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Original Research Article

Biosynthesis of silver nanoparticles using the aqueous extract of chamomile flower and their antibacterial activity against *Acinetobacter* spp.

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ABSTRACT

In this study, we have shown that biologically synthesized Silver Nanoparticles (Ag-NPs) can effectively inhibit the growth of a clinical isolate of the *Acinetobacter* spp *in vivo.* The bacterial strain was isolated from a hospitalized male burnt patient in Sulaymaniyah, Iraq. Initially, Minimum Inhibitory Concentration (MIC) of ampicillin, kanamycin, tetracycline, and azithromycine were determined for the strain using broth microdilution method. The bacterial strain was found to have the highest resistant against azithromycin (400 µg/ml), whereas the MIC for kanamycin, ampicillin and tetracycline were at 200 µg/ml, 100 µg/ml, and 100 µg/ml, respectively. Additionally, Ag-NPs were prepared using the aqueous extract of chamomile (*Matricaria chamomilla)* flowers. Scanning Electron Microscopy (SEM) image revealed the particles have spherical and irregular flower-like shapes with an average size of 18 ±3 nm based on the X-Ray Diffraction (XRD) spectrum data. Interestingly, the biosynthesized Ag-NPs shown to actively inhibit the growth of the bacterial strain with MIC at 50 µg/ml.

***Keywords*:** Multidrug resistance, silver nanoparticles, MIC

INTRODUCTION

Acinetobacter baumannii is a Gram negative, aerobic and an opportunistic human pathogen [1,2]. The bacterium can infect various human organs like skin, lungs, blood, urinary tract, eyes and brain, particularly in immunocompromised patients who are admitted in intensive card and burn units [3,4]. Recently, reports on the isolation of multi-drug resistant *A. baumannii* have increased worldwide, and

this has made the bacterium to be considered as a red alert pathogen for human beings [5]. In addition, the bacterium poses serious global threat to the healthcare system due to its unique ability to resist almost all commercially available antibiotics and limited available therapeutic options [6,7]. In 2017, the World Health Organization (WHO) ranked *A. baumannii* as a number one critical in the list of the global priority pathogens of antibiotic resistance bacteria in order to be tackled by the researchers through discovering of new antibiotics [8]. *A. baumannii* is also known as Iraqibacter since it was predominantly isolated in the wounded troops who were treated at military medical facilities in Iraq and Kuwait region during Operation Iraqi Freedom [9,10]. Several factors contributes to the virulence of *A. baumannii* such as genome flexibility, ability to produce biofilm and also colonize and invade human epithelial cells [5].

In modern medical science, application of nanotechnology has a great potential to overcome many problems including Multidrug Resistant (MDR) bacteria because of some propertie: firstly, microorganisms are unlikely to develop resistance against metals, as these metal attacks a broad range of targets in the organisms and secondly, most of these metals are nontoxic to human cells in small concentrations [11]. Green Synthesis of nanoparticles (or biosynthenthetic nanoparticles) using plant extract is particularly popular and has received a great attention worldwide. This is because the synthesis of plant-mediated nanoparticles is simple, cost-effective, eco-friendly and less toxic for human therapy [12].

Many reports on the biosynthesis of silver nanoparticles are accessible using plant extracts, such as *Lantana camara*, *Moringa oleifera*, *Catharanthus roseus*, and *Eucalyptus chapmaniana* [13]. However, the plants potential for synthesizing nanoparticles is still need to be studied.

Here, we used chamomile flowers extract to synthesize silver nanoparticles [14]. The biosynthesized nanoparticles were characterized and tested again a clinically isolate of *Acinetobacter* spp. charmo1.

