

## Synergistic Effect of Silver Nanoparticles and Polymyxin B on Multidrug-resistant *Acinetobacter baumannii* Isolated from Burn Wound Infections

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### Abstract

**Background:** Increase rate of prevalence of multidrug-resistant *Acinetobacter baumannii* in burn wound infections make treatment with traditional antibiotics more difficult and leads to severe hospital-acquired infection. Silver nanoparticles (AgNPs) show a broad spectrum antibacterial property with less toxic to mammalian cells.

**Objective:** The aim of this study was to evaluate the anti-bacterial and anti-biofilm activities of AgNPs alone and in combination with cyclic membrane-active peptide (Polymyxin B) against multidrug-resistant *Acinetobacter baumannii* isolated from burn wound infections.

**Materials and methods:** 45 clinical isolates of *Acinetobacter baumannii* tested for susceptibility tests by disk diffusion method against 12 antibiotics. The minimum inhibitory concentrations (MICs) of AgNPs and polymyxin B were performed with broth microdilution method, synergistic probability was evaluated with time kill-kinetic assays and Calgary technique applied to study biofilm formation.

**Results:** Membrane-active peptides colistin and polymyxin B were the most active antibiotics against *Acinetobacter baumannii* with sensitivity of 42 (93.4%) and 38 (84.4%) respectively, while cephalosporins (Cefotaxime) and fluoroquinolones (Ciprofloxacin) antibiotics were most resistances antibiotics with 38 (84.5%) and 35 (77.8%) resistances respectively. The data of present study indicated that the distribution of burn patients infected with *Acinetobacter baumannii* were prevalence significantly among young age groups of 15-29 years old and male / female ratio 0.64/1. AgNPs display excellent antibacterial activity against multidrug-resistant *Acinetobacter baumannii* with MIC  $2.1 \mu\text{g/ml} \pm 0.3$  comparing with polymyxin B as standard control with MIC  $0.53 \mu\text{g/ml} \pm 0.1$ . The concentration of sub-MIC ( $\frac{1}{2}$ MIC) of AgNPs exhibited bacteriostatic activity against multidrug-resistant *Acinetobacter baumannii*, while combination of  $\frac{1}{2}$  MIC with ( $\frac{1}{2}$ ) MIC of polymyxin B display bactericidal effect and complete reductions of *Acinetobacter baumannii* after 24 h. AgNPs exhibit strong anti-biofilm formation activity with inhibition of biofilm formation about 19 – 24 % at sub-MIC concentration. Moreover, combination of AgNPs with polymyxin B displayed remarkable synergistic anti-biofilm formation with 72 % inhibition of biofilm.

**Conclusions:** The data of this study indicate a strong synergistic anti-bacterial and anti-biofilm activity of combination of AgNPs with membrane-active peptide (Polymyxin B) against multidrug-resistant *Acinetobacter baumannii* isolated from burn wound infections. These results propose that AgNPs promising novel antimicrobial agent in the clinic.

