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Research Article

# Simultaneous Antibacterial and Antibiofilm Activities of three Types of Silver Nanoparticles against Human Bacterial Pathogens.

Suhad Hadi Mohammed[1*], Maysaa Saleh Mahdi[1], Abbas Matrood Bashi[1], Mohanad Mohsin Ahmed[2]

[1]*Faculty of Applied Medical Sciences, Department of Clinical Laboratories, Kerbala University, University Street 11252, Kerbala, Iraq*
[2]*Faculty of Medicine, Department of Microbiology, Kerbala University, University Street 11252, Kerbala/Iraq*

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**ABSTRACT**

Emergence of antibiotic resistant bacteria result in an approximately 550,000 deaths from bacterial infections annually. Several studies reported interesting antibacterial and anti-biofilm activities of silver nanoparticles synthesized by different physical, chemical and biological methods as an alternative to antibiotics. However, the resultant nanoparticles were varying in size, shape, and had different antibacterial and antibiofilm activities with different concentrations. Moreover, previous studies had investigated the antibacterial and antibiofilm activities in separate protocols and didn't investigate the real-time or dual effect of silver nanoparticles on both planktonic and sessile cells within single protocol. This study aimed to synthesize silver nanoparticles through three methods and analyzing the simultaneous antibacterial and antibiofilm activities against planktonic and sessile bacterial cells. Three methods were applied to analyze silver nanoparticles and used to investigate the dual effect against bacterial biomass and biofilm formation. The results showed that synthesized silver nanoparticles cause significant inhibition to bacterial cell biomass and bacterial biofilm formation when compared with controls at low concentration. Significant Higher antibiofilm activity than antibacterial activity was observed at very low concentration (0.0125 µg/ ml). The antibacterial and antibiofilm activity do not differ according to the type of bacteria. Whereas, the antibacterial effect differs significantly according to the methods of silver nanoparticles synthesis.

**Keywords**: Silver nanoparticles, Chemical synthesis, dual antibacterial and antibiofilm activities, gram positive and gram negative bacteria.

## INTRODUCTION

In recent years, Nanotechnology became as promising area for synthesis of nanoparticles with important application in medicine. Silver nanoparticles are the most popular nanoparticles and have several applications in human life[1]. Historically, silver compounds had been used widely for treatment of wounds and burns because of its antibacterial activity until the advent of antibiotics[2]. Emergence of antibiotic resistant bacteria to standard treatment has become serious threat and big challenge for human health worldwide[3]. The failure of various newly synthesized antibiotics in treatment of multidrug resistant bacteria resulted in demand for new antimicrobial alternatives which could be used in treatment[4]. Moreover, biofilm formation by different types of bacteria make the treatment more complicated and subsequently more than 550,000 deaths annually had been resulted from bacterial infections[5-7]. Biofilm is an exopolysaccharide matrix produced by bacteria in order to adapt sudden unfavorable conditions like nutrient depletion as well as host immune defense[8]. Bacteria that establish biofilm can tolerate 10-1000-times antimicrobial concentration which is necessary to kill the same planktonic bacteria[9]. Several studies reported interesting antibacterial and anti-biofilm activities of silver nanoparticles against gram positive and gram negative bacteria[10-12]. One of the most important advantages of using silver is that the bacteria can not develop resistance against it as they can do against some antibiotics[13]. There are different Physical, chemical, and biological methods for synthesis of silver nanoparticles[13,14]. The resultant nanoparticles differ in shape and size with different antibacterial and antibiofilm activities[10,11]. However, in these studies the antibacterial and antibiofilm assays were performed separately using specific technique for each assay and didn't investigate the real-time or simultaneous effect of nanoparticles on bacterial biomass and biofilm formation. The aim of this study is to investigate simultaneous antibacterial and antibiofilm activities of three forms of silver nanoparticles synthesized through three different chemical methods against biofilm forming bacteria and whether these bacteria would be affected by certain method of synthesis silver nanoparticles.

