

Bactericidal Potency of Green Synthesized Silver Nanoparticles against Waterborne *Escherichia coli* Isolates

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Abstract

In recent years, silver nanoparticles (Ag NPs) had produced by biological methods such as plant extract due to their efficiency, low cost, being non-toxic, and ecofriendly nature. Ag NPs have antibacterial, anti-mold, and anti-fungi because of their high surface area to volume ratio. In this study, *Opuntia ficus-indica* (Prickly pear) extract was used to produce green synthesized Ag NPs. Different techniques had adopted to describe the generated nanoparticles, such as an ultraviolet spectrophotometer, Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), and Zeta Potential Analysis. *Escherichia coli* (*E. coli*), separated from sediment and water of the Hillah River in Babylon city in Iraq, was utilized to estimate the antibacterial activities of Ag NPs at different concentrations. Both broth microdilution assay and well diffusion assay were applied. The Congo Red Agar implied to investigate the ability of *E. coli* isolates to form a biofilm. The TEM images of Ag NPs illustrated spherical morphology with a diameter of approximately 22 ± 4 nm. The antibacterial activity tests showed that the Minimum Inhibitory Concentration (MIC) ranged from $0.0125 \text{ mg} \cdot \text{L}^{-1}$ to $0.05 \text{ mg} \cdot \text{L}^{-1}$, whereas Minimum Bactericidal Concentration (MBC) was from $0.025 \text{ mg} \cdot \text{L}^{-1}$ to $0.05 \text{ mg} \cdot \text{L}^{-1}$. 5 h of exposure to the $0.025 \text{ mg} \cdot \text{L}^{-1}$ concentrations of the Ag NPs had a bactericidal impact on 92% of the *E. coli* isolates. In our study, we found silver nanoparticles synthesized by *Opuntia ficus-Indica* have antibacterial activity against waterborne *Escherichia coli* isolates and it could be utilized to reduce microbial growth in contaminated water.

Keywords: Bactericidal impact, Biofilm, Green synthesis, Silver nanoparticles, *Opuntia ficus-indica* extract, Waterborne *E. coli*

Introduction

The transmission of pathogens occurred by drinking and running water, remains the cause of illness and death, especially in undeveloped countries [1]. Around one billion humans lack safe and clean drinking water. Approximately two million people die each year from drinking polluted water [2]. The aquatic environment had corrupted by human

activities such as watersheds, agriculture, or rivers near wastewater discharges [3, 4]. The increased drainage of sewage directly into streams is a significant source of antibiotic-resistant bacteria. Their resistance genes had transmitted by horizontal gene transfer to bacteria in natural bacterial communities [5]. Contamination of aquatic environments with pathogens represents a health hazard and a direct threat to drinking water supply, recreational activities, and crop irrigation

[6]. *E. coli* found in the humans' feces and other warm-blooded animals stand as evidence of fecal contamination. Which can survive for a long time in river water [3, 7]. Previous studies have indicated *E. coli* is a crucial repository for Multiple drug resistance (MDR) genes, so it is an adequate agent for transferring resistance genes in the bacterial community [8, 9]. Several studies suggest that the origin of MDR genes is strains of *E. coli* isolated from animals. However, some researchers pointed out that water is a vital mediator for MDR proliferation, which leads to the prevalence of carrying-MDR bacteria in the aquatic environment [10]. Nanotechnology refers to all developed technologies in various fields such as medicine, pharmacy, and cosmetics working with nanoscale dimensions ranged from 1 nm to 100 nm [11]. Green synthesis is an alternative method of producing nanoparticles. It is cost-effective, time-consuming, and does not require toxic substances that affect living organisms and the environment. Lately, many metal nanoparticles such as gold, silver, and copper nanoparticles have been produced using phytochemical agents in plant extracts [12]. The selection of eco-friendly nanoparticles requires accurate experiments in which the shape, size, and dynamics of the interaction between the metal ions, and the reducing agents, are studied [13]. Ag NP also gained interest among nanoparticles due to their size-dependent properties, including chemical, catalytic, electrical, and antimicrobial impacts. Ag NPs had synthesized by biological methods, which have distinctive properties, such as increased surface-area, small scale, and high dispersion, which improve the antimicrobial impact of Ag NPs relative to silver elements. The production of nanoparticles in different shapes and sizes belongs to various plant essences. With the increased antimicrobial consequences of silver at the Nanoscale, Ag NPs had employed to control a wide range of pathogens [14]. Ag NP had extensively adopted in the food industry, water purification, and antimicrobial agents against MDR bacteria [15].