MATERIALS AND METHODS

Isolation and Identification of the bacterial strain

Bacterial isolation was carried out at the burn and plastic surgery emergency hospital in Sulaymaniyah city- Iraq from a male burnt patient. Identification of the strain was done by using Polymerase Chain Reaction (PCR). The bacterial total genome was extracted using Prlesto™ Mini gDNA Bacteria Kit (Geneaid). PCR reaction of

total 50 µl was prepared containing Prime Taq Premix (2x) (GeNet Bio) and 0.5 µM of 16S rRNA primers (F: 5′-AGAGTTTGATYMTGGCTCAG-3′, R: 5′-ACGGYTACCTTGTTACGACTT-3′) [15]. The PCR conditions were denaturation at 95 ºC for 30 seconds, annealing at 58 ºC for 30 seconds and extension at 72 ºC for 2minutes. The PCR products were sequenced by Sanger DNA sequencing at Macrogen - Republic of Korea. The Chromas software v1.0 (Technelysium) was used to check the quality and edit the sequence.

Construction of the Phylogenetic trees

The taxonomical identification of the newly clinical isolate was carried out by aligning the 16S rRNA sequences of the strain with the closely related species using ClustalX 2.1 [16] and then MEGA 7 programs [17]. The Neighbor-joining method (bootstrapped with 1000 replicate runs) was applied to construct Phylogenetic tree in the MEGA 7 tree-building program.

Plant collections and preparation of the chamomile aqueous extract

The flowers of the chamomile plant were used to prepare silver nanoparticles following the procedure which was previously described [18]. Briefly, the freshly flowers were collected in the Shwan region of Chamchamal, Sulaimany, Kurdistan- Iraq (Figure 1 A). The flowers were washed, cut into small pieces, and air dried for 24 h. Nearly 20 gram of the dried flowers were transferred into 500 ml conical flask containing 100 ml distilled water and then boiled for 10 minutes. The flower-water aqueous extract was filtered through 0.44 µm pore size Whatman filter paper. Flow through of the filter was collected in the 50 ml falcon tube and stored at 4 ºC refrigerator.

Biosysnthesize of the silver nanoparticles

In order to prepare silver nanoparticles, 1 ml of the chamomile flowers aqueous extract was added to 50 ml of 1 mM $AgNO_3$ in 100 ml conical flask as it was previously described [18]. The solution was heated at 75 ºC on hot plate for 20-30 minutes. The colour changes of the $AgNO_3$ from colourless to brown was used as the indication for the reduction of $AgNO_3$ to Ag^+ and the formation of nanoparticles [19].

Characterization of the silver nanoparticles

Characterization of the produced silver nanoparticles was done by using UV-visible spectroscopy, X-ray diffractometry (XRD) was used to record XRD patterns equipped with a Cu-Ka source (1.5406 Å), and Scanning Electron Microscopy (SEM).

Spectrophotometric characterization of the reduced silver nanoparticle was performed

by analyzing UV-visible light of 1 ml of the synthesized nanoparticles with UviLine 9400 (SI Analytics) in the range 200 nm to 800 wavelengths.

Aliquot (10 ml) of the synthesized silver nanoparticle was dried out in the 20 ml Petri dish under the fume hood for 4 days to be used for XRD and SEM analysis. XRD analysis was carried out X 'Pert Pro diffractometer (PanAnalytical, Almelo, Netherlands) at the fixed operating voltage and current of 45 kV and 40 mA, respectively. The XRD glancing angles were arranged in the range of $10° \leq 2\Theta \leq 70°$. The morphology of the nanoparticles was analyzed using SEM with CamScan 3200 LV using Caesium^{TM} version 6.1.10 at Kurdistan Institution for Strategic Studies and Scientific Research (KISSR) Sulaimaniya, Iraq.

Antimicrobial susceptibility testing

Prior of testing the antimicrobial activity of the biosynthesized silver nanoparticles, the MIC of the isolate was determined using broth microdilution method in the 96-well plate following the Clinical and Laboratory Standards Institute (CLSI) method [20]. The following antibiotics were used in this study: ampicillin, kanamycin, tetracycline, and azithromycine. Growth of the bacteria was monitored after 20 hours of incubation at 35 ºC with UviLine 9400 (SI Analytics) at 600 nm.

RESULTS

The problem of MDR is among the major healthcare issues worldwide. Therefore, there are several ongoing researches to overcome this global problem. Using nanoparticle, particularly silver nanoparticles, is one of the most promising area and this is due several unique physical and chemical properties of the substance [21].

