



## Antimicrobial activity of silver nanoparticles with antibiotics against clinically isolated *Acinetobacter baumannii*

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### ABSTRACT

Antibiotic-resistant bacteria are among the major healthcare problem worldwide and *Acinetobacter baumannii* is a leading threat among them. In this study, the combined effects of different antibiotics with silver nanoparticles (AgNPs) were assessed against the growth of a clinical isolate of *A. baumannii*. The bacterial strain was isolated from a hospitalized burned patient in Sulaimanyah- Iraq. Identification of the isolated bacterium was done based on the partial sequence of the 16S rRNA gene and phylogenetic analysis. The growth of the bacterium was totally inhibited by AgNPs at the concentration of (0.2 mg/ml). AgNPs treatment showed a partial synergistic effect with azithromycin (Fractional Inhibitory Concentration Index (FICI) = 0.6) and an additive effect with kanamycin (FICI = 1.67). Not a significant difference in the antimicrobial activities of either ampicillin or tetracycline was observed when they used alone or in combination with AgNPs. Overall, this study may provide a promising future use of azithromycin with AgNPs to treat *A. baumannii* superficial infections; however, a combination of kanamycin with AgNPs together should be avoided.

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Keywords: *Acinetobacter baumannii*, Multidrug resistance, Silver nanoparticles, Synergistic effect, Indifference effect.

### 1. Introduction

Since the discovery of penicillin in the early 20th century, human beings have been in a continuous race to discover new antibiotics to control microbial infectious diseases. Alongside, microorganisms are persistently developing resistance against antibiotics [1]. Currently, the issue of antibiotic-resistant bacteria is one of the most serious public health issue worldwide [2]. In the U.S alone, every year 2 million people are infected with antibiotic-resistant bacteria and around 23,000 people die from the infection [3]. This issue is escalating with irrational drug prescription, and overconsumption of antibiotics by the human [4]. Between 2000 and 2010, the global consumption of antibiotics increased by 36% with the use of nearly 73 billion standard doses per annum only in 2010 [5]. There are several known genera of antibiotic resistance bacteria, but *Acinetobacter baumannii* is considered as “red alert” human pathogen [6] and ranked as a number one critical pathogen by the World Health Organization (WHO) in the list of the global priority antibiotic-resistance bacteria [7].

*A. baumannii* is a Gram-negative, opportunistic pathogen that is accounted for nearly 2-10% of all Gram-negative nosocomial infections, particularly among the immunocompromised patients [8]. Main challenges with *A. baumannii* are an extraordinary capacity of the bacterium to quickly develop resistance against new antibiotics, and also the ability to tolerate unfavourable conditions such as surviving on the surface of the hospital equipment for weeks [9, 10].

Several methods have been developed to discover new antibiotics such as genetic engineering of the secondary metabolic pathways genes – known as combinatorial biosynthesis [11], designing antibiotics based on the structural knowledge of the target proteins [12], testing medicinal plants [13] and also natural resources for the antimicrobial compounds [14]. Recently, computation method was also developed to screen different compounds for their antimicrobial activities [15].

Using nano-antibiotic, especially the combination of silver nanoparticles (AgNPs) with antibiotics, has received great attention to overcome the problem of antibiotic-resistant bacteria [16]. This is because AgNPs have a wide range of activities as antibacterial [17, 18], antifungal [19], and even antiviral [20] agents. Besides, microorganisms are not known to develop resistance against the metals [16]. The aim of this study was to investigate the

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combination effects of AgNPs with different antibiotics were tested against a clinical isolate of *A. baumannii*.

## 2- Material and Methods

### 2.1 Isolation and Identification of the bacterial strain

A clinical specimen was collected from a burned female patient in the Burn and Plastic Surgery Emergency Hospital in Sulaimanyah city- Iraq in January 2019. Identification of the isolated bacterium was done by using Polymerase chain reaction (PCR) technique. Genomic DNA was extracted using Prlesto™ Mini gDNA Bacteria Kit (Geneaid). PCR assay performed in 50 µl final volume, composed of 25 µl Prime Taq Premix (2x) (GeNet Bio), 0.5 µM of each of the forward and reverse 16S rRNA primers (F: 5'-AGAGTTTGATYMTGGCTCAG-3'), (R: 5'-ACGGYTACCTTGTACACTT-3') [21], and 5 µl of DNA template. The volume of the reaction was completed with the addition of nuclease free water. The PCR condition was as follow: initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute, and the final extension step at 72°C for 5 minutes. Sequencing of the ~ 1.5 kbp PCR product was carried out by Sanger DNA sequencing at Macrogen - Republic of Korea. Quality and the sequence edition were performed with Chromas software version 2.6.5 (Technelysium).

