

Comparison of Antibacterial Activity of Green Synthesized Silver Nanoparticles with Different Morphologies, Using MIC Method

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In this work silver nanoparticles were produced using tannic acid solution as reducing and capping agent. Results of UV-Vis and XRD ensured the existence of silver nanoparticles in the final samples. Nanoparticles with different morphologies were detected using FE-SEM micrographs. The antibacterial activity of the obtained silver nanoparticles was investigated against six bacteria strains (Gram-positive and Gram-negative) using Minimum Inhibitory Concentration (MIC) method. The results of MIC tests for these silver nanoparticles demonstrated the inhibition of growth for most bacteria, including *Klebsiella pneumonia*, *Bacillus subtilis*, *Acinetobacter baumannii*, *Escherichia coli* and *Proteus mirabilis*.

Keywords: Silver nanoparticles, Different morphologies, Antibacterial activity, Minimum inhibitory concentration (MIC).

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1. INTRODUCTION

Silver nanoparticles are attracting a lot of attention in recent years, due to their wide applications in different fields such as catalytic materials, information storage, fabrication of polymer composites and textile product with antibacterial properties. These nanoparticles indicate great antibacterial activity against bacteria, algae and fungi. As a result, they can be used for so many medical purposes [1-3]. Wound healing is one of the most well commonly used applications of silver nanoparticles. Compared with other silver compounds, many studies have demonstrated the superior efficacy of silver nanoparticles in healing time, as well as achieving better appearance of the wounds after healing [4-5]. The antibacterial mechanism of silver nanoparticles is not completely comprehended. Yet, several studies propose that silver nanoparticles may react with or attach to the surface of the cell membrane and this will cause disturbance in permeability and vital functions of the cell. It is also possible that not only silver nanoparticles interact with the proteins and/or enzymes on the surface of membrane, but can also penetrate inside the microorganism and cause dysfunction inside the cells. Moreover, due to the large surface area of nanoparticles, the smaller silver nanoparticles would have more interaction with bacteria and have more bactericidal effect [6-8].

Chemical reduction is the most common method for producing silver nanoparticles due to its convenience. In recent years, green chemistry methods for preparing silver nanoparticles with antibacterial properties have been proved to be far more suitable than other applied methods [9]. Many methods were employed to enhance green preparation of silver nanoparticles and investigating their antibacterial activity, using natural compounds and plant extracts, including edible mushroom extract [10] honey [11], Aloe vera plant extract [12], glucose in the presence of soluble starch as a stabilizing agent [13], Hibiscus leaf extract [14], *Mimusops elengi*

leaf extract, [15], *Millingtonia hortensis* extract [16], *Artocarpus heterophyllus* seed extract [17], *Mangifera indica* extract [18], Cashew leaf [19], sucrose and maltose [20] and *Macrotyloma uniflorum* [21].

Tannic acid has a polyphenolic structure which enables it to play the role of reducing agent and capping agent simultaneously, using numerous phenol groups and steric hindrance of the molecules.

In this work, silver nanoparticles were produced as instructed in the work of Yi et al, [22] using tannic acid aquatic solution (as reducing and capping agent), for reducing silver nitrate at room temperature. Two types of morphology were detected using FE-SEM micrographs. The antibacterial activity of the obtained silver nanoparticles was also investigated against six bacteria strains (Gram-positive and Gram-negative) using Minimum Inhibitory Concentration (MIC) method.

2. EXPERIMENTAL

2.1 Materials

Silver nitrate (AgNO_3 , 99.9%), tannic acid ($\text{C}_76\text{H}_52\text{O}_{46}$) and potassium carbonate (K_2CO_3) were used in synthesizing of silver nanoparticles. Ethanol was used as solvent for washing the precipitated nanoparticles (All materials purchased from Merck Chemicals, Germany, and used without further treatments). All of the solutions were freshly made using de-ionized (D.I.) water (purchased from Zolal Company, Iran).