In this study, we used a clinical isolate, which was isolated from a hospitalized male burnt patient in the Sulaymaniyah city-Iraq. Identification of the bacterium was done by sequencing of the 16S rRNA gene. Homology sequence searching of the ~1.4 kbp sequenced gene suggested that the bacterium belongs to the genus of the *Acinetobacter*. In addition, the phylogenetic tree analysis of the 16s rRNA sequence of the bacterium confirmed that the isolate is *Acinetobacter* spp. and form a monophyletic group with several other *Acinetobacter* spps including *A. pitti* strain PgBE16 (Accession number: MH144239.1) isolated in republic of Korea and *A. calcoaceticus* strain Eu99 (Accession number JF681294) isolated in Mexico (Figure 2). The nucleotide sequence of this new strain was also uploaded to the

National Centre for Biotechnology information (www.ncbi.nlm.nih.gov) with the accession number (MK551347.1) and the strain has been named as *Acinetobacterium* sp. charmo1.

In the minimal inhibitory experiment, our clinical isolate was shown the highest resistant toward azithromycin (400 µg/ml), followed by kanamycin (200 µg/ml), and then ampicillin and tetracycline which were at 100 µg/ml. Such differences in the bacterial respond to these antibiotics could be due to the drug mode of action. Azithromycin, kanamycin, tetracycline are all affecting the bacterial growth by inhibiting the protein synthesis [22]. Ampicillin, on other hands, acts on the bacterial growth through impairing the cell wall biosynthesis [23]. The other possible explanation for the differences is azithromycin commonly prescribed as antibiotic to the patients in the local hospitals in this region.

Figure 1. Flower collections and plant extraction. A: flowers of the chamomile (*Matricaria chamomilla)* plant. B: Aqueous extract of the Chamomile flowers.

