J Med Bacteriol. *Vol. 7, No. 1, 2 (2018): pp.36-43* *jmb.tums.ac.ir*

Journal of Medical Bacteriology

Synergistic Effect between Phyto-Syntesized Silver Nanoparticles and Ciprofloxacin Antibiotic on some Pathogenic Bacterial Strains

Yaser Nikparast [1*], *Mahsa Saliani* [2]

[1] *Department of Agronomy, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.*

[2] *Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran.*

ARTICLE INFO

Article type:
Original Article

Article history:
Received: 08 Mar 2018
Revised: 11 Mar 2018
Accepted: 15 Mar 2018
Published: 15 May 2018

Keywords:
Antibacterial activity, Biosynthesis, Optimization, Plant extract, Silver nanoparticles.

ABSTRACT

Background: Plant extract as a potential phyto-reducer is used as a simple, non-toxic and ecofriendly green synthesis method of silver nanoparticles (AgNPs). In this study biosynthesis of AgNPs using leaves extract broth of *Amaranthus retroflexus* as both reducing and stabilizing agent was analyzed. Antibacterial activity toward resistant human pathogenic bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and also against plant pathogenic bacteria *Pseudomonas syringae*, *Xanthomonas oryzae*, was studied. The biosynthesized AgNPs were also evaluated for their increased antimicrobial activities with Ciprofloxacin antibiotic against some of the tested bacteria.

Methods: The formation of green synthesized nanoparticles from aqueous solution of silver nitrate was first screened by measuring the surface plasmon resonance peak at 300-800 nm using UV–vis spectroscopy. The morphology, size and Crystalline structure of the synthesized AgNPs was determined using Transmission Electron Microscope (TEM), DLS and X-ray diffraction analysis. For antibacterial studies two-fold serial dilutions were made in NB medium (Qlab Canada) and the growth of the cultures was monitored by measuring the optical density value at 630 nm (OD630) with microplate reader (Biotech ELX 800) after 24 hours of incubation to obtain the MIC of the AgNPs.

Results: The results indicated that the phyto-synthesized AgNPs were spherical with an average size of 48 nm. XRD peaks indicate the presence of a face centered cubic (fcc) structure of crystalline AgNPs. The AgNPs showed highly potent antibacterial activity toward the tested bacteria. Also the combined antibacterial activity of Ciprofloxacin with AgNPs reduced the MIC of antibiotic from 0.125 µg/ml to 0.0625 µg/ml toward *P. aeruginosa* and Ciprofloxacin MIC against *P. syringae* decreased from 0.25 to 0.0625 µg/ml in combination with 6.25, 12.5, and 25 µg/ml of AgNPs.

***Conclusion*:** Results from the current study suggested that the silver nanoparticles successfully can be synthesized using Amaranth leaf extract. The phyto-synthesized nanoparticles could have potential antibacterial applications and show synergistic effect in combination with Ciprofloxacin antibiotic.

- ***Please cite this paper as:*** Nikparast Y, Saliani M. Synergistic Effect between Phyto-Syntesized Silver Nanoparticles and Ciprofloxacin Antibiotic on some Pathogenic Bacterial Strains. *J Med Bacteriol.* 2018; **7** (1, 2): pp.36-43.

*Corresponding Author: Yaser Nikparast: Department of Agronomy, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.
Tel: +98- 051-8788974, *E-mail:* yaser.nikparast@gmail.com

Introduction

Silver nanoparticles are important materials that have been studied, extensively. They have unique physical, chemical and biological properties such as potential antibacterial activity. They can be synthesized by several physical, chemical methods. However, these methods are not environmentally friendly (1). Therefore, it is desirable to develop alternative an eco-friendly method such as utilizing the biological methods. In addition, the integration of green chemistry principles into nanotechnology is essential where the development of nanotechnology could benefit from a greener approach that promotes both performance and safety (2).

Green synthesis of AgNPs using extract of plants has been studied, extensively. The biomolecules found in plants induce the reduction of Ag+ ions from silver nitrate to AgNPs. The process of reduction is extra cellular and fast leading to the development of easy biosynthesis of silver nanoparticles (3). Some of the major active components in the extract of plants which are responsible for reduction of silver ions are nicotinamide adenine dinucleotide, water-soluble antioxidative agents like ascorbic acids (3), and certain carbohydrates (4). There are several proposed mechanisms for the production of AgNPs using Plant extracts. For example, the monovalent silver ion is primarily reduced by −OH group of the active components of the leaf extract, which is oxidized to corresponding carbonyl group (5). The fresh leaves of some plant extracts contain considerable amounts of organic compounds such as oxalic acid and tannic acid with their potential as reducing agents (4).