The bacterial strains used in this study were *Klebsiella pneumonia* ATCC 700603, *Bacillus subtilis* ATCC 19659, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Proteus mirabilis* ATCC12453, Which were supplied by Shahid Beheshti University of medical sciences (Tehran, Iran). All bacterial strains were grown and maintained on nutrient agar slants. Barium chloride (BaCl_2) and Mueller-Hinton broth (Merck chemicals, Germany) was used to prepare micro dilution broth.

2.2 Methods

Following studies of Yi et al [22] on synthesizing silver nanoparticles, the suitable pH value was reported 7 for producing silver nanoplates. Therefore, since in this study, antibacterial use of these nanoparticles was considered, they were produced in aqueous media at pH value of 7, at room temperature. Synthesis was done as illustrated in Fig. 1 and in the complete absence of light.

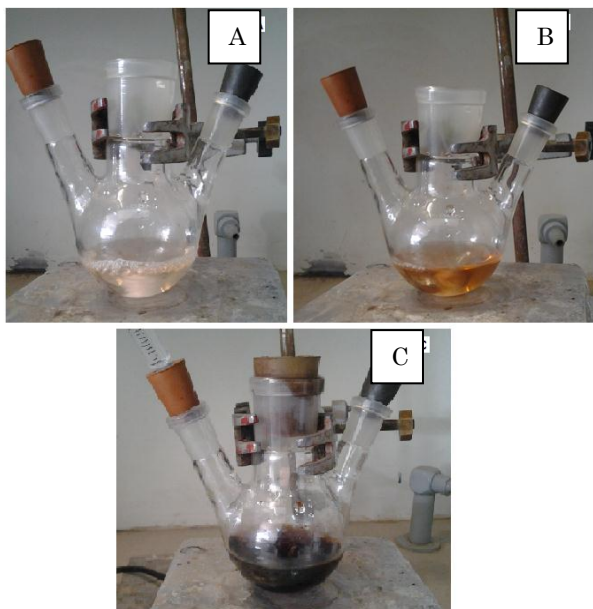


Fig. 1 – Different stages of the synthesis of the silver nanoparticles. (A) Tannic acid solution, (B) after adding silver nitrate solution and (C) after adjustment of pH value using K_2CO_3

Different concentrations of silver nitrate and tannic acid solutions were mixed in room temperature, in order to obtain different particle sizes and morphologies (Table 1). This process was done using magnetic stirring for four hours in a dark room. After that, the nanoparticles were precipitated using centrifuge (RST16, D.T.A.P Co., Iran) with 4000 rpm for 30 minutes. The final nanoparticles were obtained after washing the precipitate twice with ethanol and homogenizing in D.I. water using ultrasound (400W Topsonic, Ultrasonic Technology Development Co., Iran) with 100 Watt power for 20 minutes.

Table 1 – The recipe for Silver nanoparticle samples at pH value of 7 and room temperature

Sample	$AgNO_3$ (mM)	Tannic Acid (mM)	$AgNO_3$ /tannic Acid ratio
No.1	5	0.25	20
No.2	5	4	1.25

X-ray Diffraction patterns (D8 ADVANCE, Bruker-AXS, USA) confirmed the presence of silver crystals in obtained nanoparticles. UV-Vis spectrophotometer (JENWAY-6715, UK) was used for measuring the optical properties of final nanoparticles. Field emission scanning electron micrographs (Zeiss SIGMA/VP, Oxford Instruments, UK) were studied to characterize the

morphology and distribution of the final particles.

2.3 Antibacterial Activity

Minimum Inhibitory Concentrations (MICs) of the synthesized nanoparticles were measured against a range of Gram-positive (*B.subtilis*) and Gram-negative (*K.pneumoniae*, *A.baumannii*, *P.aeruginosa*, *E.coli* and *P.mirabilis*) bacteria. MICs were determined using the standard “two fold micro dilution method in Mueller-Hinton broth” and antibacterial susceptibilities were reported according to CLSI (2013) guidelines [23].