### 2.2 Construction of the phylogenetic trees

The taxonomical characterization of the isolate was carried out by creating a phylogenetic tree for the isolate based on the nucleotide sequence of the 16S rRNA gene as described previously [22]. Briefly, the 16S rRNA sequence of the isolate was aligned with 16S rRNA gene of the closely related species in NCBI using ClustalX 2.1 [23] and then MEGA 7 programs [24]. The phylogenetic tree was constructed using the Neighbor-joining method (Kimura 2-parameter model, bootstrapped with 1000 replicate runs) by MEGA 7 tree-building program.

### 2.3 Biosynthesize and Characterization of the silver nanoparticles

Preparation and characterization of the AgNPs that used in this study were described previously [25]. In summary, an aqueous extract of the chamomile flowers was used to reduce preheated 1mM AgNO<sub>3</sub>. Formation of AgNPs was detected by the colour change of the solution to brown. The size of synthesized nanoparticles was measured by X-ray diffractometry (XRD) (PanAnalytical; The Netherlands). Scanning electron microscopy (SEM) (CamScan 3200 LV; England) was used to analyse the morphological appearance of the particles [25].

### 2.4 Antimicrobial susceptibility test

Micro dilution method was used to determine the minimal inhibitory concentration (MIC) of the antibiotics and AgNPs against the clinical isolate. The antibiotics used in this study were ampicillin, kanamycin, and tetracycline from Sigma Aldrich-UK, and azithromycin from Pioneer- Iraq. Stock solution for each of

the antibiotic was prepared according to the Clinical and Laboratory Standards Institute (CLSI) protocol [26]. Bacterial cells were grown overnight on Mueller-Hinton broth (MHb) at 35 °C to the Optical Density 600 nm (OD 600 nm) ~ 0.8. The bacterial culture medium was diluted to OD 600 nm= 0.2, and 20 µl of the culture was added to 180 µl of MHb containing 20 µl of each of the antibiotics or AgNPs. The concentration ranges of the antibiotics, and AgNPs were 0.025 to 102.4 mg/ml and 0.025 to 0.2 mg/ml, respectively. The bacterial cultures were kept in 96-well plate at 35 °C incubator for an additional 20 hours. The effect of the antibiotics or AgNPs was assessed by measuring the growth of the bacteria at OD 600 nm and compared the result with the bacterial growth without a treatment.

### 2.5 Combination assay of antibiotics and AgNPs

In the combination assay, the bacterial cells were grown in the MHb as described above. Twenty microliters (20 µl) of 1 mg/ml of AgNPs was added to 96-well plate containing 160 µl of the media and 20 µl of the antibiotic. The cultures were kept at 35 °C for 20 hours. The bacterial growth was monitored at OD 600 nm and compared with the bacterial growth in the present or absent of the antibiotics, and the present of AgNPs alone. The antibiotics that used in this essay were ampicillin (102.4 mg/ml), kanamycin (1.6 mg/ml), azithromycin (0.025 mg/ml), and tetracycline (0.05 mg/ml). The Fractional Inhibitory Concentration Index (FICI index) of the combined effect of each antibiotic with AgNPs were calculated based on the following equation [27]:

