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ORIGINAL ARTICLE

Antibacterial action of silver nanoparticles on biofilm producing multidrug resistant *Pseudomonas aeruginosa* strains

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Pseudomonas, AgNPs, biofilm, nanoparticles.

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ABSTRACT

Background: *bacteria have acquired the ability to adapt to stress conditions and developed various mechanisms of resistance; there is a need to develop new bactericidal materials.* ***Objective:*** *The aim of this study is to test the effect of silver nanoparticles (AgNPs) on increasing the antibiotic-susceptibility of MDR biofilm-producing Pseudomonas aeruginosa.* ***Methodology****: The antibiotic susceptibility testing of the isolates were done by the Modified Kirby- Bauer method and phenotypic detection of biofilm –forming isolates was done by the Congo Red Agar method.* ***Results****: Biofilm-production was found in16.3% of isolates and 77.6% were negative. The highest resistance rate was to piperacillin (67%), followed by ticarcillin-clavulanate (63%), while the isolates were highly sensitive to colistin (73%), polymyxin B (64%), and moderately-sensitive to meropenem (58%). Ten µL of Silver nanoparticles suspensions with size ranged from 20- 40 nm and average size 30 nm were added to the used antibiotics with retesting the isolates for their antibiotic susceptibility.* ***Conclusion****: AgNPs exhibited a strong synergistic effect with antibiotics through the significant increase of the antibiotic inhibitory zones and the improvement of the susceptibility profile of the isolates.*

INTRODUCTION

At present, bacteria developed various resistance mechanisms due to the empirical excess use of antibiotics and become able to adapt to stress conditions, which represent a major health threat. Consequently, there is an increasing need to provide newer antibacterial agents [1].

Gram-negative bacteria are very important causative agents of health care associated infections, contributing in prolonged hospital stay, higher mortality rates, and increasing hospital costs. *Pseudomonas aeruginosa* is highly expressing genetic modifications leading to resistance to antibiotics and the consequent complications in hospitaized and immunocompromised patients [2].

P. aeruginosa is associated with severe health care-associated infections occur in ICUs and include sepsis, chest infection, UTI and GIT infections, endocarditis, osteomyelitis, and meningitis. They can cause opportunistic infections, especially in immune suppressed patients such as burn patients, those with cancer, and patients of cystic fibrosis. They grow readily in adverse conditions and have acquired and intrinsic-resistance mechanisms to common antimicrobials [3].

Because of its ability to adapt harsh environment, *P. aeruginosa* is known as one of the most important agents in hospital infections, and it is an important model to study the control of heath care-associated infections [4]. Large percentage of bacteria exists in the form of biofilms attached to solid surfaces. Biofilm formation on externally- attached devices such as intravascular lines, artificial heart valves, catheters, and lenses, is related to the development of chronic unresolved infections. Those biofilms are strongly resistant to antibiotics. Therefore, strategies to prevent or decrease hospital-acquired infections should focus on the prevention biofilm formation[5].

Reduction in antibiotic sensitivity of *P. aeruginosa* to antibiotics susceptibility to antibiotics has been described, highlighting the increasing resistance to broad-spectrum antibiotics, such as carbapenems, fluoroquinolones, and cephaosporins [6].

For these considerations, nanotechnology has developed nanoparticles that are capable of interacting with pathogenic micro-organisms. Among these nanoparticles, metal nanoparticles are of scientific interest, due to its bactericidal properties; gold, copper, and silver represented very interesting antimicrobial agents [7].

Silver nanoparticles are aggregates of atoms; their sizes are ranging between 1 and 100 nm. Because of their very minute sizes, AgNPs have different physical and chemical properties to that of metallic silver [8]. AgNP is the most widely explored antibacterial nano-agent due to its broad-spectrum antimicrobial properties and robust antimicrobial effectiveness against various bacteria, viruses, and fungi [9,10].

