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Green Synthesis Concept of Nanoparticles From Environmental Bacteria and Their Effects on Pathogenic Bacteria

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Abstract

Soil bacteria play an interesting role in the reduction of Ag^+ ions and the formation of silver nanoparticles (AgNPs), which may be a good source for nanoparticles and play a major role in nanotechnology applications. The concept of this project was to study the effects of these environmentally produced nanoparticles on the growth of some pathogenic bacteria. The environmental bacteria were isolated from soil, purified on broth cultures, and centrifuged, while the supernatant was extracted to detect its ability to convert silver nitrate to nanoparticles. The AgNPs was detected by Atomic Force Microscopy (AFM), while Granularity Cumulating Distribution (GCD) was employed to estimate the AgNPs sizes. The results showed the synthesis of AgNPs with sizes of 63.50nm and 45.81nm from the extracts of environmental *Pseudomonas sp.* and *Enterobacter*, respectively. The synthesized AgNPs from the extracts of all environmental bacteria showed antibacterial activity against some pathogenic bacteria (Gram positive and Negative) with variable inhibition zones. In conclusion, environmental bacteria can be a cheap source of nanoparticles.

Keywords: Silver nanoparticles, environmental bacteria, Green method, Antimicrobial activity.

مفهوم التخليق الأخضر للجزيئات النانوية من بكتيريا البيئة وتاثيرها على البكتيريا الممرضة

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الخلاصة:

تضمنت الدراسة تأثير الجسيمات النانوية المنتجة من عالق البكتيريا البيئية على نمو بعض البكتيريا المسببة للأمراض. تم عزل البكتريا البيئية من التربة ثم تنقيتها في وسط مغذي سائل , بعدها رسبت الخلايا البكتيرية بالطرد المركزي واستخدم الطاف (المستخلص) للكشف عن قدرته على تحويل نترات الفضة إلى جزيئات نانوية ، تم الكشف عنها بواسطة استخدام Atomic Force Microscope ثم قياس معدل حجم الحبيبات المتكومة بواسطة Granularity Cumulation Distribution وفحص قابلية AgNPs المتكونة على تثبيط البكتيريا الممرضة باستخدام طريقة الانتشار في الحفر . أظهرت النتائج تكون AgNPs بأحجام مختلفة (63.5 و 45.8 نانوميتر) لمستخلص بكتيريا ال *Pseudomonas sp.* و *Enterobacter* على التوالي, كذلك وجد ان AgNPs المنتج من كافة العزلات البكتيرية البيئية كان لها فعالية ضد مايكروبية ضد البكتيريا

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الموجبة والسالبة لصبغة غرام بأقطار تثبيط متفاوتة. نستنتج أن البكتيريا البيئية ممكن ان تكون مصدرا رخيصا وغير مكلف للجزيئات النانوية.

Introduction

Nanoparticles are particles with a size range of 1 to 100 nanometers (nm) and a surrounding interfacial layer. As a term in nanotechnology, a particle is defined as a small molecule that behaves as a whole unit, which is further classified according to diameter [1]. Attention has been widely focused on nanostructures due to many applications and unique properties of such structures in different fields. This resulted in the finding of simple techniques for preparation and production of nanoparticles that seem to be eco-friendly and may have solutions to the environment, as well as to medical, agricultural and technological challenges. Characteristics of such nanoparticles depend upon their overall size, shape, composition and distribution. The biosynthetic technologies represent an economic alternative for physical and chemical techniques [2].

Nanoparticles are increasingly used to eliminate bacterial cells as an alternative to antibiotics. Nanotechnology has been particularly advantageous through inactivating bacterial growth, such as the utilization of specific nanoparticles in antibacterial coatings for implantable devices and materials used in medicine to prevent infection and to promote wound healing. Other medical applications include disease treatment as in antibiotic delivery systems, microbial diagnostics by bacterial detection systems, and the control of bacterial infections through antibacterial vaccines [3].

