Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed.nahrainuniv.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2024; Vol. 22(1)

Biosynthesis of Silver Nanoparticles by *Pseudomonas aeruginosa* and Evaluation of Their Antibacterial Activity and Cytotoxicity Assay Properties

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Abstract

Background Utilizing *Pseudomonas aeruginosa* (*P. aeruginosa*) to biosynthesize silver nanoparticles (AgNPs). Antibiotic resistance poses a formidable challenge, particularly against multidrug-resistant (MDR) bacteria. The employment of supernatant as a cost-effective and straightforward approach enhances the efficacy of AgNPs biosynthesis.

Objective To explore the biosynthesis of AgNPs by *P. aeruginosa* with the aim of achieving cost-effective and environmentally friendly production methods while ensuring novel morphological characteristics of AgNPs furthermore evaluated of antimicrobial and toxicity.

Methods Preparation AgNPs by biological method (Green synthesis). Ultraviolet-visible spectrophotometer (UV–Vis), energy dispersive of X-ray spectroscopy (EDX), scanning electron microscopy (SEM), atomic force microscopic (AFM), X-ray diffraction (XRD) Zeta potential analysis. These techniques were applied for physical characterization. Disc diffusion assay and minimum inhibitory concentration (MIC) (16 µg/ml), were evaluated against different strain of Gram negative and positive bacteria, and study cytotoxicity assay normal human dermal fibroblasts (HdFn) and cell line A549.

Results The UV–Vis showed the peak absorption at 421 nm wavelength, SEM showed nanoparticles were homogeneous and spherical shape with average size 24.5 nm and energy dispersive EDX confirmed the purity of the synthesized nanoparticle, where it was observed that the AgNPs showed strong antibacterial activity against MDR bacteria and the best MIC was 32 µg/ml concentration and showed the best efficacy against resistant strains of Gram-positive and negative bacteria Furthermore, AgNPs did not show any cytotoxic effects on carcinoma cell line A549.

Conclusion The biological method used in this research is suitable to prepare AgNPs according to the size of particle and homogeny of the product and show strong antimicrobial property against MDR pathogens without having toxicity effect on cell line tissue.

Keywords Silver nanoparticles, *Pseudomonas aeruginosa*, antibacterial activity, cytotoxicity, biomedical applications

Citation Huseen DA, Ahmed ME, Hamid MK. Biosynthesis of silver nanoparticles by *Pseudomonas aeruginosa* and evaluation of their antibacterial activity and cytotoxicity assay properties. Iraqi JMS. 2024; 22(1): 154-163. doi: 10.22578/IJMS.22.1.17

List of abbreviations: AgNPs = Silver nanoparticles, EDS = Energy-dispersive X-ray spectroscopy, MDR = Multidrug resistant, MIC = Minimal concentration, MTT = Machine tool technologies, TEM = Transmission electron microscopy, UV–Vis = Ultraviolet–visible spectroscopy, VITEK-2 system = It is a fully automated system that performs bacterial identification and antibiotic susceptibility testing, WDA = Well diffusion assay, ZP = Zeta potential

Introduction

Nanotechnology is the creation of materials and devices through the control of matter at the atomic, molecular, and supramolecular (nanoscale)

level. This is the creation of large-scale new materials using very small material particles to better understand the differences between different scales [1]. About 100,000 tons of antibiotics for intended illnesses are generated globally each year. Misuse, which is this quantity of antibiotics has led for the emergence of many medicines' impedance between disease-causing strain, especially bacteria [2].

Most of these applications benefit from the unique physicochemical properties exhibited by silver (Ag) with nanoscale particle sizes. The dimensional properties that determine the design and application modes of these nanomaterial [3]. An urgent need to improve the applicability of silver nanoparticles (AgNPs) in new advanced technologies in the field of plasmonic devices and the construction of nano electronic devices [4] and the development of new applications for medical devices and the development of biomedical therapies for related diseases such as certain cancers, viral infections and pathogens [5].