Recently, there is a growing interest in the fabrication of novel AgNPs with potent antimicrobial nature. Monovalent silver compounds have been used extensively for antimicrobial treatment for decades, and recent studies suggest that the antimicrobial activity is retained in AgNPs (6). Considering the importance of toxicity of engineered nanoparticles on physiological system, Ag-NP has been found to show higher toxicity to microorganisms compared to human cells (7). AgNPs have a high specific area than their volume which leads to excellent antimicrobial activity as compared with bulk Ag metal (7). According to the advantageous applications of AgNPs as an antibacterial agent many researches focused on their biosynthesis as a new approach. In this context, the antibacterial activity of AgNPs against different bacterial species, such as *E. coli*, *Salmonella typhi*, *Proteus vulgaris*, *Yersinia enterocolitica*, *Klebsiella pneumonia*, *Micrococcus luteus*, *Enterococcus Hirae*, *Shigella flexneri* and *Streptococcus faecalis*, has been studied (6).

The present study deals with green synthesis of AgNPs using leaves extract of an important agricultural weed, *Amaranthus retroflexus*. On the basis of the available literature, we hypothesized that agricultural weeds can be utilized for the preparation of nanoparticles which shows the additional advantages of this study to make the use of agricultural waste. On the other hand, several pathogenic bacteria have developed resistance against various antibiotics and the development of resistant pathogens has become a major problem. With the prevalence and increase of microorganism's resistant to multiple antibiotics, many researchers have tried to develop new, effective antimicrobial reagents, free of resistance and cost-effective. According to such problems and needs in this study antibacterial activity of biosynthesized AgNPs and its synergistic effect with antibiotic was studied against some of the antibiotic resistant human and plant pathogenic bacteria, considering the lower propensity of AgNPs to induce microbial resistance.

Material and methods

Preparation of aqueous extract

Amaranthus retroflexus leaves were collected form Ferdowsi University campus and were thoroughly cleaned with distilled water to remove the dust and debris. The cleaned plant leaves were then crushed to small pieces and5 gr of chopped leaves were then added in to an Erlenmeyer containing 100 ml double distilled water and boiled for 5 min at 100 °C. After that, extract was filtered with Whatman filter paper no. 1. The resultant filtrates were used for the reduction of silver nitrate and synthesizing silver nanoparticles (8, 9).

Synthesis of silver nanoparticles

For reduction of silver ions, 10 mL of extract was added to 90 mL of 1 mM silver nitrate solution (pH=9) with constant stirring at room temperature. The reaction mixture turned from colorless to reddish brown color. The final nano-colloidal solution was subjected to repeat centrifugation (thrice) to get rid of any uninterested biological molecules at 12,000 rpm for 15 min and the pellet were dried in vacuum oven. The dried AgNPs were scrapped out for the further study.

Characterization of silver nanoparticles

The samples were analyzed using UV-visible spectroscopy on Agilent 8453, UV-Visible absorption spectrophotometer with a resolution of 2.0 nm between 300 to 800 nm with scanning speed of 300 nm/min. The process of reaction between metal ions and weed extract were monitored by UV–Visible spectra of silver nanoparticles in aqueous solution. To perform TEM analysis, a small aliquot of purified AgNPs were sonicated and then a thin film was prepared in a copper coated grid. The morphology and size of the AgNPs were measured at different magnification at 100 keV using Leo 912 AB high resolution transmission electron microscope. X ray diffraction (XRD) pattern analysis was done to investigate crystalline nature of the biosynthesized nanoparticles. The dried AgNPs were coated on XRD grid and the spectra was recorded by using Explorer (G.N.R., Italy) instrument operated at a voltage of 40 KV and a current of 30 mA with Cu Kα radiation. To evaluate the size of the AgNPs the DLS method (Vasco3, Cordouan, France) was used, which uses laser light diffraction to measure particle size distribution.