Silver nitrate ($AgNO_3$) is often used in the biosynthesis of Silver nanoparticles (AgNPs), which showed a non-toxic nature and safe inorganic structure with antimicrobial properties [4]. The characteristics of AgNPs made it a good candidate for many researches related to agricultural, industrial, water treatment, drug delivery and many other applications [5]. Several chemical and physical techniques were used to synthesize AgNPs [6], but, unfortunately, their high cost and other disadvantages led to the search for alternative synthesizing techniques. Studies were capable in accomplishing the successful synthesis of AgNPs using microorganisms (especially bacteria), which is considered as a biosynthetic technique [7]. Studies nowadays are interested mainly on bacteria for the synthesis of nanoparticles because of their properties of easy cultivation, short time of growth and ability to adapt to the environmental conditions. Therefore, such conditions can be controlled during the nanoparticle synthesis proses [8]. The synthesis of AgNPs by bacterial cells occur either in extracellular or intracellular manners; the extracellular synthesis is the most widely used approach, where the supernatant of the bacterial culture is treated with $AgNO_3$ aqueous solution [9]. Several studies showed evidence that the irresponsible use of antibiotics could lead to the spread of multidrug-resistant bacterial strains [10]. Bacterial resistance can develop against cell wall synthesis, translational machinery, and DNA replication machinery [11]. Studies started to prove that AgNPs are less prone to promoting resistance in bacteria as compared to antibiotics. Therefore, attention has been focused on new and exciting nanoparticle-based materials with antibacterial activity [12].

The antimicrobial mechanism of action of AgNPs is generally described as one of these models: oxidative stress induction, metal ion release, or non-oxidative mechanisms [13]. Certain studies suggested that AgNPs cause neutralization of the surface electric charge of the bacterial cell membrane and change its penetrability, finally leading to bacterial death [14]. Also, the production of reactive oxygen species (ROS) inhibits the antioxidant defense system and results in mechanical damage to the cell membrane. Nanoparticles have been considered as an alternative to antibiotics due to their ability to prevent microbial drug resistance in certain cases. The wide use of antibiotics resulted in the emergence of numerous bacterial hazards to public health, such as superbugs that do not respond to any existing antibiotic [15]. Nanoparticles were established as a promising approach to solve many problems [16] due to overcoming the existing antibiotic resistance mechanisms through the disruption of bacterial membranes and the inhibition of biofilm formation. Therefore, NPs are characterized by fighting microbes using many mechanisms simultaneously [17] and are gaining a large interest as they might fill the gaps where antibiotics frequently fail [18].

Depending on the previous information, this study aimed to synthesize AgNPs using soil borne environmental bacterial isolates and detect their antibacterial activity.

Materials and Methods

Sample collection

Ten samples of soil (approximately 10gm each) were collected from gardens of Al-Jadiriya campus, University of Baghdad, in sterile plastic bags. The samples were transported to the laboratory, placed in sterile beakers, and homogenized with sterile distilled water.

Isolation and Identification of Soil Bacteria

A volume of 50µl from the homogenized soil sample was placed in the middle of MacConkey agar and Citramide agar plates, spread by a glass spreader and incubated at 28℃ for 24h. Selective colonies from the agar plates (Fig 1) were inoculated in double nutrient broth for further purification and incubated at 28℃ for 18hr. The broth culture was then diluted (10^{-5}, 10^{-6} and 10^{-7}) then 100 µl of each was re-cultured on the previously selected agar, and incubated at 28℃ for 18hr. to ensure purification of bacteria. Finally, the isolated colonies were further identified by morphological and biochemical tests (Catalase, Oxidase, Triple sugar iron (TSI), Oxidation-fermentation test, Nitrate reduction and Carbohydrate fermentation) [19].

Figure 1-Growth of environment bacteria on MacConkey agar.

Synthesis of AgNPs using bacterial supernatant

Pure colonies of each bacterial isolate (*E.coli, Klebsiella sp. , Pseudomonas aeruginosa,* and *Enterobacter sp.*) were transferred to a flask with 250 ml nutrient broth from the previously prepared cultures and incubated at 28 C̊ for 48hr. The growth cultures were centrifuged at 10000 rpm and the supernatant was collected in a 50 ml sterile glass container. The collected supernatant (extracellular product) was further used in the synthesis of AgNPs.

Silver nitrate solution was prepared by adding 0.085 gm of $AgNO_3$ with 0.25 ml of 1%sucrose to 10 ml of distilled water. To investigate whether the bacterial supernatant has the ability to synthesize AgNPs (reducing agent), 0.2ml of $AgNO_3$ (1 mM) solution was added to 19.8 ml of each isolated bacterial supernatant separately and kept in dark for 24hr, according to a previously published method [20] with modification. The control samples contained only the supernatant without $AgNO_3$. The biosynthesis of AgNPs was detected primarily through the color change observed in the samples that contain $AgNO_3$ (1mM) from light yellow to dark brown [21].