## MATERIALS AND METHODS

*Preparation of zinc-silver nanoparticles (Xerogel).*

**Author for Correspondence: shm.med.school@gmail.com*

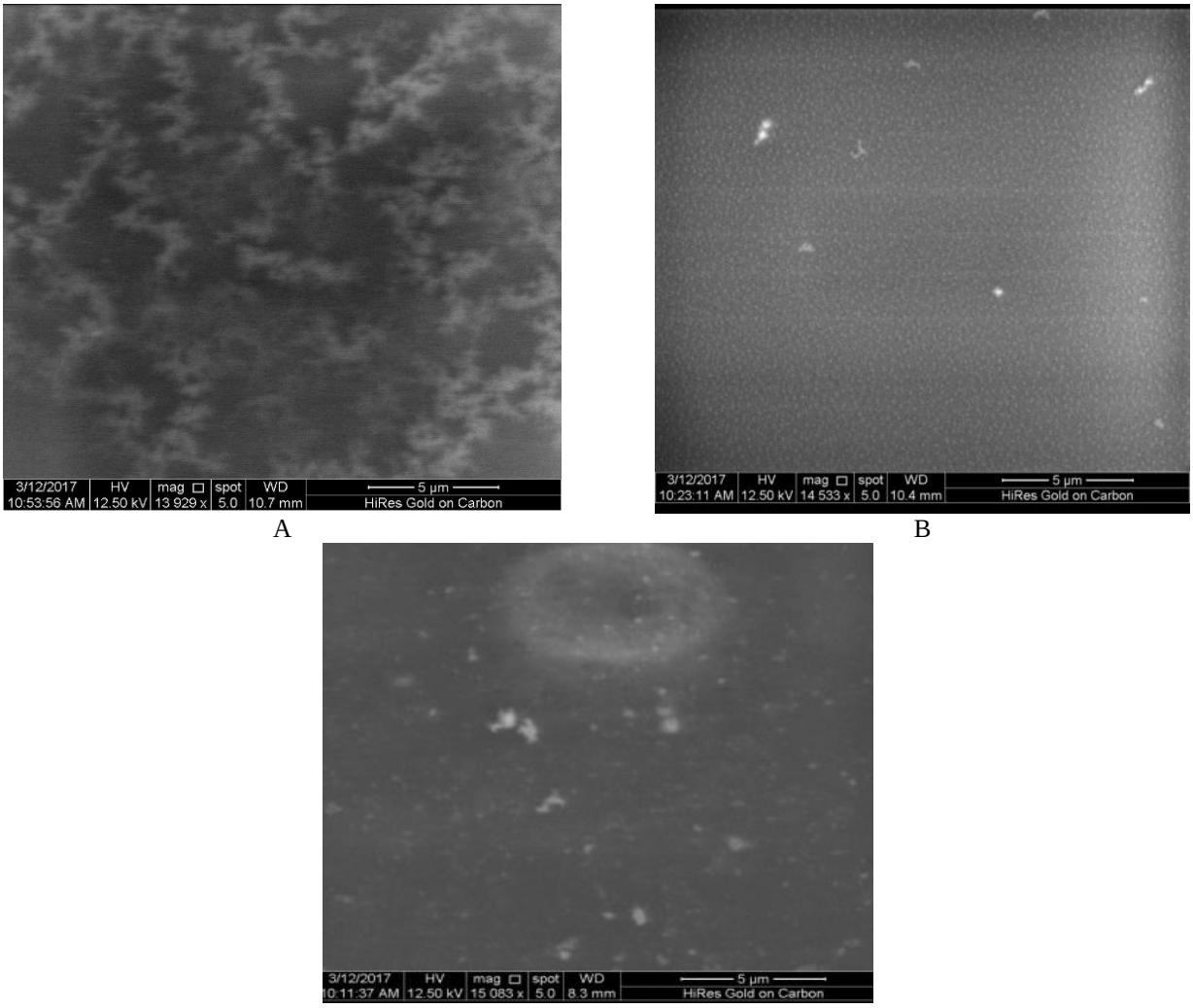



Figure 1: SEM characterization of silver nanoparticles prepared by the three methods, A. xerogel, B. NaHB4/ PVP, C. glucose.

Solution 1: A 71mg/ml Zn solution, zinc nitrate six hydrate ($Zn(NO_3)6$. H2O (Merck / pure chemical industries, 99.0%), was dissolved in ethanol ($C_2H_5OH$, BDH, 99%,) as solvent. A 5ml Ethylene glycol ($C2H_6O_2$, BDH, 99.0 %), were added in order to ensure the complete dissolution, and then solution is stirred vigorously for 1 h at 120 °C. Solution 2: a 21mg/ml $Ag^+$ solution was prepared by dissolving silver nitrate ($AgNO_3$), from BDH, 99.0 %) in distilled water and adding 5ml ethylene glycol. The solution was stirred vigorously for 1 h at 60ºC to obtain a transparent sol. Then solution 1 was added to solution 2, stirred for 1 hour at room temperature, then serve for further analysis.

*Preparation of Ag nano particles by NaBH4*

Sodium borohydride (NaBH4, 0.028gm) was dissolved in 10 ml ice-cold distilled water, and then polyvinyl pyrrolidone (PVP, 0.4 gm) was added as capping agent. Silver nitrate (AgNO3, 0.0214 gm) was dissolved in 10 ml ice-cold distilled water. Both solutions were mixed and putted on magnetic stirrer at 50-60°C and1500 rpm, for 1 h., then serve for further analysis[15,16].