Antibacterial assay

Escherichia coli (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) as human pathogenic bacteria and *Pseudomonas syringae* (*P. syringae*) and *Xanthomonas oryzae* (*X. oryzae*) as two plant pathogenic bacteria were used as sample bacteria for antibacterial studies. The two-fold serial dilutions of AgNPs (µg ml-1) were made in NB medium (Qlab Canada). 100 µl of bacterial suspensions was transferred to 700 µl of culture medium containing AgNPs-containing with final concentration of 105 CFU ml−1 (CFU: colony forming unit). Then, 200 µl of each inoculum suspension was transferred to each well of a 96-well microtiter plate in triplicate and incubated at 37 °C on a reciprocal shaker (120 rpm). The growth of the cultures was monitored by measuring the optical density value at 630 nm (OD630) with microplate reader (Biotech ELX 800) after 24 of incubation (10). The growth of bacterial suspension at the presence of AgNPs was compared with growth of bacterial cells (positive control) in the absence of the NPs. The lowest concentration of the AgNPs that inhibited growth after 24 h incubation was obtained as MIC (minimum inhibitory concentration) of the AgNPs against the tested bacteria. To avoid potential optical interference of the growing cultures caused by the light-scattering properties of the NPs, the same liquid medium without bacteria containing the same concentration of NPs of the test samples was also incubated (as blank control). To investigate the synergistic antibacterial effect of biosynthesized AgNPs with antibiotic P. aeruginosa as human pathogenic bacteria and P. syringae as plant pathogenic bacteria were selected. Ciprofloxacin was chosen to study the synergistic antibacterial effect of biosynthesized AgNPs with antibiotic. MIC of antibiotic was determined against mentioned bacteria. MIC of Ciprofloxacin toward bacterial cells in the presence of AgNPs was compared with its value in the absence of NPs (11).

Results

UV–vis spectral studies

Silver nanoparticles were synthesized by the reduction of Ag+ into Ag0 in different reaction mixtures. The colorless solutions turned reddish brown indicating the formation of AgNPs. It seemed that the AgNPs absorbed radiation in the visible regions of 300–600 nm due to the strong SPR transition and the peak observed in 420 nm. The appearance of the SPR transition in this region (Figure 1) confirmed the production of AgNPs.

XRD pattern of synthesized AgNPs

The X-ray diffractogram showed distinct peaks at 2θ values of about 38◦, 44.3◦, 64.4◦, 77.4◦ representing the {1 1 1}, {2 0 0}, {2 2 0} and {3 1 1} Braggs reflections (Figure 2). These typical XRD peaks indicate the presence of a face centered cubic (fcc) structure of crystalline AgNPs. Unidentified crystalline peaks (27.89◦, 32.30◦, 46.26◦, 54.79◦) are also apparent in many works in which the XRD pattern includes the relevant 2θ range (12).

Morphology studies of synthesized AgNPs

To investigate the morphology of synthesized nanoparticles, TEM technique was employed to visualize the shape of AgNPs. TEM image is the evident that the morphology of silver nanoparticles is nearly spherical shape. The AgNPs had different sizes ranging from 23 to 200 nm with 48 nm average.

Antibacterial activity of AgNPs and antibiotic

The antibacterial activity of AgNPs was investigated against gram negative drug resistant of two human pathogenic bacteria E. coli and Pseudomonas aeruginosa and also against two plant pathogenic bacteria *Pseudomonas syringae*, *Xanthomonas oryzae*. Various concentrations of AgNPs from 400 to 1.56 µg/ml were incubated with cells of pathogenic bacteria in liquid medium. Growth of bacterial cells at the presence of AgNPs was compared with the growth of bacterial cells (positive control) in the absence of NPs and MIC values of NPs were determined (Table. 1). Also different concentrations of Ciprofloxacin from 0.0315 to 0.25 µg/ml were used to determine MIC values of Ciprofloxacin against bacterial species as selected antibiotic (Table 1). Leaves extract of *Amaranthus retroflexus* alone did not display any antimicrobial activity against tested microorganism.