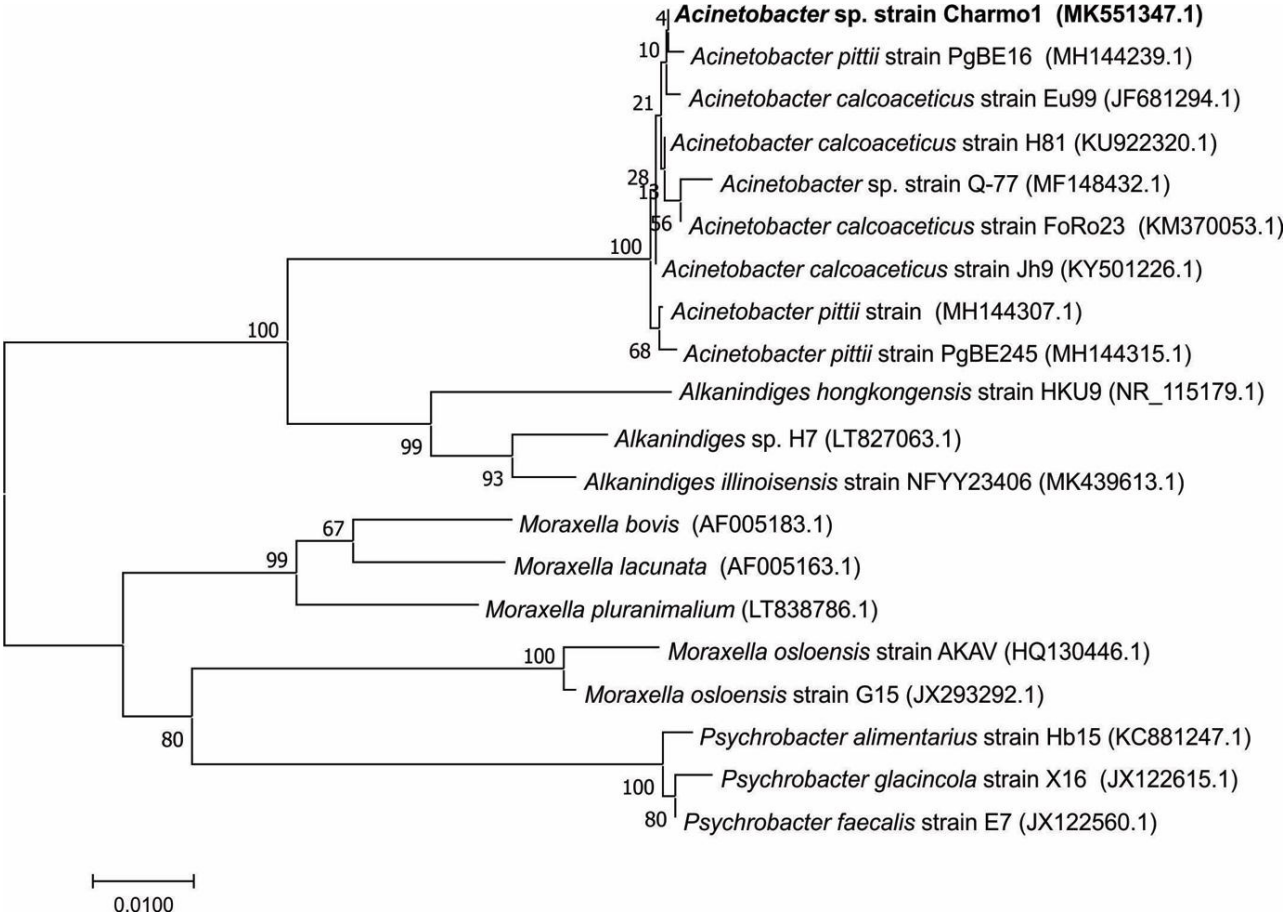

Figure 2. Phylogenetic tree of *Acinetobacterium* sp. charmo1 based on the nucleotide sequence of the 16s rRNA gene. DNA sequences were aligned using ClustalX program and the tree was created using the neighbor-joining method, bootstrapped with 1,000 replicate runs, by the MEGA 7 tree-building program.

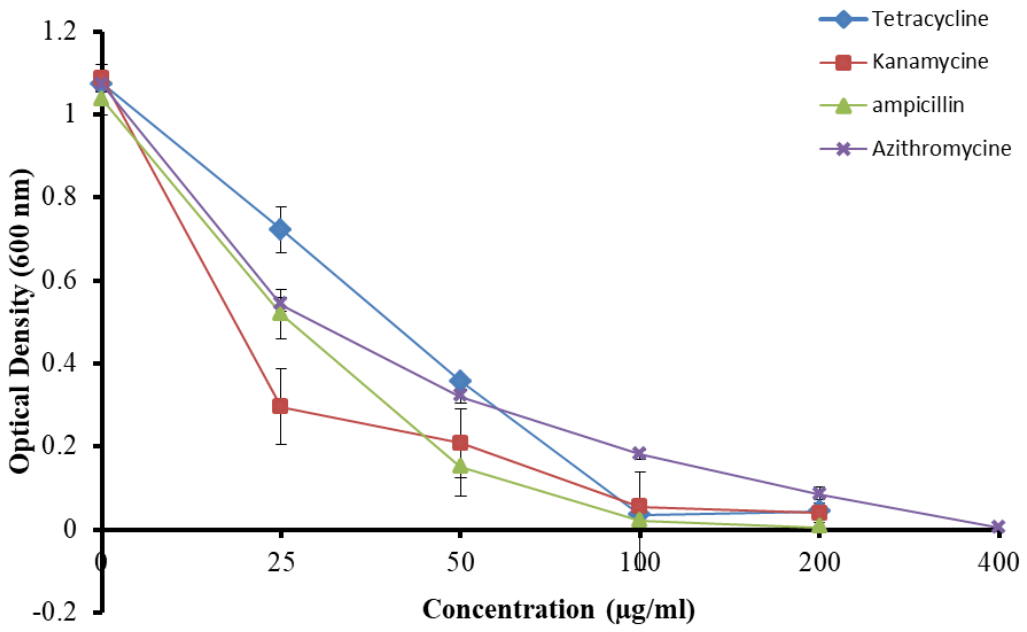

Figure 3. MIC of different antibiotics towards *Acinetobacter* sp. Charmo1. The bacterium growth was monitored after 20 hours incubation at 35 ºC with and without the antibiotics.