**Keywords:** *Acinetobacter baumannii*, Silver nanoparticles, polymyxin B, burn wound infections.

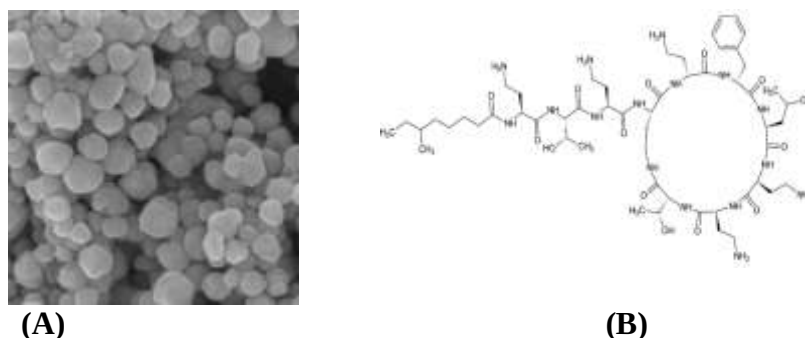
### Introduction

*Acinetobacter baumannii* is Gram-negative coccobacillus below the family of Moraxellaceae and includes strictly aerobic, catalase-positive, oxidase negative non-lactose-fermenting, and non-motile (1). *Acinetobacter baumannii* is isolated from soil, vegetables, and water. it is part of the human's opportunistic bacterial pathogen of skin, upper respiratory system, and gastrointestinal tracts (2). Multidrug -resistant strains of *Acinetobacter baumannii* have become progressively common in burns units and hospital-acquired infections associated with urinary tract, skin and soft tissue, bloodstream, and pneumonia infections. These extensively drug-resistant strains display resistance to many antibiotics including monobactams, fluoroquinolones and aminoglycosides with exclude of tigecycline and polymyxins. Polymyxins such as polymyxin B is potent antimicrobial agents against multidrug-resistant (MDR) *Acinetobacter baumannii*. Polymyxin B bind to the cell membrane of *Acinetobacter baumannii* and alter its structure, making it more permeable leads to cell death. The main side effects of polymyxin B on the human body cases toxicity for kidney and nerve system (3).

*Acinetobacter baumannii* is capable to colonize on surfaces of materials and equipment and environment of hospital burn units and development of biofilms formation which makes it difficult to eradicate from burn units or treat with traditional antibiotics, this reflected on burns patients which may lead to delayed wound healing or sepsis (4).

New strategy for control of multidrug resistant *Acinetobacter baumannii* (MDR-AB) infections developed by using silver nanoparticles (AgNPs) which showed high effectiveness against drug-resistant bacterial pathogens including Gram-negative "*Escherichia coli*, *Klebsiella aerogenes*, and *Pseudomonas aeruginosa*" and Gram-positive bacteria "*Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*"(5). Silver nanoparticles containing complexes of antibacterial agents which have different mechanism of action including degradation of bacterial enzymes, inactivation of cellular proteins and breakage of bacterial DNA (5).

The objective of this study was to evaluated synergistic antibacterial and anti-biofilm activity of the combination between silver nanoparticles and a polypeptide antibiotic (Polymyxin B) against multidrug resistant *Acinetobacter baumannii* isolated from burn wound infections.



**Figure 1. A-Morphology of Silver nanoparticles (20nm) Hongwu International Group Ltd.**

#### **B- Structures of lipopeptide polymyxin B.**

#### **Materials And Methods**

##### **Sample collection and bacterial identification**

Sterile wound swab was employ to take samples from 45 patients with burn wounds infections who were recorded in the burns center at specialist burns hospital in Baghdad (Al-Kindy Teaching Hospital and Baghdad Medical City) for the period between January-July 2017. The swabs specimens directly transfer to the laboratory for staining with Gram stain and culture on liquid media (Brain heart infusion broth) at 37 °C for 18 h, then sub-cultured of bacterial growth onto blood agar and MacConkey agar to identify cultural characteristics of *Acinetobacter baumannii*. API 20 E kit “BioMérieux, Marcy L’Etoile, France” was applied to complete biochemical tests and confirmed the final laboratory diagnosis of *Acinetobacter baumannii*.

##### **Silver nanoparticles and Antibiotics**

All Antibiotics used in this evaluation were obtained from Oxoid (UK) and Bioanalyse (Turkey) Which include “Aztreonam, amikacin, polymyxin B, ciprofloxacin, levofloxacin, colistin, ceftazidime, cefotaxime, cefepime, tetracycline, ticarcillin/clavulanic acid, piperacillin/tazobactam” Silver nanoparticles were purchased from Hongwu International Group Ltd (China) with the following properties: morphology “Spherical”, appearance “grey black powder”, purity (99.99%), particle size (20nm), apparent density (0.97g/ml), and tap density (2.16g/ml) (Fig. 1.).

