates filled with Ag-NPs as shown in figure 4.

Figure 4: Antibacterial activity of Ag-NPs against (a) *Pseudomonas aeruginosa,* (b) *Escherichia coli,* (c) *Klebsiella pneumoniae,* (d) *Serratia marcescens,* (e) *Vibrio cholerae, and* (f) *Staphylococcus aureus,* shown by well diffusion method.

The extent inhibition imposed by Ag-NPs on the growth of abovementioned pathogenic bacteria was examined by measuring the diameter of inhibition zones (mm) around each well in the culture plates. The antibiotic activity of the Ag-NPs against *Pseudomonas aeruginosa* was the maximum (27 mm) and that of *Staphylococcus aureus* was the minimum (17 mm) as shown in figure 5.

Figure 5: The antibacterial property of Ag-NPs on various human pathogenic bacteria.

Sondi and Salopek-Sondi B. (2004) reported previously that *E. coli* being the model for gram- negative bacteria was found to be susceptible for Ag-NPs, thus, confirming its antibacterial properties against human pathogenic bacteria. Gram-negative bacteria are usually resistant to antibiotics because their impermeable outer membrane to large glycopeptides molecules. The structure of the cell wall may provide resistance to drug effect. Gram- negative bacteria with their extra lipid bilayer, many antibiotics may not reach the sites of action. Therefore any antibiotic drug that is not lipid soluble enough to traverse the outer lipid bilayer and small enough to traverse the porin channel will have no effect on the microorganism. *E. coli* when treated with nanoparticles conjugated antibiotics was able to bind the cell wall and destroys the stability of the outer membrane. And it makes nanoparticles coated antibiotic easier to bind to the peptidoglycan structure as reported (Henglein, 1993).

Nanoparticles have the ability to destroy the stability of Lipopolysaccharides allowing increase in permeability of the outer membrane and the peptidoglycan structure and is recognized and captured by antibiotics immediately. The conjugation of antibiotics with silver nanoparticles helps the resistant strains to gets sensitive to antibiotics. Moreover, the membrane of the bacteria is well known to contain many sulfur-containing proteins; these might be preferential sites for the silver nanoparticles. On the other hand, nanoparticles inside the bacteria will also tend to react with other sulfur-containing proteins in the interior of the cell, as well as with phosphorus-containing compounds such as DNA. Finally the changes that occur in the morphology of the bacterial membrane as well as the possible damage caused by the nanoparticles reacting with the DNA, will affect the bacteria in processes such as the respiratory chain, and cell division, finally causing the death of the cell (Elechiguerra *et al*., 2005).

DISCUSSION

Bacteria being the major causative agents of many infectious diseases to human beings are gaining resistance to different classes of antibiotics. This may impose a greater threat to medical science in near future. The demand for newer classes of antibiotics is increasing. In the meanwhile, the use of nanoparticles as the alternative source of antibiotics against various human pathogenic bacteria can be effective. The present study was carried out as a novel approach for *Pseudomonas* sp. mediated extracellular production of Ag-NPs. The study shows that Ag-NPs exhibit broad spectrum bactericidal activity towards various human pathogenic bacteria namely *Pseudomonas aeruginosa*, *Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Vibrio cholerae, and Staphylococcus aureus.* This encourages its use in a large number of biomedical applications. This work provides a new advancement in the formation of newer types of bactericides. In future, our aim is to characterize the silver nanoparticles using TEM (transmission electron microscopy) to analyze the particle size and also to study the biochemical and molecular mechanism of nanoparticles formation and its antibacterial activities.

CONCLUSION

In conclusion, our study revealed that the silver nanoparticles have significant antibacterial effect on all the human pathogenic bacteria tested.