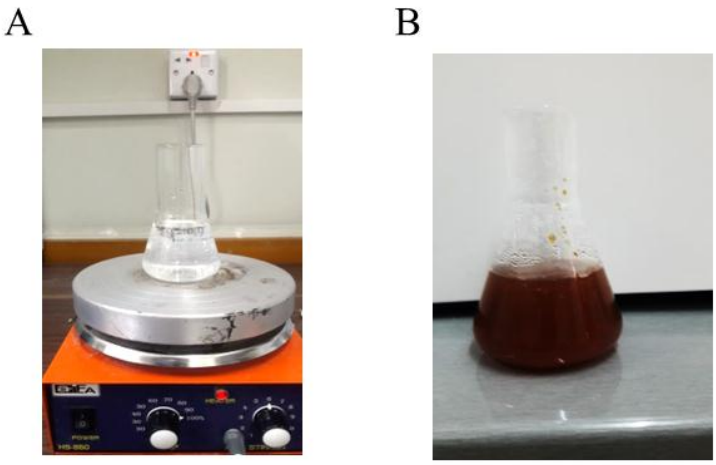

Figure 4. Preparation of the silver nanoparticle. A: Colourless solution of the $AgNO_3$ before (A) and after (B) adding of the aqueous extract of the chamomile flower.

Several studies were reported on the antimicrobial activities of silver nanoparticles (Ag-NPs) [24-27]. However, biologically synthesized Ag-NPs have been considered as a safer alternative of antibiotics to be used to treat bacterial infections [28]. In this study, we used chamomile flowers as a reducing agent to produce Ag-NPs. This is because the plant is local, widely distributed, easily accessible, and safe for handing [13].

Reduction of $AgNO_3$ to Ag^+ and productions of Ag-NPs was initially detected by visual inspection of the reaction in which the color of the solution changes from colorless into the brown due to precipitation of the silver nanoparticles (Figure 4) [18]. Such changes in the color of the solution is due to excitation of the Surface Plasmon Band (SPB) with the nanoparticles [19].

Additionally, the formation of the Ag-NPs was confirmed by UV-visible spectrophotometric analysis of the sample in the range of the 200 nm to 800 nm. The solution was shown the characteristic SPB of Ag-NPs at 450 nm (Figure 5). Kumar *et al.* was found the peak of only chemically synthesized Ag-NPs at 450 nm, whereas, the spectral peak of solutions containing biologically produced Ag-NPs was at 400 nm [18]. The SPB of biologically synthesized Ag-NPs from aqueous extract of *Ocimum gratissimum*, *Myrtus communis* L. and *Lycopersicon esculentum* extract were found at 420 nm, 370 nm, and 410 nm, respectively [26-30]. Such differences in the SPB locations are mainly related to the sizes [31], and also the shapes [32] of the particles.

Characterization of the synthesized particles was done by using XRD and SEM methods. The diffraction patterns of XRD spectrum is shown in Figure 6, confirms the crystalline nature of silver nano-particles which having face-centered cubic crystal structure. Crystal particles of the sample form six 2θ peaks were located at (27.85, 32.35, 38.25, 46.25, 54.75, 57.45). The lattice constant calculated from this pattern was a = 4.086 A° and the data obtained was matched with the database of Joint Committee on Powder Diffraction Standards (JCPDS) file No. 04-0783. The average grain sizes of the silver nanoparticles were calculated based on 2θ peaks using the following Scherrer equation and the average crystalline size (D) that obtained using this formula was 18±3 nm.

$$\text{Crystalize Size D (nm)} = \frac{K\lambda}{\beta \cos \theta}$$

D = crystallite size (nm)

K = 0.9 (Scherrer constant)

λ = 0.15406 nm (wavelength of the X-ray source)

B = FWHM (radians)

θ = Peak position (radians)

In addition, the shape and morphological appearance of the synthesized Ag-NPs were characterized by SEM analysis. The SEM morphology of Ag-NPs showed that they have spherical and irregular flower like geometry (Figure 7).