To standardize the density of inoculum for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard should be used. A 0.5 ml fractional solution of 0.048 mol/L $BaCl_2$ (1.175 % w/v $BaCl_2 \cdot 2H_2O$) was added to 99.5 ml of 0.18 mol/L H_2SO_4 (1 % v/v) with constant stirring to maintain a suspension. The inoculum of $5 \cdot 10^5$ CFU/ml was used for all MIC measurements and the MIC was taken as the lowest concentration of samples in the wells of the micro titer plate that showed no turbidity after 24 h at 37 °C [18].

3. RESULT AND DISCUSSION

3.1 Characterizations

Optical properties of the nanoparticles were investigated using UV-Vis spectrum (Fig. 2). The surface plasmon resonance peak for silver nanoparticles at 420-430 nm is clearly observed.

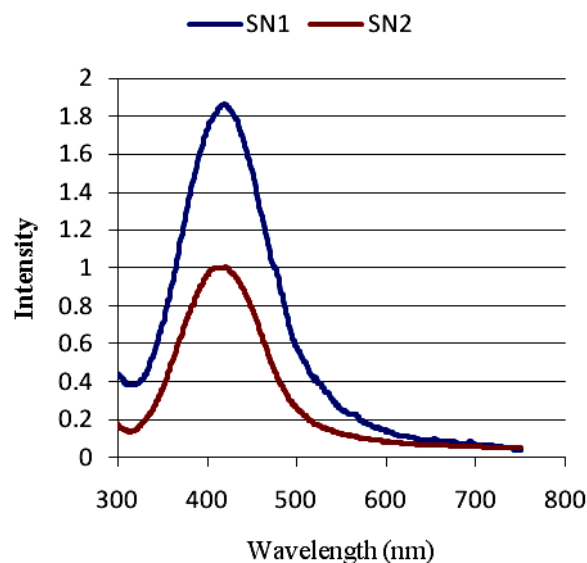


Fig. 2 – UV-Vis spectrum of the samples showing the surface plasmon resonance peak for silver nanoparticles

For further studies, XRD pattern along with FESEM micrographs were investigated. X-ray diffraction ensured the presence of silver crystals in synthesized particles. Fig. 3 shows the result for sample No. 1. The sample for XRD was prepared by drying the dispersed solution of nanoparticles in oven at low temperature (25 °C, in order to inhibit sintering of the nanoparticles), after sonication in D.I. water. (1 1 1) peak at 38.7°, (2 0 0) peak at 43.5°, (2 2 0) peak at 65° and (3 1 1) peak at 78° are in complete consistency with silver crystals identification peaks. The peaks are attributed to the diffraction of (1 1 1), (2 0 0)

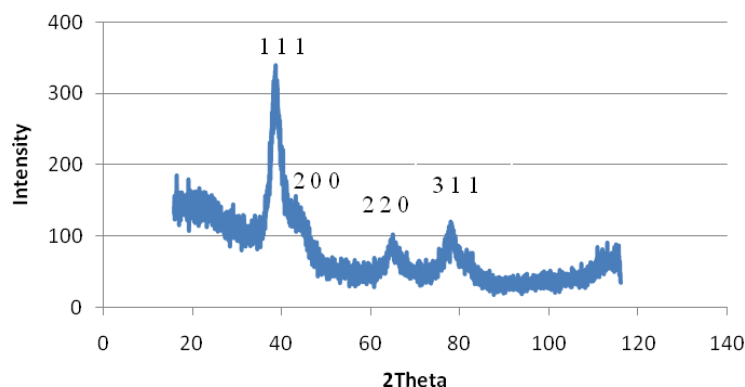


Fig. 3 – X-ray diffraction spectrum. (1 1 1) peak at 38.7°, (2 0 0) peak at 43.5°, (2 2 0) peak at 65° and (3 1 1) peak at 78°

and (2 2 0) planes of face-centered cubic (FCC) silver. The existence of (2 2 0) reflection indicates that the nanoparticles are single crystals [19].