Thus, in recent studies, a mixture of different magnetic materials added to silver–magnetite nanoparticles was studied to give a new material with unique properties in many technological applications [6]. These highly crystalline of nanoparticles showed stable and near-uniform shape. The comparison antibacterial activity of AgNPs by physicals methods permanent magnetic fields were beast than those from control [7].

Methods

Pseudomonas aeruginosa (*P. aeruginosa*) isolated from many patients suffer from wound infection were collected from burn and wound, all sample identified by biochemical method and preservation in 20% glycerol were obtained from Department of Biology, College of Science, Baghdad University, cultured in broth and cultured at 37°C for 24 hr to ban a bacterial supernatant. The bacterial solution was transferred to a tube of 10 ml and centrifuged (6000 rpm, 15 min). Transfer the supernatant to another tube and discard the compressed precipitate at the lowest of the tube [8].

Biosynthesis of AgNPs

Biological methods have received extensive attention for nanoparticle biosynthesis (green synthesis) by using bacteria [9]. Isolated strain of *P. aeruginosa* were freshly inoculated in 100 ml nutrient broth using a 250 ml Erlenmeyer flask, which put in an incubator at 37°C for 1-3 days, then centrifuge broth at 8000 rpm. For 10 min, the cell pellet was discarded and the supernatant was collected for use in silver nanoparticle biosynthesis. The supernatant (100 ml) was placed in a clean 250 mL Erlenmeyer flask, (100 ml) of (1 mM) silver nitrate was added, and the mixture was incubated at 37°C for 48 hr. Color changes were visually observed and photographed in the dark to avoid $AgNO_3$ oxidation of the biosynthesized SNPs [10] as shown in figure (1).

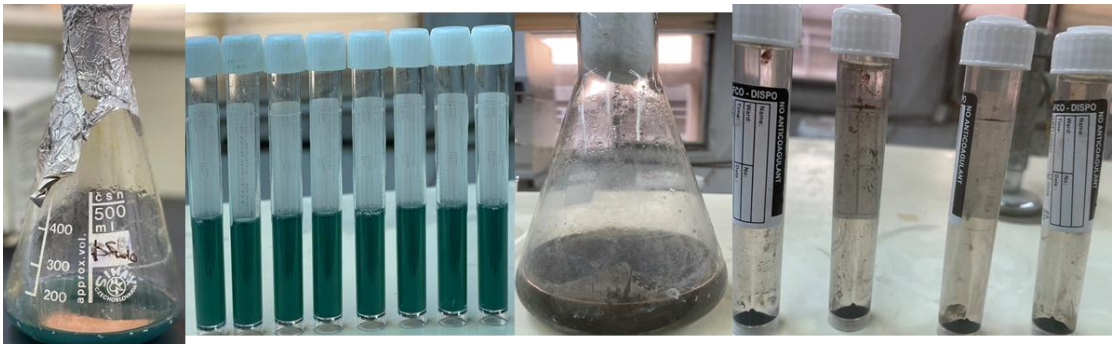


Figure 1. Steps of Biosynthesis of AgNPs

The characterization of biosynthesized AgNPs

UV–Vis spectrophotometer

A Lambda 45 UV/VIS spectrophotometer was used to search the optical properties of AgNPs; wavelength range from 200 to 700 nm [(11)].

Zeta potential (ZP)

ZP determination is a significant characterization technique of nanocrystals to estimate the surface charge, which can be employed for understanding the physical stability of Nano suspensions. ZP (positive or negative) values have a significant role in stabilizing particle suspension. This is attributed to the electrostatic repulsion between particles with the same electric charge that cause segregation of the particles [(12)].

Field emission scanning electron microscopy (FESEM)

Scanning electron microscopy (SEM) offers the topographical and elemental knowledge of NPs with an almost infinite field depth for useful amplifications. In the evaluation of elementary structure, grain size, roughness of the surface, porosity, distribution of dimensions, homogeneity, inter-metal distribution and diffusion of nanoparticles.