Antimicrobial activity of the synthesized Ag-NPs was tested against our clinical isolate *Acinetobacter* sp. charmo1. Different concentrations of Ag-NPs ranged between 12.5 µg/ml to 200 µg/ml were tested. Growth of the bacterium was monitored by spectrophotometer at 600 nm and compared to the growth of the bacterium without Ag-NPs. We found that our biosynthesized Ag-NPs has antimicrobial activity with MIC at 50 µg/ml against *Acinetobacter* sp. charmo1 (Figure 8).

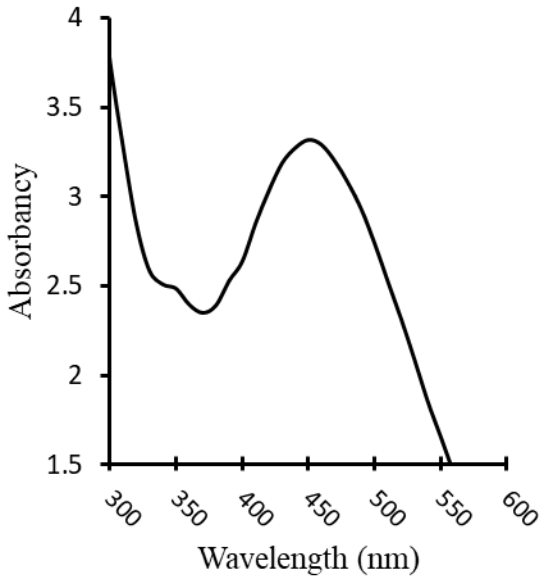

Figure 5. UV-visible spectrophotometric analysis of biologically synthesized Ag-NPs in the *Matricaria chamomilla* flower aqueous extract.

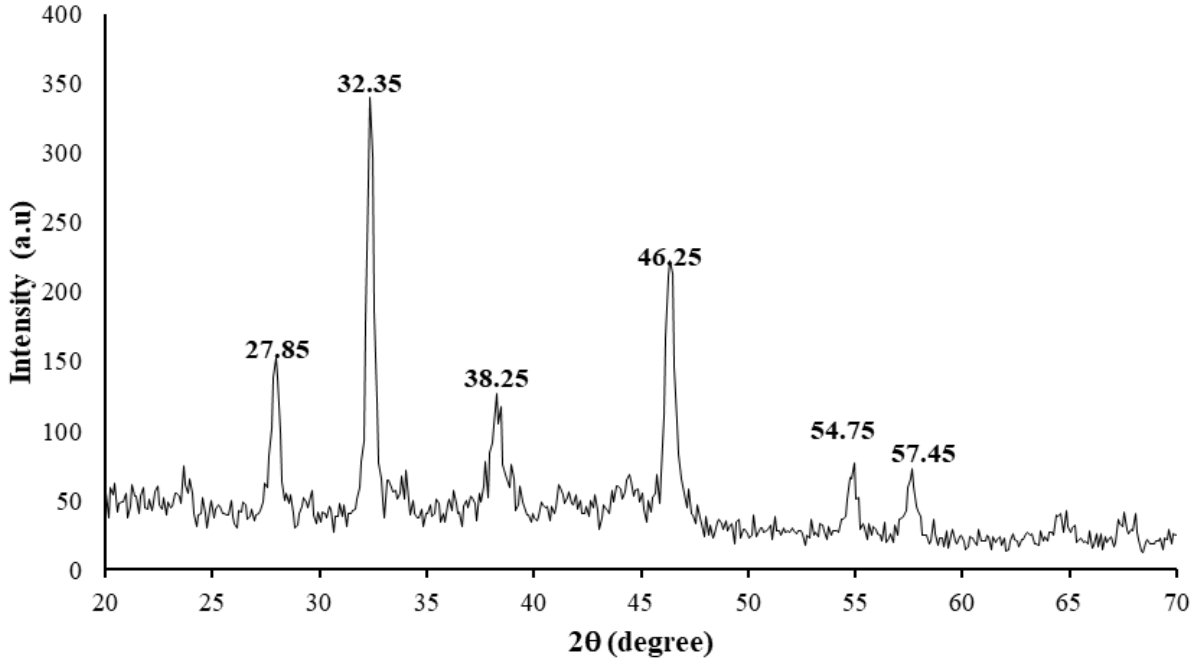

Figure 6. XRD Peaks indices and 2θ positions of biosynthesized Ag-NPs.

