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Research Article

Biosynthesized of Silver Nanoparticles from *Myrtus communis* Leaves and Investigates of their Antimicrobial Activities Against a Clinical Isolate of *Acinetobacter baumannii*

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Abstract

Multidrug resistant (MDR) bacteria are one of the major concerns of the healthcare system in the twenty-first century. Silver nanoparticles (Ag-NPs) have a great potential to be used as new antimicrobials agents to overcome this global issue. In this study, Ag-NPs were biologically synthesized using the aqueous extract of myrtus (*Myrtus communis*) leaves as a reducing agent. The biosynthesized of Ag-NPs were firstly characterized using UV-visible spectroscopy, X-ray diffractometry (XRD), and Scanning Electron Microscopy (SEM). The absorbance maximum of Ag-NPs was found at 410 nm, and the particles have spherical and/or irregular shapes with an average size of 19 ± 3 nm. The antibacterial activity of the Ag-NPs was then tested against a clinical strain of *Acinetobacter baumannii*, isolated from a hospitalized burnt patient in the city of Sulaimani-Iraq. Interestingly, the synthesized Ag-NPs were able to inhibit the growth of isolate at the concentration of 0.2 mg/ml. The results of this study suggest a potential antimicrobial activity of biologically synthesized Ag-NPs against MDR bacteria.

Keywords: *Acinetobacter baumannii*; Silver Nanoparticles; Antibiotic Resistance; Nosocomial Infections

Introduction

Antibacterial resistance is considered one of the main challenges of the 21st century to the general public health by the World Health Organization (WHO) [1]. Only in the USA, nearly three million people are infected with antibiotic-resistant bacteria every year, of which 35,000 die from the infections [2]. An opportunistic Gram-negative *Acinetobacter baumannii* is labelled as a "red alert" human pathogen for the ability to resist large classes of antibacterial agents [3-5]. In 2017, WHO top listed this bacterium as a critical pathogen to be tackled globally [6]. *A. baumannii* can survive in the hospital environment on the surface of different machines for a long period; making it a common source of nosocomial infections [7,8]. Furthermore, like other pathogenic bacteria [9,10], *A. baumannii* can infect different parts of the human body, especially in the immunocompromised patients, such as skin, respiratory tract, and urinary tract [7,11,12]. The rate of nosocomial infections caused by *A. baumannii* is estimated to be 2% in Europe and the USA, while it is about 4% in Asia and the Middle East [13]. The mechanism of antibiotic resistance in *A. baumannii* is due to enzymatic degradation of drugs, target drug modifications, multidrug efflux pumps, and permeability defects [12,14].

Since the number of multi-drug resistance bacteria is increasing, researchers have been simultaneously searching for an alternative therapy of bacterial infections. With recent advances in nanotechnology, distinct types of nanoparticles (NPs) with broader antimicrobial activities can be produced [15-17]. Using plants to produce nanoparticles have many advantageous since they are

environmentally friendly, with no toxic by-products, and also the produced nanoparticles have a greater stability [16]. In addition, plants are widely distributed, easily available, safe to handle and contain a variety range of metabolites [18,19]. Biosynthesize of Ag-NPs using the aqueous extract of different plants as reducing agents have been reported such as *Matricaria chamomilla*, *Ocimum Sanctum, Petroselinum crispum*, *Murraya koenigii*, *Coriandrum Sativum*, *Lantana camara*, *Moringa oleifera*, *Catharanthus roseus* and *Eucalyptus chapmaniana* [20-22]. The aims of this study are to biosynthesize Ag-NPs from the aqueous extract of the myrtus leaves and investigate its antimicrobial activity against a clinical isolate of *A. baumannii*.

Materials and Methods

Isolation and Identification of the bacterial strain

A clinical specimen (burn swabs) was collected from a female patient, admitted to the burn and plastic surgery unit at emergency hospital in Sulaimani city/Iraq, in February 2019. The isolate was cultured on MacConkey agar and incubated for overnight at 37°C. The non-lactose fermenting colonies were sub-cultured and incubated for another overnight. Polymerase chain reaction (PCR) was used to identify the bacterial strain.