Energy-dispersive of X-ray spectroscopy (EDS)

EDS investigates surface analysis and elemental characterization of the sample. Basic principle involves the study of the emitted X-rays of different energies coming from the sample when a beam of electron strikes its elements. The amount and composition of metal nanoparticles can be easily identified from the surface of the given sample.

Multidrug-resistant (MDR) bacteria

Detection of Gram negative MDR bacteria such as *P. aeruginosa or Klebsiella*) and Gram positive (*Staphylococcus aureus* (*S. aureus*) or *Streptococcus pyogenes*); all strain isolation from patient's urine suffer wound infection was used to select antibacterial action of AgNPs. This was the lineages acquired from Laboratory of Microbiology, Dept. of Biology, College of Science, University of Baghdad. All strain identification was done by ViteK-2 system.

Minimal concentration (MIC)

To assess the MIC, various concentrations (64, 32, 16, 8, 4) µg/ml of AgNPs extract were tested against four strains, including Gram-positive and Gram-negative [(13)]. The tubes containing the bacterial cultures were incubated at a temperature of 37°C for a duration of 24 hours in Muller Hinton Broth (MHB), the respective concentrations of AgNPs extract were containing within each of the six tubes. These concentrations were obtained from a 0.1 M of Matel nanoparticle (MNP) that it has maximum antimicrobial activity within the agar spread examination. Later, incubating the environment at 37°C for 24 hr; the tube had been observed for turbidity as increase and non-turbidity as no increase. The MIC value was exponent as the minimum sample concentration that represented a clear liquid without turbidity increase.

Well diffusion assay

Antibacterial activity was selected using a well diffusion examination [(14)]. Bacterial selection MHB cultures were freshly prepared overnight for each assay and the inoculum was clear from the surface of the coagulated medium. After drying the medium for 10 min, various concentrations (64, 32, 16, 8, 4) µg/mL of biosynthesized this AgNPs were added to the discs [(15)].

Cytotoxic by machine tool technologies (MTT) assay

Evaluation of the cytotoxic effect of three chemical concentrations of AgNPs on normal HdFn and A375 cells in 96-well plates after 24 hours of AgNPs. To determine the formula, IC50 values: cell viability = Ab S /Ab C — 100

and absorption to 540 nm were measured by usage of Omega micro lamella reader (BMGLABTECH®-FLUO star, Ortenberg, Germany) [16].

Results

All the chosen bacterial strains were MDR. The VITEK-2 system was used to confirm antibiotic susceptibility testing.

Biosynthesis of AgNPs

It could be seen by changing the color of the reaction by the biosynthesis of AgNPs indicator that the bio-formation of AgNPs from the mixture of $AgNO_3$ using microbial supernatants was monitored in the in the biosynthesis process. In contrast, the transition of the color from white to brown in the presence of *P. aeruginosa* supernatants did not affect the reduction of Ag ions.

UV–Vis spectral analysis

The UV-Vis spectrophotometer was the step to characterize the biosynthesized AgNPs. The results confirmed that biosynthesized AgNPs exhibited a maximum peak at (421 nm) as shown in figure (2).

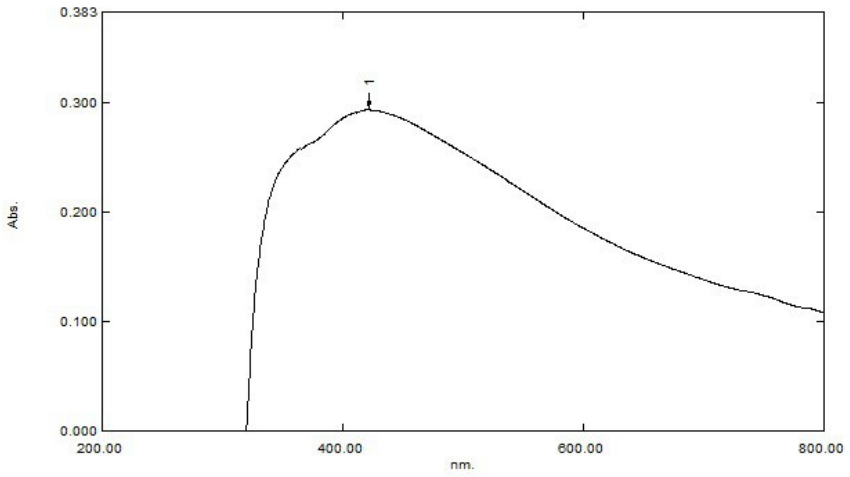


Figure 2. UV–Vis spectrophotometry of biosynthesized AgNPs

X-ray Diffraction (XRD)

