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Evaluating the Antibacterial Activity of the Nanoparticles of Silver on *Pseudomonas Aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa as one of the leading causes of nosocomial infections is a gram negative bacterium and an opportunistic pathogen. Since this bacterium is highly resistant to drugs, several new methods have been investigated to fight against it. Applying nanoparticles of silver is one of the most effective ways to tackle bacterial infections. In this research, the antibacterial properties of nano- silver, with two different sizes, was evaluated for anti-Pseudomonas aeruginosa activity. In this research, the powdered nanoparticles of silver with approximate diameters of 20 nanometers (Pishtazan Inc. Mashad, Iran) and 5 nanometers (Department of Chemistry in Maragheh University) were evaluated for antibacterial activity. The concentration of these nanoparticles was identified by (atomabsorbtion) spectroscopy method in the Faculty of Chemistry in Tabriz University, Iran. Then, the anti-Pseudomonas characteristics of nanoparticles were investigated by MIC (Minimum Inhibitory Concentration), MBC (Minimum Bactericidal Concentration) identification, disc diffusion and well diffusion methods. In this study, was investigated and compared the anti-bacterial effects of nanoparticles of silver on Pseudomonas aeruginosa PAO1 and 20 clinical strains isolated from Imam Reza Hospital in Tabriz, Iran. The findings revealed that the MIC and MBC of 20nm nanoparticles were 625 ppm and 1250 ppm respectively for Pseudomonas aeruginosa PAO1. On the other hand, the MIC and MBC of 5 nm nanoparticles were 75 ppm and 156 ppm respectively for Pseudomonas aeruginosa PAO1. According to these findings, the MIC and MBC identified for clinical Pseudomonas aeruginosa strains under study along with the PAO1 strain failed to show a significant difference. Yet the amount of inhibition for the 20nm nanoparticles in the density of 20000 ppm of clinical Pseudomonas aeruginosa and PAO1 strains was 11 millimeter with the disc diffusion method and 10 millimeters for the well diffusion method with the same concentration. The amount of inhibition of 5nm nanoparticles in the 250-ppm density with disc diffusion and well diffusion methods were 10 and 9 millimeters respectively. Results demonstrated that Pseudomonas aeruginosa is sensitive to nanoparticles of silver. Furthermore, the antibacterial effect of nanoparticles of silver increases as their size decreases. Besides, equality of MIC and MBC in numerous clinical cases reveals that there is no resistance against nanoparticles of silver in drugs resistance pathogenic bacteria.

Keywords: *Pseudomonas aeruginosa*; Nanoparticles of silver; Well diffusion; MIC and MBC

INTRODUCTION

Pseudomonas aeruginosa is gram negative and aerobic organism that moves through 2- 3 polar flagella. It could be found in soil and water. *Pseudomonas aeruginosa* is an opportunistic nosocomial pathogen that could infect patients

hospitalized in intensive care unit, burns, and other units of hospital with secondary acquisitive infections. Besides, it is the major cause of infection in Cystic fibrosis cases. This organism produces several pigments such as Pyocyanin (blue), Pyoverdine (green), Pyorubin (red) and pyomelanin (black) in the culture medium. The presence Aminoacetophenone in its colony makes it smell like grapes or jasmine. One of the most important virulence factors in this bacterium is Exotoxin A [1]. The belief that nanotechnology has commenced a new era in sciences combining engineering, biology, chemistry, medicine, and physics is generally accepted among scientists. Studies have shown that the smaller the size of the nanoparticles gives them novel activities. These characteristics have increased the rate of using nanomaterials dramatically so as various aspects of life including electronic systems, fighting microbes and diagnosis and treatment of diseases have been affected by them [2,3].