This study was conducted to test the effect of AgNPs with sizes ranging around (20 nm) on nosocomial infectious strains of *P. aeruginosa*, resistant to a large number of antibiotics and having a biofilm production ability which makes them highly resistant to the currently available antibiotics.

METHODOLOGY

This study was performed in the Medical Microbiology & Immunology department, Faculty of Medicine, Sohag University during the period from January 2019 to January 2020. A total of 43 pseudomonas isolates were collected from patients with health care associated infections (developed 48 to 72 hours after patient's admission) specially those associated with biofilm formation as; blood stream infections, infection in cystic fibrosis patients, other chronic chest infections, ventilator-associated pneumonia (VAP), urinary tract infections in catheterized patients, surgical wound infections, infected diabetic foot ulcer, and infected burn wounds. Pseudomonas strains were examined and identified by standard microbiological procedures and biochemical reactions [11].

Informed consents were taken from the patients included. The study was approved by the Faculty Local Ethical Committee.

Samples:

For infected burn wounds, infected surgical sites, and infected diabetic foot ulcers sterile cotton swabs were used for sampling. Dry sterile well-closed plastic cups were used for sputum samples either through coughing in patients with chronic chest infection or through suction catheter in patients with VAP. For urine samples: in catheterized patients with symptoms of UTI the urine sample was collected through a sterile syringe after 10 min of clamping the catheter. In urine samples bacterial counts were done using calibrated loop (10 µl). UTI was considered if there was more than 100 colonies (10^5 CFU/ ml) of un-centrifuged urine.

Culture:

Collected samples were inoculated on Cetrimide agar *(Oxoid Ltd., Basingstoke UK)* and incubated aerobically at 37°C for 24-48 hours. Colonies are smooth 2-3 mm in diameter, may be coalesced together with the detection of the yellow-green or blue-green fluorescent growth characteristic of *P. aeruginosa.*

Pseudomonas isolates were tested for susceptibility to different antibiotics using the disc diffusion method using the CLSI guidelines[12] using commercially available antibiotics ***(Oxoid Ltd., Basingstoke UK).*** (Table 1)

Table 1: Performance standards for antimicrobial sensitivity testing according to CLSI guidelines[13]

Antimicrobial agent	Disc conc.	Inhibition zone diameter (mm)		
		Sensitive	Intermediate	Resistant
Piperacillin	100 µg	≥ 21	15-20	≤14
Piperacillin-tazobactam	100/10µg	≥ 21	15-20	≤14
Ticarcillin-clavulanate	75/10 µg	≥24	16-23	≤15
Ceftazidime	30 µg	≥18	15-17	≤14
Cefepime	30 µg	≥18	15-17	≤14
Aztreonam	30 µg	≥22	16-21	≤15
Imipenem	10 µg	≥19	16-18	≤15
Meropenem	10 µg	≥19	16-18	≤15
Gentamicin	10 µg	≥15	13-14	≤12
Tobramycin	10 µg	≥15	13-14	≤12
Amikacin	30 µg	≥17	15-16	≤14
Colistin	10 µg	≥11	-----	≤10
Polymyxin B	300 units	≥12	-----	≤11
Ciprofloxacin	5 µg	≥21	16-20	≤14
Ofloxacin	5 µg	≥16	13-15	≤12
Norfloxacin	10 µg	≥17	13-16	≤12

Biofilm formation:

Phenotypic identification of biofilm-producing isolates of *P. aeruginosa* isolates was performed by Congo red agar method which is A simple qualitative method for detection of biofilm-forming isolates by using Congo Red Agar (CRA) medium which was prepared as follows: brain heart infusion agar (37 g/L) ***(Oxoid, UK)*** was prepared, with addition of (0.8 g/L) Congo red indicator (***Oxoid, UK)*** and 50 g/L of sucrose ***(Oxoid, UK).*** CRA plates were inoculated with the isolates that exhibited MDR pattern to the tested antibiotics, and incubated at 37°c for 24 h aerobically **[13].** Biofilm-forming isolates appeared as; black, dry colonies with a crystalline surface. Weak biofilm producers appeared as pink colonies, with dark centers (Figure 1)