Characterization of synthesized AgNPs

The morphology and size of AgNPs synthesized from the soil borne bacterial supernatant were analyzed by pictures taken by Atomic Force Microscope (AFM) which gave resultant microscopic information on plot topographies that show surface alleviation and structure [22]. The samples were imaged over a wide area with the detection of dimensions at higher resolution. Images were taken using Scanning Probe Microscopy (SPM).

Antibacterial activity of synthesized AgNPs

The well diffusion assay was used to detect the antibacterial activity of the synthesized AgNPs. The pathogenic bacterial isolates (*Salmonella sp., Pseudomonas aeruginosa, Escherichia coli, Klebsiella sp and Staphylococcus aureus*) were provided from the University of Baghdad/College of Science-Biology Department and tested for their multi drug resistance (MRD) against a group of antibiotics (Amoxicillin, Cefixime, Amikacin, Chloramphenicol and Tetracycline). Three serial dilutions (0.424,

0.212, and 0.101g/ml) of the synthesized AgNPs were prepared from the stock concentration (0.849 mg/ml).. Pathogenic bacterial cultures were incubated in previously prepared nutrient broth at 37Ċ for 18hr, and the concentration of each bacteria was brought to $1.5*10^8$ CFU/ml which is equivalent to a 0.5 turbidity in McFarland tube.

Mueller Hinton agar plates were seeded with the previously cultured pathogenic bacteria, using cotton swaps dipped in broth culture, and streaked on agar plates. After 5-10 minutes, the wells were made using the end of a sterile pasture pipette. Synthesized AgNPs solution (200 µL) for each concentration was added to the corresponding wells, the culture was incubated at 37Ċ for 24 hr, and the Inhibition zones were measured in mm.

Results and Discussion

Isolation and identification of soil enviromental bacteria

Four bacterial isolates were collected from selective media after purification. The colony forming units at dilutions of 10^{-5}, 10^{-6}, and 10^{-7} ranged between 161 and 32. Results of morphological and biochemical tests showed the isolation of *E.coli, Klebsiella sp., Pseudomonas sp.* and *Enterobacter sp.*.