Identification of the bacterial strain

Prlesto™ Mini gDNA Bacteria Kit (Geneaid) was used to extract the total bacterial genome. The PCR reaction of total 50 µl was prepared containing Prime Taq Premix (2x) (GeNet Bio), 5 µl of the genomic DNA extract and 0.5 µM of 16S rRNA primers (F: 5′-AGAGTTTGATYMTGGCTCAG-3′, R: 5′-ACGGYTACCTTGTTACGACTT-3′) [23]. The PCR conditions were 95°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing), and 72°C for 2 minutes (extension). PCR products were purified and sequenced by Sanger DNA sequencing at Macrogen - Republic of Korea. The Chromas software v1.0 (Technelysium) was used to analyse and assemble the sequences.

Construction of the phylogenetic trees

The taxonomical identification of the clinical isolate was carried out using previously described method [24]. Briefly, the 16S rRNA sequence of the strain was aligned with the closely related species using ClustalX 2.1 [25] and then MEGA 7 programs [26]. The Neighbor-joining method (bootstrapped with 1000 replicate runs) was used to construct the phylogenetic tree in the MEGA 7 tree-building program.

Preparation of the plants aqueous extract

The leaves of the myrtus plant were used to prepare Ag-NPs according to previously described method [20]. Briefly, the fresh leaves of the plant were collected in Shwan region of Chamchamal, Kurdistan region- Iraq (Figure 1A). The leaves were washed, cut into small pieces and air dried for 24 hours. Dried leaves (~ 20 grams) were transferred into conical flask (500 ml) containing 100 ml distilled water and then boiled for 10 minutes. The leaves-water aqueous extract was filtered through Whatman filter paper. The flow through was collected in a 50 ml falcon tube and stored at 4ºC (Figure 1B).

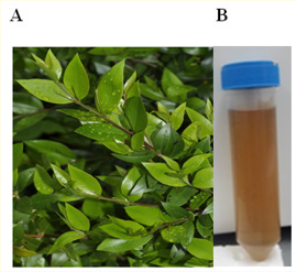

Figure 1: Collection of the leaves of myrtus plants and its extraction. A: leaves of the myrtus (*Myrtus communis*) plant. B: Aqueous extract of the myrtus leaves.

Biosynthesize of Ag-NPs

To prepare Ag-NPs, 1 ml of myrtus leaves aqueous extract was added to 50 ml of 1 mM $AgNO_3$ in a 100 ml conical flask, using previously described protocols [20,22]. In brief, the solution was heated at 75 °C on a hot plate for 25 minutes and stirred with a magnetic bar. Formation of nanoparticles was detected through the colour changes of the solution from colourless to brown [27].

Characterization of the Ag-NPs

The UV-visible spectroscopy, X-ray diffractometry (XRD), and Scanning Electron Microscopy (SEM) were used to characterized the synthesized Ag-NPs.

Spectrophotometric characterization of the reduced Ag-NPs was performed with UviLine 9400 (SI Analytics), using 1.0 ml of the reduced Ag-NPs and recording the absorbance in the range of 200 nm to 800 wavelengths. Furthermore, an aliquot (10 ml) of

the Ag-NPs was dried out in a petri dish under the fume hood for 4 days, and then it was used for SEM and XRD analysis. SEM equipped with CamScan 3200 LV using Caesium™ version 6.1.10 was used to analyze the morphology of the nanoparticles. Additionally, XRD analysis was carried out in X 'Pert Pro diffractometer (PanAnalytical, Almelo, The 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 average grain size of the Ag-NPs was calculated based on 2θ peaks using the following Scherrer equation:

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

D = Crystallite size (nm)

K = 0.9 (Scherrer constant)

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

β = FWHM (radians)

θ = Peak position (radians).

Antimicrobial susceptibility test

The microdilution method was used to determine the MIC of different antibiotics and the biosynthesized Ag-NPs as well. The antibiotics which used in this study were ampicillin, kanamycin, tetracycline, and azithromycin. Stock solutions of the antibiotics were prepared according to Clinical and Laboratory Standards Institute protocol (CLSI) [28,29]. The cells were grown overnight in Mueller-Hinton broth, then 20 µl of the cells (OD_{600} 0.2 nm) was added to the 96- wells containing 160 µl of the medium and 20 µl of different concentrations of the antibiotics or Ag-NPs using different concentrations (0.0125, 0.025, 0.05, 0.1 and 0.2 mg/ml). The cultured microplate was incubated at 35ºC for 20 hours. The optical density (OD) of the bacterial growth was recorded by a plate reader (BioTek-ELx800) at 600 nm.

Data analysis

All assays were performed in at least three biological replicates. P-value (Un-paired t-test) was done by using GraphPad Prism 8.0.1 Software Inc., La Jolla, CA, USA. Statistical significance was defined when the P-value was less than 0.01.