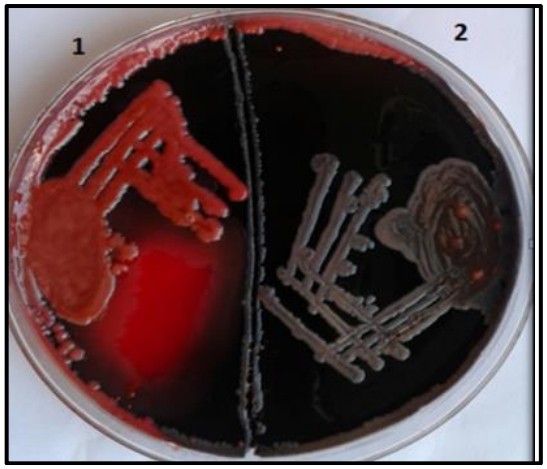

Fig. 1: CRA medium method; (1) Non biofilm forming isolate; (2) Biofilm forming isolates.

Strains that exhibited resistance to more than three antibiotic groups were considered as multidrug resistant, and strains that were positive for biofilm production were selected to be tested against silver nanoparticles as an antibacterial agent.

Characterization techniques of silver Nanoparticles (AgNPs):

Silver nanoparticles suspensions were purchased from Nano Tech (Egypt). The size of the particles ranged from 20- 40 nm with average size 30 nm coated on Polyvinylpyrrolidone (PVP) particles. V/Visible spectrophotometer double – beam spectrophotometer (Labomed spectro UV-VIS 2700, USA) was used assess the absorbance of AgNPs operated at a resolution of 2 nm. The UV/VIS absorption spectrum of the screened AgNPs was in the average of 410 nm. (Figure 2)

TEM is used to re-assess the morphology and size of the silver nanoparticles. TEM analysis of the AgNPs was performed using a JEM 2100 device operated with accelerating voltage at 200 kV (JEOL, Japan). The preparation of samples was done by drop coating on formavar- coated copper TEM grids. The diameter of nanoparticles was measured from the TEM images and the size of the AgNPs was calculated. (Figure 3)

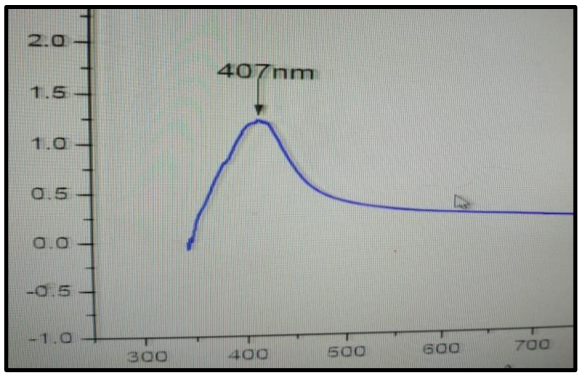

Fig. 2: UV- visible absorption spectrum of silver nanoparticles

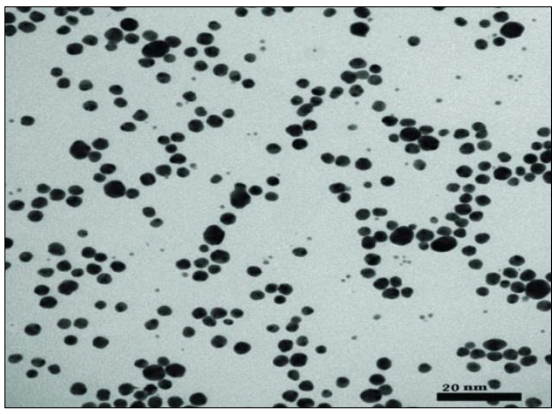

Fig.3: Transmission Electron Microscopy (TEM) image of silver nanoparticles.