The XRD spectrum of the AgNPs powder is illustrated in figure (3), and the detection of eminent peaks corresponding to the diffraction peaks of AgNPs were confirmed crystal structures of biosynthetic AgNPs by standard and by using Debay Scherrer equation ($D = K\lambda/\beta cos\theta$) have been found the nanoparticle size, which equals about 36.6 nm.

Atomic force microscopic (AFM) analysis

AFM was used as a confirmatory technique to characterize the biosynthesis of AgNPs by determining their 2D and 3D morphologies as well as their average diameters. The results obtained in this study confirmed that the AgNPs biosynthesized via *P. aeruginosa* had an average diameter of 56.76 nm as shown in figure (4).

TEM of AgNPs

The TEM images of Ag-NPS were spherical in shape as shown in figure (5). The nanoparticle diameter was measured to be (24.5-30) nm.

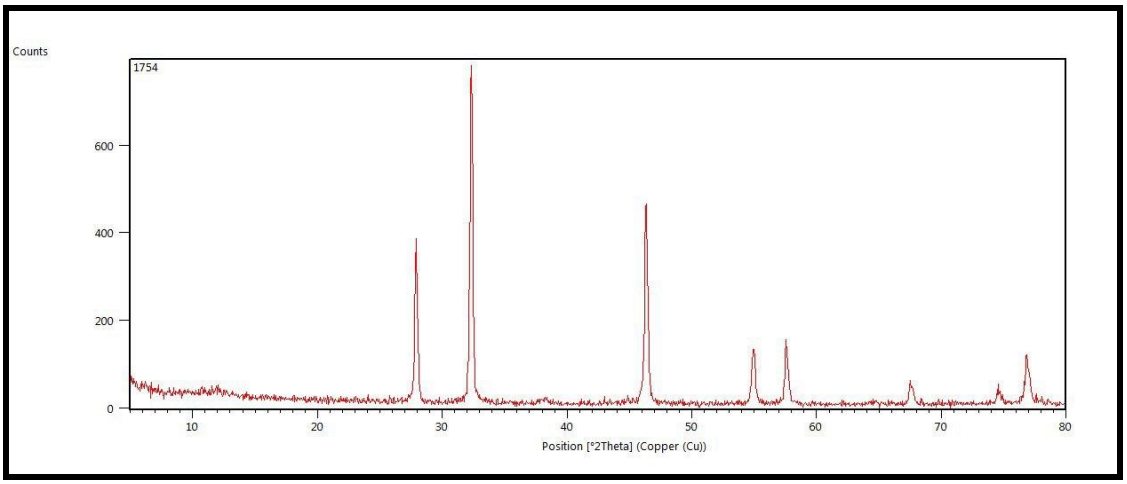
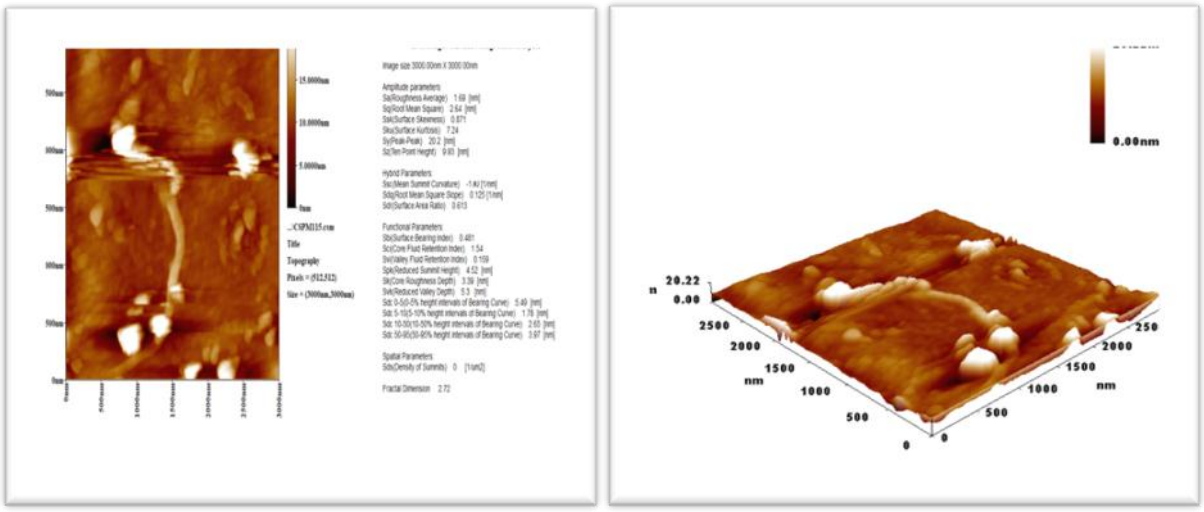