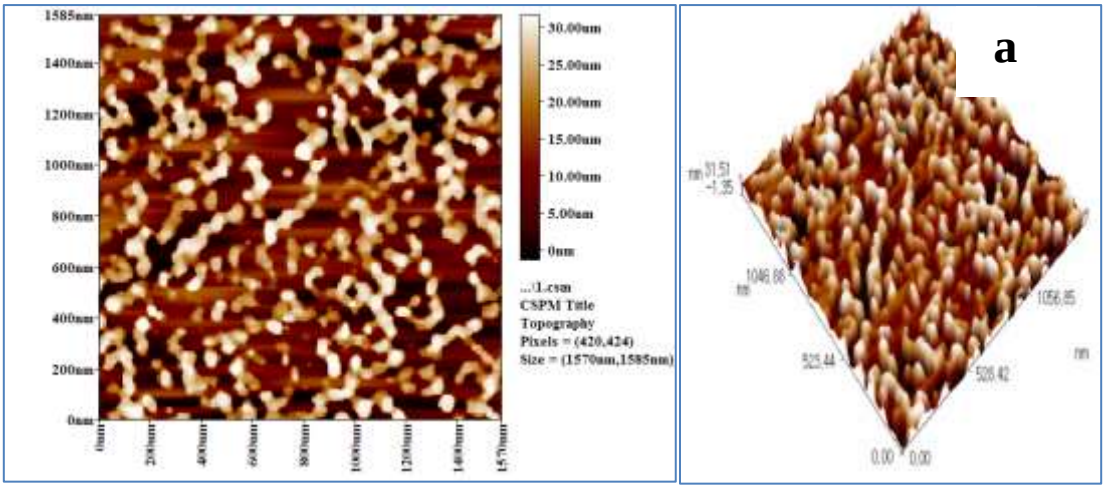
Biosynthesis of AgNPs

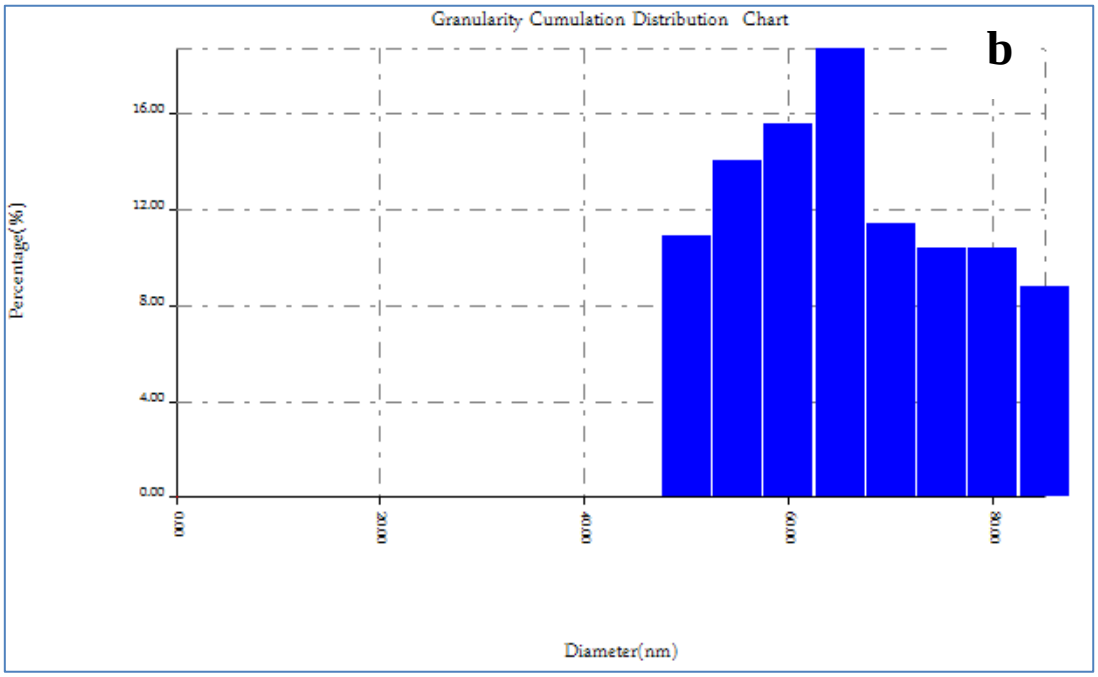
Formation of AgNPs was identified by the change in color from pale yellow Figure-(3.a) to brown color (Figure- 3.b) of the bacterial supernatant within 24hr. for each bacterial isolate of soil samples. The change of the color might be resulted from the exited surface plasmon resonance in the newly synthesized AgNPs [23]. The control, containing only distilled water with $AgNO_3$, showed no change in color Figure-(2a. and b.).

Figure-2 (a) Synthesized AgNPs characterized as light yellow color at time 0 . b) Synthesized AgNPs characterized as brown color after 24hr.

Characteristics of synthesized AgNPs

The surface features of synthesized AgNPs was measured by atomic force microscopy which typically shows digital images that allow quantitative measurements such as root mean square roughness (Rq) and average roughness (Ra), as well as the analysis of images from different perspectives, including 3D simulation [24]. Figure- 4a,b and Figure-5a,b illustrate the two and three dimensional AFM images of the AgNPs synthesized by soil bacterial strains. Average diameters of grains were 63.50 nm and 45.81 nm for AgNPs synthesized by *Pseudomonas sp.* and *Enterobacter* supernatant, respectively, as calculated by granularity cumulating distribution chart (GCDC) (Figure-3,c) and (Figure- 4.c). This average size was larger than that reported for AgNPs synthesized from algae or plant extracts (1-30 nm), while it was smaller than that of AgNPs synthesized by other bacterial sources which may reach 100nm or more [25].





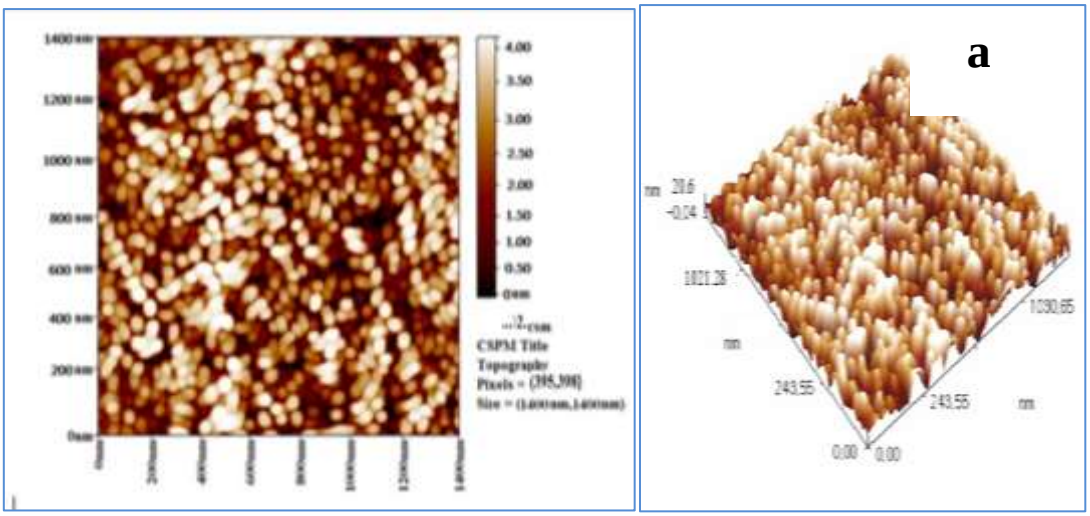
Avg. Diameter:63.50 nm **<=10% Diameter:0 nm**