Nanoparticles are particles with a diameter of about 10^{-9} meter. This extremely tiny size gives them unique physical, chemical, mechanical, magnetic and electric characteristics and let them enter the cells freely and interfere in their natural activities [4]. Nanoparticles have historically been categorized in two metallic and nonmetallic categories. Metallic nanoparticles have long been used in insecticides and bactericides. They are also known as novel developments in modern pharmacological sciences and their high potentials have drawn the attention of studies in biology, pharmacology, and several specific treatment procedures. These particles have the lowest level of toxicity in the cycle of life and ecosystem[5].

Silver is one of the most common and useful metals and possesses various antibacterial potentials. A wide range of health problems like burning infections, typhoid, anthrax and conjunctivitis in kids have always been treated with silver [6].

Different mineral antibacterial materials like copper and silver were used to treat microbial infections long before modern chemotherapy was introduced to new care systems. Recent advancements in the nanotechnology and nanoparticles have developed organic and mineral nanoparticles and this have increased their use in industry, medicine, artificial textiles and food packaging [7].

Nanoparticles have a better and different quality compared to other forms of the same element. A little bit of them can have a great deal of antibacterial effect. Therefore, nanoparticles of silver have a stronger antibacterial effect compared to silver itself [8,9].

The inhibitory effect of the nanoparticles of silver may come from their interference in the biological mechanisms of the bacteria: 1) these nanoparticles penetrate the cell wall of the bacteria and change it. This increases penetrability of cell membrane and interrupt the control of material intake and output from the cytoplasm. 2) The antibacterial mechanism of nanoparticles of silver is associated to the free radicals. 3) Their joining with the DNA does not let it replicate and inhibit cell division ending in the death of the cell. 4) Besides, the silver ion controls the enzymes of the respiratory cycle. 5) Silver ions have a great tendency toward joining to the thiol groups of the vital enzymes thanks to the presence of phosphorus [10-12].

Considering the mechanism of the function of silver nanoparticles, it could be said that they have advantages over antibiotics. Here is a list of those advantages: 1) bacteria do not gain resistance against nanoparticles of silver, because they affect several sites and different enzymes. 2) Nanoparticles of silver are effective on a wide range of bacteria. 3) Nanoparticles of silver have no bad effect on human cells because; human cells are in tissue forms. 4) Unlike antibiotics that transform after interaction with the cell and lose their power, nanoparticles of silver are released after affecting the microbe and influence other microorganisms [13].

Pseudomonas aeruginosa is one of the major causes of nosocomial infections and has a great potential in becoming resistant to drugs. This bacterium is also of numerous antibiotic resistant factors due to plasmid and chromosomes making it very difficult to treat by antibiotics. Moreover, new antibiotics have failed to reduce mortality and morbidity caused by this bacterium. Low penetrability of outer membrane and the presence of various drug efflux pumps are two of the reasons for its resistance so that various antibiotics lose their effectiveness against it.

Objectives

The present study aims to investigate the effects of nanoparticles of silver in clinical and standard strains of *Pseudomonas aeruginosa* and determination of nanoparticles of silver MIC and MBC for *Pseudomonas aeruginosa*.

Materials and methods

Nanoparticles: The present study used two types of powdered nanoparticles of silver with an approximate size of 20 nm bought from the Pishtazan Nanotechnology Co (Mashad, Iran) and nanoparticles of 5nm synthesized by the Chemistry Department of Maragheh university (Maragheh, Iran) .

Bacteria and Media: The *Pseudomonas aeruginosa* (PAO1) was obtained from Biotechnology Research Center, Tabriz University of Medical Sciences (Tabriz, Iran) , and 20 clinical strains of *Pseudomonas aeruginosa* were isolated from different samples in Imam Reza Hospital of Tabriz (Tabriz, Iran). In order to identify the bacteria, the necessary experiments including Gram Staining, catalase test and the oxidase test were conducted. Müller-Hinton agar growth medium was used in well diffusion and disc diffusion methods and the nutrient broth was used in micro dilution method.