Synergistic effect of AgNPs with antibiotics

Resistance to antimicrobial agents by pathogenic bacteria has emerged in recent years and is a major challenge for the health care industry. The other purpose of present contribution was to investigate the synergistic antibacterial effect of biosynthesized AgNPs with antibiotic (Ciprofloxacin) against drug resistant pathogenic bacteria. A broth dilution method was used to assay the synergistic effect of antibiotic with extracellularly synthesized AgNPs for bactericidal activity against tested strains. Growth of bacterial cells at different AgNPs and Ciprofloxacin concentrations was evaluated to study the combined antibacterial activity of AgNPs with antibiotic. *P. aeruginosa* as a resistant human pathogenic bacteria and *P. syringae* as a resistant plant pathogenic bacteria were selected to study the synergistic effect. Results of the study showed increased antibacterial effect of antibiotic against *P. aeruginosa* in the presence of AgNPs (Table 2). The combined antibacterial activity of Ciprofloxacin with NPs reduced the MIC of antibiotic from 0.125 µg/ml to 0.0625 µg/ml toward *P. aeruginosa* (Table 2).

Highlighted results show the synergistic effects of AgNPs and Ciprofloxacin, where the MIC of antibiotic reduced from 0.125 to 0.0315 µg/ml in presence of 12.5 µg/ml AgNPs. In addition at concentrations of 12.5 and 6.25 µg/ml of NPs, no bacterial growth is observed at 0.0625 µg/ml which show the more antimicrobial activity due to synergistic properties. Furthermore, results of study revealed intensified growth inhibitory effect on *P. syringae* originated from combined antibacterial activity of AgNPs and Ciprofloxacin (Table 3). Data indicated that MIC of antibiotic against *P. syringae* decreased from 0.25 to 0.0625 µg/ml in combination with 6.25, 12.5, and 25 µg/ml of AgNPs. Also no bacterial growth of *P. syringae* was observed at 0.125 µg/ml of antibiotic and 25 µg/ml of NPs.

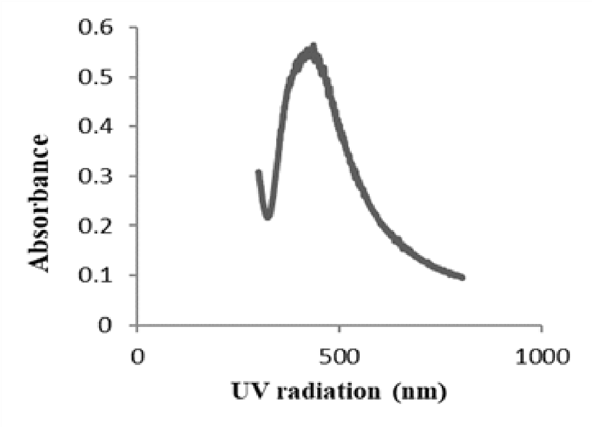

Figure 1. UV-vis spectra of reaction solution after 1 hr.

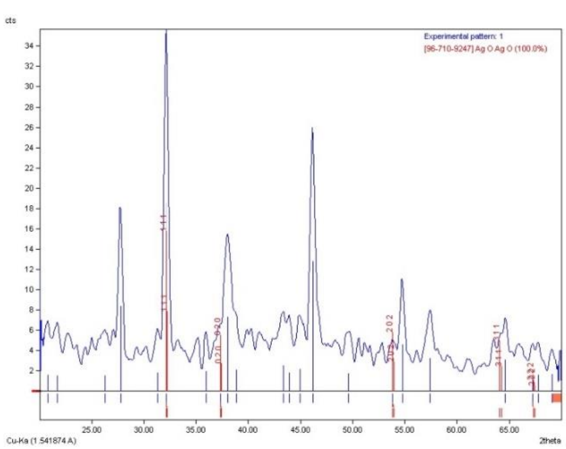

Figure 2. XRD pattern of biosynthesized of AgNPs.