In the present study, various methods have been used *in vitro* to determine the antibacterial effect of green synthesized Ag NPs. The antibacterial activity of green synthesized Ag NPs had examined against *E. coli* samples isolated from water and sediment of the Hillah River in Babylon city. Moreover, the bactericidal effect of Ag NPs was investigated against *E. coli* isolates exposed to different concentrations during the first 5 h. At the same time, the impact of different concentrations of Ag NPs on the ability of isolates to

form biofilm had also studied.

Experimental

Description of the sampling area

Although the Hillah River in Babylon, Iraq, is the foremost water source for the city's residents, pollution sources have spread along the river, such as sewage drainage and agricultural wastes. The water and sediment samples had taken from three chosen regions depending on the causes of contamination. The first region is adjacent to the farming field, the second region is near Marjan Hospital, and the third region is in urbanized areas.

Preparation of stem extract and AgNPs

Due to the existence of antioxidants in *Opuntia ficus-indica* extract (prickly pear), that was used as a reducing agent in the green synthesis of silver nanoparticles [16]. Stems of *Opuntia ficus-indica* were washed several times with sterilized deionized water to remove dirt and dust. Later 2.5 g of the stems were chopped into small pieces and boiled in 100 mL deionized water for 10 min. 13 mL of the filter-sterilized extract was diluted and pH-adjusted to the final volume of 100 mL and the pH value of 9, using deionized water and 0.1 M NaOH. Silver nitrate solution (1 mL) (0.01 M) was added to the mixture drop by drop and stirred using a magnetic stirrer with a speed of 500 rpm for 24 h. After the synthesis of Ag NP, the solution containing nanoparticles was rinsed three times with deionized water and discarded by centrifuge at 12000 RCF for 30 min to detach Ag NP. The sediment was suspended with deionized water and kept at laboratory temperature for the following experiments.

Sample characterization

The ultraviolet-visible spectrum was measured using a spectrophotometer (Analytik Jena - Specord 210) in the 200-800 nm wavelength range. The shape and size of the synthesized Ag NPs, as well as the selected area electron diffraction (SAED), were analyzed by Transmission Electron Microscopy (TEM) (JEM2000EX; JEOL, Tokyo, Japan), conducted at 200 kV. Dynamic Light Scattering (DLS) analysis (Horiba SZ-100; Kyoto, Japan) was used to define the dimensional feature and dispersity of the synthesized Ag NPs. The surface charge of Ag NPs was evaluated by determining the Zeta potential (Horiba SZ-100; Kyoto, Japan).

***E. coli* isolation**

Microorganisms of the water and sediment samples were separated by filtration using the sterilized membrane filters (0.45 μm pore size). The applied membranes were placed on *E. coli*-Enterobacteria chromogenic agar plates (Condalab, Spain). The presumptive *E. coli* colonies were further analyzed using the Enterosystem 18 R kit (Liofilchem-Italy), and the confirmed *E. coli* isolates were obtained for further experiments.

Determination of the biofilm-forming isolates

The Congo red agar (CRA) was prepared depending on the procedure stated by Freeman. et al. [17]. The components of the medium included the Brain Heart Infusion Broth (HiMedia, India) (37 $\text{g}\cdot\text{L}^{-1}$), sucrose (50 $\text{g}\cdot\text{L}^{-1}$), Agar-Agar type 1 (HiMedia, India) (10 $\text{g}\cdot\text{L}^{-1}$), and Congo red dye (HiMedia, India) (8 $\text{g}\cdot\text{L}^{-1}$). The Congo red dye was dissolved in distilled water and autoclaved separately for 15 min at 121 $^{\circ}\text{C}$. The sterilized dye solution was added to the sterilized medium, which cooled down to 55 $^{\circ}\text{C}$. The prepared CRA Petri plates were kept refrigerated until being used. The *E. coli* isolates were cultured on CRA plates and incubated at 37 $^{\circ}\text{C}$ for 24 h to evaluate their biofilm-producing ability. The presence of black colonies on CRA refers to the biofilmsformation [18].