The antibacterial effects evaluation of Nano silver

A) Determination of MIC and MBC:
First 0.08 g of each size of the nanoparticles (20 and 5 nm) were weighed and then solved in 2 ml of nutrient broth. Then serial dilutions of 312, 625, 1250, 2500, 5000, 10000, 20000, and 40000 ppm were prepared. Then, 10 µl of microbial suspension of *Pseudomonas aeruginosa* equaling 0.5 McFarland ($1.5X10^6$ CFU) was added to each tube. In this way, fixed amounts of bacteria are exposed to various densities of nanoparticles of silver. The tubes were kept for 24 hours in an incubator with shaker at 37°C . Then, 10 microliters of each tube was diluted in 15 ml of physiology serum and sub cultured in Müller-Hinton agar and incubated at 37° C for 24 hours. After incubation, the bacterial colonies were evaluated for determination of the MIC and MBC of nanosilver. The concentration in which 99.99% of the bacteria were dead was taken as the MBC and the latest transparent tube was considered to be the MIC.

B) The antibacterial effects evaluation by well diffusion and disc diffusion:
In order to evaluate the sensitivity of bacteria to nanoparticles of silver through well diffusion and disc diffusion, first the *Pseudomonas aeruginosa* was grown overnight in nutrient broth, bacteria were collected by centrifuge, and resuspended in PBS for preparation of 0.5 McFarland equal bacterial suspension. After dripping the sterilized swap in the microbial suspension, the extra solution is extracted through pressing swap into the side of the tube and the plate surface of Müller-Hinton agar was inoculated three times with a 60° by this swap. Then, 20 microliters of 20nm nanoparticles with 5000, 10000, 20000 and 40000 ppm concentrations and 20 microliters of 5 nm nanoparticles with 625, 1250, 2500, 5000 ppm concentrations were separately inoculated to the growth medium in a pointed manner. The distance between inoculated points from each other was at least 15 mm. the plates were incubated for 24 hours at 37° C . finally, the diameter of the inhibited zones was measured.

In the disc diffusion method, four blank discs were putted on inoculated Müller-Hinton agar in a standard form. Then 30 microliters of each dilution of nanoparticles were poured on each blank disc. The plates were incubated for 24 hours at 37° C and the diameter of the inhibited zones was measured. All above-mentioned stages were repeated three times and the mean of the results were recorded.

RESULTS

Results showed the MIC and MBC of silver nanoparticles (with 20 nm size) were 625 ppm and 1250 ppm and the MIC and MBC of silver nanoparticles (with 5 nm size) were 75 ppm and 156 ppm for all of clinical isolated *Pseudomonas aeruginosa* and *PAO1* strains. The MIC and MBC results obtained for clinical isolates *Pseudomonas aeruginosa* and PAO1 strains, showed no significant difference (Fig1, 2 and table 1).

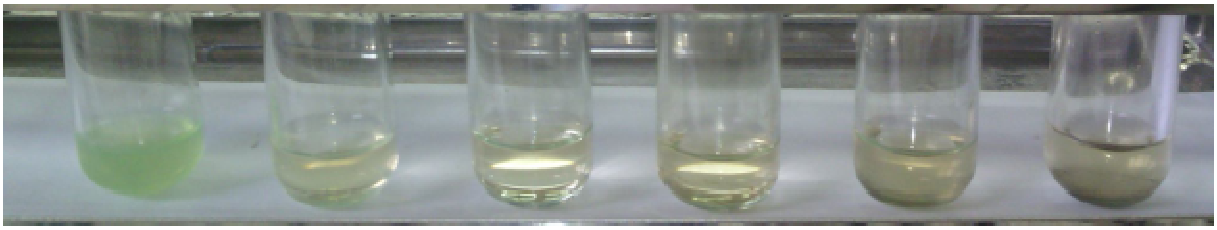

Fig1. The MIC and MBC of silver nanoparticles with 20 nm size micro dilution method