Field emission scanning electron microscopy was used in order to confirm the size of the silver nanoparticles and determine their morphologies. The micrographs indicated narrow size distribution with particle size of 10-20 nm (Fig. 4A) and also spherical morphology for sample No. 1 (Fig. 4B). In case of sample No. 2, FE-SEM micrographs indicated planar morphology. Fig. 5A shows these nanoplates or nanosheets with 150-800 nm width, and Fig. 5B specifies one of the nanoplates with width of approximately 600 nm, and other vertical plates with thicknesses less than 10 nm.

The probable reason for this difference in morphology is the low concentration of tannic acid in the synthesis medium of sample No. 1, against the high concentration used for producing sample No. 2. Tannic acid molecules have large, branched structures which due to less possibility of steric hindrance in low concentration, gives them better access to silver nitrate molecules. As a result, reduction of silver nitrate is carried out faster and the yield of reduction is much higher.

However, when the concentration of tannic acid is high, these large molecules cause extreme steric hindrance. Despite of the fact that same amount of silver nitrate was used in both samples, reduction of silver nitrate happens slowly and the process has lower yield than sample No. 1. This slow rate of reduction results in planar morphology. The steric hindrance of tannic acid molecules causes difficulty in controlling the particle size distribution and the final nano plates/sheets had width from 150 to 800 nm.

3.2 Determination of MICs

The previous works which studied the antibacterial activity of silver nanoparticles mostly used Kirby-Bauer disc diffusion method. For example Guzmán et al studied silver nanoparticles produced by using hydrazine hydrate (reducing agent), sodium citrate (reducing and stabilizing agent) and sodium dodecyl sulphate (SDS) (also as stabilizing agent) at room temperature. The results demonstrated that the colloidal silver nanoparticles inhibited the growth of the tested bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus*, *E. coli* and *P. aeruginosa* [26].

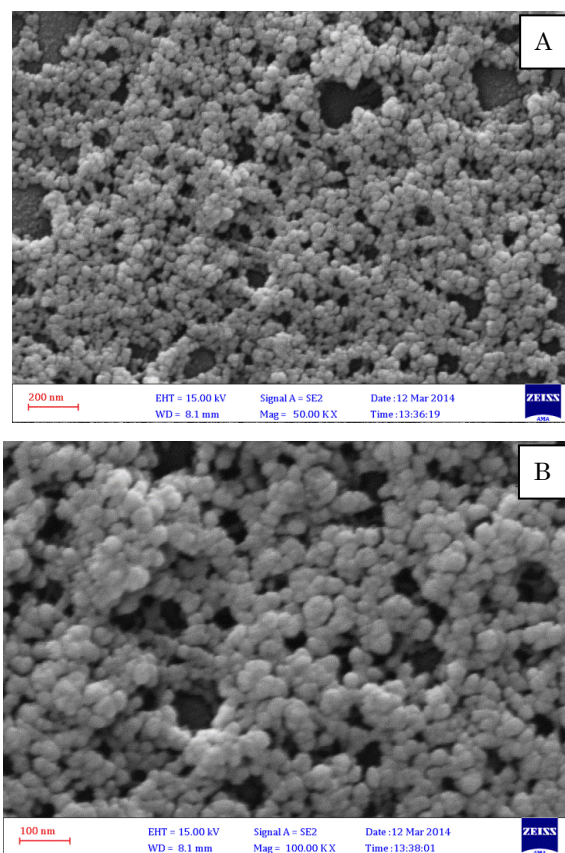


Fig. 4 – FE-SEM micrographs for sample No. 1, (A) narrow size distribution (B) spherical morphology of the nanoparticles

Saravanan et al. also investigated the antibacterial activity of silver nanoparticles which were synthesized using *Mimusops elengi* leaf extract as reducing and stabilizing agent. Outstanding antimicrobial efficiency was detected by clear zone of inhibition (Kirby-Bauer diffusion method) against *K. pneumoniae*, *M. luteus* and *S. aureus* [15].