##### **Antibiotics Susceptibility Test (AST)**

Kirby-Bauer’s disk diffusion technique was applied to test antimicrobial sensitivity against *Acinetobacter baumannii* isolates according to Clinical Laboratory Standards Institute (CLSI) guidelines. Briefly, two to three colonies of *Acinetobacter baumannii* were suspended with sterile saline to get density of  $1.5 \times 10^8$  CFU/ml “approximately 0.5 McFarland standard turbidity”. *Acinetobacter baumannii* suspension streaking on Müller-Hinton agar and antibiotic discs placed on

the agar plates and incubation at 37 °C for 24. The results recorded as resistant or sensitive after measuring the inhibition zone around the antibiotic discs according to the recommendation of CLSI (6).

##### **Minimum inhibitory concentrations of polymyxin B and AgNPs.**

The minimum inhibitory concentrations (MICs) of polymyxin B and AgNPs were performed according to CLSI standards guidelines. Briefly, two-fold serial dilution of polymyxin B or AgNPs were distribute in a 96- well microtiter plate and 100 µl of inoculum suspensions in Müller-Hinton broth of *Acinetobacter baumannii* strain were added with concentration of about  $1 \times 10^6$  “equal to 0.5 McFarland standard”. All microtiter plates incubated at optimal temperature (37 °C) for 18 h. The minimum inhibitory concentrations values were recorded with absorbance at 600 nm by enzyme-linked immunosorbent assay microtiter reader “Huma Reader-HS, Human GmbH, Wiesbaden, Germany”(7).

##### **Time kill-kinetic assays**

Time kill-kinetic assay was carried out according to recommendations of National Committee for Clinical Laboratory Standards “NCCLS” guidelines to investigate activity of AgNPs and synergistic effect in combination with polypeptide antibiotic (Polymyxin B) against *Acinetobacter baumannii* strains. bacterial suspension of *Acinetobacter baumannii* in Müller-Hinton broth were adjusted to 0.5 McFarland standard turbidity to get a final concentration about  $1 \times 10^6$  CFU/ml. Inoculum suspensions of *Acinetobacter baumannii* distributed in sterile test tubes contain polymyxin B or combination of polymyxin B with AgNPs with different concentrations. All test tubes incubated at 37 °C and aliquots of 100 µl pull out from each test tubes at specific times “0, 2, 4, and 24 h” and spread on blood agar to estimate the number of colony forming unit (CFU) and determine the synergistic activity of the combinations (6).

### Anti-biofilm formation activity

Calgary biofilm method was employed to evaluate of anti-biofilm activity of AgNPs and polymyxin B alone or in combination at sub-MIC concentrations. Briefly, *Acinetobacter baumannii* allows to grow overnight at optimal temperature (37°C) and diluted with Müller-Hinton broth to achieve a concentration of  $1 \times 10^6$  CFU/ml. Suspension 180 µl of *Acinetobacter baumannii* add to each well of a 96-well microtiter flat-bottom plate and then incubated at 37°C for 24h. AgNPs and polymyxin B alone or in combination at sub-MIC concentrations were added each well of plates and incubated at room temperature for 4-5h. then, 200 µl of phosphate buffered saline was used to wash plates three times and incubate the plates for one hour to fixe biofilms in the bottom of well. Biofilms stain with 1% crystal violet by add 200 µl of stain to each well and leave at room temperature for 30 -40 minutes and destained with 96% ethanol for 30 min at 37°C (8). The absorbance was measured at 595 nm by "Huma Reader-HS, Human GmbH, Wiesbaden, Germany". Anti-biofilm activity was calculated depending on the following equation "[1-(A 595 of cells treated with AgNPs/A 595 of non-treated control cells)] × 100". The fractional inhibitory concentration index calculations with the following equation:

$$FICI = \frac{MIC_a \text{ combination}}{MIC_a \text{ alone}} + \frac{MIC_b \text{ combination}}{MIC_b \text{ alone}}$$

Where (a) represents AgNPs and (b) represents polymyxin B. As is standard, FIC index values of  $\leq 0.5$  was considered to as synergism, values of  $>0.5$  and  $< 4$  no interaction/additivity, and values of  $\geq 4$  antagonism.