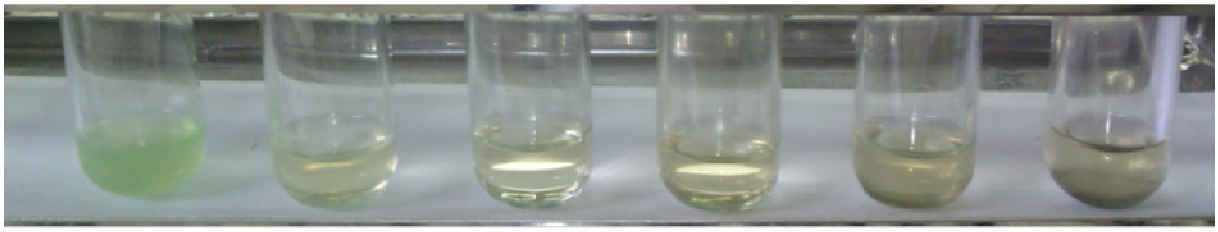

Fig2. The MIC and MBC of silver nanoparticles with 5 nm size micro dilution method

Table 1. The rate of MIC and MBC of nanoparticles of silver for 20 strains of *Pseudomonas aeruginosa* and the PAO1 strain

Strains of *Pseudomonas aeruginosa* under study	20 nm nanoparticles of silver		5 nm nanoparticles of silver	
	MIC(ppm)	MBC(ppm)	MIC(ppm)	MBC(ppm)
1	625	1250	78	156
2	625	1250	312	625
3	625	1250	78	156
4	1250	2500	156	312
5	2500	5000	78	156
6	1250	2500	39	78
7	312	625	312	625
8	625	1250	78	156
9	1250	2500	78	156
10	2500	5000	78	156
11	625	1250	312	625
12	312	625	78	156
13	625	1250	78	156
14	2500	5000	78	156
15	312	625	39	78
16	625	1250	78	156
17	625	1250	156	312
18	625	1250	78	156
19	1250	2500	78	156
20	625	1250	156	312

Besides, results from the well diffusion method demonstrated that the increase in the density of nanoparticles of silver increases the diameter of the inhibited growth zone of the bacteria. The average size of inhibited growth zone in *Pseudomonas aeruginosa* is represented in Fig 3.