AgNPs susceptibility testing:

The antimicrobial susceptibility of silver nanoparticles was evaluated using the disc diffusion. 10µL of silver nanoparticle solution were added to the following sterile antibiotic discs; Piperacillin (100 µg), Piperacillin-tazobactam (100/10µg), Ticarcillin-clavulanate (75/10µg), Ceftazidime (30µg), Norfloxacin (10 µg), Aztreonam (30 µg), cefepime (30 µg), Imipenem (10µg), Meropenem (10µg), Gentamicin (10µg), Tobramycin (10 µg), Amikacin (30µg), Colistin (10µg), Polymyxin B (300 units), Ciprofloxacin (5 µg), and Ofloxacin (5 µg), then placed in incubator at 37°C for 24 hours until dried.

Saline solution was used to suspend MDR *P. aeruginosa* isolates and adjusted to 0.5 McFarland's solution. The solution was plated on Muller Hinton's agar medium. Antibiotic discs were placed and the plates were incubated at 35 °C for 24 hours. The inhibition zones were measured. The diameter of the

inhibition zone to each antibiotic disc was measured and compared to the previous zone diameter of the same disc before addition of silver solution for all the selected MDR isolates. Synergism if present was evaluated by the formula $\{(B^2-A^2)/A^2\}$ x 100, where, A = the inhibition zone diameter of the antibiotic alone and B = the diameter of the inhibition zone to antibiotics + AgNPs. This formula was used to evaluate the increase of the inhibition zone around the bacteria caused by the antibiotic in association after addition AgNPs **[16]**. (Figure 4)

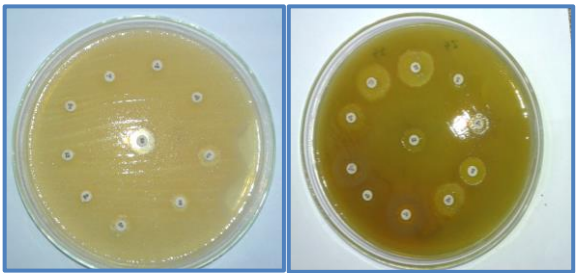

Fig.4: Susceptibility of pseudomonas isolates to the different antibiotics before and after impregnation in AgNPS solution.

RESULTS

A total of 43 *Pseudomonas aeruginosa* isolates were isolates from the previously mentioned health care-associated infections during the study period and identified by conventional and biochemical methods. The highest percentage of *P. aeruginosa* isolates were isolated from patients with infected cystic fibrosis (25%), equal percentages of isolates from patients with infected orthopedic implants (12%) and patients with ventilator-associated pneumonia (VAP) (12%) admitted in ICU, (14%) of isolates were collected from patients with UTI, (11%) from Surgical site infections, (10%) infected Diabetic foot, (10%) from patients of COPD with respiratory failure on mechanical ventilation, and (6%) from patients with infected burn.

The results of antibiotic susceptibility testing were as follows; the highest resistance pattern was to piperacillin (67%), followed by ticarcillin-clavulanate (63%), while the highest sensitivity was to colistin (73%), then polymyxin B (64%), and finally to meropenem (58%). (Table 2)

Table 2: Antibiotic susceptibility profile of pseudomonas isolates:

Antimicrobial agents	Sensitive	Intermediate	Resistant
Piperacillin	20%	13%	67%
Piperacillin-tazobactam	20%	23%	57%
Ticarcillin-clavulanate	22%	15%	63%
Ceftazidime	28%	11%	61%
Cefepime	49%	10%	41%
Aztreonam	49%	12%	39%
Imipenem	55%	22%	23%
Meropenem	58%	24	18
Gentamicin	37%	8%	55%
Tobramycin	38%	9%	53%
Amikacin	37%	13%	50%
Colistin	73%	-----	27%
Polymyxin B	64%	-----	36%
Ciprofloxacin	51%	19%	30%
Ofloxacin	49%	16%	35%
Norfloxacin	43%	13%	44%

Testing of biofilm production by CRA method revealed that 16.3% of isolates (7 isolates) were biofilm forming and 77.6% were negative. All these isolates were resistant for more than three antibiotic groups (MDR), these isolates were re-tested for susceptibility to the same antibiotics after addition of AgNPs.