<=50% Diameter:60.00 nm **<=90% Diameter:75.00 nm**

c

Diameter (nm)<	Volume(%)	Cumulation(%)	Diameter (nm)<	Volume(%)	Cumulation(%)	Diameter (nm)<	Volume(%)	Cumulation(%)
50.00	10.88	10.88	65.00	18.65	59.07	80.00	10.36	91.19
55.00	13.99	24.87	70.00	11.40	70.47	85.00	8.81	100.00
60.00	15.54	40.41	75.00	10.36	80.83			

Figure 3-AFM images of AgNPs. a)Two dimensional ; b) three dimensional ; and c) the granularity cumulating distribution chart of AgNPs synthesized by soil environment bacteria (*Pseudomonas sp.*).



Avg. Diameter:45.81 nm **<=10% Diameter:33.56 n** **c**

<=50% Diameter:40.23nm **<=90% Diameter:48.12 n**

Diameter(nm)<	Volume(%)	Cumulation(%)	Diameter(nm)<	Volume(%)	Cumulation(%)	Diameter(nm)<	Volume(%)	Cumulation(%)
35.00	22.36	22.36	45.00	33.88	73.59	55.00	26.03	100.57
40.00	35.29	45.65	50.00	35.96	88.55			

Figure 4-AFM images of AgNPs. a)Two dimensional ; b) three dimensional ; and c) the granularity cumulating distribution chart of AgNPs synthesized by soil environment bacteria (*Enterobacter*).

Antibacterial activity of synthesized AgNPs

The results showed antibacterial effects of the synthesized AgNPs product against both *P.aeruginosa* and *Staphylococcus aureus* MDR pathogenic bacteria. The highest value of inhibition zone in the Gram positive group of pathogenic bacteria (35mm) was detected against *Staphylococcus aureus* (Figure- 5.b), whereas the highest value of inhibition zone (45mm) against Gram negative bacteria was against *P.aeruginosa*; Gram positive bacteria was less affected than gram negative bacteria in this study (Figure-5,a, Table-2).