Results and Discussion

A clinical strain, isolated from a hospitalized female burnt patient, was firstly identified by sequencing of the ~1.6 kbp 16S rRNA gene. BLAST searching at National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) of the sequenced gene suggested that the bacterium belongs to the genus of the *Acinetobacter* sp. Moreover, the phylogenetic tree of the bacterial 16S rRNA sequence confirmed that the isolate is *Acinetobacter baumannii* (Figure 2). The gene sequence of the bacterium was uploaded to the NCBI with the accession number (MN326095.1) and the strain has been named as *Acinetobacter baumannii* charmo4.

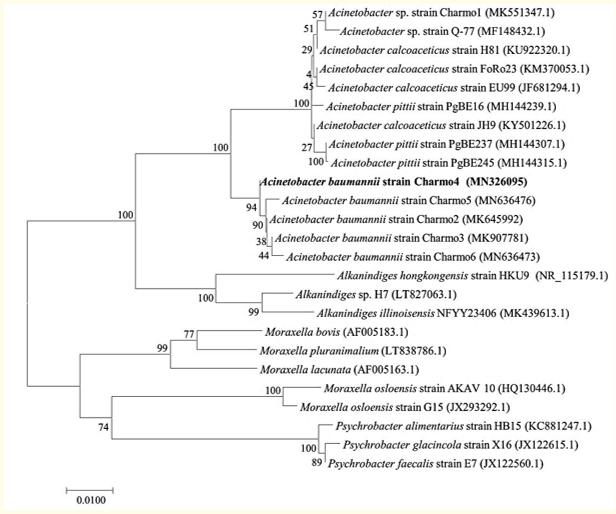

Figure 2: Phylogenetic tree of *Acinetobacter baumannii* charmo4 based on the nucleotide sequence of the 16s rRNA gene. DNA sequences were aligned using ClustalX program and the tree was created using the neighbor-joining method, bootstrapped with 1,000 replicate runs, by the MEGA 7 tree-building program.

To check the antibiotic resistance profile of the isolate, the charmo4 strain was subjected to MIC experiment (Figure 3). The result showed that the strain has the highest resistance to both ampicillin, and kanamycin, and no significant reduction in the bacterial growth was detected using 102.4 mg/ml of either antibiotic. The concentration of 102.4 mg/ml of azithromycin was shown to inhibit the bacteria growth by 60%. Tetracycline was the only effective antibiotic in our experimental setting, and the total bacterial inhibition was detected at the concentration of 0.8 mg/ml (Figure 3). The resistance of the strain kanamycin, ampicillin, and azithromycin could be due to the previously frequent use of such antibiotics in hospitals and local pharmacies that made the bacterium de-

velop resistance mechanisms against the antibiotics. These results suggest that the *Acinetobacter baumannii* charmo4 is a multi-drug resistant strain.

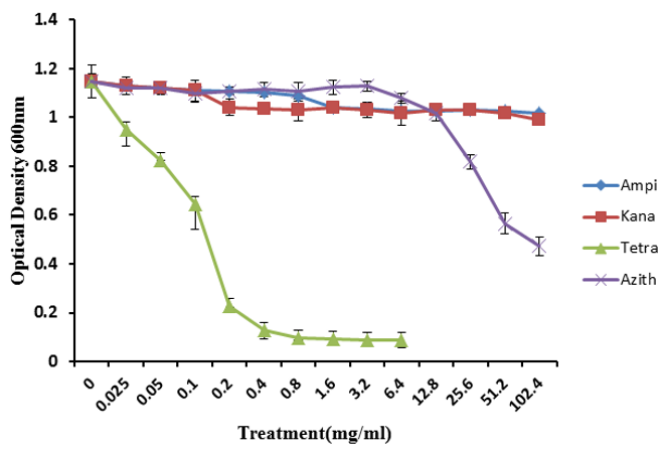

Figure 3: MIC of different antibiotics for *Acinetobacter baumannii* charmo4. The bacterial growth was monitored for 20 hours of incubation at 35ºC with and without the antibiotics. The data are means from three replicates ± standard deviations.

Nowadays, biological methods to synthesize nanoparticles through using microorganisms bioproducts (bacteria, algae and fungi) and plant extracts are compatible with the green chemistry principles because they are safe to handle and contain a wide range of secondary metabolites [18,19].