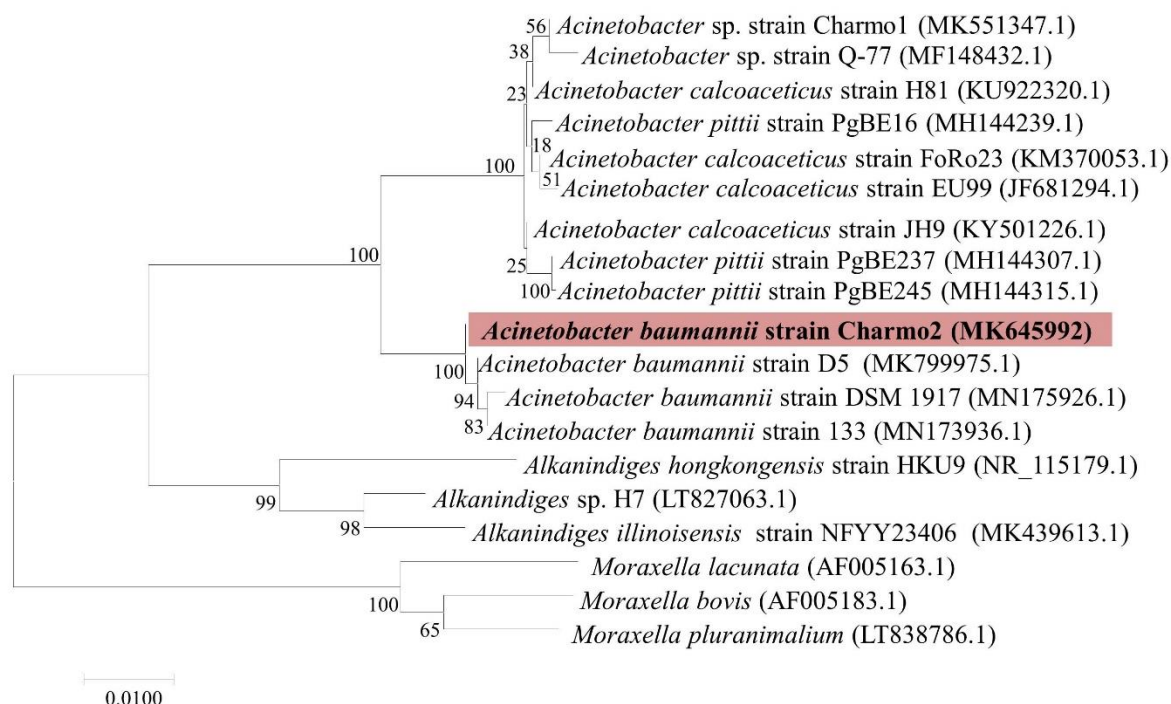
$$\text{FICI of drug A} = \frac{\text{MIC drug A in combination of AgNPs}}{\text{MIC drug A alone}}$$

### 2.6 Data Analysis

All assays were performed in at least three biological replicates. P-value (Un-paired t-test) was performed using GraphPad Prism 8.0.1 Software Inc., La Jolla, CA, USA. Statistical significance was defined when the P value was less than 0.05.

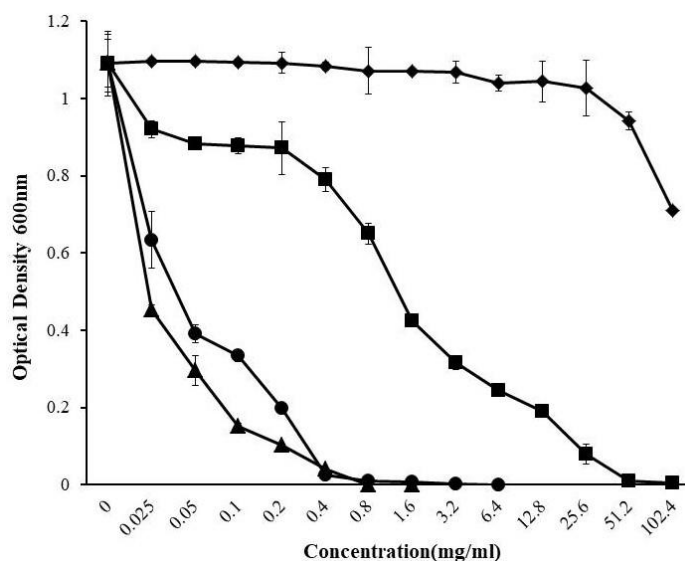
## 3. Results and Discussion

In this study, a clinical isolate of *A. baumannii* was isolated from a female burnt patient in Plastic Surgery Emergency Hospital in Sulaimanyah city- Iraq. Identification of the isolate was done based on DNA sequencing of the 16S rRNA gene. Phylogenetic tree of the isolate was constructed based on 16S rRNA gene sequence. As it shown in Figure 1, the new isolate of *A. baumannii* form a monophyletic group with other strains of *A. baumannii* such as strain D5 (Accession number: MK799975) and strain 133 (Accession number: MN173936), which were isolated from China, and the strain DSM 1917 (Accession number: MN175926) which was isolated from the USA. The nucleotide sequence of this strain was submitted to the National Centre for Biotechnology Information ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and accession number (MK645992.1) was assigned for the isolate with the strain name charmo2 (<https://www.ncbi.nlm.nih.gov/nucleotide/MK645992>).