### Statistical Analyses

All experiments in this evaluation were completed in triplicate and repeated at least twice. The data were presented as mean  $\pm$  SD "standard deviation". Graphpad PRISM ® 6 software "GraphPad Software, Inc., La Jolla, CA, USA" was used to study statistical analysis. Student's t-test was used to test P-values.  $P < 0.05$  considered as statistical significance.

### Results

During the 7-month period of this study, forty-five *Acinetobacter baumannii* isolated from patients admitted to the burns center at Al-Kindy Teaching Hospital and Baghdad Medical City. All *Acinetobacter baumannii* isolates in this study

evaluated for antimicrobial susceptibility tests by Kirby-Bauer's disk diffusion method using 12 antibiotic discs selected with different concentration and mechanism of action. Membrane-active peptides colistin and polymyxin B were the most active antibiotics against *Acinetobacter baumannii* with sensitivity of 42 (93.4%) and 38 (84.4%) respectively, while cephalosporins (Cefotaxime) and fluoroquinolones (Ciprofloxacin) antibiotics were most resistances antibiotics with 38 (84.5%) and 35 (77.8%) resistances respectively. Moreover, 31 (68.9%) were resistant to more than two antibiotics and considered as multi-drug resistant *Acinetobacter baumannii*, while the remaining 14 (31.1%) represented the sensitive strains as shown in table 1 and figure 2.

The data of present study indicated that the distribution of burn patients infected with *Acinetobacter baumannii* were prevalent significantly among young age groups of 15-29 years old and statistical analysis showed significantly difference between age groups ( $P < 0.05$ ). The patients with age below five or more than sixty 60 years old exhibited low exposure to infection with *Acinetobacter baumannii* strains and statistical analysis offers the mean age of the patients was  $(23 \pm 5)$  year.

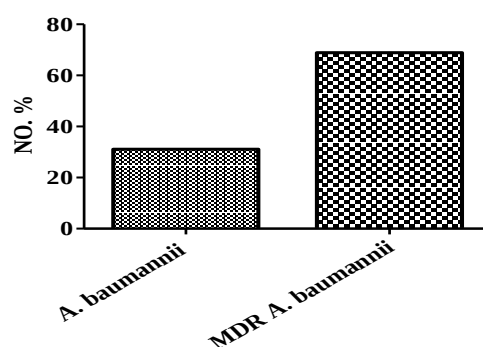
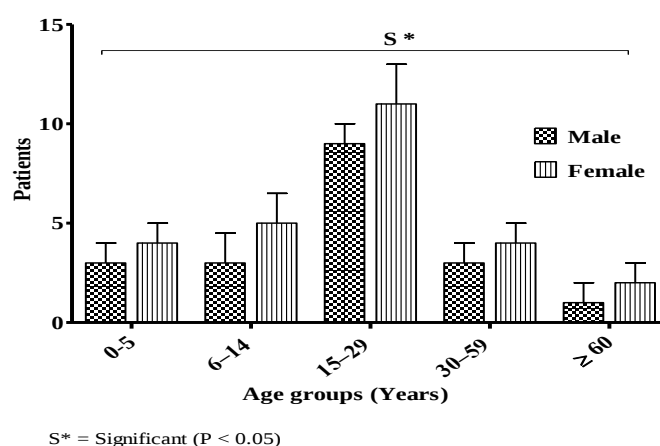
On the other hand, female patients with burn wound infections displayed high risk to be contaminated with *Acinetobacter baumannii* rather than male with male / female ratio 0. 64/1 as presented in figure 3.

In this study, AgNPs 20 nm were tested against *Acinetobacter baumannii* isolates and display excellent antimicrobial activity against with MIC  $2.1 \mu\text{g/ml} \pm 0.3$  comparing with polymyxin B as standard control with MIC  $0.53 \mu\text{g/ml} \pm 0.1$  as described in figure 4.