Figure 3. XRD analysis of synthesized AgNPs

Sample: sample Name Code sample Code
Line No: line No Grain No.: 2409
Instrument: SPM Date:2022.12.22



A B

Avg.Diameter:56.76 nm <=10%Diameter:20.00nm
<=50%Diameter:45.00nm <=90% Diameter:100.00nm

Figure 4. (A) 2D-AFM of AgNPs, (B) 3D-AFM of AgNPs

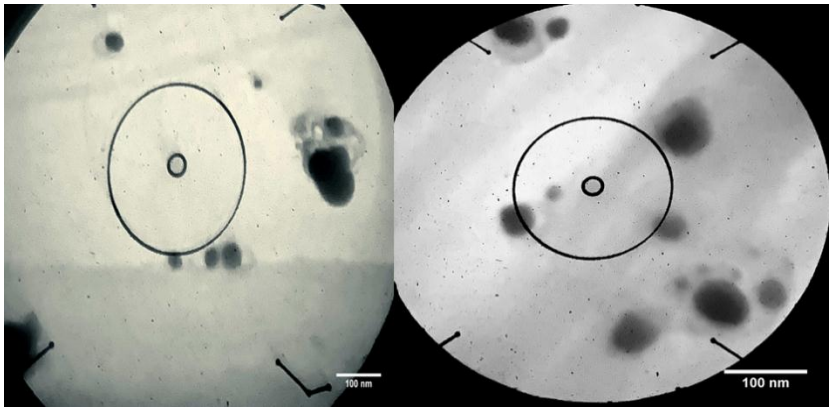


Figure 5. TEM Images of polydisperse AgNPs

EDS of AgNPs

The AgNPs components were examined using EDS analysis, which endorsed the elemental compositions and the output result of this technique is shown in the figure (6). EDS search endorsed the primary compositions and detect AgNPs and confirm the purity of synthesis nanoparticles, and EDS pattern of the AgNPs showed high emission energy at 3 keV, the presence of peaks before 5kev shows the presence of a pure silver metal ion.