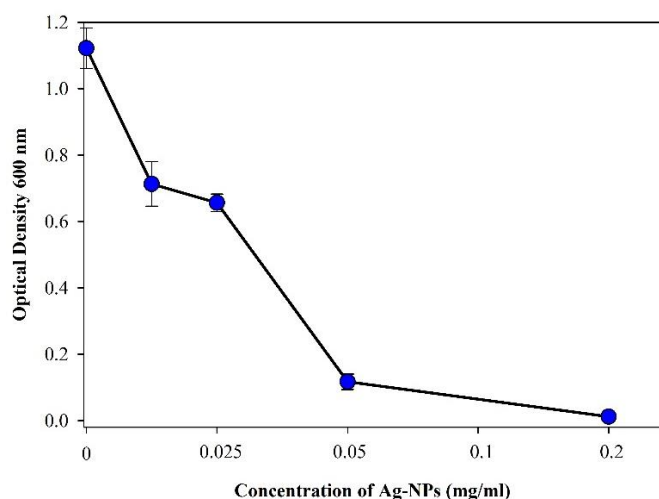
**Figure 1:** Phylogenetic tree of *A. baumannii* strain charmo2 base on the sequence of 16S rRNA gene. *A. baumannii* strain charmo2 is shown bold and highlighted red. The phylogenetic tree was created by MEGA 7 tree-building program using the neighbor-joining method, bootstrapped with 1,000 replicate runs.

In the MIC experiment, the bacterium was shown to respond differently to each of the treatments (Figure 2). Tetracycline, a broad-spectrum antibiotic which inhibits the protein synthesis in microorganisms [28], was found to have the strongest effect on the bacterial growth with MIC = 0.4 mg/ml. In a systematic review article using data of 156 patients published in 10 different research articles, the authors concluded that tetracycline, alone or in combination with other antibiotics, is an effective drug to treat *A. baumannii* infections [29]. MIC of both azithromycin and kanamycin were found at 0.8 mg/ml and 51.2 mg/ml, respectively (Figure 2). Azithromycin affects microbial growth by inhibiting the protein synthesis through binding to 50S ribosomal subunit of the bacterial ribosome [30]. Hatami was also reported that 50% of the total 50 isolates from the burning units shows resistant against azithromycin [31]. Kanamycin is also affecting the protein synthesis in microorganisms [32]. The resistance of *Acinetobacter* spp. to kanamycin was suggested to be related to the high-level activity of the aminoglycoside-modifying enzymes AAC(3)II and reduction in the intracellular accumulation of the antibiotic [33]. *A. baumannii* strain charmo2 was shown the highest resistant toward ampicillin and was able to grow in all tested concentrations of the antibiotic (Figure 2). Similarly, Rezaie Keikhaie *et al.* also reported that all 30 clinical isolates of *A. baumannii* were resistant to ampicillin [34]. Also, 98% of 48 *A. baumannii* isolates from an abattoir and 90% of 52 *A. baumannii* isolates from the aquatic environment were found to have resistant toward ampicillin [35]. In general, ampicillin is not a drug of choice to treat *A. baumannii* infections and ampicillin resistant is common among the strains of the bacterium [35].



**Figure 2:** MIC of different antibiotics towards *A. baumannii* strain charmo2. The antibiotics that used in the experiment were tetracycline (▲), azithromycin (●), kanamycin (■), and ampicillin (◆). The bacterium growth was examined after 20 hours incubation at 35 °C with and without the antibiotics. Data shown are means of data from three replicates ± standard deviations.

In addition, MIC of the previously biosynthesized AgNPs [25] was also determined to *A. baumannii* strain charmo2. A concentration range between 25 µg/ml to 200 µg/ml of AgNPs was tested and total bacterial inhibition was found at the concentration of 200 µg/ml (Figure 3).



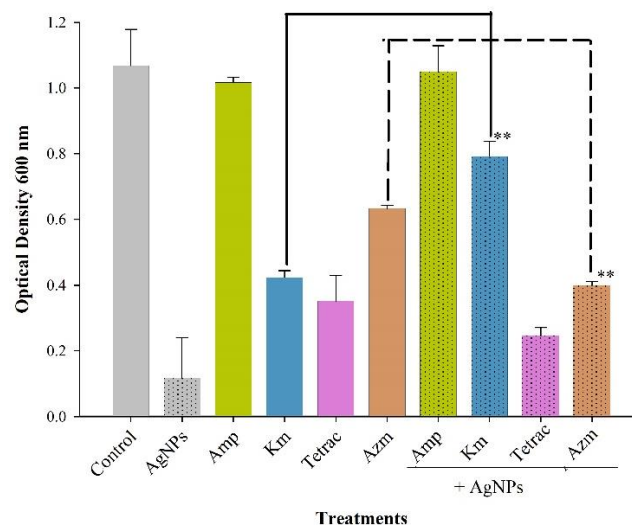
**Figure 3:** Antimicrobial activity of silver nanoparticles (AgNPs) against the growth of *A. baumannii* strain charmo2. Data shown are means of data from three replicates  $\pm$  standard deviations.