*Preparation of Ag nanoparticles by glucose*

Silver nano particles are synthesized by using glucose as oxidizing and stabilizing agent. The glucose oxidized into gluconic acid in the existence of silver nitrate, and the resultant gluconic acid will caps the silver nanoparticles. The ratio of AgNO3: glucose was 1:0.5,[15,17].

*Charactrization of Nanoparticles*

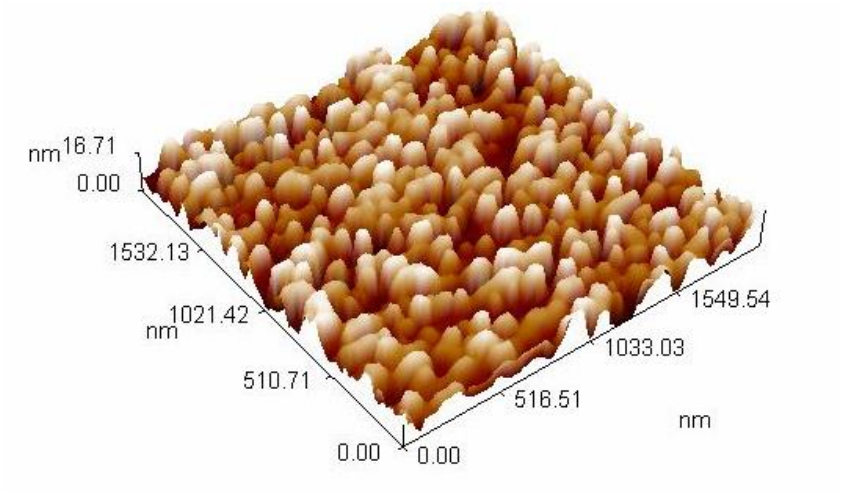
UV-visible spectrophotometer, Infrared spectra, scanning electron microscope (SEM, Philips XL-30 operated at 5 kV), Atomic force microscopy (AFM, model AA3000, Advanced Angstrom Inc- USA), FTIR (Bruker), were used to characterize the synthesized nanoparticles.

*Bacterial isolates*

Bacterial isolates were obtained from Al-Kafeel hospital after isolation and characterization from clinical samples according to standard laboratory procedures. The isolated bacteria were tested for biofilm formation and biofilm forming bacteria were used for further testing. Brain Heart Infusion (BHI, Himedia) broth was used in cultivation of the micro-organism.

*Biofilm formation Assay*

Evaluation of biofilm formation by each bacteria was performed as described previously with few

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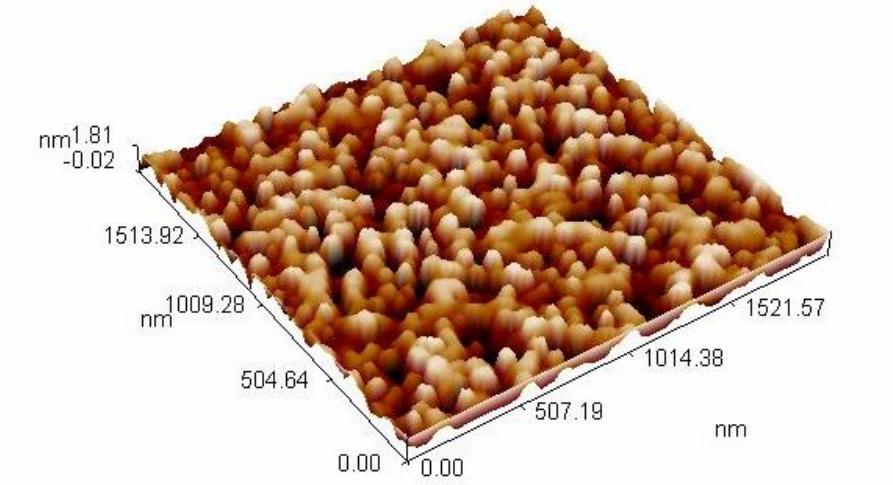