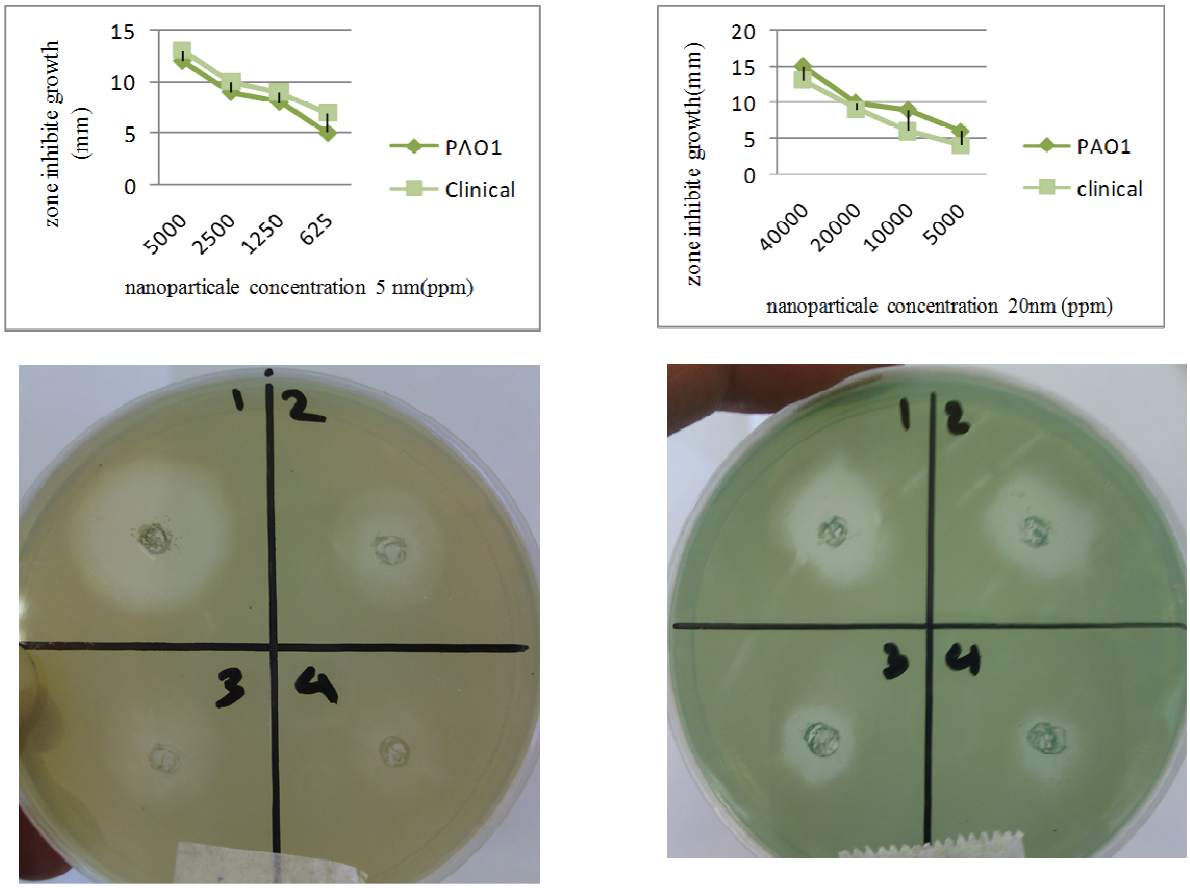

Fig 3. The comparison among the inhibited growth zone through well diffusion in different concentration of nanoparticles between *Pseudomonas aeruginosa PAO1* and clinical *Pseudomonas aeruginosa* (1. 40000, 2. 20000, 3. 10000, 4. 5000 ppm)

Moreover, the results from disc diffusion method like the ones from the well diffusion method show that the increase in the density of nanoparticles of silver increases the diameter of the inhibited growth zone. The mean of the inhibited growth zone of *Pseudomonas aeruginosa* for the prepared concentrations are represented in Figure 4.