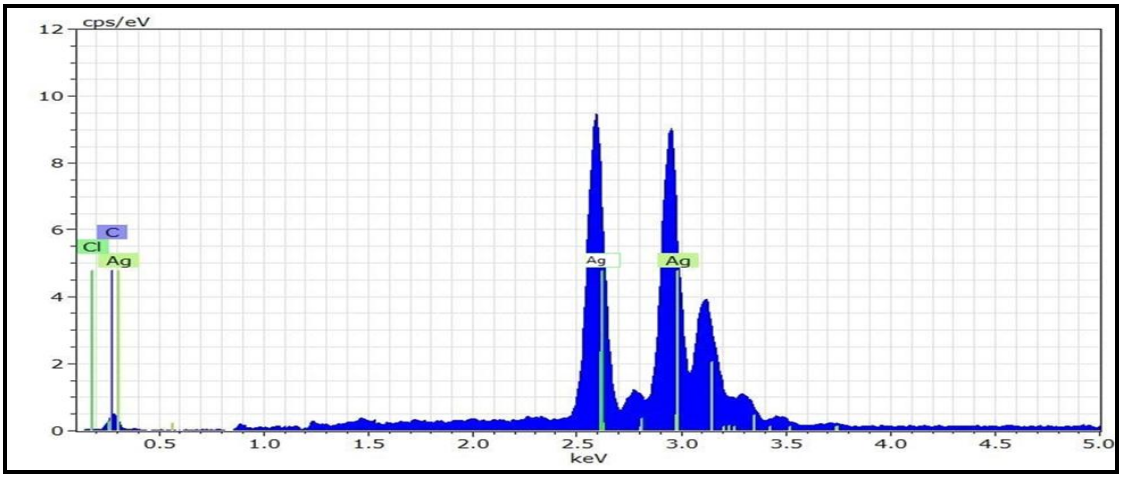


Figure 6. EDS analysis of synthesized AgNPs

FESEM of AgNPs

In FESEM the sample of Ag NPs found an irregular shape and average size of about 23 nm as shown in Figure (7).

Zeta Potential (ZP)

The ZP was found to be -29.30 mv for AgNPs as shown in figure (8). ZP describes the stability of nanoformulations. Particles smaller than 20 nm have higher mobility, less light scattering, and a narrower concentration spectrum. The minimum concentration used to measure particle size affects the ZP test because it affects the particle surface charge.