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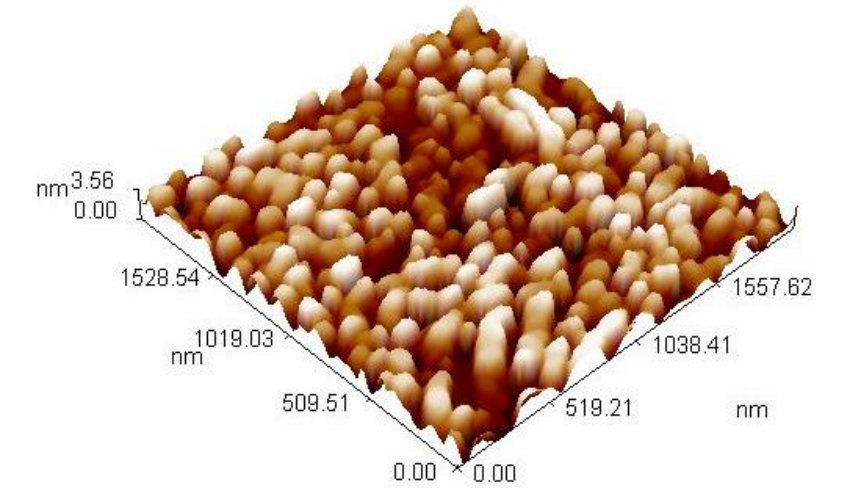


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Figure 2: AFM characterization of silver nanoparticles prepared by the three methods, A. xerogel, B. NaHB4/ PVP, C. glucose.

modifications[18-20]. Briefly, 200 µl of overnight bacterial suspension with approximately $10^{-8}$ CFU was added to each well in a microtiter plate in a sterile condition. Control wells contain only BHI broth without bacteria. The plate was incubated at 37ºc for 24 h. After 24 h, the non-adhered planktonic cells were removed and the wells were washed with 200 µl of 0.9 % (W/V) sterile normal saline for three times and the wells left to dry at room temperature (RT). The created biofilm was fixed with 200 µl methanol and left at room temperature for 15 minutes and then stained with 200 µl of 0.1% (V/V) crystal violet solution for 15 minutes. After that, the wells were washed

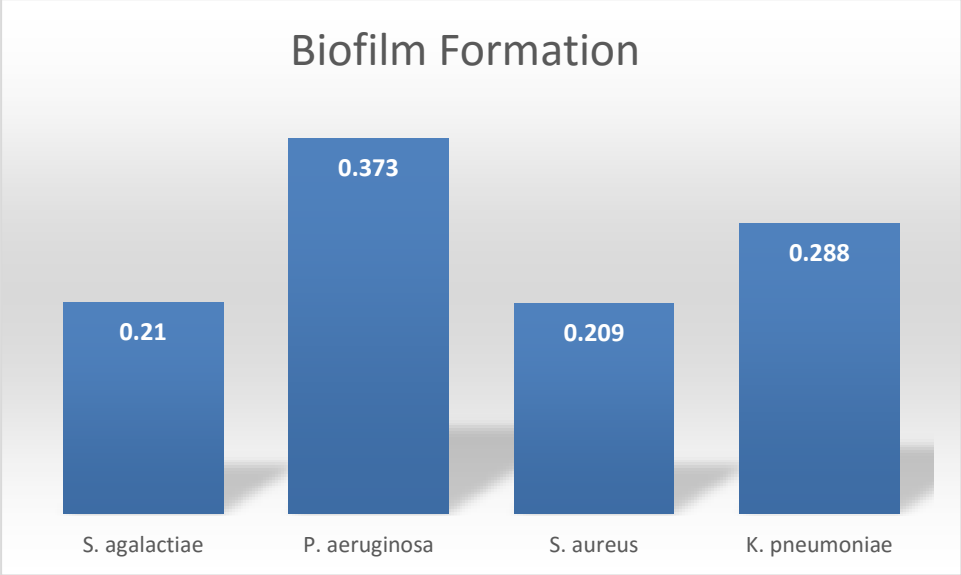


Figure 3: Biofilm forming bacteria.

Table 1: Measurement of biofilm production by bacteria.

| Type of bacteria | Mean ± SD[a] | Biofilm formation[b] | *P- value* |
|---|---|---|---|
| *Klebsiella peumoniae* | 0.348 ± 0.105 | 0.288 | 0.533 |
| *Staphylococcu aureus* | 0.269 ± 0.102 | 0.209 | |
| *Pseudomonas aeruginosa* | 0.433 ± 0.168 | 0.373 | |
| *Streptococcus agalactiae* | 0.270 ± 0.058 | 0.21 | |