Re-assessment of antimicrobial sensitivity profile after addition of AgNPs:

The combined effect of the formed nanoparticles with different antimicrobial agents was investigated against the biofilm-forming Pseudomonas isolates using the disc diffusion method. The diameter of inhibition zones (in millimeters) around all the tested antibiotic discs before and after addition of Ag-NPs solution were measured and reported. The results of antibiotic susceptibility to the antibiotics with high resistance rates; piperacillin, piperacillin/tazobactam, ticarcillin/clavulonate, ceftazedime, gentamycin, tobramycin, amikacin, norfloxacin, and cefipeme, after addition of AgNPS are presented in (Tables 3, 4, 5).

Table 3: Zone of inhibition (mm) of the tested biofim-forming *pseudomonas aeruginosa* isolates against piperacillin, piperacillin-tazobactam, ticarcillin-clavuonate in presence and absence of AgNPs.

Isolate	Piperacillin	AgNPs+ Piperacillin	Increase in fold area	Piperacillin-tazobactam	AgNPs+ Piperacillin-tazobactam	Increase in fold area	Ticarcillin-clavulanate	AgNPs+ Ticarcillin-clavulanate	Increase in fold area
Ps 2	0	17	7.43	12	21	0.72	0	17	7.43
Ps 7	10	22	1.25	14	25	0.79	11	22	1.00
Ps 13	0	12	2.45	0	19	9.11	0	12	2.45
Ps 22	0	13	3.69	13	23	0.76	12	23	0.91
Ps 29	9	22	1.44	8	20	1.5	9	22	1.44
Ps 35	0	9	9.3	14	22	0.57	0	15	5.31
Ps 42	9	25	1.7	0	16	6.13	0	13	3.72

Table 4: Zone of inhibition (mm) of the tested biofim-forming *pseudomonas aeruginosa* isolates against ceftazidime, gentamicin, and tobramycin in presence and absence of AgNPs.

Isolate	Ceftazidime	AgNPs + Ceftazidime	Increase in fold area	Gentamicin	AgNPs+ Gentamicin	Increase in fold area	Tobramycin	AgNPs + Tobramycin	Increase in fold area
Ps 2	0	17	7.43	0	14	4.32	0	15	5.31
Ps 7	14	20	0.30	0	0	0.00	0	17	7.43
Ps 13	14	22	0.57	12	17	0.42	0	0	0.00
Ps 22	0	18	8.52	0	12	2.45	0	17	7.43
Ps 29	0	16	6.32	0	12	2.45	0	15	5.31
Ps 35	11	17	0.24	11	14	0.27	0	12	2.45
Ps 42	17	21	0.55	0	0	0.00	0	0	0.00

Table 5: Zone of inhibition (mm) of the tested biofim-forming pseudomonas *aeruginosa* isolates against amikacin, norfloxacin, and cefipeme in presence and absence of AgNPs.

Isolate	Amikacin	AgNPs+ Amikacin	Increase in fold area	Norfloxacin	AgNPs+ Norfloxacin	Increase in fold area	cefipeme	AgNPs+ cefipeme	Increase in fold area
Ps 2	**0**	**16**	**6.32**	**0**	**14**	**4.32**	**0**	**17**	**7.43**
Ps 7	**0**	**0**	**0.00**	**16**	**20**	**0.25**	**15**	**20**	**0.33**
Ps 13	**14**	**20**	**0.43**	**17**	**22**	**0.29**	**15**	**22**	**0.46**
Ps 22	**0**	**15**	**5.31**	**0**	**12**	**2.45**	**0**	**18**	**8.52**
Ps 29	**0**	**16**	**6.32**	**0**	**12**	**2.45**	**0**	**16**	**6.32**
Ps 35	**12**	**18**	**0.50**	**14**	**19**	**0.36**	**17**	**22**	**0.29**
Ps 42	**0**	**0**	**0.0**	**12**	**19**	**0.58**	**16**	**21**	**0.31**