Time-kill assay was employed to study kinetic activity of silver nanoparticles alone and in combination with cyclic membrane-active peptide (Polymyxin B) multidrug-resistant *Acinetobacter baumannii* isolated from burn wound infections. The results presented in figure 5. show that the concentration of sub-MIC ( $\frac{1}{2}$ MIC) of AgNPs exhibited bacteriostatic activity against multidrug-resistant *Acinetobacter baumannii*, while combination of  $\frac{1}{2}$  MIC with ( $\frac{1}{2}$ ) MIC of polymyxin B display bactericidal effect on multi-drug resistant *Acinetobacter baumannii* and complete reductions of bacteria after 24 h.

**Table 1. Antibiotic susceptibility test of *Acinetobacter baumannii* isolated from burn wound infection**

| NO. | Antibiotic                  | Symbol | Conc. (mcg) | Sensitive No. (%) | Intermediate No. (%) | Resistant No. (%) |
|-----|-----------------------------|--------|-------------|-------------------|----------------------|-------------------|
| 1.  | Aztreonam                   | ATM    | 30          | 8 (17.8)          | 3 (6.6)              | 34 (75.6)         |
| 2.  | Amikacin                    | AMI    | 30          | 9 (20)            | 3 (6.6)              | 33 (73.4)         |
| 3.  | Polymyxin B                 | PB     | 300 units   | 38 (84.4)         | 0                    | 7 (15.6)          |
| 4.  | Ciprofloxacin               | CIP    | 5           | 6 (13.3)          | 4 (8.9)              | 35 (77.8)         |
| 5.  | Levofloxacin                | LVF    | 5           | 11 (24.5)         | 2 (4.4)              | 32 (71.1)         |
| 6.  | Colistin                    | COL    | 10          | 42 (93.4)         | 1 (2.2)              | 2 (4.4)           |
| 7.  | Ceftazidime                 | CAZ    | 30          | 10 (22.3)         | 6 (13.3)             | 29 (64.4)         |
| 8.  | Cefotaxime                  | CTX    | 30          | 4 (8.9)           | 3 (6.6)              | 38 (84.5)         |
| 9.  | Cefepime                    | FEP    | 10          | 18 (40)           | 3 (6.6)              | 24 (53.4)         |
| 10. | Tetracycline                | TET    | 10          | 19 (42.2)         | 1 (2.2)              | 25 (55.6)         |
| 11. | Ticarcillin/Clavulanic acid | TIM    | 75/10       | 11 (24.4)         | 4 (8.9)              | 30 (66.7)         |
| 12. | Piperacillin/Tazobactam     | TZP    | 20/10       | 13 (28.9)         | 3 (6.6)              | 29 (64.5)         |

**Figure 2. Distribution of *Acinetobacter baumannii* and multi-drug resistant *Acinetobacter baumannii* isolated from burn wound infections.****Figure 3. Distribution of the burn patients infected with *A. baumannii* according to sex and age groups**

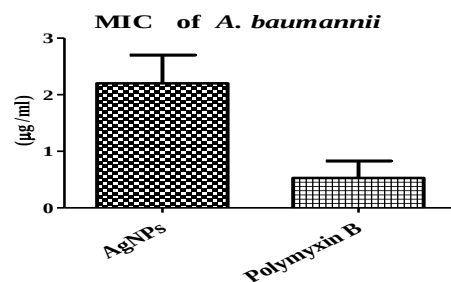


Figure 4. Minimum inhibitory concentrations of AgNPs and polymyxin B for *A. baumannii*.