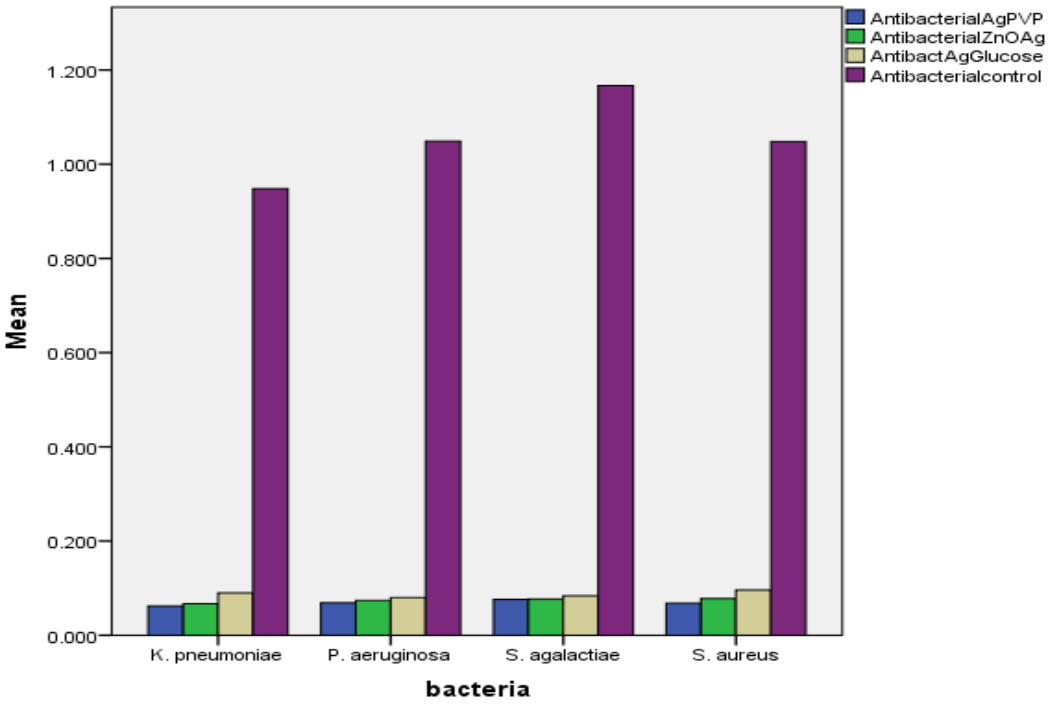


Figure 4: Inhibition of bacterial biomass by silver nanoparticles.

three times with sterile normal saline. Finally, the wells were treated with absolute ethanol for extraction of the absorbed dye and measure at 595 nm spectrophotometer SP-300 (OPTIMA). For each bacterium, biofilm formation assay was performed by seeding more than 24 wells and the experiment was repeated three times. The ability of bacteria to form biofilm was calculated according to the equation:

Biofilm formation= the OD at 595 nm of the stain extracted from attached cells – the OD at 595 nm of

Table 2: Percentage of Inhibition of bacterial biomass.

| Silver nanoparticle | *K. pneumoniae* | *S. aureus* | *P. aeruginosa* | *S. agalactiae* |
|---|---|---|---|---|
| Ag-NaHB4/PVP | 93.4 | 93.5 | 93.4 | 93.4 |
| ZnO-Ag | 92.9 | 92.5 | 92.9 | 93.4 |
| Ag-glucose | 90.5 | 90.3 | 92.3 | 92.8 |
| *P- value* | | | 0.004 | |

control wells.

*Dual Antibacterial and antibiofilm activity of silver nanoparticles*

Both antibacterial and antibiofilm activities of silver nanoparticles was assessed simultaneously according to previous studies[11,12,21-24] by measuring turbidity of the cell biomass and tissue culture plate method (TCP). Briefly, 200 µl was added in 96-well microtiter plate (100 µl of overnight bacterial culture □$10^{-4}$-$10^{-6}$ CFU with equal volume of different concentrations of silver nanoparticles prepared by different methods. The plate was incubated at 37 ºc for 24 h. In the following day, cell biomass was measured using ELISA reader (BioTek, ELX 800) at 630 nm. After reading the optical density, the created biofilm was measured. The mixture within wells were removed and the wells were washed with 200 µl of 0.9 % (W/V) sterile normal saline for three times and left to dry at room temperature (RT). The created biofilm was fixed with 200 µl methanol and left at room temperature for 15 minutes and then stained with 200 µl of 0.1% (V/V) crystal violet solution for 15 minutes. After that, the wells were washed three times with sterile normal saline. Finally, the wells were treated with absolute ethanol for extraction of the absorbed dye and measured at 595 nm. Control wells include wells with BHI media alone, wells with bacterial suspension and BHI media. The whole procedure was repeated three times and each well was triplicated. The three forms of silver nanoparticles were tested using five concentrations (0.0125, 0.025, 0.03, 0.04, 0.05 µg/ml).