Bapat et al studied the same properties for the silver nanoparticles which were produced by using *Artocarpus heterophyllus* Lam. seed extract. The inhibition zones of agar diffusion method were reported for several Gram-positive (*B. cereus*, *B. subtilis*, *S. aureus* and *S. typhomurium*) and Gram-negative (*P. aeruginosa* and *P. vulgaris*) pathogenic bacteria. The results indicated no antibacterial activity against *S. typhomurium* and *P. vulgaris* [17].

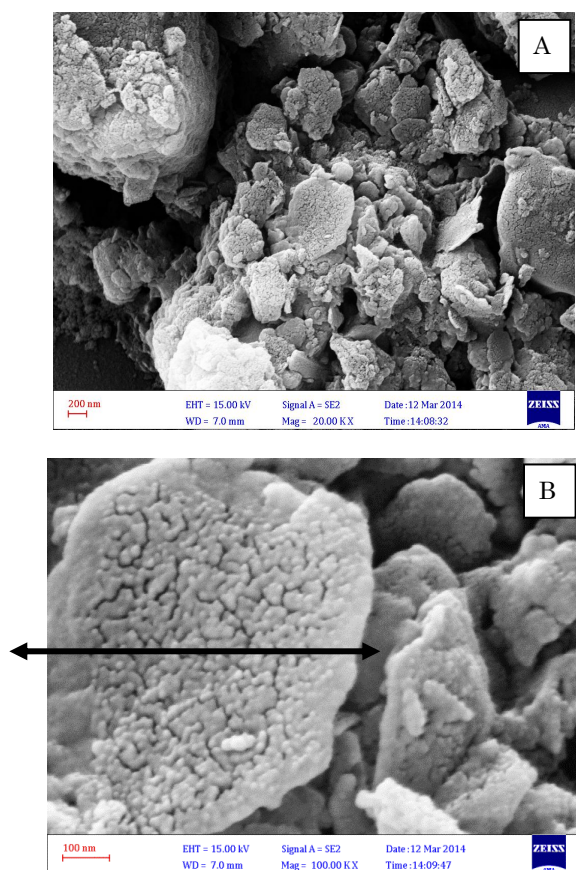


Fig. 5 – FE-SEM micrographs for sample No. 2, (A) planar morphology of the nanoparticles. (B) A nanoplate with width of approximately 600 nm

MIC results for different concentrations of silver nanoparticle samples in the wells of the micro titer plate showed different turbidities after 24 h in 37 °C. This haziness of the solution indicated the growth and multiplication of the bacteria. Both samples had acceptable results, but in case of *P. aeruginosa* there was no display of antimicrobial activity for both samples.

Table 2 also demonstrates the minimum concentrations in which the bacterial growth was stopped, as fraction of concentration 1 which was 100 µg/ml for both samples. As reported in Fig. 6, sample No. 2 had the same MIC as sample No. 1, except in cases of *K. pneumonia* and *B. subtilis*. Sample No. 1, which had spherical morphology, showed better results in these two strains.

As it was mentioned, the mechanisms of the bactericidal effect of silver nanoparticles against

bacteria are not completely known. Inhibition of bacterial growth can be caused by interaction with surface of the cell membrane and disturbing its nutritional function such as permeability and respiration [27]. It is reasonable that the attachment of the particles to the bacteria would depend on the surface area of nanoparticle available for interaction/adsorption. Smaller nanoparticles with morphologies closer to spherical shape have larger surface area available for interacting with bacteria. As a result, they will have more bactericidal effect than the larger particles.

The cell wall of Gram-negative bacteria is thinner than Gram-positive. The cell wall in Gram-negative bacteria composed of single or bi-layer Peptidoglycan while the cell wall of Gram-positive bacteria is composed of multiple layers of Peptidoglycan. This causes complications in diffusion and penetration of silver nanoparticles. Certainly the basic reason for antibacterial activity of the nanoparticles is release of silver cations from nanoparticles. Due to the toxicity of these cations for bacteria, their presence could change the permeability of the bacterial membranes, which would lead to their extermination [28]. Currently, silver nanoparticle based wound dressings are used in the clinics and these have been commonly used for many years with no reported systemic toxicity. As a result, it would seem that silver nanoparticles would be safe for humans to use at low doses [29].