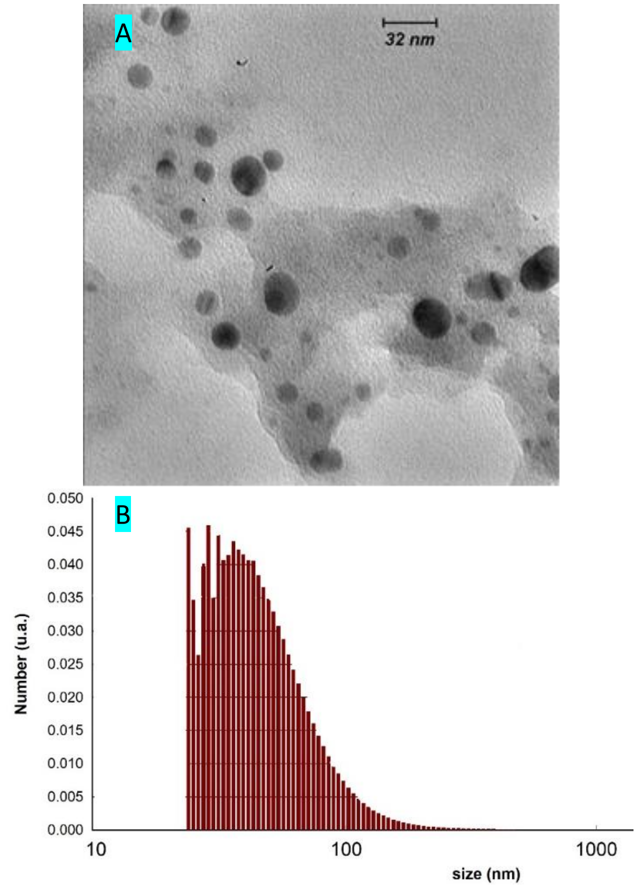

Figure 3: A .TEM image of biosynthesized Ag nanoparticles. B. Size distribution of biosynthesized AgNP.

Table 1: Independent MIC of AgNPs and antibiotic against tested microorganisms.

Microorganism	MIC of AgNPs (µg/ml)	MIC of antibiotic (µg/ml)
E. coli	12.5	0.0625
P. aeruginosa	25	0.125
P. syringae	50	0.25
X. oryzae	50	0.25

Discussion

The results showed that *Amaranthus retroflexus* can be an effective component for Phyto-synthesize of stable AgNP. The reduction of the Ag+ ions during the exposure to Amaranth leaf extract can be easily monitored by visual observation and UV-vis spectroscopy. Other researches have confirmed that plant secondary metabolites, polysaccharides and proteins can be responsible for the reduction process (13, 14).

Several hypotheses have been proposed in the literature to explain the mechanism of antibacterial activity of AgNPs. Among different suggested mechanisms distribution of bacterial cell membrane is highly accepted. Guzman et al, reported that during antibacterial effect, biosynthesized AgNPs highly interacted with sulfur and phosphorus, which are found in abundance throughout the cell membrane and utilize these essential elements for displaying antibacterial properties (15). The results of other study showed that the microbicidal activity is due to the silver cations released from AgNPs pertaining to changes in the membrane structure of microbes, which lead to the increased membrane permeability of the bacteria and finally cell death (5). Furthermore, it is believed that the attachment of AgNPs to the bacterial cell membrane causes the formation of “pits” on the bacterial cell walls, damage of cell membrane, thereby allowing the nanoparticle to enter the periplasm of the bacterial cells and release of cell contents (17) Also it is reported that the attachment of AgNPs to the cell surface alter the physical and chemical properties of the cell membranes and the cell wall and disturb important functions such as permeability, osmoregulation, electron transport and respiration (18).

Ciprofloxacin is a broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, necessary to separate bacterial DNA, thereby inhibiting cell division (19). According to the several studies on disruption of g4tbacterial cell membrane by AgNPs, integration of these NPs with Ciprofloxacin would facilitate the entrance of antibiotic into bacterial cells leading to inhibition of DNA gyrase and bacterial death. Thus, the combination of AgNPs and Ciprofloxacin yield novel combination of antimicrobial agents with synergistic properties which could be exploited for higher antibacterial activity.

Conclusion

In this study, we have developed a fast, eco-friendly and cheap method for the synthesis of spherical silver nanoparticles using *Amaranthus retroflexus* leaf extract with an average diameter of 48 nm. No chemical reagent or surfactant was necessary in this method, which consequently make this bioprocess environmentally benign. The antibacterial activity of phyto-synthesized silver nanoparticles was evaluated against *E. coli*, *P. aeruginosa*, *P. syringae* and *X. oryzae*, showing effective bactericidal activity. The results also showed that AgNP have a synergistic effect on P. aeruginosa as and *P. syringae* in combination with Ciprofloxacin as a common antibiotic. Biological silver nanoparticles synthesized by Amaranth could be of immense use in the medical field for their efficient antimicrobial function.

Conflict of interest

There is no conflict of interest.