The percentage of antibacterial effect of nanoparticles was calculated according to the equation:

Percentage of Antibacterial effect = (Absorbance at 630 nm of control wells – Absorbance at 630 nm of treated wells) / Absorbance at 630 nm of control * 100

The percentage of inhibition of biofilm formation was calculated according to the equation:

Percent of inhibition = {1-(absorbance at 595 nm of treated wells/ Absorbance at 595 nm of non-treated control wells) × 100}

*Statistical Analysis*

SPSS software version 20.00 was used to calculate the significant difference among the three types of silver nanoparticles against biofilm forming bacteria. ANOVA test was used to analyze the presence of any significance for antibiofilm and antibacterial activity of silver nanoparticles. Post Hoc multiple comparisons was used to analyze LSD and Dunnett tests to determine the presence of significant difference between groups if the p-value of ANOVA test was significant. The mean difference is significant at the 0.05 level.

## RESULTS

*Characterization of Nanoparticles*

characterization of the three forms of silver nanoparticles by using UV-visible spectrophotometer, Infrared spectra, FTIR (Data not shown), the results of SEM and AFM were as follows, Figure (1), (2).

*SEM*

For ZnO-Ag nanoparticles: the results showed that the Ag nanoparticle (triangular shape) distributed overall the surface of ZnO particles. The size of the observed particles was below 20nm with the existence of nanopores. The measured particle size is about 20 ± 16 nm, and the surface of the films is very smooth, since the calculated roughness for the nanoparticles was 1.5 nm dispersed over the surface of the film, the distribution of Ag nano particles is with agreement of xerogel form.

The result of SEM for silver nanoparticles prepared by NaBH4 with PVP and that prepared by glucose showed aggregation of silver nanoparticles on the surface. The shape and size of the nanoparticles was (rod, 54nm) for nanoparticles prepared by NaHB4 with PVP and (spherical, 40-80nm) for nanoparticles prepared by glucose, respectively.

*AFM analysis revealed the following*

ZnO-Ag: The average of diameter, roughness, and square root were 100nm, 0.327nm, 0.377nm, respectively.

Ag- NaBH4/PVP: The height of the nanoparticles was within 1.8nm, with roughness of about 0.4nm and mean square root is 0.471nm which is evidence of high crystallography.

Ag-Glucose: The average diameter was 5.25nm, square root was 0.919nm, and roughness was 0.786 nm.

*Biofilm formation*

There were different strategies for defining the bacteria that can produce biofilm from non-producing bacteria. Some researchers calculate the cut-off value according to the average of the OD + 3SD of the standard non-biofilm producing bacterial strains[25]. Others were use 0.1 as cut-off value[26,28]. Naves et al., 2008[19] reported that the extent of biofilm formation could be calculated according to three equations. In the current study, two strategies were applied to define biofilm producing bacteria (by considering 0.1 as a cut-off value and the second strategy is applying the equation: Biofilm formation= the OD at 595 nm of the stain extracted from attached cells – the OD at 595 nm of control wells). The results showed that four types of bacteria produce biofilm namely, *Pseudomonas aeruginosa* which produce higher biofilm layer followed by *Klebsiella pneuminiae* (this is indicated by higher optical density value). The other biofilm producing bacteria were *Staphylococcus aureus* and *Streptococcus agalactiae*. ANOVA test was applied to investigate if there is significant difference in biofilm