In this study, we used myrtus leaves as a reducing agent to produce Ag-NPs. This is because the plant is widely locally distributed [30]. The colour change of the solution from colourless to brown was used as indication to produced Ag-NPs (Figure 4) [20]. This changes in the color of the solution is due to excitation of the Surface Plasmon Band (SPB) with the nanoparticles [27].

The production of the Ag-NPs was further confirmed by UV-visible spectrophotometric analysis of the sample in the range of the 200 nm to 800 nm. The solution showed the characteristic SPB of Ag-NPs at 410 nm (Figure 5). Previously, the peak of chemically synthesized Ag-NPs is reported to be at 450 nm, however, the peak of solutions containing biologically produced Ag-NPs was found at 400 nm [20]. The SPB of biosynthesized Ag-NPs from aqueous

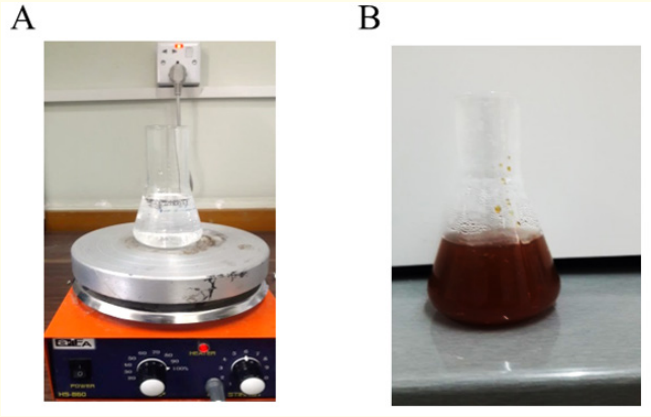

Figure 4: Preparation of the silver nanoparticle. The Colourless solution of AgNO3 before (A) and after (B) adding of the aqueous extract of the myrtus leaves.

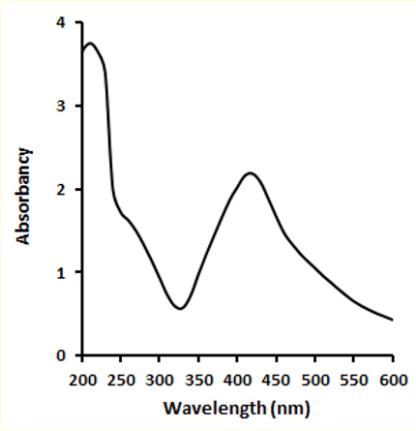

Figure 5: UV-visible spectrophotometric analysis of biologically synthesized Ag-NPs in the aqueous extract of *Myrtus communis* leaves.

extracts of *Ocimum gratissimum*, *Myrtus communis*, *Lycopersicon esculentum* and *Matricaria chamomilla* flowers were found at 420 nm [31], 370 nm [32], and 410 nm [33], 450nm [22] respectively. These differences in the SPB can be related to the sizes [34], and the shapes [35] of the nanoparticles.

Moreover, both XRD and SEM were used to characterize biosynthesized Ag-NPs. The diffraction pattern of XRD spectrum confirms the crystalline nature of silver nanoparticles that have face-

centered cubic crystal structure (Figure 6). Crystal particles of the sample form seven 2θ peaks were located at (32.25, 38.14, 44.27, 46.27, 27.84, 54.75 and 57.45). The lattice constant calculated from the pattern was a = 4.086 A°. Based on the Scherrer equation, the average crystalline size (D) of the synthesized Ag-NPs was found to be 19 ± 3 nm.

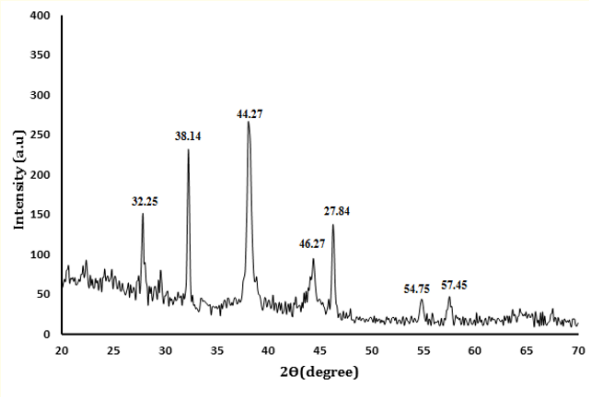

Figure 6: XRD Peaks indices and 2θ positions of biosynthesized Ag-NPs. The shape and morphological appearance of the synthesized Ag-NPs were characterized by using SEM analysis.