Identification of the antimicrobial activity by the well diffusion assay

The antibacterial activity of green synthesized Ag NPs was estimated using the well-diffusion method. The *E. coli* isolates were cultivated on a Brain Heart Infusion Agar (BHIA) (HiMedia, India) at 37 $^{\circ}\text{C}$ for 18 h. Three to five single colonies were transferred into normal saline to obtain bacterial suspension equal to the 0.5 McFarland. Shortly, Mueller-Hinton agar (MHA) (HiMedia, India) plates were cultured with 0.5 McFarland bacterial suspensions under aseptic conditions. Six pits with 5 mm diameter were made on the inoculated MHA, and 50 μL of sequentially decreasing concentrations of the Ag NPs were transferred into the wells as the following: 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 $\text{mg}\cdot\text{L}^{-1}$. Imipenem disc (10 μg) was used as negative control, and distilled water as positive control. Finally, inoculated plates were incubated for 24 h at 37 $^{\circ}\text{C}$. The diameter of the inhibition zone around every pit reflects the green synthesized Ag NPs' antimicrobial activity.

Identification of the minimum inhibitory concentration and the minimum bactericidal concentration of green synthesized silver nanoparticles

Broth microdilution technique was performed according to the CLSI standard (M07A9, 2012) to determine The MIC and MBC of the green synthesized Ag NPs. Firstly, broth serial dilutions of Ag NPs were achieved in (50 μL) sterilized Müller Hinton broth (HiMedia, India) in the wells of microtiterplate to obtain the following concentrations: 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 $\text{mg}\cdot\text{L}^{-1}$. A 50 μL of bacterial suspension with 1×10^6 CFU/mL concentration was transferred to each well containing Ag NPs. Imipenem was used as negative control, and distilled water as positive control. The inoculated microtiter plates were incubated at 37 for 24 h. The lowest concentration of Ag NPs, which resulted in no visible growth, was regarded as MIC. MBC was determined as the lowest concentration of Ag NPs resulting in deletion of 99.9% of initial inoculum and was detected by the cultivation of the contents of every growth-inhibited well in BHI agar and colony counting after 24 h incubation at 37 $^{\circ}\text{C}$.

Evaluation of the viability and biofilm formation ability during 5h exposure to the green synthesized Ag NPs

Bactericidal and anti-biofilm potencies of various concentrations of Ag NPs were determined in 1-h intervals during a 5-h exposure time. The broth microdilution method was applied according to the CLSI standard (M07A9, 2012). Broth serial dilutions of Ag NPs were made in (50 μL) sterilized Müller Hinton broth (HI Media, India) in the wells of microtiterplate to obtain the following concentrations: 0.4, 0.2, 0.1, 0.05, 0.025 and 0.0125 $\text{mg}\cdot\text{L}^{-1}$. A 50 μL of bacterial suspension with 1×10^6 CFU/mL concentration was transferred to each well containing Ag NPs. A 10-microliter portion of every well was transferred and spread on TSA plates at 1-h intervals. The TSA plates were incubated at 37 $^{\circ}\text{C}$ for 24 h and colony counted to find out the MBC at the related time of exposure. Also, a 5-microliter portion was taken from every well at the 1-h intervals and streaked on CRA plates to investigate the capacity of biofilm formation of the *E. coli* isolates exposed to the various concentrations of Ag NPs for 1 to 5 h. The CRA plates were incubated at 37 $^{\circ}\text{C}$ for 24 h. The appearance of black colonies was regarded as the biofilm formation ability, according to Freeman, et al. [17].

Results and Discussion

Spectrophotometer analysis

Adding silver nitrate solution to *Opuntia ficus-indica* extract changes the solution color from light yellow to dark brown. The emergence of the brown color belongs to the excitation of the Surface Plasmon Resonance (SPR) and the reduction of silver nitrate solution by the plant extract [19]. As depicted in (Fig. 1), the typical absorption peak at 420 nm in the ultraviolet-visible spectrum showed the formation of Ag NPs.

Transmission electron microscopy (TEM) analysis

The TEM image of the green synthesized Ag NPs sample displayed spherical morphology of NPs, and a bit of agglomeration (Fig. 2). The average diameter of Ag NP was estimated at 22 ± 4 nm. The selected area electron diffraction (SAED) pattern, illustrated in (Fig. 2(d)), was indexed for Ag NPs. (ICDD no: 05-0664)

Dynamic light scattering (DLS) analysis

DLS analysis was performed to estimate the dispersion size of Ag NPs in the aquatic environment.