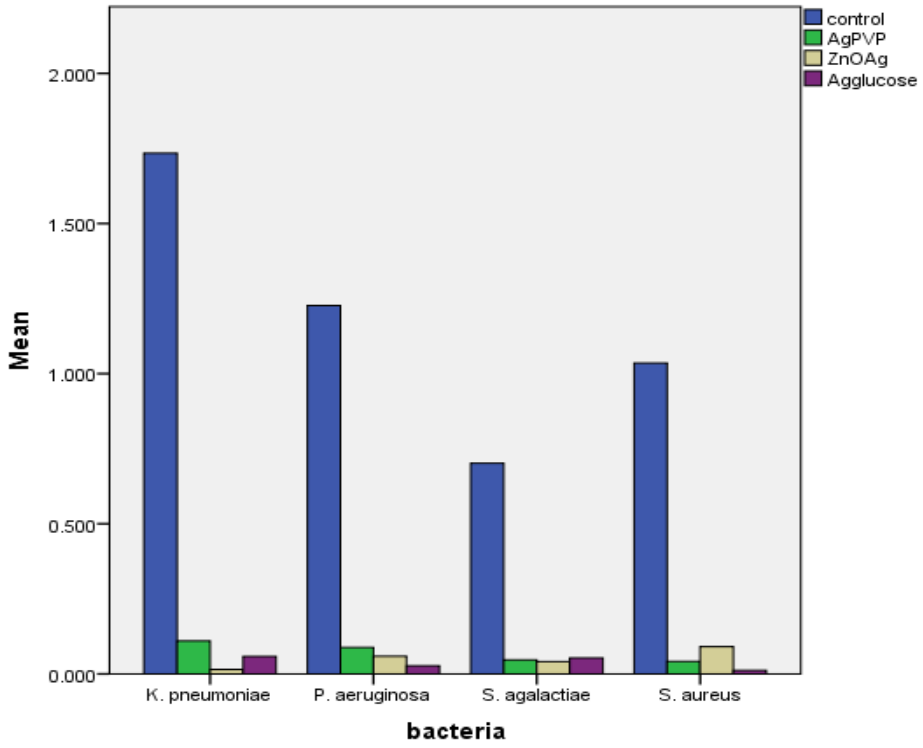


Figure 5: Inhibition of biofilm formation by silver nanoparticles.

Table 3: Percentage of Biofilm Inhibition.

| Silver nanoparticle | *K. pneumoniae* | *S. aureus* | *P. aeruginosa* | *S. agalactiae* |
|---|---|---|---|---|
| Ag-NaHB4/PVP | 93.7 | 96 | 92.9 | 93.4 |
| ZnO-Ag | 99.2 | 91.3 | 95.2 | 94.2 |
| Ag-glucose | 96.7 | 98.8 | 97.8 | 93.5 |
| P- value | 0.299 | | | |

formation among biofilm forming bacteria and the results revealed that there was no statistically significant difference, figure (3), table (1).

a represent average of absorbance at 595 nm ± SD, b represents absorbance after applying the equation (BF= OD at 595 of wells inoculated with bacteria – OD at 595 of control wells).

*Simultaneous Antibacterial and antibiofilm activities*

Several previous studies had reported antibacterial and antibiofilm activities of silver nanoparticles, we aimed to investigate the effect of silver nanoparticles on both the planktonic and sessile cells at different concentrations (0.0125, 0.025, 0.03, 0.04, 0.05 µg /ml) and the results showed significant inhibition at concentration 0.0125 µg/ml for both antibacterial and antibiofilm activities.

Concerning the antibacterial effect, the results demonstrated that the three forms of silver nanoparticles Significantly reduce the absorbance of bacterial biomass for biofilm producing bacterial isolates as compared with their controls. Additionally, when we compared the antibacterial efficacy of the three forms of silver nanoparticles against bacterial isolates by using ANOVA test, the results showed the presence of significant difference. Post Hoc multiple comparisons was used to analyze LSD and Dunnett tests to determine the significant difference between groups and the results revealed that silver nanoparticles prepared by glucose had statistically significant lower antibacterial effect, figure (4), and table (2).

For antibiofilm activity, the results showed that there was statistically significant inhibition of biofilm formation of the biofilm producing bacteria when it compared with control (*P value* ≤0.05) at concentration 0.0125 µg /ml. However, there were no significant difference among the tested bacteria but at the same time the results revealed that *K. pneumoniae* and *S. agalactiae* biofilm formation is inhibited with ZnO-Ag nanoparticles more than other nanoparticles (99.2%, 94.2%, respectively). Whereas, Pseudomonas and Staphylococcus biofilm formation were greatly inhibited by silver nanoparticles prepared by glucose as compared with other types of nanoparticles (98.8%, 97.8%, respectively), figure (5), table (3).

In the current study, higher percentages of biofilm inhibition were observed than percentages of antibacterial effect for the tested bacteria.

## DISCUSSION

In recent years, there was great interest in nanoparticles with antibacterial and antibiofilm activity as new strategy against multidrug resistant bacteria[11,29]. Silver nanoparticles are known to have antibacterial and antibiofilm activities. Several methods used for synthesis

silver nanoparticles has been reported in several previous studies[11]. The aim of this study is to synthesize silver nanoparticles using three different chemical procedures and investigating their dual antibacterial and antibiofim activities on both planktonic and sessile bacterial isolates and whether the effect differ according to the type of method or to the bacteria.

Synthesis of silver nanoparticles was made by three ways, ZnO-Ag Xerogel, Ag-NaHB4 / PVP, Ag-Glucose). After synthesis, characterization of nanoparticles was made using UV-visible spectrophotometer, infrared spectra, FTIR (data not shown) and by SEM and AFM. The results revealed that size and shape of nanoparticles were (rod 54 nm, spherical 20 ± 16nm, and spherical 40-80nm, for silver nanoparticles with NaHB4/PVP, Zno-Ag, and Ag with glucose, respectively).