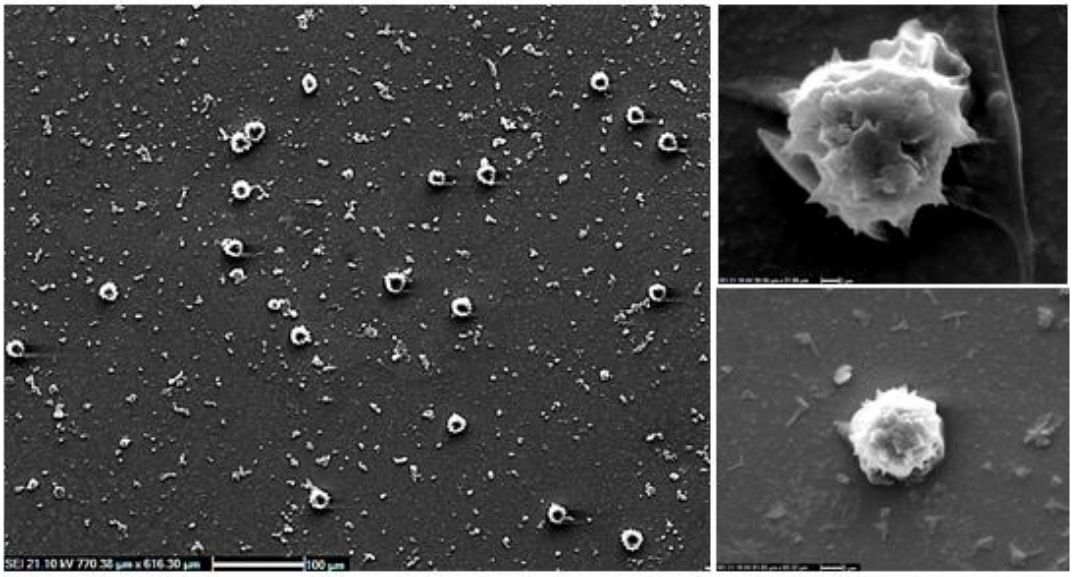

Figure 7. SEM photos of biosynthesized Ag-NPs.