This procedure was done at 25 °C in a standard dispersed medium, at a viscosity of 0.892 mPa·s. As shown in (Fig. 3), a particle size distribution of obtained Ag NPs was ranging between 60 nm to 110 nm with an average peak value of 76 nm. The results of the DLS analysis revealed the polydispersity index (PDI) of green synthesized Ag NPs was 0.716, indicating broad range of different size of generated Ag NPs.

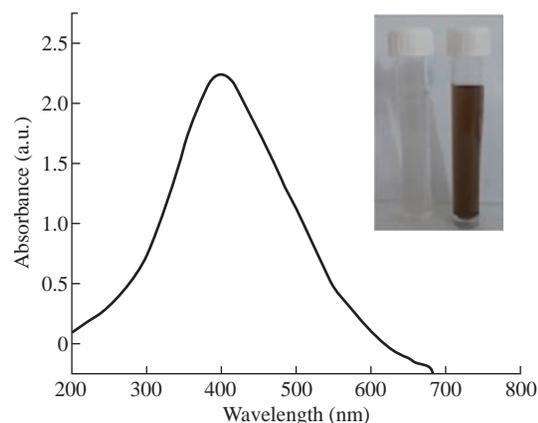


Fig. 1 UV-visible spectra of the green synthesized silver nanoparticles.

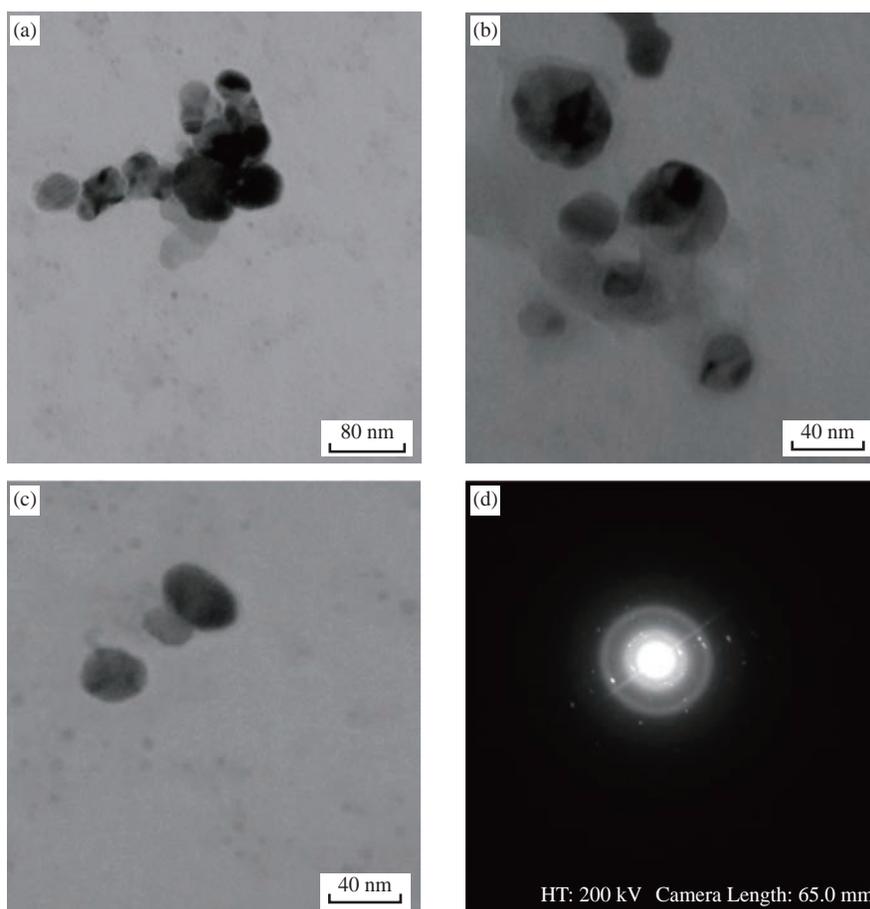


Fig. 2 Transmission electronic microscope image of green synthesized silver nanoparticles in different sizes. (a) 80 nm, (b) 40 nm, (c) 40 nm, and (d) SAED pattern.