Paired T test was used to measure the statistical difference between the inhibition zone diameter before and after addition of AgNPs and it was found that the inhibition zone diameter is higher after addition of AgNPs to all used antibiotic with statistically-significant difference (*P value* < 0.05), and high statistically-significant difference in some strains like Ps 22, Ps29, and Ps 35, (*P value* < 0.005). (Table 6)

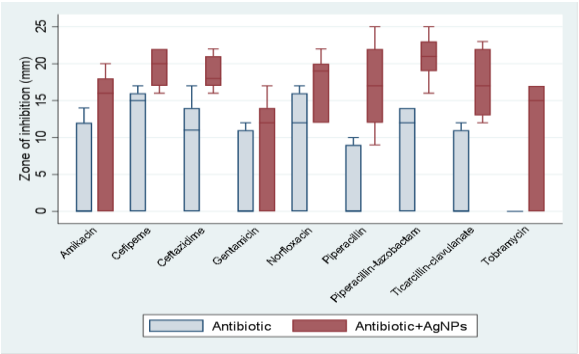

Fig. 4: Mean value of Zone of inhibition before and after addition of AgNPs

Table 6: mean value of Zone of inhibition before and after addition of AgNPs

Antibiotic	Zone of inhibition			P value
	Antibiotic alone	Antibiotic+ AgNPs	Difference	
	Mean ± SD Median (range)	Mean ± SD Median (range)	Mean ± SD (95% CI)	
Piperacillin	4±5 0 (0:10)	17.14±6.04 17 (9:25)	13.14±2.67 (10.67:15.61)	0.02
Piperacillin-tazobactam	8.71±6.29 12 (0:14)	20.86±2.91 21 (16:25)	12.14±3.98 (8.47:15.82)	0.02
Ticarcillin-clavulanate	4.57±5.77 0 (0:12)	17.71±4.61 17 (12:23)	13.14±2.19 (11.11:15.17)	0.02
Ceftazidime	8±7.68 11 (0:17)	18.71±2.29 18 (16:22)	10.71±6.02 (5.15:16.28)	0.02
Gentamicin	3.29±5.62 0 (0:12)	9.86±6.94 12 (0:17)	6.57±5.99 (1.03:12.12)	0.03
Tobramycin	0	10.86±7.60 15 (0:17)	10.86±7.60 (3.83:17.89)	0.03
Amikacin	3.71±6.37 0 (0:14)	12.14±8.45 16 (0:20)	8.43±7.21 (1.76:15.09)	0.03
Norfloxacin	8.43±8.04 12 (0:17)	16.86±4.10 19 (12:22)	8.43±4.12 (4.62:12.34)	0.02
Cefipeme	9±8.45 15 (0:17)	19.43±2.43 20 (16:22)	10.43±6.21 (4.68:16.18)	0.02

DISCUSSION

Treatment and prevention of *P. aeruginosa* infection become increasingly challenging, due to its acquired and intrinsic drug-resistance abilities [14]. Due to the increasing number of severe hospital-acquired infections caused by *P. aeruginosa;* there is a rising need to develop novel strategies which deal with this phenomenon. Biofim-formation is a strategy of survival for *P. aeruginosa* to tolerate their environment. Inside the protecting environment of biofilm, microorganisms tolerate the antibiotics and become resistant to the immune responses, which increase the difficulties for the clinical treatment of biofilm-associated infections -.

The infections with drug-resistant microorganisms result in spending more time in the hospital and require a form of treatment that uses two or three different antibiotics and is less effective, more toxic, and more expensive. Nanotechnology provides the re-assessment of the biological criteria of the known antimicrobial compounds by manipulating and altering the effect [16].