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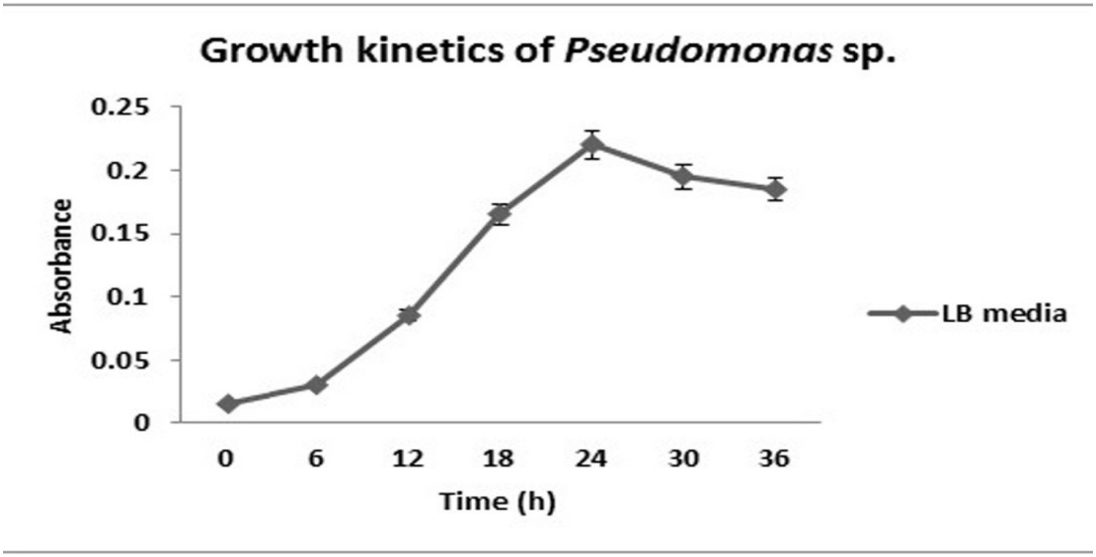
Figures



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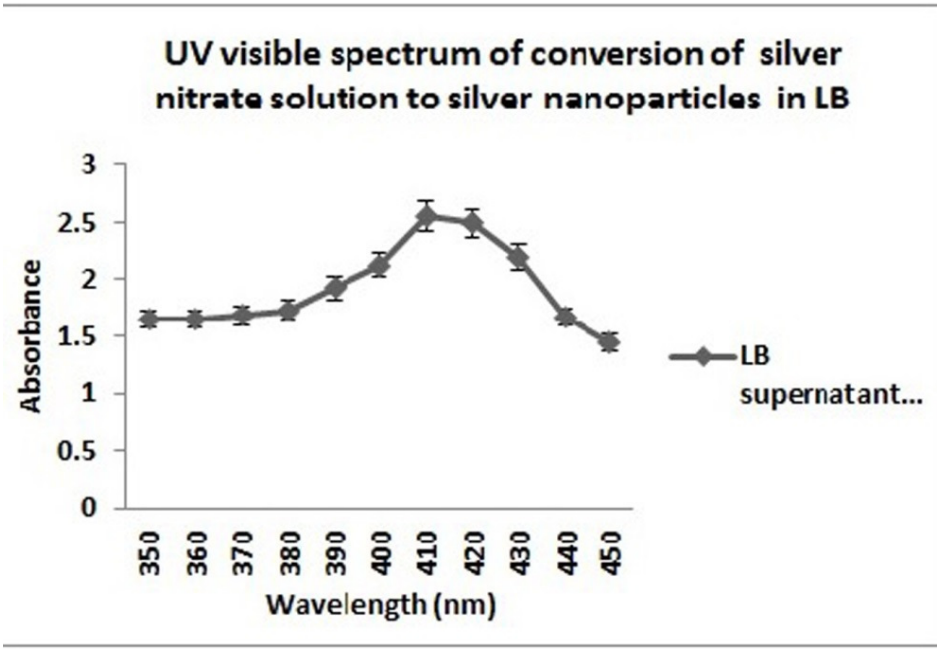
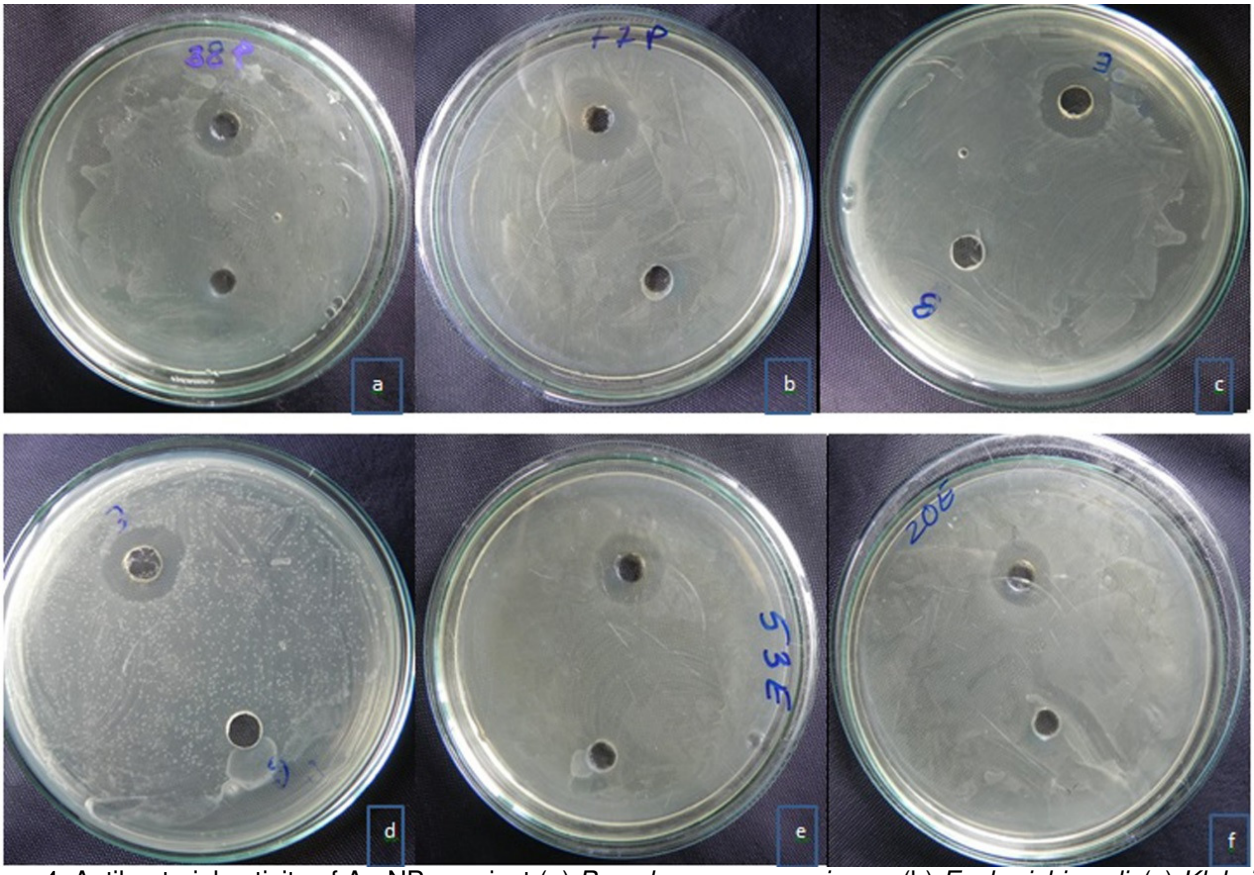