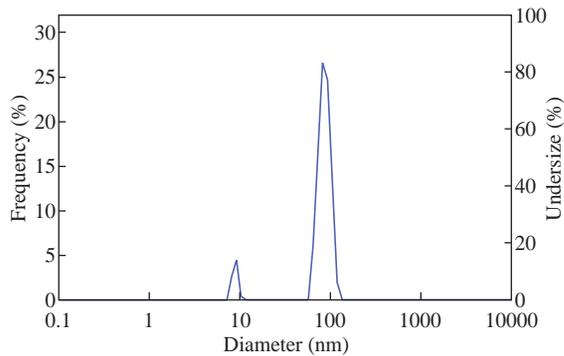


Fig. 3 Dynamic light scattering pattern of the green synthesized silver nanoparticles.

Zeta potential analysis

Zeta potential of Ag NPs was analyzed to evaluate the stability of suspension. It is well known that NP with a surface charge higher than +30 mV or less than -30 mV exhibit more stability and dispersion in liquid [20]. As a result, the zeta potential diagram of Ag NPs revealed that the surface charge of Ag NPs is -66.3 mV (Fig. 4). The high negativity of the zeta potential value might be relating to the capping agent existing in the stem extract of the *Opuntia ficus-indica*.

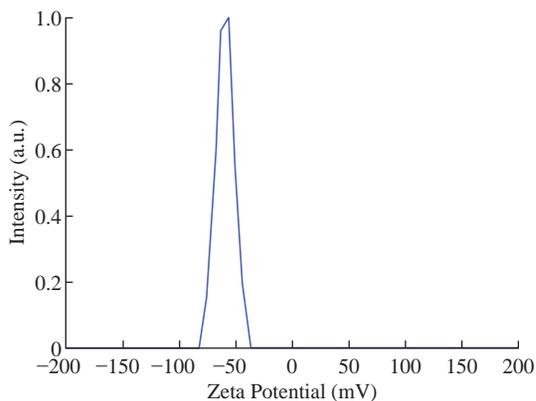
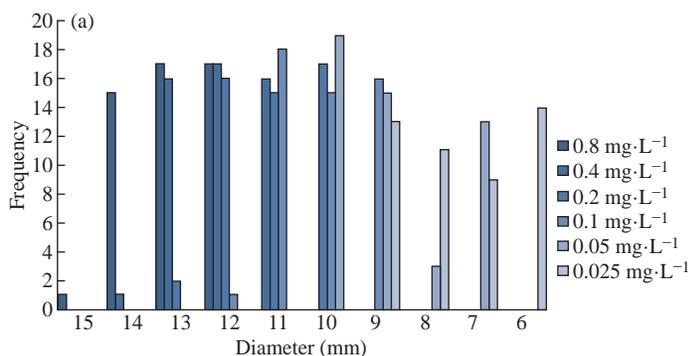


Fig. 4 Zeta potential of the green synthesized silver nanoparticles.



Determination of the biofilm-forming isolates

Based on CRA culture results, 85% of the *E. coli* isolate (50 out of 59) had black colonies with a crystalline appearance and were regarded as the biofilm-forming isolates. The non-biofilm-forming isolates were distinguished as white colonies on CRA.

Identification of the antimicrobial activity by the well diffusion assay

The well-diffusion assay was conducted to evaluate the susceptibility of the *E. coli* isolates against various concentrations of the green synthesized Ag NPs. The inhibition zone diameter ranged from 2 to 15 mm, 11 to 14 mm, 10 to 13 mm, 9 to 12 mm, 7 to 10 mm, and 6 to 9 mm by the Ag NPs concentration of 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 mg·L⁻¹ respectively, as depicted in (Fig. 5(a)). The inhibition zone ranged from 12 to 15 mm, 11 to 14 mm, 10 to 13 mm, 9 to 12 mm, 7 to 10 mm, and 6 to 9 mm at 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 mg·L⁻¹ respectively. Based on the results of the repeated measures ANOVA with a Greenhouse-Geisser correction determined that the mean inhibition zone varied statically significantly between different concentrations ($F(3.224, 157.955) = 1604.807, P < 0.0005$). Post hoc tests using the Bonferroni revealed a significant difference at 0.05 level between the diameters of inhibition zones at various concentrations as shown in (Fig. 5(b)). The results demonstrated that the diameters of the inhibition zone decreased when the concentrations of Ag NP decreased. These results had confirmed by the results of Pal et al. [21].