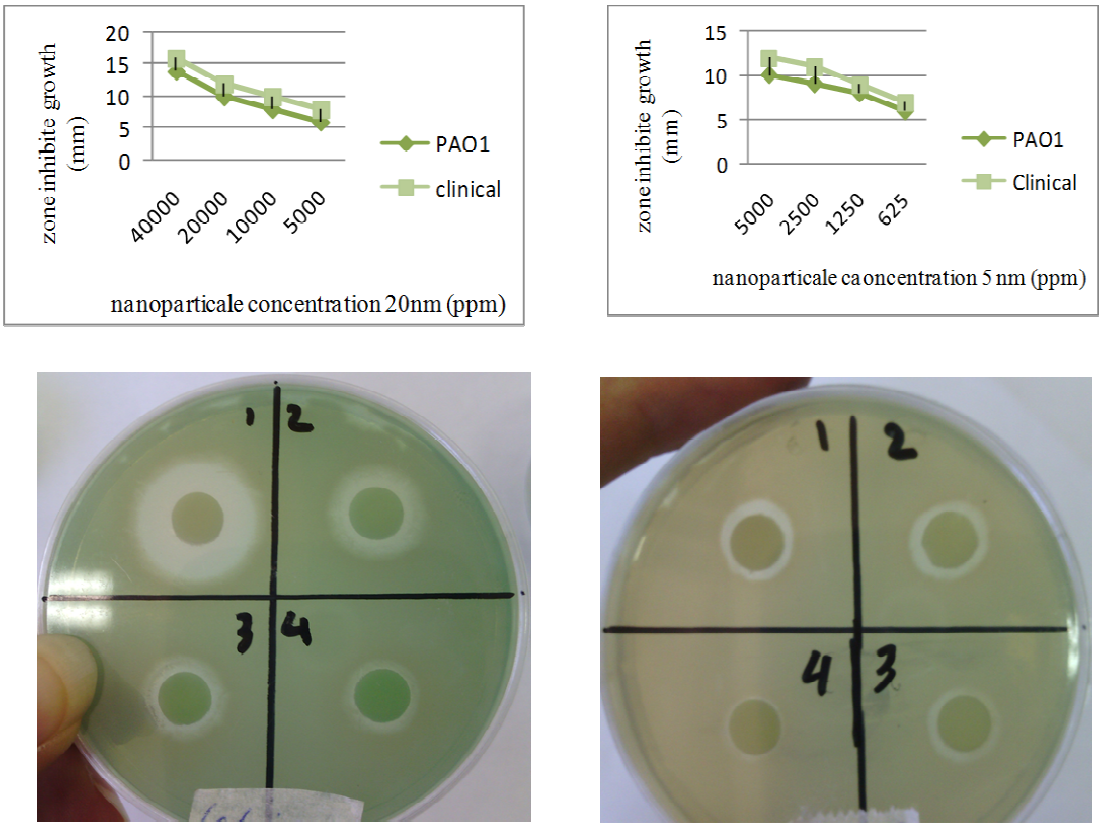

Fig 4. The comparison among the inhibited growth zone through disc diffusion in different concentration of nanoparticles between Pseudomonas aeruginosa PAO1 and clinical *Pseudomonas aeruginosa*. (1. 5000, 2. 2500, 3. 1250, 4. 625)

DISCUSSION

Nowadays, nosocomial infections have become a serious problem in the health and hygiene as the hospitals begin to develop. *Pseudomonas aeruginosa* is one of the most important causes of nosocomial infection that has a high potential of gaining resistance against drugs thus several antibiotics lose their effectiveness day by day. Using nanoparticles against bacterial infections has proven to be one of the most effective methods. The present study used the bactericidal effects of nanoparticles of rolled silver. Considering the results from microdilution method, it could be said that nanoparticles of silver could inhibit the growth of *Pseudomonas aeruginosa* in low densities. This concords with the results from Sondi et. al. working on *Escherichia coli* [14]. They also concord with the studies conducted by Athirah et. al. on drug resistant *Pseudomonas aeruginosa* [6].

In both well diffusion and disc diffusion methods, results revealed that the increase in the concentration of the nanoparticles of silver enlarges the diameter of the inhibited growth zone of the *Pseudomonas aeruginosa*. The reason for this may be the inhibited dispersion in solid environment for nanoparticles of silver swiftly connect to each other and do not contact with the microbes. Yet the diameter of the inhibited growth zone greatly depends on the dose of nanoparticles of silver used. This concords with the results from Kim et. al. studying *Escherichia coli*, *Staphylococcus aureus* and the yeast in 2007 [10].

The study conducted by Humberto et.al. in 2010 the inhibitory effect of nanoparticles of silver were investigated on the *Pseudomonas Aeruginosa* and *Escherichia coli* resistant to Ampicillin and the *Streptococcus pyogenes* resistant to Erythromycin. They approved of the bacteriostatic effects of nanoparticles of silver on bacteria. *Pseudomonas aeruginosa* has several antibiotic resistant factors due to its plasmid and chromosome making its antibiotic treatment very difficult. Thus, new antibiotics failed to stop the mortality and morbidity it causes. The low penetrability of exterior membrane of the bacteria and the presence of drug efflux pumps are among the mechanisms causing its drug resistance [15]. Furthermore, in 2005, Morones et.al investigated the bactericidal effects of nanoparticles of

silver on four gram-negative bacteria including *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Escherichia coli and Salmonella*. They discovered that nanoparticles of silver stick to the cell membrane of the bacteria degenerate the membrane and disrupt the penetrability of membrane through releasing silver ion [16].

CONCLUSION

Nanoparticles of silver have similar bactericidal effect on standard and clinical *Pseudomonas aeruginosa*. Moreover, they have similar effects on the drug resistant and drug sensitive bacteria.

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