Biofilm formation assay was used to determine the ability of bacteria to form biofilm and the results showed four types of bacteria were able to form biofilm (two of them were gram positive- *S. aurues, and S. agaglactaie*- and the other two were gram negative- *Pseudomonas aeruginosa, and Klebsiella pneumonaie*). There were different strategies for defining the bacteria that can produce biofilm, two strategies were applied in the current study (the first is considering 0.1 as a cut-off value and the second is applying the equation: Biofilm formation= the OD at 595 nm of the stain extracted from attached cells – the OD at 595 nm of control wells). The results showed that *Pseudomonas* produce higher biofilm layer followed by *Klebsiella* which is indicated by higher optical density value. However, there were no significant difference in biofilm formation among the four bacteria.

The synthesized nanoparticles were tested against biofilm producing bacteria to investigate the effect on planktonic cell and sessile cells. Regarding the antibacterial activity of silver nanoparticles, the results demonstrated that the three forms of silver nanoparticles Significantly reduce the absorbance of the biomass for the tested bacterial isolates as compared with their controls. Additionally, when we compare the antibacterial efficacy of the three forms of silver nanoparticles against bacterial isolates the results showed that silver nanoparticles prepared by glucose had statistically significant lowest effect.

Concerning the antibiofilm activity, the results showed that there was significant inhibition of biofilm formation for all tested bacteria when it compared with control (*P value* ≤0.05). However, there were no significant difference in inhibition among the type of bacteria. This mean that the antibiofilm activity of different silver nanoparticles does not differ according to the type of bacterial isolates but at the same time the results revealed that *K. pneumoniae* and *S. agalactiae* biofilm formation is inhibited more likely with ZnO-Ag nanoparticles more than other nanoparticles (99.2%, 94.2%, respectively). Whereas, *P. aeruginosa* and *S. aureus* biofilm formation were greatly inhibited by silver nanoparticles prepared by glucose as compared with other types of nanoparticles (98.8%, 97.8%, respectively).

Moreover, the results indicated that 0.0125 µg/ ml concentration for the three types of silver nanoparticles can cause significant inhibition for more than  93 % of biofilm and 90% of cell biomass for all types of bacterial isolates. Lower percentage of antibacterial and antibiofilm activity with higher concentration were reported in previous studies. Palanisamy et al., showed that the antibiofilm activity of chemically synthesized silver nanoparticles (at concentration of 20 µg/ml and size of nanoparticles is about 20-30 nm) was 67% at bacterial concentration of $10^{-4}$ for sensitive *Pseudomonas* and 56% at bacterial concentration $10^{-5}$ and $10^{-6}$ for multidrug resistant *Pseudomonas* and there was no inhibition at concentration $10^{-8}$ for both strains[10]. In other previous study, biologically synthesized silver nanoparticles had antibiofilm activity (more than 90 %) against *P. aeruginosa* and *S. flexneri* at the concentration of 0.5 µg/ml and against *S. aureus* and *S. pneumonaie* with the concentration 0.7 µg/ml[11]. In the same study, 0.6 µg/ml cause 95% reduction in cell viability of *P. aeruginosa* and *S. flexneri* and that the concentration of about 0.5 µg/ml cause 50% inhibition for *S. aureus* and *S. pneumonia* (the nanoparticle size was 2-10nm).

Amany et al. showed that MIC of silver nanoparticles prepared by glucose and NaHB4/PVP were 30 µg/mL, 40 µg/mL, respectively against S. aureus ATCC 6538[15].

Its proposed that the antibacterial effect of silver result from the inhibition of enzymatic system of respiratory chain and interference with DNA synthesis[30]. Another proposed mechanism is by pore formation in cell membrane, result in cell destruction[31].

Yammato et al. and Sawai et al. suggested that the inhibitory effect of ZnO-Ag nanoparticles result from hydrogen peroxide generated from surface of ZnO[32,33].

Previous study documented that different antibacterial effect of silver nanoparticles against bacteria might possibly leads to various levels of biofilm inhibition[10]. In the current study, higher antibiofilm activity was observed than antibacterial effect for the tested bacteria. This mean that silver nanoparticles affect sessile cells than planktonic cells when they tested simultaneously.

## CONCLUSION

Silver nanoparticles have significant antibiofilm activity regardless the type of bacteria and the method used for nanoparticle synthesis. Whereas, the antibacterial effect depends upon the method used for nanoparticle synthesis.

Lower concentration with higher antibacterial and antibiofilm activity was observed with the current method for synthesis of silver nanoparticles. Higher antibiofilm activity was observed than antibacterial effect for the tested bacteria.

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