Furthermore, the shaped of the biosynthesized particles were appeared to be spherical and irregular shapes by using Scanning electron microscopy (Figure 7).

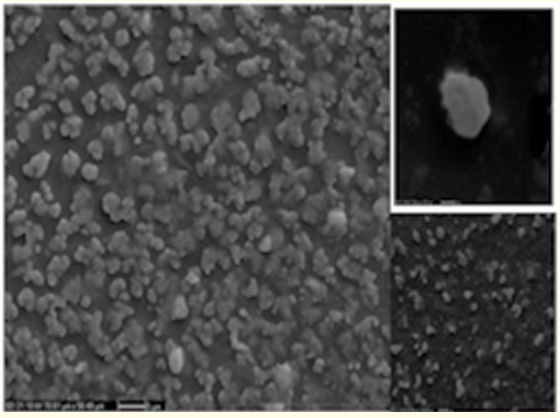

Figure 7: The Scanning electron microscopy for the synthesized Ag-NPs showed that they have spherical and irregular shapes.

The antibacterial activity of produced Ag-NPs was then tested against the clinical isolate of *A. baumannii* charmo4 (Figure 8). The concentrations ranged between0.0125 to 0.2 mg/ml of Ag-NPs was tested. Bacterial growth inhibition was investigated using spectrophotometer method at OD 600 nm and compared to the growth of the bacterium without Ag-NPs. The results showed that the biosynthesized Ag-NPs has antibacterial activity and MIC of 0.2 mg/ml against *Acinetobacter baumannii* charmo4 (Figure 8). Therefore, it appears that the growth of this isolate can be inhibited by higher MIC of Ag-NPs compared to previous studies [22,33,36].

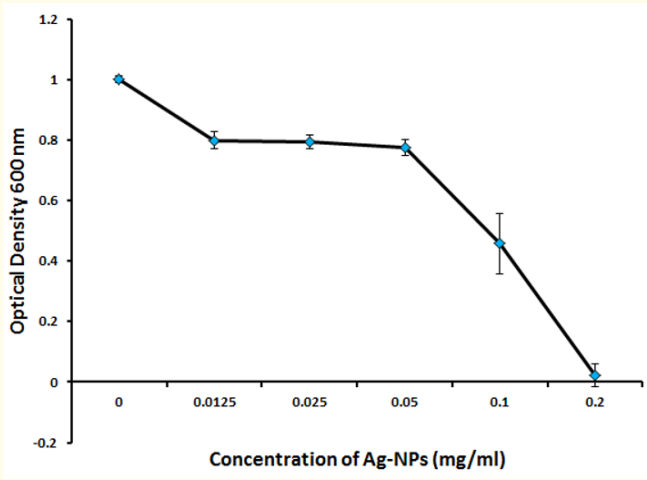

Figure 8: Effect of different concentration of biologically synthesized Ag-NPS on the growth of *Acinetobacter baumannii* charmo4. Data shown are means of data from three replicates ±.

Previously, the MIC of biosynthesized Ag-NPs from *Lycopersicon esculentum* against *Escherichia coli* was found at 50 µg/ml [33] and MIC of Ag-NPs produced from *Murraya koenigii* against methicillin-sensitive *Staphylococcus aureus* was at 32 µg/ml [36]. In addition, The MIC of Ag-NPs by using aqueous extract of *Matricaria chamomilla* flowers against a clinical isolated *A.baumannii* charmo1 was 50 µg/ml [22]. It has been shown that the shape and the size of particles can also affect the efficiency of antimicrobial activity of the Ag-NPs [37-39]. With reducing in the particle size, the antimicrobial activities of the parties are increased [39]. It was also shown that the spherical shaped Ag-NPs have a stronger antimicrobial activity against *E. coli*, *S. aureus* and *P. aeruginosa* than the triangle shape [40]. The multi antibiotic resistance property of the bacterial strains may also affect the MIC range of Ag-NPs.

Conclusion

In this study, we have prepared biologically synthesized Ag-NPs by using aqueous extract of myrtus leaves. The antibacterial activity of the synthesized Ag-NPs was tested against an antibiotic resistant clinical isolate of *Acinetobacter baumannii* charmo4. The result showed that the total bacterial strain inhibition by the synthesized Ag-NPs at the concentration of 0.2 mg/ml. The potential antimicrobial activity of the Ag-NPs should be further investigated so as to be used as an alternative of antibiotic to treat infectious diseases, caused by antibiotic resistant bacteria.

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Conflicts of Interest

None.

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