Previous studies have shown that the MIC of the biosynthesized AgNPs from *Lycopersicon esculentum* and *Murraya koenigii* against *Escherichia coli* and *Staphylococcus aureus* were found at 0.5 mg/ml [36] 0.032 mg/ml [37], respectively. It was also reported that both shapes and the sizes of nanoparticles affect the antimicrobial activity of the AgNPs [38-40]. For instance; the spherical-shaped AgNPs shown a stronger antimicrobial activity against *E. coli*, *S. aureus* and *P. aeruginosa* than the triangle shape [41]. Moreover, the smaller nanoparticles size have a stronger antimicrobial activity [40]. The antimicrobial activity of the AgNPs that used in this study could be driven from the effect of nanoparticles, which had an average size of  $18 \pm 3$  nm, and also the effect of different terpenoids and flavonoids compounds that naturally found in chamomile flowers [42].

In the combination assay, AgNPs (0.1 mg/ml) was tested with each of the ampicillin (102.4 mg/ml), tetracycline (0.05 mg/ml), kanamycin (1.6 mg/ml), and azithromycin (0.025 mg/ml). To analyse the combination effect, the result of each of the treatments was compared with the growth of the bacterium in the presence of the antibiotic alone, AgNPs alone, and without any treatment (Figure 4). Statistical analysis of the results show that there is not a significant difference ( $p$ -values:  $P \geq 0.05$ ) in the antimicrobial activity of ampicillin and tetracycline when they were used alone or in combination with AgNPs (Figure 4). Contrary, Fayaz *et al.* reported a synergistic effect when ampicillin and AgNPs used together against a number of Gram-positive and Gram-negative bacteria [43]. In addition, synergistic effect was reported when tetracycline and AgNPs used together against *Salmonella typhimurium* DT 104 [44].

Adding AgNPs to either of kanamycin or azithromycin shown a significant difference in the antimicrobial activity of each of the antibiotics ( $p$ -value  $< 0.005$ ) in comparison with using the antibiotic alone. Based on FICI analysis, there was a partial synergy effect (FICI= 0.6) when azithromycin and AgNPs used together and the antimicrobial activity of the antibiotic was

increased by 1.6 folds, whereas, indifference effect (FICI=1.87) was observed in case of using kanamycin with AgNPs (Figure 4).



**Figure 4:** Combination effects of antibiotics with AgNPs (0.1 mg/ml) against *A. baumannii*. The concentration of ampicillin, kanamycin, azithromycin, and tetracycline were equal to 102.4 mg/ml, 1.6 mg/ml, 0.025 mg/ml, and 0.05 mg/ml, respectively. Data shown are means of data from three replicates  $\pm$  standard deviations.

Numbers of studies reported synergistic effect of combined AgNPs with azithromycin [45] or kanamycin [43, 44, 46] against different bacterial genera.

The mechanism of the combination effect of antibiotics with AgNPs is not well understood. But, it has been proposed the synergistic effect occur because antibiotic enhances the attachment of Ag to the bacterial cells and increase the release of  $Ag^+$  to the bacterial cells [44].

#### 4. Conclusion

*A. baumannii* charmo2 was isolated from a burn patient in Sulaimanyah/ Iraq. The bacterium was shown to be resistant to ampicillin, kanamycin, azithromycin, and tetracycline. The growth of *A. baumannii* charmo2 was totally inhibited by biologically synthesized AgNPs at the concentration at 0.2 mg/ml. Combination of AgNPs with azithromycin were shown to improve the antimicrobial activity of the antibiotic by 1.6 folds. However, not a significant difference in the antimicrobial activity of neither ampicillin nor tetracycline was observed when they were used alone or with AgNPs. Indifference combination effect was observed when kanamycin was used with AgNPs.

#### Conflict of interest

None.

#### Author contributions

DKS was supervising the project and responsible for designing the experiments. PMM was conducting the experiment in the lab. Manuscript was prepared and written by both PMM and DKS.

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