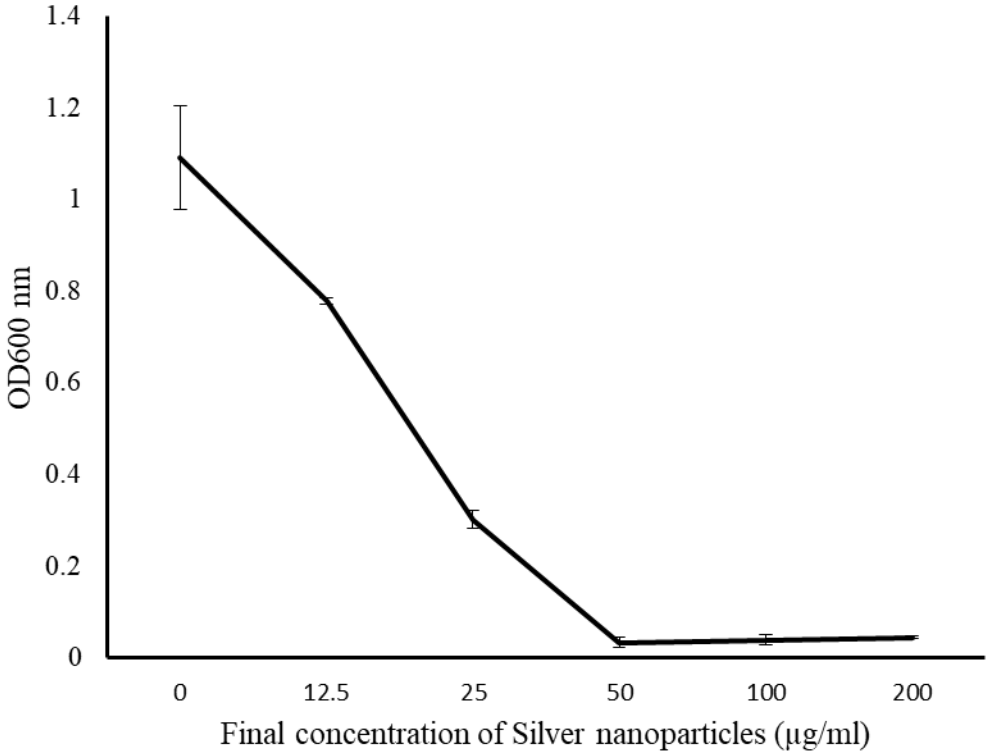

Figure 8. Effect of different concentration of biologically synthesized silver nanoparticles (Ag-NPS) on the growth of *Acinetobacter* sp. charmo1.

DISCUSSION

The problem of multidrug resistant bacteria is among the major healthcare issues worldwide. Therefore, there are several ongoing research to overcome this global problem. Nanoparticle is one of these substances [21]. *Acinetobacter* sp. charmo1 was isolated from a burn patient. The bacterium was shown to have the highest resistant toward azithromycin (400 µg/ml), followed by kanamycin (200 µg/ml), and then ampicillin and tetracycline (100 µg/ml). These differences in the bacterial respond to the antibiotics could be related to the drug mode of action. Azithromycin, kanamycin, tetracycline are all affecting the bacterial growth by inhibiting the protein synthesis [22]. Ampicillin acts on the bacterial growth through impairing the cell wall biosynthesis [23]. Studies have reported the antimicrobial activities of Ag-NPs [24-27] and other nanomaterial [2,28]. However, the biologically synthesized Ag-NPs have been considered as a safer alternative of antibiotics to be used to treat

bacterial infections [29]. In this study, the aqueous extract of the chamomile flowers was used as a reducing agent to produce Ag-NPs. This is because the plant is local, widely distributed, easily accessible, and safe for handing [13]. The UV-visible spectrophotometric analysis of biosynthesized Ag-NPs was shown the SPB of Ag-NPs at 450 nm. The spectral peak of solutions containing biologically produced Ag-NPs was at 400 nm [18]. The SPB of biologically synthesized Ag-NPs from aqueous extract of *Ocimum gratissimum, Myrtus communis* L. and *Lycopersicon esculentum* extract were found at 420, 370, 410 nm [26,30,31], respectively. Such differences in the SPB locations are mainly related to the size and shape of the particles [32,33]. Based on the XRD spectrum (Figure 5), it was confirmed the sample is Ag-NPs with average size of 18±3 nm. The SEM study was also found that the shape of the particles is flower shape (Figure 6). Benakashani *et al.* were also reported the biosynthesized Ag-NPs from *Capparis spinosa* leaf extract have spherical shape with an average particle size 20 nm [34]. The antimicrobial activity of the synthesized Ag-NPs was tested. Different concentrations of the Ag-NPs were tested and relatd MIC of the particles were found 50 µg/ml against *Acinetobacter* sp. charmo1 (Figure 8). Similarly, MIC of biosynthesized Ag-NPs from *Lycopersicon esculentum* against *Escherichia coli* was also reported 50 µg/ml [31], whereas Ag-NPs produced from *Murraya koenigii* showed the MIC against methicillin-sensitive *Staphylococcus aureus* (MSSA) 32 µg/ml [35]. Shape and size of the particles affect the antimicrobial activity of the Ag-NPs [11,36,37]. It is also suggested that Ag-NPs have strong antibacterial activity against Gram negative in comparison to the Gram positive bacteria [34]. Moreover, the smaller nanoparticles size have a stronger antimicrobial activity [37]. It showed that the spherical shap of Ag-NPs has stronger antimicrobial activity against *E. coli, S. aureus* and *P. aeruginosa* than the triangle shape [38].

CONCLUSION

In conclusion, the bio-synthesized Ag-NPs using aqueous extract of chamomile flowers have antimicrobial activity against the clinically isolated *Acinetobacter* spp. This method can provide an eco-friendly and cost efficient approach to produce Ag-NPs. In addition, the potential antimicrobial activity of the Ag-NPs should be further investigated for

controlling the problem of the antibiotic resistant bacteria.

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