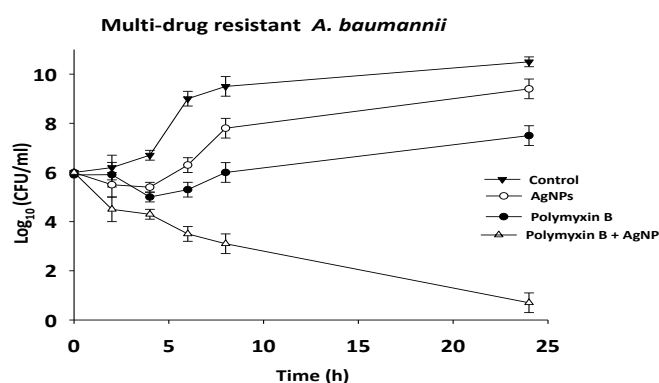
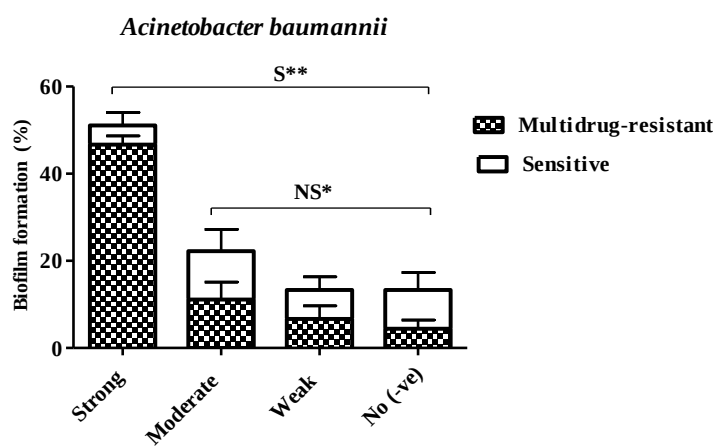


Figure 5. Kill kinetics of AgNPs alone and in combination with polymyxin B against multidrug-resistant *Acinetobacter baumannii*.

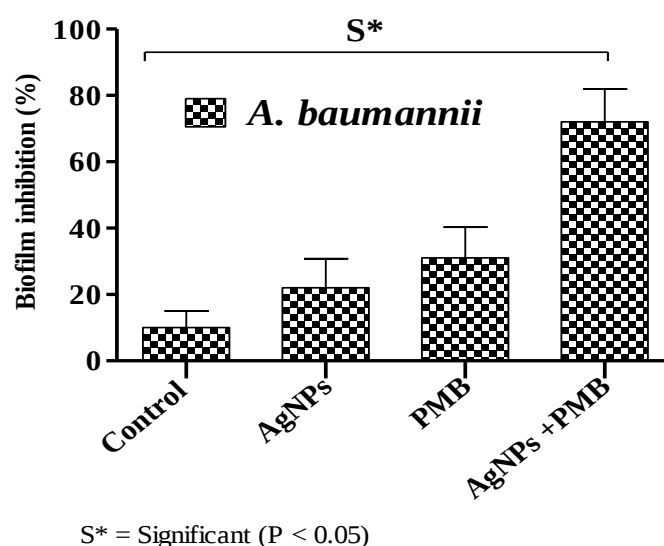


S\* = Significant ( $P < 0.05$ )  
 NS\* = Non-significant ( $P > 0.05$ )

Figure 6. Anti-biofilm inhibitory activity of AgNPs against *Acinetobacter baumannii*.

**Table 2. Fractional inhibitory concentration index (FICI) values of two-drug combination between Polymyxin B and AgNPs that inhibited biofilm formation of *Acinetobacter baumannii*.**

| Microorganism       | Compounds   | MIC ( $\mu\text{g/ml}$ ) Range | FIC index Range | Interaction |
|---------------------|-------------|--------------------------------|-----------------|-------------|
| <i>A. baumannii</i> | Polymyxin B | 0.25- 0.5                      | 0.24 –0.51      | Synergistic |
|                     | AgNPs       | 2.5- 5                         |                 |             |

**Figure 7. Anti-biofilm inhibitory activity of AgNPs alone and in combination with polymyxin B against multidrug-resistant *Acinetobacter baumannii*.**