Figure 3: UV–vis absorption spectra of silver nanoparticles synthesized by *Pseudomonas* sp. culture.



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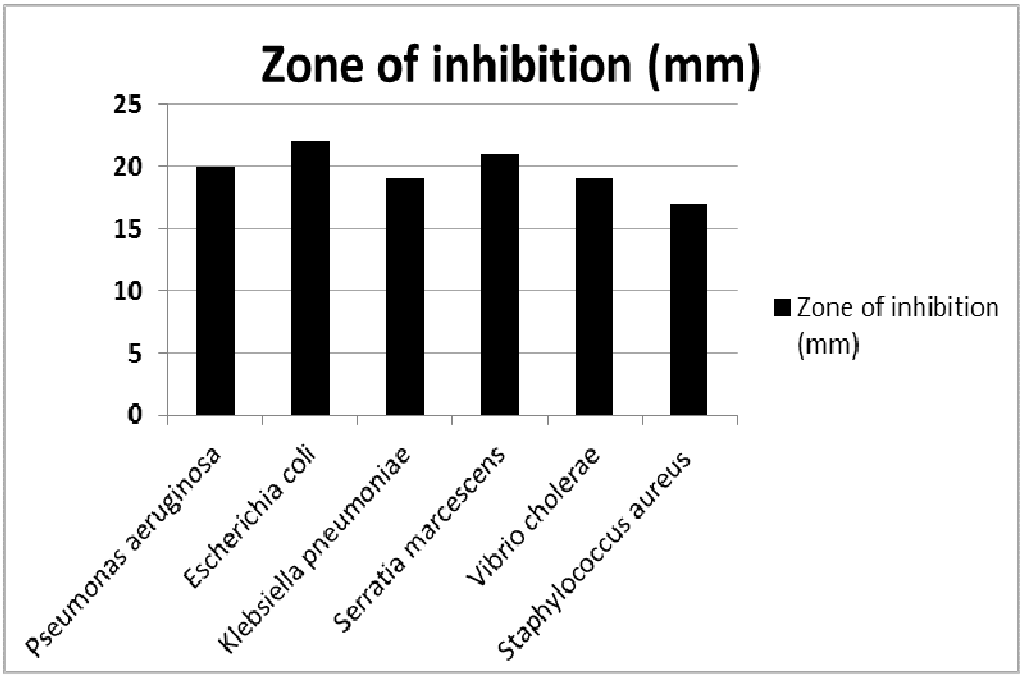

Figure 5: The antibacterial property of Ag-NPs on various human pathogenic bacteria.

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