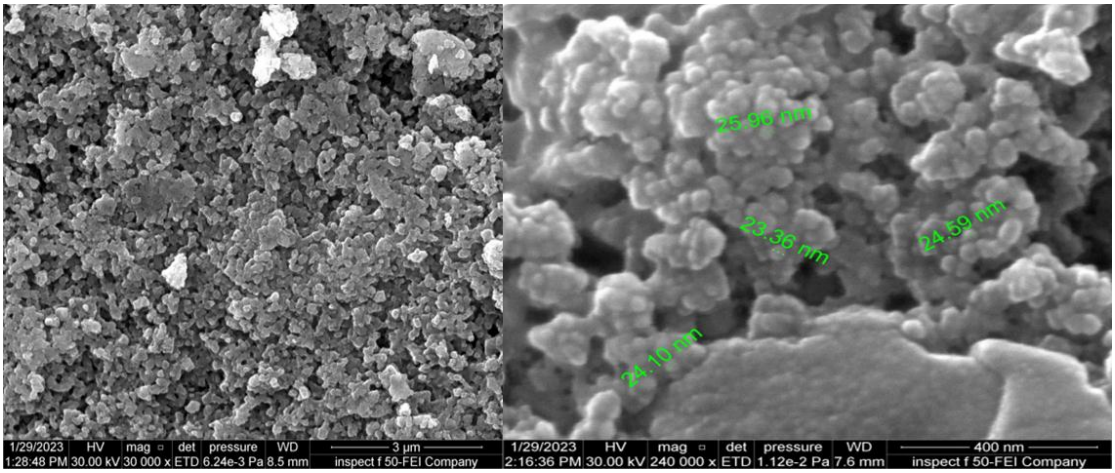


Figure 7. FESEM images of AgNPs sample

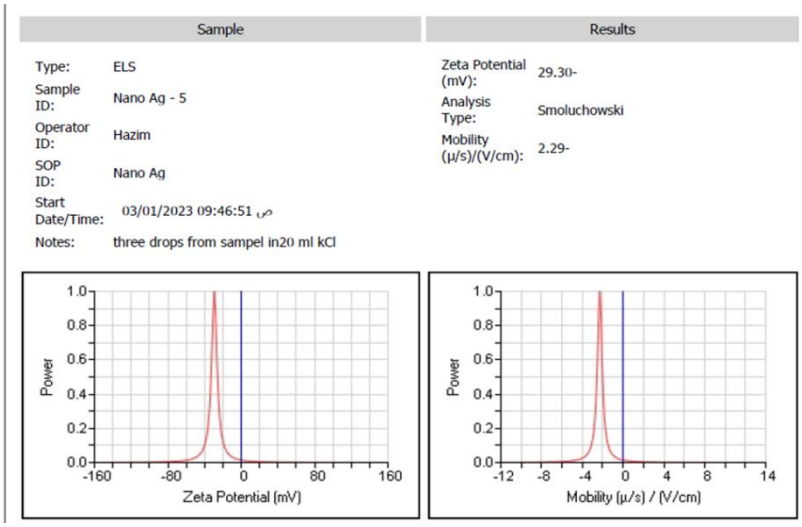


Figure 8. ZP analysis of synthesized AgNPs

Antibacterial activities of AgNPs

The antimicrobial capacity of the prepared AgNPs was evaluated by a biological method and well diffusion assay against the fourth bacterium *S. aureus*. After plate incubation, inhibition zone diameter was observed as an affirmation of antibacterial capacity (Table 1). This was a higher concentration (64 µg/ml) than the maximum. In this regard, the effective dose of the preceding antibacterial examination, which reached 19 mm, was used as the baseline concentration for measuring the MIC assay.

Cytotoxicity of assay - Cell line maintenance

The results obtained showed that the cells viability reduced as concentration of $AgNO_3$ increased. Use the cytotoxicity effect of AgNPs that synthesis by biological method in normal cell line (HdFn) and tumor cell line (A375) at concentration of range 100-500 µg/ml, as shown in figure (9). The half-maximal inhibitory concentration (IC50) values of the HdFn (161.6 µg/ml). The IC50 values of the A375 (121.7 µg/mL) cells viability was increasing the concentration of the AgNPs and displayed dose-dependent sequence of progressive cytotoxicity beginning at lower concentration

to its maximum inhibition (40%) inhibition of HdFn cells and (55%) inhibition of A375 cells.

Table 1. Results for AgNPs effects on bacterial strain

Different concentrations of AgNPs dimeter of inhibition zone (mm)					
Bacterial strains	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml
S. aureus	11	10	9	8	5
Streptococcus pyogenes	14	12	8	6	4
E. coli	19	14	9	6	3
P. aeruginosa	13	11	8	6	4