In this study we used AgNPs ranged from 20- 40 nm with average size 30 nm coated on Polyvinylpyrrolidone (PVP) particles to test its antibacterial effect against MDR, biofim-forming pseudomonas aeruginosa isoates.

The main advantage of using smaller AgNPs (<50 nm) is their easy permeability through Gram-negative membranes. Furthermore, contact surface is bigger due to their small size. Incorporation of AgNPs into polymermatrices has been researched in order to obtain a prolonged bacterial inhibitory effect[10]. In a study conducted by Jaiswal et al.[17] on the antibiofilm activity of AgNPs has been demonstrated against *Pseudomonas putida* biofilms, the results suggested that biofilms are impacted by the treatment with AgNPs of average size less than 50 nm.

We used Polyvinylpyrrolidone (PVP) as a coating material for AgNPs. Polyvinylpyrrolidone (PVP) has good antibacterial effect against *S. aureus*, *E. coli*, *P. aeruginosa*, *Bacillus subtilis*, and good antifungal activity against various yeasts and molds [18].

In our study, we isolated and tested 43 *P. aeruginosa* strains. Among them, 17 were drug-resistant and 7 were multidrug-resistant and biofilm-forming. We retested their antibiotic susceptibility after addition of 10 µg of AgNPs the previously used antibiotic discs. The diameters of inhibition were measured and reported and the increase in the zone diameter is present was calculated.

Paired T test was used to measure the statistical difference between the inhibition zone diameter before and after addition of AgNPs and it was found that the inhibition zone diameter is higher after addition of AgNPs to all used antibiotic with statistically-significant difference (*P value* < 0.05), and high statistically-significant difference in some strains like Ps 22, Ps29, and Ps 35, (*P value* < 0.005).

The increase in inhibitory zone-diameter after addition AgNPs was highly significant with Gentamycin, Tobramycin, and Amikacin with a *p value* of 0.01, 0.003, and 0.001 respectively in (100 %) of the tested MDR strains. The antibacterial effect of AgNPs

by its characters used in this study and its effect on improving the sensitivity results of the used antibiotics was in agreement with similar studies conducted by Tien et al.[19], Athirah et al.[20].

Tien et al.[19] studied the antibacterial activity of AgNPs on *pseudomonas aeruginosa* in well diffusion assay; suppression of growth was detected in plates supplemented with silver nanoparticles after incubation at 37°C for 24 hours. While, control plate with sterile saline did not show any zone of inhibition. The diameter of the inhibition zone was around 5 mm in the plate loaded with 4 µg of nanosilver. The zone of inhibition increased linearly with increasing amount (1–5 µg) of nanoparticles.

In another study by Athirah et al.[20], approximately 10^8 CFU/ml of Pseudomonas colonies were used on Muller Hinton agar plates. Sterile discs loaded with 10 µg of AgNPs were placed onto plates. Discs containing 10 µg gentamicin and water were used as a positive and negative control, respectively. The plates were incubated for 24 hours at 37°C. The inhibition zones for all samples were of approximately 11.6 mm when the organism was tested against 10 µg of nanoparticles. The inhibition zone-diameter around gentamicin was larger among susceptible strains but it was slightly reduced among the MDR strains as compared to the silver nanoparticles, which demonstrated that the Ag nanoparticles have antimicrobial activity against the *P. aeruginosa* strains.

From this study we can say that silver nanoparticles have significant antibacterial effect on *P. aeruginosa* and also can its susceptibility to antibiotics. The data presented in this work could lead to new advances in development of antibacterial material that could overcome the multidrug resistance burden. However, further studies have to be conducted on the toxicity level and possible adverse effects that could arise in the use of Ag nanoparticles as an effective antibacterial agent either alone or in combination with the already used antibiotics.

Conflicts of interest: The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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