Identification of the minimum inhibitory concentration and the minimum bactericidal concentration of green synthesized silver nanoparticles

The green synthesized Ag NPs exhibited

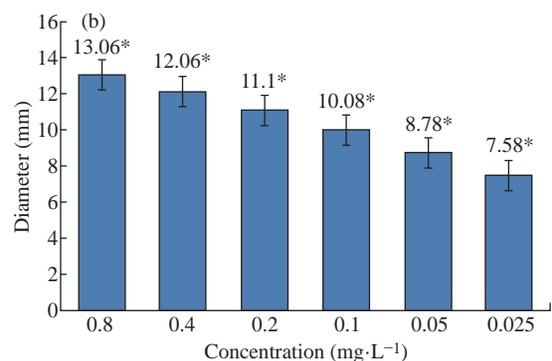


Fig. 5 Antimicrobial activity the green synthesized silver nanoparticles by the well diffusion assay. (a) Inhibition zone of the green synthesized silver nanoparticles at various concentrations. (b) Average diameter of inhibition zone of the green synthesized silver nanoparticles at various concentrations. (The small stars represented significant difference at the 0.05 level.)

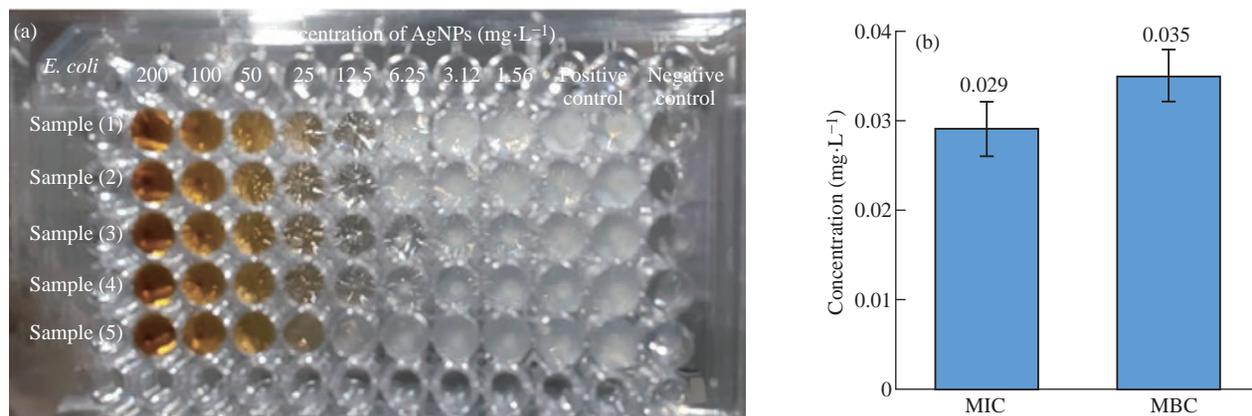


Fig. 6 (a) The antibacterial activity of the green synthesized Ag NPs against *E. coli* isolates. (b) Average MIC&MBC of Ag NPs.

antibacterial activity against *E. coli* isolates. The lowest concentration with no visible growth and the lowest concentration eliminating 99.9% of the inoculum has counted MIC and MBC of the Ag NP, respectively. The MIC of Ag NPs against the *E. coli* isolates ranged from 0.0125 to 0.05 mg·L⁻¹. The Ag NPs revealed bactericidal characteristics and had MBC values of 0.025 and 0.05 mg/L against the *E. coli* isolates (Fig. 6). The small size of Ag NPs displays a much higher dissolution rate that leads to a greater level of toxicity. The antibacterial activity mechanism of Ag NPs involves the incorporation of silver ions with thiol groups in biological molecules such as enzymes and other proteins. That prevents cell division and destroys the cell envelope. Also, silver ions have high affinity to react with sulfur-containing bases than phosphate groups in nucleic acids [22]. Silver ions released from Ag NPs can tightly attach to cells, suppress enzyme activity, damage cell membranes, and drove cell death [23]. Antimicrobial activity with a ratio of MBC to MIC ≤ 4 is described as a bactericidal effect [24]. The green synthesized Ag NPs revealed a bactericidal feature against 100% of the *E. coli* isolates. Values of the MBC to MIC ratio exhibited the bactericidal impact of that green synthesized Ag NPs against 100% of the *E. coli* isolates. The previous studies revealed that antibacterial agents with bactericidal impact work on the rapid elimination of the infectious agents preventing the spread of infection and growth of the resistant strains [25]. The different MIC and MBC values against the *E. coli* isolates in this study may be attributable to the developed resistance against Ag NPs. The constant exposure of *E. coli* against silver nanoparticles creates resistance to silver nanoparticles. This resistance is due to the generation of the flagellin protein, which causes accumulation of the