References

1. Ghorbani HR, Safekordi AA, Attar H, et al. Biological and non-biological methods for silver nanoparticles synthesis. Chemical and Biochemical Engineering Quarterly, 2011; **25**(3):317-326.
2. Mittal AK, Bhaumik J, Kumar S, Banerjee UC. Biosynthesis of silver nanoparticles: Elucidation of prospective mechanism and therapeutic potential. *J Colloid Interface Sci* 2014a; **415**:39-47.
3. Roopan SM, Rohit, Madhumitha G, et al. Low-cost and eco-friendly phyto-synthesis of silver nanoparticles using Cocos nucifera coir extract and its larvicidal activity. *Ind Crops Prod* 2013; **43**:631-5.
4. Mittal J, Batra A, Singh A, Sharma MM. Phytofabrication of nanoparticles through plant as nanofactories. *Adv Nat Sci Nanosci Nanotechnol* 2014b; **5**:43002.
5. Durán N, Marcato PD, Durán M, et al. Mechanistic aspects in the biogenic synthesis of extracellular metal nanoparticles by peptides, bacteria, fungi, and plants. *Appl Microbiol Biotechnol* 2011; **90**:1609-24.
6. Azócar MI, Tamayo L, Vejar N, et al. A systematic study of antibacterial silver nanoparticles: Efficiency, enhanced permeability, and cytotoxic effects. *J Nanoparticle Res* 2014; **16**.
7. Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. *Biotechnol Adv* 2009; **27**:76-83.
8. Vijayakumar M, Priya K, Nancy FT, et al (2013) Biosynthesis, characterisation and anti-bacterial effect of plant-mediated silver nanoparticles using Artemisia nilagirica. Ind Crops Prod **41**:235-40.
9. Das S, Das J, Samadder A, et al. Biosynthesized silver nanoparticles by ethanolic extracts of *Phytolacca decandra*, *Gelsemium sempervirens*, *Hydrastis canadensis* and *Thuja occidentalis* induce differential cytotoxicity through G2/M arrest in A375 cells. *Colloids Surfaces B Biointerfaces* 2013; **101**:325-36.
10. Logeswari P, Silambarasan S, Abraham J (2013) Ecofriendly synthesis of silver nanoparticles from commercially available plant powders and their antibacterial properties. *Sci Iran* **20**:1049-54.
11. Zarei, M., Jamnejad, A. and Khajehali, E., 2014. Antibacterial effect of silver nanoparticles against four foodborne pathogens. *Jundi J Microbiol* **7**(1).
12. Jyoti K, Baunthiyal M, Singh A. Characterization of silver nanoparticles synthesized using *Urtica dioica* Linn. leaves and their synergistic effects with antibiotics. *J Radiat Res Appl* Sci 2016; **9**:217-27.
13. Saxena A, Tripathi RM, Zafar F, et al. Green synthesis of silver nanoparticles using aqueous solution of Ficus benghalensis leaf extract and characterization of their antibacterial activity. *Mater Lett* 2012; **67**:91-94.
14. Ramesh PS, Kokila T, Geetha D. Plant mediated green synthesis and antibacterial activity of silver nanoparticles using Emblica officinalis fruit extract. *Spectrochim Acta Part A Mol Biomol Spectrosc* 2015; **142**:339-43.
15. Guzman M, Dille J, Godet S. Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria. *Nanomedicine Nanotechnology, Biol. Med* 2012; **8**:37-45
16. Durán N, Durán M, de Jesus MB, et al

(2016) Silver nanoparticles: A new view on mechanistic aspects on antimicrobial activity. *Nanomedicine Nanotechnology, Biol Med* **12**:789-99.

17. Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: A case study on E. coli as a model for Gram-negative bacteria. *J Colloid Interface Sci* 2004; **275**:177-82.
18. Franci, G., Falanga, A., Galdiero, S., Palomba, L., Rai, M., Morelli, G. and Galdiero, M. Silver nanoparticles as potential antibacterial agents. Molecules, 2015; **20**(5): 8856-74.
19. LeBel M. Ciprofloxacin: Chemistry, Mechanism of Action, Resistance, Antimicrobial Spectrum, Pharmacokinetics, Clinical Trials, and Adverse Reactions. *Pharmacother J Hum Pharmacol Drug Ther* 1988; **8**:3-30.