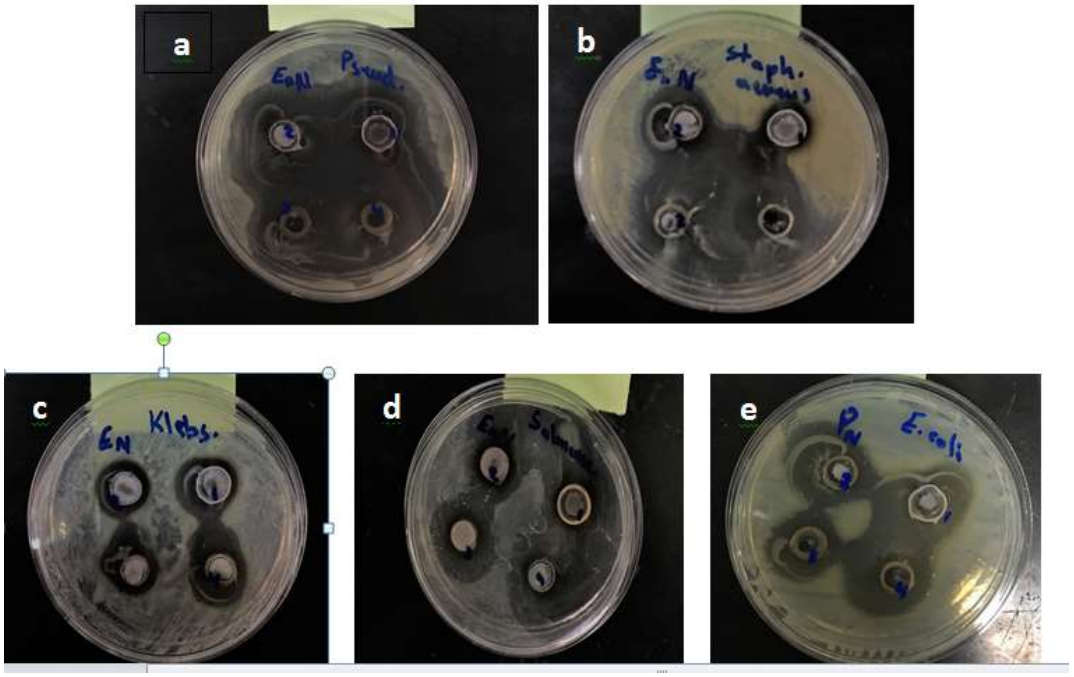


Figure 5-Antibacterial inhibition zones of AgNPs against: a) *Pseudomonas sp.* , b) *S.aureus*, c) *Klebsiella*, d) *Salmonella*, and e) *E.coli*

The results of antibacterial effects showed that the synthesized AgNPs by the supernatant of *Enterobacter sp*. caused the highest values of inhibition zones against MDR pathogenic bacteria , while AgNPs synthesized by *Klebsiella sp*. showed the lower effect (Table-2). These results provide support to those from other studies which emphasized the antibacterial activity of AgNPs synthesized by bacterial sources [26]. Synthesized AgNPs are known to be diverse depending on the source and kind of technique used in the synthesis as well as other factors which result in specific characteristics of AgNPs with variations in the grain size and shape of the newly synthesized nanoparticles [27, 28, 29].

Table 2-Inhibition zones (mm) of AgNPs synthesized by supernatant of siol bacterial isolates against pathogenic bacteria.

Soil isolates / MDR pathogenic bacteria	**Inhibtion zones (mm), AgNPs mg/ml**															
	Enterobacter sp.				***E.coli***				***Pseudomonas sp.***				***Klebsiella sp.***			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Salmonella sp.	**25**	**23**	**21**	**12**	**16**	**15**	**15**	**13**	**25**	**22**	**20**	**12**	**15**	**12**	**10**	**R**
E.coli	**35**	**24**	**20**	**16**	**25**	**20**	**15**	**11**	**27**	**25**	**21**	**18**	**11**	**9**	**R**	**R**
Pseudomonas sp.	**45**	**43**	**40**	**36**	**35**	**31**	**28**	**20**	**33**	**29**	**26**	**19**	**17**	**11**	**9**	**R**
Klebsiella sp.	**12**	**10**	**8.5**	**7.5**	**21**	**15**	**12**	**9**	**15**	**11**	**9**	**8**	**14**	**14**	**10**	**8**
Staphylococcus aureus	**35**	**32**	**21**	**10**	**22**	**20**	**12**	**8**	**22**	**21**	**12**	**9**	**11**	**8**	**R**	**R**

MDR: Multi drug resistance; mm: milimiter; AgNPs: Silver nanoparticals; (1: 0.84mg/ml, 2: 0.42mg/ml, 3: 0.21mg/ml, 4: 0.10mg/ml); R: resistant.

The mechanisms responsible for these bactericidal effects of AgNPs are not well-known; they may attach to the surface of bacterial cell membrane and disturb its physiological functions or enter the bacterial cell and cause the destructive effect [30].

Nanoparticles have been shown to be in contact with bacterial cell walls to achieve successful antibacterial function. The forms of contact may be through hydrophobic interactions, electrostatic attraction and van der Waals forces [31, 32, 33]. After contact of AgNPs with bacterial cell, they may enter the cell membrane and disrupt metabolic pathways of the cell, as a result influencing function and shape of the cell. Therefore, it is clear that AgNPs interact with basic components of the bacterial cell, such as enzymes, ribosomes and DNA, leading to permeability changes of the membrane, oxidative stress, electrolyte imbalance, enzyme malfunction, and finally gene expression changes [34,35]. The AgNPs have the ability to enter biofilms, which provides a practical method to inhibit biofilm formation [36]. The different simultaneous mechanisms of action that AgNPs use against bacteria would need many gene mutations to be developed in the same bacterial cell for antibacterial resistance; therefore, it is suggested to be difficult for bacterial cells to become resistant to AgNPs [37]. The effect of AgNPs has been detected in animals as well, studies have been noting to its effect on albino rats especially at histogenesis of cerebellum in post-implantation of embryo rats [38].

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