nanoparticles and decreases their suspension stability and results in diminishing their antibacterial activity [26]. The *mutY*, *mutS*, *mutM*, and *mutT* genes in *E. coli* are thought to be responsible for the regeneration of Ag NP-induced DNA damage. As a result, the mutation in these genes made the bacteria more susceptible to Ag NP. Mutations in the *OmpF* and *OmpC* purine coding genes, which involve the formation of water-filled channels in the outer membrane of *Escherichia coli*, lead to Ag NPs resistance [27].

Evaluation of the viability and biofilm formation ability during 5-h exposure to the green synthesized Ag NPs

In this study, the *E. coli* isolates were exposed to different concentrations of Ag NPs for 5 h, and the bactericidal and anti-biofilm properties were determined at various times of exposure to the different Ag NPs concentrations by culturing on TSA and CRA, respectively. The Ag NPs revealed antibacterial effect in a time and dose-dependent manner. After 1-h exposure, the 0.4, 0.2, and 0.1 mg·L⁻¹ concentrations of the Ag NPs resulted in at least 3-log reduction in the bacterial count of 46, 22, and 12% of the *E. coli* isolates. The lower concentrations neither resulted in a 3-log reduction in the bacterial count nor exhibited the anti-biofilm activity. The longer the time of exposure, the higher the frequency of the affected *E. coli* isolates. Finally, 92, 84, 58, and 38% of the *E. coli* isolates experienced at least a 3-log population reduction, after 5 h of exposure to the 0.4, 0.2, 0.1, 0.05, and 0.025 mg·L⁻¹ Ag NPs concentrations (Fig. 7). None of the growing *E. coli* isolates exhibited loss of biofilm formation ability due to the various exposure times to the different concentrations of the Ag NPs.

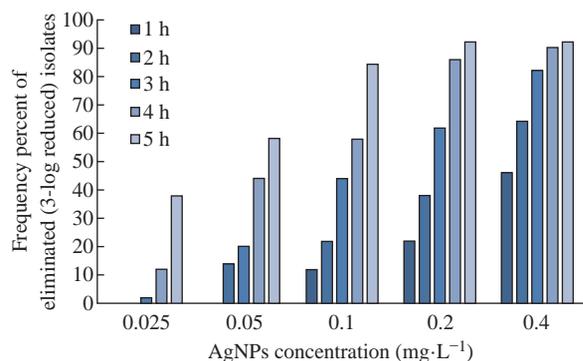


Fig. 7 The bactericidal activity of different concentrations of the green synthesized Ag NPs against the *E. coli* isolates during the 5-h period.

Conclusions

The green synthesis of nanoparticles from plant extract is considered a promising low-cost, environmentally friendly method, which does not need any poisonous chemicals for synthesis. The leaf extract of *Opuntia ficus-indica* was utilized to produce the silver nanoparticles in a spherical shape with an average size of 20 nm. The green synthesized silver nanoparticles exhibited significant antibacterial activity against *E. coli* isolates in the range of 0.0125-0.05 mg·L⁻¹. The current study showed green synthesized silver nanoparticles do not affect the ability of *E. coli* isolates to generate biofilm, even in low concentrations. The use of green synthesized silver nanoparticles in the wastewater treatment process may effectively eradicate the pathogens by releasing the silver ions from the surface of the nanoparticles in aquatic environments. The silver ions are involved in the production of reactive oxygen species (ROS), leading to the destruction of the bacterial genome, protein degradation, and the permeability of cell membranes.

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Abbreviations

Ag NPs: Silver nanoparticles; BHIA: Brain heart infusion agar; CRA: Congo red agar; DLS: Dynamic light scattering; *E. coli*: *Escherichia coli*; MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration; MDR: Multiple

drug resistance; MHA: Mueller-Hinton agar; SAED: Selected area electron diffraction; SPR: Surface Plasmon resonance; TSA: Tryptic soy agar; TEM: Transmission electron microscopy.

Conflicts of Interests

The authors declare no conflicts of interests.

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