## Discussion

*Acinetobacter baumannii* is the most frequently bacterial pathogens isolated from patients with burns and commonly affecting immunocompromised patients in intensive care units or burn centers (9). The Increase rate of prevalence of multidrug-resistant *Acinetobacter baumannii* in burn wound infections make treatment with traditional antibiotics more complex and leads to severe hospital-acquired infections(10). Several studies have indicated that multidrug resistant Gram-negative bacteria including *Acinetobacter baumannii* were the most predominant bacterial pathogens isolated from patients with burns. In a recent study by Rashid et al (11), and Aziz et al(12) , they found that high prevalence rate of multidrug-resistant *Acinetobacter baumannii* isolated from different intensive care units or burn centers in Iraq. In addition, Ghaima et al (13), report that the most *Acinetobacter baumannii* isolated from patients with burns in Baghdad hospital resistant to piperacillin, gentamicin and cefotaxime. The results of this study confirmed that the majority of *Acinetobacter baumannii* were resistant to  $\beta$ -lactam and aminoglycoside

antibiotic. It has been documented that AgNPs showed broad spectrum activity against Gram-negative bacteria “*Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*” and Gram-positive bacteria “*Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*”(14, 15). Moreover, a number of studies report that AgNPs display excellent antimicrobial activity against multidrug-resistant pathogenic bacterial such as “Methicillin-resistant *Staphylococcus aureus*, Vancomycin-resistant enterococci, and drug resistant tuberculosis (15, 16). The result of this study supports these findings, AgNPs exhibited remarkable antibacterial activity against multidrug-resistant *Acinetobacter baumannii* isolated from burn wound infections. On the other hand, Wan et al (5), and Ghosh et al (17), found that AgNPs enhanced antimicrobial activity of antibiotics and showed synergistic activity in combination with rifampicin and Beta-lactam (piperacillin) against carbapenem-resistant *Acinetobacter baumannii*.

In addition, Hendiani et al (18), report that two-drug combination of AgNPs with lower concentration of imipenem displayed synergistic

anti-biofilm activity of against *Acinetobacter baumannii*.

In the present study, AgNPs exhibited synergistic bactericidal activity in combination with sub-MIC levels of active peptide "Polymyxin B" against multidrug-resistant *Acinetobacter baumannii* isolated from burn wound infections. Moreover, synergistic anti-biofilm activity was observed in this combination with % 70 biofilms inhibition *Acinetobacter baumannii*.

The wide spectrum bactericidal properties of AgNPs against multidrug-resistant *Acinetobacter baumannii* may be attributed to interfere of AgNPs with main mechanisms resistance of *Acinetobacter baumannii* which include "Production of  $\beta$  lactamases, efflux pumps, lower permeability of the outer membrane, mutations in antibiotic targets (e.g, for quinolones), and production of enzymes inactivating aminoglycosides"(19). Moreover, AgNPs can damage the membrane potential, prevent adenosine triphosphate production, increase the level of reactive oxygen species, and damage the membrane lipids and DNA (20). The data of this study exhibited strong synergistic bactericidal effect of combination between AgNPs and cyclic antimicrobial peptides (Polymyxin B) multidrug-resistant *Acinetobacter baumannii*. The possible role of polymyxin B in this synergism is it polypeptides that interact with the lipopolysaccharide layer of *Acinetobacter baumannii* and binds with lipid A component, rapid permeabilization of the outer cell membrane that permitting improved penetration of AgNPs, polymyxin B also disturb the balance of magnesium ions ( $Mg^{2+}$ ) and calcium ions ( $Ca^{2+}$ ) leading to disturbing the integrity and function of the outer membranes of *Acinetobacter baumannii* (21). On the other hand, polymyxin B can inhibit the formation of biofilm by reduction in the planktonic population, inhibition of the initial adhesion of bacteria to the surface, and suppression of the established biofilm(22).

In conclusions, the data of this study indicate a strong synergistic bactericidal and anti-biofilm activity of combination of AgNPs with membrane-active peptide (Polymyxin B) against multidrug-resistant *Acinetobacter baumannii* isolated from burn wound infections. These results propose that AgNPs can employ as novel antimicrobial agent in the clinic.

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