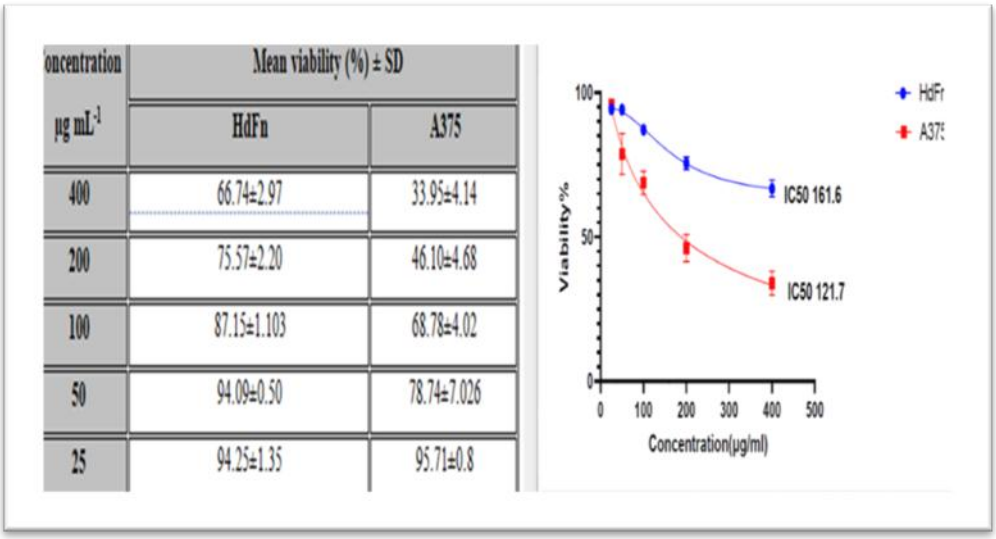


Figure 9. The cytotoxic effect of on HdFn and A375 cell line

Discussion

The synthesis of AgNPs using biological methods, particularly employing *P. aeruginosa*, presents a promising path for the development of Gram-negative drug alternatives. Antibiotic resistance, especially among MDR bacteria, poses a significant challenge in healthcare. This research aimed to explore the biosynthesis of AgNPs by *P. aeruginosa*, emphasizing cost-effective and environmentally friendly production methods while evaluating novel morphological characteristics and assessing antimicrobial and cytotoxic properties. UV-Vis spectrophotometry revealed a peak absorption at 421 nm, consistent with previous studies, indicating the formation of AgNPs and other studies found biosynthesis process of AgNPs by using the supernatant of *P. aeruginosa* peak at

300 to 400 nm [17]. XRD analysis confirmed the crystal structure of biosynthesized AgNPs, with an average size of approximately 36.6 nm, suggesting the suitability of the biological method employed agree with [18]. The value of 2θ between 20° to 82° confirmed the crystalline structure of the biosynthesized AgNPs according to the standards spectrum. Morphological characterization using TEM and AFM revealed spherical-shaped AgNPs with average diameters of 24.5-30 nm and 56.76 nm, respectively. These results corroborate with existing literature, demonstrating the reproducibility and reliability of the biosynthetic process [19]. EDS analysis confirmed the purity of synthesized AgNPs, further validating the effectiveness of the biological synthesis approach. According to [20], ZP expressed the stability of nano formulations particles below 20 nm show high mobility, low light diffusion, and a narrow concentration spectrum. The minimum concentration to perform particle size influences the ZP test because it affects the particle surface charge. Antibacterial assays, including well diffusion and MIC tests, demonstrated the strong antimicrobial activity of AgNPs against MDR bacteria, including Gram-positive and Gram-negative strains. The smaller the size of the AgNPs, the more pronounced the effects on causing cell death by adhering to the cell surface and rapidly spreading across the membrane of the microorganisms [21].

The MIC value of 32 µg/ml indicates the potent bactericidal effects of AgNPs, highlighting their potential as alternative antimicrobial agents against resistant pathogens. Moreover, cytotoxicity assays using the MTT method revealed no significant reduction in viability of human lymphocytes treated with AgNPs, indicating their biocompatibility and potential safety for therapeutic applications. These findings suggest that the biosynthesized AgNPs exhibited promising antibacterial efficacy against MDR pathogens without eliciting cytotoxic effects on mammalian cells. According to Wan et al. [22], the effect of $AgNO_3$ at the MIC concentration on the AgNPs and their cytotoxic effects on cell growth insignificant cytotoxicity in A549 and HL-7702 cells, especially at higher concentrations than 10 µg/ml.

In conclusion, the biological synthesis method employed in this research offers a viable approach for the preparation of AgNPs with desired characteristics, including size uniformity and strong antimicrobial properties against MDR bacteria. The findings underscore the potential of AgNPs as effective and safe alternatives to conventional antibiotics, emphasizing their importance in addressing the growing threat of antibiotic resistance. Further research is necessary to explore the clinical applications and potential toxicity profiles of biosynthesized AgNPs, paving the way for their integration into medical therapeutics and biomedical technologies. Further research is warranted to explore the clinical applications and potential toxicity profiles of biosynthesized AgNPs, paving the way for their integration into medical therapeutics and biomedical technologies.

Acknowledgement

The authors would like to thank the staff who work in the Dept. of Biology, College of Science, University of Baghdad for their effort in the preparation of nanoparticles.

Author contribution

Dr. Hamid and Dr. Ahmed: designed and performed the experiments. Huseen performed the clinical isolation of bacteria and prepared the nanoparticles, wrote the article.

Conflict of interest

The author declares that there is no conflict of Interest.

Funding

Self-Finding.

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Received May 4th 2023
Accepted Sep. 10th 2023