4. CONCLUSION

In this study, silver nanoparticles were produced using tannic acid aquatic solution for reducing silver nitrate at room temperature at pH 7. UV-Vis spectrum and XRD pattern ensured the existence of silver nanoparticles in final samples. Two types of morphology were detected using FE-SEM micrographs.

Additionally the antibacterial activity of the nanoparticles was measured against Gram-positive and Gram-negative bacteria, using MIC method. The results demonstrated the inhibition of growth for most bacteria, including *Klebsiella pneumonia*, *Bacillus subtilis*, *Acinetobacter baumannii*, *Escherichia coli* and *Proteus mirabilis*. The lowest MIC of both samples were against *E. coli* and *P. mirabilis* in 3.12 µg/ml. Sample No.2 (with planar morphology) showed less antibacterial activity against *K. pneumonia* and *B. subtilis*. This could be due to the lower surface area of the nanoplates/sheets, and their larger width in two dimensions. But the nano spheres produced using nontoxic materials showed exceptional antibacterial

Table 2 – The results for MIC test for bacteria strains

Bacteria Strain	Gram nature	MIC Sample No. 1 (fraction of 1) (µg/ml)		MIC Sample No. 2 (fraction of 1) (µg/ml)	
<i>K. pneumonia</i> ATCC 700603	Negative	1/16	12.5	1/8	25
<i>B. subtilis</i> ATCC 19659	Positive	1/16	12.5	1/8	25
<i>A. baumannii</i> ATCC 19606	Negative	1/32	6.25	1/32	6.25
<i>P. aeruginosa</i> ATCC 27853	Negative	–	–	–	–
<i>E. coli</i> ATCC 25922	Negative	1/64	3.12	1/64	3.12
<i>P. mirabilis</i> ATCC12453	Negative	1/64	3.12	1/64	3.12

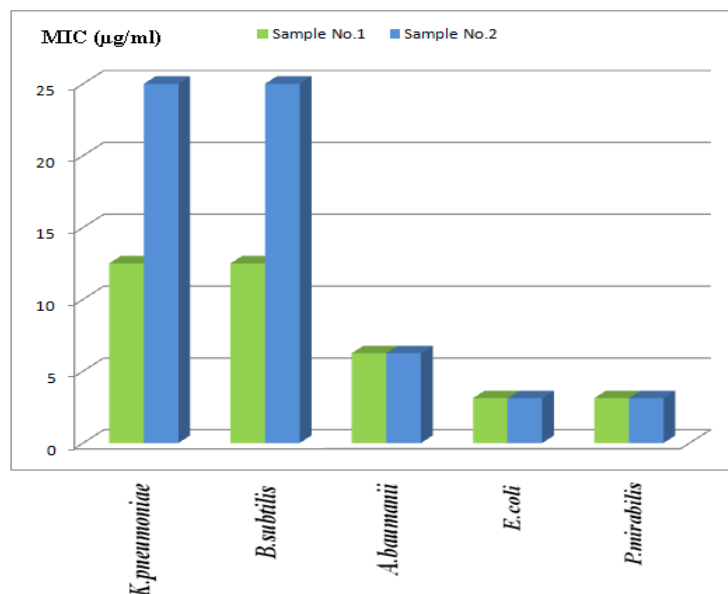


Fig. 6 – Comparing the MIC results for sample No. 1 (green columns) and sample No. 2 (blue columns)

activity because of their very high surface area and better ability to interact with bacteria.

Using “green” materials for synthesizing these

silver nanoparticles opens up the opportunities to use them for various biomedical applications such as hospital textile products or open wound dressings.

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